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Linkage Analysis by Two-Dimensional DNA Typing

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Summary

In two-dimensional (2-D) DNA typing, genomic DNA fragments are separated, first according to size by electrophoresis in a neutral polyacrylamide gel and second according to sequence by denaturing gradient gel electrophoresis, followed by hybridization analysis using micro- and minisatellite core probes. The 2-D DNA typing method generates a large amount of information on polymorphic loci per gel. Here we demonstrate the potential usefulness of 2-D DNA typing in an empirical linkage study on the red factor in cattle, and we show an example of the 2-D DNA typing analysis of a human pedigree. The power efficiency of 2-D DNA typing in general is compared with that of single-locus typing by simulation. The results indicate that, although 2-D DNA typing is very efficient in generating data on polymorphic loci, its power to detect linkage is lower than single-locus typing, because it is not obvious whether a spot represents the presence of one or two alleles. It is possible to compensate for this lower informativeness by increasing the sample size. Genome scanning by 2-D DNA typing has the potential to be more efficient than current genotyping methods in scoring polymorphic loci. Hence, it could become a method of choice in mapping genetic traits in humans and animals.

Introduction

In recent years a number of genes have been isolated from humans and other higher organisms by positional cloning. Positional cloning employs a DNA-based map of polymorphic markers to localize genes that are only known by their phenotype; knowing the position of such genes allows their ultimate isolation by walking from adjacent markers (e.g., see Rommens et al. 1989). Thus far, positional cloning efforts have been based on the serial analysis of large numbers of (polymorphic) marker loci. Both the discovery of highly polymorphic VNTR-type markers and technical improvements, such as PCR and multiplexing, as well as the use of robotics and other methods of scale, have considerably compressed the time period necessary to successfully complete a positional cloning project (Todd 1992). Nevertheless, the amount of work is still substantial, especially when very large numbers of markers must be screened, i.e., in studying complex traits by linkage and/or association methods (Clerget-Darpoux and Bonaïti-Pellié 1992; Todd 1992).

Two-dimensional (2-D) DNA typing represents a parallel approach to positional cloning, in that hundreds of informative marker loci are analyzed simultaneously in a single separation pattern (Uitterlinden et al. 1989). 2-D DNA typing is based on the electrophoretic resolution of genomic restriction-enzyme digests, first on the basis of size in native polyacrylamide gels and then according to sequence in denaturing gradient gels (Fischer and Lerman 1979). Subsequent rounds of hybridization with micro- and minisatellite core probes allows the rapid screening of hundreds of polymorphic VNTR loci in a short period of time. It was demonstrated that excellent separation patterns could be obtained when G+C-rich core probes are used, allowing the target sequence to act as a stable GC-clamp (Uitterlinden and Vijg 1991). Recently, a routine protocol for the method was developed for producing standardized separation patterns to allow intergel comparison (Uitterlinden and Vijg 1993).

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Each spot in a 2-D DNA typing pattern represents a single or double copy of a specific allele or represents several unrelated alleles which fortuitously comigrate in the 2-D gel. In comparison, standard RFLPs will in most cases reveal information about all specific alleles at a certain locus, except when alleles comigrate with a constant band. 2-D DNA typing data behave as phenotypic observations on a dominant gene: the presence of a spot does not reveal whether heterozygosity or homozygosity exists for the presence of an allele. Linkage analysis of 2-D DNA typing data by computing pedigree likelihoods would require that single unknown alleles can be specified. Nevertheless, 2-D DNA typing patterns contain genetic information: for example, when two parents have, at a specific position in the 2-D gel, a spot which is absent in a child, it can be inferred that the parents are identical in state for one allele. Compared with a fully informative RFLP marker, less information is available, but it is presumed to be enough to be useful for linkage analysis. The amount of genetic information can be expressed in an expected lod score which can be compared for two fully informative markers and for a fully informative marker against a semi-informative 2-D marker.

Here we present some empirical data and simulation data elucidating the practical informativity of 2-D DNA typing for genetic mapping studies. The major question addressed in this study is, How efficient is the mapping of genetic traits by means of 2-D DNA markers, compared with fully informative locus-specific markers? This study was performed on empirical data of the red/black factor in cattle, which in the chosen design is inherited as a dominant trait. The methods used for power calculations were (*a*) simulation with markers, given the observed segregation of the red/black trait, and (*b*) analytic derivation of the influence of allele frequency on expected lod scores. Examples presented on the segregation of spot variants in Centre d'Étude du Polymorphisme Humain (CEPH) pedigrees illustrate that, with respect to power, the results from this model study are equally relevant for applications in human pedigrees with dominant diseases.

Material and Methods

2-D DNA Typing

2-D DNA typing of humans and cattle was performed essentially as described elsewhere (Uitterlinden and Vijg 1993). High-molecular-weight genomic DNA was isolated from white blood cells according to standard procedures and was digested with *Hae*III restriction enzyme according to the manufacturer's (BRL, USA) recommendations. Separations of 10 μg of restriction enzyme–digested genomic DNA were performed in polyacrylamide (PAA) gels (acrylamide:bisacrylamide = 37:1) by using a gel apparatus originally described by Fisher and Lerman (1979). The first dimension was run in a 0.75-mm-thick neutral 6% gel at 50°C for 3 h at 200 V in 0.5 × TAE (1 × TAE = 40 mM Tris-HAC pH 7.4, 20 mM NaAc, 1 mM Na_2EDTA). The separation patterns were visualized by staining the gel with ethidium bromide (0.1 μg/ml) for 30 min, followed by destaining for at least 10 min. From a given lane, the 0.4–4-kb region was used for 2-D separation. Lanes were cut out of the 1-D gel and were applied to a 1-mm-thick 6% PAA gel containing a 10%–75% linear concentration gradient of denaturant (100% denaturant = 7.0 M urea, 40% formamide) parallel to the direction of electrophoresis. Gels were poured by mixing two solutions, containing the desired boundary denaturant concentrations, into a gradient maker (Pharmacia) with a peristaltic pump (BioRad). Electrophoresis at 200 V (12 V/cm) was performed for 13.5 h at 60°C. After 2-D electrophoresis, the DNA fragments in the separation patterns were first fragmented by irradiating the gel with 302-nm UV light (UVP transilluminator) for 4 min. Transfer to a nylon membrane (Hybond N^+; Amersham) was achieved by semidry electroblotting at 400 mA (6–28 V), between a horizontal ceramic (anode) and stainless steel (cathode) plate with the cathode on top. Electroblotting was performed for 1.5 h, between 6 Whatman 3 MM paper sheets which were soaked in 0.5 × TBE (1 × TBE = 89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA). For denaturation of membrane-bound DNA fragments, filters were posttreated twice, for 2 h each time, by incubation in 0.6 N NaOH, 1.5 M NaCl and subsequently were neutralized in 2.5 × SSC (1 × SSC = 150 mM NaCl, 15 mM Na citrate). The filter was air-dried and irradiated for 1 min with 302-nm UV light to cross-link the DNA fragments to the filter. Hybridization analysis with micro- and minisatellite core probes was as described elsewhere (Uitterlinden and Vijg 1993).

Interpretation of Spot Patterns

2-D DNA typing patterns were compared by eye, independently by two investigators, to detect any differences between individual samples. Each sample was analyzed at least twice. For comparing spot patterns, use is made of a mixture of marker fragments (consisting of lambda DNA fragments generated with different restriction enzymes) added to each genomic digest. In

addition, the patterns are compared using spots, detected by the micro- or minisatellite core probe, which occur in all individuals (constant spots). The reproducibility of the method is discussed extensively elsewhere (Uitterlinden and Vijg 1993). In general, it appears that the position of spots varies about 2 mm in x and y direction and that 1 of 100 spots cannot be reproduced.

Lod-Score Analysis

A series of programs have been developed for general pedigree calculations, with the specific objective of improving adaptability of genetic analysis programs to different genetic models and analysis of complex inbred pedigrees (te Meerman 1991; te Meerman, submitted). One version (GRONLOD) is suitable for linkage analysis and duplicates the functions of the LINKAGE group of programs (Lathrop and Lalouel 1984). For general aspects of power analysis for linkage studies, we refer to Ott (1991). In LINKAGE, 2-D DNA data can be analyzed as data on a dominant gene. For the present analysis, using the GRONLOD programs had the advantage that a close tie exists between the versions for lod-score and expected-lod-score analysis. In GRONLOD, single unknown allelic phenotypes are permitted, and it can analyze a marker's lod score against itself (auto lod score). This auto lod score is always computed for zero recombination frequency, since a marker always cosegregates with itself without recombinants. The higher the auto lod score is, the more informative a marker will be. We use computing auto lod scores to analyze the informativity of a marker in a given pedigree, as compared with the PIC value, which is useful for estimating a marker's informativity in general. Another particular advantage of GRONLOD is that recoding alleles (as is typically necessary for VNTRs when different pedigrees are added) is not required for computing even complicated pedigrees. GRONLOD also has specific options, not present in LINKAGE, for analyzing imprinting. Specific additions in GRONLOD made it possible to study, by simulation, how 2-D DNA typing markers performed in linkage analysis with codominant RFLP markers.

Results

Linkage Analysis by 2-D DNA Typing of Cattle

Empirical data.—An empirical data set relates to a linkage study of 2-D markers with the red/black factor in cattle. This study will be discussed in more detail elsewhere (E. Mullaart, unpublished data), but some linkage results will be used here as a model. The red/black factor is recessive for the red color and was analyzed by crossing a black sire, who was heterozygous for the color factor, with red dams. A pedigree was used with one sire and 12 dams having 16 offspring (fig. 1). The pedigree is informative for linkage because the design makes it possible to follow the male meiosis for 16 offspring. To preselect for spots potentially linked to the black factor, we performed 2-D DNA typing of pools of DNA derived from either red or black sibs in the pedigree. Spots found only in the black pool are candidate markers potentially linked to the red/black locus. To confirm results of the pool experiment, segregation of candidate spots was followed in the individual sibs. An example of the segregation of (CAC) spot 3 in some members of the pedigree is shown in figure 3. Figure 2 shows the 2-D DNA typing patterns for the black and red pools. Candidate markers (2–6) were found only with microsatellite core probe $(CAC)_n$; for two other core probes, minisatellite core probes INS and 33.15, no linked markers were found. Genetic analysis of the segregation in the cattle pedigree for $(CAC)_n$ spot 3 (fig. 1) shows that the sire is evidently heterozygous for the red allele and for this spot. Dam Truus-6, for example, is heterozygous for this spot, and the "+" allele is in phase with the black allele (alternatively, a recombinant in the sire may have occurred). In the offspring $(CAC)_n$ spot 3 (see fig. 1) segregates with the red/black phenotype, such that this spot is in phase with the "black" factor. This explains the excess of this allele in the black pool and the absence in both the red pool and the individual animals from the pools. No recombinant is required for explanation of the segregation of the marker.

Two-point lod scores were computed between the red/black phenotype and the four selected 2-D spots detected with core probe $(CAC)_n$ (table 1). When one meiosis is used for phase determination, in this pedigree a maximum lod score of 15 log(2) = 4.5 is possible for a fully informative marker. The auto lod score of the red locus has such a lod score. The matrix of two-point lod scores between each of the candidate spots and the red/black factor, including auto lod scores and recombination fractions (θ's) required to obtain the maximum lod score, is shown in table 1. On the diagonal the auto lod scores are shown at $\theta = 0$. The entries below the diagonal are the maximum lod scores; that is, column 1 contains lod scores of each of the candidate spots with the red/black factor. The entries above the diagonal are the θ's required for obtaining the maximum lod score; that is, row 1 shows all θ's for the red/black factor that are required for obtaining a maxi-

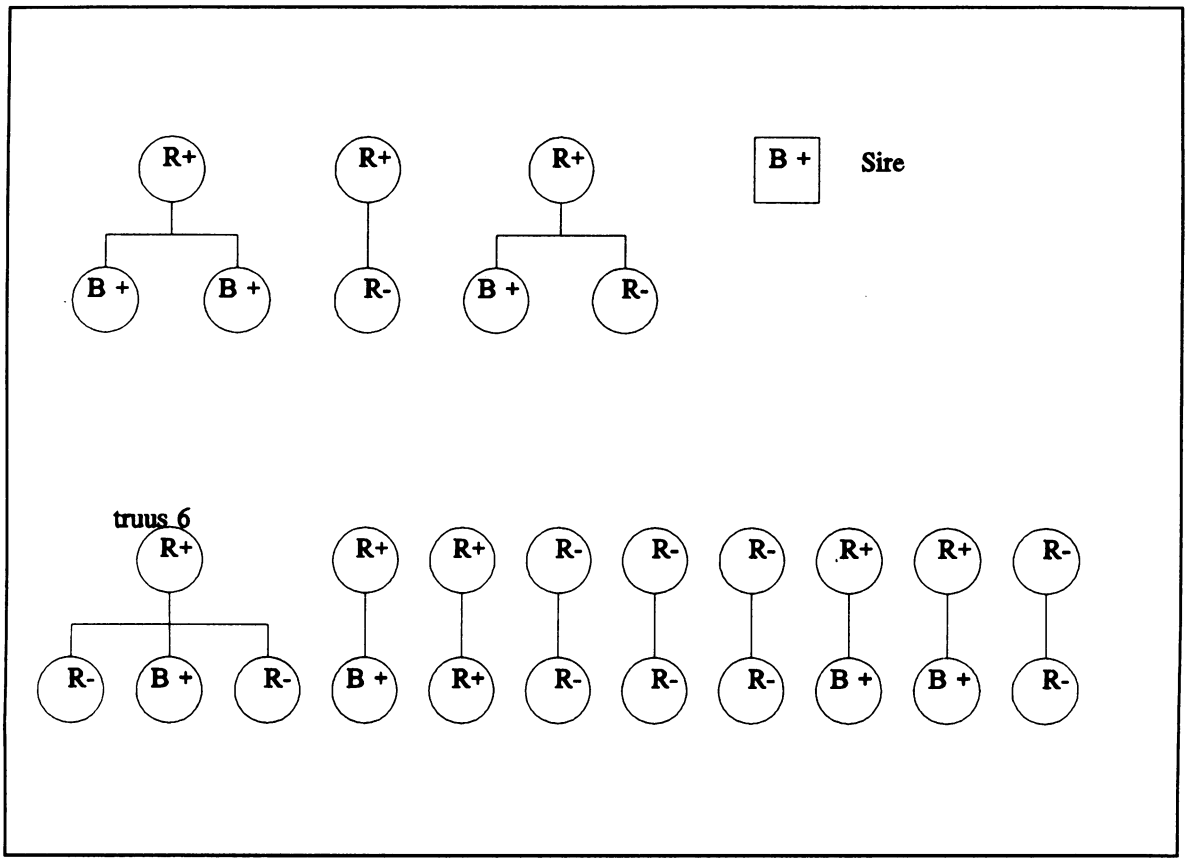

Figure 1 Cattle pedigree, consisting of 1 sire, 12 dams, and 16 offspring, which was used to identify cosegregation of 2-D DNA typing spots with the red/black phenotype. All dams are red, and the sire is black. The segregation of the best-linked marker (i.e., $(CAC)_n$ spot 3) is indicated. B = black phenotype; R = red phenotype; + = presence of spot; and − = absence of the spot.

mum lod score with any one of the candidate spots. It appears that the highest lod score (2.64) is obtained with $(CAC)_n$ spot 3 (segregation is shown in fig. 1) at $\theta = 0$. The second highest maximum lod score (0.95 for $(CAC)_n$ spot 2) was obtained at a $\theta = .15$. All recombinants are observed in the father; this is not surprising, because the mothers are all homozygous red and because only those with at least two offspring could theoretically show recombinants between marker alleles.

Simulation.—A simulation study was performed with 400 replicates per condition to evaluate the sensitivity of the red-factor linkage study to variations in allele frequency and genetic distance of the marker expressed by the θ value between a given 2-D spot variant and the trait. This means that 16 meioses were randomly generated in 400 pedigrees by using allele frequencies of .3, .5, and .7 and θ's of 0, .05, .15, and .30. The pedigree structure and the phenotype information were taken from the empirical example described under "Empirical data." For this purpose, simulation with a locus-specific two-allele RFLP was compared with simulation with a 2-D spot variant. Markers were randomly assigned to both Orkan (the father of all calves) and the mothers, according to three allele frequencies, at varying θ's and under the assumption of linkage equilibrium. For the offspring, random segregation of parental alleles was performed.

The results for a two-allele locus-specific RFLP marker are shown in table 2. The median of the observed lod score is in all cases 0 (data not shown), indicating that most of the simulation conditions in the pedigree are not informative, because the marker alleles in the sire do not segregate in an informative way. As expected, the maximum number of informative pedigrees and the highest mean lod score are found for a marker frequency of .5.

For a presumed 2-D DNA typing marker, the effect

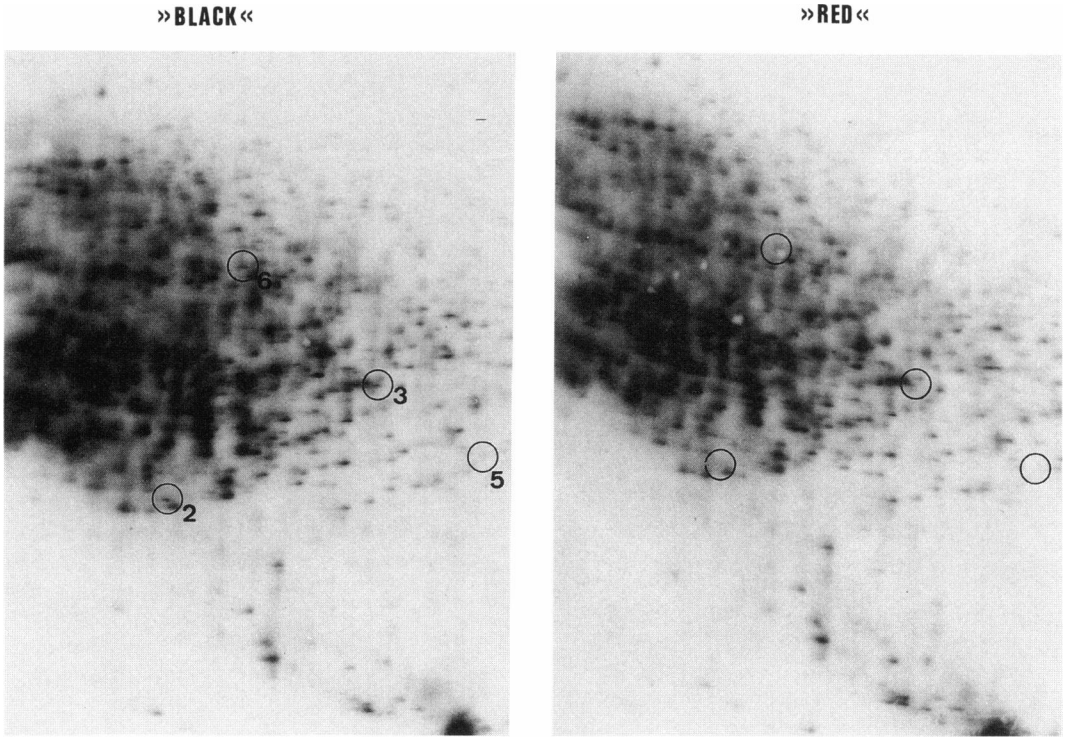

Figure 2 2-D DNA typing patterns obtained with microsatellite core probe $(CAC)_n$ of the black pool and the red pool. Candidate 2-D DNA typing markers (i.e., those present in the black pool but not in the red pool) are indicated. Of these, spot 3 had the highest lod score.

of allele frequency variations of the spot-giving allele is not symmetric around .5. A higher spot frequency will be less informative because the chance of being homozygous for the spot will be higher, and, thus, informative segregation for the paternal alleles will be masked more often by maternal alleles. The mean and the median of the observed lod score (always 0; data not shown) reflect that most of the simulation conditions in the pedigree are not informative, leading to results where the lod score is either rather high or 0 when the sire is not informative. When tables 2 and 3 are compared, it can be seen that for 2-D DNA typing, on average, two times as many individuals in a pedigree should be analyzed, in comparison to a two-allele marker. The optimal allele frequency for 2-D analysis in this respect is closer to 30% for spot-giving alleles than to the 50% seen for two-allele RFLPs. This asymmetry is caused by the fact that absence of a spot is more informative than the presence of a spot, because the presence can only indirectly be interpreted as a single or double allele. This is numerically explained in table 4, where the probability of an informative meiosis is calculated for different allele frequencies.

Applications in humans: CEPH pedigree analysis.— To illustrate the applicability of 2-D DNA typing for performing linkage analysis in human pedigrees, we analyzed 2-D DNA typing patterns obtained with core probe 33.6 from CEPH pedigree 1377. Figure 4 shows an example of a segregating spot variant and a constant spot observed in this pedigree. About 80% of 264 spots observed in this and other pedigrees are spot variants, segregation patterns of which have been analyzed. The results of this study will be described elsewhere (E. Mullaart, unpublished data).

Discussion

The results presented on the genetic informativity of 2-D DNA typing data demonstrate that this system can be used for performing genetic linkage analysis. This is illustrated by the genetic linkage which we observed for a 2-D spot detected with core probe $(CAC)_n$ and the red/black phenotype in a small cattle pedigree with only 16 male meioses which were analyzed. The high a priori probability against linkage and the fact that multiple testing was performed make independent

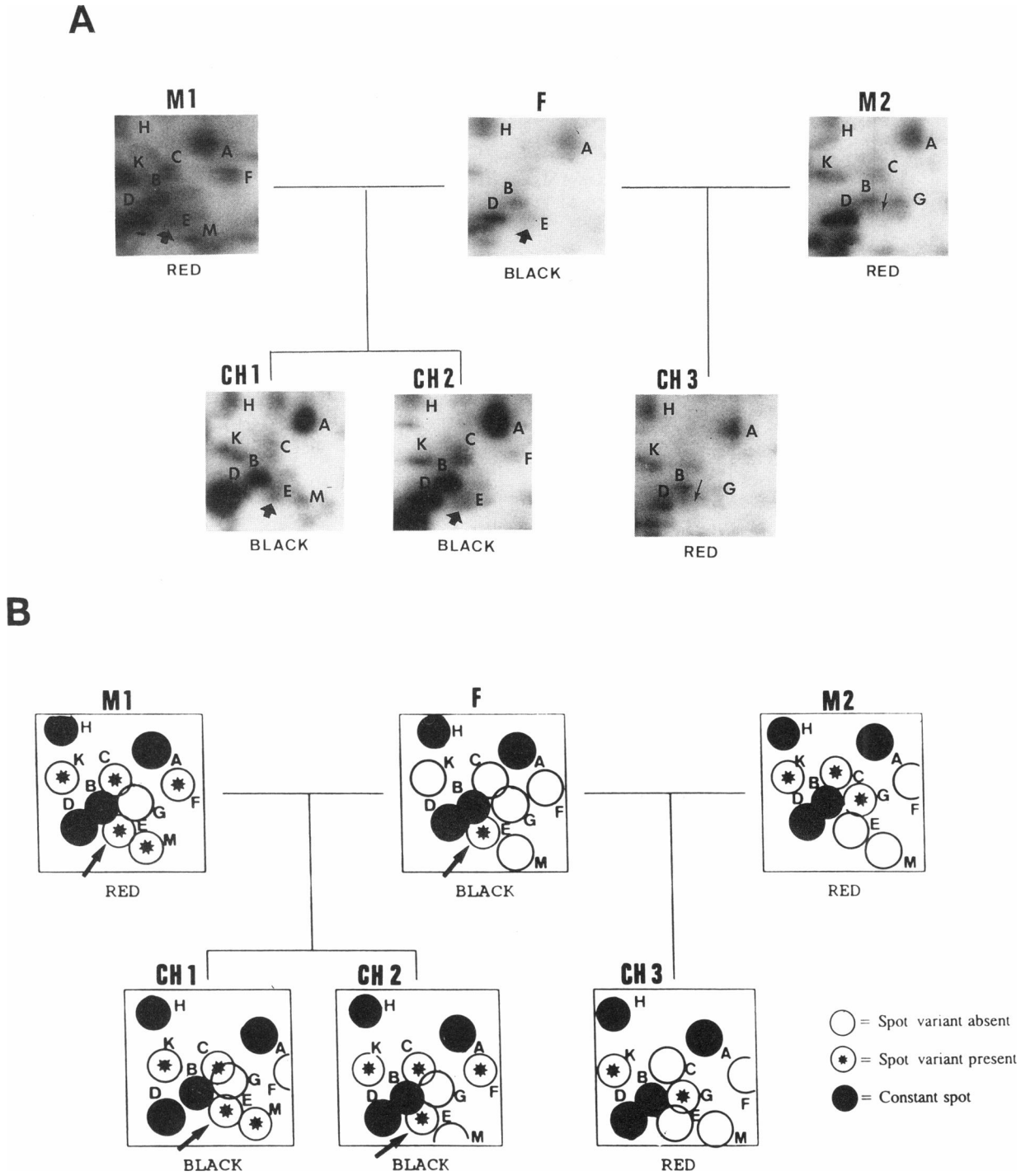

Figure 3 *A*, Details of corresponding areas in 2-D DNA typing patterns obtained with probe $(CAC)_n$ of some sibs of the pedigree shown in fig. 1. Spot 3 is indicated by an arrow; a thick arrow indicates presence of the spot, whereas a thin arrow indicates absence of the spot. F = father; M = mother; and CH = child. The phenotype is indicated at the bottom. *B*, Schematic representation of the area shown in *A*. The arrow indicates presence of spot 3.

Table 1

θ's at Maximum Lod Scores (above the Diagonal), Auto Lod Scores (on the Diagonal), and Maximum Two-Point Lod Scores (below the Diagonal) between the Red/Black Phenotype (R/B) and Four Candidate 2-D Spots Detected with $(CAC)_n$

	R/B	2	3	5	6
R/B	4.5	.15	.0	.45	.2
2	.95	3.35	.0	.0	.2
3	2.64	1.48	2.70	.5	.2
5	.0	.14	.0	.99	.5
6	.59	.30	.20	.0	2.75

Table 3

Lod Score Means and SDs for 400 Simulation Replicates per Condition, for Variations in p and q[a] and in Θ, between a Simulated 2-D DNA Marker Spot and the Observed Trait

	MEAN LOD SCORE (SD) AT Θ =			
q/p	0	.05	.15	.30
.7/.3	.265 (.501)	.196 (.425)	.077 (.301)	.013 (.097)
.5/.5	.760 (.919)	.561 (.825)	.283 (.550)	.017 (.186)
.3/.7	.086 (1.396)	.767 (1.162)	.341 (.691)	.053 (.256)

[a] q is the frequency of the spot-giving allele.

confirmation of the linkage observed necessary. Such final proof can be obtained by analyzing more pedigrees and/or by developing a locus-specific marker for the 2-D spot variant of interest and analyzing the resulting marker on this and other pedigrees. The 2-D DNA typing system can also be used to perform linkage analysis in human pedigrees, as is illustrated by the example, which we provide, of a segregating spot variant in a CEPH pedigree.

The simulation results for the red/black pedigree show that, for the 2-D DNA typing method, the same power to detect linkage can be obtained by using slightly more than twice as many subjects as are used with fully informative locus-specific markers. The relation between the expected lod score and the θ shows that, although a noninformative result is most likely (median = 0), a lod score as high as the one found can be obtained in a sizable proportion of pedigrees.

In comparison with putatively autosomal spots, X-linked spots will be more informative because in that case all sons will be informative when the mother is heterozygous, and, if the father does not have the spot, all daughters will also be informative. For example, when a spot frequency of 30% is assumed, the informativity of putatively X-linked spots will be better than 75%, in comparison with codominant markers (for which all daughters will be informative).

When 2-D DNA typing is compared with locus-specific markers, such as RFLPs and $(GT)_n$ markers, for performing genetic linkage studies the informativity per spot in a 2-D DNA typing pattern is lower. This is due to the lack of information on the complementary allele in the individual analyzed. To bring 2-D DNA typing to the same level of informativity as is given in single-locus typing, two possibilities can be envisaged. First, spots of interest can be directly isolated from the 2-D DNA typing pattern and can be developed into locus-specific markers. The use of such markers as probes onto 2-D DNA typing blots will subsequently reveal the position of complementary alleles. In principle, a particular spot variant on a 2-D gel may have a complementary spot

Table 2

Lod-Score Means and SDs for 400 Simulation Replicates per Condition, for Variations in Allele Frequency (p and q[a]) and Θ, between a Simulated Two-Allele Marker and the Black/Red Phenotype in the Pedigree Shown in Figure 1

	MEAN LOD SCORE (SD) AT Θ =			
q/p	0	.05	.15	.30
.7/.3	1.294 (1.700)	.959 (1.361)	.419 (.808)	.108 (.354)
.5/.5	1.614 (1.704)	1.116 (1.329)	.594 (.856)	.084 (.329)
.3/.7	1.410 (1.759)	1.014 (1.418)	.459 (.827)	.098 (.335)

[a] $q = (1-p)$.

Table 4

Proportion of Informative Meioses in 2-D DNA Typing, Depending on q[a]

		FREQUENCY FOR q/p =		
	FORMULA	.7/.3	.5/.5	.3/.7
Informative sires	$2pq$ (=a)	.42	.5	.42
Dams with absent spot	p^2 (=b)	.09	.25	.49
Informative dams with spot ..	$.5 \times 2pq$ (=c)	.21	.25	.21
Proportion of informative meioses	$a(b + c)$	.126	.25	.294

[a] q is the frequency of the spot-giving allele.

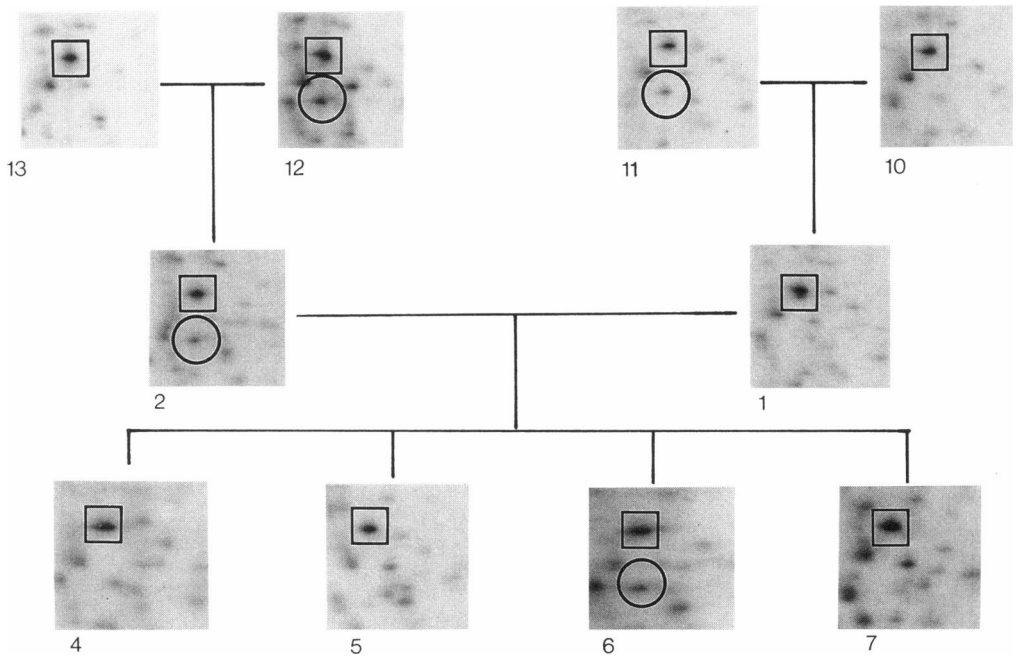

Figure 4 Example of the segregation of a spot variant detected with core probe 33.6 in CEPH pedigree 1377. Each square indicates a constant spot, and each circle indicates the presence of a segregating spot variant. Pedigree members are as follows: 10–13 = grandparents; 1 and 2 = parents; and 4–7 = children.

with the same first-melting domain, but with a different base-pair length. It has been shown elsewhere (Uitterlinden and Vijg 1991; Hovig et al. 1993) that VNTR alleles from the same locus show such electrophoretic behavior in DGGE gels, except when one or more VNTR repeat units constituting the allele contain a recognition site of the restriction enzyme used to digest the genomic DNA.

Second, segregation analysis of 2-D DNA typing spots and available codominant markers (e.g., from CEPH) in large pedigrees will allow the construction of genetic maps. These approaches will result in a database of genetic 2-D DNA typing markers as spots defined by (1) restriction enzyme, (2) length, (3) melting temperature, and (4) core probe. A multilaboratory collaborative research project toward that aim is currently in progress.

2-D DNA typing can be applied in linkage analysis of genetic diseases when enough informative meioses are available, as is the case for many currently studied monogenic disorders in humans. In light of the high density of polymorphic marker loci which can be detected by 2-D analysis, the method has potential to be an efficient tool for (*a*) detecting linkage, especially in species for which no genetic maps are available, (*b*) filling in gaps in existing maps, and (*c*) association studies where close linkage between marker loci and genes is required. The latter application can be compromised by the relatively high mutation rate observed for some VNTR loci (especially those with large alleles) (Jeffreys et al. 1988). However, such elevated mutation rates have not been observed for the relatively small alleles analyzed in 2-D DNA typing (E. Mullaart, unpublished data).

Software Availability

The programs able to handle data with single unknown loci as found in 2-D DNA typing data, as well as other types of linkage problems, with examples and user documentation on diskette (3.5 inch only), are available free of charge from the first (corresponding) author and can be requested by fax, mail, or E-mail (G.J.TE.MEERMAN@MED.RUG.NL). Documented sources are available in conjunction with te Meerman (1991).

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2-D markers can be analyzed using LINKAGE. We thank Dr. Howard Cann (CEPH, Paris) for providing us with DNAs. We would like to express our appreciation for the comments from the reviewers. 2-D DNA typing is covered by U.S. patent 5,068,176. Inquiries concerning commercial applications should be directed to the senior author of this paper at INGENY B.V.

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