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Inverse sequence-tagged repeat (ISTR) analysis, a novel and universal PCR-based technique for genome analysis in the plant and animal kingdom

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ABSTRACT

The previously described amplification of a subset of *copia*-like repetitive sequence elements in the coconut genome by inverse sequence-tagged repeat analysis (ISTR) was extended into a generally applicable strategy for plant and animal genome analysis. A wide range of genomic DNAs was amplified in a polymerase chain reaction in the presence of identical *copia* sequence-derived primers or primer pairs. The number of loci detected by a single ISTR analysis is large and ranges between 20 and 100, a value which is comparable to that obtained by the AFLP (amplified fragment length polymorphism) technique. Depending on the species and the PCR primers, also the number of DNA polymorphisms (at an average 5-50) equals that of AFLP analyses. A modification of the experimental approach allowed the nonradioactive detection of DNA fragments in the sequence gel without membrane transfer thereby allowing for the widespread use of the technology. In addition, specific PCR fragments of interest can be reisolated from the gel, reamplified, sequenced and thus converted for PCR-based sequence-tagged site (STS) analysis.

Key words : Biodiversity, DNA fingerprinting, Molecular markers, Polymorphisms

INTRODUCTION

The advent of DNA marker technology designed to detect naturally occurring polymorphisms at the DNA level has become an invaluable and in part revolutionizing tool for both applied and basic diagnostic studies of plant and animal genomes as well as for microorganisms (BURR, 1994). These studies range from the examination of the origin of biological diversity in systematic biology to practical application as for example in gene isolation by map-based cloning, in breeding by marker-assisted selection or in clinical diagnostics by DNA fingerprinting. One of the most important findings of DNA marker-based studies first observed in the *Solanaceae* (BONIERBALE *et al.*, 1988) and more recently described for cereals (MOORE, 1995) is the phenomenon of synteny which refers to partial genome co-linearity between distantly related species.

For the genomes of various crop plants, physical maps have been constructed by the incorporation of DNA markers accessible by different novel techniques of molecular biology (PHILLIPS and VASIL, 1994). These include markers detecting restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and mini- and microsatellite DNAs termed variable number tandem repeats (VNTRs) or simple sequence repeats (SSRs). Other, more specific approaches exploit polymorphisms located on mitochondrial, chloroplast or ribosomal DNAs. All of these techniques offer a variety of advantages as well as drawbacks for their application in plant breeding (HAYWARD *et al.*, 1993; RAFALSKI and TINGEY, 1993; POWELL *et al.*, 1994; PHILLIPS and VASIL, 1994), where the requirements for automated genome analysis of large populations have to be met. Clearly, strategies based on the polymerase chain reaction (PCR) have the highest potential for routine diagnosis, and consequently efforts have been undertaken to convert interesting RFLP or other markers into specific oligodeoxynucleotide primers for PCR-based diagnosis.

In addition to the repetition of simple sequence motifs like in mini- and microsatellite DNAs, plant and animal genomes contain extended repetitive elements many of which are mobile genetic elements capable of transposition (FINNEGAN, 1989). Among these transposable elements, the retrotransposons constitute the largest group. They are divided into two main classes depending on the presence or absence of flanking long terminal direct repeats (LTRs). The LTR-retrotransposons (*gypsy-/copia*-like retrotransposons) are apparently universally distributed among eukaryotic genomes and occur in high copy numbers (VOYTAS *et al.*, 1992; FLAVELL *et al.*, 1992; HIROCHIKA and HIROCHIKA, 1993; WESSLER *et al.*, 1995). Truncated versions of *copia*-like elements are also abundantly present in the coconut genome (ROHDE *et al.*, 1992), and subfamilies of clustered *copia*-like sequences were recently characterized in this plant species (ROHDE *et al.*, 1995). These subfamilies have been the basis to establish a PCR-based approach with *copia*-like sequence-derived oligodeoxynucleotide primers for coconut genome analysis designated inverse sequence-tagged repeat (ISTR) analysis.

Here I describe that such clustered *copia*-like sequences (irrespective of their length) are apparently ubiquitously dispersed among eukaryotic genomes and that identical, i.e. universal primers derived from coconut *copia*-like sequences amplify homo- and polymorphic DNA segments in the analysis of plant, animal and human genomes.

MATERIALS AND METHODS

Plant material

Leaf material from species of the *Palmae* were kindly provided by J. Dowe (Townsville, Australia). Material of sisal and the Milala and Borassus palms were collected on site in Tanzania. Seeds for wild-growing *Hordeum spontaneum* populations were supplied by Dr. E. Nevo (Haifa, Israel). All other plants were grown at the greenhouse facilities of MPIZ.

Isolation of genomic DNAs

Genomic DNAs (if not otherwise supplied) were isolated in a two-step procedure by the recently described modification (ROHDE *et al.*, 1995) of the method of DOYLE and DOYLE (1990). DNA was used for PCR amplification directly after the RNAse digestion step without further purification by reversible adsorption to QIAEX.

Amplification and analysis of polymorphic DNA

PCR primers for forward and backward reactions during ISTR analysis were designated ISTR-F# (Forward primer) and ISTR-B# (Backward primer), respectively (Fig. 1B), and generally ranged between 18 and 22 nucleotides in length. For radioactive ISTR analysis, the primers were labelled with polynucleotide kinase and [γ-^{33}P]ATP. Primers labelled with digoxigenin (DIG) were synthesized by MWG (Germany) and purified by HPLC before use.

PCR reactions were performed according to standard protocols in a final volume of 25 µl containing 25 ng of genomic DNA, 200 µM dNTPs, 2.5 mM $MgCl_2$, 1x PCR buffer (Gibco/BRL), 2.5 pmoles of each primer, and 1 unit of Taq DNA polymerase (Gibco BRL). The programme consisted of the following steps: step 1, 95°C/3 min; step 2, 95°C/30 sec; step 3, 45°C/30 sec; step 4, 72°C/2 min; step 5, 72°C/10 min; with 40 cycles of steps 2 to 4.

Dye markers in formamide (3 µl) were added to the reaction mixture and after denaturation at 100° C for 1 min, 1 µl-aliquots were separated on a 4% polyacrylamide gel. Radioactive-labelled DNA bands were made visible by autoradiography, DIG-labelled DNAs were detected directly in the gel using the DIG detection kit with 5-bromo-4-chloro-3-indolyl (X) phosphate/nitroblue tetrazolium (NBT) as the substrates (Boehringer, Mannheim) and following the protocol of the supplier.

Analysis of data

Autoradiograms or stained DIG gels were directly scanned into a suitable computer for fine analysis and data exchange. The gels were analyzed visually for the presence (1) or absence (0) of polymorphic DNA fragments and the constructed matrices were processed by the NTSYS-pc (version 1.80) software package (Exeter Software, Setauket, USA). Simple matching coefficients were calculated between each pair of genotypes using the quotient number of matches/total sample size. Based on the matrix of these coefficients between all genotypes, the cluster analysis was performed by the UPGMA method.

RESULTS

ISTR primers derived from copia-like sequences are apparently universally applicable in plant genome analysis

Previously it has been described (ROHDE *et al.*, 1995) that subsets of the *copia*-like *Eco*RI elements of coconut (ROHDE *et al.*, 1992) exist as clusters in the coconut genome. These conclusions resulted from the PCR amplification of genomic DNA with the outward directed primer

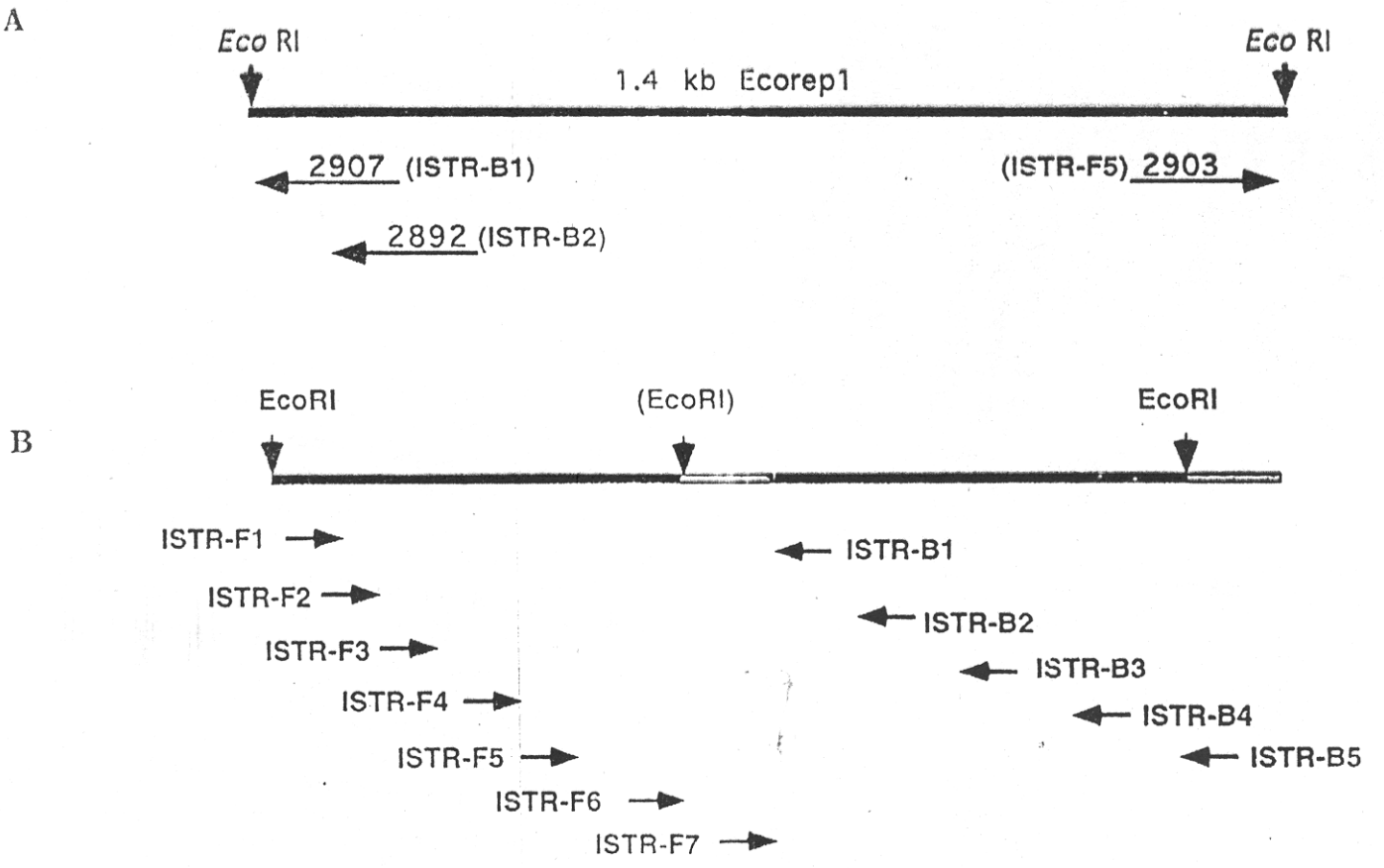


FIGURE 1 - Schematic representation of ISTR analysis: amplification of genomic DNA by coconut *copia* sequence-derived PCR primers.

(A) Original primers used for amplification of «spacer» regions separating the 1.3-1.4 kb *Eco*RI elements on coconut genomic DNA.

(B) Selected *copia* sequence-derived primers used for the ISTR analysis of plant and animal genomes. The direction of arrowheads designates the 5' 3' orientation of the PCR primers. Forward primers are given the letter F, backward primers the letter B.

pairs 2903 (ISTR-F5) / 2907 (ISTR-B1) and 2903 (ISTR-F5) / 2892 (ISTR-B2) (Fig. 1). Reexamination of the 400 bp «spacer» region (Fig. 1B; shaded areas) separating the 1.3-1.4 kb *Eco*RI elements revealed that it was highly homologous to part of the *copia*-like BARE-1 element from barley (MANNINEN and SCHULMAN, 1993). Thus *Eco*RI element plus «spacer» represented a continuous subgenomic fragment homologous to part of the endonuclease, the entire reverse transcriptase and the N-terminal half of the RNase H region of a *copia*-like element (data not shown) with the entire sequence being directly repeated as illustrated in Fig. 1B.

Based on the ubiquitous presence of *copia*-like sequences in the plant kingdom, plant DNAs other than coconut DNA were examined for the existence of clustered *copia*-like sequences similar to those described for the coconut genome (see above). As shown in Fig. 2, DNAs from yeast as well as from various mono- and dicotyledonous plants served as templates for the amplification of distinct DNA fragments when tested with the standard primer pair ISTR-F5/B2. In addition to the initial ISTR primer pairs ISTR-F5/B1 and ISTR-F5/B2, further primers were synthesized in order to evaluate the entire region of the coconut *Eco*RI elements for ISTR primers producing DNA polymorphisms. These additional ISTR primers (Fig. 1B) were designed such that a general annealing temperature of 45°C was applicable with all PCR amplifications.

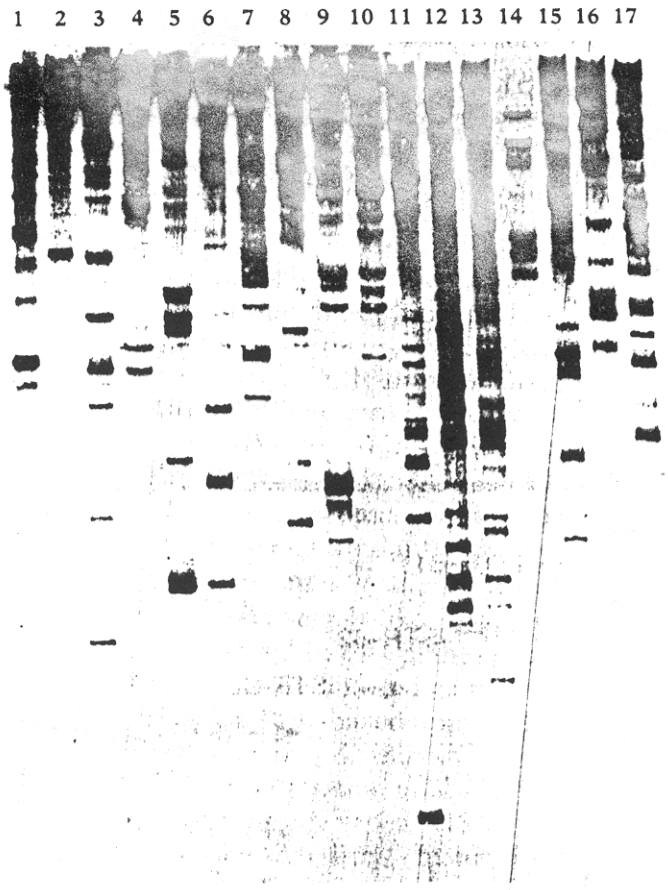


FIGURE 2 - General application of ISTR primers in the plant kingdom. Plant DNAs were amplified in the presence of the ^{33}P-labelled primer pair ISTR-F5/B2, the reaction products were denatured, separated on a 4% sequence gel and made visible by autoradiography. Lane1: tobacco, 2: barley, 3: potato, 4: maize, 5: snapdragon, 6: *Arabidopsis*, 7: rapeseed, 8: *Craterostigma*, 9: petunia, 10: parsley, 11: sisal, 12: Milala palm (Tanzania), 13: Borassus palm (Tanzania), 14: coconut palm, 15: sugar beet, 16: *Cuphea*, 17: yeast.

All 35 possible primer pair combinations were evaluated on the barley cultivars Proctor and Nudinka for which random lines as mapping populations were available as well as on two coconut types growing in Africa (East African Tall, EAT; Pemba Red Dwarf, PRD; ROHDE *et al.*, 1995). Every single primer combination gave rise to PCR products, and nested primers did not repeat a pattern, but resulted in different PCR patterns with new polymorphisms (data not shown; see also Fig. 6). While for the two genetically diverse coconut types which belong to the African (EAT) and Pacific (PRD) germplasm, respectively, all tested primer combinations produced at least five polymorphic bands, several of the primer combinations did not easily distinguish the barley cultivars Proctor and Nudinka. These data, however, demonstrated that the entire 1.3-1.4 kb coconut *Eco*RI sequence could be exploited for the synthesis of ISTR primers and the generation of polymorphic DNA. Secondly, *copia* like sequences, irrespective of their total length, are apparently distributed across eukaryotic genomes in an arrangement such that PCR amplification with 2 ISTR primers results in distinct and reproducible fingerprint patterns.

Examples for ISTR analysis in the plant kingdom

As *copia*-like sequences are ubiquitous in the plant kingdom, various DNAs of plants rangeing from yeast to exotic palm trees sampled in Tanzania were tested for the presence of clustered *copia*-related sequences that would allow amplification of DNA by one of the primer pairs (ISTR-F5/B2) depicted in Fig. 1B. The results (Fig. 2) for the 17 different species demonstrated that all tested DNAs served as templates for PCR amplification.

A similar standard ISTR analysis (primer pair ISTR-F5/B2) was then performed for 20 members of the *Arecaceae (Palmae)* in an effort to evaluate the ISTR technology for taxonomic purposes. In fact all 20 *Palmae* were amenable to ISTR analysis with hardly any particular PCR fragment being common to two or more palm species (Fig. 3A). For broad-range application of the ISTR technology, the ^{33}P-labelled primers were substituted by primers labelled with dioxigenin (DIG), and the identical palm DNAs were amplified. As depicted in Fig. 3B, such gels could be stained directly for the visualization of the DIG-labelled PCR fragments without the need of membrane transfer prior to the reaction with phosphatase-coupled DIG-antisera and the Xphosphate/NBT substrate.

ISTR analysis was further extended to high cultivated *(Hordeum vulgare)* and wild-growi barley *(Hordeum spontaneum)*. The analy (Fig. 4) of 34 *H. vulgare* and 31 *H. spontane* genotypes (belonging to four populations) v a single primer pair (ISTR-F6/B2) revealed both *H. vulgare* and *H. spontaneum* share s ral homomorphic DNA fragments. For exar the two doublets at the bottom of the two are invariably present in all barley geno examined to date. Visual inspection as w

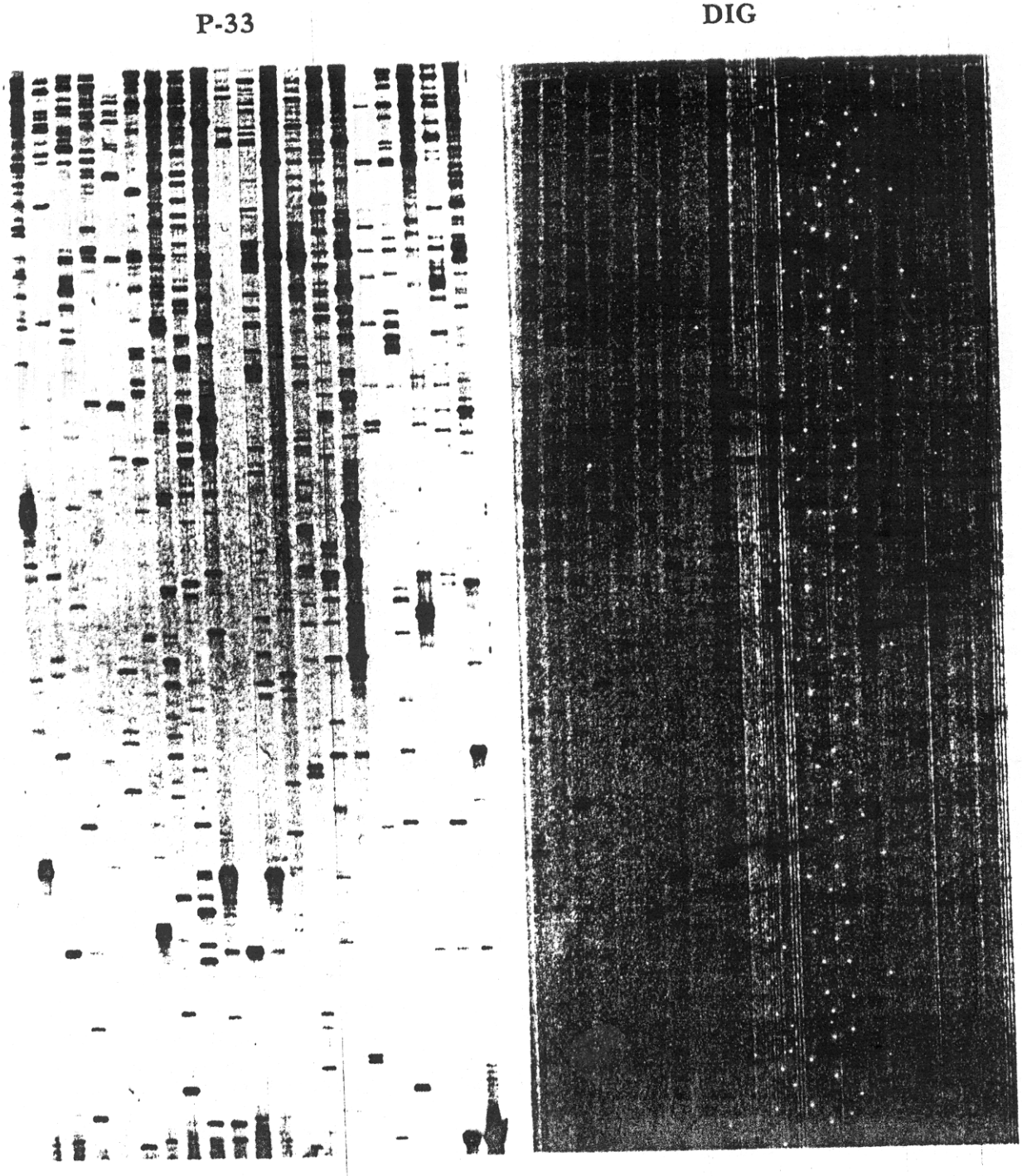


FIGURE 3 - ISTR analysis (primer pair ISTR-F5/B2) of some members of the *Arecaceae* (*Palmae*). The analysis was done as described in the legend to Fig. 2.
Lane 1: *Hyphaene petersiana* Mart.; 2: *Bismarckia nobilis* Hildebrandt & H. Wendl.; 3: *Eugeissona utilis* Becc.; 4: *Korthalsia echinometra* Becc.; 5: *Mauritiella aculeata* (H.B. & K.) Burret; 6: *Nypa fruticans* Wurmb.; 7: *Pseudophoenix sargentii* H. Wendl. ex Sarg.; 8: *Oraniopsis appendiculata* (F.M.Bailey) J.Dransf., Irvine and N.W.Uhl; 9: *Socratea exorhizza* (Mart.) H.Wendl.; 10: *Halmoorea tripatha* J. Dransf. & N.W.Uhl.; 11: *Cyrtostachys peckeliana* Becc.; 12: *Deckenia nobilis* H.Wendl.; 13: *Oncosperma tigillarium* (Jack) Ridley; 14: *Syagrus amara* (Jacq.f.) Mart.; 15: *Attalea allenii* H.E.Moore ex L.H.Bailey; 16: *Scheelea insignis* (Mart.) Karsten; 17: *Asterogyne martiana* (H.Wendl.) H.Wendl. ex Hemsley; 18: *Calyptrogyne sarapiquensis* H.Wendl. ex Burret; 19: *Polyandrococos caudescens* (Mart.)Barb.Rodr.; 20: *Reinhardtia gracilis* var. *gracilior* (Burret) H.E.Moore. Left panel: Autoradiogram of ISTR analysis with ^{33}P-labelled primers; right panel: ISTR analysis with DIG-labelled primers and direct staining in the gel.

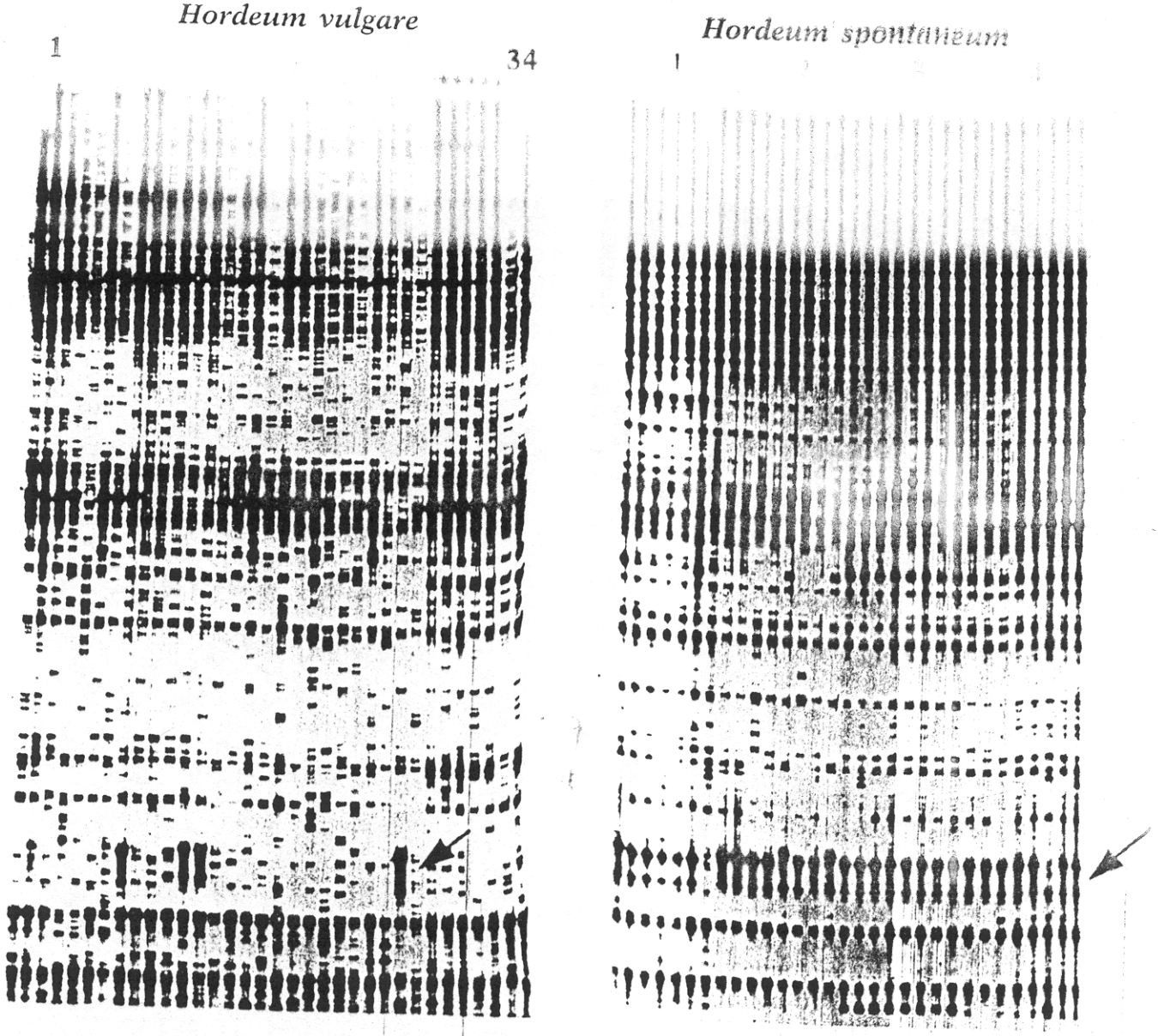


FIGURE 4 - ISTR analysis (primer pair ISTR-F6/B2) of *Hordeum vulgare* (left panel) and *Hordeum spontaneum* (right panel) genotypes. Left panel: DNAs of 34 barley (*H. vulgare*) genotypes (varieties, accessions or lines) were amplified in a standard PCR reaction and separated on a 4% polyacrylamide gel. Lane 1: Fiction; 2: Kaskade; 3: Red; 4: Georgie; 5: Alexis; 6: Marinka; 7: Flash; 8: Portikos; 9: Aura; 10: Gimpel; 11: Prisma; 12: Gitane; 13: Gavotte; 14: Manila; 15: Pilastro; 16: Masto; 17: Torrent; 18: Thibault; 19: Onice; 20: Mette; 21: Robur; 22: Probidor; 23: Mario Otter; 24: Nico; 25: Magie; 26: Tehri (accession from Nepal); 27: Asse; 28: *Calcaroides-C15* (ex Bonus); 29: *calcaroides-b2* (ex Bonus); 30: *calcaroides-b19* (ex Bonus); 31: Bonus; 32: Kristina; 33: Nudinka; 34: «Proctor». Asterices denote lanes 28-32. Right panel: DNAs from seven and eight, respectively, genotypes belonging to four wild-growing *H. spontaneum* populations from Israel were analysed as described above.

Arrow points out cluster of ISTR markers represented in most *H. spontaneum* genotypes and in four of the *H. vulgare* genotypes (Portikos, Gitane, Gavotte, Tehri).

superpositon of the two autoradiograms demonstrate that a cluster of 5-6 fragments present in almost all *H. spontaneum* genotypes (arrow in Fig. 4, right panel) is perfectly conserved in one *H. vulgare* genotype (arrow in Fig. 4, left panel). This genotype is an accession (Tehri) from the Nepal region. Also, the barley cultivars Portikos, Prisma, Gitane, and Gavotte show a si-

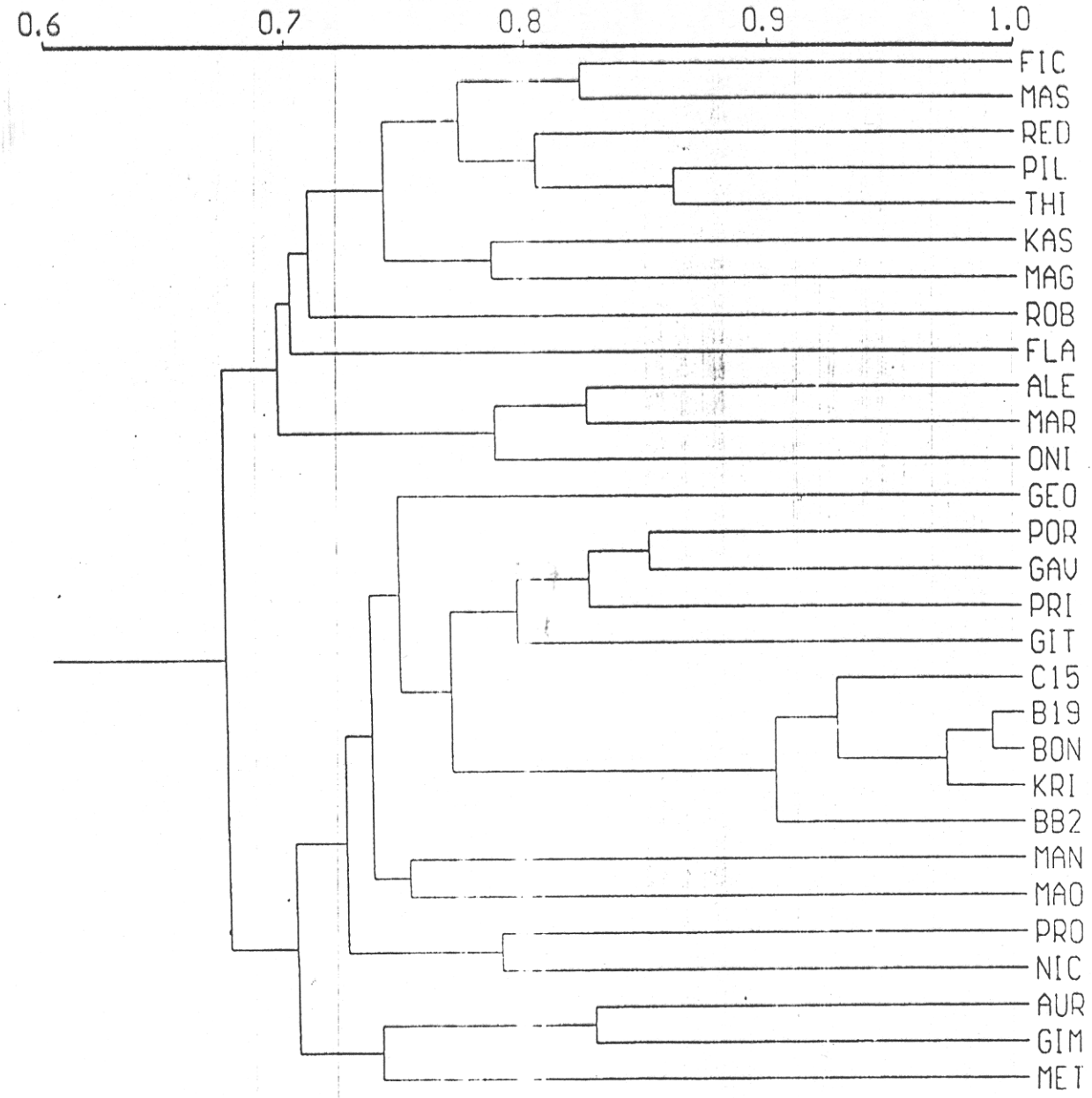


FIGURE 5 - Dendrogram of the *H. vulgare* ISTR analysis represented in Fig. 4 (left panel).
ALE: Alexis; ASS: Asse; AUR: Aura; B19: *calcaroides-b19* (ex Bonus); BB2: *calcaroides-b2* (ex Bonus); BON: Bonus; C15: *Calcaroides-C15* (ex Bonus); ETR: Etrusco; EXP: Express; FIC: Fiction; FLA: Flash; GAV: Gavotte; GEO: Georgie; GIM: Gimpel; GIT: Gitane; KAS: Kaskade; KRI: Kristina; MAG: Magie; MAN: Manila; MAO: Mario Otter; MAR: Marinka; MAS: Masto; MET: Mette; NIC: Nico; NUD: Nudinka; ONI: Onice; PAN: Panda; PIL: Pilastro, POR: Portikos, PRI: Prisma; PRO: Probidor; RED: Red, ROB: Robur, THI: Thibault; TIM: Timura; TOR: Torrent. The accession Tehri and the variety «Proctor» were not included in the dendrogram.

milar, but not identical pattern in this region. In addition, the genotypes Bonus, Kristina, *Cal-C15*, *cal-b2* and *cal-b19* (asterices in Fig. 4) are highly related as deduced from the ISTR pattern with this primer pair (see also below). In addition, this analysis as well as previous AFLP studies reveal that the cultivar designated «Proctor» is not identical to the original cv. Proctor received from a germplasm collection, and that it has been mislabelled during propagation in the breeding garden.

For *H. vulgare* a total of 79 polymorphic bands obtained from the analysis with ISTR-F6/B2 (Fig. 4, left panel) and ISTR-F5/B2 (not

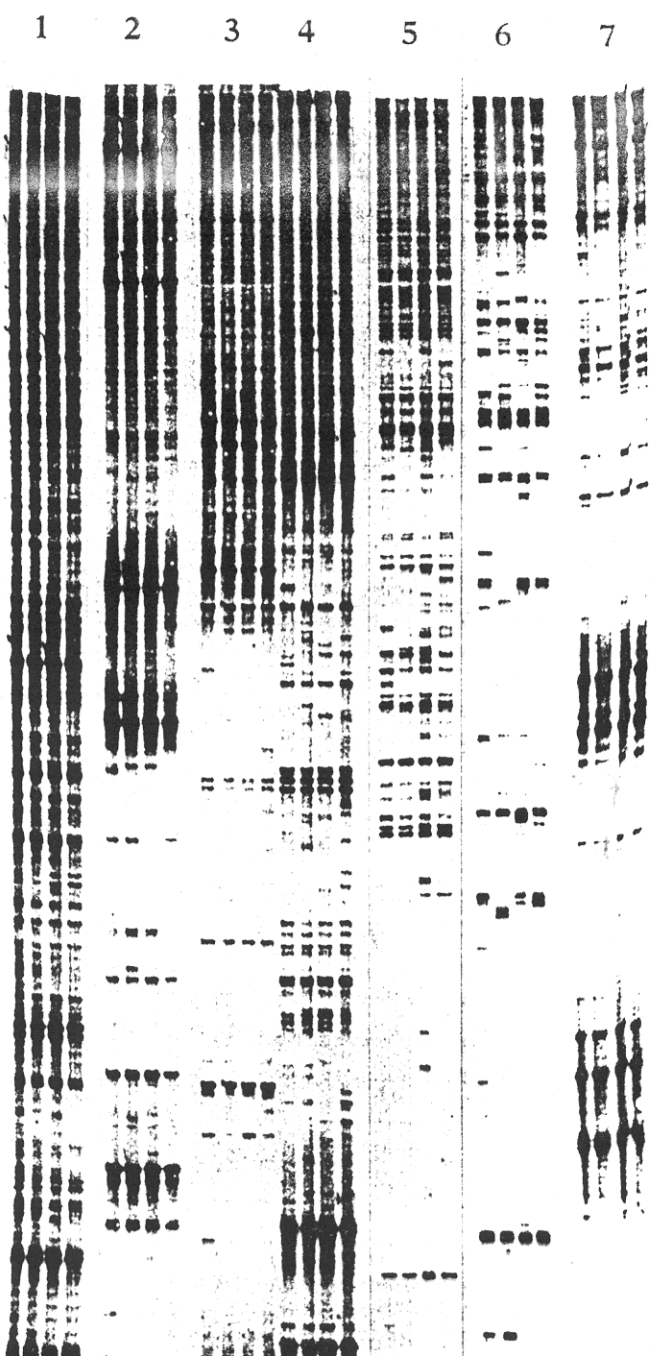


FIGURE 6 - ISTR analysis on barley genotypes with a common genetic background.
Nested primer pairs were used in the characterization of the barley variety Bonus and the *calcaroides* mutants *Cal-C15*, *cal-b2* and *cal-b19* (from left to right with each primer combination) obtained in the Bonus background by mutagenic treatment with sodium azide (*cal-b19*), Xray (*cal-b2*) and neutrons (*Cal-C15*). Primer combinations were: 1 (ISTR-F6/B2); 2 (ISTR-F7/B2); 3 (ISTR-F8/B2); 4 (ISTR-F5/B1); 5 (ISTR-F6/B1); 6 (ISTR-F7/B1); 7 (ISTR-F8/B1).

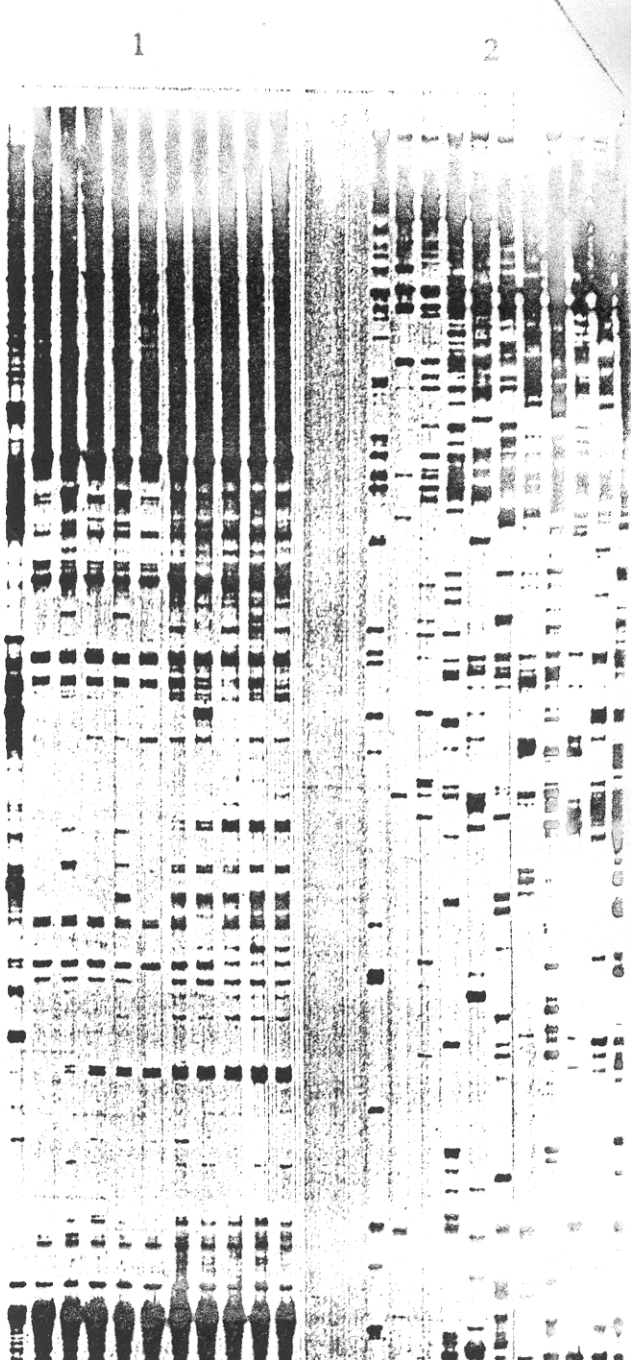
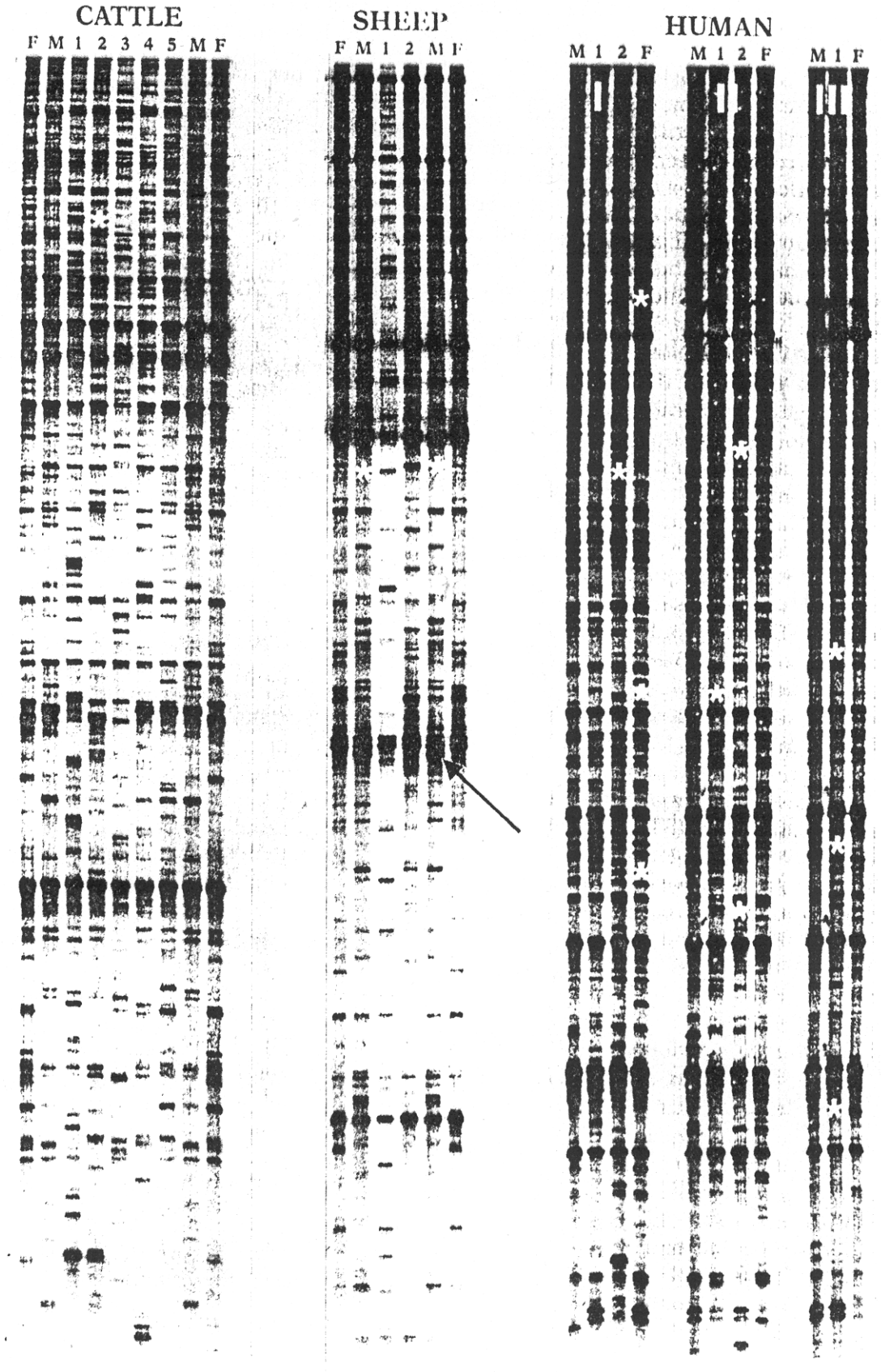


FIGURE 7 - Tomato ISTR analysis with *Lycopersicon pennellii* (lane 1) and *L. esculentum* (lanes 2-11) genotypes with primer combinations 1 (ISTR-F6/B2) and 2 (ISTR-F7/B1).
Lanes 1: *L. pennellii*; 2: FM6203 (processing variety); 3: P111B (processing variety); 4: A049 (advanced breeding line); 5: 1211 (parent in hybrid production); 6: var. Castlerock; 7: var. Master #2; 8: var. Estriella; 9: var. Frembgen Rheinlands Ruhm; 10: var. Vollendung; 11: var. Moneymaker



URE 8 - ISTR analysis of animal (primer pair ISTR-F5/B2) and human (primer pair ISTR-F6/B1) families. M: mother; F: ier; offsprings are numbered. Asterices indicate some of the individual-specific DNA fragments. The arrow points out a sible sex-specific marker in the sheep family.

shown) formed the matrix for the construction of the dendrogram depicted in Fig. 5. The barley cultivars are distinguished into two main groups with the previously mentioned cvs Prisma, Gitane, Gavotte, and Portikos clustering together as one subgroup. A second distinct cluster is formed by Bonus, Kristina, and the calcaroides mutants *Cal-C15, cal-b2* and *cal-b19*. This clustering reflects the pedigree for these genotypes: cv. Bonus has been the progenitor in a mutation programme from which the three *calcaroides* mutants as well as cv. Marie were derived. Marie together with the Norwegian cultivar Domen served as a parent in the generation of cv. Kristina. Such genetic relationships are also apparent for the four *H. spontaneum* populations which, with single exceptions, form four distinct clusters of genotypes (data not shown).

The genetically highly related genotypes Bonus, *Cal-C15, cal-b2* and *cal-b19* were then analyzed by several ISTR primer pairs in order to ascertain whether mutants raised in the same genetic background (Bonus) could be distinguished from the progenitor by ISTR fingerprinting. Seven different primer pairs were randomly chosen for ISTR analysis of the four genotypes. As shown in Fig. 6, primer pairs 1 to 3 and 7 show few differences in the PCR patterns, while primer pairs 4 to 6 detect a remarkably high degree of polymorphism. Thus ISTR can discriminate between highly related germplasm depending on the primer pair employed. One additional observation should be stressed: In the primer pairs 6 (ISTR-F7/B1) and 7 (ISTR-F8/B1) the primers ISTR-F7 and ISTR-F8 (not shown in Fig. 1B) are both derived from *copia*-like sequences in the genome of the PRD coconut type and exhibit the same orientation, but are located just 24 nucleotides apart on the *copia*-like sequence. Nevertheless, both primer pairs resulted in totally different fingerprints (Fig. 6, panels 6 and 7) suggesting that the primers do not prime on the *copia*-like subfamilies described earlier (ROHDE et al., 1995), but on short, widely dispersed *copia*-like sequences.

The potential of ISTR fingerprinting was demonstrated by another example. With tomato cultivars the currently available DNA marker technologies produce few, if any, polymorphic DNA fragments. Only about 7% of all RFLP markers in tomato will reveal polymorphisms among varieties (P. Jack, personal communication). Therefore, 10 tomato cultivars and one alien species were analyzed by two randomly chosen primer pairs. Primer combination 1 (ISTR-F6/B2) clearly distinguishes the alien species (*Lycopersicon pennellii*; Fig. 7, left panel, lane 1) from the tomato cultivars and produces an almost *Lycopersicon esculentum*-specific fingerprint for the 10 cultivars or parents in hybrid production (lanes 2-11) with few apparent polymorphisms. On the contrary, primer combination 2 (ISTR-F7/B1) produces an abundance of polymorphisms with only few homomorphic bands (Fig. 7, right panel). This example demonstrates that just two ISTR primer combinations may be sufficient in the characterization of a particular crop, one yielding a «species-specific» fingerprint and one differentiating between the different varieties or parents for hybrid production.

Examples for ISTR analysis in the animal kingdom and in human

Copia elements and *copia*-like sequences have been first discovered in *Drosophila melanogaster* (FINNEGAN, 1985), and they are apparently widely distributed in the animal kingdom and in human. On this basis, ISTR analysis was applied to selected human and animal families by the use of ISTR primers synthesized from coconut *copia*-like sequences (Fig. 1B). Two animal families (cattle and sheep) were selected, as in both cases the parents were known (*in vitro* fertilization). For humans, DNAs were available for the three families I, II and III: the two children of families I and II were homozygous twins.

The analysis of these families (Fig. 8) reveals that ISTR primers derived from plant sequences amplify animal and human DNA. Besides homomorphic bands, some polymorphic parental ISTR markers appear in the progeny. For example, in sheep a doublet is present in all four individuals, while the female contains an additional smaller fragment (Fig. 8, arrow) which is also present in the mother of a second sheep family (data not shown): All male animals only contain the double band. Similarly, the primer combination ISTR-F7/B2 (data not shown) detects a fragment only present in the three fathers, but not in the mothers nor in the five children: the children are in fact all daughters.

that these ISTR markers would invariably cosegregate with a specific sex phenotype. In addition to these segregating markers, some of the parental ISTR markers are not present in the offspring and vice versa as indicated for some of these markers by asterices in Fig. 8.

DISCUSSION

Here I describe a novel PCR-based DNA marker technique (ISTR) in the fingerprinting of eukaryotic genomes which on the basis of all the results available for the time being is universally applicable by using identical primers derived from coconut *copia*-like sequences (ROHDE *et al.*, 1992, 1995). This assumption is not only justified by the data presented in this paper, but is further supported by unpublished data on genotypes from populations of or breeding programmes with oil palm, plum trees, pine trees, wheat, potato, grapevine etc. where all determinations of genetic biodiversity by ISTR analyses have resulted in meaningful relationships backed up for example by the pedigree of varieties.

As the advantages and drawbacks of the most important molecular marker techniques like SSRP, RFLP, RAPD and AFLP have been discussed (see for example POWELL *et al.*, 1994), I will concentrate on a comparison of ISTR and AFLP, as these techniques make use of the identical analytical procedure (sequence gels). The large number of loci detected in a single ISTR analysis together with the high percentage of polymorphic fragments (Figs. 3-8) compares favourably with the recently developed AFLP technology. The advantages of ISTR over AFLP are at least fourfold: (i) After DNA isolation further manipulations are not necessary in the case of ISTR; (ii) the restriction of genomic DNA by two different restriction enzymes, the T4 DNA ligase-catalyzed addition of adapters, and the preamplification as part of the AFLP protocol are not only time-consuming, but at least in the case of the ligation reaction, which requires very pure DNA, technically difficult; (iii) the addition of single terminal nucleotides to the AFLP adapter/primers for discriminatory PCR amplification calls for a robust and highly accurate PCR equipment, if reproducible results should be obtained and (iv) the three additional steps (restriction, ligation, preamplification) are much more expensive than ISTR.

We have recently completed a comparison of AFLP and ISTR with respect to their applicability to the characterization of 19 Italian grapevine accessions (SENSI *et al.*, 1996). Both techniques resulted in almost identical results on the genetic biodiversity among the examined genotypes, with ISTR detecting more genetic diversity within the various accessions for one of the grapevine cultivars (var. Sangiovese). Thus, like other DNA marker technologies, ISTR can serve for practical application. Although more intensive studies will be needed, current experience also suggests that the reproducibility of the ISTR technique is high: eight individual DNA preparations (four with identical material, the other half with different leaf material) from the same coconut tree resulted in identical ISTR patterns, although the material was handled at ambient temperatures for 2-3 weeks before storage at - 20°C. The fact that DIG-labelled ISTR primers can substitute for radioactive labelling (Fig. 3) allows for its widespread use with a minimum of technical equipment and manipulation and without restriction by the availability of radioactivity or the presence of radioisotope laboratories. Current efforts are directed towards the use of fluorescence-labelled ISTR primers for the establishment of large scale analyses.

The practical applications of ISTR are apparently manyfold and would range (in the plant field) from the general determination of biodiversity (Figs. 2-7), the characterization of wild-growing species (Fig. 4), genebank management (Fig. 4), studies on population genetics (Fig. 4), fingerprinting of varieties for identification (Fig. 4, 7), following the introgression of genes (Fig. 4), systematic studies (Fig. 3) to possibly marker-assisted selection in breeding by the identification of markers cosegregating with desirable traits. The establishment of an ISTR molecular map for barley (*Hordeum vulgare* L.) is under way. Preliminary results from the ISTR analysis of doubled haploid barley mapping populations show that the ISTR markers segregate and are distributed across the seven chromosomes. Important conclusions may also be drawn from the ISTR analysis of wild-growing plants. Analyses of Israelian *H. spontaneum* populations like the one depicted in Fig. 4 (right panel) indicate a clear division between xeric (steppic and desert)

and mesic (Mediterranean mountainous) populations (E. Nevo, personal communication). Furthermore, within such populations occasionally individual genotypes can be differentiated by their different fingerprint pattern. This has important consequences as to the selection of specific genotypes from wild-growing populations for germplasm conservation (ENGELS, 1995).

The possible applications in the animal and human field remain to be determined. The fact that ISTR detects differences between homozygous twins in human families (Fig. 8) is not surprising, as the DNA was isolated from blood samples, and white blood cells undergo DNA rearrangements during differentiation (METCALF, 1994). In addition, genome instability as reflected by microsatellite expansion is associated with several human diseases (reviewed by ASHLEY and WARREN, 1995) including epithelial tumours (DUTRILLAUX, 1995). This finding of instability with repetitive sequences like microsatellites corroborates results from the plant field (unpublished) which clearly demonstrate that DNA samples from different tissues and organs of the identical plant (tobacco) will result in differences in the ISTR patterns when specific ISTR primer combinations are used. Similarly, ISTR patterns of plants (potato) generated from the identical genotype via tissue culture vary as well (unpublished). This observation is in agreement with the well-established fact that tissue culture stress can give rise to karyotypic changes, chromosomal changes (mutations, translocations, deletions, inversions, gene amplification etc.), differences in methylation patterns and the activation of transposable elements (for reviews see KUMAR, 1994; PESCHKE and PHILLIPS, 1992). Further investigations on the application of the ISTR technology will focus on the identification of ISTR primer combinations that will yield (i) a stable species- or crop-specific fingerprint (Fig. 7, left panel), (ii) a stable genotype-specific fingerprint (possibly Fig. 7, right panel) and (iii) fingerprints which reflect processes of differentiation within an indidual plant. While for practical application group (i) and (ii) primer combinations will be of greatest use, group (iii) primer combinations will open a novel route to the study of molecular processes involved in differentiation, not by relating to expression regulation, but by studying the fluidity of the plant genome.

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