

Oligonucleotide Fingerprinting Using Simple Repeat Motifs: A Convenient, Ubiquitously Applicable Method to Detect Hypervariability for Multiple Purposes

J. T. Epplen^a, H. Ammer^a, C. Epplen^a, C. Kammerbauer^a, R. Mitreiter^a, L. Roewer^a, W. Schwaiger^a, V. Steimle^a, H. Zischler^a, E. Albert^b, A. Andreas^b, B. Beyermann^c, W. Meyer^c, J. Buitkamp^d, I. Nanda^e, M. Schmid^e, P. Nürnberg^f, S. D. J. Pena^g, H. Pöche^h, W. Sprecherⁱ, M. Schartl^j, K. Weising^k and A. Yassouridis^l

^aMax-Planck-Institute for Psychiatry, W-8033 Martinsried, Germany; ^bKinderpoliklinik University, W-8000 München, Germany; ^cBiology Section Humboldt University, O-1040 Berlin, Germany; ^dInstitute for Animal Husbandry and Breeding, Hohenheim University, W-7000 Stuttgart, Germany; ^eHuman Genetics Institute University, W-8700 Würzburg, Germany; ^fMedical Genetics Institute Humboldt University (Charité), O-1040 Berlin, Germany; ^gBiochemistry Department, Federal University Minas Gerais, 30161 Belo Horizonte, Brazil; ^hInstitute for Legal Medicine Free University, W-1000 Berlin, Germany; ⁱInstitute for Legal Medicine University, W-3400 Göttingen, Germany; ^jGene Center, W-8033 Martinsried, FRG; ^kPlant Molecular Biology Group, Institute for Botany University, W-6000 Frankfurt, Germany; ^lMax-Planck-Institute for Psychiatry, W-8000 Munich, Germany

Summary

A panel of simple repetitive oligonucleotide probes has been designed and tested for multilocus DNA fingerprinting in some 200 fungal, plant and animal species as well as man. To date at least one of the probes has been found to be informative in each species. The human genome, however, has been the major target of many fingerprinting studies. Using the probe (CAC)₅ or (GTG)₅, individualization of all humans is possible except for monozygotic twins. Paternity analyses are now performed on a routine basis by the use of multilocus fingerprints, including also cases of deficiency, i.e. where one of the parents is not available for analysis. In forensic science stain analysis is feasible in all tissue remains containing nucleated cells. Depending on the degree of DNA degradation a variety of oligonucleotides are informative, and they have been proven useful in actual case work. Advantages in comparison to other methods including enzymatic DNA amplification techniques (PCR) are evident. Fingerprint patterns of tumors may be changed due to the gain or loss of chromosomes and/or intrachromosomal deletion and amplification events. Locus-specific probes were isolated from the human (CAC)₅/(GTG)₅ fingerprint with a varying degree of informativeness (monomorphic versus truly hypervariable markers). The feasibility of three different approaches for the isolation of hypervariable mono-locus probes was evaluated. Finally, one particular mixed simple (gt)_n(ga)_m repeat locus in the second intron of the HLA-DRB genes has been scrutinized to allow comparison of the extent of exon-encoded (protein-) polymorphisms versus intronic hypervariability of simple repeats: adjacent to a single gene sequence (e.g. HLA-DRB1*0401) many different length alleles were found. Group-specific structures of basic repeats were identified within the evolutionarily related DRB alleles. As a further application it is suggested here that due to the ubiquitous interspersions of their targets, short probes for simple repeat sequences are especially useful tools for ordering genomic cosmid, yeast artificial chromosome and phage banks.

Oligonucleotides Specific for Simple Repeat Motifs are Informative for all Species Tested So Far in the Fungal, Plant and Animal Kingdoms

The existence of repetitive DNA sequences in eukaryotic genomes has been known for more than two decades (Britten and Kohne, 1968). Even after all these years of intense experimentation and scholastic reflection the *raison d'être* of most repetitive elements in the eukaryotic genomes remains elusive. Particularly with respect to the generation and/or maintenance of the class of tandemly organized simple repeat motifs, a coherent or thoroughly convincing concept is lacking. This failure is certainly also due to the fact that these elements exert in almost all cases no sequence-dependent function whatsoever (for a discussion see Epplen, 1988). Bearing this primary shortcoming in mind, simple sequences are evaluated here exclusively as a tool.

In hindsight the design and testing of simple repetitive oligonucleotide probes for DNA profiling could and should have been initiated as early as 1981/82. By then it was already clear that *cloned* simple repetitive (gata)_n and (gaca)_m sequences showed extensive restriction fragment length polymorphisms (RFLP's), e.g. among human individuals (Epplen *et al.*, 1982). Meanwhile Alec Jeffreys and coworkers (1985a, b) have described the "minisatellites" and have established the whole concept of DNA profiling. Finally, in 1986 Ali *et al.* reported on initial successful efforts to differentiate human genomes using (GATA)₄ and (GACA)₄ as well as other sequentially related simple quadruplet repeat (*sqr*) oligonucleotides. Additional probes harboring basic repeat motifs of two to six nucleotides were subsequently synthesized and hybridized to DNAs from a variety of sources (Arnemann *et al.*, 1989; Beyermann *et al.*, in press; Buitkamp *et al.*, 1990, 1991; Epplen, 1988; Epplen *et al.*, 1988, 1989; Nanda *et al.*, 1988, 1990a, 1988b, in press; Vogel *et al.*, 1988; Weising *et al.*, 1989, 1990, 1991 (Fig. 1)).

The mere presence and quantity of these simple tandem repeats in the genome can be assessed by a convenient slot-blot hybridization method (Epplen, 1988). This, however, does not in itself prove the suitability of a given probe for DNA fingerprinting. The latter has to be investigated with restriction enzyme digested and electrophoretically separated DNA of the respective species. Even though it is best to start out on the basis of previous experiences using, for example (GGAT)₄ for individualization in a novel fish species, the principle of trial and error has to be adopted. Table 1 lists the panel of oligonucleotide probes that have been chemically synthesized for DNA profiling. In common to all the probes are simple repeat motifs of 2–6 bases. The total lengths range from 15 to 24 bases thus allowing the calculation of hybridization and stringent wash temperatures according to the formula T_m (°C) = [(A + T) × 2 + (C + G) × 4] – 5 (Miyada *et al.*, 1985). This rule applies for a salt concentration of 1M NaCl. In practice, all hybridization and washing

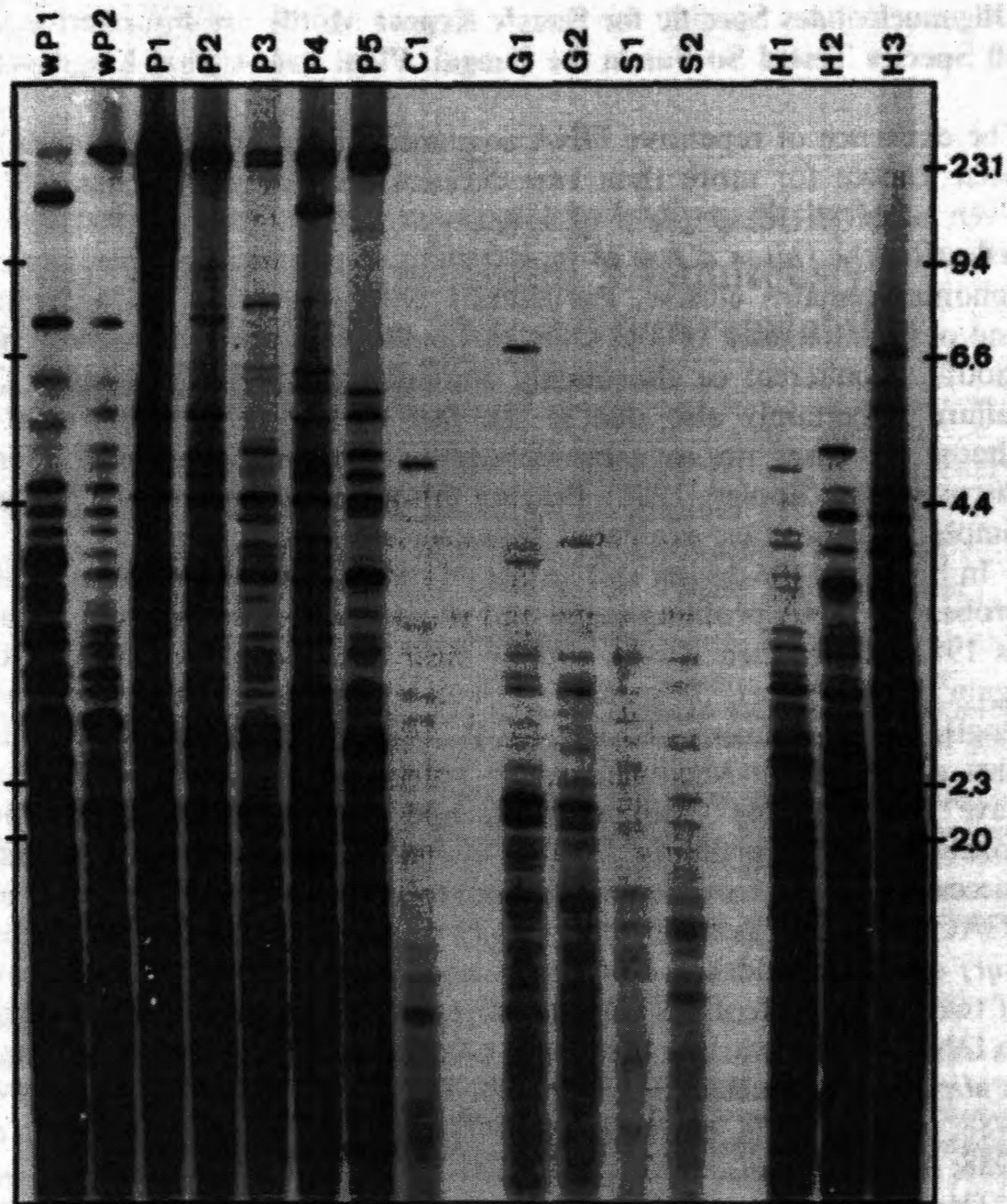


Figure 1. Oligonucleotide fingerprints as obtained with the probe $(GGAT)_4$ in wild boar (wP1-wP2), domestic pigs (P1-P5), camel (C1), goat (G1-G2), sheep (S1-S2) and horse (H1-H3) DNA after *Hinf*I digestion. DNA was prepared from peripheral blood leukocytes, digested and separated by agarose gel electrophoresis (0.7% in Tris-acetate buffer). The gel was dried and hybridized with the ^{32}P -labeled probe under the stringent conditions described previously (Schäfer *et al.*, 1988). Exposure was performed overnight without intensifying screens. Note the inter-individual variability in each species and the species-specific amount and distribution of the ranges of fragment lengths. Molecular weight markers are given in kilobases on the right. Approximately equal amounts of DNA have been applied with the exception of lane P1, which is overloaded. The relationships of the individuals in each species are not known. As can be inferred from our other family data in these species, they appear to include close relatives.

Table 1. Simple repetitive oligonucleotide probes for DNA fingerprinting

Probes	Sequences
(AT) ₈	atatatatatatat
(CA) ₈ [*]	cacacacacacaca
(CAC) ₅ ^{*°}	caccaccaccaccac
(CT) ₈	ctctctctctctct
(CT) ₄ (CA) ₅	ctctctctcacacaca
(GAA) ₆ ^{*°}	gaagaagaagaaga
(GACA) ₄ [°]	gacagacagacagaca
(GATA) ₂ (GACA)(GATA) ₂	gatagatagacagatagata
(GATA) ₃ (GACA) ₂	gatagatagatagacagaca
(GATA) ₄ ^{*°}	gatagatagatagata
(GATA) _{4,5} , (GATA) ₅ ; (GATA) _{5,75} ^{*°}	18 mer, 20 mer, 23 mer
(GGAT) ₄ [°]	ggatggatggatggat
(GGGCA) ₃	gggcagggcagggca
(TCC) ₅ [°]	tctctctctctcc
(TTAGGG) ₃	ttagggttagggttaggg

*complementary probe available

°available also in digoxigenated form

steps are conveniently performed in a temperature range from 35°C to 65°C, solely depending on the length and the base composition of the probe.

In order to give a rough overview from our previous experiences, Table 2 lists many of the fungal, plant and animal species that have so far been investigated by oligonucleotide fingerprinting. Depending on the species of interest, different oligonucleotide probes are informative for various purposes. The number of probes, harboring principally different simple repeat sequence motifs, necessary for the study of almost all eukaryotic genomes is 20 or less. It should be mentioned here that no emphasis has been laid on a systematically ordered phylogenetic compilation. In addition to individuality testing in several species, the accumulation of simple repetitive sequences on the heteromorphic sex chromosomes can at the same time be demonstrated by sex-specific hybridization patterns (Nanda *et al.*, 1990a; see also below). Finally, in primates including man (and in several fungi) certain simple sequence motifs are associated with the nucleolus organizer regions (NORs), probably due to their accumulation in the spacer regions between the ribosomal RNA genes (Nanda *et al.*, 1990b; Beyermann *et al.*, unpubl. data).

Oligonucleotide Fingerprinting in Man with Special Reference to Mutation Rates, Tumors and Statistical Evaluations of Paternity Disputes

Following the initial efforts which consecutively used combinations of the (GATA)₄ and (GACA)₄ and related "mixed" probes, optimal

Table 2. A comprehensive list of eukaryote species fingerprinted by oligonucleotide probes

Species investigated	Informative probes for individualization or identification*, sex chromosome status †
Fungi	
<i>Phycomyces blakesleeana</i> (Zygomycetes)	
<i>Ascochyta rabiei</i> (Ascomycetes)	(GTG) ₅ *, (GATA) ₄ *, (CA) ₈ *
<i>Saccharomyces cerevisiae</i> (Ascomycetes)	
<i>Penicillium janthinellum</i> (Ascomycetes)	(GTG) ₅ *, (CT) ₈ *, (GACA) ₄ *
<i>Penicillium citrorividae</i> (Ascomycetes)	(GTG) ₅ *, (CT) ₈ *, (GACA) ₄ *
<i>Penicillium chrysogenum</i> (Ascomycetes)	(GTG) ₅ *, (CT) ₈ *, (GACA) ₄ *
<i>Aspergillus niger</i> (Ascomycetes)	(GTG) ₅ *, (GACA) ₄ *, (GACA) ₄ *
<i>Aspergillus flavus</i> (Ascomycetes)	(GTG) ₅ *, (GACA) ₄ *
<i>Aspergillus oryzae</i> (Ascomycetes)	(GTG) ₅ *, (GACA) ₄ *
<i>Aspergillus batatae</i> (Ascomycetes)	(GTG) ₅ *, (GACA) ₄ *
<i>Aspergillus awamori</i> (Ascomycetes)	(GTG) ₅ *, (GACA) ₄ *
<i>Trichoderma harzianum</i> (Ascomycetes)	(GTG) ₅ *, (CT) ₈ *, (GACA) ₄ *
<i>Candida maltosa</i> (Deuteromycetes)	(GTG) ₅ *, (GACA) ₄ *
<i>Candida utilis</i> (Deuteromycetes)	(GTG) ₅ *, (GACA) ₄ *
<i>Coprinus comatus</i> (Basidiomycetes)	
Plants	
Algae	
<i>Oedogonium spec.</i> (Chlorophyceae)	
<i>Stenogramme interrupta</i> (Rhodophyceae)	
<i>Fucus serratus</i> (Phaeophyceae)	
Liveworts and Mosses	
<i>Marchantia polymorpha</i> (Hepaticae)	
<i>Polytrichum formosum</i> (Musci)	
Ferns	
<i>Equisetum arvense</i> (Equisetatae)	
<i>Polypodium vulgare</i> (Filicatae)	
<i>Osmunda regalis</i> (Filicatae)	
<i>Dodlia caudata</i> (Filicatae)	
Gymnosperms	
<i>Taxus baccata</i> (Pinatae)	
<i>Juniperus communis</i> (Pinatae)	
Monocotyledonous Angiosperms	
<i>Echinodorus osiris</i> (Alismatidae)	
<i>Musa acuminata</i> (Liliidae)	(CA) ₈ *, (GATA) ₄ *, (GTG) ₅ *, (GACA) ₄ *, (GATA) ₄ *
<i>Hordeum vulgare</i> (Liliidae)	(GACA) ₄ *
<i>Hordeum spontaneum</i> (Liliidae)	(GACA) ₄ *
<i>Asparagus densiflorus</i> (Liliidae)	
<i>Zea mays</i> (Liliidae)	(GAA) ₆ *
<i>Triticum aestivum</i> (Liliidae)	(GTG) ₅ *, (GATA) ₄ *
<i>Secale cereale</i> (Liliidae)	(GTG) ₅ *, (GATA) ₄ *
<i>Chamaedorea cataracterum</i> (Arecidae)	
Dicotyledonous Angiosperms	
<i>Helleborus niger</i> (Magnoliidae)	
<i>Urtica dioica</i> (Hamameliidae)	
<i>Ficus benjamina</i> (Hamameliidae)	
<i>Humulus lupulus</i> (Hamameliidae)	
<i>Aruncus silvester</i> (Rosidae)	
<i>Cicer arletinum</i> (Rosidae)	(GATA) ₄ *, (CA) ₈ *, (GACA) ₄ *, (GTG) ₅ *, (GGAT) ₄ *
and 7 additional species of <i>Cicer</i>	
<i>Lens culinaris</i> (Rosidae)	(GATA) ₄ *, (GACA) ₄ *, (CA) ₈ *
and additional species	
<i>Simmondsia sinensis</i> (Rosidae)	
<i>Vicia faba</i> (Rosidae)	(GATA) ₄ *, (GGAT) ₄ *

Table 2. (continued)

Species investigated	Informative probes for individualization or identification*, sex chromosome status ⁺
<i>Daucus carota sativus</i> (Rosidae)	(GACA) ₄ *, (GATA) ₄ *
<i>Camellia sinensis</i> (Dilleniidae)	
<i>Brassica oleracea</i> (Dilleniidae)	(GATA) ₄ *, (GTG) ₅ *
<i>Brassica napus</i> (Dilleniidae)	(GATA) ₄ *, (GTG) ₅ *
<i>Nicotiana tabacum</i> (Asteridae)	(GGAT) ₄ *, (TCC) ₅ *, (GTG) ₅ *, (GATA) ₄ *
<i>Nicotiana paniculata</i> (Asteridae)	
<i>Nicotiana glutinosa</i> (Asteridae)	
<i>Nicotiana acuminata</i> (Asteridae)	
<i>Lycopersicum esculentum</i> (Asteridae)	(GGAT) ₄ *, (GTG) ₅ *, (GATA) ₄ *
<i>Lycopersicum hirsutum</i> (Asteridae)	(GGAT) ₄ *, (GAA) ₆ *, (GATA) ₄ *
<i>Lycopersicum pimpinellifolium</i> (Asteridae)	(GGAT) ₄ *, (GAA) ₆ *, (GATA) ₄ *
<i>Lycopersicum peruvianum</i> (Asteridae)	(GGAT) ₄ *, (GAA) ₆ *, (GATA) ₄ *
<i>Solanum tuberosum</i> (Asteridae)	(GATA) ₄ *, (GTG) ₅ *, (GGAT) ₄ *, (TCC) ₅ *, (GACA) ₄ *
<i>Helianthus annuus</i> (Asteridae)	
<i>Lactuca sativa</i> (Asteridae)	
<i>Petunia hybrida</i> (Asteridae)	(GTG) ₅ *, (GATA) ₄ *
<i>Silene alba</i> (Carophyllaceae)	
<i>Arabidopsis thaliana</i> (Brassicaceae)	(GATA) ₄ *
<i>Rumex acetosella</i> (Polygonaceae)	
<i>Beta vulgaris</i> (Chenopodiaceae)	(GTG) ₅ *, (GATA) ₄ *
<i>Beta maritima</i> (Chenopodiaceae)	(GATA) ₄ *, (GGAT) ₄ *
<i>Microseris pygmaea</i> (Asteraceae)	(GACA) ₄ *, (GATA) ₄ *
<i>Microseris bigelovii</i> (Asteraceae)	(GACA) ₄ *, (GATA) ₄ *
Animals	
Ciliates	
<i>Stylonychia lemnae</i>	(GATA) ₄ *
Protozoa	
<i>Leishmania brasiliensis</i>	(CAC) ₅ *
<i>Leishmania guyanensis</i>	(CAC) ₅ *
<i>Leishmania mexicana</i>	(CAC) ₅ *
<i>Trypanosoma cruzi</i>	(CAC) ₅ *
Insects	
<i>Poecillimon tessalicus</i>	(GACA) ₄ *, (GATA) ₄ *
<i>Macrosiphum rosae</i>	(GATA) ₄ *
Vertebrates	
Fish	
<i>Latimeria chalumnae</i>	(CA) ₈ *, (CT) ₄ (CA) ₅ *
<i>Poecilia reticulata</i>	(GGAT) ₄ *, (GATA) ₄ *, (GACA) ₄ ⁺
<i>Poecilia sphenops</i>	(GGAT) ₄ *, (CA) ₈ ⁺
<i>Poecilia velifera</i>	(GGAT) ₄ *, (CA) ₈ ⁺
<i>Poecilia perugiae</i>	(GGAT) ₄ *, (GACA) ₄
<i>Poecilia formosa</i>	(GGAT) ₄ *, (GACA) ₄
<i>Poecilia latipinna</i>	(GGAT) ₄ *, (GACA) ₄ *
<i>Xiphophorus maculatus</i>	(GGAT) ₄ *, (GACA) ₄ *
<i>Xiphophorus helleri</i>	(GATA) ₄ *
<i>Xiphophorus montezumae</i>	(GACA) ₄ *
<i>Xiphophorus cortesi</i>	(GACA) ₄ *
<i>Chupea harengus</i>	
<i>Salmo trutta</i>	
<i>Leporinus obtusidens</i>	(GACA) ₄ *
<i>Leporinus elongatus</i>	(GACA) ₄ *
Amphibians	
<i>Bufo melanostictus</i>	(GACA) ₄ *, (CA) ₈ *, (TCC) ₅ *
<i>Necturus maculatus</i>	

Table 2. (continued)

Species investigated	Informative probes for individualization or identification*, sex chromosome status ⁺
<i>Triturus cristatus carnifex</i>	(GACA) ₄ [*] , (CA) ₈ [*]
<i>Triturus vulgaris</i>	(GACA) ₄ [*] , (CA) ₈ [*]
<i>Gastrotheca riobambae</i>	(TCC) ₅ [*]
<i>Pyxicephalus adspersus</i>	(GACA) ₄ [*] , (GATA) ₄ [*] , (CA) ₈ [*] , (TCC) ₅ [*]
Reptiles	
<i>Elaphe radiata</i>	(GACA) ₄ ⁺ , (GATA) ₄ ⁺
<i>Elaphe bimulata</i>	(GACA) ₄ ⁺ , (GATA) ₄ ⁺ , (GTG) ₅ [*]
<i>Elaphe taeniura</i>	(GACA) ₄ ⁺ , (GATA) ₄ ⁺ , (GTG) ₅ [*]
<i>Python regius</i>	(GATA) ₄ [*]
<i>Natrix fasciatus</i>	(GTG) ₅ [*]
<i>Boa constrictor imperatus</i>	(GATA) ₄ [*]
Birds	
<i>Buteo buteo</i>	(GGAT) ₄ [*]
<i>Accipiter nisus</i>	(GGAT) ₄ [*]
<i>Accipiter gentilis</i>	(GGAT) ₄ [*]
<i>Nyctea scandiaca</i>	(GGAT) ₄ [*]
<i>Tinnunculus tinnunculus</i>	(GGAT) ₄ [*]
<i>Hierofalco peregrinus</i>	(GACA) ₄ [*]
<i>Hierofalco rusticolus</i>	(GACA) ₄ [*]
<i>Hierofalco cherrug</i>	(GACA) ₄ [*]
<i>Hierofalco biarmicus</i>	(GACA) ₄ [*]
<i>Bubo bubo</i>	(GACA) ₄ [*]
<i>Milvus milvus</i>	(GGAT) ₄ [*]
<i>Aquila chrysaetos</i>	(GACA) ₄ [*] , (GGAT) ₄ [*]
<i>Gallus domesticus</i>	(GGAT) ₄ [*] , (GTG) ₅ [*] , (TCC) ₅ ⁺
<i>Columba livia domestica</i>	(GGAT) ₄ [*] , (TCC) ₅ ⁺
<i>Phylloscopus collybita</i>	(GACA) ₄ [*] , (GGAT) ₄ [*]
<i>Periparus ater</i>	(GACA) ₄ [*] , (GGAT) ₄ [*]
<i>Cyanistes caeruleus</i>	(GACA) ₄ [*] , (GGAT) ₄ [*]
<i>Erithacus rubecula</i>	(GACA) ₄ [*] , (GGAT) ₄ [*]
<i>Fringilla coelebs</i>	(GACA) ₄ [*] , (GGAT) ₄ [*]
<i>Coccothraustes coccothraustes</i>	(GACA) ₄ [*] , (GGAT) ₄ [*]
<i>Pyrrhula pyrrhula</i>	(GACA) ₄ [*] , (GGAT) ₄ [*]
Mammals	
<i>Canis lupus familiaris</i>	(CA) ₈ [*] , (GTG) ₅ [*]
<i>Canis lupus familiaris Sapsari</i>	(GTG) ₅ [*]
<i>Equus p. caballus</i>	(CA) ₈ [*] , (GGAT) ₄ [*] , (GTG) ₅ [*]
<i>Sus scrofa domesticus</i>	(AT) ₈ [*] , (GGAT) ₄ [*] , (TCC) ₅ [*]
<i>Ontotragus megaceros</i>	(GTG) ₅ [*]
<i>Odocoileus dichotomus (Blastoceros)</i>	(GTG) ₅ [*]
<i>Odocoileus bezoarticus (Blastoceros)</i>	(GTG) ₅ [*]
<i>Cervus duvauceli</i>	(GTG) ₅ [*]
<i>Tragelaphus spekei</i>	(GTG) ₅ [*]
<i>Bos taurus</i>	(GTG) ₅ [*] , (GGAT) ₄ [*] , (CA) ₈ [*]
<i>Capra aegagrus hircus</i>	(GACA) ₄ [*] , (CA) ₈ [*]
<i>Ovis ammon aries</i>	(GACA) ₄ [*] , (CA) ₈ [*]
<i>Ellobius lutescens</i>	
<i>Clethrionomys glareolus</i>	(GACA) ₄ [*]
<i>Apodemus flavicollis</i>	(GACA) ₄ [*]
<i>Apodemus sylvaticus</i>	(GACA) ₄ [*]
<i>Microtus agrestis</i>	(GGAT) ₄ [*]
<i>Rattus norvegicus</i>	(GTG) ₅ [*] , (GATA) ₄ [*]
<i>Mus musculus</i>	(GGGCA) ₃ [*] , (GATA) ₄ [*] , (GACA) ₄ ⁺
<i>Cricetulus griseus</i>	(GACA) ₄ [*]

Table 2. (continued)

Species investigated	Informative probes for individualization or identification*, sex chromosome status [†]
<i>Nesokia indica</i>	
<i>Nyctalus noctula</i>	(GATA) ₄
<i>Hylobates syndactylus</i>	(GTG) ₅ [*]
<i>Macaca mulatta</i>	(GTG) ₅ [*] , (GACA) ₄ ^{*‡} , (GATA) ₄ [*]
<i>Aotus trivirgatus</i>	(GACA) ₄ [‡]
<i>Pongo pygmaeus</i>	(GTG) ₅ [*] , (GACA) ₄ [‡]
<i>Pan troglodytes</i>	(GTG) ₅ [*] , (GACA) ₄ [‡]
<i>Gorilla gorilla</i>	(GTG) ₅ [*] , (GACA) ₄ [‡]
<i>Homo sapiens</i>	(GTG) ₅ [*] , (GACA) ₄ [‡]

[†] sex differences depending on the population investigated

[‡] associated with nucleolus organizers

genetic individualization in the human was subsequently achieved in a single hybridization step using (CAC)₅ (Schäfer *et al.*, 1988). Solely depending on the resolving power of the gel system used, all human individuals can be distinguished with the only exception of genetically identical, monozygotic twins. In practice one single case of questionable paternity could not be cleared up because the putative fathers were monozygotic brothers (Pöche *et al.*, in press). The only possibility of solving such a problem would have occurred if one of the men carried a somatic mutation that the other one lacked. Using several informative probes this rare event was not encountered. So far only one case of monozygotic twins with differences in their (CAC)₅/(GTG)₅ fingerprints has been encountered (Hundrieser *et al.*, unpubl. data). On the other hand, even questionable paternity cases can be solved beyond any doubt, whereas enzyme polymorphism studies alone would have had insufficient discrimination power or could have yielded false exclusions (Bender *et al.*, 1991).

Nürnberg *et al.* (1989) established the somatic stability of (CAC)₅/(GTG)₅ fingerprints in human tissues and determined the mutation rate to be 0.001 per fragment per gamete (from more than 350 meioses). Meanwhile, this rate has been confirmed further by increasing the sample size to over 1500 meioses (unpubl. data). Somatic stability and similar or even reduced mutation rates have been determined for the (GACA)₄ fingerprint bands (Roewer *et al.*, 1990). One exception to the rule of somatic stability has been provided by the study of transformed tissues (Lagoda *et al.*, 1989). Indeed, in an extended parallel fingerprint and cytogenetic study on human gliomas versus peripheral blood, (CAC)₅/(GTG)₅ proved to be a superior probe for the detection of genomic alterations (Nürnberg *et al.*, in press). Changes of the constitutional patterns were observed in nearly 80% of the gliomas, and subsequent investigation using additional probes detected an additional

15% alterations in the fingerprints. These changed patterns mostly reflect the corresponding chromosomal aberrations seen in a majority of the cells. Concomitantly it was observed that particularly strong fingerprint bands appeared in about 30% of the gliomas when probed with independent simple repeat oligonucleotides. After digestion with several restriction enzymes that recognize unrelated target sequences, consecutive hybridizations with the $(CA)_8$ probe and a cDNA clone containing epidermal growth factor (EGF) receptor gene sequences revealed the comigration of one simple repeat band with one of the amplified EGF receptor gene fragments (Nürnberg *et al.*, in press). Thus gene amplification events can readily be monitored by the set of simple repeat probes due to their ubiquitous distribution all over the human chromosomal complement. During the same investigation it was also noted that the telomere lengths increased during the progression of some glioma tumors as revealed by the telomere repeat probe $(ttaggg)_3$. This is in contrast to the findings of Hastie *et al.* (1990), who described recently shortened telomeres in colon carcinomas and aged fibroblasts. In conclusion, oligonucleotide fingerprinting is a valuable asset whenever cultured cells of various sources need to be identified (Speth *et al.*, in press).

Since it is not known which bands are allelic to one another in a complex fingerprint pattern, formal genetic models and the corresponding classical statistics cannot be used for the evaluation of *multilocus* fingerprints in paternity analysis. In addition, one or both of the alleles at a locus might well be hidden in the smeared signal below the 2–3 kilobase (kb) range, which is not resolved in routine fingerprint gels. Various new statistical approaches have been worked out for calculating the probability of paternity from multilocus fingerprint profiles (for a discussion see Fimmers *et al.*, 1990; Yassouridis and Eppel, 1991). The primary method relies on the calculation of likelihood ratios based on Bayes' theorem (Evetts *et al.*, 1989). This likelihood approach can also be adopted for use in deficiency cases (Yassouridis, in preparation). Despite quite promising results and comparatively widespread recognition – also due to good experiences with the method in practical case work – some restrictions and compromises affect the practicability of the approach of Evetts *et al.* It is not yet known if simpler methods which depend on fewer assumptions will finally prove practicable and if they will meet the necessary standards.

Advantages of the Demonstration of Genetic Individuality by Oligonucleotide Fingerprinting

It has been repeatedly stated that oligonucleotide fingerprinting is advantageous over related methodologies for a number of reasons: (i) the constant probe quality for up to a million hybridizations from a

single batch of chemically synthesized oligonucleotide; (ii) no need for blotting, and therefore no loss of DNA due to non-optimal transfer, by simply fixing the DNA in the dried gel matrix; (iii) the least time-consuming procedure, additionally due to short hybridization times with higher probe concentrations and limited, standardized washing steps; (iv) the possibility of repeated hybridizations of the same gel-fixed DNA – especially in comparison to filter-bound nucleic acids; (v) convenient non-radioactively labeled probes that can also be hybridized directly in the gel (Zischler *et al.*, 1989). Above all, the most significant advantage over other fingerprint probes is the base-specific hybridization under the appropriate conditions: mismatch abrogates hybridization. Therefore, if one follows the established protocols, almost absolute reproducibility is obtained.

Forensic Applications of Genetic Fingerprints

The forensic sciences represent an immediately obvious and broad field of application for individualization techniques, i.e. analyses of stains from spots of blood and sperm, vaginal swabs, sputum, urine, hair roots, decayed tissue remains, parts of bodies, etc. (Pöche *et al.*, 1990; Roewer *et al.*, 1990; Sprecher *et al.*, 1990). Oligonucleotide fingerprinting has been successfully used in actual casework including the identification of burnt bodies (Sprecher *et al.*, in press; Pöche *et al.*, in press). During the successful identification from the DNA of one of the burnt bodies, a remarkable phenomenon was observed that should be brought to the attention of other investigators whilst requiring further experimental clarification: informative (GTG)₅ fingerprints of normal intensity could be established, whereas when using the probe (GACA)₄ in a rehybridization only extremely weak patterns were observed. This feature is absolutely reproducible using the DNA from the one burnt body. Upon the next hybridization with (GATA)₄ the result was again negative, yet (TCC)₅ on the other hand yielded the expected intense bands for identification. It thus appears as if the oligonucleotide probes with a high GC content were still hybridizing, whereas this was not true when the GC content was 50% or less. One working hypothesis to explain this probe hybridization behaviour relates to the possible partial denaturation of DNA into single strands under high temperatures depending on the base composition: (i) single-stranded DNA was much more amenable to degradation in the burnt tissue remains, generating at random broken short fragments, (ii) single-stranded DNA would not be cut (as efficiently) and (iii) the subsequent agarose gel electrophoretic separation of unpaired single DNA strands would not result in a distinct pattern but rather in a smeary, background-like signal. These possibilities are considered in ongoing *in vitro* experiments by heating

tissues to different temperatures and demonstrating the fingerprint patterns obtained using a variety of simple repetitive oligonucleotides with different GC content. Finally, another source of artifacts has not yet received sufficient attention: the "hidden partial" phenomenon (Nürnberg and Epplen, 1989), where some sites in the genomic DNA need, for completely unknown reasons, an exorbitant amount of enzymatic activity and are not digested.

In this context Roewer *et al.* (1990) have recently established some general rules for oligonucleotide fingerprinting with respect to the amount and the quality of the DNA samples available, as well as the informativeness of several probes for each purpose. Because of the possibility of repeated rehybridizations, for complicated cases, including those with low amounts of degraded DNA, the results of different oligonucleotides can preferably be combined. Though the virtually absolute sensitivity of the polymerase chain reaction (PCR) cannot be reached by conventional oligonucleotide fingerprinting, the inherent and partly uncontrollable problems of PCR concerning minute contaminations are almost completely avoided. Because of the species-specific accumulation and organisation of these repetitive elements, any large scale contamination of the stain by, for example, blood from a different animal species or fungal overgrowth, can readily be sorted out by hybridizing with the panel of additional simple repeat oligonucleotides identifying the foreign additive. Almost all prokaryotic DNA contaminations would not contain any simple repetitive motifs.

Variation of Simple Repeats in Clonal Vertebrates

Questions concerning genetic identity or relatedness are being asked more and more in the different biological disciplines. In unisexual fish like *Poecilia formosa* (Telostei, Poeciliidae) a single genome is transmitted from mothers to daughters through several generations without genetic recombination (Monaco *et al.*, 1984). For the apomictic breeding of the laboratory lines a single male of an ornamental black molly strain is used which gives the physiological stimulus for gynogenesis, but which does apparently not contribute to the gene pool of *P. formosa*. One of us (MScha) has been keeping two laboratory strains that had been separated for at least seven years. When the fingerprint patterns were compared between several individuals from both strains, a band-sharing rate of 80% or more was observed between the strains (depending on the restriction enzyme/probe combination). Individuals from sibships and their respective mothers show variation limited mostly to very few, truly hypervariable loci (Schartl *et al.*, 1990; Schartl *et al.*, submitted). These results stand in clear contrast to those of a recent study of wild populations by Turner *et al.* (1990), where the fingerprints

of specimens caught at identical and at different locations were described to be comparatively variable.

Heterochromatin and Sex Chromosome Evolution as Studied by Fingerprinting and *in situ* Hybridization Using Non-isotopically Labeled Oligonucleotide Probes

Animal (and plant) sex chromosome evolution probably started on different occasions with a homologous pair of autosomes leading to morphologically differentiated sex chromosomes. In fishes (and reptiles) a range from poorly established to thoroughly heteromorphic gonosomes is sometimes found in closely related species. The guppy fish *Poecilia reticulata* is characterized by the XX/XY sex chromosome mechanism. Yet the Y chromosome can only be identified cytologically on the basis of (i) its heterochromatin staining, (ii) via its Y-chromosomally accumulated (gaca)_n sequences by hybridization *in situ* or (iii) the male-specific high molecular weight smear after electrophoretic separation and hybridization with this same probe (Nanda *et al.*, 1990). All these recent investigations were considerably ameliorated by the fact that non-radioactively labeled oligonucleotide (digoxigenin or biotin) probes could be developed for the *in situ* hybridizations to metaphase chromosomes (Zischler *et al.*, 1989). In addition, the probe (GATA)₄ reveals a Y chromosomal polymorphism in the commercially available guppy strains. In contrast, in the closely related genus *Xiphophorus* none of the genetically defined heterogametic situations could be verified consistently with simple repeat probes. Only particular *Xiphophorus* populations produced consistent sex-specific hybridization patterns with (GATA)₄. Other poeciliid species (*P. sphenops* var. *melanistica* and *P. velifera*) harbour different sex-specifically organized repeat motifs in females on the W chromosome (Nanda *et al.*, in press). Thus the accumulation of various diverse simple repeats often accompanies the initial steps of sex chromosome differentiation, leading finally either to male or female heterogamety and the XX/XY or ZZ/ZW sex chromosome mechanism.

Development of Single Copy Probes for Individual Hypervariable Loci from a (CAC)₅/(GTG)₅ Human Fingerprint

Multilocus fingerprints in humans are composed of many hypervariable bands originating from different loci spread more or less evenly over the genome. Initially, evidence for dispersion originated mainly from *in situ* hybridization data (Zischler *et al.*, 1989; Nanda *et al.*, in press) and from segregation analyses in a limited number of other species (Epplen *et al.*, unpublished). In very few species like, for example, *Ephestia kuehniella* only two or three chromosomal loci harbour long stretches of particular

simple repeat motifs (Traut *et al.*, submitted). Whether the allelic signals from one and the same locus in humans appear in the resolvable part of the complex fingerprint pattern or not depends on the multilocus probe used and length variations of the target fragments. As there is much overlap in the size range of alleles at different loci, multilocus profiles do not reveal an individual's genotype at any locus and so maintain genetic confidentiality. The main advantage of locus-specific probes is clearly the defined formal genetic model (and therefore the associated accepted statistical treatment). For other reasons we have tried several approaches to generate such locus-specific markers from the human (CAC)₅/(GTG)₅ fingerprint: (i) screening of a complete genomic phage library after partial Sau3AI digestion; (ii) screening of a (partial) phage library containing 3–10 kb fragments after complete MboI digestion; (iii) semi-specific ligation-mediated polymerase chain reaction (PCR), which involves the ligation of adaptors to isolated 3–10 kb fragments followed by PCR using primers specific for the adaptor and the simple (cac/gtg)_n repeat (Zischler *et al.*, 1991; Zischler *et al.*, in preparation). Thereafter the PCR products are conveniently cloned into plasmid vectors.

A major disadvantage of the PCR approach is the comparatively time-consuming evaluation of the informativeness of the isolated probes, which has to be done by hybridization to restriction enzyme digested human DNA from different individuals. In addition, in (i) and (ii) the phages containing simple repeats often showed cloning artifacts in the prokaryotic hosts due to deletions and other complex rearrangements. Approach (iii) also involved prescreening of the clones obtained for the presence of other repetitive elements by separate dot blot hybridization with, for example, human Alu repeats and total human DNA. One of us (HZ) came up with at least four hypervariable locus probes spread over the human genome (heterozygosity rate >90%), several informative probes (heterozygosity >50%) and many clones (25–30) that are less informative or contain additional repeats that confound their exact evaluation even after competition hybridizations, specific oligonucleotide syntheses and hybridizations and other procedures. The apparent evolutionary conservation of individual human hypervariable loci ranges from those found to hybridize to taxa from fish (and chicken) to primates, even under moderately stringent conditions, to no detectable conservation at all. The potential expression of the informative probes is presently being investigated at the mRNA level.

Exonic Polymorphism Versus Intronic Hypervariability: A Mixed Simple Repeat Locus in *HLA-DRB* and Related Genes

Recently, several hypervariable, highly informative single copy loci have been described (see previous section; Nakamura *et al.*, 1987; Jeffreys *et*

to slipping of the Taq polymerase in the simple repeat region. It has been shown previously by electroblot hybridization and sequence determination that these amplified exon-intron region clusters are inherited according to Mendelian laws (Riess *et al.*, 1990). For example, among ten randomly sampled, unrelated individuals typed serologically as DR4 and subtype Dw4 (all *HLA-DRB1*0401* exonic sequences), the basic simple repeat structure is completely maintained. Variations occur exclusively in the numbers of the dinucleotide unit copy numbers: In the $(gt)_n$ stretch, n ranges from 19–23, whereas m in $(ga)_m$ ranges from 14–16. In the DRw52 group, a more complex basic repeat structure is found: $(gt)_n(ga)_mca(ga)_3[ca(ga)_3]$. Here, there is abundant variation in the $(gt)_n$ sequence, but significantly less in the first $(ga)_m$ stretch. Table 3 summarizes the group-specific architecture of the simple repeat blocks including data from a baboon (*Papio hamadryas*, Paha) and cattle (*Bos taurus*, Bota). Throughout this table only one intermediately long version is given from several (between 1 and 12) investigated for each *HLA-DRB* gene sequence: invariably the stretch commences with $(gt)_n$, whereby n ranges from 6 in a DR1 pseudogene intron to 26 in a DRB1*1401 individual. This simple dinucleotide organization of the 5' stretch is interrupted only in two categories: DRw53 pseudogenes and DRB4 introns. On the other hand the following $(ga)_m$ stretches of dinucleotides exhibit numerous deviations, depending on the *HLA-DR* gene investigated. Simple $(ga)_m$ repeats appear in DRB1*0401, in the pseudogene associated repeats and in the only baboon sequence. Group-specific simple repeat organization could also be demonstrated across species barriers, for example in a parallel study on Bota-DR intronic simple repeats from cattle (H. Ammer, W. Schwaiger, C. Kammerbauer, and J. T. Epplen, submitted). We were particularly surprised by the repeat composition in the Bota-DR sequence: the exon exhibits 95% homology to the protein domains of the DRw52 supergroup and, also, the intronic repeat is virtually indistinguishable from its human counterparts (H. Ammer, W. Schwaiger, C. Kammerbauer, and J. T. Epplen, unpubl. data; genomic Bota-DR DNA clone obtained from Groenen *et al.*, 1990; there were several differences in the sequences obtained from the same isolate in independent analyses in two laboratories). Attempts to perform a phylogenetic analysis on the simple repeats for comparison with the results obtained from the *HLA* protein sequences are hampered by the inability of the available alignment programs to perform (meaningful) simple sequence alignments. If the alignments are done "by eye", i.e. introducing gaps to separate the pure $(gt)_n$ and $(ga)_m$ repeats, and thereafter one applies the Felsenstein programs (1989), then more closely related groups are found to cluster together (Epplen, unpublished). Attempts to establish respective algorithms for "meaningful" alignments of simple sequences are presently being evaluated (Zucker *et al.*, in preparation).

Table 3. Intronic simple (gt)_n(ga)_m repeats in DRB and related genes

"DRB" allele/ No. individ./ RFLP	Total repeat length	Repeat composition
HLA-DRB1*0101/2	44 (gt) ₁₅	aa (ga) ₁ aa(ga) ₄
HLA-DRB1*0301/7/3b	64 (gt) ₁₅	(ga) ₇ ca(ga) ₃ ca(ga) ₅
HLA-DRB1*0301/1/3a	66 (gt) ₁₇	(ga) ₆ ca(ga) ₃ ca(ga) ₅
HLA-DRB1*0302/1/3c	68 (gt) ₁₉	(ga) ₆ ca(ga) ₃ ca(ga) ₄
HLA-DRB1*0401/12	74 (gt) ₂₂	(ga) ₁₅
HLA-DRB1*0404/1	90 (gt) ₂₂	(ga) ₂₃
HLA-DRB1*0405/4	70 (gt) ₁₉	(ga) ₁₆
HLA-DRB1*0407/1	76 (gt) ₂₁	(ga) ₁₇
HLA-DRB1*0801/4	62 (gt) ₁₈	(ga) ₇ ca(ga) ₅
HLA-DRB1*1001/1	48 (gt) ₁₆	(ga) ₈
HLA-DRB1*1101/2/5c	74 (gt) ₂₀	(ga) ₆ ca(ga) ₃ ct(ga) ₆
HLA-DRB1*1102/1/5c	72 (gt) ₂₂	(ga) ₃ ca(ga) ₃ ca(ga) ₆
HLA-DRB1*1103/3/5c	88 (gt) ₂₂	(ga) ₁₁ ca(ga) ₃ ca(ga) ₆
HLA-DRB1*1104/1/5c	76 (gt) ₂₂	(ga) ₅ ca(ga) ₃ ca(ga) ₆
HLA-DRB1*1104/1/5c	80 (gt) ₂₅	(ga) ₅ ca(ga) ₃ ca(ga) ₅
HLA-DRB1*1301/1/6b	76 (gt) ₁₇	(ga) ₁₀ ca(ga) ₃ cagc(ga) ₅
HLA-DRB1*1301/3/6a	88 (gt) ₂₃	(ga) ₁₀ ca(ga) ₃ ca(ga) ₆
HLA-DRB1*1301/1	92 (gt) ₂₅	(ga) ₁₀ ca(ga) ₃ ca(ga) ₆
HLA-DRB1*1401/1	88 (gt) ₂₆	(ga) ₁₁ ca(ga) ₆
HLA-DRB1*1501/7	82 (gt) ₁₉	(ga) ₅ ca(ga) ₄ ca(ga) ₃ ggaa(ga) ₆
HLA-DRB4*0101/2	70 (gt) ₂ at(gt) ₁₁	(ga) ₁₂ ca(ga) ₁ ggaa(ga) ₅
HLA-DRB5*0101/9	74 (gt) ₂₁	(ga) ₅ ggaa(ga) ₄ ca(ga) ₂ gg(ga) ₁
HLA-DR1ψ/3	32 (gt) ₆	(ga) ₁₀
HLA-DR2ψ/3	48 (gt) ₁₃	(ga) ₁₁
HLA-DR4ψ/2	37 (gt) ₂ tt(gt) ₃	t(ga) ₅
HLA-DR7ψ/1	35 (gt) ₂ tt(gt) ₃	t(ga) ₅
Paha/1	42 (gt) ₁₄	(ga) ₇
Bota*/1	86 (gt) ₂₀	(ga) ₉ ca(ga) ₂ ca(ga) ₂ [ca(ga) ₂] ₂

*Ammer, corrected sequence; but see Groenen *et al.* (1990)

Finally, apart from these purely evolutionary aspects, our studies also aim at a better understanding of the significance of the invariantly present (gt)_n(ga)_m simple repeat locus, since the latter has been identified in such diverse vertebrate species. Is this ubiquitous presence just a mere coincidence? Does the repeat represent a DNase hypersensitive site? Do particular proteins bind to this DNA region? Are there secondary structures like, for example, Z DNA (Rich *et al.*, 1984) or H DNA (Mirkin *et al.*, 1987) possible in this part of the intron?

In an initial attempt to make use of the information provided by this simple repeat locus, rheumatoid arthritis (RA) patients were also included in the panel of the aforementioned *DRB1*0401* cohort. RA has been found previously to be associated with *HLA-DR4*, especially, for example, with the *DR4Dw4* subtype. In the limited number of probands tested, the structure and the lengths of the simple repeats of RA patients and healthy control individuals did not differ. In principle the same result was found for multiple sclerosis (MS) patients, a disease which is associated with the serologically determined *HLA-DR2* haplotype. The comparison of the *DR2* gene adjacent simple repeats from healthy donors and MS patients revealed neither particular sequence lengths nor configurations (Eppelen *et al.*, unpublished).

Future Prospects

It is now well established that loci containing tandem simple repeat sequences are a practically unrestricted source of highly informative markers in eukaryote genomes. Therefore they appear to be *the* tools for a diverse range of applications. In addition to their already recognized utility for individualization, the simple repeat sequences will have an especially major impact in large scale mapping projects, for example in interrelating and ordering cosmids and yeast artificial chromosomes from genomic libraries. One particularly interesting development concerns a veritable hotspot of recombination in the chicken genome, which has initially been identified using the $(GAA)_6$ simple repeat probe: more than 80% of the length alleles in true offspring cannot be matched to either the paternal or to the maternal genotype (Nanda *et al.*, submitted). Meanwhile, from this simple repeat locus an adjacent single copy probe has been isolated by semispecific PCR (see above) as well as genomic phage clones, with the aid of which a detailed, targeted analysis will be performed. But apart from these problem-centred considerations there are also solid grounds to expect, in due course, major breakthroughs in the quest for the actual significance of these enigmatic simple tandem elements. It has been proposed that simple repetitive sequences have weak enhancer-like activity in viral expression systems. Certainly, at least *in vitro*, they are able to occur in special confirmations, i.e. secondary structures, depending on the purine/pyrimidine composition. Yet whether the outcome will be that they represent merely junk and jumble from the evolutionary past or that they bear present day function(s), only the results of diligent experimentation and insightful interpretation in a broad and open conceptual framework will tell.

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