Instituto Juan March
de Estudios e Investigaciones

20 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on
Genomic Fingerprinting

Organized by

M. McClelland and X. Estivill

G. Baranton
D. E. Berg
X. Estivill
J. L. Guénet
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INDEX

PROGRAMME

GENOMIC FINGERPRINTING

MONDAY, October 25th, 1993

Registration.

Chair: J.-L. Guénet

M. McClelland- Arbitrarily Primed PCR.

M. Perucho - A New Mechanism for Sporadic and Hereditary Colorectal Cancer Revealed by AP-PCR DNA Fingerprinting.

Chair: J.H. Nadeau

J.A. Rafalski- DNA-Amplification Based Genetic Mapping and Fingerprinting.

J. Welsh - RNA Fingerprinting by Arbitrarily Primed PCR.

P. Liang - Differential Display of Messenger RNAs, a New Approach of Cloning Differentially Expressed Genes.

Chair: P. Liang

D.L. Nelson - Alu PCR: A Method for Rapid Access to Human DNA of Unknown Sequence.

K. Hayashi - Collection of Polymorphic Sequence-Tagged Sites.

X. Estivill - Microsatellite Markers: Tracers of Gene Mutations and Evolution.

Chair: X. Estivill

J.-L. Guénet - Establishing a High Resolution/High Density Molecular Genetic Map of the Mouse Genome.

J.H. Nadeau - DNA Fingerprinting for Mouse Genome Analysis: Applications for Genetic and Physical Mapping, Genetic Quality Control Programs, and Developing a Gene Mapping Resource Panel.

TUESDAY, October 26th, 1993

Chair: D.L. Nelson

G. Baranton - Fingerprinting of Two Genera of Pathogenic Spirochetes (*Leptospira* and *Borrelia*).

D.E. Berg - Studies of Molecular Epidemiology and Microbial Genome Organization: *Escherichia Coli* and *Helicobacter Pylori*.

A.J.G.Simpson - Sequence-Specific "Gene Signatures" Can be Obtained by PCR with Single Specific Primers at Low Stringency.

Chair: D.E. Berg

A.van Belkum - Comparison of Phage Typing and DNA Fingerprinting By Polymerase Chain Reaction for Discrimination of Methicillin-Resistant *Staphylococcus Aureus* Strains.

A.J.Martínez-Murcia - Genomic Typing of *Haloferax Mediterranei* Strains Using Random Primer-Polymerase Chain Reaction.

N.B. Murphy - Application of Arbitrarily Primed PCR Fingerprinting to Molecular Studies on Trypanosomes.

J. Stanley - Systematic Genotypic Typing and Fingerprinting of *Salmonella* with rRNA Gene and Insertion Sequence Probes: Applications to Epidemiology and Phylogenetics.

I. Martínez-Galarza - Species Identification by Arbitrary amplification of Polymorphic DNA.

Chair: J.A. Rafalski

H.S. Kwan - Application of Arbitrarily-Primed Polymerase Chain Reaction Genomic Fingerprinting in Molecular Studies of Basidiomycetous Fungi.

R. Sederoff - Genomic Mapping in Woody Plants with RAPD Markers.

W. Powell - PCR Based Assays for Plant Germplasm Characterisation.

Chair: W. Powell

P.C. Sharma - Oligonucleotide Fingerprinting in Chickpea and Identification of Molecular Markers Linked with *Ascochyta* Resistance.

K. Wolf - The Use of RAPDs as Genetic Markers in Chrysanthemum.

E.Zietkiewicz- Fingerprinting Eukaryotic Genomes by PCR Using Primers Specific to Ubiquitous Genomic Repeats.

E. Rogaev - Genomic Fingerprinting for Genetic Analysis of Alzheimer's Disease.

WEDNESDAY, October 27th, 1993

Chair: R. Sederoff

H. Zischler - Mutational Mechanisms in Mitochondrial DNA.

O.A. Ryder - Investigation of Intra-Specific Genomic Variation Utilizing Molecular Genetics Technologies: Application to Endangered Species Conservation.

Chair: O.A. Ryder

J.A. Melero - Molecular Epidemiology of Human Respiratory Syncytial Virus Studied By RNase A Fingerprinting and Sequence Analysis.

J. Dopazo - Estimates by Computer Simulation of Genetic Distances from Comparisons of RNAse A Mismatch Cleavage Patterns.

C. López-Galíndez - The RNAse A Mismatch Cleavage Method in the Characterization of HIV-1 Isolates from Different Countries.

G. Dorado - Restriction Site Mutagenesis (RSM): a PCR-Based Genotypic Mutation Assay.

M.A. Peinado - Determination of Genomic Instability in Cancer Cells by DNA Fingerprinting.

Summary. X. Estivill.

INTRODUCTION

M. McClelland

The development of new methods to generate molecular data has opened up many opportunities in areas of genetic mapping, epidemiology, genealogy and phylogenetics. Many of these advances have been due to genomic fingerprinting and, most recently, the development of methods based on PCR.

The workshop provided an opportunity for researchers using different biological systems to discuss common themes in methodology and the interpretation of data. Because the backgrounds of the attendees were so diverse, a particular effort was made to ensure that all the attendees had an opportunity to speak or, if they preferred, present a poster.

Most PCR-based techniques were represented at the workshop. We were fortunate to have among the speakers the inventors of Alu-PCR, SSCP, AP-PCR, RAPDs, RAP and DD. There was a particular emphasis on presentations from people working on arbitrarily primed PCR (AP-PCR/RAPDs) and the extension of this methodology to RNA fingerprinting (RAP/DD). Speakers also included world experts on the use of various fingerprinting technologies, including genetic mapping of plants and animals, epidemiology of bacterial infections, cancer, phylogenetics, and the use of AP-PCR to detect differentially expressed RNAs.

The participants presented work on a very wide array of biological systems, including condors, trypanosomes, pine trees and mice! This diversity was quite unusual for a workshop, and reflected the broad technological theme. The number of participants was large enough that sessions could be grouped into broad areas of biology.

It was exciting to bring together such a diverse set of participants and yet to have common ground for discussion. I thank all the attendees for their enthusiastic participation that was essential for the success of a meeting with 50 participants. I certainly gained a considerable amount from the workshop and I am sure many others did also.

MONDAY, October 25th

Arbitrarily Primed PCR

Michael McClelland and John Welsh. California Institute of Biological Research. 11099 North Torrey Pines Road. La Jolla. CA 92037.

Arbitrarily Primed PCR (AP-PCR) generates a fingerprint often referred to as Random Amplified Polymorphic DNA (RAPDs) (Welsh and McClelland, 1990; Williams et al., 1990). Arbitrarily primed PCR is a modification of PCR that provides an information rich fingerprint of genomic DNA. This method is based on the selective amplification of genomic sequences that, by chance, are flanked by adequate matches to an arbitrarily chosen primer. If two template genomic DNA sequences are different, their arbitrarily primed PCR reaction products display different banding patterns. Such differences can be exploited in ways largely analogous to the uses of restriction fragment length polymorphisms, including genetic mapping, taxonomy, phylogenetics and the detection of mutations. This abstract provides references to some published examples, with emphasis on work from our own laboratory.

One of the most frequent uses of arbitrarily primed PCR methods has been the detection of dominant polymorphic markers in genetic mapping experiments (Williams et al., 1990). Using DNA from a family of recombinant inbred mice, we and others have placed several hundred anonymous markers derived from arbitrarily primed PCR on the mouse genetic map (Nadeau et al., 1992; Serikawa et al., 1993; Woodward et al., 1993). Arbitrarily primed PCR methods have found wide use in the molecular genetics of plants. Sobral and colleagues have placed 200 markers on the sugar cane genetic map (Al-Janabi et al., 1993), while similar mapping projects are on-going in several laboratories on many other plants, for instance, pine (Neale and Sederoff, 1991), and Arabadopsis (Reiter et al., 1992). Genetic markers that are linked to a measurable phenotype can be detected with these methods by bulked segregant analysis (Michelmore et al., 1991). In another system, Kubota et al. (1992) used arbitrarily primed PCR to detect gamma ray-induced damage in fish embryos.

Polymorphisms detected by Arbitrarily Primed PCR can also be used as taxonomic markers in population studies of a wide variety of organisms (Chalmers et al., 1992; Welsh et al., 1992). In this context, the method is directly applicable to epidemiology and historical ecology. The application of arbitrarily primed PCR to the taxonomy and phylogeny of microorganisms, such as *Staphylococcus* and *Borrelia burgdorferi*, is essentially the same and has been described elsewhere (Welsh et al., 1992; Welsh and McClelland, 1993).

Arbitrarily primed PCR can be used to detect somatic genomic changes that occur in anonymous sequences in certain tumors. Such somatic genetic alterations include allelic losses or gains (Peinado et al., 1992) and deletion or insertion mutations of one or a few nucleotides (Ionov et al., 1993) which occur during tumor development and progression.

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A NEW MECHANISM FOR SPORADIC AND HEREDITARY COLORECTAL CANCER REVEALED BY AP-PCR DNA FINGERPRINTING.

Manuel Perucho, Yurij Ionov, Sergei Malkhosyan, Antonia Velazquez, Miguel Angel Peinado[1], Sofia Casares and Darryl Shibata[2].

California Institute of Biological Research. 11099 North Torrey Pines Road. La Jolla, CA 92037. USA. 1: Present address: Institut de Recerca Oncologica. Hospital Duran i Reynals, Autovia Castelldefels Km 2,7. Hospitalet 08907 Barcelona, Spain. 2: Department of Pathology. University of Southern California School of Medicine. Los Angeles, CA 90033. USA.

That spontaneous errors in DNA replication may be substantial in transformation was proposed in an attempt to explain the chromosomal alterations of cancer cells (Nelson and Mason, J. Theoret. Biol., *37*, 197,1972; Loeb et al, Cancer Research *34*, 2311,1974.; Cairns, Nature, *255*, 197, 1975). A replication defective factor could generate an enhanced error rate in the clonal variants arising during tumor progression. However, increased mutation rate in tumor cells has not been demonstrated despite intensive efforts (Harris, Cancer Res. *51*, 5023, 1991).

The reproducible and quantitative amplification of many genomic sequences in a single and simple experiment by the Arbitrarily Primed PCR or AP-PCR (Welsh & McClelland, NAR *18*, 7213, 1990), is a powerful tool for the unbiased analysis of the genetic alterations accompanying malignancy. We have shown that both allelic losses and gains can be easily detected and characterized by AP-PCR genomic fingerprinting which thus provides an alternative molecular approach for cancer cytogenetics (Peinado et al, PNAS *89*, 10065, 1992).

Using the AP-PCR we have found that 12% of colorectal carcinomas have sustained somatic mutations in numbers that in some cases can be estimated by extrapolation to reach the million (Ionov *et al*, Nature, *363*, 558-561,1993). These mutations are deletions of one or a few nucleotides in monotonic runs of dA:dT base pairs, including the poly(A) tails of repetitive *Alu* sequences, and deletions and also insertions of a few base pairs in other simple repeats such as the polymorphic CA repeats and other microsatellite sequences. Somatic instability in microsatellites is significantly associated with an early cancer onset, with poorly differentiated carcinomas of the proximal colon, with tumors of blacks, and with carcinomas with low incidence of metastases at diagnosis and of *ras* and p53 gene mutations.

We concluded from these results that Ubiquitous Somatic Mutations (USM) in Simple Repeated Sequences (SRS) reveal a new molecular genetic mechanism for oncogenesis, corresponding to the *"cancer as a mutator phenotype"* hypothesis (Nelson and Mason, 1972; Loeb et al, 1974; Cairns, 1975). Because these clonal mutations were present from the most superficial to the most invasive regions of each of multiple tumors from the same individual, including adenomas, we concluded 1) that they are the consequence of a mutation in a gene coding for a DNA replication or repair factor that results in

decreased fidelity in any of these processes (a *"mutator mutation"*); 2) that this mutator mutation plays an ultimate causal role in tumorigenesis, and 3) that the USM tumors overlapped with tumors from the Hereditary Non-Polyposis Colorectal Cancer (HNPCC) Syndrome (Ionov et al, Nature, *363*, 558-561,1993).

How widespread are the USM tumors? HNPCC is not restricted to the colon but also occur (with less frequency) at other sites, mainly endometrium, ovary, stomach, esophagus and others (Lynch et al, Cancer Genet. Cytogenet. *143*, 160-170, 1991). In this context, we have found USM in simple repeats in carcinomas of the stomach and pancreas, but not in lung tumors. This negative result strongly supports the view that USM in simple repeats are the genomic phenotypic manifestations of the HNPCC Syndrome, because individuals from these hereditary cancer families show a lower risk for developing lung cancer (Lynch et al, *ibid*, 1991).

While the genomic instability in simple repeated sequences is likely due to a defect in replication/repair, the gene(s) responsible for this massive accumulation of mutations and the exact timing of their occurrence are unknown. It is also not clear whether this defect in replication or repair continues during tumorigenesis or whether the mutator phenotype could be deletereous to the cancer cell and therefore be selected against during tumor progression (a "hit and run" hypothesis).

We have determined the mutation rates of these sequences *in vitro* and *in vivo*. We have found that the instability in these repeated sequences persists in malignant cells of the mutator phenotype and that while USM in SRS are very early events in tumor development, they continue to accumulate after neoplastic transformation. Therefore, the "hit and run" hypothesis for the origin and maintainance of these mutations was ruled out. Because mutations in monotonic runs of As were always deletions of a single base pair, and in dinucleotide repeats the deletions and insertions of a single dinucleotide were more often than those of two, and these more frequent than those of three and so on, these results reinforce our interpretation (Ionov *et al*, 1993) that these mutations are generated by the slippage by strand misalignement mechanism (Streisinger *et al*, Cold Spring Harbor Sympos. Quant. Biol., *31*, 77-84, 1966).

We also have found that 1) Repeated sequences that undergo considerable expansions in some tumors behave identically as above, with deletions more frequent than insertions, and mutations of one repeat unit more frequent that those of more than one repeat unit; 2) The same CA repeat underwent deletions in other tumors; 3) The tumor with expansions in a particular microsatellite sequence had deletions in others; and 4) Trinucleotide repeats also showed the same behavior in cultured cells. Based on these results, we hypothesize that slippage is also the underlying mechanism responsible for the expansions of trinucleotide repeated sequences observed in hereditary triplet diseases such as fragile X, Huntington's disease and myotonic dystrophy (Richards and Sutherland, Cell, *70*, 709, 1992).

DNA-Amplification based genetic mapping and fingerprinting

J. Antoni Rafalski

Biotechnology Research, Agricultural Products Department, E.I. du Pont de Nemours and Co. (Inc.) P.O. Box 80402 Experimental Station, Wilmington, DE 19880-0402, Tel. (302) 695-4348

Several DNA amplification-based mapping, fingerprinting and diagnostic technologies became available, including random primer amplification (RAPD), microsatellite markers, alumorphs, AFLPs, etc. Utility and limitations of these different techniques will be reviewed, with emphasis on applications to plant breeding and germplasm analysis (Rafalski and Tingey, 1993).

RAPD Markers

DNA-amplification based RAPD markers were developed recently (Williams et al. 1990; Welsh and McClelland 1990; Caetano-Anolles et al. 1991)), and have been widely used for the construction of genetic maps (Reiter et al. 1992), for simplified identification of markers linked to traits of interest (Michelmore et al. 1991), for genetic fingerprinting (Welsh et al. 1991), for genetic diagnostics (Horn and Rafalski 1992) and in population genetics (Arnold et al. 1991). Several reviews (Rafalski et al. 1991; Waugh and Powell 1992; Tingey and del Tufo 1993) and a detailed discussion of the RAPD methodology (Williams et al. 1993) have appeared recently.

The RAPD reaction performed on genomic DNA with an arbitrary oligonucleotide primer results in the amplification of several discrete DNA products. These are usually separated on agarose gels and visualized by ethidium bromide staining. Silver staining or autoradiography of 33P or 32P-labeled RAPDs could also be used. The polymorphisms between individuals result from sequence differences in one or both of the primer binding sites, and are visible as presence or absence of a particular RAPD band. Such polymorphisms behave as dominant genetic markers. The requirement for small amounts of DNA (5-25 ng), non-radioactive assay that can be performed in several hours, and simple experimental set-up make RAPD markers attractive for automated breeding applications. The dominant nature of the RAPD markers has to be taken into account in designing experiments. A "prenatal" genetic diagnostic assay for oilseed rape breeding utilizing microspore-derived embryos has been described (Horn et al. 1992). RAPD allele identification techniques that do not require gel separation have been developed (our unpublished work).

RAPD markers are currently used in several map-based cloning efforts. Bulk-segregant analysis (Michelmore et al. 1991) permits identification of markers linked to a locus of interest. These markers are then ordered using fine recombinational and physical mapping. The RAPD bands of interest may then be cloned and partially sequenced (Paran and Michelmore 1993), permitting a PCR-based search for YAC, P1 or cosmid clones of interest.

Several conditions must be met to assure reproducible RAPD amplification: The concentration of the genomic DNA should be determined accurately, and the amount of DNA used in the assay should be uniform and well within the experimentally determined reproducibility range (usually 5-15 ng/20 µl reaction). The RAPD assay is sensitive to exact thermal profile of the thermocycler: If it is necessary to reproduce the results on a different machine, the actual thermal profile should be measure with a thermocouple, and the setting of both machines adjusted until the profiles are nearly identical. Quality of the polymerase (Taq or Stoffel fragment) should be consistent. Pipetting errors should be minimized, by avoiding pipetting volumes smaller then 5 ul.

It is relatively easy to turn the RAPD assay into a secondary PCR assay through DNA sequencing of the RAPD band of interest and conversion to allele-specific PCR (Wu et al. 1989), allele-specific ligation (Landegren et al. 1988; Nickerson et al. 1990) or a sequence characterized amplified region (SCAR) assay (Paran et al. 1993) Additional sequence polymorphisms may be detected in RAPD bands using restriction enzyme digestion (Williams et al. 1993). Techniques that scan for DNA sequence divergence, single strand conformational polymorphism assay (SSCP, Orita et al. 1989), or denaturing gradient gel electrophoresis (DGGE, Myers et al. 1987) may be applied to individual RAPD bands.

Microsatellites

Microsatellites are DNA sequences in the genome of many higher organisms, including humans and plants, composed of a tandem repetition of a simple short sequence motif, for example "CACACACACACACACACACA" . The most common are dinucleotide repeats, (CA)n, (CT)n, (AT)n. Microsatellites are very common, for example more then 30 000 copies of the (CA)n repeat are present in the genome of mammals. Microsatellites evolve faster then surrounding DNA, and as a result of this, they are very polymorphic. Providing the DNA sequence of the DNA surrounding a microsatellite is known and suitable PCR primers can be designed, the stretch of DNA incorporating the microsatellite can be amplified and its length determined by electrophoresis in acrylamide or agarose (Weber and May 1989; Litt and Luty 1989). Such experiments have shown that multiple allelic length variants can be identified at most microsatellite loci. This holds true even in notoriously non-polymorphic species, like soybean (Akkaya et al. 1992; Morgante and Olivieri 1993). Therefore, microsatellites provide a very powerful tool for genetic mapping and genetic diagnostics. Efforts are under way in several laboratories to develop microsatellite markers in plants. One of the advantages of microsatellite markers is they can be shared between laboratories by exchanging primer DNA sequences. Thus, microsatellite markers should provide for better information exchange. Microsatellite markers are also perfectly suited for use in map-based cloning, because identification of marker-anchored clones could be conveniently carried out by PCR.

Multiplex techniques

Several newly developed techniques, including alumorphs (Sinnett et al. 1990) and AFLPs (Zabeau and Voss 1993) permit simultaneous sampling of multiple loci distributed throughout the genome. The "multiplex ratio" of these techniques is high (50-100), higher then that of RAPDs (about 10). The advantage of high multiplexing has to weighted against the disadvantage of the need for a high-resolution separation method, like a sequencing gel, and relative complexity of the procedure (AFLP).

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RNA fingerprinting by Arbitrarily Primed PCR

John Welsh, David Ralph, Kwong Kwok Wong and Michael McClelland. California Institute of Biological Research. 11099 North Torrey Pines Road. La Jolla. CA 92037/

We have developed a novel approach to the detection and cloning of differentially expressed genes. This method is based on a modification of arbitrarily primed PCR (Welsh and McClelland, 1990; Williams et al., 1990). Here, we describe the methods and rationale behind the adaptation of arbitrarily primed PCR to the fingerprinting of RNA, thereby facilitating the detection of RNA transcripts that are differentially expressed. A similar method has been developed by Liang and Pardee (1992).

In RNA arbitrarily primed PCR (RAP), first strand synthesis is initiated from an arbitrarily chosen primer at those sites in the RNA that best match the primer. DNA synthesis from these priming sites is performed by reverse transcription. Second strand synthesis is achieved by adding Taq polymerase and the appropriate buffer to the reaction mixture. Once again, priming occurs at the sites where the primer finds the best matches. These products serve as templates for high stringency PCR amplification, which can be performed in the presence of radioactive label such that the products can be displayed on a sequencing-type polyacrylamide gel, as in arbitrarily primed PCR fingerprinting of genomic DNA (Welsh et al., 1992; Wong et al., 1993). Because priming is arbitrary in the first step the lack of poly-A tails in most bacterial RNAs is also not a problem for this method (Wong and McClelland, 1993).

Given two or more RNA populations, differences in the fingerprints will result when corresponding templates are represented in different amounts. However, while these fingerprints contain anywhere from 10 to 40 bands typical RNA populations for eukaryotic cells have complexities in the tens of thousands of molecules. Therefore, searching for a *particular* differentially expressed gene would be futile. Rather, the method is more appropriate for problems where many differentially expressed genes are anticipated. This is not so great a limitation as it might initially appear. First of all, many developmental and pathological phenomena are accompanied by many dozens or even hundreds of alterations in gene expression. Secondly, much of the technological development associated with sequencing, such as fluorescent tagged primers and automated gel reading, and capillary electrophoresis, is readily adaptable to RAP. Therefore, a large fraction of the genes expressed in many situations can be surveyed given existing technology, and this capability will be greatly enhanced by further technological

developments. There are, however, other intrinsic limitations that should be considered in the design of an experiment based on RNA arbitrarily primed PCR. Primarily, the influence of the abundance of messenger RNA on the fingerprint must be considered.

We have performed four demonstration projects using RAP:

(1) Differentially expressed RNA were identified in human ovarian carcinoma cells (Wong et al., 1993).

(2) Differentially expressed genes were isolated that were affected by treatment of mink lung epithelial cells with TGF-β, a treatment that stops cell division. Among the first three differentially expressed genes isolated (Ralph et al., 1993) was a homologue of a rat extracellular matrix protein, osteonectin, which had been previously demonstrated to be regulated by TGF-β and the homologue of cyclin-A, required for passage of mammalian fibroblasts through S-phase. This finding further supports a link between cyclin-A and TGF-β.

(3) Thirty genes that are differentially expressed in the mouse brain were cloned. We have further investigated two of these genes and confirmed that they are spatially and temporally regulated during brain development (Chada et al., submitted).

(4) Finally, we have used the method to isolate a gene that responds to peroxide stress in *Salmonella*. Peroxide stress is a method used by macrophage to kill invading bacteria. The gene occurs twice in the *Salmonella* genome and may be a surface protein involved in pathogenesis (Wong and McClelland, 1994).

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Differential Display of Messenger RNAs, a New Approach of Cloning Differentially Expressed Genes

Peng Liang

Dana-Farber Cancer Institute, Harvard Medical School, Boston, USA

Current methods to distinguish mRNA in comparative studies rely largely on subtractive hybridization. This technique, although it has been used successfully in isolating a number of important genes, is rather difficult to establish, irreproducible, and requires large amounts of RNA. We have recently developed an alternative approach, named mRNA differential display which circumvents these problems (1, 2, 3). The essence of differential display method is to use for reverse transcription an anchored oligo-dT primer which anneals to the beginning of a subpopulation of the poly(A) tails of mRNAs. The anchored oligo-dT primers consist of 11 or 12 T's plus two additional 3' bases which provide specificity. These are used in conjunction with a decamer oligodeoxynucleotide of arbitrarily defined sequence for the subsequent PCR amplification, as has been used previously for detecting DNA polymorphism by PCR (4). Amplified cDNA fragments of 3' termini of mRNAs were thus separated by size up to 500 bp on a denaturing polyacrylamide gel.

we examined in more detail the specificity of the anchored oligo-dT primers, the sensitivity limit of the method in terms of the amount of input RNA, reproducibility from run to run; and we compared total RNA versus poly(A) RNA as templates. We describe and demonstrate that highly reproducible patterns of differential display can be achieved using conditions optimized here with DNA-free total RNA samples. In addition, we incorporate as a built-in control for reproducibility of amplified cDNA band pattern displays of more than two relevant RNA samples simultaneously. Therefore, genes specific to a process, as an example, cell transformation caused by cooperation of oncogene ras and inactivation of tumor suppressor gene p53, can be readily identified.

The differential display method can offer several technical advantages over existing methods such as subtractive hybridization. 1. Simplicity: technically it is based on PCR and DNA sequencing gel electrophoresis. 2. Sensitivity: 2 μg of total RNA is enough to cover the four anchored oligo-dT primers used in all combinations with 20 arbitrary 10mers. Statistically, this would cover about 50% of the mRNA. 3. Reproducibility: up to 95% of the bands of mRNA display are reproducible from run to run. 4. Versatility: more than two RNA samples can be compared at one time, revealing genes unique to a process, such as a type of cancer, instead of cell type specific genes. characterization of the gene is therefore built-in. Also both dominant genes such as oncogenes or recessive genes such as tumor supressor genes can be detected simultaneously. 5. Speed: within two days the pattern of mRNA display can be obtained. Reamplification of probes and Northern blot confirmation take another week. 6. Most importantly, since the assay can be checked at each step, it is no longer necessary, as with other methods, to wait until the end of the procedure to determine whether it worked.

With continuing refinements and optimization of the method and great enthusiasm from the research community at large, it is expected that many important new genes will be discovered by differential display.

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Alu PCR: A Method for Rapid Access to Human DNA of Unknown Sequence.

David L. Nelson
Institute for Molecular Genetics
Human Genome Center
Baylor College of Medicine
Houston, TX 77030 USA

The development of the polymerase chain reaction (PCR) has revolutionized the practice of molecular biology. Amplification using known sequences from complex genomes allows ready access to the nucleic acids without the tedium of preparation and screening of recombinant libraries. This aspect of the PCR has expanded greatly our ability to sample numerous genomes for variability at the DNA sequence level. However, the requirement of specific oligonucleotide primers directed to known DNA sequence limited the PCR to regions previously characterized in fine detail. The development of oligonucleotide primers directed to repetitive sequences has allowed PCR-based amplification of DNA fragments with no prior knowledge of the region to be isolated. In the case of the human, this is known in the short-hand as "Alu PCR" (Alu is the most common repetitive element found in the human genome, and the sequence that has received the most attention for this purpose), although a more formal term, "Interspersed Repetitive Sequence PCR" (IRS-PCR) has also been proposed.

Repeated DNA sequences comprise between 25 and 30% of the human genome. Two elements have been used in the studies described here to generate oligonucleotide primers for PCR amplification. These are the Alu and LINE-1 families of repeats.

The Alu repeat sequence has been extensively studied, as it alone composes ~10% of the human genome. Approximately 900,000 copies of the Alu repeat are present in the haploid genome, with each copy slightly less than 300 nucleotides in length. Thus, nearly 3 x 10^8 base pairs of human DNA are Alu repeats, with one repeat found every 4000 bp on average. The repeat is a dimer of two ~130 bp monomers, usually separated by a poly A sequence. The second monomer in human and other primate Alu repeats contains a 31 bp insertion not found in the first monomer. The 3' end of the Alu also demonstrates a poly A tract. Alu-equivalent repetitive sequences are also found in other mammalian genomes. In the mouse genome, the B1 and B2 repeats have been best characterized. These are single 130 bp repeats, and are also polyadenylated. These mouse repeats occur less frequently than the human Alu, with one found on average every 8-10 kb.

The human LINE-1 element (L1Hs), also referred to as the Kpn or Hind III (1.8 kb) family, is repeated 10,000 to 100,000 times in the haploid genome in either a complete or truncated form of a 6.4 kb sequence. Due to the frequent truncation, fewer copies of the 5' end of the repeat are found, so that probes derived from the two ends of a complete copy recognize a variable number of fragments in the genome. As with the Alu repeat, the likely mode of dispersal of elements is reverse transcription of RNA transcripts derived from active copies of the repeat followed by integration into novel genomic sites. The presence of poly A sequences at the 3' end of L1Hs members supports this explanation. Significant sequence divergence is found between L1Hs and its equivalents in rodent genomes (65% identity between mouse and human consensus), however the general organization is similar. A 208 bp region in the 3' end of the human L1Hs is conserved among copies of the human repeat, but is not found in repeats from other species.

The original inspiration for Alu PCR was to enable direct amplification and isolation of human DNA sequences contained in somatic cell hybrids retaining specific human chromosomes in rodent cell backgrounds. Prior to the development of PCR, access to human DNAs in a chromosome or region specific manner was achieved through preparation of recombinant libraries from DNA

prepared from somatic cell hybrids followed by identification of human clones using species-specific repeat sequence probes or by sorting of specific chromosomes and library preparation. While the former method is limited to specific chromosome regions only by the hybrid cell lines available, it does require the preparation of a complete library for each hybrid cell line. Chromosome sorting can provide whole chromosome libraries; however it requires very specialized resources. Alu PCR allows direct access to a portion of the sequences present in somatic cell hybrids, and is limited by the available hybrids. The ability to identify DNA fragments rapidly from specific chromosomal regions is an important and necessary tool for positional cloning of disease and other loci.

Alu PCR products are being used increasingly to identify large insert clones from total human libraries. Recent efforts have allowed isolation of yeast artificial chromosome (YAC) and cosmid clones in a region-specific manner using complex probes composed of Alu PCR products derived from somatic cell hybrids. New methods for isolation of clones using pooling schemes for library screening have also been developed revolving around the Alu PCR technique. These offer rapid and inexpensive access to YAC libraries as well as a facile method for walking in YACs.

The extension of the Alu PCR method to cloned DNA allows more rapid analysis of clones and provides ready access to the cloned material without the need for DNA preparation or subcloning. This is most readily seen in the case of yeast artificial chromosomes (YAC), where large fragments (100-1000 kb) of human DNA can be isolated, but are difficult to work with due to the complexity of the host cell (15 Mbp), and the lack of efficient means to separate the cloned DNA from that of the host. Alu PCR allows human insert DNA to be isolated from crude lysates or purified DNA from YAC-containing yeast cells. With the use of primers directed to vector sequences, vector-adjacent fragments can be generated from a high percentage of YAC inserts. Once again, Alu PCR only provides a small fraction of the insert sequences, but these fragments can serve as probes for a variety of purposes, such as clone localization in somatic cell hybrids or by *in situ* hybridization and identification of overlapping clones.

While the number of Alu repeats in the human genome predicts the presence of an element roughly every 4 kb, this is high enough to have been useful in the identification of human-containing lambda clones from recombinant libraries derived from somatic cell hybrids. An estimated 95% of 18 kb inserts contains one or more Alu repeats. Taking advantage of this frequency, Alu-vector PCR in lambda and cosmid clones has also been developed. Again, the method provides sub-fragments of the insert in the recombinant clone that can be used for a number of purposes. While methods for the isolation of insert sequences from lambda and cosmid clones exist in abundance, the Alu-vector PCR method is very fast and simple, and can be accomplished with very crude materials (such as scraped colonies or picked plaques).

Continued development of the PCR and Alu PCR will clearly continue to advance our ability to isolate and analyze human DNAs in a variety of settings. The recognition that repetitive sequences can be used to advantage in PCR has also led to applications in species other than human. One example is repetitive element sequence (rep-PCR) in bacteria. Using interspersed repeats, it is now possible to rapidly type bacterial isolates for strain differences. Rep- PCR will be an important adjunct to standard microbiological methods for identification of bacterial infections.

COLLECTION OF POLYMORPHIC SEQUENCE-TAGGED SITES

Kenshi Hayashi
Division of Genome Analysis, Institute of Genetic Information
Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka, Japan

1. PCR-SSCP: ITS SENSITIVITY

In PCR-SSCP, mutations are detected as difference in gel-electrophoretic mobility between sample and reference DNAs which are PCR-amplified and single-stranded. The mobility shift by mutation is believed to be caused by altered conformation of the single stranded mutated DNA. Experiences in several laboratories suggested that most one-base substitutions are detected by this technique, but the sensitivity of PCR-SSCP remained to be evaluated. Main difficulty in this evaluation is that the sensitivity should depend on the context of target sequence, and it is unclear how many sequence contexts (and how many mutations) need to be tested before the sensitivity for the new can be estimated in reasonable accuracy. Within this limitation, I tried to collect available data on percentage detection of mutations which has been known by other techniques, from various laboratories that have positive results with this technique. From these data, I conclude that for the fragments shorter than 300 bp, PCR-SSCP will detect more than 90 % of mutations.

Data on sensitivity of PCR-SSCP are still limited for longer fragments. We, in collaboration with Dr. S. Sommer, Mayor Clinic, tested PCR-SSCP using 31 mutations in 6 different sequence contexts in 339 to 797 bp fragments, all within the Factor IX gene. We could detect more than 70 % of the mutations in fragments longer than 500 bp (16 mutations in 3 sequence contexts) using one electrophoretic condition.

PCR-SSCP is a powerful tool in estimating possible diversity of human genome. Sequence difference between individual is infrequent, and a large genomic area of many individuals must be searched for reliable estimation of overall variability of the genome. PCR-SSCP can survey about 10 kb of the genomic sequence for possible mutations in a few hours of electrophoresis (30 lanes, each 300 bp DNA). We examined diversities of a total of 525 kb of *Alu* repetitive sequences and 102 kb of non-transcribed single copy sequences, and obtained 0.06 % and 0.03 %, respectively, of heterozygosity at the nucleotide level. These values are at least one order of magnitude lower than the previously estimated values.

2. OBTAINING POLYMORPHIC SIMPLE-REPEAT MARKERS

Construction of detailed linkage map heavily depends on efficient collection of PCRable polymorphic DNA markers. CA-repeats

are the major source of such markers because they are abundant in the human genome and many of them, when they are long, are highly polymorphic in their lengths. Typically, primer sequences that amplify individual $(CA)_n$ are obtained by first screening genomic clones carrying the repeats by hybridization, and secondly sequencing flanking regions of these repeats. We found that under conventional conditions of hybridization, most of the obtained clones have unexpectedly short and monomorphic $(CA)_n$. By using a long $(CA)_n$ probe and increasing stringency of hybridization we could obtain clones, virtually all of them carrying $(CA)_{14}$ or longer. We also established an efficient strategy for determining the flanking sequences using mixed primers. These screening and sequencing methods should also be applicable for retrieval of polymorphic simple-repeat sequences other than $(CA)_n$.

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Microsatellite markers: tracers of gene mutations and evolution

X. Estivill, N. Morral, M.I. Banchs, A. Bosch, J. Guimerà, A. Puig, R. Llevadot
Molecular Genetics Dept., Institut de Recerca Oncològica, Barcelona.

Microsatellite markers, or short tandem repeat polymorphisms (STRPs), have proved to be excellent tools in genetic analysis. The large number of alleles detected at microsatellite loci, their high heterozygosity and the easy and uniform method of analysis, by means of the polymerase chain reaction (PCR), have made these markers extremely useful in human gene mapping, paternity testing, forensic analysis, diagnosis of genetic disorders by linkage, loss of heterozygosity studies in cancer, and hemizygosity detection of deletions in inherited genetic disorders. Although tetranucleotide repeats are less represented in the genome than CA-repeats, the interpretation of allele sizes is easier with tetranucleotides, than with dinucleotide repeats. However, the generation of human genetic linkage maps is mainly being accomplished using CA-repeats, analysed in the large three-generation families distributed by CEPH (Centre d'Etude du Polymorphisme Humain).

One interesting point about microsatellites is the mechanism of generating variability and their mutation rates. We have analysed 40 CEPH families with 29 microsatellites (26 CA-repeats, a TCTA-repeat, a TTA-repeat and an AAAT-repeat) and have detected 21 cases of abnormal segregation of alleles for a total of 14 markers (48%). In 11 cases the abnormal transmissions were of somatic origin, 10 of which (91%) occurred in the lymphoblastoid cell lines. In 9 other cases it was not possible to determine the germline or somatic origin of the new alleles, and in one case hemizygosity in several family members was observed, which was germline in origin. The 20 new mutations detected in the 22,852 meiosis analysed represent a mutation frequency of 8.7×10^{-4} per locus/gamete/generation. The germline mutation rate could be as high as 3.9×10^{-4} (from 0 to 3.9×10^{-4}), but the rate of somatic mutations detected in the study was much higher (4.8×10^{-4} to 8.7×10^{-4}). Individual mutation rates ranged from 0 to 3.8×10^{-3}. Among the markers analysed, all three that were tri- or tetra-nucleotide repeats showed one or two new alleles, compared to only 15 of the 26 (58%) CA-repeats showing mutations. These results and previous data, suggest that the mutation rate at microsatellite loci within families, using DNA directly obtained from cells from the individual, is of less than 1×10^{-4} (true germline mutation rate), which should not affect the use of these markers in diagnosis and linkage. However, for DNA obtained from cell lines mutations are much more frequent (1×10^{-2} to 1×10^{-3}).

Another issue of interest for microsatellite markers, is their potential use in tracing the origin of disease mutations and their application to evolution studies. We have addressed this to the study of the most common mutation in the Caucasoid population: cystic fibrosis (CF), which affects 1 in 2,000 to 4,000 individuals. More than 350 mutations have been detected in the *CFTR* gene, most of which being uncommon in the world population. However, a deletion of 3 base pairs at codon 508 (ΔF508) is the most frequent one and accounts for about 70% of worldwide CF chromosomes. The high frequency of the ΔF508 mutation (which has a single origin) and the variability observed at three highly polymorphic microsatellite markers, within the *CFTR* gene (IVS8CA, IVS17BTA and IVS17BCA), provide an exceptional resource for human evolution studies. We have analysed 1,738 ΔF508 chromosomes from 15 European regions, for *CFTR* microsatellites identifying a total of 54 haplotypes, of which only 4 (23–31–13, 17–31–13, 17–32–13, and 23–32–13) are common and present in almost all populations. The reconstruction of the evolution of ΔF508 chromosomes by microsatellite haplotype variation shows that haplotypes derive from the original ones by slippage and mispairing at one of the three loci or, exceptionally, by recombination. The evolution of ΔF508 haplotypes was traced from the original through a maximum parsimonia analysis, showing that the ancestral ΔF508 haplotype was 23–31–13. Different mutation rates at microsatellite loci allowed us to calculate that the ΔF508 mutation arose at least between 65,000 and 215,000 years ago. Comparison of microsatellite haplotypes for ΔF508 and normal chromosomes shows that genetic distances are high, suggesting that the ΔF508 originated in a population different from the actual European. Haplotypes show two geographical groups that are genetically different: a) the Mediterranean area and the British Isles; and b) the Central and North Europe. The mutation was introduced in Europe at different periods. The first (about 40,000 years ago) was mainly associated with the ancestral haplotype 23–31–13, but also with haplotypes 17–31–13, 17–32–13, and 23–32–13. A second expansion (probably at the Neolithic age) gave rise to the pattern observed in Central and Northern Europe, mainly associated with haplotypes 17–31–13 and 17–32–13, and to a lesser extent with haplotypes 23–31–13, and 23–32–13. The origin of other common CF mutations can also be traced using microsatellite haplotypes. Haplotype changes for G542X (haplotype 23–33–13) and N1303K (23–31–13) suggest that they originated at least 35,000 years ago, whereas other mutations (R1162X, 1609delCA or 3601-111G→C) have suffered less variation at their microsatellite haplotypes, suggesting a more recent origin.

Establishing a high resolution/high density molecular genetic map of the mouse genome

Jean-Louis Guénet[1], Xavier Montagutelli[1], Dominique Simon-Chazottes[1], Cécile Julier[2], Rachel D. Sheppard[3], Lee M. Silver[1,3] and Tadao Serikawa[1,4].
[1]Unité de Génétique des Mammifères, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France. [2]Centre d'études du polymorphisme humain, 27 rue Juliette Dodu, 75018 Paris France. [3]Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, U.S.A. [4]Institute of Laboratory Animals, Faculty of Medicine, Kyoto University, Sakyo-ku, Kyoto 606, Japan.

Over the last twelve years our laboratory has been much involved in the development of a high resolution, high density genetic map of the mouse genome. Such a map is essential for many purposes and in particular for the subsequent establishment of the physical map which requires that as many molecular markers as possible be precisely ordered on each of the 20/21 mouse chromosomes. The ideal situation would be attained if the average distance between any two contiguous markers was in the range of 0.2 to 0.3 cM because this distance is equivalent (in the mouse) to the average size of a standard Yeast Artificial Chromosome (i.e. 400/600 kbp). However such an ideal map is difficult and expensive to achieve, even with the mouse, because it requires that 1000 to 1600 offspring of highly polymorphic crosses be typed for thousands of molecular markers. Mouse geneticists consider nowadays that a map having 2800/3000 well spread markers would be of considerable help. To reach this goal, and given that the laboratory mice are closely related to each other, we have first developed a strategy based on an interspecific backcross with mice of the wild species *Mus spretus*; then we have applied to the offspring of this cross some of the techniques used for detecting polymorphisms which had already been successfully used in other species.

These techniques were of four kind:

1°- RAPDs. We have used short sized oligonucleotides (10-mers), with 50-70 percent (G+C) content, to perform single primer PCR amplification of genomic DNA templates and we have analysed each of these primers independently, or by pairs with several DNA samples. We have found that half of the primers generated strain or species specific polymorphic products (ranging from 0.3 to 0.6 kb) when used individually and we have been able to localise most of them on the mouse genome. We have also found that when used by pairs these primers generated additional polymorphic products.

This technique offers the major advantage of providing geneticists with an almost unlimited number of markers, at very low cost, but it has the drawback that a given product may be observed in one strain or species, without any

equivalent detected in others. We also found that PCR conditions have a great influence on the results and should be kept constant to obtain reliable and reproducible results.

2°- MINISATELLITES. We have tested several human variable number of tandem repeat probes and we have found that, among laboratory strains, about half of the latter produced fingerprint patterns potentially useful for the establishment of genetic maps. The combination of several restriction enzymes indicated that polymorphic markers could be observed for at least 90% of the linkage groups.

3°- MODERATELY REPEATED SEQUENCES. Proviral copies and probes identifying pseudogenes have proved useful tools for the localisation of new mutations but exhibit few advantages for the establishment of molecular maps.

4°- 2-DIMENSIONS ANALYSIS OF DNA WITH HIGHLY REPEATED PROBES. We have experimented with a specially designed electrophoretic apparatus and an experimental protocol making possible the analysis of individual members of complex gene families by gel fractionation in two dimensions. This technique has proved reliable and easily applicable to resolve gene families with tens of copies within the genome. With highly repeated probes, such as retroviral like DNA families, the situation is more complex and requires the help of computer program for the analysis of the autoradiograms. It is also similar to what we observed with the VNTR or RAPDs in the sense that the identification of equivalent spots in different experiments requires internal controls.

CONCLUSION. With the exception of the moderately repeated sequences (proviral copies and probes identifying pseudogenes), which are helpful only for the rapid assignment of new mutations in the mouse, all the techniques for genomic fingerprinting which we have used have proved relatively reliable and useful to some extent for the establishment of genetic maps. However, as we shall discuss, none of them can seriously compete with microsatellites in the present status of development of the mouse map.

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DNA fingerprinting for mouse genome analysis: applications for genetic and physical mapping, genetic quality control programs, and developing a Gene Mapping Resource Panel

Joseph H. Nadeau, Hedrick Bedigian, Terese Denial, Beverly Paigen, Lucy B. Rowe, Rowena Turner, Edward H. Birkenmeier. Jackson Laboratory, Bar Harbor, Maine

Introduction Short PCR primers of arbitrary nucleotide sequence have many applications for mouse genome analysis. We continue to explore new ways of using these primers and we have applied several of these to enhance genetic and physical mapping studies, genetic quality control programs, and gene mapping resource panels. These assays and resources are a valuable complement to the existing repetoire of mapping reagents and methods.

Genetic mapping with arbitrary primer PCR We previously demonstrated the utility of these markers for enhancing the genetic map of the mouse genome. This well-documented approach will be reviewed briefly.

Optimized PCR conditions We explored alternative primer characteristics and PCR conditions. Longer primers (16-18 bp) tended to give more reproducible results than shorter primers (10-12 bp). We also found that different PCR products could be obtained at different annealing temperatures. Some products were obtained at all temperatures tested, whereas occurrence of other products depended strongly on annealing temperature. In general, longer primers and higher temperatures gave more reproducible results without significantly reducing the number of PCR products.

Physical mapping Because of their ability to amplify genomic segments regardless of genome size, we tested whether short arbitrary primers could be used for physical mapping. For example, if PCR products could be obtained from individual large insert clones such as YACs, they could could be used for establishing patterns of clone overlap and for obtaining mapping reagents directly from clones of interest. We tested this approach by determining whether unique PCR products could be obtained from mouse YACs. Individual YACs were isolated from PFGE gels and used as template for PCR reactions. PCR products were readily obtained and each YAC yielded a unique combination of products. In principal, this approach should provide a rich resource of mapping reagents for positional cloning projects.

Genetic quality control programs An important limitation of existing methods for genetic quality control programs is their ability to distinguish closely related strains. Isozyme variants are powerful, inexpensive reagents that distinguish most strains of mice. Histocompatibility tests distinguish many strains. Simple sequence length polymorphisms (SSLPs) distinguish most strains, but substrains often share indistinguishable variants. These reagents rarely if ever distinguish closely related substrains. To test the utility of short arbitrary primers, we used a panel of primers to survey representative inbred strains of laboratory mice. With a modest number of primers, we identified a combination of PCR products that defined a unique fingerprint

for most strains. For example, variants were identifed that distinguished the A/HeJ and A/J substrains and the CBA/CaJ and CBA/J substrains. Surveys are underway to identify primers that distinguish the remaining strains and to define a set of primers that maximize informativeness.

MOTIF PCR

The Birkenmeier laboratory modified arbitrarily-primed PCR by using sequence-specific oligonucleotide primers derived from conserved promoter elements and protein motifs. The primary consideration in primer selection was conservation of sequence and function in evolution. Examples were promoter elements of the glycerol-3-phosphate dehydrogenase gene, the TATA box region, fat specific elements (FSE), and a C/EBP alpha transcription factor footprint site. Also included were protein structural motifs such as the rat *Isl-1* LIM motif and homeodomain, the mouse and rat CRIP zinc finger, and the leucine zipper and basic regions of transcription factors from the C/EBP gene family. Inheritance of 27 polymorphic fragments was followed in recombinant inbred strains and linkage was readily detected, demonstrating that these follow Mendelian inheritance. It remains to be determined whether these are amplifying random sites or instead whether they show annealing preferences for specific sequence motif elements.

Jackson Laboratory Mapping Resource

We have established a pair of gene mapping resource panels for the biomedical research community. These panels consist of genomic DNAs (~30 mg DNA per mouse) for 188 progeny of complementary interspecific backcrosses - (C57BL/6J x *Mus spretus*)F1 females crossed to C57BL/6J males (N=94) and (C57BL/6J x SPRET/Ei)F1 females crossed to SPRET/Ei males (N=94). These panels have already been typed for selected SSLP loci from the MIT Center for Genome Research (N=43, 49), genes and anonymous loci (N=32, 114), and MOTIF PCR loci (N=140, 276). Aliquots of DNA from this panel are being distributed to the community without obligation of coauthorship. All loci recently analyzed showed very strong linkage (≤ 3 recombinants), demonstrating that marker density is adequate for reliably detecting linkage. Map density is also sufficient to reliably identify anomalous results that probably indicate typing errors. Each newly mapped locus contributed to the database engenders a report to the investigator including relevant closely linked loci in the public database, a graphical representation of the chromosomal segment, and a statistical analysis of the mapping data. Data privacy is provided on request. Information about using these panels for mapping projects can be obtained from Lucy B. Rowe at the Jackson Laboratory (email address: lbr@aretha.jax.org).

TUESDAY, October 26th

Fingerprinting of two genera of pathogenic spirochetes (*Leptospira* and *Borrelia*)

par G. Baranton (*)

Introduction

Pathogenic *Leptospira* and Lyme disease *Borrelia* are very different spirochetes. *Leptospira* are strictly aerobic bacteria susceptible to long term survival in fresh aquatic environment. Their nutritional requirements are limited to fatty acids for carbon and energy sources and ammonium salts for nitrogen requirements. *Leptospira* are usually responsible for an inapparent infection among wild animals (mainly rodents) leading to a chronic bacterial urinary excretion, while they cause an acute disease in humans and cattle. From the taxonomic point of view, pathogenic *Leptospira*, known since the First World War, constituted a nomenspecies, *Leptospira interrogans*, further divided into more than 200 serovars. Since 1987, seven genomic species have been delineated.

In contrast, *Borrelia burgdorferi* is a strictly parasitic microaerophilic bacterium transmitted by acarian vectors. The disease is clinically inapparent among wild animals. In humans, *B. burgdorferi* leads to a potentially chronical and polymorphic disease. It is a highly exacting bacterium with regards to in vitro growth. The bacterium, first isolated in 1982, was until recently considered as constituting a unique species. In order to characterize and type fresh isolates, many strains were submitted to diverse molecular typing methods.

Ribotyping

The first genetic typing method used was restriction enzyme analysis of chromosomal DNA by fixed-field gel electrophoresis. However, Restriction Fragments Length Polymorphism consisted of such a large number of bands that it hampered its usefulness. A significant improvement was the use of ribosomal ribonucleic acid (rRNA) gene restriction patterns. The conserved nature of rRNA genes allowed the use of a single universal probe such as (16S+23S) RNA.

Among the 50 *Borrelia* strains DNA tested (restriction by 4 enzymes), 3 main ribotypes were recorded.

For *Leptospira*, results were surprising since the 169 studied serovars exhibited 98 distinct profiles with *Eco*RI. Strikingly, there was a high heterogeneity in the size of the hybridized fragments. This seemed to be inconsistent with the usually characteristic number of *rrn* operons in a bacterial species. Along these lines, a Japanese team demonstrated that indeed no ribosomal operons were found in both *Leptospira* and *Borrelia* but instead diversely associated (or not) sets of each RNA genes.

(*) Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 28, rue du Docteur Roux, 75724 Paris Cedex 15, France.

Pulsed Field Gel Electrophoresis

The advantage of Pulsed Field Gel Electrophoresis after restriction by a rare-cutting enzyme is the obtention of Large Restriction Fragment Patterns consisting in a small number of bands quite amenable to analysis. Furthermore, the study of undigested spirochetal DNA by Pulsed Field Gel Electrophoresis allowed the discovery of the linearity of the small Borrelial chromosome (950 kbp) and of the presence of two chromosomes (4500 and 350 kbp) for *Leptospira*.

*Mlu*I was the enzyme of choice for *Borrelia* DNA. 15 different profiles were obtained for 46 strains analysed. For *Leptospira*, the largest chromosome was analysed by *Not*I. The results showed an extreme diversity since 162 distinct profiles corresponded to the analysis of 170 serovars. In addition, there was, for a given serovar, a high level of genome conservation in space and time, providing thus a reproducible typing system.

Arbitrarily primed PCR was then applied to both genera. The method consists of 2 low stringency amplification cycles with a unique arbitrarily chosen primer followed by the usual further 30 cycles with radiolabelled nucleotides. With such conditions, reproducible amplicons are generated even when cross-matching with the target DNA is imperfect.

Thirty *Borrelia* strains studied with 3 primers led to 54 characters, 34 of which were shared by 2 or more strains. A phylogenic analysis concluded that there were 3 clusters of strains which were shown to correspond to a species level by DNA/DNA hybridization.

Concerning *Leptospira*, 48 serovars representative strains exhibited higher pattern diversity, so that apomorphic characters were insufficient to draw phylogenic trees. Since, whatever the method used, it was not possible to infer phylogenical or taxonomical conclusions for *Leptospira*, the following technique was used.

Mapped Restriction Site Polymorphism (MRSP)

By using bacterial RNA consensus primers for *rrs* and *rrl* *Leptospira* genes, amplification of the concerned genes (without flanking regions) was obtained and submitted to restriction enzymes known to cut ribosomal genes. Since *rrs* and *rrl* sequences are available, site positions could be precisely localized and therefore analysed by phylogeny software. Among the 172 sites (throughout 700 bp) examined, 22 were polymorphic. The data lead to a phylogenetic cladogram which correlated with the seven genomic species now described. A subgrouping inside species is also obtained.

Two polymorphic characters were not due to point mutations but to pure length polymorphism. They correspond to insertion sequences ranging from 485 to 740 bp at base 1230 in the *rrl* gene. Being present only in some strains of some species, it suggests that the intervening sequences are specific mobile elements.

In conclusion, using genomic typing methods to identify spirochetes strains, it was possible to solve classification and phylogenic problems at different levels (species and infraspecies). In addition, unexpectedly some paradigmatic features of bacteria, such as the presence of a single circular chromosome, the organisation of ribosomal genes in operons, were shaken by the results obtained.

Studies of molecular epidemiology and microbial genome organization: *Escherichia coli* and *Helicobacter pylori*.

Douglas E. Berg, Natalia Akopyanz and Nick O. Bukanov. Dept. of Molecular Microbiology, Washington Univ. Medical School, St. Louis, MO 63110 USA

We wish to better understand microbial evolution and population structure and also factors governing bacterial genome organization. In the case of pathogens, we wish to gain new insights into how they spread among susceptible hosts, their modes of colonization, and events and mechanisms leading to fulminant or long term chronic infections. The arbitrary primer PCR (AP-PCR or RAPD) DNA fingerpinting method (Welsh and McClelland, 1990; Williams et al., 1990), often in combination with restriction analysis of specific PCR amplified DNA segments (PCR-RFLP), have been immensely useful for these studies.

Much of the prior work in bacterial population genetics has been based on multilocus enzyme electrophoresis (MLEE), which detects a significant fraction of allelic differences among genes encoding housekeeping enzymes. In collaboration with T. Whittam and C. M. Berg, we calibrated RAPD fingerprinting vs. MLEE typing using diarrheagenic *Escherichia coli* strains of known MLEE type. *E. coli* strains from 15 different groups, each group consisting of isolates that were not distinguishable by MLEE typing with 20 different enzymes (Whittam et al., 1993), were studied with five arbitrary primers. Each primer reproducibly distinguished at least 53 different types. The results with the five primers distinguished 74 of the 75 strains, overall. This indicates that RAPD typing is much more sensitive than MLEE typing. Analysis of polymorphic RAPD fragments may provide a critical test of models in which bacterial (*E. coli*) populations that are postulated to be essentially clonal, with no significant transfer of chromosomal genes among lineages.

Derivatives of *E. coli* K-12 representing the divergent branches of its pedigree (Bachmann, 1987) have been studied in collaboration with I. Brikun. The ancestry of these strains is known, and they can be considered an example of "epidemic" spread and divergence from a single source beginning some 50 years ago, without gene transfer from other lineages. In RAPD tests, eight polymorphisms were found among 150 bands scored; six bands were in accord with the pedigree, but two seemed to disappear and then reappear in individual lineages. These anomalous bands may reflect evolution in stab cultures in the early years of bacterial genetics. Additional tests were carried out with isogenic strains that differed by up to ~ 11% in genome size, due to a 340 kb chromosomal deletion and two ~100 kb plasmids. Of 78 bands scored in the profiles from the strain with the larger genome, nine were absent from profiles of the strain with the smaller genome. Three of these bands were plasmid-derived, and six were from the deletable chromosomal segment.

Most of our other work has focused on *Helicobacter pylori*, a bacterium that eventually establishes chronic infection in the stomachs of most humans. Although such infections are often asymptomatic, they can also lead to severe gastritis, and constitute a major risk factor for peptic ulcers and gastric cancer (for reviews, Blaser, 1993; Graham and Go, 1993).

RAPD analysis of independent *H. pylori* isolates from patients at a single hospital indicated an amazingly high level of diversity: each of 60 isolates was distinguishable with just a single primer. Restriction analysis of the 2.4 kb *ureAB* segment distinguished 45 different types among these 60 isolates. Most isolates not distinguished using this segment could be distinguished easily using other DNA segments (e.g., *flaA*, *ureCD*) (Akopyanz et al., 1992b).

Classical fulminant infections are generally considered to result from clonal proliferation of just a single bacterial cell that possesses appropriate virulence traits, and that by chance breached or avoided host defenses. In the case of chronic long term infections, such as those caused by *H. pylori*, evolutionary considerations make it important to learn whether these too are dominated by just one clone. Or, do they often reflect colonization by multiple strains, at least among persons likely to be exposed repeatedly (e.g., in developing countries, and among the very poor in our own society). We have thus begun to characterize *H. pylori* isolates taken from initial and followup biopsies of the same person. In experiments to date:
(i) the same strain was found in each of two biopsies from each of three patients in West Virginia (Akopyanz et al., 1992a,b);
(ii) two strains were found in sequential biopsies of each of three of four low-income patients in Lima, Peru (collaboration with R. Gilman, PRISMA); and
(iii) two different strains were found in three of seven patients from southern Sweden (their surnames suggested that they were from Eastern Europe) (collaboration with J. Lelwala-Guruge and T. Wadström). One pair of these strains exhibited RAPD and PCR-RFLP patterns suggesting earlier horizontal DNA transfer; and
(iv) in a gnotobiotic piglet experimental infection model (Eaton et al., 1992), two strains were detected in eight of eleven pigs infected with these two strains, but three piglets seemed to carry just one strain. Some biopsies from mixedly infected pigs seemed to contain just one strain, suggesting bacterial growth as microcolonies with relatively little mixing (collaboration with K. Eaton).

We conclude that mixed *H. pylori* infections are commonplace when the risk of infection is high, and that the critical stage of establishment of mixed infections can be modeled in the gnotobiotic piglet. Given the diversity of natually occurring *H. pylori* isolates, the recently demonstrated ease of DNA transformation in vitro (Haas et al., 1993; Segal and Tompkins, 1993; Suerbaum et al., 1993), and the ability of multiple strains to colonize a given host, we propose that horizontal gene transfer in mixed infections in vivo may contribute significantly to the long term adaption of *H. pylori* to individual hosts and the emergence of drug resistance.

In related studies, we constructed a high resolution genetic map of *H. pylori* strain NCTC11638 by: (i) generating a 20-fold redundant library of ~40 kb cosmid clones from genomic DNA of this *H. pylori* strain; (ii) ordering the cosmids, primarily by hybridization to cosmid-containing colonies and to pulse field gel separated genomic NotI fragments; and (iii) mapping all known *H. pylori* genes to these cosmids. The library was inferred to cover more than 90% of the genome, and a 68-member cosmid miniset that is representative of the entire library was identified (Bukanov and Berg, 1993). Interestingly, the order of *H. pylori* genes in the strain we mapped was quite different from the order in another unrelated strain reported by Taylor et al. (1992).

We are beginning to use DNAs from the cosmid library to examine growth phase regulation of transcript abundance. In initial experiments we have hybridized Southern blots of HindIII digested DNA from each cosmid in the 68-member miniset with labelled RNA from exponential and from stationary phase *H. pylori* cells. More than 20 DNA fragments have been identified whose corresponding transcripts are either significantly more or less abundant in stationary phase than in exponentially growing cells. This result illustrates that in the case of small bacterial genomes, a direct hybridization approach constitutes a useful alternative to the technically demanding method of arbitrary primer PCR fingerprinting of RNA (Welsh et al., 1993). More generally, we anticipate that sequence and mutational analysis of differentially expressed DNA segments will help identify factors important in colonization and disease.

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SEQUENCE-SPECIFIC "GENE SIGNATURES" CAN BE OBTAINED BY PCR WITH SINGLE SPECIFIC PRIMERS AT LOW STRINGENCY

Andrew J.G. Simpson[1], Guillermo Barreto[2], Annamaria R. Vago[2], Emmanuel Dias Neto[1], Otavia L.S. Damas de Caballero[1,3], Luisa L. Villa[4] and Sergio D.J. Pena[2,5].

1-Centro de Pesquisas "René Rachou", Fundação Oswaldo Cruz, 31090-002 Belo Horizonte, Brazil; 2-Departamento de Bioquímica, Universidade Federal de Minas Gerais, 30270 - Belo Horizonte, Brazil ; 3- Departamento de Ciências Biológicas, Faculdade do Triângulo Mineiro, 38015-050 - Uberaba, Brazil; 4-Instituto Ludwig de Pesquisa Sobre Câncer, 01509-010 - São Paulo, Brazil; 5-Núcleo de Genética Médica de Minas Gerais (GENE/MG), Av. Afonso Pena 3111/9, 30130-909 Belo Horizonte, Brazil.

There are a number of PCR based techniques that permit comparison of genomic polymorphisms, one of the most powerful and generally useful of these is AP-PCR (1). We have been attempting to discover an equally powerful PCR based methodology capable of rapidly and simply identifying sequence variations is gene sized DNA fragments. Existing techniques for detection of sequence variation include allele-specific hybridization (2,3) and shot gun strategies such as SSCP (4) and DGGE (5). The former relies on prior knowledge of the exact base alterations and becomes awkward when a large number of possibilities exist. The latter can only be applied to small fragments and give no information about the type or position of the mutation. The outcome of our investigations has been the development of a novel technique that we have called LSSP-PCR (Low-Stringency Single Specific Primer PCR). When LSSP-PCR is applied to gene sized DNA fragments (of at least up to 1 Kb) it translates the underlying DNA sequence into a unique multiband "gene signature". We have found that even changes in sequence as small as one nucleotide in a one kb fragment produce alterations in the banding pattern and each variant of a gene has its own signature. Importantly, no prior knowledge of the position, number or type of mutations present is required.

To undertake LSSP-PCR, a purified DNA fragment is submitted to PCR using very high concentrations of Taq polymerase and a single oligonucleotide primer specific for a sequence close to one of the extremities. Under these conditions the primer hybridizes not only to its complementary region but also to multiple sites within the fragment, in a sequence-dependent manner, producing a complex set of reaction products separable by electrophoresis.

LSSP-PCR has innumerable applications in all fields where the rapid identification of gene polymorphisms is required. Obvious major applications include the study and diagnosis of genetic and infectious diseases, cancer, and identity testing. We are pursuing the use of the technique with selected topics in all of these areas. In the case of identity testing, the application of LSSP-PCR for the detection of mt-DNA D-loop variants is being investigated (6-8). The protocol involves the preparation of a 1,021 bp portion of human mtDNA, containing the D-loop region, by specific PCR from blood derived genomic DNA. The PCR product is then gel purified and submitted to LSSP-PCR which produces complex D-loop signatures consisting of dozens of bands. We have compared 24 unrelated individuals and found different patterns in all of them. In contrast, mother-

child pairs are always identical, as expected from the matrilineal inheritance of mitochondrial DNA. As an alternative we have also produced D-loop signatures with a fluorescent primer and run the products on an automated DNA sequencer (Pharmacia A.L.F) with a view to increasing the throughput and accuracy of LSSP-PCR. The molecular size range scrutinized in the fluorescent run is much more restricted (250 to 550 bp) than that with the silver stain (200 to >2,000 bp) but the resolution is superior. Nevertheless, even in small details the traces obtained with the ALF show identity of mtDNA in mothers and their children and variation from the mtDNA of their respective fathers in all cases.

In the study and diagnosis of infectious diseases identity of the strain or subtype of the infecting organism is often of paramount importance. In the case of human papillomavirus, for example, over 20 virus types infect the genital and anal mucosa. Some types such as 16 and 18 are highly associated with the development of anogenital cancer whereas other types such as 6 and 11 are commonly associated with benign warts (9). Using a pair of degenerate primers to amplify a 450bp region of the L1 gene we have been able to distinguish all virus types examined to date using for LSSP-PCR a third internal primer specific for a relatively conserved region. In a further example, we have used LSSP-PCR for serovar identification of *Leptospira*, a highly variable spirochete infectious for man and animals (10). Using LSSP-PCR with either one of the two primers used for the specific amplification of a 290bp fragment we are able to identify the infecting serovar avoiding the necessity of prolonged culture of this slow growing organism.

In all the cases we have so far investigated LSSP-PCR has always produced complex patterns of fragments that are sensitive to sequence changes. We see no reason to suppose that it will not be universally applicable.

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Comparison of Phage Typing and DNA Fingerprinting by Polymerase Chain Reaction for Discrimination of Methicillin-Resistant *Staphylococcus aureus* Strains

ALEX VAN BELKUM,[1]* RENÉ BAX,[1] PAUL PEERBOOMS,[2] WIL H. F. GOESSENS,[3] NAN VAN LEEUWEN,[4] AND WIM G. V. QUINT[1,3]

Diagnostic Centre SSDZ, P.O. Box 5010, 2600 GA Delft,[1] Municipal Health Service Amsterdam, 1018 WT Amsterdam,[2] Academic Hospital Dijkzigt, Erasmus University Rotterdam, 3015 GD Rotterdam,[3] and National Institute of Public Health and Environmental Protection, 8720 BA Bilthoven,[4] The Netherlands

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A typing procedure for methicillin-resistant *Staphylococcus aureus* (MRSA) based on the polymerase chain reaction (PCR) amplification of both *mecA* sequences and variable DNA sequences as present in the prokaryotic genome has been developed. Two primers based on the sequences of DNA repeats as discovered in gram-negative members of the family *Enterobacteriaceae* allow detection of variable regions in the genome of a gram-positive bacterium such as *S. aureus*, as does a newly described arbitrary primer. This procedure, enabling the detection of 23 different genotypes in a collection of 48 MRSA isolates, was validated by comparisons with phage typing studies. It appeared that within the same group of isolates only 13 different phagovars could be identified. Combination of the results from both phage typing and genotyping allowed the discrimination of 34 of 48 isolates. However, depending on the primer-variable complexity of the PCR fingerprints, which could also be modulated by combination of PCR primers, clear homologies between the groups defined by either phage typing or fingerprinting were observed. An analysis of an MRSA outbreak in a geriatric institution showed a collection of genetically homogeneous isolates. In agreement with phage typing, PCR fingerprinting revealed the identical natures of the MRSA strains isolated from all patients.

GENOMIC TYPING OF *Haloferax mediterranei* STRAINS USING RANDOM PRIMER-POLYMERASE CHAIN REACTION.

Martínez-Murcia, A.J.* and Rodríguez-Valera, F.

Departamento de Genética y Microbiología, Campus de San Juan, Apdo. 374, Universidad de E-03080 Alicante, Spain.

Bacterial classification has been historically based on expressed or phenotypic cellular characters. Over the last two decades, systematics has focused very much on bacterial genotype. For instance, DNA/DNA hybridization technology has notably contributed to the classification of organisms at the species level, although several discrepancies for splitting still exist. It is noteworthy that the methods used in DNA/DNA pairing just give a "rough", "crude" measure, in part due to some technical limitations. Ribosomal RNAs (or genes) are considered as the best molecular chronometers known to determine bacterial phylogenetic relationships, however the RNA of the small ribosomal subunit does not always differentiate significantly between closely related, but considered different, genospecies. The identification of a new isolate requires a high information content if predictive capacity about the characteristics and behaviour of the microorganism is desired. The total genome of the bacteria could be an ideal tool to develop rapid identifications methods as it contains an enormous volume of information which is highly related with the phenotype. Therefore, this kind of data is very much more predictive of the bacterial properties than that obtained from the phylogenetic tree based on the sequences of one, or a few genes. In addition, information about the total genome may be more useful for measuring particularly close bacterial relationships if the 16S rRNA is too conserved. Although it is not virtually possible to sequence such amount of nucleotides, there are some methods to analyze microbial genomes by fragmentation of the DNA. The development of pulsed-field gel electrophoresis (PFGE) allows the fractionation of macro-restriction fragments generated by endonucleases, nevertheless it remains a laborious and slow technique. Random amplified polymorphic DNA (RAPD) is an alternative method to characterize closely related bacterial strains. There is very little information in the literature comparing both methods (PFGE and RAPD) of total genome typification. Genomic characterization of strains M2a, M2b, M4, M6, M10, M14 and the type strain R4 (ATCC 33500) belonging to the halophilic archaeobacteria *Haloferax mediterranei* has been carried out by López-García and colleagues using PFGE. In the present study, we have applied the RAPD to the taxonomy of these strains in an attempt to evaluate the newly developed method and to assess congruence with results from the PFGE studies.

PCR protocol was as the standard with minor modifications. Annealing temperatures on the PCR cycles were 4 to 6 ºC above the melting temperature to avoid non-specific

hybridizations of the primers. At these conditions RAPD patterns were repetitive. Resulting PCR products (ranging *ca.* 300 to 3000 bp) were analyzed by simple agarose gel electrophoreses. Ten arbitrary primers from all those evaluated produced information to analyze genomic relationships between the type strain and other isolates of *Hf. mediterranei*. DNA samples from *Haloferax volcanii*, *Halobacterium salinarium* and *Haloarcula marismortui* were included in this study as species and genus references.

The RAPD patterns obtained in this study were of good value to ascertain some genomic relatedness between the organisms under investigation. For instance strain M14 showed RAPD patterns completely different to these obtained from strains M2a, M2b, M4, M10 and the type strain of the species ATCC 33500 (R4). Several primers that produced bands with DNA from M14 did not hybridize with any of these from strains M2a, M2b, M4, M10 and R4. These results indicated that strain M14 does not belong to the species *Hf. mediterranei* as previously proposed on the basis of PFGE. Most bands from RAPDs of R4 migrated the same distances that these of strain M10 indicating that they may be the same strain. Only very few differences were found between the RAPDs of these strains; this agrees with the PFGE and those additional bands could be explained by the presence of a extra 66Kb genomic element in strain M10. Strains M2a, M2b, M4 and M6 form a coherent group whose members were well related between each other in terms of the RAPDs. Some oligonucleotides produced species-specific bands that serve to distinguish *Hf. mediterranei sensu stricto* strains from other organisms.

In this investigation the value of RAPD patterns for classification and identification purposes was newly demonstrated. Results of this approach correlated very well with those from PFGE. Future work will include the generation of a number of RAPDs from many strains of all species of several genera (viz: *Haloferax*, *Halobacterium* and *Haloarcula*) in order to determine valuable bands for the identification of species and, perhaps, genera.

Noel B. MURPHY
International Laboratory for Research on Animal Diseases
ILRAD - P.O. Box 30709 - NAIROBI (Kenya)

Application of arbitrarily primed PCR fingerprinting to molecular studies on trypanosomes

Protozoan parasites of the genus *Trypanosoma* are responsible for a group of diseases (trypanosomiases) in man and his domesticated livestock. These parasites are grouped into five well-differentiated subgenera (*Nannomonas, Duttonella, Trypanozoon, Pycnomonas* and *Schizotrypanum*) and ten less well-defined species. Three of these subgenera (*Nannomonas, Duttonella* and *Trypanozoon*) are widespread in Africa where they cause considerable losses in productivity in domestic animals. Two of the subspecies in the *Trypanozoon* subgenus (*Trypanosoma brucei rhodesiense* and *T. b. gambiense*) are, in addition, responsible for life-threatening diseases in man. Trypanosome infections are complicated by the existence of many disease-causing subgenera, species and subspecies, in addition to the occurrence of several apparently harmless trypanosome populations which, simultaneously, can infect an animal. Rapid, efficient characterisation and diagnosis of such infections is required to optimise the use of chemotherapy and to obtain sound epidemiological data. Several different methods are presently applied to distinguish the different populations of trypanosomes that infect people and their domestic animals. Each one has inherent strengths and weaknesses, and often there is a requirement for the application of a number of different tests to gain a proper understanding of the disease complex in a given locality. Arbitrary primers (10-mers) have recently been applied to the differentiation and characterisation of trypanosomes. From over 100 primers tested, one, ILO525, has enabled the differentiation of the different subgenera, species and individual isolates from Africa and South America. This finding greatly simplifies and extends the possibilities in studies of the epidemiology of trypanosomiasis.

Phenotypic traits considered to be of most importance to the epidemiology of trypanosomiasis are (i) drug resistance, (ii) virulence or pathogenicity and (iii) tsetse transmissibility. Mutants for (i) and (ii) are available and arbitrarily primed PCR (AP-PCR) techniques are being applied to identify genes involved in determining these phenotypes. The recent development of transfection systems for *T. b. brucei*, in combination with AP-PCR, make a powerful combination for the identification of genes controlling such traits. For example, a number of attenuated GUTat 3.1 *T. b. brucei* mutants which can be propagated *in vitro*, but are unable to establish an infection in a mammalian host, have been generated through extended growth *in vitro,* and manipulation of the *in vitro* incubation temperature and hemen concentration. These mutants represent ideal material for producing and characterising revertants, since there is a direct selection system through re-establishment of growth in a mammalian host. The generation of mutants with easily identifiable phenotypes is an important tool in genetic analysis. However, the ability to generate revertants and map the genomic location at which an alteration has occurred, which

correlates with the change in phenotype, is considerably more powerful. Transfection of total DNA, with an average size of 30 kbp, from a virulent *T. b. brucei* ILTat 1.1 parasite resulted in three independent revertants whose chromosome profiles appeared identical to the parental attenuated population. In order to identify the genomic location of the alteration, AP-PCR of genomic DNA from the virulent parental, the avirulent mutant and the three revertant transfectants was applied. One of the primers used amplified a fragment in the virulent parental and the three transfectants, but not in the avirulent mutant. This PCR product has been cloned and sequenced at both ends. One end of the fragment contains sequences of the TRS1/ingi transposable element, but the sequence identity of the other end has not yet been established. In similar studies, mutants which are resistant to the commonly used anti-trypanosomal compounds have been generated from a sensitive population. Again, AP-PCR has been applied to identify regions in the genome which have altered in the derivation of the resistant populations. Several alterations have been observed, but the use of populations expressing intermediate levels of resistance has helped in focusing on alterations which correlate with the acquisition of the resistance phenotype.

Trypanosome species capable of undergoing cyclical development are digenetic and can undergo a complex development cycle involving both a mammalian host and tsetse insect vector. Prior to transfer between their host and vector, and vice-versa, these organisms can undergo a developmental change to become non-dividing and pre-adapted for the new host. These alterations are important in the establishment and maintenance of an infection and offer possible target points for intervention and control of the disease. The isolation and characterisation of regulatory genes involved in these differentiation processes is therefore of importance. Since AP-PCR products are generally less than 1 kb in size, the use of arbitrary primers on cDNA should generate fingerprints which are characteristic of expressed genes. Two model systems were employed to assess this; one in which actively-dividing *T. b. brucei* bloodstream forms differentiate to non-dividing forms, and the second in which non-dividing metacyclic tsetse fly forms of *T. congolense* differentiate to actively-dividing bloodstream forms. mRNA from each differentiated form was reverse transcribed into cDNA which was then used as the template in the PCR. The resultant products were examined by agarose gel electrophoresis. As few as 10^3 trypanosomes were sufficient for the generation of a fingerprint after first amplifying the total cDNA through exploitation of the fixed 3' and 5' ends of trypanosome nuclear mRNAs. Differences in fingerprint patterns between the differentiated forms examined were mainly due to differences in gene expression. The technique can rapidly identify genes expressed at very low levels and which are up or down regulated in the different forms examined. PCR products of interest are easily purified from the agarose gels for direct cloning and complete sequence determination due to their relatively small size (0.1 to 1 kb).

Systematic genotypic typing and fingerprinting of *Salmonella* with rRNA gene and insertion sequence probes: applications to epidemiology and phylogenetics.

John Stanley
NCTC Molecular Genetics Unit
Central Public Health Laboratory
London NW9 5HT
UK

In the Enterobacteriaceae, classical serotyping is highly developed for the Salmonellae, and is the basis of epidemiological studies. In addition, intra-serovar subtyping with phages is made for certain important serovars like *S. enteritidis*, *S. typhimurium* or *S. typhi*. In *Salmonella* in particular therefore, novel genotypic typing approaches may be directly compared with existing and efficient phenotypic typing systems. We have developed a 'universal' methodology to subtype *Salmonella* serovars which carry the 708bp DNA insertion sequence IS*200*, the smallest known IS element (Lam & Roth, 1983; 1986). IS*200* is specific to *Salmonella* and to a few clones of *E. coli* (Biseric & Ochman, 1993). Since it is a mobile element, variation arises both from DNA base substitution and from rearrangements consequent upon transposition. The number of IS*200* bands can be related to its apparent copy number, and characteristic IS*200* profiles are generated from genomic Southern blots. This can be efficiently characterized with PCR-generated probes (Baquar *et al.*, 1993), for the purposes both of fine-structure phylogenetic studies or of genotypic typing for epidemiology (*e.g.* Stanley *et al.*, 1992; 1993).

In our laboratory 'IS*200* profiling' has been integrated with 16S rRNA gene profiling and plasmid screening to analyze evolutionary and epidemiological relationships among strains of various *Salmonella* serovars of public health importance. Genotypic subtyping of *S. enteritidis*, *S. bovismorbificans*, *S. heidelberg*, *S. typhimurium* and *S. paratyphi* B/*java* have been described. In serovars where the copy number is greater than three, such as *S. heidelberg* or *S. typhimurium*, IS*200* profiles provide highly discriminatory molecular fingerprints of the chromosome. In *S. typhimurium*, for example, one observes serovar-specific IS*200* bands, and phage-type-specific IS*200* profiles. Furthermore, single phage types can be 'split' into a number of IS*200* profile subtypes (Baquar *et al.*, 1993). Such profiles can be displayed, photographically or graphically, in band-matching databases. They are composed of chromosomal insertion sites, and are sensitive markers of common chromosomal genetic origin, *i.e.* of clonality, for epidemiological purposes. It is instructive to compare the results so obtained with the IS6110-based molecular typing scheme recently adopted internationally for *Mycobacterium tuberculosis* (van Embden *et al.*, 1992).

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Species identification by arbitrary amplification of polymorphic DNA[1]

Iciar Martinez and Audny Johansen

Norwegian Institute of Fisheries and Aquaculture, PO Box 2511, 9002-Tromsø, NORWAY

Arbitrarily primed polymerase chain reaction (acronym: AP-PCR) (Welsh & McClelland, 1990, *Nucleic Acids Research*, **18**: 7213-721) or random amplification of polymorphic DNA, called RAPD for short (Williams *et al.*, 1990, *Nucleic Acids Research*, **18**: 6531-6535), both of which names refer to the same principle, is a technique used to generate fingerprints of complex genomes. The conditions are usually optimized to detect divergences of even a fraction of a percent between two genomes, in order to use those PCR products that are shared between only some individuals as polymorphic markers.

We show here the validity of this technique also for species identification. In the fish industry, fast and reliable fish species identification is necessary for the correct labelling (and pricing) of manufactured products, such as canned, minced, ready-to-serve, and alike commodities, in which the species used is no longer identifiable.

We have tested arbitrary ten-mer primers S-01 to S-12 (Operon technologies) with Arctic charr and Atlantic salmon, two species of salmonid fish. The fingerprint patterns obtained with either of the 12 primers were useful for species identification. In addition, the fingerprints of different individuals of the same species did reveal some differences due to individual-specific polymorphisms.

A second situation in which fast species identification is necessary in the fish industry includes monitoring of processing lines to assess the presence of pathogenic bacteria, such as *Listeria monocytogenes*. Detection of this species, no matter which strain, will implicate closing down the production line, checking of all the critical production points for their reevaluation and possible relocation, possibly change of the production process line, and thorough cleaning and decontamination, before new production resumes. Due to the easy spreading and strong resistance of *Listeria*, it is important to detect the source of contamination and follow the above procedure as soon as possible, and before the microorganism is spread all over the production line.

2 and 20ng of DNA extracted from 23 strains of *Listeria* (5 strains of *L. innocua*, 2 of *L. ivanovii*, 5 of *L. seeligeeri*, 3 of *L. welshineri*, 7 of *L. monocytogenes* and one unknown) were fingerprinted using arbitrary ten-mer primers T-01 to T-08 (Operon Technologies). In this case, those primers which did not render good strain-characteristic polymorphic profiles, were the most useful to differentiate individual species. Thus,

[1]The part of this work concerning *Listeria* is being carried out in collaboration with Dr. Liv Marit Rørvik, Norges Veterinær Høgskole, Insititutt for Næringsmiddlehygiene, Oslo.

primer T-01 gave characteristic profiles for *L. innocua*, *L. seeligeeri* and *L. monocytogenes*, T-02 for *L. monocytogenes*, T-03 for *L. seeligeeri* and T-05 for *L. innocua* and *L. seeligeeri*. Primer T-07, on the other hand, which was useful to differentiate the individual strains analyzed of *L. monocytogenes*, did not render a common profile for all of them which could be used for a fast identification of the species. The unknown sample was readily identify as a *L. seeligeeri*, closely related to one of the provided strains (L-808) by all primers.

It is worth mentioning that, in contradiction with several published papers and personal communications from other researchers, we were not able to lyse any of the *Listeria* strains by boiling for over 30min. After treatment with lysozyme, however, genomic DNA was satisfactorily extracted by a modification of the procedure described by Miller *et al.* (1988, *Nucleic Acids Research*, **16**: 1215). This may be due to differences in susceptibility to lysis depending on the state of growth of the bacteria. Therefore, we strongly advise to use the latest procedure to identify *Listeria* from clinical specimens or from samples taken from processing lines, in order to avoid false negatives.

If these results are further sustained by the analysis of more strains of *L. monocytogenes* isolated from a variety of sources, AP-PCR analysis could be routinarily used without the need for specific primers. This has several advantages: *i*) since it is a general pattern, and not a single band what we are looking at, the results are more reliable than with specific primers (mutants of *L. monocytogenes*, extra bands, etc.) and the likelihood for false negatives diminished, *ii*) it is possible to identify the presence of other *Listeria* species, which it is not with specific primers, and *iii*) arbitrary ten-mer primers are inexpensive.

Application of Arbitrarily-primed Polymerase Chain Reaction Genomic Fingerprinting in Molecular Studies of Basidiomycetous Fungi

Hoi Shan KWAN

Department of Biology, The Chinese University of Hong Kong,
Shatin, N.T., Hong Kong

The genetics and molecular biology of most basidiomycetous fungi have not been extensively studied. Only scanty genetic information and few genetic markers are available for most of these fungi. Such lack of information is especially serious for mushrooms that breeding of mushrooms has relied on trial and error approaches. My laboratory has been interested in developing molecular markers for the genetic characterization of mushrooms. We have been concentrated on generating arbitrarily primed polymerase chain reaction (AP-PCR) markers for the Shiitake mushroom *Lentinula edodes*, the second most cultivated basidiomycetous mushroom. Initially we compared the use of the sequences from PCR-amplified ITS2 regions of rRNA genes and AP-PCR patterns for strain typing of this mushroom. Shiitake strains could be distinguished more easily by their AP-PCR patterns than by ITS2 sequence comparisons (Kwan *et al.*, 1992).

Protoplast fusion technology has been widely used for intraspecific and inter-specific mushroom hybrid formation. However, it is not always possible to demonstrate that hybrids are formed after protoplasts from two different strains or species are fused. The use of genetic markers is the main means to demonstrate hybrids but classical genetic markers in mushrooms are rare and often unstable. AP-PCR generated markers could be used as markers to distinguish fusion products from their parents. Nevertheless, the use of AP-PCR markers for this purpose is still poorly studied (Chiu *et al.*, 1993).

Basidiomycetous fungi have distinct dikaryotic (containing two different kinds of nuclei, one from each parent) and monokaryotic (containing one kind of nuclei only) stages in their life cycles. In many cultivated mushrooms, only the dikaryotic form is known. However, for breeding of mushrooms, it is necessary to use monokaryons for crosses. The two major ways of generating monokaryons from dikaryons (dedikaryotization) are fragmentation and protoplast generation. AP-PCR profiles of the putative monokaryons can be used to distinguish monokaryons from dikaryons. AP-PCR profiles of the monokaryons contains a subset of bands of the dikaryons (Chiu *et al.*, 1993).

The most important application of AP-PCR markers in Basidiomycetous fungi is the generation of molecular markers which can be used to construct a genetic map and tester strains for mapping agronomically important phenotypic traits. We tested this application in generating AP-PCR markers from parents and progenies of *Lentinula edodes* strain L40, a strain used for cultivation in Japan. DNA samples were isolated from L40, its dedikaryotization products, and 18 single spore isolates from the meiotic progenies of L40. Fresh mycelium from a liquid culture was freeze-dried with liquid nitrogen and ground to powder with precooled mortar and pestle. The powder was extracted with a lysis buffer that contained 50mM Tris-HCl, pH7.2, 50mM EDTA, 3% SDS and 1% 2-mercaptoethanol.

After one hour of incubation at 65°C, the samples were extracted with equal volumes of chloroform. DNA in the aqueous phase after centrifugation was precipitated with isopropanol in 0.3M sodium acetate, pH 5.2, washed, and resuspended in TE buffer. Because of the frequent contamination of carbohydrates and phenolic compounds in the DNA samples from mushrooms, it is necessary to purify the DNA sample by a cesium chloride method to avoid AP-PCR interference of these compounds . In this method, 100 mg cesium chloride was added to the mushroom DNA sample, mixed and centrifuged at 13,500xg for 20 minutes. The DNA in the supernatant was free of carbohydrate and phenolic compound contamination and was then precipitated, washed, and used for AP-PCR.

The DNA samples from the 21 parents and progenies of strain L40 were AP-PCR amplified with 6 primers singly and 4 pairwise combinations. The length of the primers ranged from 20 to 30 bases. The GC contents were from 42 to 70%. The AP-PCR temperature profile was two cycles of 94°C, 5 minutes, 35°C, 5 minutes, 72°C, 5 minutes; and 35 to 40 cycles of 94°C, 1 minute, 55°C, 1 minute, and 72°C, 2 minutes. The final elongation was 72°C, 10 minutes. The AP-PCR products were analyzed with ethidium bromide staining after electrophoresis in 4% NuSieve-SeaKem agarose gel or silver staining after polyacrylamide gel electrophoresis.

Polymorphic AP-PCR bands were first identified in the monokaryons obtained by dedikaryotization of L40. A total of 10 AP-PCR amplifications were analyzed and 6 of them produced, altogether, 14 scorable markers in the size range of 200 to 850 base pairs. Segregation patterns of these 14 markers in the 18 progenies were analyzed with the Chi-square test. Only two deviated significantly at $p=0.05$ from Mendelian segregation of 1:1. One of the 12 markers in 5 strains was analyzed by the method of single stranded conformation polymorphism. This marker appeared to be a single species among the AP-PCR products of the 5 strains. Linkages among the 12 markers were determined by the LOD score method. Only two pairs of markers were shown to be linked with the remaining 8 markers unlinked to any other markers.

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Genomic Mapping in Woody Plants with RAPD Markers:

Ron Sederoff[1,3], Dario Grattapaglia[1], Jose Chaparro[2], Phillip Wilcox[3], Susan McCord[3], Barbara Crane[3], Dennis Werner[2], Henry Amerson[3], Steve McKeand[3], Floyd Bridgwater[3], William Dvorak,[1,4] Ross Whetten[3], Ben-Hui Liu[3,4], David O'Malley[3], Departments of Genetics[1], Horticulture[2], Forestry,[3], CAMCORE[4] and Statistics[5], North Carolina State University, Raleigh, NC. 27695.

Genetic analysis of trees is limited by long generation times, and their large size. Most forest trees are undomesticated plants and have high levels of genetic diversity. The most advanced tree breeding programs are only in the first few generations of selection in contrast to our more familiar crops where cultivar development has been conducted for hundreds or thousands of years. For most traits of interest in forest trees, little is known about their mode of inheritance.

RAPD markers are particularly useful for forest genetics because prior knowledge of DNA sequence is not necessary and there is little or no sequence information for most forest tree species. High levels of heritable RAPD polymophisms have been observed in many outcrossing tree species. The amount of DNA required for RAPD marker amplification is about one thousand fold less than is needed for RFLP analysis.

A special feature of the genetic biology of gymnosperms is the haploid megagametophyte tissue in the seeds. Megagametophytes in conifers are mitotic derivatives of a single haploid megaspore and are derived from the same megaspore that gives rise to the maternal gamete. A heterozygous marker locus in a diploid individual seed parent gives rise to megagametophytes in seeds that show 1:1 segregation and are equivalent to a testcross. A single megagametophyte can provide more than 1ug of DNA, enough for many hundreds of RAPD reactions. It is therefore, possible to carry out genetic analysis on small amounts of haploid megagametophyte tissue.

The mapping strategy using megagametophytes from the seed parent (half sib mapping) has now been applied to several forest tree species. The half sib mapping strategy depends on individual trees that are heterozygous for many loci, and on examining the genotypes of the haploid megagametophytes. Extended pedigrees are not needed, and mapping can be done on the seed parent using controlled cross or open pollinated seed. RAPD markers show high levels of polymorphism in the conifers we have examined, including loblolly pine (*Pinus taeda* L.), monterey pine (*Pinus radiata* D.Don), and several Central American pine species. In *Eucalyptus*, high levels of polymorphism were observed in both species examined, *E. grandis* W. Hill ex Maiden, and *E. urophylla* S.T.Blake. Peach (*Prunus persica* [L.] Batsch), on the other hand, has very low levels of polymorphism. Peach is a self-pollinating domesticated species, with a narrow genetic base. Several of the forest trees we have examined are essentially from natural populations.

The ability to screen in advance for informative markers provides a method for genetic analysis in diploids with RAPD markers that approaches the kind of analysis that is possible with conifers using the haploid tissue of the megagametophyte. The major advantages of the haploid approach are that segregation ratios are 1:1 and that complete information is available even though the markers are dominant. The same situation is achieved in a diploid system in a testcross where a heterozygous F1 is crossed to a homozygous recessive parent. In genetically heterogeneous forest trees such as loblolly pine or *Eucalyptus*, a typical tree is highly heterozygous. The equivalent

situation to a testcross can be achieved simply if informative markers are identified by prescreening primers. If one parent in a controlled full sib cross is heterozygous for a given marker and the other parent is homozygous null, the resulting progeny will segregate1:1. The genetic situation mimics a testcross and is therefore called a pseudotestcross. Markers in the required configuration will occur often in a wide cross of highly heterozygous individuals. The major advantage of RAPD markers lies in the opportunity to screen large numbers of oligonucleotides for such informative markers. Enough markers can be obtained in an informative configuration to construct genetic maps and to locate QTLs.

QTL mapping could be particularly valuable for the breeding of trees. For inbred crop populations, a specific combination of marker alleles and a tightly linked useful gene may be strongly associated due to linkage. Tree species, however, are genetically heterogeneous and highly outcrossed so linkage equilibrium is expected. We expect linkage of specific markers and useful QTLs to be different in any individual tree, therefore the ability to create maps for many individual trees is essential. Experiments in progress and the strategies for application of genomic mapping in tree breeding will be discussed.

A population bulking approach has been used with RAPD markers to examine the genetic relationships of Eucalypt species and clones. Preliminary results indicate that outcrossing rate in a seed orchard can be estimated. RAPD markers would be attractive for studies of mating systems in tropical tree populations. Similarly, a RAPD marker study of the closed cone pines showed showed promise for the identification of species specific markers in *Pinus*.

These results lead to several conclusions. (1) RAPD markers are useful for a wide variety of genetic studies in forest trees. (2) Mapping using RAPD markers is feasible in individual trees. The levels of polymorphism in individual trees is often sufficient to create genomic maps using several hundred oligonucleotides for initial screening and a subset of about 100 oligonucleotides for QTL mapping. (3) Detailed mapping can be carried out in conifers, without extensive pedigrees, on individual trees that are producing seed. (4) Mapping can be done either on single open pollinated trees or on trees in full sib crosses. Many genetic studies in forest genetics and tree breeding are carried out using families derived from open pollinated select individuals. In full sib crosses, it is possible to obtain genetic maps for each parent. (5) The ability to map any sexually mature tree from collected open pollinated seed allows the study of variation, recombination and linkage in individual trees directly in natural populations.

PCR–Based Assays For Plant Germplasm Characterisation

W. Powell, K.J. Chalmers, C. Orozco–Castillo, U.M. Barua & R. Waugh
Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland, UK

J.A. Rafalski, M. Morgante & C. André
E.I. DuPont de Nemours and Company, DuPont Agricultural Products, Wilmington, Delaware 19880–0402, USA

Developments in molecular biology have provided a new repertoire of polymorphic assay procedures for use in genetic analysis and DNA fingerprinting. Molecular marker systems are being used to create linkage maps, in studies of biodiversity and to analyse phylogenetic relationships. However, when considering the use of molecular assay procedures for plant genome analysis it is important to recognise features which distinguish plants from animals. These differences provide opportunities (and some challenges) for the exploitation of molecular markers in germplasm evaluation and improvement.

The plasticity of the plant genome is reflected in the tolerance of many plants to interspecific hybridisation. In addition to being a feature of crop improvement, gene introgression is a major factor in angiosperm evolution. The capacity of RAPD markers to identify different taxonomic units has resulted in RAPDs being used to identify species specific amplification products.

We have used RAPD markers to detect genetic polymorphisms between coffee species and between *Coffea arabica* genotypes. RAPD profiles were used to derive phylogenetic relationships and these were consistent with the known history and evolution of *Coffea arabica*. Material originating from Ethiopia and the *arabica* sub–groups were clearly distinguished. RAPD analysis therefore reflects morphological differences between sub–groups and the geographical origin of the coffee material. Species specific amplification products were also identified, but, more importantly, amplification products specific to *C. canephora* were identified in two *Coffea arabica* genotypes Rume Sudan and Catimore 5175. This diagnostic product is therefore indicative of interspecific gene flow in coffee and has biological implications for selective introgressive hybridisation in coffee.

Large numbers of progeny from highly polymorphic crosses can facilitate the creation and exploitation of genetic maps. Polyploidy (auto– and allopolyploidy) which is far more common in the plant that animal kingdom has allowed the creation and exploitation of aneuploid genetic stocks to rapidly locate genes to specific chromosomes. Plant biologists

have also pioneered gamete based regeneration of viable plants from microspores. This technology can therefore be used in some plant species to rapidly obtain fixed homozygous families for genetic analysis.

This process of plant regeneration from microspore or anther culture represents a developmental switch from gametophytic to sporophytic mode of development. We have used this technology to produce a doubled haploid population for genetic mapping studies in barley. Mapping the components of polygenic systems is an important objective in both basic and applied genetic research programmes. The identification of specific regions of the genome which enhance the expression of quantitatively controlled characters may allow the development of more efficient plant breeding strategies. Our approach has been to use doubled haploids of barley in conjunction with RFLP and RAPD markers to locate genes controlling quantitative traits to specific regions of the genome. Simply monitoring the segregation of genetic markers and searching for associations with quantitative traits is, however, a relatively inefficient procedure and strategies are needed to target markers to regions of the genome which are important in controlling the expression of quantitative traits.

Continuous distributions that often characterise quantitative traits are represented by a range of individual genotypes each segregating for genes controlling the trait of interest. Genotypes found in the tails of the distribution are expected to differ at most of the loci controlling the character so that the alleles (increasing and decreasing) are highly associated. In addition to differing for QTL these genotypes are also expected to differ for individual loci linked to the quantitative trait of interest. This observation therefore provides a strategy to quickly map QTL. DNA from the extreme members of the distribution can be pooled to form two bulked samples which can be screened with genetic markers to identify those which are polymorphic. We have used this approach to identify RAPDs linked to a QTL controlling milling energy in barley. Four random primers were identified which detect polymorphism in the bulks and these were shown to be linked to the *Rrn2* locus on the short arm of chromosome 5H.

There are, however, limitations in the use of RAPD markers for this purpose. These fall into two categories: technical and genetical. In our experience the technical problems can be resolved by careful standardisation and optimisation of experimental conditions. The genetical limitation of the RAPD approach relates to their dominant mode of transmission, the extent to which two products can be considered allelic when compared across genotypes and species and their tendency to amplify sequences homologous to repetitive DNA in species with large complex genomes.

Oligonucleotide fingerprinting in chickpea and identification of molecular markers linked with Ascochyta resistance

P.C. Sharma[1], B. Hüttel, P. Winter, T. Bünger, F. Weigand[2], K. Weising and G. Kahl

Plant Molecular Biology Group, Department of Biology, Frankfurt University, Frankfurt/M., Germany
[1]Department of Agricultural Botany, Meerut University, Meerut, India
[2]ICARDA, Aleppo, Syria

Chickpea (Cicer arietinum L.) is an important pulse crop for semi-arid regions in the world. The crop yields are significantly decreased by the disease Ascochyta blight caused by the fungus Ascochyta rabiei. The development of genetic markers linked to Ascochyta resistance would be desirable for at least two purposes: (1) marker-assisted selection in breeding programs, and (2) isolation of resistance genes via map-based cloning. Both strategies usually require the establishment of a genetic map from polymorphic markers. Unfortunately, commonly used marker systems such as isozymes and RFLPs do not reveal sufficient polymorphism in intraspecific chickpea crosses. On the other hand, our previous results demonstrated that DNA fingerprinting using simple repetitive oligonucleotide probes yielded highly polymorphic hybridization patterns in different chickpea accessions (Weising et al.; Genome 35, 436-442, 1992). Therefore, we are currently investigating the potential of oligonucleotide fingerprinting for the establishment of a genetic map of chickpea, marker-assisted selection for Ascochyta resistance, and the isolation of putative resistance genes. Our strategy includes:

1. Screening for informative oligonucleotide/enzyme combinations for fingerprinting chickpea genomes

DNA isolation: A fast and convenient method was developed for isolation of DNA via intact nuclei. The method yields satisfactory amounts of DNA from fresh leaves, suitable for DNA fingerprinting without any further purification steps.
Oligonucleotide fingerprinting: DNA was isolated from bulked material as well as from individual plants of four chickpea accessions. Bulked DNA and individual plant DNA was digested with 12 and four restriction enzymes, respectively. The restricted DNAs were in gel-hybridized to the complete panel of possible di-, tri- and tetranucleotide repeat probes. Hybridization patterns with bulked DNA revealed stable and distinct bands for particular accessions. On the other hand hybridization patterns with individual plant DNAs also revealed bands which are polymorphic within an accession. The number of bands was more or less independent of the enzyme and solely depended on the probe. However, in some cases differences in the number of bands were observed for four-base-cutter versus

six-base-cutter enzymes indicating some clustering of these simple sequence repeat (SSR) loci in the chickpea genome. Out of 12 enzymes tested, HinfI, TaqI, DraI and EcoRI were found to be most informative, DraI being the most suitable. The different oligonucleotide probes studied could be classified in three broad categories: 1) probes yielding distinct, polymorphic bands (e.g. $(GATA)_4$, $(GAAT)_4$, $(CAA)_5$ and $(CAT)_5$) useful for mapping purposes. 2) probes yielding many diffuse monomorphic bands or smear (e.g. $(GTG)_5$, $(CAG)_5$ and $(GTAA)_4$). 3) probes yielding a few, strong, moderately polymorphic bands in a smear background (e.g. $(GTGA)_4$ and $(GGTA)_4$). The results obtained demonstrate that oligonucleotide fingerprinting can be employed to distinguish different chickpea accessions and even individuals within an accession, thus providing sufficient polymorphism to be used for analysing the segregating progeny of an intraspecific cross.

2. Genome mapping

Two F_2 populations from a cross between ILC 3279 (Ascochyta tolerant) and ILC 1272 (Ascochyta susceptible) were screened for segregation and linkage of fingerprint bands in order to identify an SSR marker associated with Ascochyta tolerance. TaqI digested DNAs of individual parents and F_2's were hybridized to different oligonucleotide probes. The hybridization patterns from four probes which showed polymorphism between parents were subjected to linkage analysis. In the majority of cases a 3:1 segregation for the presence or absence of a band, respectively, was noticed, suggesting dominant behaviour of these markers, although distorted segregation was also noted for a few bands. The fingerprint data obtained are currently being analysed by suitable statistics in order to develop a tentative genetic map for chickpea.

3. Cloning of Simple Sequence Repeat loci

Our strategy of cloning SSR loci from the chickpea genome includes the isolation of size-selected DNA fractions from the gel regions harbouring the different fingerprint bands, ligation into a phagemid vector, transformation into E.coli SURE strain and identification of positive clones by colony hybridization to the respective oligonucleotide probe. The desired clones will be tested for polymorphism by hybridization to genomic DNA of different chickpea accessions. Analysis of the flanking regions of the SSR loci may provide an insight into the mechanism of sequence evolution and conservedness of these loci and related genomes to study the generation mechanisms of simple sequence length polymorphisms. The cloned SSR loci can be transformed into RFLP and STS markers. Following hybridization or PCR analysis they can serve as anchor points for a chickpea genetic map.

The use of RAPDs as genetic markers in chrysanthemum

Kirsten Wolf. TNO - Dept. of Microbiology
PO Box 360, 3700 AJ Zeist, the Netherlands

Chrysanthemum (*Dendranthema grandiflora*) is a hexaploid species, bred for over 3000 years and a very popular cutflower with high economic value. A major problem in chrysanthemum growing is the large quantity of insectides needed. This should be lowered for economical and environmental reasons. The aim of the study is to find and characterize genetic markers in order to localize genes involved in insect resistance. The hexaploid nature of chrysanthemum makes it difficult to make maps and to localize QTLs as many more markers are needed than in a diploid species. Furthermore, chrysanthemum is strongly heterozygous because of the strong self-incompatibility system and the purposely outcrossing of the breeders. Therefore, no inbred lines exist or can be made. Fortunately chrysanthemum has an extremely high level of polymorphism and a F_1 generation resulting from a biparental cross can be used as the segregating generation.

Of the 60 RAPD primers tested sofar approximately 70% give polymorphisms in the F_1 of the testcross, with on average 3 polymorphic fragments each. In this way presence and absence of 150 polymorphic markers will be scored and a "map", of both parents separately, will be constructed. RAPD fragments that are related to insect resistance will be sequenced and specific primers will be designed to be able to transfer these insect resistance markers to other chrysanthemum crosses.

Patterns obtained with the RAPD technique are highly variable when several cultivars are tested; mean band sharing is 66%. This means that two or three primers are enough to be able to discern at least 30 cultivars. Variability among species related to chrysanthemum is extremely high with a low band sharing: 49% of the scorable patterns. Some RAPD primers give extreme variable patterns with almost all bands being present in only one individual and were, therefore, not scored.

To check for homology of RAPD fragments with similar size we used 15 fragments as

probes in a hybridization with a blot of a RAPD gel and with a blot of digested genomic DNA. It appeared that these fragments consisted of low copy sequences and that no polymorphisms were found on the genomic blots. On the RAPD blots the fragments hybridized to two to four bands per individual. Within the species chrysanthemum these bands had the same size. Among species, however, these fragments sometimes appeared to hybridize to several fragments of variable size.

The use of RAPDs as a taxonomic tool among species related to chrysanthemum is not correct for the three reasons mentioned above: 1) variability within species is too high to find one or a few representative individuals 2) band sharing is too low and there are many non-informative bands 3) homology between bands of the same molecular weight is questionable in many cases.

In chrysanthemum cultivars are not only developed from biparental crosses, but also by (somatic) mutation in vegetatively propagated material. In this way families of cultivars are developed that differ mainly for flower color. Among these family members we have found no differences in RAPD pattern. This means that the mutations causing the flower color difference are mutations in a small part of the genome and probably not the loss of a chromosome and that RAPD patterns are stable over many generations of vegetative propagation. The cultivars belonging to one family and additional cultivars with multiple origins, will also be tested using microsatellite fingerprinting.

The use of RAPDs seems highly valuable in chrysanthemu as molecular markers and also for cultivar identification.

FINGERPRINTING EUKARYOTIC GENOMES BY PCR USING PRIMERS SPECIFIC TO UBIQUITOUS GENOMIC REPEATS

Ewa Zietkiewicz and Damian Labuda

Service de génétique médicale, Centre de recherche, Hôpital Ste-Justine,
Département de pédiatrie, Université de Montréal,
Montréal (Québec) Canada H3T 1C5

Polymerase chain reaction (PCR) selectively amplifies DNA fragments flanked by sequences complementary to oligonucleotide primers used (Saiki *et al.*, 1985). Given the size of a mammalian genome (3×10^9 bp per haploid equivalent) one expects a 16-mer (or longer) oligonucleotide to prime DNA polymerization at a single genomic site ($4^{16} = 4 \times 10^9$); the selectivity of PCR increases if two distinct flanking primers are used. Typically the knowledge of the sequence at the locus of interest is required to design the primers. However, if sufficiently short primers of an "arbitrary" sequence are used, the amplification from numerous random loci may occur. For instance, one expects a ten-nucleotides long sequence to occur once per every million nucleotides, i.e. several thousand times in a mammalian genome. When two such sequences are present within an amplifiable distance and in an inverted orientation, a single complementary decanucleotide will prime amplification of the intervening DNA segment. A characteristic pattern of PCR products thus obtained can be considered as a signature or fingerprint of the analyzed DNA template (Welsh *et al.*, 1990; Williams *et al.*, 1990). DNA polymorphism among individuals of the same population can be also detected because the analysis is sensitive (i) to mismatches at priming sites (or to the presence/absence of a genomic element recognized by the primer, see below) and (ii) to the length of the amplified intervening sequence. The informativity of such a PCR experiment, measured as a number of amplified DNA segments, can be substantially enhanced if, instead of an arbitrary sequence, the primer recognizes interspersed repetitive elements that are found in all eukaryotic genomes.

The application of arbitrary primers provided new genetic markers, so called **RAPDs** (Random Amplified Polymorphic DNA) successfully used as mapping tools (Williams *et al.*, 1990), as well as in genomic fingerprinting in a variety of organisms (Welsh *et al.*, 1990; Caetano-Anolles *et al.*, 1991; Nadeau *et al.*, 1992; Weber *et al.*, 1989). Our approach was different: we used primers complementary to ubiquitous human Alu repeats. The underlying technique is known as Alu-PCR and was originally introduced to extract human DNA segments from heterologous sources (Nelson 1991). In this way we could reveal genomic polymorphisms, so called alumorphs (Sinnett *et al.*, 1990) later used as multiple-loci genetic markers to map hereditary

disorders (Zietkiewicz *et al.* 1992). To include amplification with primers complementary to other than Alu interspersed repetitive sequences (IRS), Alu-PCR was renamed **IRS-PCR** (Ledbetter *et al.*, 1990) and the term **SINE-morphs** was coined to describe DNA polymorphisms involving short interspersed repeats (SINEs) other than Alu and in other species (Kaukinen *et al.*, 1992).

However, the use of IRS-PCR and of SINE-morphs is limited to those species where abundant repeat families have been identified. Considering lineage-specific repeats such as Alu, a single IRS-PCR system is only applicable in related species colonized by the same family of repeats. An improved system would ideally have (i) the broadest taxonomical applicability, comparable with RAPDs (which do not require any prior knowledge of the analyzed genomes) and (ii) the SINE-morphs advantage of simultaneously targetting multiple genomic loci.

In this communication we report our experience with alumorphs (Sinnett *et al.* 1990; Zietkiewicz *et al.* 1992) used for inter-species comparisons among primates, and the development of other fingerprinting systems based on similar principles. We have recently characterized a ubiquitous family of repeats present in all mammalian genomes so called mammalian interspersed repeats, MIRs (Jurka, Zietkiewicz and Labuda, in preparation). The use of MIR-specific primers to fingerprint a great variety of vertebrate species indicates their utility in the studies of mammalian genomes.

We have explored a class of ubiquitously distributed repeats, called microsatellites or SSR (simple sequence repeats) that are spread in genomes of very distant species. These sequence elements consist of tandemly repeated short sequence motifs such as penta-, tetra-, tri- and dinucleotide repeats. The latter, and particularly $(CA)_n$-repeats (typically n = 10-60), are the most frequent in the human genome with an estimated copy number of more than 50,000 elements per haploid equivalent (Hamada *et al.*, 1982). In an attempt to design a "taxonomically generalized" approach allowing for simultaneous examination of a variety of genomic loci, we investigated the possibility to utilize $(CA)_n$ repeats as priming sites for inter-SSR PCR or inter-microsatellite PCR (Zietkiewicz, Rafalski & Labuda, submitted). Here again no sequencing is required to design the oligonucleotide primers. A complex, species-specific pattern of amplification products separated on non-denaturing acrylamide gel was obtained with all species examined. Polymorphisms between individuals within a species were also observed. These inter-repeat length polymorphisms may be used for genetic mapping. This novel fingerprinting method is applicable to taxonomic and phylogenetic purposes in a wide range of organisms, and provides information on multiple genomic loci in a single gel lane. This study was supported by Fonds de recherche en santé du Québec.

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GENOMIC FINGERPRINTING FOR GENETIC ANALYSIS OF ALZHEIMER'S DISEASE.

Rogaev E.I.*, Lukiw W J., Chumakov I, Rogaeva E.A. ST George-Hyslop P..
Laboratory of Molecular Brain Genetics, National Research Center for Mental Health, Academy of Medical Sciences, Zagorodnoe sh. 2/2, Moscow, Russia, 113152.
University of Toronto, Departments of Medicine and Neurology, Tanz Neuroscience Building, Center for Research in Neurodegenerative Disease, 6 Queen's Park Crescent West, Toronto, Canada, M5S 1A8.

Large collections of Alzheimer disease (AD) families (12 families) and sporadic cases from ethnic populations (>180 patients) were genotyped using a genomic variability.
(a) A comparison of chromosomal telomeric and peritelomeric DNA repeat length for a collection of elderly AD and age-matched normal brain samples has been carried out;
(b) A search for informative markers for gene- candidate regions has been performed and microsatellite compounds isolated from NFNL and Amiloid Precursor gene regions were used for allelic association analysis for both sporadic and familial Alzheimer's diseases cases;
(c) A set of random microsatellite markers with H>75% for most chromosomes was selected and developed for linkage analysis as "multiplex PCR markers". An early-onset FAD gene for set of families was linked to 14q23-24 (St. George-Hyslop P., Haines J, Rogaev E.I. et al., Nature genetics, 1992 and Rogaev E.I., Lukiw W.J., Vaula G. et al., Neurology, 1993);
(d) For isolating of FAD gene the minimum contig for FAD locus has been constructed by YAC clones. Method of the linear amplification of the repeats (LAR) was developed and YAC clones were overlapped by the LAR fingerprinting. Three known genes and > 100 transcribed sequences were isolated from this region by screening of the brain cDNA. Arbitrarily primed system will be developing for the screening of these sequences and detection of the FAD mutation.

MUTATIONAL MECHANISMS IN MITOCHONDRIAL DNA

H. Zischler

The determination of mitochondrial DNA sequences is especially suited for phylogenetic analyses. Firstly mitochondrial DNA can be regarded as an effectively haploid genome lacking recombination. Secondly the rate of evolution is different for various mitochondrial genes, thus allowing on one hand to use "slow" evolving sequences for reconstructing deep branches in a phylogenetic tree. On the other hand hypervariable regions of the only non-coding region of vertebrate mitochondrial DNA, the so called Control-region (or alternatively D-loop), exhibit an extraordinarily high rate of evolution, which is about 100 times faster than nuclear pseudogenes. Therefore these sequences could be succesfully used to infer intraspecific phylogenetic relationships, e.g. in human populations.
A difficulty however is, that the evolution rate is not homogeneous among different sites, with some positions undergoing a high rate of substitutions in evolutionary time scales, while others do not.
This creates a multiple hit phenomenon for "fast" evolving sites leading to faulty estimations of branching dates among the taxa in question, although there are statistical methods to correct for this phenomenon.
There are two components, which determine the site specific evolution rate: Site specific selection and site specific mutation rate.
Our aim is to tackle the problem of site specific mutation rates using biochemical approaches.
Our working hypothesis is based on mainly one hallmark of mitochondrial DNA-evolution, which is the fact, that transition-substitutions occur far more often than transversions. Beside the possible influence of the asymmetric replication mode of mitochondrial DNA on the transition-bias, which will be discussed in more detail, polymerase errors preferentially result in transitions. We therefore focus our research on the mitochondrial DNA-

replicating enzyme γ –polymerase as a possible generator of sequence changes.
In general γ -polymerase from various organisms seems to be a very reliable enzyme, as tested by conventional M13 mutational analyses. We are currently building up an assay system, in which we combine our knowledge about demographic data with *in vitro* extension analyses on cloned human D-loop templates. Hitherto we are using the best characterized γ -polymerase, which is isolated from Drosophila embryos (Wernette and Kaguni JBC 261, 1987).
The aim is to test if hot and cold spots of mutation, as revealed by sequence analysis of different human populations, correlate with site specific misincorporation- or pausing - tendencies of the γ-polymerase.
In future we will try to extend these analyses on human γ-polymerase, thus creating an assay system exclusively based on human specific components.

Investigation of Intra-specific Genomic Variation Utilizing Molecular Genetics Technologies: Application to Endangered Species Conservation

Oliver A. Ryder, Center for Reproduction of Endangered Species
Zoological Society of San Diego, San Diego, California 92112

Utilizing new technologies in molecular genetic analysis including multi-locus DNA fingerprinting, direct sequencing of PCR amplification products, and analysis of highly polymorphic microsatellite loci amplified by PCR, information obtained from model systems and human medical research has provided new insights into the population genetics and phylogenetics of these species. Knowledge of comparative aspects of genome organization and dynamics is based on a relative few examples. Examination of exotic species provides not only additional insights into evolutionary processes but, also, provides information unavailable from ecological and behavioral studies relevant to conservation of species' gene pools.

The analysis of genomic variation at the nucleotide sequence level may contribute to a broader understanding of the biology of a species and contribute to the conservation of biological diversity at several levels. Identification of phylogenetic species or evolutionarily significant units is aided by information from studies of genomic variation that infer rates of gene flow or the presence of isolation mechanisms. Concordance (or the lack thereof) between morphological or ecological discontinuities between populations with patterns of mitochondrial and/or nuclear genome variation provide support for (or refute) the use of traditional subspecies as the focus of conservation efforts for species that are threatened or endangered. Retention of heterozygosity and maintenance of genetic variation in isolated populations is limited by genetic drift, suggesting that determination of pedigrees through methods of ancestral inference and qualification of progeny provides a powerful means for assessing the vulnerability of isolated populations to losses in genetic variation. Recent investigations undertaken at the Genetics Laboratories of the San Diego Zoo's the Center for Reproduction of Endangered Species illustrate these points.

The California condor is the largest bird in North America. Reduced to a non-viable wild population, the species survived through captive breading and enhancement of reproduction. At the time that the last wild California condor was brought into captivity in November, 1988, there were at most only 14 potential unrelated wild condors that could form the basis of the program to preserve the species and its gene pool. While there seemed little likelihood that the surviving founders were unrelated, the lack of genetic information applicable to inferring kinship was of concern because the potential for loss of chicks through inbreeding as a result of pairing closely related birds was considered a threat to efforts to expand the population. Multi-locus mini-satellite probes were utilized with several restriction endonucleases to produce DNA fingerprints. A statistical model producing inferences about the genetic relationships between individuals was compared with estimates of relatedness based upon pairwise determinations of bandsharing. The results of both analyses were consistent, but the inferences based on the model were not obvious from the raw pairwise bandsharing data. Three "clans" of California condors, apparently approximately as closely related as second-degree relatives were identified by this analysis and the implications of this new information have been incorporated into the breeding plan for recovery of the species, including its release back into the wild.

Mitochondrial DNA D-loop sequence comparisons have been undertaken to assess genetic variability within and between populations of gorillas by PCR

amplification of DNA in follicles of shed hairs collected from night nests after the gorillas had departed. The samples were obtained non-invasively and could be easily stored and shipped. Because the DNA we obtained from hairs was degraded and only short regions could be amplified, a 250 basepair "hypervariable" region was selected for study. Surviving populations of gorillas are distributed in equatorial forests in West and Central Africa from Cameroon to Gabon as well as in eastern populations in Rwanda, Zaire and Uganda. Three subspecies are recognized, but there have been no genetic studies that add insights into the evolutionary separation of these populations. The high rate of nucleotide substitution in the gorilla D-loop hypervariable region complicates comparisons between species and to some extent, subspecies. However, this high variability is useful for detection of relatedness within and between gorilla populations. Assuming a relatively constant rate of nucleotide substitution, the genetic distance between western lowland gorillas and mountain or eastern lowland gorillas is approximately equal to the distance between the two recognized species of chimpanzees.

A common, although controversial, application of molecular data concerns estimating divergent times based on the "molecular clock", particularly where there are insufficient data from stratigraphy and paleontology. In some cases, the estimates of time have provided important biogeographic and evolutionary insights. Therefore, it is necessary to carefully evaluate the possible extent and reliability of the molecular clock. Recent results of different nucleotide substitution rates in both nuclear and mitochondrial genomes among divergent taxonomic groups raise cautions when applying the molecular clock among divergent groups. However, the constant-rate hypothesis within species or among closely related species of vertebrates is still generally accepted, even though it has never been rigorously tested. We have investigated evolutionary rates of the mitochondrial genome among individuals of a species of diminutive antelope, Kirk's dik-dik, using the relative rate test. Our results demonstrate that individuals of two chromosome races, East African cytotype A and Southwest African cytotype D evolve about 2.3 times faster than East African cytotype B. This could not be explained by current hypotheses for differential rates of nucleotide substitution. The rate heterogeneity in Kirk's dik-dik has important implications for molecular and evolutionary biology. First, estimates of divergence times based upon the inappropriate calibration from DNA sequence data will be misleading. Consequently, inferences concerning biogeographic, evolutionary and demographic history based on the time or rate calibrations could be erroneous. Second, multiple factors, including some hitherto unrecognized factors, may have an effect on DNA substitution rate in vertebrates. Detection of rate differences in nucleotide substitution within species requires an appropriate evolutionary context that includes the availability of at least two lineages (e.g. subspecies) which evolve at different rates as well as a recently diverged representative outgroup. If the representative outgroup has diverged too distantly in the past, any rate difference may be obscured. Not all species provide the necessary evolutionary features to detect rate differences by the relative rate test.

While molecular genetic studies in support of planning and implementing efforts for conserving species and their gene pools are being undertaken in laboratories located in universities, research institutes and zoological parks, at the same time the rate of loss of species and habitats throughout the world assures that characterization of many populations and species of interest and conservation concern will be unrealizable. While PCR methodologies can even allow insights into extinct species and ancient populations, notions of resurrection of species are as improbably as the notion of perpetual motion machines.

MOLECULAR EPIDEMIOLOGY OF HUMAN RESPIRATORY SYNCYTIAL VIRUS STUDIED BY RNase A FINGERPRINTING AND SEQUENCE ANALYSIS.

José A. Melero.- Instituto de Salud Carlos III,- Majadahonda, 28220- Madrid,- Spain.

Human respiratory syncytial (RS) virus is the leading cause of lower tract respiratory infections in very young children. Antigenic variation has been proposed as one of the factors that facilitates RS virus reinfections in the presence of circulating antibodies. In this sense, the G glycoprotein, responsible for virus attachment to the cell surface receptor, is the most variable antigen among human isolates. Consequently, we have oriented our studies to analyze in detail the degree of antigenic variation of the G glycoprotein and the genetic mechanisms that generate such antigenic diversity. The G protein gene of many RS virus isolates was compared by the RNase mismatch cleavage method of RNA; RNA heteroduplexes, using a radioactive RNA probe of the Long strain. The results obtained allowed us to classify the human RS viruses into two major genetic subgroups, which correlated with two previously described antigenic subgroups, identified by reactivity with a panel of monoclonal antibodies. Within each subgroup, multiple viral lineages were identified by their patterns of RNase A fingerprints. These genetic differences correlated with minor antigenic changes detected with monoclonal antibodies; however, some viral strains, which were antigenically identical, produced different fingerprints, emphasiging the discriminating potential of the RNase a method.

Sequence analysis of the G protein gene from representative strains that generated different fingerprints confirmed our previous clasification based on the RNase A mismatch cleavage method. In addition, it allowed us to establish a quantitative correlation between the fingerprint similarities (expressed as similarity coefficients) and genetic relatedness of the viral isolates. These parameters

have been used to construct evolutionary trees that illustrate the degree of genetic variability among viruses and the mode of RS virus evolution.

The sequence data identified variable and conserved regions of the G protein gene and certain correlations could be established between sequence and antigenic changes, suggesting that immune presure could be indeed a driven force in RS virus evolution.

ESTIMATES BY COMPUTER SIMULATION OF GENETIC DISTANCES FROM COMPARISONS OF RNAse A MISMATCH CLEAVAGE PATTERNS.

Joaquín Dopazo[1], Francisco Sobrino[1] and Cecilio López-Galíndez[2].

[1]Centro de Investigación en Sanidad Animal, INIA. 28130 Valdeolmos, Madrid, Spain.

[2]Instituto de Salud Carlos III. Centro Nacional de Biología Celular y Retrovirus. 28220, Majadahonda, Madrid, Spain.

Genetic distances between organisms have commonly been obtained by comparison of sequences. However, the number of individuals as well as the length of the fragments which can be analyzed by this approach are limited. Other approaches such as restriction fragment length polymorphism (RPFL), permit the analysis of many individuals, although they lack the degree of resolution provided by nucleotide sequencing. The RNAse A mismatch cleavage method (Myers et al., 1985; Winter et al., 1985) combines the advantages of both methods: many individuals can be analyzed with sufficient detail and it is highly sensitive to nucleotide sequence variations over large genomic lengths. Therefore, it can be considered as a good method for genomic fingerprinting and rapid sample screening.

This technique, based on the detection of point mutations in heteroduplexes, has been widely used to analyze genomic variability in RNA and DNA systems. In spite of its sensitivity and reproducibility, it was unclear how to relate the observed differences between two patterns of cleavage fragments to the number of nucleotide differences between the compared sequences. Here we present a computer simulation of the results of the digestion with RNAse A of heteroduplexes composed of a defined riboprobe and different, related sequences. From this analysis we conclude that the number of non shared fragments ($\mathbf{F_{ns}}$), generated by two different sequences hybridyzated with the same riboprobe, constitutes a good estimator of the genetic distance in terms of number of nucleotide differences between the two sequences. (Dopazo et al., 1993). This prediction is supported by the comparison of the RNAse A mismatch patterns and the actual nucleotide sequences of a set of influenza A (H3N2) haemagglutinin genes.

The procedure allows for the first time a quantitative use of the RNAse A mismatch cleavage method.

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"THE RNASE A MISMATCH CLEAVAGE METHOD IN THE CHARACTERIZATION OF HIV-1 ISOLATES FROM DIFFERENT COUNTRIES".

S. Sánchez-Palomino[1], I. Olivares[1], M.J. Martin[2], J. Dopazo[2], C. López Galíndez[1].
1-CNBCR, Instituto Carlos III, Majadahonda, Madrid 28220.
2-CISA, I.N.I.A., Valdeolmos, Madrid 28130.

The Rnase A mismatch cleavage method (RMCM) was developed to detect activating point mutation in K-ras genes (Winter et al 1985). It takes advantage of the ability of RNase A to digest mismatches in heterohybrids. We applied the technique to study genetic variability of RNA viruses applying the Influenza virus as a model (López-Galíndez et al 1988). The digestion of hybrids of a labelled RNA from a reference strain and the viral RNA from an isolate gives a pattern of digestion bands that is specific for each strain as a fingerprint. Then the method can be used to genetically analyze and to stablish relations between viral isolates in molecular epidemiology studies.

The technique was then used to study genetic variability of HIV-1 isolates obtained after primary co-culture and growth in MT-2 cells. We were able to demonstrate that two distinct genotypes were circulating among the HIV-1 isolates obtained from San Diego. We also detected the presence of strains that were resistant to AZT by the presence of the characteristic mutations in the 3'end of RT (López-Galíndez et al 1991).

Recently we have undertaken the analysis of HIV-1 strains from different countries in the world starting from PBMC material. We analyzed the samples in two genomic regions in RT and ENV gene. Better result for classification purposes came from data in the ENV gene, spanning mostly over the GP41. By the presence of common bands in the digestion pattern we have been able to stablish similarities among the strains in comparison with the patterns displayed by reference strains of different subgroups.

Following the theoretical model for the RNase A mismatch cleavage method, the number of bands in the digestion pattern is proportional to the genetic distance between strains (Dopazo et al 1993). By this approach we have obtained a better classifications of the strains clearly identifying the Brazil isolates as B type and Thailand as E type.

In summary RMDM is a technique very helpful for genetic screening of viral isolates and could help in the selection of strains for further characterization.

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Restriction Site Mutagenesis (RSM): a PCR–based genotypic mutation assay

Dorado G, Leal JFM, López–Barea J

Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, 14071 Córdoba (Spain)
Tel: +57 218689; Fax: +57 218666; e–mail: dopeg@uco

Classical mutation assays are based on the phenotypic expression of mutations. Due to its nature, these methods are unable to detect mutations that do not affect the coding and/or functional properties of the products of gene expression. The new advances in molecular biology offer the possibility to overcome such limitations, allowing the development of real genotypic mutation assays. Restriction Site Mutagenesis (RSM) is a PCR–based genotypic mutation assay currently under optimization. RSM has been designed to detect any mutation at any restriction site. It can be applied to any DNA sequence derived from any virus, prokaryotic or eukaryotic cell. In short, the DNA to be analyzed is first digested with a restriction endonuclease of choice. Wild type sequences for the restriction site will be digested; only mutated ones remaining uncut. These sequences are further amplified exponentially by PCR using oligonucleotides flanking the restriction site. The outcome of RSM is first monitored by agarose gel electroforesis and further detected by Southern blotting. The molecular nature of the mutations is finally determined by sequencing of the PCR products.

While the RSM methodology is quite straightforward, a series of problems must be solved if this new technology should become useful for general screening of human populations. Among them are partial digestion of the DNA, DNA polymerase errors, presence of multiple mutated sequences and DNA carry–over contamination. These problems could be overcome, respectively, digesting three times with 10–fold excess enzyme, using a high fidelity polymerase like Pfu, diluting or subcloning mixtures of mutated sequences and following strict laboratory procedures (like separate locations for pre– and post–PCR experiment setup). Furthermore, the level of sensitivity of the system is a keystone in all the process. Assuming a mutation frequency of, say, 10^{-6}, that would mean 1 mutant sequence in 10 ng, 100 ng or 10 µg of total DNA for bacteria, yeast or human cells, respectively. Thus, it is of paramount importance to accomplish a high sensitivity of the RSM system when applied to human DNA.

To develop and optimize the RSM system we have chosen a 857 pb sequence of the *araA* gene of *Salmonella typhimurium*. This sequence has five single–site restriction sites specially suited for RSM analysis (i.e., cut by highly processive and cheap restrictases). By ethyl methane sulfonate and PCR mutagenesis, five mutant sequences were obtained for the sites *Bss*HII, *Eco*RI, *Eco*RV, *Mlu*I and *Rsa*I. The mutant sequences were then cloned into pBluescript vector and used for the RSM optimization experiments.

We have carried out reconstruction experiments to determine the level of sensitivity of the RSM approach. Different amounts of mutant DNA were mixed with a fixed amount of λ or wild type DNA. The mix was exhaustively digested with the restrictase of choice and amplified by PCR. The amplification products were subjected to agarose gel electrophoresis and Southern blotted to nylon membranes. A DIG/AMPPD–based nonisotopic DNA detection system was used to determine the sensitivity of the methodology. We were able to detect up to 1–3 copies of mutant DNA when mixed with either 1 µg of λ as accompanying DNA, or (using more restrictive conditions) in the presence of 100 pg (10^7 copies) of wild type DNA, thus paving the way to its application for human biomonitoring.

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DETERMINATION OF GENOMIC INSTABILITY IN CANCER CELLS BY DNA FINGERPRINTING

Miguel A. Peinado[1], Antonia Velázquez[2], Laurie Wilson[2], and Manuel Perucho[2].
[1]Institut de Recerca Oncològica, Autovia Castelldefels km 2,7, L'Hospitalet, 08907 Barcelona, Spain and [2]California Institute of Biological Research, 11099 N-Torrey Pines Rd, La Jolla CA 92037, USA.

INTRODUCTION

It is generally accepted that genetic alterations are directly involved in the initiation and progression of tumors. The accumulation of different somatic mutations plays a key role in the invasiveness and eventual dissemination of tumor cells (reviewed in Fearon & Vogelstein, 1990; Fearon & Jones, 1992). Genomic instability is a typical feature of many cancer cells which may be the underlying cause for the accumulation of genetic alterations including imbalances in chromosome number that result in aneuploidy.

Measurement of DNA content (ploidy) and determination of fractional allelic losses (FAL) (Kern et al, 1989; Vogelstein et al, 1989; Laurent-Puig et al, 1992) provide an estimation of the extent of the genetic damage sustained by the tumor cells. Both parameters have been used as prognostic factors.

We have used a DNA fingerprinting technique to identify sequences that have been altered in the tumor cells versus the corresponding normal tissue.The method is based on the amplification by the Polymerase Chain Reaction (PCR) of multiple and anonymous sequences by using an arbitrary primer (Arbitrarily Primed PCR or AP-PCR, Welsh & McClelland, 1990) Previously, we have demonstrated that AP-PCR allows the detection of allelic losses and gains (Peinado et al, 1992) and microdeletions (Ionov et al, 1993) involved in the tumorigenic process. In addition, theoretical and experimental evidence shows that it is also possible to detect other more infrequent alterations such as chromosome rearrangements.

We postulate that genetic alterations detected by AP-PCR directly reflect the genomic damage sustained by the tumor cell and, consequently, quantification of these alterations may be a reliable indicator of genomic instability. In addition, we propose that measurement of genomic instability by AP-PCR may be useful as a prognostic indicator in colorectal cancers.

RESULTS

In preliminary studies we have analyzed by AP-PCR 70 colorectal tumors including 7 adenomas (all of them with their corresponding normal tissue DNA) with three different arbitrary primers. Since this method provides an unbiased examination of the genome, it can be expected that a random representation of the total number of alterations should be obtained.

When normal and tumor tissue fingerprints were compared, four types of alterations were detected. These include decreases and increases of intensity (losses and gains/amplifications respectively), microdeletions (deletions of a few nucleotides) and new bands that appear in the tumor tissue fingerprint but are neither present in its corresponding normal pair nor any other normal tissue (possibly corresponding to chromosomal rearrangements). All these alterations have been taken into account for the determination of genomic instability by AP-PCR, with the exception of microdeletions. Because microdeletions in simple repeated sequences are ubiquitous in a subset of colorectal tumors with differential molecular and biological characteristics (Ionov et al, 1993), they have not been considered in this preliminary study.

When tumors were classified according to the number of alterations, a bimodal distribution was observed (figure 1). Hence, we classified the tumors into two groups, tumors containing zero or one alteration and tumors with more than one (cumulative data of three different experiments). With current data, we can hypothesize that these two groups correspond to tumors with low genomic instability (≤1 alteration) and high instability (>1 alteration). Obviously, confirmation of this hypothesis and this bimodal distribution will require more experiments with different primers.

Interestingly, in spite of the few primes used and of the short series of samples analyzed, we observed that tumors with high number of alterations, as detected by AP-PCR, correlated with a shorter disease free survival. The number of alterations is higher in more advanced tumors (classified according to the Duke's stage). When tumors were separated depending on the presence of mutations in *ras* oncogene (K-*ras* and N-*ras*) this tendency was only manifested in those cases negative for *ras* mutation (p=0.032) (figure 2), indicating that in tumors that have progressed in the absence of *ras* mutations, genomic instability may play a more important role to produce an increasing malignancy than in tumors in which the *ras* mutation has occurred in earlier stages.

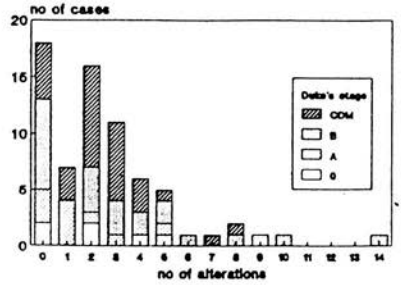

Figure1. Distribution of tumors according to the number of alterations detected after three AP-PCR experiments. Column filling pattern indicates Duke's stage. A test for deviation of the Poisson distribution indicated that alterations are not randomly distributed in this series of colorectal tumors (p=0.036).

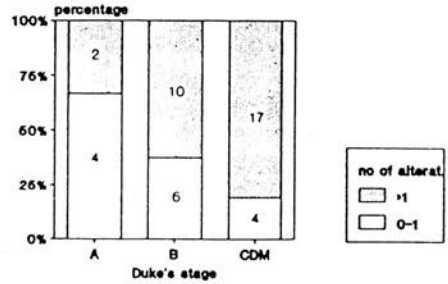

Figure 2. Relative distribution of tumors negative for mutations at the K-*ras* and N-*ras* oncogenes according to the Duke's stage and genomic instability as measured by AP-PCR. Shadowed areas indicate percentage of tumors with more than one genetic alteration and, therefore, considered positive for genomic instability. Numbers in columns indicate number of cases.

In contrast to previous hypotheses suggesting the importance of the accumulation of different genetic alterations rather than the order, our results point out that pathways for colorectal tumorigenesis may be distinctive, and that the presence and timing of some alterations might condition the *"necessity"* and/or the probability that other alterations occur. These data also suggest that determination of genomic damage by AP-PCR can be used as an independent prognostic factor.

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POSTERS

LOCALISED MAPPING OF SPECIFIC CHROMOSOMAL REGIONS CONFERRING DISEASE RESISTANCE IN POTATO

E. Baird, R. Waugh, C. Gebhardt, W. Powell

Scottish Crop Research Institute,
Invergowrie, Dundee DD2 5DA, Scotland, UK

Potato is an important crop plant world-wide however significant yield losses are caused by disease, pests and parasites.

It has become an important aim in potato research to identify genetic markers linked to disease/pest resistance traits and thus enable the use of marker based selection schemes in breeding programmes.

RFLPs have already been extensively used to construct genetic linkage maps of potato (Gebhardt *et al.*, 1989). Several resistance loci have subsequently been mapped to chromosomal regions.

In order to more precisely identify the chromosomal area associated with a particular disease resistance trait we attempted to saturate targeted areas of the chromosome with RAPD markers; in effect creating a more localised genetic map.

Two chromosomal regions were chosen, one on chromosome V targeting the area in which the R1 locus, which codes for *Phytophthora infestans*, had previously been mapped (Leonards-Schippers *et al.*, 1992). The other area was situated on chromosome VII where the *Gro*1 locus had been located (Barone *et al.*, 1990). This gene confers resistance to potato cyst nematode *Globodera rostochiensis*.

In order to screen the target areas quickly for RAPD markers two pooled DNA samples for each chromosomal region were created using known RFLP genotypes as described by Giovannoni *et al.* (1991). Amplification of DNA and identification of RAPD markers was carried out as described by Baird *et al.* (1992).

When the markers were mapped two useful markers were identified, one at each of the targeted areas. One of these markers was found to be more tightly linked to the chromosome VII trait than the existing RFLP marker and could itself be used in a selection scheme. Alternatively the markers could be sequenced and then the sequence information used to generate SCAR probes with which to screen populations.

The use of RAPD marker techniques in conjunction with pooled DNA strategy provides a quick and simple method of screening large numbers of potential markers in a short space of time. It is a method which is ideally suited to saturation mapping and to localised mapping of the genome.

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Polymerase Chain Reaction Mediated DNA Typing for Epidemiological Studies on *Campylobacter Spp.*

BELINDA A.J. GIESENDORF[1,2*], HERMAN GOOSSENS[4], HUBERT G.M. NIESTERS[1,3], ALEX VAN BELKUM[1], ANKIE KOEKEN[1], HENK STEGEMAN[2] AND WIM G.V. QUINT[1,3]

1 Diagnostic Centre SSDZ, Department of Molecular Biology
PO Box 5010, 2600 GA Delft, The Netherlands
2 State Institute for Quality control of Agricultural Products
Department of Microbiology
PO Box 230, 6700 AE Wageningen, The Netherlands
3 University Hospital Rotterdam-Dijkzigt
Department of Clinical Microbiology and Virology
Dr. Molewaterplein 40, 3015 GD Rotterdam, The Netherlands
4 Laboratory of Clinical Microbiology, University Hospital Antwerp
2650 Edegem, Belgium

In order to study *Campylobacter* epidemiology, the applicability of PCR mediated DNA typing, using primers complementary to dispersed repetitive DNA sequences and arbitrary DNA motifs, was evaluated. With a single PCR reaction and simple gel electrophoresis, strain specific DNA banding patterns were observed for all 33 *Campylobacter jejuni* and 85 *Campylobacter upsaliensis* strains. Strains isolated during an outbreak of *C. jejuni* meningitis all led to the generation of identical banding patterns and could be distinguished from randomly isolated strains. Strains from a community outbreak of *C. upsaliensis*, all identical based on conventional typing methods, could be divided into two genetically typeable groups. This report illustrates that PCR fingerprinting can be successfully applied in epidemiological investigations on *Campylobacter* infections.

ALU-PCR FINGERPRINTING OF YEAST ARTIFICIAL CHROMOSOMES. Alison Coffey[1], Charlotte Cole[1], Simon Gregory[2], Violet Trim[1], Michael Yee[1], Peter Little[2], John Collins[1], Ian Dunham[1], David Bentley[1]. [1]Division of Medical and Molecular Genetics, UMDS of Guy's and St. Thomas's Hospitals, London, UK; [2]Department of Biochemistry, Imperial College of Science Technology and Medicine, London, UK.

Despite the recent success for both the human Y chromosome and chromosome 21 of an STS approach to constructing YAC contigs, it would be more convenient to be able to map large regions of the genome without the need to generate hundreds of STSs. Random fingerprinting approaches applied to unordered collections of clones have been very successful at assembling contigs in several smaller genomes. The success of these approaches has been facilitated by the ability to generate and resolve sufficient numbers of bands in each fingerprint to detect as many as possible of the potential overlaps. However, the application of random fingerprinting to human YACs has thus far been limited to detection of restriction fragments containing human-specific repetitive sequence by Southern blotting. We have sought to increase both the resolution and informativeness of fingerprints obtained from human YACs using a differernt method.

We have developed a rapid and efficient high resolution YAC fingerprinting system based on *Alu*-PCR performed directly on yeast colonies. The primers used direct amplification from the left and right hand monomers of the *Alu*-element (ALE1 and ALE3 respectively). In order to maximise the number of *Alu*-PCR products and hence the information content of the fingerprint, they are used together giving an average of 33 bands amplified per YAC. This represents a five- to ten-fold improvement when compared with other previously published primer combinations.

Colonies are resuspended and an aliquot of the suspension is used in a primary PCR in the presence of both two primers (ALE1 and ALE3) for 30 cycles. The primary PCR products are diluted and subjected to a second round of PCR in the presence of both ^{32}P end-labelled primers for 10 cycles. The products are electrophoresed on high resolution denaturing polyacrylamide gels with ^{35}S labelled *Sau*3AI digested lambda DNA markers run every seventh lane to allow for effective gel-to-gel comparisons. The resulting autoradiograms are digitally scanned and the data entered using a semi-automatic data entry system then analysed using CONTIG 9 as successfully applied to the *C. elegans* genome project.

To test the feasibility of this approach in generating contigs de novo, a 2.5-3 Mb YAC contig in human Xq26 was used as a model system. All but two overlaps were identified: these were in regions known to amplify few *Alu*-PCR products and which may be *Alu* poor

or the region of overlap may be small.

We then applied this approach to a collection of 733 YACs selected with human chromosome 22 probes. In an initial analysis at a random matching probability of 10^{-5} or less, 353 YACs have been assembled into 89 contigs of up to 21 clones. The validity of the assembled contigs was confirmed by comparison with STS and probe content data. On further examination of the data, approximately one-third of the clones which remained unattached after the initial analysis were found to hybridise to repetitive probes or have no probe data associated with them and are therefore possibly not chromosome 22 specific. Since this initial analysis a further 300 YACs from chromosome 22 have been fingerprinted and are currently being incorporated into the analysis.

The *Alu*-PCR fingerprint can be used in the detection of chimaeric clones within a contig of sufficent depth. While novel single bands may be the result of polymorphism, multiple bands which are not found to be present in YACs spanning the clone in question are very likely to come from a chimaeric portion of the clone.

The total *Alu*-PCR products generated for fingerprinting are also a source of hybridisation probes which can be used to detect or confirm potential overlaps. Alternatively, individual bands can be purified for use as landmarks. Following autoradiography the region of the gel containing the appropriate PCR product is cut out and a toothpick used to transfer a small aliquot into a secondary PCR for a further 20 cycles in the presence of ALE1 and ALE3. This approach has been used to isolate a landmark to confirm the overlap between two contigs suggested by fingerprinting. The fingerprint also allows the identification of candidates for "surrogate end-probes" of YAC clones for walking.

Producing Randomly Amplified Polymorphic DNAs (RAPDs) from degraded DNA.

Emmanuel Dias Neto(1), Otavia Luísa S. D. Caballero(1, 3), Teofânia H. D. A. Vidigal (1), Sérgio D.J. Pena(2) and Andrew J. G. Simpson (1).
1- Centro de Pesquisas "René Rachou"- FIOCRUZ. Av. Augusto de Lima, 1715-Belo Horizonte, MG - 30.190.002 - Brazil. 2- Laboratório de Genética/Bioquímica - Depto de Bioquímica e Imunologia - ICB - UFMG Belo Horizonte, MG Brazil. 3- Departamento de Ciências Biológicas - Faculdade de Medicina do Triângulo Mineiro, Uberaba - Minas Gerais, Brazil.

The amplification of genomic DNA with either arbitrarily selected [*Nucleic Acids Res.* 18 :7213-7218 *Nucleic Acids Res.* **18**:6531-6535] or specific [*Nucleic Acids Res.* **21**:763-764] primers under low stringency conditions produces complex patterns of DNA fragments (termed RAPDs, Randomly Amplified Polymorphic DNAs, or LSPs, Low Stringency Products) that can be resolved by gel electrophoresis. These patterns provide information concerning the sequence arrangements of the genome and can detect polymorphisms among closely related genomes. We have been using low stringency PCR technologies in studies involving parasites and other infectious microorganisms [*Mol. Biochem. Parasitol.* **57**:83-88]; when working with such organisms, sometimes, the biological specimens are not well conserved and the DNA is partially degraded. We thus investigated whether it is still possible to obtain meaningful and reproducible PCR fingerprints in these situations and whether the fingerprints can be compared with those obtained with high molecular weight DNA.

For this study we extracted total nucleic acids from adult worms of the trematode parasite *Schistosoma mansoni* and from the bacteria *Leptospira interrogans.* To obtain the degraded material, nucleic acids were sonicated by a ultrasonic processor, 50 watt model (Tekmar Co., USA) at maximum power and examined by agarose gel electrophesis. The initial DNA was of high molecular weight and contaminated by RNA; after sonication for two minutes no DNA fragments of greater than 500 bp were detectable. This time point was chosen as the end point as the DNA fragments produced were similar to those obtained from poorly preserved specimens.

The use of degraded DNA as template for the amplification of specific sequences has been previously demonstrated [*Bio Technology* **6**:943-947]. However, this is the first demonstration of the use of degraded DNA for the generation of complex DNA banding

patterns. The main features of the results are the overall preservation of the DNA profile, the reproducibilility of the duplicate samples taken at each time point of sonication and an increase in the relative intensity of the low molecular weight bands with increasing DNA degradation. Most significantly, even the most degraded sample tested produced a profile that was directly comparable with that obtained from high molecular weight DNA. In general using acrylamide gels, which we favour because of their increased sensitvity, we use DNA fragments of 1.0 kb or less for analysis. Longer fragments are of little informative value due to the poor resolving power of the gel system but often tend to be more intenseand dominate RAPD profiles. The reduction of the DNA to small fragments prevents the priming events that result in higher molecular weight products from being productive so that in later rounds of amplification they are not depleting reagents (primers and enzyme). The net result is that weaker bands in the lower half of the gel, that are of great value as markers because they are well resolved, are more efficiently amplified and more easily scored on the gels.

In face of the reproducibility of the profiles, including the intensity of the low molecular weight bands, we have found that the sonication of the DNA prior to testing can be a advantage. For example, we have found that the use of primers specific for *Leptospira* DNA under low stringency conditions (manuscript in preparation), produces a complex pattern of low stringency products (LSPs) that are highly polymorphic and serve to distinguish all the biological variants (serovars) that we have examined to date. In this case the most useful fragments are all of 0.2kb or less. Frequently, these bands were extremely weak requiring repeated amplification with higher volumes so that greater quantities of reaction products can be loaded on the gel. More uniform and easily interpreted results are obtained if we sonicate the DNA to an average size of 0.2 kb (2.0 minutes of sonication) before amplification. In conclusion, we find that degraded DNA can be analyzed by low stringency PCR protocols and the profiles can be directly compared with those obtained with high molecular weight DNA. Furthermore sonication prior to amplification increases the informative value of one DNA profile because of the shift of intensity to lower molecular weight bands that are better resolved using acrylamide gels.

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Unpredictably Primed PCR for gene walking (UP PCR)

Orlando Domínguez and Carlos López-Larrea

Inmunología, HCA, E-33006 Oviedo

Introduction

Several modifications on standard PCR have been proposed to isolate unknown sequences next to a known locus. The problem to solve is how to anchor a primer at an unknown sequence stretch in a distal position to a locus specific primer. Some have avoided this problem by using PCR primers specific to the known locus on original orientations (IPCR, panhandle). Others have faced the problem by attaching linkers or tails to the ends of digested DNA (vectorette), they had prepared oligos complementary to those adaptors. A third approach has employed primers of arbitrary sequence either alone or in conjunction to locus specific primers (targeted gene walking).

Their overall applications either use lower complexity "easy" genomes (bacteria) or require extensive manipulation to get a dubiously clean walk.

We present a method that yields neat gene walks with the aid of multipurpose arbitrary sequence oligonucleotides.

Methods

UP PCR consists of two rounds of amplification preceded by a dedicated primer extension step and employs four different primers.

The locus specific and inner locus specific primers (SP and iSP, respectively) are oligos complementary to the known sequence from which one intends to walk. Both have their 3' end oriented toward the desired sequence. The SP is 25 to 30-mer to help in the specificity. The iSP gives the advantages of a nested primer and can be shorter than the outer one.

The walking primers (WP and short WP or sWP) are oligos of arbitrary sequence. The recommended WPs have the same basic design and have been used successfully on a variety of templates: T_mG_nT. The one to be used at the 1st PCR (WP) is chosen in function of the SP's Tm. Whereas the one of the nested reaction (sWP) is included on the WP and balanced to the iSP.

The 1st primer extension reaction affects to only the WP which is allowed to

anneal anywhere in the sample at low stringency

In a fraction of cases it anneals on the proximity of the desired locus and its extension copy the known sequence. This brand new strand includes the complementary sequence to the SP.

Then the SP is included at high temperature and after denaturation it is allowed to anneal at high stringency. It will extend both from natural complementary targets and from strands just synthesised: former extensions from the WP that copied the known locus. When copying a new synthesised strand the sequence complementary to the WP will be generated at its 3' end. These latter extensions are the desired ones, in which the WP is at one end and the SP at the other. Ideally and keeping this temperature as annealing temperature (Ta) on PCR the WP does not find any other complementary site from where to extend.

Anyway a nested PCR is needed to enrich in specific walks. It employs sWP and iSP as primers, in otherwise standard conditions.

Results and Discussion

The Unpredictably Primed PCR (UP PCR) permits the enzymatic amplification of unknown sequences flanking a known one within a sample of high complexity, i.e., mammalian genome. The unpredictable feature applies to the position from which a PCR primer of arbitrary sequence extends distally to a locus specific oligonucleotide.

Although the WP extends ubiquitously: the same WP can be used for virtually any gene walk, the experimental conditions select the extensions selected by the SP.

Following two rounds of amplification single specific products are seen under ethidium bromide staining. The product length is usually around 500 bp.

In order to get an specific walk and depending on SP's Tm, two to three trial reactions might be needed.

Results will be shown illustrating the reliability of UP PCR. It extends to every locus tested up to date: Ki-ras, murine complement factor H, a locus of Lambda among a background of human genomic DNA and other unpublished human complement sequences. It has been used to access to both promoter and intronic sequences.

MOLECULAR CHARACTERIZATION OF A SUGAR BEET CROPS PEST, *Aubeonymus mariaefranciscae*, BY AP-PCR ANALYSIS. PRELIMINARY RESULTS.

Ana Taberner[1], Joaquín Dopazo[2], Ana Rodríguez[2], Francisco Sobrino[2] y Pedro Castañera[1].

[1] Departamento de Biología de Plantas, Centro de Investigaciones Biológicas, CSIC. Velázquez 144. 28006 Madrid, Spain.
[2] Centro de Investigación en Sanidad Animal, INIA. 28130 Valdeolmos, Madrid, Spain.

Aubeonymus mariaefranciscae Roudier (AMF) is a curculionid, first detected as a new pest of sugar beet in Andalucia (Spain) in 1979. Later, in 1981, was described as a new species (Roudier, 1981). At present, economic damages have been reported in about 3000ha in Córdoba, Sevilla and Jaén provinces, with a clear trend to expand to other areas.

Despite of the time elapsed since its was first reported, there is little information on the biology and ecology of AMF, partially due to its telluric habits. Among the various causes of AMF outbreaks, we are considering two alternatives hypothesis: i) the independent colonization of sugar beet crops by a wild type of AMF, already present in the field, with potential risk of new reintroductions in other areas which overlap the natural ecological niches of AMF (still unknown); ii) a unique colonization event, followed by its disperssion to the other affected areas.

The study of molecular markers can help in the characterization of the different AMF populations. From the study of the degree of genetic relationship among them, information about the origin and dispersion of the AMF outbreaks might be drawn, which is essential for the control of this pest. We have chosen AP-PCR fingerprinting because it is more informative than isoenzyme analysis or RFPL (see Welsh and McClelland, 1990), specially in populations composed of insects where a high degree of genetic relationship might be expected.

Preliminary results included the detection of variability within and between AMF populations. This confirms AP-PCR as a promising technique for the study of AMF from an ecological and evolutionary point of view

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GENOME ORGANIZATION OF THE CONIFER *LARIX DECIDUA* USING PCR-BASED MARKERS.

Keith Hutchison[1], Patricia Singer[1], Antoinette Decker[1], Colleen Skelly[1], Hugo Volkaert[2] and Michael Greenwood[2], [1]Department of Biochemistry, Microbiology and Molecular Biology, and [2]Department of Forest Biology, The University of Maine, Orono, Maine 04469

Conifers, as a group, represent one of the major plant crops of the world. However, until recently there has been little progress in our overall understanding of the organization of conifer genomes, and little use of genetic mapping data for the genetic improvement of conifers relative to the herbaceous crop plants. Genetic analysis of conifers has been impeded, of course, by their slow growth, long generation times, and poor juvenile-to-mature correlations. The genetic maps that do exist for this group of plants have been generally limited to a small set of loci defined by isozymes of proteins expressed in the haploid megagametophyte. Interestingly, this limited set of data suggests a strong syntenic relationship among the various conifers, even after a long evolutionary time scale. Most conifers have the same number of chromosomes (n=12). Genetic maps in different genera tend to show co-linearity and similar frequencies of recombination between loci. It is not clear, however whether the syntenic relationships are true for the entire genome or only this small set of loci. To address the question of conifer genome organization and evolution, genetic maps of much greater detail are needed for a variety of species.

PCR-based strategies for scoring segregation of alleles at informative loci now allows a nearly unlimited number of loci to be mapped. And though conifer genomes are physically large, often greater than 10^{10}bp, it does not appear that they are genetically large to the same degree. That is, the number of markers required will not be proportionally greater than other plant, or animal species.

Though conifers have long generation times, rapid construction of molecular genetic maps in these trees is possible due to two major characteristics. The first of these is that the tissue surrounding the embryo in the developing seed, the megagametophyte, is haploid of maternal origin. DNA extracted from megagametophyte provides enough material for up to 2000 PCR reactions. The second characteristic is that conifer genomes are highly heterozygous and highly polymorphic. Therefore, two or more alleles are relatively easy to find at any locus.

We are developing a "fingerprint" for the genome of the conifer *Larix decidua*, (European larch) using a combination of PCR-based strategies. We have initiated this work by amplifying megagametophye DNA using RAPD primers (Operon). Radiolabelled RAPD-PCR products are separated on non-denaturing acrylamide gels. We have found that >90% of RAPD primers produce

polymorphic bands, with an average of 5 polymorphic bands per primer. Approximately 5% of the polymorphic bands appear to be co-dominant, that is, the alleles are bands of clearly distinguishable size differences.

Whereas the highly polymorphic nature of conifer genomes has meant that RAPD-based maps are quickly and easily obtained, it has also a significant disadvantage. Work on RAPD-based maps in a number of pine species has shown that such maps are specific for individual trees and cannot even serve as anchor points for genetic analysis within a species. Therefore, we are using our RAPD-based molecular genetic map to link other PCR-based markers. For this purpose, a genomic library of a complete Mbo I digest of European larch genomic DNA was constructed. By cloning small fragments of genomic DNA, few clones will contain both single-copy and repetitive sequences. Furthermore, we took advantage of the fact that in conifer genomes 20-30% of the cytosine residues are methylated. By cloning the library in *mcr*A$^+$-*mcr*B$^+$ *E. coli* host, sequences containing highly methylated DNA were eliminated. This produce a genomic library which was nearly completely single copy sequences. The DNA sequence for the ends of each of the single-copy clones are being determined and used for the construction of specific primer sets. We are using single-stranded conformational polymorphisms of PCR-amplified genomic DNA to score loci. DNA from either the haploid megagametophyte, or individual pollen grains can be used in these reactions. We have also cloned and sequenced a number of loci containing micro-satellite DNA. We have loci containing both $(GT)_n$ repeats and $(AG)_n$ repeats. We are currently analyzing these loci for the level of polymorphism. All non-RAPD-based markers will then be used to compare genome structure across species and genera.

Molecular Analysis of *Salmonella typhi* by Pulsed-Field Gel Electrophoresis

Kwai-Lin Thong, S.D. Puthucheary[1], Chong-Lek Koh[2], Yuet-Meng Cheong[3], M. Jegathesan[3], and Tikki Pang

Institute for Advanced Studies, [1]Dept. of Medical Microbiology, and [2]Dept. of Genetics & Cellular Biology, University of Malaya, 59100 Kuala Lumpur; [3]Bacteriology Division, Institute for Medical Research, 50588 Kuala Lumpur, Malaysia.

DNA profiles generated by pulsed-field gel electrophoresis (PFGE) are now widely used in analysing and comparing genetic identities of bacterial pathogens. *Salmonella typhi,* the causative agent of typhoid fever, remains an important public health problem in many parts of the world, including Malaysia, an endemic country. We report here the usefulness of PFGE in assessing the extent of genetic variation among *S. typhi* strains isolated in Malaysia and the determination of the genome size of *S. typhi.*

A total of 158 *S. typhi* were isolated from blood or stool of patients. They were: (a) from patients admitted to the University Hospital between 1987 to 1991; (b) outbreak strains from well-defined areas between 1989 to 1991; and (c) sporadic strains isolated at about the same time as the outbreaks. The strains were phage-typed at the Institute for Medical Research, Kuala Lumpur, Malaysia.

Initially 23 restriction endonucleases were used to digest *S. typhi* genomic DNA to assess their usefulness in generating DNA fragments suitable for PFGE analysis with a CHEF-DRII System (Bio-Rad). Among them, *Avr*II, *Sfi*I, *Spe*I, *Xba*I, and *Xho*I generated distinct and suitable number of bands. Within each outbreak location, the genomic fingerprints of *S. typhi* isolates showed no or slight variation. Comparison of representative outbreak *S. typhi* strains from different locations showed that a particular genomic fingerprint was associated with each of the outbreaks. However, the genomic fingerprints of the sporadic strains, isolated at about the same time as the outbreaks, showed considerable variation. Each genomic fingerprint pattern differed with respect to the number of bands generated and the electrophoretic mobility of the bands. A dendrogram, computed by using the neighbour-joining method (1), showed that the sporadic strains were more variable compared to the outbreak strains.

Using *Avr*II, *Sfi*I, *Spe*I, *Xba*I, and *Xho*I to digest DNA from 8, 7, 37, 48, and 10 *S. typhi* isolates, respectively, to generate 11 to 24 DNA fragments ranging from 20 to 630 kbp, we estimated the mean genome size of *S. typhi* to be 4.38 mbp. This genome size is comparable to that of *Salmonella typhimurium*, reported to be 4.8 mbp (2).

To the best of our knowledge, this represents the first report of the genome size and analysis of *S. typhi* by PFGE. It should be noted, however, that PFGE has some inherent limitations related to a degree of subjectivity in assessing the existence of bands, and the possibility of two or more fragments co-migrating in one band cannot be excluded in this study.

This study, with *S. typhi* isolated in an endemic area, supports other studies (e.g., 3,4) that PFGE is a useful technique in differentiating individual strains of pathogenic bacteria and may complement phage typing and other molecular approaches for epidemiological purposes. PFGE is more convenient to perform compared to other methods (e.g., ribotyping) of assessing genetic variation as it does not require the transfer of DNA fragments from agarose gels to solid supports or the use of labelled nucleic acid probes. In addition, pattern differences observed reflect differences over the entire genome, rather than polymorphisms detected with a specific nucleic acid probe.

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CHARACTERIZATION OF *Leptospira* ISOLATES FROM SEROVAR HARDJO USING ARBITRARILY PRIMED PCR AND MAPPED RESTRICTION SITE POLYMORPHISMS.

PHILIPPE PEROLAT [1*], FABRICE MERIEN [1], WILLIAM A. ELLIS [2] AND GUY BARANTON [3].

Laboratoire des Leptospires, Institut Pasteur, B.P. 61, Nouméa, Nouvelle-Calédonie,1 Veterinary Research Laboratory, Stoney Road, Stormont, Belfast BT4 3SD, Northern Ireland, United Kingdom,2 and Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 75724 Paris Cedex 15. 3

Leptospirosis, caused by *Leptospira interrogans*, is a zooanthroponosis which has a worldwide distribution. For taxonomic purposes the parasitic leptospires have been subdivided into serogroups on the basis of antigenic relationships, as determined by cross agglutination reactions, and further subdivided into serovars by agglutination-absorption patterns. There are some 23 serogroups recognised, containing approximately 212 serovars.
The advent of genetic typing methods has thrown the taxonomy of the genus into some confusion, but has provided rapid, reproducible typing protocols. Seven genospecies of parasitic leptospires have been described - based on DNA relatedness and guanine + cytosine (G + C) content studies (3, 7). The value of this system with respect to epidemiology is limited. Cattle act as maintenance hosts for serovar hardjo and hardjo is endemic in the cattle populations of most countries as a major factor in bovine abortion. It is probable that strain differences make an important contribution to differences in epidemiological situations of hardjo infection throughout the world. The genomic heterogeneity of strains grouped as serovar hardjo was first demonstrated by using REA (1), leading to the delineation of two subtypes, Hardjoprajitno and Hardjobovis.
Polymerase chain reaction (PCR) based characterisation methods use much less DNA - an important consideration when studying such slow growing bacteria as leptospires. Arbitrarily primed PCR (AP-PCR) (5) generates fingerprints that can be used to compare microorganisms at the species level (6) and within a species. This technique has recently been applied to the study of selected *Leptospira* strains (4). A new PCR strategy, based on the study of mapped restriction site polymorphisms (MRSP) in PCR amplified *rrs* and *rrl* ribosomal genes, was developed and tested on *Leptospira* (4) allowed the grouping of reference strains into 16 MRSP profiles which correlated with genomic as species defined in DNA relatedness studies (3,7). The purpose of this study was to characterise a significant set of Hardjoprajitno and Hardjobovis isolates from various geographical origins using AP-PCR and MRSP in PCR amplified rRNA genes in order to evaluate these techniques as methods for characterising and differentiating strains within a single important *Leptospira* serovar.

Thirty-nine isolates from serovar hardjo were studied. These strains, were previously typed by restriction enzyme analysis : 27 belong to Hardjobovis type and 12 to Hardjoprajitno type. AP-PCR fingerprints were obtained as previously described (5,6) with 7 primers KF, KG, KN, KpnR, KZ, RSP, SP. Mapped restriction site polymorphisms (MRSP) in PCR-amplified *rrs* and *rrl* genes analysis was performed according to Ralph *et al* (4). Arbitrarily primed PCR leptospiral fingerprints were obtained with the seven primers tested. The level of complexity of the patterns was variable , according to the primer used. It was simple with few different products using KpnR and SP and relatively complex with KF, KG, KN, KZ and RSP . In each case, strains belonging respectively to the Hardjoprajitno and Hardjobovis groups could be clearly separated on the presence of one to nine proeminent products specific to each of the two group. As demonstrated with primers KF and KG (4) and RSP (2) on reference strains of *Leptospira*

genospecies, proeminent species specific products (not shared with the other leptospiral genospecies) could be oberved . The Hardjoprajitno group included *Leptospira interrogans* strains; conversly the Hardjobovis group exibited the *Leptospira borgpetersenii* species specific fragments. In addition to these species specific AP-PCR products, variations in other products were observed on sequencing gels between strains of the two groups and within each group. As previously demonstrated (4), the primers used allowed amplification of conserved portions of the *rrs* and *rrl* genes. The *rrs* (with *Dde*I, *Hha*I, *Nla*IV and *Taq*I) and *rrl* (with *Hha*I, *Hph*I and *Mnl*I) restriction data allow the assignation of Hardjoprajitno and Hardjobovis isolates respectively to the *L. interrogans* MRSP profile B and *L. borgpetersenii* MRSP profile C.

Analysis of arbitrarily primed PCR fingerprints and mapped restriction polymorphisms in *rrs* and *rrl* ribosomal genes allowed us to categorize a significative set of leptospiral field isolates from serovar hardjo at the genospecies and subspecies levels. Furthermore, on the basis of AP-PCR fingerprints, a genomic heterogeneity was evidenced among the strains belonging to the Hardjobovis group commonly associated with bovine leptospirosis throughout the world. Using both approaches, analyzing respectively highly conserved genes (MRSP) and the whole genome (AP-PCR), which converge is significant. Differences in the pathogenicity of the two groups Hardjoprajitno and Hardjobovis. have been suggested (1) and the epidemiology of Hardjobovis zoonotic infections is very variable according to the geographical region which is considered (8 and references therein). This report conducted on a significant set of serovar hardjo strains from various geographic origins confirms the hetererogeneity of this serovar at the genospecies level. The dual usefulness of arbitrarily primed-PCR fingerprints for the characterization of leptospiral isolates is also illustrated in this study. At the species level, the presence of specific proeminent PCR products allowed a simple and reliable categorization. In addition the sensitivity of AP-PCR for sequencing divergence made possible intraspecific comparisons . However, significant relations between geographical areas and particular fingerprints could not be demonstrated : this requires further investigations based on more isolates from each zoonotic focus during a given epidemic period. These data have to be correlated to the recent evidence of the heterogenous distribution of the repetitive sequence IS 1533 among the genome of Hardjobovis strains which led to the delineation of 10 genetic groups (8): the invariability of a number of Hardjobovis specific AP-PCR products could suggest that these portions of the genome are stable while the other regions are submitted to genetic rearrangements based on insertion sequences. This genomic plasticity does not seem constant among *Leptospira* as the reference strains of the serovars icterohaemorrhagiae and copenhageni, serotypically closely related, present similar AP-PCR fingerprints (4).
This study also reports the practical interest of the PCR-based strategies to classify *Leptospira* isolates at the species level and to study infra specific population structures. More, for slow growing bacteria such as leptospires, PCR needs much less cells or DNA : in the case of serovar hardjo isolates, MRSP profiles and arbitrarily primed PCR fingerprints categorized quickly a significative number of strains and stated their species assignation. In addition MRSP does not require srain isolation which is not always possible for *Leptospira* . The detailed study of arbitrarily primed PCR fingerprints allows an accurate comparison of strains isolated in different geographic areas. As the questions of the clonal nature or of the level of genomic heterogeneity of strains isolated inside a delimited enzootic focus remain, AP-PCR fingerprinting may contribute to the knowledge of the molecular epidemiology of hardjo infections and could be applied to other *Leptospira* serovars of public health importance.

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A newly available Mouse mapping tool: Interspecific backcross DNA panels typed with motif-primed PCR and anchored with SSLP and proviral loci

Lucy B. Rowe, Minoru S. H. Ko, Wayne N. Frankel, Verity Letts,

J. H. Nadeau, and Edward H. Birkenmeier

The study of genome organization and function has been recently given high priority in biomedical research programs throughout the world. High resolution genetic and physical maps are the focus of intensive efforts in many laboratories. The rapid development of new techniques for genetic analysis has permitted an exponential increase in the density of loci placed on the genetic maps of many species. Comparative mapping of evolutionarily conserved chromosomal segments between mouse and human have expedited the placement of many important loci on the human map. For most laboratories, however, the process of mapping genes of interest in the mouse has remained cumbersome. Readily available DNAs from recombinant inbred strains permitted typing of DNA polymorphisms between some closely related inbred mouse strains, but the number of available strains is small, as is the degree of polymorphism between founders. Interspecific backcrosses have recently been exploited in several laboratories to overcome these two limitations. Since every backcross animal is genetically unique, however, the density of genetic markers on these maps will ultimately be limited by the quantity of DNA originally isolated. Therefore the use of these mapping tools has been restricted to a small number of specialized laboratories, and the availability of the data from these crosses has been limited.

We have prepared DNA from whole animals which are progeny of the two reciprocal interspecific backcrosses of (C57BL/6J x SPRET/Ei) inbred mouse strains. The high yields (25 to 50 mg of DNA per animal) have allowed us to establish a pair of DNA panels available for use in many laboratories for the purpose of mapping large numbers of genetic loci. The panels are aliquotted in microtiter format, with 94 backcross DNAs and two parental controls, at dilutions convenient for either Southern blot RFLP or PCR polymorphism typing.

Initial characterization of the genetic maps of both panels has been completed, so that new loci will be mapped with a high probability and certainty. We have used the SSLP primers from Research Genetics to place a minimum of two anchor loci per chromosome, establishing chromosome identity and centromeric orientation. These loci also serve to connect our backcross panel maps to the established detailed maps of the mouse. In addition, we have typed the panel DNAs for a large number of proviral loci, many of which have been previously mapped using recombinant inbred strains. Several other sequence-defined genes have also been mapped on these panels by several different laboratories, using either traditional cDNA probing of Southern blots, or specific PCR with primers derived from known gene sequences. Thus, the Panel 1 map (N2 from the F1 backcrossed to C57BL/6J) is anchored by 44 SSLP and 28 gene sequence loci, and the Panel 2 map (N2 from the F1 backcrossed to SPRET/Ei) contains 48 SSLP loci, 119 proviral loci, and 61 gene sequence loci.

We have used motif-primed PCR to "fingerprint" these panel DNAs, allowing the scoring of 10 to 30 different polymorphic loci in each PCR reaction. Since so many loci are easily scored, we have used this method to

fill in the maps with a high density of markers. The primers are chosen from conserved DNA sequence motifs important in gene regulation and function. 15 to 18-mers were selected from GPDH fat-specific elements, Isl-1 LIM motif and homeodomain, C/EBP gene family promoters, leucine zipper, and basic region, CRIP zinc finger domain, regulatory domains from c-myb, various Pax class consensus sequences, and consensus from conserved regions of potassium channel gene families. These primers were used in both logical and random pairwise combinations to generate highly reproducible complex patterns of radiolabelled PCR products visualized on films from non-denaturing sequencing gels. Although the exact genetic function of the sequences represented by these polymorphisms is not yet elucidated, even as anonymous DNA sequence tags they provide the marker density to allow new loci to be placed on these maps with high certainty. Because of the multiplicity of bands in each lane on the films, the fingerprinting method allows a greater degree of internal control for band intensity and detection of possible rare misloadings which would otherwise cause artifactual scorings in the data set. Thus the reliability of the maps is enhanced by the use of MP-PCR to generate a high density of markers. To date we have placed 141 MP-PCR loci on the C57BL/6J backcross Panel 1 map, and 271 MP-PCR loci on the SPRET/Ei backcross Panel 2 map. We have selected the Panel 2 DNAs for our more intense effort since polymorphisms most easily mapped on this backcross will be C57BL/6J-specific, and thus are more generally applicable to other molecular analysis such as library screening or mapping in other crosses.

There are two individual maps currently available, each representing one of the two panels. All mapping data are listed in the resource cross_matrix database at The Jackson Laboratory. Each newly mapped locus contributed to the database engenders a report to the investigator including relevant closely linked typings in the public database, a graphic representation from RIManager of the mapped region, and statistical analysis of relevant map distances including 95% confidence limits also from RIManager. Virtually all new loci can be located with a high degree of certainty on the maps at their current marker density.

Examining the overall patterns of segregation revealed by these data allows detection of some interesting genetic phenomena. There appear to be regions of segregation distortion, some driven by heterozygote advantage, and some by a specific allele preference. In addition, the frequency and distribution of crossovers on individual chromosomes can be examined. It is clear that this kind of database will be increasingly useful for addressing questions about genome behavior in meiosis, fertilization, and development.

Future experiments will be designed to simultaneously address remaining questions about the use of the motif primers to detect functional gene sequences, and to continue to improve the density and comparability of these interspecific backcross maps to the other existing mouse genetic information.

Sampling of nucleic acids sequences to study the molecular evolution of foot-and-mouth disease virus.

J.C. Sáiz, E. C. Carrillo, A. M. Martín-Hernández, A. Holguín, B. Borrego, N. Sevilla, E. Martínez-Salas, E. Escarmís and E. Domingo.

Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid, Cantoblanco, 28049-Madrid, Spain.

We have been interested in the molecular basis and biological implications of the quasispecies structure (extreme genetic heterogeneity) of RNA viruses, using mainly foot-and-mouth disease virus (FMDV) as a model system. This picornavirus causes an acute debilitating infection of cattle, but it also may produce asymptomatic persistent infections in animals. Many natural isolates and laboratory variants of this pathogen are available and have been used to study evolution and machanisms of antigenic diversification of a viral quasispecies.

In the last decade, sampling of nucleotide sequences of genomic FMDV RNA has involved a number of techniques: T1 oligonucleotide fingerprinting of unfractionated genomic RNA or of defined genomic fragments isolated by hibridization to cDNA representing relevant genomic regions (Sobrino *et al.* , 1986). More recently, RNA and cDNA sequencing have been applied to determine average or consensus sequences in FMDV quasispecies; in addition, cloned cDNA sequences have been determined to study mutant spectra in individual populations. Application of the polymerase chain reaction (PCR) has facilitated sequence determinations from minute amounts of viruses such as those present in individual plaques or those that survive in a few cells at the onset of a persistent infection in cell culture. These analyses

have documented the quasispecies structure of FMDV and its influence in the antigenic diversification of this virus (for review see Mateu *et al.* 1989; Domingo et al., 1992; Borrego *et al.* , 1993) .

Continuous viral genomic changes were also documented with BHK-21 cell lines persistently infected with FMDV. In spite of initiating persistence with cloned cells and plaque-purified virus , rapid generation of heterogeneity occurred both in the virus and in the host cells. Upon passage of the carrier cultures the virus became increasingly virulent for the cells and the cells, in turn, became increasingly resistant to the virus (de la Torre *et al.* , 1988) A typical gradual change towards a round cell morphology with passage number, as well as the restoration of the BHK-like morphology after ribavirin treatment. The cells reached an extremely high degree of resistence to FMDV and they did so by generating diverse cell subpopulations differing in growth properties and resistance to FMDV (de la Torre *et al,*. 1989) Furthermore, recent analyses have indicated that rapid cell variation — rather than virus variation— was the main determinat of the initiation of persistence (A. M. Martín-Hernández *et al.* 1993). This was surprising in view of the adaptability generally attributed to viral quasispecies. Sequence analysis of coding and non-coding FMDV genomic sequences (a total of 46,490 nucleotides) at the onset of persistence revealed a complex quasispecies (heterogeneity of 1.6×10^{-3} to 1.6×10^{-4} substitutions per nucleotide) and rapid evolution (4×10^{-5} to 6×10^{-5} substitutions per nucleotide per day). However, the quasispecies evolved towards increased virulence rather than attenuation.

Given the profound phenotypic alterations observed in carrier BHK-21 cells —even when virus was eliminated by ribavirin

treatment— it was of interest to sample the differences in gene expression between the evolved and the parental BHK-21 cells. The RNA arbitrarily primed polymerase chain reaction (AP-PCR) fingerprinting method described by Liang and Pardee (1992) and Welsh et al. (1992) was used with several primers of 10 to 14 nucleotides in length. Preliminary results using RNA extracted from the two types of cells produced an average of 4 band differences, out of about 180 bands visualized per primer pair. The differences observed between the two types of cells with this assay probably reflect differences in gene expresion and/or RNA stability. The results suggest that up to 1 to 2% of the RNA molecules visualized differ in the two types of cells. This may reflect a quite high degree of genetic and/or functional diversification of BHK-21 cells promoted by their coevolution with resident FMDV. In addition to providing some quantification of the differences in cellular RNA, it is hoped that this fingerprinting procedure may lead to identification of cellular gene functions relevant to initiation and/or maintenance of persistence, or able to mediate resistance to FMDV.

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SUMMARY

X. Estivill

Genome fingerprinting has been an area of rapid development in recent years. Polymerase chain reaction (PCR) based technology has provided the appropriate tools for the study of genomes and for analysis of gene expression.

The genomic fingerprinting workshop provided the opportunity for interaction between groups with different interests, using similar or different approaches, but with a common aim, the analysis of diversity and similarities among organisms, species and individuals.

Some of the work presented emphasised the use of RAPDs/AP-PCR for mapping, taxonomy, phylogenetics and mutation analysis in several organisms, including human, mouse, fish, insects, plants (woody plants, plant germplasms, chickpea, potato, chrysanthemum), and microorganisms (spirochetes, *E. coli*, *Helicobacter pylori*, *Staphylococcus aureus*, *Listeria*, *Haloferax mediterranei*, *Salmonella*, *Trypanosomes*, and Fungui),

Work was presented on the mouse genome using RAPDs/AP-PCR and pulsed field gel electrophoresis for the construction of physical maps. Also microsatellite and minisatellite (VNTRs) studies were reported. The analysis of long repeat sequences (Alu and LINE-1) and microsatellites in humans were shown to be useful for different types of studies, including the identification of unknown DNA sequences, development of STS's (sequence tagged sites), characterization of YACs, isolation of microsatellites, and identification of new genes. The loss and gain of DNA fragments, generated by AP-PCR, demonstrated its power in the analysis of the somatic instability of repeat sequences in cancer. Modifications of the RAPDs/AP-PCR were shown to be of great value for the analysis of mRNA in studies on levels of expression, regulation of genes and identification of new genes.

In addition to the RAPDs/AP-PCR technology other methods for genomic fingerprinting were presented and discussed. These included LSSP-PCR (low stringency single specific primer), insertion sequence probes, ribotyping, PFGE, MRSP (mapped restriction site polymorphisms), MLEE (multilocus enzyme electrophoresis), QTL, oligonucleotide fingerprinting, RNAse mismatch cleavage, RSM (restriction site mutagenesis), UP-PCR (unpredictable primer PCR), and SSCP (single strand conformation analysis).

Data was presented on the use of microsatellites for the localisation of genes, the detection of deletions, and for studies on evolution. Methods for the

detection of new microsatellite sequences were presented, as well as the use of SSCP for genomic fingerprinting and the isolation of new CA-repeats.

Discussions were held on several specific topics: the use of mitochondrial DNA and microsatellites for the characterisation of endangered species, new mutation mechanisms in cancer and maps for tree species. Finally, the application of robotics to the study of plant genomes was demonstrated.

It was clear from data presented at the meeting that an universal method for genomic fingerprinting does not exist, but tools have been developed that allow genetic mapping of a very high standard. RAPDs/AP-PCR has become the most powerful method for fingerprinting most organisms, but from the work presented at the workshop and also with recent progress, microsatellites are the optimal and universal tools for mapping the mouse and human genomes.

List of Invited Speakers

Workshop on

GENOMIC FINGERPRINTING

List Invited Speakers

G. Baranton — Unité de Bactériologie Moléculaire et Médicale, Institut Pasteur, 28 rue du Dr. Roux, 75724 Paris Cedex 15, (France).
Tel.: 33 1 45 68 83 37
Fax : 33 1 40 61 30 01

D.E. Berg — Department of Molecular Microbiology, Campus Box 8230, Washington University Medical School, St. Louis, MO. 63110 (USA).
Tel.: 1 314 362 27 72
Fax : 1 314 362 12 32

X. Estivill — Departamento de Genética Molecular, Instituto de Investigación Oncológica, Autovía de Castelldefels Km. 2,7, 08907 Hospitalet de Llobregat, Barcelona (Spain).
Tel.: 34 3 335 71 52
Fax : 34 3 263 22 51

J.L. Guénet — Unité de Génétique des Mammifères de l'Institut Pasteur, 25 rue du Dr. Roux, 75724 Paris Cedex 15 (France).
Fax : 33 1 45 68 86 39

K. Hayashi — Division of Genome Analysis, Institute of Genetic Information Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka (Japan).
Tel.: 81 92 641 11 51 Ext. 3465
Fax : 81 92 632 23 75

H.S. Kwan — Department of Biology, The Chinese University of Hong Kong, Shatin, N.T. (Hong Kong).
Tel.: 852 609 62 85
Fax : 852 603 56 46

P. Liang — Dana-Farber Cancer Institute, Harvard Medical School, 44 Binney Street, Boston, MA. 02115 (USA).
Tel.: 1 617 632 46 83
Fax : 1 617 632 46 80

M. McClelland — California Institute of Biological Research, CIBR, 11099 North Torrey Pines Road, La Jolla, Ca. 92037 (USA).
Tel.: 1 619 535 54 71
Fax : 1 619 535 54 72

J.H. Nadeau — The Jackson Laboratory, 600 Main Street, Bar Harbor, Maine 04609 (USA).
Tel.: 1 207 288 33 71
Fax : 1 207 288 89 82

D.L. Nelson — Institute for Molecular Genetics, Human Genome Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030 (USA).
Tel.: 1 713 798 47 87
Fax : 1 713 798 63 70 or 53 86

M. Perucho — California Institute of Biological Research, CIBR, 11099 North Torrey Pines Road, La Jolla, Ca. 92037 (USA).
Tel.: 1 619 535 54 71
Fax : 1 619 535 54 72

W. Powell — Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA Scotland (U.K.)
Tel.: 44 382 56 27 31
Fax : 44 382 56 24 26

J.A. Rafalski — Biotechnology Research, Agricultural Products Department, E.I. du Pont de Nemours and Co. (Inc), P.O. Box 80402 Experimental Station, Wilmington, DE 19880-0402 (USA).
Tel.: 1 302 695 43 48
Fax : 1 302 695 42 96

O.A. Ryder — Center for Reproduction of Endangered Species, Zoological Society of San Diego, P.O. Box 551, San Diego, CA. 92112 (USA).
Tel.: 1 619 557 39 50
Fax : 1 619 557 39 58

R. Sederoff — Department of Forestry, North Carolina State University, Raleigh, NC. 27695 (USA).
Tel.: 1 919 515 78 00
Fax : 1 919 515 78 01

A.J.G. Simpson

Centro de Pesquisas "René Rachou", Fundação Oswaldo Cruz, Av. Augusto de Lima 1715, Belo Horizonte, 31090-002 Minas Gerais (Brazil)
Tel.: 55 31 295 35 66
Fax : 55 31 295 31 15

J. Welsh

California Institute of Biological Research, CIBR, 11099 North Torrey Pines Road, La Jolla, CA. 92037 (USA).
Tel.: 1 619 535 54 71
Fax . 1 619 535 54 72

H. Zischler

Institute of Zoology, University of Münich, Luisenstraße 14, W-8000 Münich 2 (Germany).
Tel.: 49 89 218 00
Fax : 49 89 590 24 74

List of Participants

Workshop on

GENOMIC FINGERPRINTING

List of Participants

F.J. Avalos — Departamento de Genética, Facultad de Biología, Universidad de Sevilla, Apartado 1095, 41080 Sevilla (Spain).
Tel.: 34 5 455 71 10
Fax : 34 5 455 71 04

E. Baird — Cell and Molecular Genetics Department, Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA, Scotland (U.K.).
Tel.: 44 382 56 27 31
Fax : 44 382 56 24 26

A. van Belkum — Diagnostic Center SSDZ, Department of Molecular Biology, P.O. Box 5010, 2600 GA Delft (The Netherlands).
Tel.: 31 15 60 30 50
Fax : 31 15 56 81 03

A. Blanchetot — Department of Biochemistry, University of Saskatchewan, Saskatoon, Saskatchewan, Canada S7N OWO.
Tel.: 1 306 966 43 44
Fax : 1 306 966 87 18

A. Castagnaro — Departamento de Bioquímica y Biología Molecular, E.T.S.I., Agrónomos, Universidad Politécnica, 28040 Madrid (Spain).
Fax : 34 1 336 57 57

I. del Castillo — Unidad de Genética Molecular, Hospital Ramón y Cajal, Ctra. Colmenar Km 9,1, 28034 Madrid (Spain).
Tel.: 34 1 336 85 41
Fax : 34 1 336 90 16

A. Coffey — Division of Medical and Molecular Genetics, UMDS of Guy's and St. Thomas Hospitals, London, SE1 9RT (U.K.).
Tel.: 44 71 955 50 00 ext 2524
Fax : 44 71 955 46 44

E. Dias Neto — Centro de Pesquisas "René Rachou", Fundação Oswaldo Cruz, FIOCRUZ, Av. Augusto de Lima 1715, Barro Preto Belo Horizonte, Minas Gerais, (Brazil).
Tel.: 55 31 295 35 66
Fax : 55 31 295 31 15

O. Domínguez	Servicio de Inmunología, Hospital Central de Asturias, c/Celestino Villamil s/nº, 33006 Oviedo (Spain). Tel.: 34 8 510 61 00 ext. 36395 Fax : 34 8 525 45 09
J. Dopazo	INIA, Centro de Investigación en Sanidad Animal, 28130 Valdeolmos, Madrid (Spain). Tel.: 34 1 620 23 00 Fax : 34 1 620 22 47
G. Dorado	Departamento de Bioquímica y Biología Molecular, Facultad de Veterinaria, Universidad de Córdoba, Avda. Medina Azahara s/nº, 14071 Córdoba (Spain). Tel.: 34 57 21 86 89 Fax : 34 57 21 86 66
P. García	Area de Genética, Facultad de Biología, Universidad de León, 24071 León (Spain). Tel.: 34 87 29 15 48 Fax : 34 87 29 14 79
K. Hutchison	Department of Biochemistry, Microbiology and Molecular Biology, University of Maine, 5735 Hitchner Hall, Orono, Maine 04469-5735 (USA). Tel.: 1 207 581 28 27 Fax : 1 207 581 28 01
Ch.-L. Koh	Department of Genetics and Cellular Biology, University of Malaya, 59100 Kuala Lampur (Malaysia). Tel.: 603 755 27 44 Ext. 237 Fax : 603 757 64 78
C. López Galíndez	Centro Nacional de Biología Celular y Retrovirus, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km. 2, 28220 Majadahonda, Madrid (Spain). Tel.: 34 1 638 00 11 Fax : 34 1 638 82 06
I. Martínez Galarza	Norwegian Institute of Fisheries and Aquaculture, P.O. Box 2511, N-9002 Tromsø (Norway). Tel.: 47 83 29 000 Fax : 47 83 29 100
A.J. Martínez-Murcia	Departamento de Genética y Microbiología, Universidad de Alicante, Campus de San Juan, Apartado 374, 03080 Alicante (Spain). Tel.: 34 6 565 85 54 Fax : 34 6 594 17 87

J.A. Melero — Centro Nacional de Biología Celular y Retrovirus, Instituto de Salud Carlos III, Ctra. Majadahonda-Pozuelo Km. 2, 28220 Majadahonda, Madrid (Spain).
Tel.: 34 1 638 24 39
Fax : 34 1 638 06 13

N.B. Murphy — International Laboratory for Research on Animal Diseases (ILRAD), P.O. Box 30709, Nairobi (Kenya).
Tel.: 254 2 63 23 11
Fax : 254 2 63 14 99

M.A. Peinado — Instituto de Investigación Oncológica, Autovía Castelldefels Km. 2,7, 08907 Hospitalet de Llobregat, Barcelona (Spain).
Tel.: 34 3 335 71 52
Fax : 34 3 263 22 51

P. Perolat — Institut Pasteur, Laboratoire des Leptospires, B.P. 61, Nouméa (New Caledonia).
Tel.: 1 687 27 26 66
Fax : 1 687 27 33 90

E. Rogaev — Centre for Research in Neurodegenerative Diseases University of Toronto, Tanz Neuroscience Building, 6 Queen's Park Crescent West, Toronto, ON. Canada M5S 1A8.
Tel.: 1 416 978 74 61
Fax : 1 416 978 18 78

L.B. Rowe — Jackson Lab B6xSpret Backcross DNA Panels, 600 Main Street, Bar Harbor, Maine 04609 (USA).
Tel.: 1 207 288 33 71
Fax : 1 207 288 50 79

J.C. Sáiz — Centro de Biología Molecular, (CSIC), Facultad de Ciencias, Universidad Autónoma, 28049 Madrid (Spain).
Tel.: 34 1 397 84 77
Fax : 34 1 397 47 99

M.P. Sánchez de la Hoz — Departamento de Biología Celular y Genética, Facultad de Ciencias, Universidad de Alcalá, Campus Universitario, 28870 Alcalá de Henares, Madrid (Spain).
Tel.: 34 1 885 47 99
Fax : 34 1 885 47 99

P.C. Sharma — Department of Agricultural Botany, Meerut University, 250 004-Meerut (India).
Tel.: 0091 121 75 021

J. Stanley — NCTC Molecular Genetics Unit, Central Public Health Laboratory, 61 Colindale Avenue, London NW9 5HT (U.K.).
Tel.: 44 81 200 44 00
Fax : 49 81 200 78 74

D.J. Takemoto — Department of Biochemistry, Kansas State University, Willard Hall, Manhattan, KS. 66506 (USA)
Tel.: 1 913 532 70 09
Fax : 1 913 532 72 78

T.M. Thomson — Departamento de Biología Molecular, Facultad de Medicina, Universidad de Cantabria, Cardenal Herrera Oria s/nº, 39011 Santander (Spain).
Tel.: 34 42 20 19 40
Fax : 34 42 20 19 45

K. Wolff — TNO Nutrition and Food Research, Department of Microbiology, Utrechtseweg 48, P.O. Box 360, 3700 AJ Zeist (The Netherlands).
Tel.: 31 34 04 4 41 44
Fax : 31 34 04 5 41 86

E. Zietkiewicz — Département de Pédiatrie, Service de Génétique Médicale, Centre de Recherche, Hôpital Ste-Justine, Université de Montréal. Montréal, H3T-1C5 PQ. (Canada).
Tel.: 1 514 345 46 91
Fax : 1 514 345 48 01

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248 Beato, M.:
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