

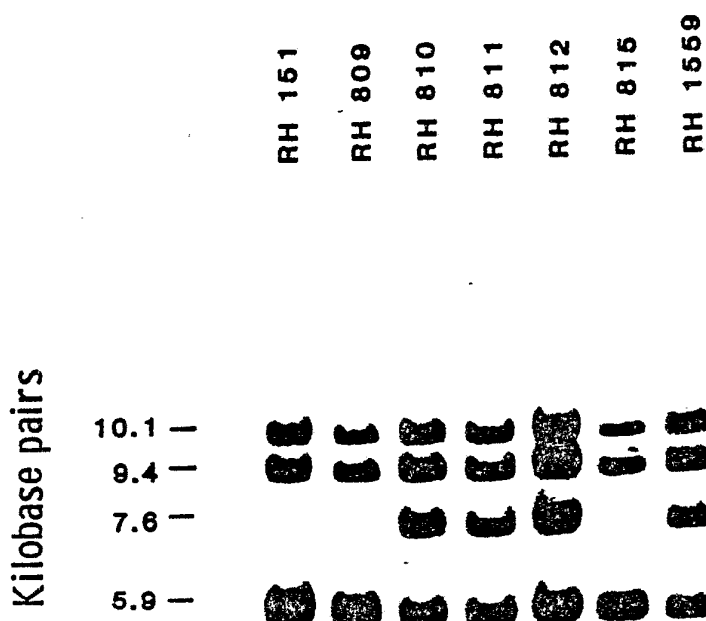


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(54) Title: METHOD FOR IDENTIFYING AND CHARACTERIZING ORGANISMS**(57) Abstract**

A method of characterizing an unknown organism species comprises determining the position of part or whole of evolutionarily conserved sequences in genetic material of said organism, relative to a known position of restriction endonuclease cleavage sites in said genetic material (other than by determining the chromatographic pattern of restriction endonuclease digested DNA from said unknown organism, which digested DNA has been hybridized or reassociated with ribosomal RNA information containing nucleic acid from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of said unknown organism, and comparing said characterization with information from at least two sets of identifying genetic characterizations derived from the same conserved sequences, each of said sets defining a known organism species.

EcoR I digested DNA
from P. aeruginosa strains

identifying genetic characterization of said unknown organism, and comparing said characterization with information from at least two sets of identifying genetic characterizations derived from the same conserved sequences, each of said sets defining a known organism species.

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METHOD FOR IDENTIFYING AND CHARACTERIZING ORGANISMS

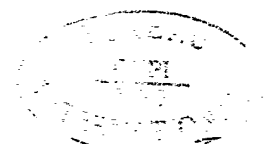
The present invention relates to a method for the rapid and accurate characterization and identification of organisms, including prokaryotic and eukaryotic organisms, such as bacteria, plants, and animals.

5. The classification of living organisms has traditionally been done along more or less arbitrary and somewhat artificial lines. For example, the living world has been divided into two kingdoms: Plantae (plants) and Animalia (animals). This classification
10. works well for generally familiar organisms, but becomes difficult for such organisms as unicellular ones (e.g., green flagellates, bacteria, blue-green algae), since these differ in fundamental ways from the "plants" and "animals".
15. It has been suggested to simply divide organisms with respect to the internal architecture of the cell. In this scheme, all cellular organisms are either prokaryotic or eukaryotic. Prokaryotes are less complex than eukaryotes, they lack internal compart-

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- mentalization by unit membrane systems, and lack a defined nucleus. Prokaryotic genetic information is carried in the cytoplasm on double-stranded, circular DNA; no other DNA is present in cells (except for the
5. possible presence of phage, bacterial viruses, and circular DNA plasmids, capable of autonomous replication). Eukaryotes, on the other hand, have a multiplicity of unit membrane systems which serve to segregate many of the functional components into
10. specialized and isolated regions. For example, genetic information (DNA) can be found in a well-compartmentalized nucleus and also in organelles: mitochondria and (in photosynthetic organisms) chloroplasts. The replication, transcription, and translation of the eukaryotic genome occurs at either two or
15. three distinct sites within the cell: in the nucleocytoplasmic region, in the mitochondrion, and in the chloroplast.

- The differences between prokaryotes and
20. eukaryotes, however, breaks down when a comparison of mitochondria and chloroplasts is carried out with prokaryotes: these organelles are today considered to have been derived from free-living prokaryotes, which entered into an endosymbiotic relation with primitive
25. eukaryotes, and eventually became closely integrated with the machinery of the host cell and incapable of independent existence (see e.g., Fox, G.E. et



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- al, Science 209:457-463 (1980), at 462; Stanier, R. Y. et al, "The Microbial World," Fourth Edition, Prentice-Hall, Inc., 1976, at p. 86). For example, it has been demonstrated that DNA from mouse L cell mitochondria
5. carrying the ribosomal RNA gene region exhibits notable sequence homologies to Escherichia coli ribosomal RNA, thus providing strong support for the endosymbiotic model (Van Etten, R. A. et al, Cell, 22:157-170 (1980)). It has also been shown that the nucleotide
10. sequence of 23S ribosomal DNA from Zea mays chloroplast has 71% homology with 23S ribosomal DNA from E. coli (Edwards, K. and Kossel, H., Nucleic Acids Research, 9:2853-2869 (1981)); other related work (Bonen, L. and Gray, M. W., ibid, 8:319-335 (1980)) also further
15. supports the general concept.

- In this model the eukaryotic cell is a phylogenetic "chimera" with organelle components that are clearly prokaryotic in nature. The "prokaryotic-eukaryotic" dichotomy then, also has drawbacks, even as
20. a broad classification method.

- Where classification of organisms becomes more than a scientific exercise is in the identification of plants and animals for hybridization and breeding purposes, and in the accurate and reliable identification
25. of microorganisms which may infect so-called "higher" organisms or other media. For example, the plant-breeder, cattle breeder, or fish breeder may wish to



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have a quick and reliable means of identifying different species and strains of their subjects. The veterinarian, physician, or horticulturist may wish to have an accurate identification of any infectious organisms (parasites, fungi, bacteria, etc.) and viruses present in examined plant or animal tissues. The correct identification of species of these organisms and viruses is of particular importance.

The problem can best be illustrated by referring to the identification of bacteria. Names of bacterial species usually represent many strains, and a strain is considered to be a population derived from a single cell. Bacterial species are usually defined by describing the degree of homogeneity and diversity of attributes in representative samples of strains of species. Precise definitions of bacterial species are difficult to express because subjective limits to strain diversity within species are required to define species boundaries. (Buchanan, R. E., International Bulletin of Bacteriological Nomenclature and Taxonomy, 15:25-32 (1965)). The practical application of definitions of species to the identification of an unknown bacterial strain requires the selection of relevant probes, such as substrates and conditions to detect phenotypic attributes, and radioactively-labeled DNA from the same species. Because of the diversity of bacterial species, a screening procedure is the primary



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tool used in the classical, progressive method for identification of a strain. Results of the screening procedure are then used to predict which other laboratory methods and reagents are relevant for

5. definitive identification of the strain.

Identification is ultimately based on certain phenotypic and genotypic similarities between the unidentified strain and characterized species. The challenge is to precisely define the boundaries of

10. species, preferably in terms of a standard probe which reveals species-specific information, so that definitions of species can be directly and equally applied to the identification of unknown strains.

Bergey's Manual of Determinative Bacteriology

15. (Buchanan, R. E. and Gibbons, N. E., Editors, 1974, 8th Edition, The Williams & Wilkins Company, Baltimore) provides the most comprehensive treatment of bacterial classification particularly for nomenclature, type strains, pertinent literature, and the like. It is,
20. however, only a starting point for the identification of any species since, inter alia, it is normally out of date, and is limited in space to describing species quite briefly. (See for example, Brenner, D. J., "Manual of Clinical Microbiology," 3rd Edition,
25. American Society of Microbiology, Washington, D.C., 1980, pages 1-6.)



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The term "species", as applied to bacteria, has been defined as a distinct kind of organism, having certain distinguishing features, and as a group of organisms which generally bear a close resemblance to one another in the more essential features of their organization. The problem with these definitions is that they are subjective; Brenner, supra, at page 2. Species have also been defined solely on the basis of criteria such as host range, pathogenicity, ability or inability to produce gas in the fermentation of a given sugar, and rapid or delayed fermentation of sugars.

In the 1960's, numerical bacterial taxonomy (also called computer or phenetic taxonomy) became widely used. Numerical taxonomy is based on an examination of as much of the organism's genetic potential as possible. By classifying on the basis of a large number of characteristics, it is possible to form groups of strains with a stated degree of similarity and consider them species. Tests which are valuable for the characterization of one species, however, may not be useful for the next, so this means to define species is not directly and practically applicable to the identification of unknown strains. Although this may be overcome in part by selecting attributes which seem to be species specific, when these attributes are used to identify unknown strains, the species definition is applied indirectly. See for example Brenner, supra, at



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pages 2-6. The general method, furthermore, suffers from several problems when it is used as the sole basis for defining a species, among them the number and nature of the tests to be used, whether the tests should be weighted and how, what level of similarity should be chosen to reflect relatedness, whether the same level of similarities is applicable to all groups, etc.

- Hugh, R. H. and Giliardi, G. L., "Manual of
10. Clinical Microbiology," 2nd Edition, American Society for Microbiology, Washington, D.C., 1974, pages 250-269, list minimal phenotypic characters as a means to define bacterial species that makes use of fractions of genomes. By studying a large, randomly selected sample
 15. of strains of a species, the attributes most highly conserved or common to a vast majority of the strains can be selected to define the species. The use of minimal characters is progressive and begins with a screening procedure to presumptively identify a strain,
 20. so that the appropriate additional media can be selected. Then the known conserved attributes of the species are studied with the expectation that the strain will have most of the minimal characters. Some of the minimal characters do not occur in all strains
 25. of the species. A related concept is the comparative study of the type, the neo-type, or a recognized reference strain of the species. This control is neces-



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sary because media and procedures may differ among laboratories, and it is the strain, not the procedure, that is the standard for the species.

- A molecular approach to bacterial classification
5. is to compare two genomes by DNA-DNA reassociation. A genetic definition of species includes the provision that strains of species are 70% or more related. With DNA-DNA reassociation a strain can be identified only if the radioactively labeled DNA probe and unknown DNA
10. are from the same species. The practical application of this 70% species definition however is limited by selection of an appropriate probe. This may be overcome in part by selecting phenotypic attributes which seem to correlate with the reassociation group, but
15. when these are used alone the DNA-DNA reassociation species definition is also applied indirectly.

Brenner, supra, at page 3, states that

- the ideal means of identifying bacterial species would be a 'black box' which
20. would separate genes, and instantly compare the nucleic acid sequences in a given strain with a standard pattern for every known species-something akin to mass spectrophotometric analysis.
25. Brenner, however, concedes that although restriction endonuclease analysis can be done to determine common sequences in isolated genes, "we are not at all close to



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having an appropriate black box, especially one suited for clinical laboratory use." His words could be equally applied to any species of organism.

- This brief review of the prior art leads to the
5. conclusion that there presently exists a need for a rapid, accurate, and reliable means for identifying unknown bacteria and other organisms, and to quickly classify the same, especially to identify the organism of a disease, or of a desirable biochemical reaction. The
10. method should be generally and readily useful in clinical laboratories, should not be dependent on the number of tests done, on the subject prejudices of the clinician, nor the fortuitous or unfortuitous trial and error methods of the past. Further, a need also exists for a
15. method useful for identifying and distinguishing genera and species of any living organism, which can be readily and reliably used by veterinarians, plant-breeders, toxicologists, animal breeders, entomologists and in other related areas, where such identification is necessary.
20. It is therefore an object of the invention to provide a method which may be found to be quick, reliable and accurate of objectively identifying organisms, especially - but not limited to - microorganisms.

- Yet another object of the invention is to provide a
25. method of identifying organisms such as bacteria which utilizes the organisms' genome.

Another object of the invention is to provide a method of characterizing and identifying species and



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genera of pathogenic organisms in the clinical laboratory, so as to provide the capability of characterizing and identifying the cause of any given animal or plant disease.

Still another object of the invention is to provide
5. various products useful in the aforementioned methodologies.

These and other objects of the invention, as will hereinafter become more readily apparent, have been attained by providing:

A method of characterizing an unknown organism species
10. which comprises determining the position of part or whole of evolutionarily conserved sequences in genetic material of said organism, relative to a known position of restriction endonuclease cleavage sites in said genetic material (other than by determining the chromatographic pattern of restric-
15. tion endonuclease digested DNA from said unknown organism, which digested DNA has been hybridized or reassociated with ribosomal RNA information containing nucleic acid from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of said unknown
20. organism, and comparing said characterization with information from at least two sets of identifying genetic characterizations derived from the same conserved sequences, each of said sets defining a known organism species.

Still another object of the invention has been
25. attained by providing:

A method of diagnosing a pathogenic organism infection in a sample which comprises identifying the organism in said sample by the aforementioned method.



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For a better understanding of the invention, and to show how it may be put into effect, reference will now be made, by way of example, to the accompanying drawings in which:

FIGURE 1 shows the EcoR I restriction endonuclease

5. digest of DNA isolated from strains of Pseudomonas aeruginosa, using cDNA to 16S and 23S ribosomal RNA (rRNA) of E. coli as the probe.

FIGURE 2 shows the Pst I restriction endonuclease digest of DNA isolated from strains of P. aeruginosa,

10. using cDNA to 16S and 23S rRNA of E. coli as the probe.

FIGURE 3 shows the EcoR I restriction endonuclease digest of DNA isolated from species of glucose-nonfermenting, gram-negative rods, using cDNA to 16S and 23S rRNA of E. coli as the probe.

15. FIGURE 4 shows the Pst I restriction endonuclease digest of DNA isolated from species of glucose-nonfermenting, gram-negative rods using cDNA to 16S and 23S rRNA of E. coli as the probe.

- FIGURE 5 shows the EcoR I restriction endonuclease
20. digest of DNA isolated from various Bacillus subtilis strains, using cDNA to 16S and 23S rRNA of E. coli as the probe.

FIGURE 6 shows the Pst I data for the same strains as in FIGURE 5, with the same probe.

25. FIGURE 7 shows the Bgl II data for the same strains as in FIGURES 5 and 6, with the same probe.

FIGURE 8 shows the Sac I data for the same strains as in FIGURES 5-7, with the same probe.



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FIGURE 9 shows the EcoR I restriction endonuclease digest of DNA isolated from B. subtilis and B. polymyxa, using cDNA to 16S and 23S rRNA from E. coli as the probe.

FIGURE 10 shows the Pst I data for the same strains
5. as in FIGURE 9 with the same probe.

FIGURE 11 shows the Bgl II and Sac I data for the same strains as in FIGURES 9 and 10, with the same probe.

FIGURE 12 shows the detection of Streptococcus pneumoniae in EcoR I digested DNA from infected mouse
10. tissues using cDNA from 16S and 23S rRNA from E. coli as the probe.

FIGURE 13 shows the identification of a mouse species by comparing Pst I digests of DNA isolated from mammalian tissues, using cDNA to 18S and 28S rRNA from
15. cytoplasmic ribosomes of Mus musculus domesticus (mouse).

FIGURE 14 shows the EcoR I digested DNA from mouse and cat tissues hybridized with Mus musculus domesticus 28S rRNA cDNA probe.

FIGURE 15 shows Sac I digested DNA from mammalian
20. tissues hybridized with Mus musculus domesticus 18S and 28S rRNA cDNA probe.

FIGURE 16 shows EcoR I digested DNA from mammalian tissues and cell cultures hybridized with Mus musculus domesticus 18S and 28S rRNA cDNA probe.



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- This invention is based on the inventor's realization that, if species are discrete clusters of strains related to a common speciation event, there should be, despite divergence, a likeness shared by strains that
5. objectively defines the species boundary; strains of species should contain structural information which is a clue to their common origin. The greatest amount of an organism's past history survives in semantides, DNA and RNA, (Zuckerkandle, E. and Pauling, L., Journal of
10. Theoretical Biology, 8:357-366 (1965)).

- In European Patent Application (EP-A-) No.0076123 (herein incorporated by reference) the inventor described a system for the definition of species and characterization of organisms which makes use of
15. information contained in ribosomal RNA genes (rRNA). Ribosomal RNA has a structural and functional role in protein synthesis (Schaup, Journal of Theoretical Biology, 70:215-224 (1978)), and the general conclusion from rRNA-DNA hybridization studies, is that the base
20. sequences of ribosomal RNA genes are less likely to change, or are more conserved during evolution, than are the majority of other genes (Moore, R. L., Current Topics In Microbiology and Immunobiology, Vol. 64:105-128
- (1974), Springer-Verlag, New York). For example, the
25. primary structure of 16S rRNA from a number of bacterial species has been inferred from oligonucleotide analysis



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- (Fox, G. E. et al, International Journal of Systematic Bacteriology, 27: 44-57 (1977)). There are negligible differences in the 16S oligomer catalogs of several strains of E. coli (Uchida, T. et al, Journal of Molecular Evolution, 3:63-77 (1974)); the substantial differences among species, however, can be used for a scheme of bacterial phylogeny (Fox, G.E., Science, 209:457-463 (1980)). Different strains of a bacterial species are not necessarily identical; restriction enzyme
5. maps show that different EcoR I sites occur in rRNA genes in two strains of E. coli (Boros, I.A. et al, Nucleic Acids Research 6:1817-1830 (1979)). Bacteria appear to share conserved rRNA gene sequences and the other sequences are variable (Fox, 1977, supra).
- 10.
15. The present inventor had thus discovered that restriction endonuclease digests of DNA have sets of fragments containing conserved sequences that are similar in strains of a species of organism (e.g., bacteria), but different in strains of other species of the organism;
20. i.e., despite strain variation, enzyme specific sets of restriction fragments with high frequencies of occurrence, minimal genotypic characters, define the species. This is the essence of the invention described in EP-A-0076123. and also that of the invention
25. described herein.

The present invention constitutes an extension of the concepts developed in EP-A-0076123, in that



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it has further been discovered that there exist sequences, in addition to those of rRNA, which are highly conserved through evolution and which may be as useful as rRNA sequences in the identification system. In other words,

5. the present invention provides means for carrying out the identification and characterization techniques of EP-A-0076123, using any probe which is conserved, other than rRNA. The present invention also provides additional examples of methods which may be used in the identification

10. process. The present inventor has also discovered that the method is general, in that it is applicable to both eukaryotic and prokaryotic DNA, using a conserved nucleic acid probe from any organism, prokaryotic or eukaryotic, of the same or different (classic) taxonomic classification

15. than the organism being identified.

The invention offers an objective method of defining organisms based on conserved sequences of DNA or other genetic material in relation to known positions such as restriction endonuclease sites. The detection of re-

20. striction fragments containing a conserved sequence may be carried out by hybridizing or reassociating DNA segments with nucleic acid containing conserved sequence information from a probe organism.

By the "organism" which can be characterized (which

25. term is meant to include "identified") by the process of the invention, it is meant to include virtually any organism which, by definition, contains DNA or RNA in its genome.



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In this respect it is useful to refer to a classical taxonomic scheme as a point of reference.

- All organisms belonging to the Kingdoms Monera, Plantae and Animalia are included. For example, among
5. those of the Kingdom Monera can be mentioned the Schizomycetes (Bacteria) of the classes myxobacteria, spirochetes, eubacteria, rickettsiae, and the cyanophyta (blue green algae). Among those of the Kingdom Plantae can be mentioned the Division Euglenophyta (Euglenoids),
 10. Division Chlorophyta (green-algae) classes chlorophyceae and charophyceae, Division Chrysophyta, classes xanthophyceae; chrysophyseae, bacillariophyceae; Division Pyrrophyta (Dinoflagellates); Division Phaeophyta (Brown algae); Division Rhodophyta (Red algae); Division
 15. Myxomycophyta (slime molds), classes myxomycetes, acrasiae, plasmodiophoreae, labyrinthuleae; Division Eumycophyta (true fungi), classes phycomycetes, ascomycetes, and basidiomycetes; Division Bryophyta, classes hepaticae, anthocerotae, and musci; Division Tracheophyta
 20. (Vascular plants), subdivisions psilopsida, lycopsida, sphenopsida, pteropsida, spermopsida classes cycadae, ginkgoae, coniferae, gnetae and angiospermae subclasses dicotyledoneae, monocotyledoneae. Among those of the Kingdom Animalia can be mentioned the Subkingdom Proto-
 25. zoa, Phylum Protozoa (Acellular animals) subphylum plasmodroma, classes flagellata, sarcodina and sporozoa; subphylum ciliophora, class ciliata; the Subkingdom



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- Parazoa, Phylum porifera (Sponges), class calcarea, hexactinellida, and desmospongiae; the Subkingdom Mesozoa, Phylum mesozoa; the Subkingdom Metazoa, Section Radiata, Phylum coelenterata, classes hydrozoa, scyphozoa, anthozoa, Phylum ctenophora, classes tentaculata and nuda; Section Protostomia Phylum platyhelminthes (flatworms) classes tubellana, trematoda, and cestoda; Phylum nemertina; Phylum acanthocephala; Phylum aschelminthes, classes rotifera, gastrotricha, kinorhyncha, priapulida,
5. nematoda and nematomorpha; Phylum entoprocta; Phylum ectoprocta, classes gymnolaemata and phylactolaemata; Phylum phoronida; Phylum brachiopoda, classes inarticulata and articulata; Phylum mollusca (molluscs) classes
10. amphineura, monoplacophora, gastropoda, scaphopoda, pelecypoda, and cephalopoda; Phylum sipunculida; Phylum echiurida; Phylum annelida, classes polychaeta, oligochaeta and hirudinea; Phylum onychophora; Phylum tardigrada; Phylum pentastomida; Phylum arthropoda,
20. subphylum trylobita, subphylum chelicerata classes xiphosura, arachnida, pycnogomida, subphylum mandibulata classes crustacea, chilopoda, diplopoda, pauropoda, symphyla, insecta of the orders collembola, protura, diplura, thysanura, ephemerida, odonata, orthoptera,
25. dermaptera, embiana, plecoptera, zoraptera, corrodentia, mallophaga, anoplura, thysanoptera, hemiptera, neuroptera, coleoptera, hymenoptera, mecoptera, siphonaptera, diptera, trichoptera and lepidoptera; those of the



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Section Deuterostomia, phylum chaetognatha, phylum echinodermata, classes crinoidea, asterordea, ophiuroidea, echinoidea, and holoturoidea, phylum pogonophora; phylum hemichordata, classes enteropneusta, and

5. pterobranchia; phylum chordata, subphylum urochordata, classes ascidiacidae, thaliaceae, larvacea; subphylum cephalochordata, subphylum vertebrata, classes agnatha, chondrichthyes, osteichthyes (subclass saccopterygii orders crossopterygii and dipnoi), amphibia, reptilia.
10. aves and mammalia, subclass prototheria, subclass theria, orders marsupialia, insectivora, dermoptera, chiroptera, primates, edentata, pholidota, lagomorpha, rodentia, cetaceae, carnivora, tubulidentata, proboscidea, hyracoidea, sirenia, perissodactyla and artiodactyla.

15. It is understood that beyond the order, the organisms are still classified according to their families, tribes, genus and species, and even subspecies, infra-specific taxons, and strains or individuals. In addition, cell cultures (plant or animal), as well as

20. viruses can also be identified. These classifications are used in this application for illustrative purposes only, and are not to be taken as exclusive. The organism is either known or unknown, most commonly the organism is an unknown being identified.

25. Functionally, for the purposes of this invention, it is convenient to divide all organisms into the eukaryotes and the prokaryotes. When identifying a prokaryotic



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organism, the DNA to be analyzed is that present in the cells or in the non-compartmentalized chromosomes. When identifying a eukaryotic organism one may either use the nuclear DNA or the organelle DNA (mitochondrial DNA or

5. chloroplast DNA).

Briefly, high molecular weight DNA and/or small circular DNAs are isolated from the organism to be identified in order to analyze the conserved sequences (and possibly sequences that could be used to create a taxon

10. below the rank of species or infrasubspecific subdivisions.) The DNA's are extracted by methods which are well-known to the art.

The DNA's are analyzed to ascertain both 1) the presence and position of the conserved sequences and 2)

15. their position relative to endonuclease restriction sites. The easiest way to analyze for the presence of the conserved sequences is to utilize a polynucleotide probe capable of hybridizing with the conserved DNA sequence. However, direct sequence information as

20. obtained by chemical sequence determination and analysis thereof could also be utilized. In EP-A-0076123 the probe utilized was an rRNA information containing-probe; in this case any other probe having conserved sequences could be used. In an analogous manner, the easiest way

25. of finding a given set of endonuclease restriction sites is to cleave the DNA with the appropriate restriction enzymes. (This, indeed, is the manner taught and



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practised in EP-A-0076123. However, alternative methods, such as sequence information coupled with known restriction site sequences, or cleavage and partial sequencing could also be used.

5. Most commonly DNA's are going to be cut at specific sites into fragments by restriction endonucleases. The fragments are separated according to size by a chromatographic system. In EP-A-0076123 gel chromatography was used as an example of a useful
10. chromatographic system. However, other systems can also be used, such as high pressure liquid chromatography, capillary zone electrophoresis, or other separation techniques. In using gel chromatography, the fragments are separated, the gels are stained, as is otherwise
15. well-known in the art, and standardized as to the fragment sizes using standards curves constructed with fragments of known sizes. The separated fragments may then be transferred to cellulose nitrite paper by the Southern blot technique (Southern, E. M., Journal of
20. Molecular Biology, 38:503-517 (1975), herein incorporated by reference), and covalently bound thereto by heating. The fragments containing the conserved sequences are then located by their capacity to hybridize with a nucleic acid probe containing conserved sequence information.
25. Alternatively, hybridization can occur after digestion but before separation; or restriction cleavage can occur after hybridization, followed by separation of the



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fragments.

- The nucleic acid probe can either be non-radioactively labeled or, preferably, radioactively labeled. When radioactively labeled, the probe can be
5. RNA, or preferably DNA which is complementary to RNA (cDNA), either synthesized by reverse transcription or contained on a cloned fragment, which can be labeled, for example, by nick translation. Also, synthetic oligodeoxyribonucleotides may be prepared with labeled
10. nucleotides.

- The well-defined probe is derived from an arbitrarily chosen organism, see infra, or may be a consensus sequence. Once hybridization has occurred, the hybridized fragments are detected by selectively
15. detecting double stranded nucleic acid (non-radiolabeled probe), or visualized by, e.g., autoradiography (radio-labeled probe). The size of each fragment which has been hybridized is relative to the restriction sites and is determined from the distance traveled using standard
20. curves, as described previously. The amount of hybridization, the pattern of hybridization, and the sizes of the hybridized fragments, which are relative to restriction sites, can be used individually or in conjunction to identify the organism.
25. The genetic characterization that emerges from this technique can be readily compared to equivalent characterizations derived from at least two and up to a



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multiplicity of known, standard organisms, genera or species. After a preliminary broad classification has already been carried out (using, for example, classical taxonomy), the comparison can be either by visual inspection and matching of appropriate chromatographic patterns, (as in EP-A-0076123) by comparison of hybridized restriction fragment sizes, by band intensity (amount of hybridization) or by any combination thereof. Ideally, the comparison is carried out with a one-dimensional computer-based pattern recognition system, such as those used in point-of-sale transactions.

The present inventor discovered that when using the aforementioned method, the genetic characterizations for organisms of the same species are substantially similar, with minor variations allowed for intraspecies differences due to strain variations, whereas differences between species, and differences between genera (and higher classifications) are maximal.

The use of enzyme-specific fragment variations among strains of a species permits the typing of strains for various purposes; e.g. in the case of bacteria, for epidemiological purposes. In fact, restriction enzymes can be chosen for their ability to distinguish strains within species.

The "probe organism" used in the present invention, and from which is obtained the nucleic acid probe, can also be any of the aforementioned organisms; it can be



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either eukaryotic or prokaryotic. The only limitation is given by the fact that the conserved sequence-containing probe should hybridize maximally with the unknown organism's DNA.

5. There are four types of conserved sequence information-containing probes: 1) prokaryotic probes (especially bacterial-derived), 2) eukaryotic mitochondrial probes, 3) eukaryotic chloroplast probes, and 4) eukaryotic non-organelle probes. There are also four
10. sources of DNA (to be endonuclease digested): 1) prokaryotic cellular DNA, 2) eukaryotic mitochondrial DNA, 3) eukaryotic chloroplast DNA, and 4) eukaryotic nuclear DNA. The following hybridization table can thus be constructed (Table 1).



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Table 1

Hybridization Table

Conserved Gene Sequence Probe

Unknown organism				
DNA	Prokaryotic	Eukaryotic		
		Mito- chondrial	Chloro- plast	Non- organelle
Prokaryotic	+	+	+	-
Eu. (1) Mitochondria	+	+	+	-
Eu. Chloroplast	+	+	+	-
Eu. Nuclear	-(2)	-	-	+

(1) Eu = Eukaryotic

(2) = refers to generally less effective hybridization,
see Example. 4, *infra*.



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- The Table shows which probes can generally be maximally hybridized with which unknown DNA. For example, one can identify a eukaryotic organism by extracting species specific mitochondrial or chloroplast
5. DNA, endonuclease-digesting it and hybridizing the digest with either a prokaryotic probe, or with an organelle derived eukaryotic probe. In the same manner, one can identify a prokaryotic organism by extracting species-specific cellular DNA, endonuclease-digesting it, and
 10. hybridizing the digest with either a prokaryotic probe, or an organelle-derived eukaryotic RNA probe. Also, one can identify a eukaryotic organism by extracting and digesting species-specific nuclear DNA, and hybridizing it with a non-organelle derived eukaryotic probe.
 15. Eukaryotes could be defined by one or any combination of the nuclear, mitochondria, or in some cases chloroplast systems. These cross-hybridizations are based on the fact that nucleic acid derived from eukaryotic organelles has extensive homology with evolutionarily conserved
 20. sequences from prokaryotic nucleic acid, but that the same homologies are generally not present to such extent between nuclear-derived eukaryotic DNA and prokaryotic DNA.

- The choice of any pair of DNA to be digested and
25. accompanying probe is arbitrary, and will depend on the organism being identified, i.e. it will depend on the question asked. For example, in detecting a prokaryotic



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- species (e.g. bacteria) present in or together with a eukaryotic cell (e.g. animal or plant) for purposes of detecting and identifying an infecting agent, one may choose a prokaryotic probe and work under conditions
5. where organelle-derived DNA is not extracted or only minimally extracted. In this manner one assures that interference between organelle-derived DNA and prokaryotic DNA is minimal. In identifying a eukaryotic species (which is not infected with a prokaryote) with a
 10. prokaryotic probe, it is best to maximize the concentration of organelle-derived DNA, as for example by separating organelles from nuclei, and then extracting only organelle DNA. If one wishes to identify a eukaryotic organism which has been infected with a
 15. prokaryotic organism, it is best to use a non-organelle, non-prokaryotic derived probe since it will generally not hybridize well with the DNA from the prokaryote.

- It is preferred to use a pair (DNA and probe) from the same kingdom, or same subkingdom, or same section, or
20. same phylum, or same subphylum, or same class, or same subclass, or same order, or same family or same tribe or same genus. It is particularly preferred to use prokaryotic probe (e.g. bacterial probe) to hybridize with prokaryotic DNA. In this manner one could detect,
 25. quantify, and identify genera, species, and strains of prokaryotic organisms. One of the most preferred prokaryotic probes is derived from bacteria, and further,



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- because of the ease and availability, from E. coli. The probe from E. coli can be used to identify any organism, especially any prokaryotic organism, most preferably a strain of any bacterial species. Another particularly
5. preferred embodiment is to use eukaryotic probe derived from a given class to identify eukaryotic organisms of the same class (e.g. mammalian probe to identify mammalian organism). Most preferred is to use probe and DNA from the same subclass and/or order and/or family of organisms,
10. (e.g. if identifying a species of mouse, it is preferred to use mouse-derived probe).

The most sensitive and useful pair systems are those where there is less evolutionary distance or diversity between the source of the probe and the unknown DNA.

15. The phrase "evolutionarily conserved genetic material sequence" is used in this invention to denote genetic material, e.g DNA, sequences that show homology between at least two different species of plants, animals or micro-organisms. The homology between two conserved sequences
20. is to be such that, if one of such DNA molecules were to be detectably labelled, sufficient hybridization or annealing would occur if both single stranded DNA molecules or fragments thereof were placed together under hybridization conditions, thereby to produce a duplex of sufficient
25. stability to be detectable by standard methodology (i.e., radiolabelling, enzyme labelling, and the like).

In EP-A-0076123 the evolutionarily conserved



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sequence exemplified was that of ribosomal RNA genes. This is still a highly preferred gene sequence. However, it has been discovered that other gene sequences exist which are sufficiently conserved across the

5. evolutionary span to be useful.

Examples of such additional sequences are those of genes or portions thereof coding for transfer RNA, or proteins denoted as belonging to the same Superfamily, or same Family, preferably same Subfamily or even same

10. entry in Dayhoff's "Atlas of Protein Sequence and Structure", Volume 5, Supplement 3, 1978, NBR, 1979, pages 9-24, herein incorporated by reference. A Family of proteins is one wherein any two proteins differ from each other by less than 50% amino acid residues in their
15. sequence. A Subfamily of proteins is one wherein any two proteins differ from each other by less than 20% amino acid residues in their sequence. An "Entry" is one wherein any two proteins differ from each other by less than 5% amino acid residues in their sequence.

20. Specific examples of gene sequences or appropriate portions thereof which can be used are: cytochrome C related genes, cytochrome C₃ related genes, cytochrome c₁ related, cytochrome b₅ related, ferredoxin related, rebreodoxin related, flavodoxin related, alcohol
25. dehydrogenase related, lactate dehydrogenase related, peroxidase related, adenylate kinase related, phospholipase A₂ related, tryptophan operon related,



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- carboxypeptidase related, subtilisin related,
penicillinase related, protease inhibitor related,
somatotropin related, corticotropin related, lipotropin
related, glucagon related, snake venom toxin related,
5. plant toxin related, antibacterial toxin related,
immunoglobulin related gene, ribosomal other than rRNA-
related genes, heme carrier genes, chromosomal protein
genes, fibrous protein genes, and the like.

- The conservation of some of these additional DNA
10. sequences is not as widespread throughout the animal,
plant or microbiological domains as is that of the rRNA
genes. (Thus the still preferred use of rRNA). This,
however, does not constitute a serious impediment to
their use since it may be possible to utilize such
15. additional sequences to identify or characterize
organisms within more limited ranges or subdomains. For
example it may be possible to utilize trp D gene
sequences from bacteria to generate a trp D bacterial
probe and then use this probe to test within the
20. bacterial domain. In fact, it may be possible to use a
trp D probe within an even narrower domain (e.g., test
for the presence of Enterobacteriaceae, or of Bacillus,
etc.) with a trp D probe from the same order, family or
genus. Thus, while the range of applicability of some of
25. the additional probe sequences may not be as broad as
that of rRNA probes, their applicability will
nevertheless be quite effective within narrower domains.



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A probe containing the conserved DNA sequence information is prepared in the same manner as the preparation of rRNA information containing probe exemplified in EP-A-0076123. The probe can thus be

5. RNA, DNA or cDNA, and the like.

The individual steps involved in the technique will be described hereinafter broadly with reference to both eukaryotic and prokaryotic cells when applicable, or specifically for each type of cell if some difference in
10. technique exists.

The first step is extraction of the DNA from the unknown organisms. Nuclear DNA from eukaryotic cells can be selectively extracted by standard methodology well-known to the art (see for example, Drohan, W. et al,
15. Biochem. Biophys. Acta, 521 (1978), 1-15, herein incorporated by reference). Because organelle DNA is small and circular, spooling techniques serve to separate the non-circular nuclear DNA from the circular, organelle-derived DNA. As a corollary, the non-spooled
20. material contains the organelle-derived DNA which can separately be isolated by density gradient centrifugation. Alternatively, mitochondria (or chloroplasts) are separated from a mixture of disrupted cells; the purified mitochondrial (or chloroplast) fraction is used
25. for the preparation of organelle-derived DNA while the purified nuclear fraction is used to prepare nuclear DNA. (See for example Bonen L. and Gray, M. W., Nucleic Acids Research, 8:319-335 (1980)).



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- Prokaryotic DNA extraction is also well-known in the art. Thus, for example, an unknown bacterium present in any medium, such as an industrial fermentation suspension, agar medium, plant or animal tissue or sample or
5. the like, is treated under well-known conditions designed to extract high molecular weight DNA therefrom. For example, cells of the unknown organism can be suspended in extraction buffer, lysozyme added thereto, and the suspension incubated. Cell disruption can be further
10. accelerated by addition of detergents, and/or by increase in temperature. Protease digestion followed by chloroform/phenol extraction and ethanol precipitation can be used to finalize the extraction of DNA. An alternative method of extraction, which is much faster
15. than phenol/chloroform extraction, is rapid isolation of DNA using ethanol precipitation. This method is preferably used to isolate DNA directly from colonies or small, liquid cultures. The method is described in Davis, R. W. et al: "A Manual for Genetic Engineering,
20. Advanced Bacterial Genetics," (hereinafter "Davis"), Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1980, pp. 120-121, herein incorporated by reference.

- The DNA (prokaryotic or eukaryotic (nuclear or non-nuclear)) is dissolved in physiological buffer for the
25. next step. There are a variety of possible steps to be followed after isolation of the desired DNA. One of these steps is endonuclease digestion.



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Digestion of extracted DNA is carried out with restriction endonuclease enzymes. Any restriction endonuclease enzyme can be used. Preferably it is not from the same organism species as that being identified, since

5. otherwise, the DNA may remain intact. (This may, in any event, identify the organism, since the enzymes are not expected to cut DNA from the species of their origin.) Since the organism species being characterized may be unknown, obtaining a suitable digest of fragments may

10. entail a minimum amount of trial and error, which can routinely be carried out by those skilled in the art without undue experimentation. Examples of possible restriction endonuclease enzymes are Bgl I, BamH I, EcoR I, Pst I, Hind III, Bal I, Hga I, Sal I, Xba I, Sac I,

15. Sst I, Bcl I, Xho I, Kpn I, Pvu II, Sau IIIa, or the like. See also Davis, supra, at pp. 228-230, herein incorporated by reference. A mixture of one or more endonucleases can also be used for the digestion.

Normally, DNA and endonuclease are incubated together in

20. an appropriate buffer for an appropriate period of time (ranging from 1 to 48 hours, at temperatures ranging from 25°C-65°C, preferably 37°C).

The resulting identifying genetic characterization will depend on the type or types of endonucleases

25. utilized, and will be endonuclease-specific. It is therefore necessary to note which enzyme or enzymes have been used for the digestion since comparative character-



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izations used in a catalog should have been prepared using the same enzyme or enzymes.

- An alternative step is to define endonuclease sites on the desired DNA molecules without digestion thereof,
5. for example, by sequencing and reference to a restriction site library. Obviously, digestion is the more efficient method of noting such sites, but the method need not be limited thereto. The essence of the invention is the discovery that the position of conserved sequences along
10. DNA, relative to the position of endonuclease restriction sites, forms a set which is characteristic for each species. Thus, any technique which yields the desired information (position of the genes vis a vis position of
15. the sites) will be useful in the invention.

- Also, the position of the conserved sequences along the DNA molecule is best noted by use of a hybridization probe. This probe is allowed to anneal to restriction fragments of the unknown's DNA. However, any other
20. method that would allow the determination of the conserved DNA sequences, such as sequencing, would also be useful. When using the hybridization probe it is preferred to first digest and separate DNA fragments according to size, and then to hybridize the separated
25. fragments. However, it is possible to first digest and anneal DNA with a molar excess of probe and/or sequences complementary to probe and then separate the mixture. For example, unknown DNA can be digested with a



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- restriction endonuclease, denatured, and hybridized in liquid with a molar excess of one or more small detectably labeled DNA fragments or synthetic oligodeoxyribonucleotides complementary to a portion or
5. portions of the conserved sequence of interest. Since most restriction enzymes cut fairly infrequently in DNA, in most cases the double-stranded region or regions of the hybrid will be small relative to the size of the restriction fragment. The hybridization reaction is
10. conducted under conditions where only the oligodeoxyribonucleotides hybridize. The unreacted, single-stranded DNA fragments, and the DNA fragments containing the hybridized oligodeoxyribonucleotides are separated by conventional chromatographic techniques.
15. The labeled DNA fragments will appear in predictably sized fractions. It is also possible to first anneal DNA with a molar excess of probe, then digest, and then separate the mixture. When the solution is incubated for a short time period or to a low C_0t , the restriction
20. sites will be limited to the hybridized, double-stranded regions. When the solution is incubated for a long time period or to a high C_0t , the unknown DNA will anneal, thus creating a labeled duplex susceptible to restriction endonuclease cleavage. Unreassociated single-stranded
25. tails may be removed with a nuclease such as S1. Unpaired bases may be filled in using DNA polymerase I or T4 polymerase.



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Alternatively, one could find subsequences within the conserved sequence information (e.g. 20-, 30-, or 50-mers), which are more highly conserved than the remainder of the conserved region chosen as the probe. Those

5. "shorter" sequences can be made synthetically or enzymatically, if desired, and may incorporate labeled nucleotides. Single-stranded, predigested DNA from the unknown is allowed to incubate with these shorter, highly conserved fragments and allowed to hybridize thereto.
10. Separation would then be carried out on the digest mixture containing fragments partly annealed to the shorter labeled probes. (Thus, separation would occur after hybridization.) Separation could be by liquid chromatography, since the digest mixture would for all
15. practical purposes behave as a mixture of essentially single-stranded fragments.

As indicated, a preferred method is to first digest, then separate, and then hybridize. Thus, after endonuclease digestion, the incubation mixture, which

20. contains fragments of varying sizes, is preferably separated thereinto by an appropriate chromatographic method. Any method which is capable of separating nucleic acid digests according to size, and which allows the eventual hybridization with the nucleic acid probe
25. when hybridization is the last step, can be used. For example, gel electrophoresis, high pressure liquid chromatography or capillary zone electrophoresis can be

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- used. (Jorgenson, J.W., J. of HRC and CC, 4: 230-231 (1981)). Presently preferred is gel electrophoresis, most preferred is agarose gel electrophoresis. In this system, the DNA digests are normally electrophoresed in
5. an appropriate buffer, the gels are normally immersed in an ethidium bromide solution, and placed on a UV-light box to visualize standard marker fragments which may have been added. Detectably labeled standard marker fragments may be used as well.
10. After separation and visualization, the DNA fragments are transferred onto nitrocellulose filter paper or onto charge-modified nylon membranes by the method of Southern (Journal of Molecular Biology, 38:503-517 (1975)). The transfer can be carried out after
15. denaturation and neutralization steps, and is usually done for long periods of time (approximately 10-20 hours) or, alternatively by means of an electrically driven transfer from gel to paper. Instruments used to accelerate the transfer from gel to paper are com-
20. mercially available. The receiving nitrocellulose filter papers are then normally baked at high temperatures (60-80°C) for several hours, to bind the DNA to the filter.
- Alternatively, transfer can be avoided by using the recent method of direct hybridization of Purrello, M. et
25. al, Anal. Biochem., 128: 393-397 (1983).

The probe utilized for the hybridization of the paper-bound DNA digest fragments is a defined nucleic



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acid probe preferably from or derived from a given well-defined organism or the base sequence is known.

- Alternatively, the probe sequence may not have a natural counterpart; i.e., it may be a consensus sequence
5. with a base at each position that is most commonly present at that residue in a number of equivalent sequences in different species. The consensus sequence is then generally able to form a more stable hybrid than any one of the naturally occurring sequences. The probe
 10. may be a synthetic oligodeoxyribonucleotide molecule made by covalently attaching individual nucleotides in a predetermined sequence. Synthetic molecules may be prepared, for example, by the triphosphate method of synthesis (Alvarado-Urbina et al, Science 214: 270-274
 15. (1981)). The probe molecules may be of any useful size, and more than one sequence may be in the probe solution. For example, several 20 base sequences might be used to detect several highly conserved regions in rRNA genes. It may be detectably labeled or non-labeled,
 20. preferably detectably labeled. In such case, it is either detectably labeled RNA, but preferably nick-translated labeled DNA, cloned DNA, or detectably labeled DNA which is complementary to the RNA from the probe organism (cDNA), all of which contain highly conserved
 25. DNA sequence information. Synthetic oligodeoxyribonucleotides may be prepared with detectably labeled nucleotides, so the molecule is labeled by



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incorporating labeled nucleotide residues. Depending on the choice of pair, the probe may be from a prokaryote, or from a eukaryote (cytoplasm-derived, or organelle derived). Most preferably, the detectable label is a

5. radioactive label such as radioactive phosphorus (e.g., ^{32}P , ^3H or ^{14}C) or a biotin/avidin-based system. The nucleic acid probe may also be labeled with metal atoms. For example, uridine and cytidine nucleotides can form covalent mercury derivatives. Mercurated nucleoside
10. triphosphates are good substrates for many nucleic acid polymerases, including reverse transcriptase (Dale et al, Proceedings of the National Academy of Sciences 70:2238-2242, 1973). Direct covalent mercuration of natural nucleic acids has been described. (Dale et al,
15. Biochemistry 14:2447-2457). Reannealing properties of mercurated polymers resemble those of the corresponding nonmercurated polymers (Dale and Ward, Biochemistry 14:2458-2469). Metal labelled probes can be detected, for example, by photo-acoustic spectroscopy, x-ray
20. spectroscopy, e.g., x-ray fluorescence, x-ray absorbance, or photon spectroscopy.

- The isolation and preparation of any desired conserved DNA sequence-containing probe is within the skill of the art. For example, the isolation of rRNA
25. from eukaryotes or prokaryotes is well-known in the art. Thus, to prepare rRNA from eukaryotic cytoplasmic ribosomes, RNA can be extracted from whole cells or



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- ribosomes, separated by sucrose gradient centrifugation, and the 18S and 28S fractions can be collected using known molecular weight markers. (See for example, Perry, R. P. and Kelly, D. E., "Persistent Synthesis of 5S RNA When Production of 28S and 18S Ribosomal RNA is Inhibited by Low Doses of Actinomycin D," J. Cell. Physiol., 72:235-246 (1968), herein incorporated by reference). As a corollary, organelle-derived rRNA is isolated and purified from the organelle fractions in the same manner
10. (see e.g. Van Etten, R. A. et al, Cell, 22:157-170 (1980), or Edwards, K. et al, Nucleic Acids Research, 9:2853-2869 (1981)).

- If radioactively labeled probe is used, the same is isolated from the probe organism after growth or cultivation of the organism with nutrients or in culture media containing appropriately radioactive compounds. When the probe is complementary DNA (cDNA), the same is prepared by reverse transcribing isolated RNA from the probe organism, in the presence of radioactive nucleoside
20. triphosphates (e.g., ^{32}P -nucleosides or ^3H -nucleosides).

- The labeled probe may also be a nick-translated DNA molecule, especially one obtained from organelle-derived whole circular DNA. In this embodiment, chloroplast or mitochondrial DNA is nick-translated in the presence of
25. radiolabel, and a labeled DNA probe is thereby obtained. The chloroplast labeled probe will hybridize best with chloroplast DNA, and the mitochondrial labeled



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- probe will hybridize best with mitochondrial DNA. The chloroplast (or mitochondrial) nick-translated labeled probe will hybridize second best with mitochondrial (or chloroplast) DNA; it will also hybridize, albeit
5. generally in less favorable fashion, with whole plant (or animal) DNA. The probe may also be obtained from eukaryotic nuclear DNA by nick-translation, although practical considerations would rule against this mode. A more useful approach in this embodiment is to cut out the
10. highly conserved genes from the nuclear eukaryotic DNA (by restriction enzymes), separate the fragments, identify the gene sequences (as by hybridization), and isolate said gene sequences (as by electrophoresis). The isolated sequences may then be recombined into a plasmid
15. or other vector, and after transformation of an appropriate host, cloned in ^{32}P -containing media. Alternatively, the transformed host is grown, and the DNA is then isolated and labeled by nick-translation; or the DNA is isolated, the sequences are cut out and then
20. labeled. The resulting ribosomal probe will hybridize in the same instances as cDNA (see infra).

The preferred nucleic acid probe is radioactively labeled DNA complementary to RNA from the probe organism. The RNA is usually messenger RNA coding for a

25. conserved gene and is substantially free of other RNA's such as transfer RNA (tRNA) or ribosomal RNA (rRNA) (unless rRNA is used). If rRNA were to be used,



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- prokaryotic rRNA normally contains three subspecies: the so-called 5S, 16S and 23S fragments. The reverse transcription into cDNA can be carried out with a mixture of all three, or alternatively, with a mixture of 16S and
5. 23S fragments. It is less preferred to carry out the reverse transcription with only one of the rRNA components, although under certain conditions this may be feasible. Eukaryotic rRNA normally contains two subspecies: 18S and 28S, and the reverse transcription
10. into cDNA can be carried out with a mixture of 18S and 28S fragments or with each.

- The pure RNA, substantially free of other types of RNA, is incubated with any reverse transcriptase capable of reverse transcribing it into cDNA, preferably with
15. reverse transcriptase from avian myeloblastosis virus (AMV) in the presence of a primer such as calf thymus DNA hydrolysate. The mixture should contain appropriate deoxynucleoside triphosphates, wherein at least one of said nucleosides is radioactively labeled, for example
20. with ^{32}P . For example, deoxycytidine 5'-(^{32}P), deoxythymidine 5'-(^{32}P), deoxyadenine 5'-(^{32}P), or deoxyguanine 5'-(^{32}P) triphosphates can be used as the radioactive nucleosides. After incubation, from 30
25. minutes to 5 hours at 25°C-40°C, extraction with chloroform and phenol, and centrifugation as well as chromatography, the radioactively labeled fractions are pooled, and constitute the cDNA probe. The radioactively labeled



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- cdNA probe containing conserved DNA information in substantially purified form, i.e., free of non-labeled molecules, free of cDNA which is complementary to other types of RNA, free of proteinaceous materials as well as
5. free of cellular components such as membranes, organelles and the like, also constitutes an aspect of the present invention. A preferred probe is prokaryotic labelled cDNA, most preferred being the bacterial labelled cDNA. The probe species can be any bacterial microorganism,
 10. such as those of the family Enterobacteriaceae, Brucella, Bacillus, Pseudomonas, Lactobacillus, Haemophilus, Mycobacterium, Vibrio, Neisseria, Bactroides and other anaerobic groups, Legionella, and the like. Although the prokaryotic examples in the present application are
 15. limited to the use of E. coli as a bacterial prokaryotic probe organism, this aspect of the invention is by no means limited to this microorganism. The use of cDNA in radioactively labeled form as the probe is preferred to
 20. the use of radioactively labeled RNA because DNA has greater stability during hybridization.

- It is important to recognize that the labeled cDNA probe should be a faithful copy of the RNA, i.e. be one wherein all nucleotide sequences of the template RNA are
25. transcribed each time the synthesis is carried out. The use of a primer is essential in this respect. That the cDNA is a faithful copy can be demonstrated by the fact that it should have two properties following hybridization:



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1. The cDNA should protect 100% of labeled RNA from ribonuclease digestion; and
 2. The labeled cDNA should specifically anneal to the RNA as shown by resistance to S1 nuclease.
5. Beljanski M. M. et al, C.R. Acad. Sc Paris t 286, Serie D. p. 1825-1828 (1978), described ^3H radioactively labeled cDNA derived from E. coli rRNA. The cDNA in this work was not prepared with reverse transcriptase in the presence of a primer as in the present invention, but was
10. prepared with a DNA polymerase I, using as a template rRNA which had been pre-cleaved using ribonuclease U_2 . The rRNA digestion product (with RNase U_2) of Beljanski et al has a different base ratio from the initial rRNA, indicating a loss of bases and/or loss of short frag-
15. ments. Thus the cDNA obtained therefrom is not a faithful copy. In addition, the use of DNA polymerase I used by Beljanski is known to favor predominance of homopolymeric over heteropolymeric transcription of rRNA (see Sarin, P. S. et al, Biochem. Biophys. Res. Comm., 59:202-
20. 214 (1974)).

In sum, the probe can be seen as being derived a) from genome DNA containing conserved sequences, e.g. genes, by cloning and/or nick-translation, b) from RNA itself or c) from cDNA by reverse transcription of RNA.

25. Normally, the next step in the process of the invention is the hybridization of the separated DNA digest from the unknown organism with the unlabeled or



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(preferably) radioactively labeled RNA or DNA probe. Hybridization is carried out by contacting the paper containing covalently labeled DNA digest from the unknown, with a hybridization mix containing the probe.

5. Incubation is carried out at elevated temperatures (50-70°C) for long periods of time, filter papers are then washed to remove unbound radioactivity (if needed), air dried and readied for detection. An alternative, highly preferred hybridization, which is much more rapid than
10. the one described above, is the room temperature phenol emulsion reassociation technique of Kohne, D. E. et al, Biochemistry, 16:5329-5341 (1977), which is herein incorporated by reference.

- After hybridization, the technique requires selective
15. tive detection of the appropriately hybridized fragments. This detection can be carried out by taking advantage of the double strandedness of the hybridized fragments and using a selective method therefor (for non-labeled probe), or by autoradiography or by an appropriate
 20. radiation scanner which may or may not be computerized, and which may increase the speed of detection (for labeled probe). These techniques are well known to those skilled in the art and will not be further described at this point.
 25. The end product of the technique is an identifying genetic characterization, such as a chromatographic band pattern having peaks and troughs, or preferably, light



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and dark regions of various intensities, at specific locations. These locations can be readily matched to specific fragments sizes (in kilobase pairs) by introduction into the separation technique of a marker, such as EcoR I digested λ bacteriophage DNA. In this manner, both the relative position of the bands to each other, as well as the absolute size of each band can be readily ascertained. The identifying genetic characterization for the unknown is then compared with characterizations present in a catalog or library. The catalog or library can consist of a book containing characterizations for at least two, and up to a virtually unlimited number of defined different organisms genera and species. For example, the number of pathologically relevant bacteria that cause human disease is estimated to be about 100, so it is estimated that a standard catalog of pathogenic bacteria would contain anywhere between 50 and 150 such characterizations. A catalog of types of bacterial strains for epidemiological typing systems can also be included.

The characterizations will depend on the type or types of endonuclease enzymes selected, possibly on the particular organism used as the source for the radioactively labeled probe (the probe organism), and on the composition of the conserved DNA sequence information nucleic acids utilized to prepare the probe (e.g. containing either prokaryotic rRNA 5S, 16S or 23S



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- subtypes, or only 16S and 23S, or consensus sequences or the like). Thus, the catalog may, for each probe, contain a variety of enzyme-specific characterizations, with the size of each band listed, and with the relative
5. intensity noted. As the concentration of the bound DNA bound to the filter decreases, only the most intense bands can be seen, and the size of this band or bands can thus identify species. Any variation or permutation of the above can of course be used for the library.
10. Additionally, for a eukaryotic organism the library may contain patterns that result from the use of one type of DNA or any combination of organelle and/or nuclear DNA. The pattern for each DNA digest will depend on the probe composition. The catalog may be arranged so that if more
15. than one strain or species is present in the extracted sample and detected by the probe, the resulting characterization can be interpreted.

- A user can either compare the obtained characterization, e.g., band pattern, visually, or by aid of a one-
20. dimensional, computer assisted, digital scanner programmed for recognition of patterns. These computer scanners are well known in the art of the time-of-sale transactions (the commonly utilized "supermarket" check-out bar code or pattern readers). Ideally, the library or catalog
25. is present in a computer memory both in terms of the relative characterizations for a plurality of organisms, and in terms of the absolute values of molecular weight



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- or size of the fragments. The catalog comparison then consists of matching the unknown characterization with one of the characterizations present in the library by means of either one or both of the stored information elements (relative characterizations and/or absolute size elements). The intensity of each band when compared to a standard can also reveal the amount of bound DNA hybridized, and thus can be used to estimate the extent of the presence of an organism, for example a prokaryote in a eukaryote.

- If a user wishes to further confirm the nature and identification of a given organism, such user can digest the unknown with a second, different endonuclease, and compare the resulting characterization to catalog characterizations of the organism for the second chosen endonuclease. This process can be repeated as many times as necessary to get an accurate identification.

Normally, however, a single analysis with a single probe would be sufficient in most instances.

- The present invention and its variations can be used for a myriad of applications. It may be used by plant or animal breeders to correctly identify their subjects, or it may be used by clinical and microbiological laboratories to identify bacteria, parasites or fungi present in any medium, including in eukaryotic cells. In this latter use, the method is preferred to the standard microbiological assays, since it does not require



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- microbiological assays, since it does not require isolation and growth of the microbes. In vitro growth and characterization is now either impossible for some microorganisms such as Mycobacterium leprae (agent of leprosy), impossible on standard media for some microorganisms such as the obligate intracellular bacteria (e.g. rickettsia, chlamydia, etc), or highly dangerous (e.g. B anthracis (agent of anthrax)). The present method depends on the isolation of nucleic acid and
5. avoids these problems since it avoids conventional bacterial isolation and characterization. The method is expected to detect microorganisms that have not yet been conventionally described. In addition, the present method allows distinguishing different strains of
10. species, and this can be useful, for example, for epidemiological typing in bacteriology. The method can be used by forensic laboratories to correctly and unambiguously identify plant or animal tissues in criminal investigations. It can also be used by entomologists to
15. quickly identify insect species, when ascertaining the nature of crop infestations.
- 20.

- In addition, upon the conjunction of the method with the identification of infrasubspecific taxons (such as e.g., nitrogenase genes in plant roots, see Hennecke, H.
25. 291 Nature 354 (1981)), the methodology can be utilized to search for and identify the genotypes of individual strains.



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The method of this invention is preferably used for the identification of microorganisms wherever they may be found. These microorganisms may be found in physiological as well as non-physiological materials. They may

5. be found in industrial growth media, culture broths, or the like, and may be concentrated for example by centrifugation. Preferably, the microorganisms are found in physiological media, most preferably they are found in animal sources infected therewith. In this latter

10. embodiment, the method is used to diagnose bacterial infections in animals, most preferably in humans. The detection and identification of bacterial DNA with a prokaryotic probe is highly selective and occurs without hindrance, even in the presence of animal, (e.g., mam-

15. malian) DNA. If a prokaryotic probe is used, conditions can be selected which minimize hybridization with mitochondrial DNA, or mitochondrial bands can be subtracted from the pattern. The technique can thus be used in clinical laboratories, bacterial depositories, industrial

20. fermentation laboratories, and the like.

Of particular interest is the possibility of detecting, in addition to the species and strain identity of the infecting microorganism, the presence in the microorganism of any specific genetic sequences. For example,

25. it is possible to detect the presence of antibiotic resistance sequences found on R factors, which are transmissible plasmids mediating drug resistance. One



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- can add labeled R-factor DNA or cloned labeled antibiotic resistance sequences to the hybridization mixture in order to correctly determine the antibiotic resistance of the organism, (an extra band or bands would appear), or
5. one can rehybridize the once hybridized filter in the presence of added antibiotic resistance sequence probe or probes. Alternatively one could separate the unknown DNA into aliquots, and test the first aliquot for identification, the second for the presence of drug resistance
 10. sequences, the third for toxin genes, etc. Alternatively, one could use conserved gene information containing probe labeled with one radionuclide (e.g. ^{32}P) in a hybridization mixture with added R-factor probe labeled with a different radionuclide (e.g. ^3H or ^{14}C).
 15. After hybridization, the presence of R-factor DNA in the unknown DNA can be tested by scanning with two different scanners: one for species and strain identification (e.g. ^{32}P), the other for drug resistance, or the like (e.g. ^3H or ^{14}C). In this manner the lab can, without
 20. isolating and characterizing the microorganism, identify the genus and species, type the strain and test for drug resistance, possible toxin production or any other character or taxon below the rank of species that can be detected with a labeled nucleic acid sequence or probe,
 25. all in one experiment.

The R-factors are universal and cross species boundaries, so that identification can be carried out in



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any bacterial genus or species with the same R-factor probe (see Tomkins, L. S. et al, J. Inf. Dis., 141:625-636 (1981))).

- In addition, the presence of viruses or virus-related sequences in eukaryotes or prokaryotes can also be detected and identified in conjunction with the method of the invention: Any of the viruses described in "Manual of Clinical Microbiology", 3d edition, edited by Lennette, E.H., Amer. Soc. Microb., 1980, 774-778 can be identified, e.g., picornaviridae, caliciviridae, reoviridae, togaviridae, orthomyxoviridae, paramyxoviridae, rhabdoviridae, retroviridae, arenaviridae, coronaviridae, bunyaviridae, parvoviridae, papovaviridae, adenoviridae, herpesviridae, vidoviridae and poxviridae.

- A) When the viral genome is integrated into host DNA (as with DNA viruses, for example members of Papovaviridae, and RNA viruses, for example, members of Retroviridae), high molecular weight DNA is extracted from the tissue and digested with restriction endonucleases. The overall procedure is the same as used for bacteria. The choice of a viral probe again depends on the question asked, and on the extent of homology between the "probe virus" and the viral related sequences to be detected. In order to have suitable sequence homology, it may be necessary that the probe and tissue sequences are related to the same family, genus, or species of



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virus. In addition to the extent of conserved sequences, whether or not a viral probe hybridizes to viral related sequences in host DNA may be determined by the hybridization conditions, which can be stringent or relaxed.

5. The result of the hybridization will be a band or a pattern of bands showing that there are viral sequences incorporated into the host DNA. This information may be useful in helping to predict the occurrence of cancer. The probe can be any labelled complementary nucleic acid
10. probe including cloned viral sequences. For RNA viruses, for example viral RNA can be used to make a DNA with reverse transcriptase; for DNA viruses, for example, viral DNA labelled by nick translation can be used. Again multiple probes can be used, especially with
15. different labels.

Same general features apply equally to DNA and RNA viruses. Viral genomes are relatively small, so the precipitated nucleic acid is preferably collected by centrifugation; all of the procedures can use the total

20. nucleic acid or the various procedures can be run separately. It is expected that viral nucleic acid can be concentrated by spooling cellular DNA to remove it before centrifugation. This can also be used to determine if the viral genome is integrated.

25. For the viral probe to hybridize, it may be necessary and at least most preferred that the probe be from the same family, genus, or species as the unknown.



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Reaction conditions, stringent or relaxed, may determine whether or not a given probe hybridizes a distantly related genome. The probe may be cloned viral sequences that are labeled, or may be the complete genome or a

5. portion of it.

The technique described by Southern, supra is useful for the transfer of large DNA fragments (greater than about 0.5 kilobases) to nitrocellulose paper after alkali denaturation. This technique might be useful for DNA

10. viruses but not for RNA viruses. RNA has been transferred and covalently coupled to activated cellulose paper (diazobenzyloxymethyl-paper), and this can be used for RNA viruses. The modification of the Southern

technique by Thomas (Thomas, P., Proc. Nat. Acad. Sci., USA, 77:5201-5205 (1980)) can be used for the efficient transfer of RNA, and small DNA fragments to nitrocellulose paper for hybridization. RNA and small DNA fragments are denatured with glyoxal and dimethyl sulfoxide, and electrophoresed in agarose gel. This

20. procedure transfers DNA fragments between 100 and 2000 nucleotides and RNA efficiently, and they are retained on the nitrocellulose paper during hybridization. This is useful for small ribosomal DNA fragments as well. So it is most preferred to divide the restriction-enzyme

25. digested specimen and denature the nucleic acid in one portion with glyoxal. The Southern and Thomas procedures would yield a maximum amount of information.



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- B) For DNA viruses, restriction analysis can be carried out with double-stranded (DS) viral DNA's to identify viruses present. Single-stranded (SS) DNA viruses will have different genome lengths. The probe
5. (the sequence information could be converted to DS DNA) that hybridizes, the hybridized fragment pattern and/or the sizes or size can be used to identify viruses. There are again a number of ways to obtain complementary nucleic acid probes. For example, for DS DNA nick-
10. translation can be used; for SS DNA, DNA polymerase can be used to synthesize a cDNA.

- C) For RNA viruses, RNA is not digested by restriction endonucleases (the sequence information could be converted to DS DNA). The genomes of different RNA
15. viruses are of different sizes, and some RNA viruses have more than 1 molecule in their genome. This, along with the base sequences detected by certain probes or pooled probes allows the RNA viruses to be identified. An example of a probe would be cDNA synthesized using viral
20. RNA.

- When searching for infectious agents in specimens it is possible to search directly by extracting nucleic acid from the specimen, or by culturing first in media or cells to increase the number of agents, or by using a
25. concentration step such as centrifugation, or by trying all approaches.



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The present invention lends itself readily to the preparation of "kits" containing the elements necessary to carry out the process. Such a kit may comprise a carrier being compartmentalized to receive in close confinement therein one or more container means, such as tubes or vials. One of said container means may contain unlabeled or detectably labeled nucleic acid probe, such as for example the radioactively labeled cDNA to RNA from the organism probe, (preferably prokaryotic cDNA in the case of a kit to identify bacteria). The labeled nucleic acid probe may be present in lyophilized form, or in an appropriate buffer as necessary. One or more container means may contain one or more endonuclease enzymes to be utilized in digesting the DNA from the unknown organism. These enzymes may be present by themselves or in admixtures, in lyophilized form or in appropriate buffers. Ideally, the enzymes utilized in the kit are those for which corresponding catalogs have been prepared. Nothing stops the user, however, from preparing his or her own comparative standard at the moment of experiment. Thus, if a user suspects that an unknown is in fact of a given genus or species, he or she may prepare the identifying characteristics for the known and compare it with the characterization for the unknown. The kit may thus also contain all of the elements necessary in order to carry out this sub-process. These elements may include one or more known organisms, (such



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- as bacteria), or isolated DNA from known organisms. In addition, the kit may also contain a "catalog", defined broadly as a booklet, or book, or pamphlet, or computer tape or disk, or computer access number, or the like,
5. having the identifying characterizations for a variety of organisms of a certain group, such as plant species, mammal species, microbe species, especially pathologically important bacteria, insect species or the like. In this mode, a user would only need to prepare
 10. the characterization for the unknown organism, and then visually (or by computer) compare the obtained characterization with the characterizations in the catalog. The kit may also contain in one container probe RNA for probe synthesis, in another container radio-
 15. labeled deoxyribonucleoside triphosphate, and in another container primer. In this manner the user can prepare his or her own probe cDNA.

- Finally, the kit may contain all of the additional elements necessary to carry out the technique of the
20. invention, such as buffers, growth media, enzymes, pipettes, plates, nucleic acids, nucleoside triphosphates, filter paper, gel materials, transfer materials, autoradiography supplies, and the like. It may also contain antibiotic resistance sequence probes, viral
 25. probes, or other specific character probes.

Having now generally described this invention, the same will be better understood by reference to certain



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reference experiments and specific examples which are included herein for purposes of illustration only and are not intended to be limiting of the invention, unless specified.



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MATERIALS AND METHODSA. BacterialExtraction of High Molecular Weight DNA

Bacterial broth cultures were centrifuged and the cells were washed with cold saline. The cells were

5. suspended in a volume measured in ml of extraction buffer (0.15 M sodium chloride, 0.1 M EDTA, 0.03 M tris pH 8.5) approximately 10 times the gram weight of the packed cells. Lysozyme at 10 mg/ml was added to 0.5 mg/ml final concentration. The suspension was incubated at 37°C for

10. 30 minutes. Cell disruption was completed by the addition of 25% SDS to 2.5% final concentration, and raising the temperature to 60°C for 10 minutes. After cooling in a tap water bath, mercaptoethanol was added to

15. 1% final concentration. Pronase® at 20 mg/ml in 0.02 M tris pH 7.4 was predigested at 37°C for 2 hours and then added to 1 mg/ml final concentration. The solution was incubated at 37°C for 18 hours. Phenol was prepared by mixing one liter redistilled phenol, 2.5 liters double

20. distilled water, 270 ml saturated Tris base, 12 ml mercaptoethanol, and EDTA to 10^{-3} M final concentration and allowing the mixture to separate at 4°C. The phenol was washed with wash buffer (10^{-1} M sodium chloride, 10^{-3} M EDTA, 10mM tris pH 8.5). Then an equal volume of fresh

25. buffer was added. Mercaptoethanol was added to 0.1% final concentration. The solution was mixed and stored at 4°C. One half volume prepared phenol and one half



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volume chloroform was added to the lysed cell solution. This was shaken for approximately 10 minutes and centrifuged at 3,400 x g for 15 minutes. The aqueous phase was removed with an inverted 25 ml glass pipette. The

5. extraction procedure was repeated until there was little precipitate at the interface. One-ninth volume 2 N sodium acetate pH 5.5 was added to the aqueous phase. Two times volume of 95% ethyl alcohol at -20°C was poured slowly down the side of the flask. The end of a Pasteur

10. pipette was melted close and used to spool the precipitated DNA. High molecular weight DNA was dissolved in buffer (10^{-3} EDTA, 10^{-2} M tris pH 7.4). The concentration of DNA was determined by absorbance at 260 nm using 30 micrograms per absorbance unit as conversion factor.

15.

Restriction Endonuclease Digestion of DNA.

EcoR I restriction endonuclease reactions were performed in 0.1 M tris-HCl pH 7.5, 0.05 M NaCl, 0.005 M $MgCl_2$, and 100 micrograms per ml bovine serum albumin.

20. EcoR I reaction mixtures contained 5 units of enzyme per microgram of DNA, and were incubated four hours at 37°C. PST I restriction endonuclease reactions were performed in 0.006 M tris-HCl pH 7.4, 0.05 M sodium chloride, 0.006 M magnesium chloride, 0.006 M 2-

25. mercaptoethanol, and 100 micrograms per ml of bovine serum albumin. PST I reaction mixtures contained 2 units of enzyme per microgram DNA, and were incubated four



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- hours at 37°C. Usually 10 micrograms DNA was digested in a final volume of 40 microliters. Ten times concentration buffers were added. Sterile distilled water was added depending on the volume of DNA. λ Bacteriophage
5. DNA was restricted with EcoR I to provide marker bands for fragment size determinations. Usually 2 micrograms λ DNA was digested with 20 units EcoR I in a final volume of 20 microliters.

10. Gel Electrophoresis and DNA Transfer.

- DNA digests were supplemented with glycerol, to about 20%, and bromophenol blue tracking dye. In the case of λ DNA digests, 20 microliters of 1x EcoR I buffer was added to each 20 microliter reaction mixture.
15. Usually 15 microliters 75% glycerol and 5 microliters 0.5% bromophenol blue were added to each 40 microliter reaction mixture.

- 10 micrograms digested bacterial DNA or 2 micrograms digested λ DNA were loaded per well and overlaid with
20. molten agarose. Digests were electrophoresed in 0.8% agarose with 0.02 M sodium acetate, 0.002 M EDTA, 0.018 M tris base, and 0.028 M tris HCl pH 8.05 at 35 V until the dye migrated 13 to 16 cm. Gels were then immersed in ethidium bromide (0.005 mg/ml) and placed on a UV-light
25. box to visualize the λ fragments. DNA was transferred to nitrocellulose filter paper by the method of Southern, supra. Gels were treated with denaturing solution (1.5 M



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- sodium chloride, 0.5 M sodium hydroxide) on a rocker table for 20 min. Denaturing solution was replaced with neutralization solution (3.0 M sodium chloride, 0.5 M tris HCl, pH 7.5), and after 40 minutes the gels were
5. checked with pH paper. Following neutralization, the gels were treated with 6 x SSC buffer (SSC = 0.15 M sodium chloride, 0.015 M sodium citrate) for 10 minutes. DNA fragments were transferred from the gel to the nitrocellulose paper by drawing 6 X SSC through the
10. gel and nitrocellulose paper with a stack of paper towels for 15 hours. Filters were placed between two sheets of 3 MM chromatography paper, wrapped in aluminum foil, shiny side out, and dried in a vacuum oven at 80°C for 4 hours.
- 15.

Synthesis of ^{32}P ribosomal RNA Complementary DNA (^{32}P rRNA cDNA).

- ^{32}P -labeled DNA complementary to E. coli R-13 23S and 16S ribosomal RNA was synthesized using reverse
20. transcriptase from avian myeloblastosis virus (AMV). The reaction mixture contained 5 microliters 0.2 M dithiothreitol, 25 microliters 1 M tris pH 8.0, 8.3 microliters 3 M potassium chloride, 40 microliters 0.1 M magnesium chloride, 70 micrograms actinomycin, 14 micro-
25. liters 0.04 M dATP, 14 microliters 0.04 M dGDP, 14 microliters 0.04 M dTTP and 96.7 microliters H_2O . The following were added to a plastic tube: 137.5 microliters



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- reaction mixture, 15 microliters calf thymus primer (10 mg/ml), 7 microliters H 20, 3 microliters rRNA (using 40 micrograms /OD unit concentration, is 2.76 micrograms/microliters), 40 microliters deoxycytidine 5'-
5. (^{32}P) triphosphate (10 mCi/ml), and 13 microliters AMV polymerase (6,900 units μl . The enzymatic reaction was incubated 1.5 hours at 37°C. Then the solution was extracted in 5 ml each of chloroform and prepared phenol. After centrifugation (JS 13,600 RPM), the aqueous phase
10. was layered directly on a Sephadex® G-50 column (1.5 x 22 cm). A plastic 10 ml pipette was used for the column. A small glass bead was placed in the tip, rubber tubing with a pinch clamp was attached, and degassed G-50 swelled in 0.05% SDS overnight was added. The aqueous
15. phase wall allowed to flow directly into the G-50 and was then eluted with 0.05% SDS. 20 fractions at 0.5 ml each were collected in plastic vials. Tubes containing peak fractions were detected by Cerenkov counting using a ^3H discriminator, counting for 0.1 min. per sample and re-
20. cording total counts. Peak fractions were pooled. Aliquots were added to Aquesol® (commercially available), and the CPM of ^{32}P per ml was determined by scintillation counting.



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Hybridization and Autoradiography.

Fragments containing ribosomal RNA gene sequences were detected by autoradiography after hybridization of the DNA on the filters to ^{32}P -rRNA cDNA. Filters were

5. soaked in hybridization mix (3 x SSC, 0.1% SDS, 100 micrograms/ml denatured and sonicated canine DNA, and Deinhart's solution (0.2% each of bovine serum albumen, Ficoll, and polyvinyl pyrrolidone)), for 1 hour at 68°C. ^{32}P rRNA cDNA was added at 4×10^6 CPM/ml, and the

10. hybridization reaction was incubated at 68°C for 48 hours. Filters were then washed in 3 x SSC, and 0.1% SDS at 15 min. intervals for 2 hours or until the wash solution contained about 3,000 cpm ^{32}P per ml. Filters were air dried, wrapped in plastic wrap and autoradiographed

15. approximately 1 hour with Kodak X-OMAT R film at -70°C.

B. Mammalian experiments. Mus musculus domesticus (mouse) rRNA probes were synthesized from 18S and 28S, and only 28S rRNA. Nucleic acid was extracted from mouse liver and precipitated. High molecular weight DNA was

20. spooled and removed. The remaining nucleic acid was collected by centrifugation and dissolved in buffer, 50 mM MgCl_2 and 100 mM Tris pH 7.4. DNase (RNase free) was added to a concentration of 50 $\mu\text{g}/\text{ml}$. The mixture was incubated at 37°C for 30 min. The resulting RNA was

25. reextracted, ethanol precipitated, and dissolved in 1mM sodium phosphate buffer pH 6.8. A 5 to 20% sucrose gradient in 0.1M Tris pH 7.4 and 0.01M EDTA was pre-



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pared. The sample was added and the gradients spun in an SW40 rotor 7 hr. at 35K RPM. Fractions were collected by optical density. The 18S and 28S fractions were selected by comparison to known molecular weight markers.

5. For all of the mammalian experiments relaxed hybridization conditions were used, 54°C. The washing procedure, carried out at 54°C, was 3 separate washes with 3xSSC with 0.05% SDS for 15 min. each.

- Reference Experiments 1-8 describe experiments carried out with rRNA-information containing probes. Examples 1 to 3 describe computer simulations utilizing a histone gene information-containing probe, a tryptophan operon trp D gene-information containing probe, and an α -fetoprotein gene information-containing probe, respectively.

15.

Reference Experiment 1

Bacterial Species are Defined by Restriction

Endonuclease Analysis of Ribosomal RNA Genes

- The several strains of P. aeruginosa used in this example have the minimal phenotypic characters which identify the species (Hugh R.H., et al, in: Manual of Clinical Microbiology, 2d Ed. ASM, 1974, pp. 250-269). (Table 2). Strains of three other Pseudomonas and two Acinetobacter species were selected to compare species and genera (Table 3).



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TABLE 2

Corresponding strain numbers of isolates with the
minimal phenotypic characters of P. aeruginosa
for the comparison of Strains.

	<u>RH</u>	<u>ATCC</u>
5.	151	10752
	809	7701
	810	8689
	811	8707
10.	812	8709
	815	10145
	1559	14425

15. Strains used for comparison of Pseudomonas and
Acinetobacter species are listed in Table 3.

20.



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TABLE 3

Corresponding strain numbers of type, neotype and reference strains for the comparison of species and genera

5.	<u>Species</u>	<u>RH</u>	<u>ATCC</u>	<u>NCTC</u>	<u>Strain Status</u>
	<u>P. aeruginosa</u>	815	10145	10332	type
	<u>P. stutzeri</u>	2601	17588		neotype
	<u>P. fluorescens</u>	818	13525	10038	neotype
	<u>P. putida</u>	827	12633		neotype
10.	<u>A. anitratus</u>	2208	19606		type
	<u>A. lwoffii</u>	462	7976		reference

Acinetobacter species were selected for comparison of genera because they share certain attributes with many Pseudomonas species.

The sizes (kilobase pairs) of fragments in EcoR I digests are: P. stutzeri 16.0, 12.0, 9.4; P. fluorescens 16.0, 10.0, 8.6, 7.8, 7.0; P. putida 24.0, 15.0, 10.0, 8.9; A. anitratus 20.0, 15.0, 12.5, 9.8, 7.8, 6.1, 5.2, 4.8, 3.8, 2.8 (size of the smallest 3 fragments not calculated); A. lwoffii 12.0, 10.0, 9.1, 7.0, 6.4, 5.7, 5.5, 5.3, 4.8, 4.4, 3.6, 3.2, 2.9 (size of the smallest 3 fragments not calculated). The sizes (kilobase pairs) of fragments in PST I digests are; P. stutzeri 6.7, 6.1, 5.5; P. fluorescens 10.0, 9.4, 7.8, 7.0; P. putida 10.5,



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9.9, 6.8, 6.3, 4.4; A. anitratus 36.0, 28.0, 20.5, 12.0, 10.0, 5.8, 3.7, 2.6, 2.4; A. lwoffii 9.9, 8.7, 7.2, 5.7, 4.0, 3.6, 3.2, 2.7.

- Comparison of the hybridized restriction fragments
5. from the seven strains of P. aeruginosa leads to the conclusion that this species can be defined by an EcoR I specific set of fragments containing rRNA gene sequences, 10.1, 9.4, 7.6, and 5.9 kilobase pairs (KBP) (FIGURE 1). The 7.6 KBP EcoR I fragment occurs in 4 of the 7
10. strains in this sample. An analogous situation occurs among certain phenotypic characters of strains of species. The fact that the EcoR I sets of fragments from the 7 strains can be used to separate the strains into two groups prompts speculation that there may be two
15. species with the minimal phenotypic characters of P. aeruginosa. The results of experiments in which DNA was digested with PST I (FIGURE 2) lead to the conclusion that the strain variation shown by the EcoR I 7.6 KBP fragment represents variation within the species, since
20. there is a single conserved set of PST I fragments, 9.4, 7.1, 6.6, and 6.4 KBP, that define the species. The 9.4 and 6.6 KBP Pst I fragments occur in 6 of the 7 strains of P. aeruginosa; the 7.1 and 6.4 KBP PST I fragments occur in all of the strains sampled. PST I fragment
25. variation occurs in strains that do not contain an EcoR I 7.6 KBP fragment; RH 151 has 10.1 and 8.2 KBP fragments, RH 809 does not contain a 9.4 KBP fragment and has a 6.0

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KBP fragment, and RH 815; the type strain, does not contain a 6.6 KBP fragment. The patterns of hybridized fragments support the conclusion that enzyme specific, conserved sets can be used to define species. Strains of

5. a species probably have a majority of the fragments in the conserved set. The occurrence of fragment variations in some strains does not prevent identification and may prove useful in epidemiological studies.

The occurrence of variation, EcoR I 7.6 KBP fragment

10. in P. aeruginosa strains, may be put into perspective by examining hybridized EcoR I fragments found in the type strains of other Pseudomonas species (FIGURE 3). The type strains of P. stutzeri, P. fluorescens, and P. putida do not contain a 7.6 KBP fragment, but do have

15. EcoR I fragments of the same size in common; P. aeruginosa and P. stutzeri each have a 9.4 KBP fragment, P. stutzeri and P. fluorescens each have a 16 KBP fragment, and P. fluorescens and P. putida each have a 10 KBP

20. unique in the type strains of each of the 4 Pseudomonas species; and the type strain of each species has a different size range of fragments. These general comments are also true for the PST I digests (FIGURE 4).

When the fragment patterns of one strain of each of

25. the 4 Pseudomonas and 2 Acinetobacter species are compared, it can be concluded that the species of each genus are similar, but the genera differ. The 2 Acinetobacter

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species have a greater range of hybridized fragment sizes than do the 4 Pseudomonas species.

- Without the aid of restriction enzyme maps such as those available for E. coli, Bacillus thuringiensis and B. subtilis, it is not possible to predict where enzymes cut rRNA genes, the number of copies per genome, whether there are heterologous flanking regions between genes or gene heterogeneity. The E. coli rRNA cDNA probe may fail to hybridize with some restriction fragments containing
5. rRNA gene sequences, and if so, this reflects the evolutionary distance or diversity between the test organism and E. coli. The conserved nature of rRNA can be used to argue that this is not the case. However, this is a minor problem compared to the advantage of having a standard probe that can be equally applied to any unknown species.
- 10.
- 15.

Reference Experiment 2

Comparison of Restriction Analysis with DNA-DNA

20. Liquid Hybridization:

The strains used in this study are listed in Tables 4 and 5.



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Table 4

Corresponding Strain Numbers
of Neotype strains of B. subtilis
and type strains of junior synonyms

5.

			Strain
<u>Species</u>	<u>RH</u>	<u>ATCC</u>	<u>Status</u>
<u>B. subtilis</u>	3021	6051	neotype
<u>B. uniflagellatus</u>	2990	15134	type
10. <u>B. amyloliquafaciens</u>	3061	23350	type



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Table 5. Corresponding strain number of
strains of B. subtilis

	<u>RH</u>	<u>NRRL</u>	<u>ATCC</u>
5.	3063	B-354(NRS-231)	6633
	3064	B-356(NRS-238)	7067
	3065	NRS-265	6455
	3066	NRS-659	7060
10.	3067	NRS-730	7003
	3068	NRS-737	943
	3069	NRS-741	4344
	3070	NRS-773	8188
	3071	NRS-1106	4944
15.	3072	NRS-1107	7480

High molecular weight DNA was isolated from each of the strains. Liquid DNA-DNA hybridization data was collected using RH 3021 and RH 2990 labeled DNAs and results are shown in Table 6.



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TABLE 6

Percent hybridization between labeled
DNA probe and DNA from strains of B. subtilis

5.

Labeled DNA

<u>probe</u>	<u>RH 3063</u>	<u>RH 3064</u>	<u>RH 3066</u>	<u>RH 3067</u>	<u>RH 3068</u>	<u>RH 3065</u>
RH 3021	61	77	51	96	84	18
RH 2990	12	10	13	15	16	50

10.

	<u>RH 3069</u>	<u>RH 3070</u>	<u>RH 3071</u>	<u>RH 3072</u>	<u>RH 3021</u>	<u>RH 2990</u>
RH 3021	14	—	93	15	100	20
RH 2990	100	—	17	100	20	100

15.

	<u>RH 3061</u>
RH 3021	11
RH 2990	70



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- The data shows there are two hybridization groups. Similar data is reported in the literature for B. subtilis (Seki et al, International Journal of Systematic Bacteriology 25:258-270 (1975)). These two groups can be
5. represented by RH 3021 and RH 2990. When restriction endonuclease analysis of ribosomal RNA genes is carried out, EcoR I digests (FIGURE 5) can be separated into two groups. The group represented by RH 3021 has two intensely hybridized fragments (2.15 and 2.1 KBP). The
10. group represented by RH 2990 has two intensely hybridized fragments (2.6 and 2.5 KBP). The EcoR I data can be used to place B. subtilis strains in appropriate DNA-DNA hybridization groups. According to the DNA-DNA hybridization 70% rule, B. subtilis is actually two
15. species. However, when the PST I data (FIGURE 6) is considered, it is possible to think of the groups as two divergent populations related to a common ancestor or speciation event. The conclusion that B. subtilis is one species correlates with phenotypic data. The strains
20. listed in Table 5 are identified as B. subtilis in Gordon, R. E. et al "The Genus Bacillus", Agriculture Handbook No. 427, Agricultural Research Service, U.S. Dept. of Agriculture, Washington, D.C. pp. 36-41.
- Restriction analysis can provide data which is comparable
25. to DNA-DNA hybridization data, or by selecting the proper enzyme, restriction analysis can adequately define species despite divergence. RH 3061 has lost PST I sites. How-

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ever, the EcoR I data suggests that the strain is B. subtilis. The same is concluded from the Bgl II data (FIGURE 7) and Sac I data (FIGURE 8).

Reference Experiment 3

5.

Stability of the Restriction Analysis

Pattern and Other

Bacillus polymyxa Experiments

TABLE 7

10. Neotype strains of B. subtilis and B. polymyxa

	Species	RH	ACTT	NRRL	Comments
	<u>B. subtilis</u>	3021	6051		neotype
15.	<u>B. polymyxa</u>	3074	842		neotype
	<u>B. polymyxa</u>	3033			asporogenous mutant derived from RH 3074
	<u>B. polymyxa</u>	3062		NRS-1105	neotype
20.	<u>B. polymyxa</u>	3073			asporogenous mutant derived from NRS-1105



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- B. subtilis and B. polymyxa can be distinguished by EcoR I data (FIGURE 9), PST I data (FIGURE 10) Bgl II data (FIGURE 11, left) and Sac I data (FIGURE 11, right). It can be concluded from the major differences in the PST I
5. band patterns that bacillus polymyxa is in the wrong genus. While both species produce spores, they are not phenotypically similar. It is reassuring that the type strain of B. polymyxa from both culture collections, ATCC and NRRL have the same band patterns. The important
10. data, however, is that the asporogenous mutants can be identified. It is very difficult, perhaps impossible, to identify Bacillus species if they fail to form spores.

Reference Experiment 4

15. Identification of a Bacterial Species in Mouse Tissue without Isolation

- A Swiss mouse, Mus musculus domesticus (inbred strain), was inoculated intraperitoneally with 0.5 ml of a turbid suspension of Streptococcus pneumoniae RH 3077
20. (ATCC 6303). When the mouse became moribund, the heart, lungs, and liver were removed. High molecular weight DNA was isolated from these tissues, S. pneumoniae RH 3077 and Swiss mouse organs, and the procedure for restriction endonuclease analysis of rRNA genes was carried out using
25. EcoR I to digest the DNAs. In addition to washing the filters in 3 X SSC, they were washed for 2 X 15 minute periods in 0.3 X SSC and 0.05% SDS. Autoradiography was

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- carried out for 48 hr. The data (FIGURE 12) shows that S. pneumoniae can be defined by seven hybridized fragments (17.0, 8.0, 6.0, 4.0, 3.3, 2.6 and 1.8 KBP). The bacterial cDNA probe hybridizes poorly to two mouse
5. DNA fragments (14.0 and 6.8 KBP). Hybridized fragments signal the presence of S. pneumoniae in the infected tissues. All seven bands can be seen in the heart DNA extract. They are less intense in the liver DNA extract, but all can be seen in the autoradiograph. Only the 6.0
10. KBP band appears in the lung DNA extract. The lesser number of bacteria in the lungs can be explained by the mouse having septicemia rather than pneumonia. The lungs showed no consolidation at autopsy. In order to determine the sensitivity of the assay, bacterial DNA was
15. diluted with mouse DNA and electrophoresed. All seven bands can be seen in the autoradiograph when 0.1 micrograms of bacterial DNA is used. The 17.0, 8.0 and 6.0 KBP bands can be seen with 10^{-3} μ g of bacterial DNA. If the figure of 5×10^{-3} μ g DNA per 10^6 S. pneumoniae cells
20. is used (Biochim Biophys Acta, 26:68), 10^{-1} μ g is equivalent to 2×10^7 cells. The present technique is thus useful for diagnosing infections at this level of sensitivity.

- This reference experiment also demonstrates that the
25. bacterial probe hybridizes with mouse-specific EcoR I fragments (see FIGURE 9, fragments having 14.0 and 6.8 KBP). These fragments correspond to EcoR I fragments detected by

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- mouse 18S and 28S ribosomal RNA probe (FIGURE 14 infra shows that the 6.8 KBP fragment contains the 28S rRNA sequences). The bacterial probe does not hybridize as well to mammalian ribosomal RNA gene sequences, so the
5. bands are less intense, the system of bacterial probe and nuclear mammalian DNA is less sensitive, and selectivity for DNA from infecting prokaryotes is clearly demonstrated. In experiments where bacterial probe was hybridized to 10 µg digested bacterial DNA per lane, no
10. hybridization to 10 µg digested human or mouse DNA per lane was detected on the autoradiographs when the bacterial bands were clearly seen.

Reference Experiments 5-8

15. Mammalian experiments

These reference experiments illustrate that the concept of rRNA restriction analysis to identify organisms can be successfully applied not only to bacteria but to complex, eukaryotic organisms.

20. FIGURE 13 shows that mammalian genera can be recognized with Mus musculus domesticus 18S and 28S rRNA probe, and that several species of Mus can be distinguished. In this figure, the enzyme is PST I and the subjects and corresponding bands are as follows:

25. 1. Mus musculus melosinus (mouse) 14.5, 13.5, 2.6
2. Mus musculus domesticus (mouse) 13.5, 2.6
3. Canis familiaris (dog) 12.0

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4. Cavia porcellus (guinea pig) 17.0, 14.0, 13.0, 8.8, 5.7, 4.7, and one band less than 3.0
5. Cricetulus griseus (hamster) 25.0, 4.7
6. Homo sapiens (human) 15.0, 5.7
5. 7. Felis catus (cat) 20.0, 9.7
8. Ratus norvegicus (rat) 12.5
9. Mus musculus domesticus (mouse) 13.5, 2.6
10. 10. Mus cervicolor cervicolor (mouse) 14.0, 2.7
11. Mus cervicolor papeus (mouse) 13.5, 2.6
10. 12. Mus pahari (mouse) 13.0, 3.7
13. Mus cookii (mouse) 13.5, 2.6

FIGURE 14 shows that mouse and cat DNA can be distinguished by the 28S rRNA cDNA alone, and that the pattern of hybridized bands is dependent on the composition of the probe sequences. In Figure 14 the enzyme is EcoR I, and the subjects and bands are as follows:

1. Mus musculus domesticus (mouse) 6.8 KBP
20. 2. Felis catus (cat) 8.3 KBP

In Figure 15 the enzyme is Sac I, and the subjects and bands are as follows:

1. Erythrocebus patas (patas monkey) 8.5, 3.7, <3.0
2. Ratus norvegicus (rat) 25.0, 9.5, 3.6, <3.0
25. 3. Mus musculus domesticus (mouse) 6.8, <3.0
4. Felis catus (cat) 9.5, 5.3, 4.0, <3.0, <3.0
5. Homo sapiens (human) 10.5, <3.0



6. Macaca mulatta (rhesus monkey) 9.8, <3.0 When Figure 15 (Sac I digests) is compared to the other mammalian figures it can be seen the the hybridized pattern is enzyme specific.
5. Figure 16 shows that primates can be distinguished. Cell cultures have bands in common with tissue from the species of origin, and different human cell cultures can be distinguished by additions and deletions of bands. In this figure, the enzyme is EcoR I, and the
10. subjects and bands are as follows:
1. Erythrocebus patas (patas monkey) >22.0, 11.0, 7.6, 2.6
 2. Macaca mulatta (rhesus monkey) 22.0, 11.5, 7.6
 15. 3. Homo sapiens (human) >22.0, 22.0, 16.0, 8.1, 6.6
 4. M 241/88 (langur monkey cell culture) 14.0, 7.2, 5.7
 20. 5. HeLa (human cell culture) >8.1, 6.6
 6. J96(human cell culture) > 22.0, 22.0, 16.0, 11.0, 8.1, 6.6
 25. 7. AO (human cell culture) 22.0, 16.0, 8.1, 6.6
 8. X-381 (rhesus monkey) 22.0, 11.5, 7.6



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Example 1Use of an H4 Histone Gene Probe

- A computer simulation of the identification and characterization of two animal species (sea urchin and mouse) was carried out using a conserved DNA sequence derived from the H4 histone gene.

- The histone H4 gene sequence for sea urchin (*Psammechinus miliaris*) is shown below, where A, T, C, G, represent the known nucleotides, and N represent a presently unknown position (788 base pairs).

	10	20	30	40	50
	CAACATATTA	GAGGAAGGGA	GAGAGAGAGA	GAGAGAGAGA	GAGAGAGAGA
	GTTGTATAAT	CTCCTTCCCT	CTCTCTCTCT	CTCTCTCTCT	CTCTCTCTCT
	60	70	80	90	100
15.	GGGGGGGGGG	GAGGGAGAAT	TGCCCAAAC	ACTGTAAATG	TAGCGTTAAT
	CCCCCCCC	CTCCCTCTTA	ACGGGTTTTG	TGACATTTAC	ATCGCAATTA
	110	120	130	140	150
	GAACTTTTCA	TCTCATCGAC	TGCGCGTGTA	TAAGGATGAT	TATAAGCTTT
	CTTGAAAAGT	AGAGTAGCTG	ACGCGCACAT	ATTCCTACTA	ATATTCGAAA
	160	170	180	190	200
	TTTTCAATTT	ACAGGCACTA	CGTTACATTC	AAATCCAATC	AATCATTTGA
	AAAAGTTAAA	TGTCCGTGAT	GCAATGTAAG	TTTAGGTTAG	TTAGTAAACT
20.	210	220	230	240	250
	ATCACCGTCG	CAAAAGGCAG	ATGTAAACTG	TCAAGTTGTC	AGATTGTGTG
	TAGTGGCAGC	GTTTTCCGTC	TACATTGAC	AGTTCAACAG	TCTAACACAC
	260	270	280	290	300
	CGCGGCCTCC	AGTGAGCTAC	CCACCGGGCC	GTCGCGGAGG	GGCGCACCTG
	GCGCCGGAGG	TCACTCGATG	GGTGGCCCGG	CAGCGCCTCC	CCGCGTGGAC
	310	320	330	340	350
	TGCGGGAGGG	GTCATCGGAG	GGCGATCGAG	CCTCGTCATC	CAAGTCCGCA
25.	ACGCCCTCCC	CAGTAGCCTC	CCGCTAGCTC	GGAGCAGTAG	GTTCAGGCGT
	360	370	380	390	400
	TACGGGTGAC	AATACCCCCG	CTCACCAGGA	GGGTTGGTCA	ATCCCTCAGC
	ATGCCCACTG	TTATGGGGGC	GAGTGGCCCT	CCCAACCACT	TAGCGAGTCG

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410 420 430 440 450
 GAAACGTCCA GTCGTCAGCA TCGCACTAAG ACTCTCTCTC AATCTCCATA
 CTTTGCAGGT CAGCAGTCGT AGCGTGATTG TGAGAGAGAG TTAGAGGTAT

460 470 480 490 500
 ATGTCAGGCC GTGGTAAAGG AGGCAAGGGG CTCGGAAAGG GAGGCGCCAA
 TACAGTCCGG CACCATTTC TCCGTTCCCC GAGCCTTTCC CTCCGCGGTT

5. 510 520 530 540 550
 GCGTCATCGC AAGGTCCTAC GAGACAACAT CCAGGGCATC ACCAAGCCTG
 CGCAGTAGCG TTCCAGGATG CTCTGTTGTA GGTCCCCTAG TGGTTCGGAC

560 570 580 590 600
 CAATCCGCCG ACTCNNNNNN NNNNNNNNNN NNNNNNGAAT CTCTGGTCTT
 GTTAGGCGGC TGAGNNNNNN NNNNNNNNNN NNNNNNCTTA GAGACCAGAA

610 620 630 640 650
 ATCTACGAGG AGACACGAGG GGTGCTGAAG GNNNNNNNNN NNNNNNNNNN
 10. TAGATGCTCC TCTGTGCTCC CCACGACTTC CNNNNNNNNN NNNNNNNNNN

660 670 680 690 700
 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN
 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN

710 720 730 740 750
 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNGGCCGAAC ACTGTACGGC
 NNNNNNNNNN NNNNNNNNNN NNNNNNNNNN NNCCGGCTTG TGACATGCCG

15. 760 770 780
 TTCGGCGGCT AAGTGAAGCA GACTTGGCTA GAATAACG
 AAGCCGCCGA TTCACTTCGT CTGAACCGAT CTTATTGC



The analogous mouse H4 gene sequence is shown below
(968 base pairs):

```

      10      20      30      40      50
5. GAATTCTCCG AGGGACTTCG GCACCATAAT TAAGAAAATC GAAAATAAAA
   CTTAAGAGGC TCCCTGAAGC CGTGGTATTA ATTCTTTTAG CTTTTATTTT

      60      70      80      90     100
   AAATAAAGGC TTGAGACTGT AAGGAACCGG TAGAGGGCAG AGAAGAGAAA
   TTTATTTCCG AACTCTGACA TTCCTTGGCC ATCTCCCGTC TCTTCTCTTT

      110     120     130     140     150
   AGAAAAACAG GAAGATGATG CAACATCCAG AGCCCGGATA ATTTAGAAAG
   TCTTTTTGTC CTTCTACTAC GTTGTAGGTC TCGGGCCTAT TAAATCTTTC

      160     170     180     190     200
10. GTTCCCGCCC GCGCGCTTTC AGTTTTCAAT CTGGTCCGAT CCTCTCATAT
   CAAGGGCGGG GCGCGGAAAG TCAAAAGTTA GACCAGGCTA GGAGAGTATA

      210     220     230     240     250
   ATTAGTGGCA CTCCACCTCC AATGCCTCAC CAGCTGGTGT TTCAGATTAC
   TAATCACCGT GAGGTGGAGG TTACGGAGTG GTCGACCACA AAGTCTAATG

      260     270     280     290     300
   ATTAGCTATG TCTGGCAGAG GAAAGGGTGG AAAGGGTCTA GGCAAGGGTG
15. TAATCGATAC AGACCGTCTC CTTTCCCACC TTTCCCAGAT CCGTTCCCAC

      310     320     330     340     350
   GCGCCAAGCG CCATCGCAAA GTCTTGCGTG ACAACATCCA GGGTATCACC
   GCGGGTTCGC GGTAGCGTTT CAGAACGCAC TGTGTAGGTT CCCATAGTGG

      360     370     380     390     400
   AAGCCCGCCA TCCGCCGCCT GGCTCGGCGC GGTGGGGTCA AGCGCATCTC
   TTCGGGCGGT AGGCGGCGGA CCGAGCCGCG CCACCCAGT TCGCGTAGAG

      410     420     430     440     450
20. CGGCCTCATC TACGAGGAGA CCCGTGGTGT GCTGAAGGTG TTCCTGGAGA
   GCCGGAGTAG ATGCTCCTCT GGGCACCACA CGACTTCCAC AAGGACCTCT

      460     470     480     490     500
   ACGTCATCCG CGACGCAGTC ACCTACACCG AGCACGCCAA GCGCAAGACC
   TGCAGTAGGC GCTGCGTCAG TGGATGTGGC TCGTGCGGTT CCGGTTCTGG

      510     520     530     540     550
   GTCACCGCTA TGGATGTGGT GTACGCTCTC AAGCGCCAGG GCCGCACCCT
25. CAGTGGCGAT ACCTACACCA CATGCGAGAG TTCGCGGTCC CGGCGTGGGA

      560     570     580     590     600
   CTACGGCTTC GGAGGCTAGA CGCCGCGGCT TCAATTCCCC CCCCCCCCCC
   GATGCCGAAG CCTCCGATCT GCGGCGGCGA AGTTAAGGGG GGGGGGGGGG

```



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- 610 620 630 640 650
 ATCCCTAACG GCCCTTTTGA GGGCCAACCA CAGTCTCTTC AGGAGAGCTG
 TAGGGATTGC CGGGAAAAAT CCCGGTTGGT GTCAGAGAAG TCCTCTCGAC
- 660 670 680 690 700
 ACACTGACTT GGGTCGTACA GGTAAATAACC GCGGGTTTAG GACTCACGCT
 TGTGACTGAA CCCAGCATGT CCATTATTGG CGCCCAAATC CTGAGTGCGA
5. 710 720 730 740 750
 ACTAGGTGTT CCGCTTTTAG AGCCATCCAC TTAAGTTTCT ATACCACGGC
 TGATCCACAA GGCAGAAATC TCGGTAGGTG AATTCAAAGA TATGGTGCCG
- 760 770 780 790 800
 GGATAGATAG CATCCAGCAG GGTCTGCTCA CACTGGGAAT TTTAATTCTT
 CCTATCTATC GTAGGTCGTC CCAGACGAGT GTGACCCTTA AAATTAAGGA
10. 810 820 830 840 850
 ACTTAGGGTG TGAGCTGGTT GTCAGGTCAA GAGACTGGCT-AAGATTTTCT
 TGAATCCAC ACTCGACCAA CAGTCCAGTT CTCTGACCGA TTCTAAAGA
- 860 870 880 890 900
 TTAGCTCGTT TGGAGCAGAA TTGCAATAAG GAGACCCTTT GGATGGGATG
 AATCGAGCAA ACCTCGTCTT AACGTTATTC CTCTGGGAAA CCTACCCTAC
- 910 920 930 940 950
 ACCTATGTCC ACACATCAAA TGGCTATGTG GCTGTGTCCC TGTGTTTCCA
 TGGATACAGG TGTGTAGTTT ACCGATACAC CGACACAGGG ACACAAAGGT
15. 960
 ATGAGTGGCT GTGCTTGA
 TACTCACCGA CACGAACT



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The region of homology for both aforementioned sequences is shown below, where asterisks denote not homologous portions. Within the region shown, the first 118 base pairs have 80.5% homology and are used as a conserved DNA sequence probe in this example (sea urchin (top) base positions 449 to 567, mouse (bottom) base positions 257 to 375):

```

      *  *  *  *  *  *  *  *  *  *  *  *  *
449TAATGTCAGGCCGTGGTAAAGG-AGGCAA-GGGGCTCGG-AAAGGGAGGCGCCAAGCGTC
257TA-TGTCTGGCAGAGG-AAAGGGTGG-AAAGGGTCTAGGCAA-GGGTGGCGCCAAGCGCC

```

```

      *  *  *  *  *  *  *  *  *  *  *  *  *
ATCGCAAGGTCCTACGAGACAACATCCAGGGCATCACCAAGCCTGCAATCCGCCGACT-C
ATCGCAAAGTCTTGCGTGACAACATCCAGGGTATCACCAAGCCCGCCATCCGCCGCTGG

```

```

      *  *  *  *  *  *  *  *  *  *  *  *  *
NNNNNNNNNNNNNNNNNNNNNNNGAATCTCTGGTCTT-ATCTACGAGGAGACACGAGGG-G
CTCGGCGCGGTGGGGTCAAGCG-CATCTCCGG-CCTCATCTACGAGGAGACCCG-TGGTG

```

```

TGCTGAAGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
TGCTGAAGGTGTTCTTGAGAACGTATCCGCGACGCAGTCACCTACACCGAGCACGCCA

```

```

      *  *  *
NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNGGCCG-AAC-
AGCGCAAGACCGTCACCGCTATGGATGTGGTGTACGCTCTCAAGCGCCAGGGCCGACCC

```

```

*  *  *  *  *  *  *  *  *  *  *  *  *
ACTGTACGGCTTCGGCGGCTAAGTGAAGCAGAC--TTGGCTA      780
TCT--ACGGCTTCGGAGGCTA--GACGCCGCCGCTT--CAA      584

```

% = 84.503

P(342, 289) = .000E+00

E = .000



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Restriction endonuclease cleavage sites were determined from the two sequences. A list of cleavage sites for the sea urchin and mouse sequences is shown below. Numbers indicate the 5' side of the cleavage site, unless site name is in brackets, which indicates that only the recognition site is known.

SEA URCHIN

	Sequence	Appears at position		
10.				
	AcyI (GPCGQC)	495		
	AluI (AGCT)	147	267	
	AsuI (GGNCC)	277	514	
	AvaII (GGLCC)	514		
15.	CauII (CCMGG)	276	377	
	DdeI (CTNAG)	396	427	
	DpnI (GATC)	326		
	EcoRI* (PPATRQ)	184		
	EcoRII (CCLGG)	531		
20.	Fnu4HI (GCNGC)	254		
	FnuDII (CGCG)	125	253	285
	FokI (GGATG)	148		
	FokI (CATCC)	324	515	
	HaeII (PGCGCQ)	498		
25.	HaeIII (GGCC)	256	279	459
	HgaI (GCGTC)	491		
	HsiCI (GGGACC)	494		

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HsiJII (GPGCQC)	483						
HhaI (GCGC)	125	253	295	497			
HindIII (AAGCTT)	145						
HinfI (GATC)	200	431	561				
5. HpaII (CCGG)	275	376					
HphI (GGTGA)	368						
HphI (TCACC)	195	365	532				
MboI (GATC)	324						
10. MnlI (CCTC)	267	342					
MnlI (GAGG)	4	42	54	280	299	311	
	372	463	484				
NarI (GGCGCC)	495						
NspBII (GCMGC)	256						
15. PvuI (CGATCG)	327						
SerFI (CCNGG)	276	377	533				
SfaNI (GCATC)	409	527					
TaqI (TCGA)	117	327					

-87- MOUSE

Sequence	Appears at position							
AcyI (GFCGQC)	302	571						
AflIII (CTTAAG)	731							
AluI (AGCT)	234	256	648	815	855			
AsuI (GGNCC)	184	540	611	622				
AvaII (GGLCC)	184							
BssHII (GCGCGC)	162							
CanII (CCMGG)	135							
DdeI (CTNAG)	803	840						
DpnI (GATC)	190							
[EcoBI] (AGCANNNNNNNTCA)	766							
[EcoPI] (AGACC)	418	496	882					
[EcoPI] (GGTCT)	285	771						
[EcoPI5] (CAGCAG)	765							
EcoRI (GAATTC)	2							
EcoRI* (PFATQQ)	4	790	845					
EcoRII (CCLGG)	338	368	443	536				
Fnu4HI (GCNGC)	366	543	574	577				
FnuDII (CGCG)	162	164	380	461	682			
FokI (GGATG)	526	905	910					
FokI (CATCC)	111	322	346	442	587	711	748	
HaeII (FGCGCQ)	305	312	537					
HaeIII (GGCC)	404	542	612	624				
HsaI (GACGC)	472	579						



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	HsiAI (GLGCLC)	485					
	HsiCI (GGQFCC)	21	301				
	HsiJII (GPGCQC)	135					
	HhaI (GCGC)	164	166	304	311	380	395
5.		494	536				
	HinfI (GANTC)	692					
	HpaII (CCGG)	78	135	401			
	HphI (TCACC)	220	339	462	495		
10.	MboI (GATC)	188					
	MboII (GAAGA)	105	124				
	MboII (TCTTC)	629					
	MnII (CCTC)	202	227	236	415	559	
	MnII (GAGG)	3	76	261	407	555	
15.	NarI (GGCGCC)	302					
	NspBII (GCMGC)	368	545	576	579		
	PvuII (CAGCTG)	234					
	RsaI (GTAC)	523	668				
	SacII (CCGCGG)	683					
20.	SerFI (CCNGG)	135	340	370	445	538	
	SfaNI (GATGC)	127					
	SfaNI (GCATC)	385	751				
	TaqI (TCGA)	40					
	Tth111I (GACHNNGTC)	466					
25.	Tth111II (TGQTTG)	953					
	XmnI (GAANNHNTTC)	439					



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- The sea urchin and mouse sequences are compared with Hha I (GCGC) and the described probe sequence. The sea urchin sequence has cleavage sites at positions 295 and 497, thus creating a 202 bp fragment, which, if
5. denatured, would hybridize with the probe sequence. Hha I (GCGC) sites in the mouse sequence, 166, 304, 311 and 380, indicate that fragments of 69 and 138 could be detected with the probe sequence.

- Thus the genetic characterization for sea urchin is
10. 202
- while that for mouse is
- 69
- 138

15. Example 2

Use of trp D Gene of the Tryptophan Operon as a Probe

- The same type of computer simulation as in Example 1 was carried out using trp D gene as a probe. This allows
20. the conclusion that E. coli and Salmonella typhimurium can be distinguished by restriction fragments containing a conserved sequence.

The E. coli trp D gene with 684 bp's is shown below:



-90-

	10	20	30	40	50
	GAAGCCGACG	AAACCCGTAA	CAAAGCTCGC	GCCGTACTGC	GCGCTATTGC
	CTTCGGCTGC	TTTGGGCATT	GTTTCGAGCG	CGGCATGACG	CGCGATAACG
	60	70	80	90	100
	CACCGCGCAT	CATGCACAGG	AGACTTTCTG	ATGGCTGACA	TTCTGCTGCT
	GTGGCGCGTA	GTACGTGTCC	TCTGAAAGAC	TACCGACTGT	AAGACGACGA
	110	120	130	140	150
5.	CGATAATATC	GACTCTTTTA	CGTACAACCT	GGCAGATCAG	TTGCGCAGCA
	GCTATTATAG	CTGAGAAAAT	GCATGTTGGA	CCGTCTAGTC	AACGCGTCGT
	160	170	180	190	200
	ATGGGCATAA	CGTGGTGATT	TACCGCAACC	ATATACCGGC	GCAAACCTTA
	TACCCGTATT	GCACCACTAA	ATGGCGTTGG	TATATGGCCG	CGTTTGGAAAT
	210	220	230	240	250
	ATTGAACGCT	TGGCGACCAT	GAGTAATCCG	GTGCTGATGC	TTTCTCCTGG
10.	TAACCTGCGA	ACCGCTGGTA	CTCATTAGGC	CACGACTACG	AAAGAGGACC
	260	270	280	290	300
	CCCCGGTGTG	CCGAGCGAAG	CCGGTTGTAT	GCCGGAACTC	CTCACCCGCT
	GGGGCCACAC	GGCTCGCTTC	GGCCAACATA	CGGCCTTGAG	GAGTGGGCGA
	310	320	330	340	350
	TGCGTGCGAA	GCTGCCCAT	ATTGGCATT	GCCTCGGACA	TCAGGCGATT
	ACGCACCGTT	CGACGGGTAA	TAACCGTAA	CGGAGCCTGT	AGTCCGCTAA
	360	370	380	390	400
15.	GTCGAAGCTT	ACGGGGGCTA	TGTCGGTCAG	GCGGGCGAAA	TTCTCCACGG
	CAGCTTCGAA	TGCCCCCGAT	ACAGCCAGTC	CGCCCCGCTT	AAGAGGTGCC
	410	420	430	440	450
	TAAAGCCTCC	AGCATTGAAC	ATGACGGTCA	GGCGATGTTT	GCCGGATTAA
	ATTTCGGAGG	TCGTAACCTG	TACTGCCAGT	CCGCTACAAA	CGGCCTAATT
	460	470	480	490	500
	CAAACCCGCT	GCCGGTGCGG	CGTTATCACT	CGCTGGTTGG	CAGTAACATT
	GTTTGGGCGA	CGGCCACCGC	GCAATAGTGA	GCGACCAACC	GTCATTGTAA
20.	510	520	530	540	550
	CCGGCCGGTT	TAACCATCAA	CGCCCATTTT	AATGGCATGG	TGATGGCAGT
	GGCCGGCCAA	ATTGGTAGTT	GCGGGTAAAA	TTACCGTACC	ACTACCGTCA
	560	570	580	590	600
	ACGTCACGAT	GCGGATCGCG	TTTGTGGATT	CCAGTTCCAT	CCGGAATCCA
	TGCAGTGCTA	CGCCTAGCGC	AAACACCTAA	GGTCAAGGTA	GGCCTTAGGT
	610	620	630	640	650
25.	TTCTCACCAC	CCAGGGCGCT	CGCCTGCTGG	AACAAACGCT	GGCCTGGGCG
	AAGAGTGGTG	GGTCCCGCGA	GCGGACGACC	TTGTTTGCGA	CCGGACCCGC
	660	670	680		
	CAGCATAAAC	TAGAGCCAGC	CAACACGCTG	CAA	
	GTCGTATTTG	ATCTCGGTGG	GTTGTGCGAC	GTT	



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The trp D gene, 683 base pairs, for S. typhimurium
is shown below:

```

      10      20      30      40      50
5. GAAGCCGATG AAACCCGTAA TAAAGCGCGC GCCGTATTGC GTGCTATCGC
   CTTCGGCTAC TTTGGGCATT ATTTGCGCGC CGGCATAACG CACGATAGCG

      60      70      80      90     100
   CACCGCGCAT CATGCACAGG AGACCTTCTG ATGGCTGATA TTCTGCTGCT
   GTGGCGCGTA GTACGTGTCC TCTGGAAGAC TACCGACTAT AAGACGACGA

      110     120     130     140     150
   CGATAACATC GACTCGTTCA CTTGGAACCT GGCAGATCAG CTACGAACCA
   GCTATTGTAG CTGAGCAAGT GAACCTTGGA CCGTCTAGTC GATGCTTGGT
10.

      160     170     180     190     200
   ACGGTCATAA CGTGGTGATT TACCGTAACC ATATTCCGGC GCAGACGCTT
   TGCCAGTATT GCACCACTAA ATGGCATTGG TATAAGGCCG CGTCTGCGAA

      210     220     230     240     250
   ATCGATCGCC TGGCGACAAT GAAAAATCCT GTGCTAATGC TCTCCCCCGG
   TAGCTAGCGG ACCGCTGTTA CTTTTTAGGA CACGATTACG AGAGGGGGCC

      260     270     280     290     300
15. CCCGGGTGTT CCCAGCGAGG CAGGTTGTAT GCCGGAGCTG CTGACCCGAC
   GGGCCCACTA GGGTCGCTCC GTCCAACATA CGGCCTCGAC GACTGGGCTG

      310     320     330     340     350
   TACGCGGCAA GTTACCGATC ATCGGCATTT GTCTGGGGCA TCAGGCGATT
   ATGCGCCGTT CAATGGCTAG TAGCCGTAAA CAGACCCCGT AGTCCGCTAA

      360     370     380     390     400
   GTCGAAGCTT ACGGCGGTTA CGTCGGTCAG GCGGGAGAAA TCCTGCATGG
   CAGCTTCGAA TGCCGCCAAT GCAGCCAGTC CGCCCTCTTT AGGACGTACC
20.

      410     420     430     440     450
   CAAAGCCTCC AGCATTGAGC ATGACGGTCA GGCATGTTT GCCGGGCTCG
   GTTTCGGAGG TCGTAACTCG TACTGCCAGT CCGCTACAAG CGGCCCCGAGC

```



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460 470 480 490 500
 CGAATCCGCT ACCGGTCGCG CGTTATCATT CGCTGGTCGG CAGTAATGTT
 GCTTAGGCGA TGGCCAGCGC GCAATAGTAA GCGACCAGCC GTCATTACAA

510 520 530 540 550
 CCTGCCGGGGC-TGACCATTAAG-GGCCCCATTTC-AACGGCATGG-TGATGGCGGT
 GGACGGCCCCG ACTGGTAATT GCGGGTAAAG TTGCCGTACC ACTACCGCCA

5.

560 570 580 590 600
 ACGTCATGAT GCGGATCGCG TTTGCGGTTT TCAATTTTCAT CCCGAGTCCA
 TGCAGTACTA CGCCTAGCGC AAACGCCAAA AGTTAAAGTA GGGCTCAGGT

610 620 630 640 650
 TCCTGACGAC ACAGGGCGCG CGTCTACTGG AGCAAACATT AGCCTGGGCG
 AGGACTGCTG TGTCCCGCGC GCAGATGACC TCGTTTGTAA TCGGACCCGC

10. 660 670 680
 CTGGCGAAGC TGGAAACGAC CAACACCCTA CAG
 GACCGCTTCG ACCTTGCGTG GTTGTGGGAT GTC



-93-

Two homology regions between both sequences were next established, where the upper sequence is for E. coli and the lower one for S. typhimurium:

Region I

```

      **      *      *      *      *      *      **      * * * **
452  AAACCCGCTGCCGGTGGCGCGTTATCACTCGCTGGTTGGCAGTAACATTCC-GGCCGG-T
453  AA-TCCGCTACCGGTCGCGCGTTATCATTGCTGGTCGGCAGTAATGTTCTGCC-GGGC

      * *      *      * *      *      *
      TTAACCATCAACGCCCATTTTAATGGCATGGTGATGGCAGTACGTCACGATGCGGATCGC
      TGA-CCATTAAACGCCCATTTCACGGCATGGTGATGGCGGTACGTCATGATGCGGATCGC

      * **      * *      * *      * *      * *      * *      * *
/ GTTTGTGG-ATTCAGTTCATCCGGAATCCATTGTCACCAACCCAGGGCGCTCGGCTGCT
  GTTTGCGGTTTTTC-AATTTTCATCCCGAGTCCATCCTGACGACACAGGGCGCGCGTCTACT

      *      * * *      * *      * *      * *      * *      * *
  GGAACAAACGCTGGCCTGGGCGC-AGCATAAACTAGAGCC-AGCCAACACGCTGCA      682
  GGAGCAAACATTAGCCTGGGCGCTGGC-GAAGCTGGAACCGACC-AACACCCTACA      682

```

 $\% = 78.390$
 $P(236, 185) = .000E+00$
 $E = .000$


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Region II

* ** * ** * * *

1 GAAGCCGACGAAACCCGTAA-CAA-AGCTCGCGCCGTACTGCGCGCTATTGCCACCGCGC
 1 GAAGCCGATGAAACCCGTAATAAAGCGCGCGC--CGTATTGCGTGCTATCGCCACCGCGC

* * * *

ATCATGCACAGGAGAC-TTTCTGATGGCTGACATTCTGCTGCTCGATAATATCGACTC-T
 ATCATGCACAGGAGACCTT-CTGATGGCTGATATTCTGCTGCTCGATAACATCGACTCGT

** ** * * * * * * * *

TTTAC--GTACAACCTGGCAGATCAGTTGCGCAGC-AATGGG-CATAACGTGGTGATTTA
 T-CACTTGGA-ACC-TGGCAGATCAGCTACG-AACCAA-CGGTCATAACGTGGTGATTTA

* * * * * * * *

CCGCAACCATATACCGGCGCAAAC-CTTAATTGAACGC-TTGGCGACCATGAGTAA-TCC-
 CCGTAACCATATTCCGGCGCAGACGCTTA-TCGATCGCCTGG-CGACAATGA-AAAATCC

* * * * * * * *

GGTGCTGATGCT-TTCTCCTGGCCCCGG-TGT-GCC-GAGCGAAGCCGGTTGTATGCCGG
 TGTGCTAATGCTCTCC-CCCGGCC-GGGTGTTCCAGCG--AGGCAGGTTGTATGCCGG

* * * * * * * *

AACTCCTCACCCG-CT-TGCGTGGAAGCTGCCCATTATTGGCATTGCTTGCCT-CGGACATC
 AGCTGCTGACCCGACTACGCG--GCAAGTTACCGATCATCGGCATTGCTGCTGGGG-CATC

* * * * *

AGGCGATTGTGCGAAGCTTACGG-GGGCTATGTCGGTCAGGCGGGCGAAATTCTCCACGG-
 AGGCGATTGTGCGAAGCTTACGGCGG-TTACGTCGGTCAGGCGGGAGAAATCCTGCATGGC

* * * *

TAAAGCCTCCAGCATTGACATGACGGTCAGGCGATGTTTGCCGG 445
 AAA-GCCTCCAGCATTGACATGACGGTCAGGCGATGTTTGCCGG 445

Z = 80.215

P(465, 373) = .000E+00 E = .000



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Restriction sites in both sequences are shown as follows:

E. coli

HpaII (CCGG)	187	229	254	272	283	443	463	502
	506	592						
HphI (GGTGA)	177	552						
HphI (TCACC)	285	597						
MboI (GATC)	135	564						

S. typhimurium

HpaII (CCGG)	187	248	253	283	443	463	506
HphI (GGTGA)	177	552					
MboI (GATC)	135	204	317	564			
MnII (CCTC)	417						



-96-

- The E. coli sequence has Mbo I (GATC) sites at 135 and 564. There is a 429 bp fragment that can be detected by both Region 1 and 2 probes. The same enzyme has sites at 135, 204, 317, and 564 in the S. typhimurium sequence. A probe of the two homology regions would detect fragments of 69, 113 and 247 bp.
- 5.

Thus the identifying genetic characterization for E. coli with this probe and enzyme is

429

10. whereas that for S. typhimurium is

69

113

247



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Example 3Use of α -Fetoprotein Gene as a Probe

- This example shows the use of a region of homology
5. in the α -fetoprotein gene sequence of human and rat, and the endonuclease MnlI (GAGG).

The human α -fetoprotein message cDNA (1578 bp's) is as follows:

```

10.      10      20      30      40      50
      AGCTTGGCAT AGCTACCATC ACCTTTACCC AGTTTGTTC GGAAGCCACC
      TCGAACCGTA TCGATGGTAG TGGAAATGGG TCAAACAAGG CCTTCGGTGG

      60      70      80      90      100
      GAGGAGGAAG TGAACAAAAT GACTAGCGAT GTGTTGGCTG CAATGAAGAA
      CTCCTCCTTC ACTTGTTTTA CTGATCGCTA CACAACCGAC GTTACTTCTT

      110     120     130     140     150
      AAAGTCTGGC GATGGGTGTT TAGAAAGCCA GCTATCTGTG TTTCTGGATG
      TTTGAGACCG CTACCCACAA ATCTTTCGGT CGATAGACAC AAAGACCTAC

15.      160     170     180     190     200
      AAATTTGCCA TGAGACGGAA CTCTCTAACA AGTATGGACT CTCAGGCTGC
      TTTAAACGGT ACTCTGCCTT GAGAGATTGT TCATACCTGA GAGTCCGACG

      210     220     230     240     250
      TGCAGCCAAA GTGGAGTGGA AAGACATCAG TGTCTGCTGG CACGCAAGAA
      ACGTCGGTTT CACCTCACCT TTCTGTAGTC ACAGACGACC GTGCGTTCTT

      260     270     280     290     300
20.      GACTGCTCCG GCCTCTGTCC CACCCTTCCA GTTTCCAGAA CCTGCCGAGA
      CTGACGAGGC CGGAGACAGG GTGGGAAGGT CAAAGGTCTT GGACGGCTCT

      310     320     330     340     350
      GTTGCAAAGC ACATGAAGAA AACAGGGCAG TGTTTCATGAA CAGGTTTCATC
      CAACGTTTCG TGTACTTCTT TTGTCCCGTC ACAAGTACTT GTCCAAGTAG

      360     370     380     390     400
      TATGAAGTGT CAAGGAGGAA CCCCTTCATG TATGCCCCAG CCATTCTGTC
      ATACTTCACA GTTCCTCCTT GGGGAAGTAC ATACGGGGTC GGTAAGACAG

25.      410     420     430     440     450
      CTTGGCTGCT CAGTACGACA AGGTCGTTCT GGCATGCTGC AAAGCTGACA
      GAACCGACGA GTCATGCTGT TCCAGCAAGA CCGTACGACG TTTCGACTGT

```



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460 470 480 490 500
 ACAAGGAGGA GTGCTTCCAG ACAAAGAGAG CATCCATTGC AAAGGAATTA
 TGTTCCTCCT CACGAAGGTC TGTTCCTCTC GTAGGTAACG TTTCCTTAAT
 510 520 530 540 550
 AGAGAAGGAA GCATGTAAAT TGAGCATGTA TGTTCAGTGA TAAGAAAATT
 TCTCTTCCTT CGTACAATTT ACTCGTACAT ACAAGTCACT ATTCTTTTAA
 5. 560 570 580 590 600
 TGGATCCCAG AACCTCCAGG CAACAACCAT TATTAAGCTA AGTCAAAAGT
 ACCTAGGGGCT TTGGAGGTCC GTTGTGGTA ATAATTGAT TCAGTTTTCA
 610 620 630 640 650
 TAACTGAAGC AAATTTTACT GAGATTCAGA AGCTGGCCCT GGATGTGGCT
 ATTGACTTCG TTAAAATGA CTCTAAGTCT TCGACCGGGA CCTACACCGA
 660 670 680 690 700
 10. CACATCCACG AGGAGTGTG CCAAGGAAAC TCGCTGGAGT GTCTGCAGGA
 GTGTAGGTGC TCCTCACAAC GGTTCCTTTG AGCGACCTCA CAGACGTCTT
 710 720 730 740 750
 TGGGGAAAAA GTCATGACAT ATATATGTTT TCAACAAAAT ATTCTGTCAA
 ACCCCTTTTT CAGTACTGTA TATATACAAG AGTTGTTTTA TAAGACAGTT
 760 770 780 790 800
 GCAAAATAGC AGAGTGCTGC AAATTACCCA TGATCCAACCT AGGCTTCTGC
 CGTTTTATCG TCTCAGGACG TTTAATGGGT ACTAGGTTGA TCCGAAGACG
 15. 810 820 830 840 850
 ATAATTCACG CAGAGAATGG CGTCAAACCT GAAGGCTTAT CTCTAAATCC
 TATTAAGTGC GTCTCTTACC GCAGTTTGGA CTTCCGAATA GAGATTTAGG
 860 870 880 890 900
 AAGCCAGTTT TTGGGAGACA GAAATTTTGC CCAATTTTCT TCAGAGGAAA
 TTCGGTCAAA AACCTCTGT CTTTAAACG GGTAAAGA AGTCTCCTTT
 910 920 930 940 950
 20. AAATCATGTT CATGGCAAGC TTTCTTCATG AATACTCAAG AACTCACCCC
 TTTAGTACAA GTACCGTTCC AAAGAAGTAC TTATGAGTTC TTGAGTGGGG
 960 970 980 990 1000
 AACCTTCCTG TCTCAGTCAT TCTAAGAATT GCTAAAACGT ACCAGGAAAT
 TTGGAAGGAC AGAGTCAGTA AGATTCTTAA CGATTTTGCA TGGTCCTTTA
 1010 1020 1030 1040 1050
 ATTGGAGAAG TGTTCCCAAG CTGGAAATCT ACCTGGATGT CAGGACAATC
 TAACCTCTTC ACAAGGGTCA GACCTTTAGA TGGACCTACA GTCTGTAG



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1060 1070 1080 1090 1100
 TGGAGAAGA ATTGCAGAAA CACATCGAGG AGAGCCAGGC ACTGTCCAAG
 ACCTTCTTCT TAACGTCTTT GTGTAGCTCC TCTCGGTCCG TGACAGGTTT

1110 1120 1130 1140 1150
 CAAAGCTGCG CTCTCTACCA GACCTTAGGA GACTACAAAT TACAAAATCT
 GTTTCGACGC GAGAGATGGT CTGGAATCCT CTGATGTTTA ATGTTTTAGA

5. 1160 1170 1180 1190 1200
 GTTCCTTATT GGTACACGA GGAAAGCCCC TCAGCTGACC TCAGCAGAGC
 CAAGGAATAA CCAATGTGCT CCTTTCGGGG AGTCGACTGG AGTCGTCTCG

1210 1220 1230 1240 1250
 TGATCGACCT CACCGGGAAG ATGGTGAGCA TTGCCTCCAC GTGCTGCCAG
 ACTAGCTGGA GTGGCCCTTC TACCACTCGT AACGGAGGTG CACGACGGTC

1260 1270 1280 1290 1300
 CTCAGCGAGG AGAAATGGTC CGGCTGTGGT GAGGGAATGG CCGACATTTT
 10. GAGTCGCTCC TCTTTACCAG GCCGACACCA CTCCCTTACC GGCTGTAAAA

1310 1320 1330 1340 1350
 CATTGGACAT TTGTGTATAA GGAATGAAGC AAGCCCTGTG AACTCTGGTA
 GTAACCTGTA AACACATATT CCTTACTTCG TTCGGGACAC TTGAGACCAT

1360 1370 1380 1390 1400
 TCAGCCACTG CTGCAACTCT TCGTATTCCA ACAGGAGGCT ATGCATCACC
 AGTCGGTGAC GACGTTGAGA AGCATAAGGT TGTCTCCGA TACGTAGTGG

15. 1410 1420 1430 1440 1450
 AGTTTTCTGA GGGATGAAAC CTATGCCCTT CCCCCATTCT CTGAGGATAA
 TCAAAGACT CCCTACTTTG GATACGGGGA GGGGGTAAGA GACTCCTATT

1460 1470 1480 1490 1500
 ATTCATCTTC CACAAGGATC GTGCCAAGCT CGGCAAAGCC CTACAGACCA
 TAAGTAGAAG GTGTTCTAG CACGGTTCTGA GCCGTTTCGG GATGTCTGGT

1510 1520 1530 1540 1550
 20. TGAAACAAGA GCTTCTCATT AACCTGGTGA AGCAAAAGCC TGAAGTACA
 ACTTTGTTCT CGAAGAGTAA TTGGACCACT TCGTTTTCGG ACTTGACTGT

1560 1570
 GAGGAGCAGC TGGCGGCTGT CACTGCAG
 CTCCTCGTCG ACCGCCGACA GTGACGTC

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The rat α -fetoprotein 3' end cDNA is as follows (540 bp's):

```

      10      20      30      40      50
5.  GAGGGACTGG CCGACATTTA CATTGGACAC TTGTGTTTAA GACATGAGGC
    CTCCTTGACC GGCTGTAAAT GTAACCTGTG AACACAAATT CTGTACTCCG

      60      70      80      90      100
    AAACCCTGTG AACTCCGGTA TCAACCACTG CTGCAGTTCC TCGTATTCCA
    TTTGGGACAC TTGAGGCCAT AGTTGGTGAC GACGTCAAGG AGCATAAGGT

      110     120     130     140     150
    ACAGGAGGCT CTGCATCACC AGCTTTCTGA GGGACGAAAC CTACGTCCCT
    TGTCTCCGA  GACGTAGTGG TCGAAAGACT CCCTGCTTTG GATGCAGGGA

10.

      160     170     180     190     200
    CTACCATTCT CTGCGACAAA TTCATCTTCC ACAAGGAATC TGTGCCAAGC
    GATGGTAAGA GACGCTGTTT AAGTAGAAGG TGTTCCTTAG ACACGGTTCC

      210     220     230     240     250
    TCAGGGCCGA GCACCACAGA CCATGAAACA AGAGCTTCTC ATTAACCTAG
    AGTCCC GGCT CGTGGTGTCT GGTACTTTGT TCTCGAAGAG TAATTGGATC

      260     270     280     290     300
15. TGAACAACAAA GCCTGAAATG ACAGAGGAGC AGCACGCGGC TGTCACTGCT
    ACTTTGTTTT CGGACTTTAC TGTCTCCTCG TCGTGCGCCG ACAGTGACGA

      310     320     330     340     350
    GATTTCTCTG GCCTCTTGGA GAAGTGCTGC AAAGACCAGG ATCAGGAAGC
    CTAAGAGAC  CGGAGAACCT CTTACGACG TTTCTGGTCC TAGTCTTCCG

      360     370     380     390     400
    CTGTTTCGCA AAAGAGGTCC AAGTTGATTT CCAAACCTCGT GAGGCTTTGG
    GACAAAGCGT TTTCTCCAGG TTCAACTAAA GGTTCGAGCA CTCCGAAACC

20.

      410     420     430     440     450
    GGGTTTAAAC ATCTCCAAGA GGAAGAAAGG ACAAAAAAAT GTGTCGACGC
    CCCAAATTTG TAGAGGTTCT CCTTCTTTCC TGTTTTTTTA CACAGCTGCG

      460     470     480     490     500
    TTTGGTGTGA GCTTTTCGGT TTGATGGTAA CTGGTGGAGA CTTCCATGTG
    AAACCACACT CGAAAAGCCA AACTACCATT GACCACCTCT GAAGGTACAC

      510     520     530     540
25. GGATTTCTAT GCCTAAGGAA TAAAGACTTT TCAACTGTTA
    CCTAAAGATA CGGATTCTTT ATTTCTGAAA AGTTGACAAT
  
```

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The homologous regions between both is as follows,
(human: upper; rat: lower):

*** * * * *
1367 CTCTTCGTATTCCAACAGGAGGCTATGCATCACCAG-TTTTCTGAGGGATGAAACCTATG
90 CTC---GTATTCCAACAGGAGGCTCTGCATCACCAGCTTT-CTGAGGGACGAAACCTACG

* ** * ** * * * *
CCCCTC-CCCCATTCTCTGAGGA-TAAATTTCATCTTCCACAAGGA-TC-GTGCCAAGCTC
TCCCTCTACC-ATTCTCTG-CGACAAA-TTCATCTTCCACAAGGAATCTGTGCCAAGCTC

* ** * * * * * *
-GG--CAAAGC-CCTACAGACCATGAAACAAGAGCTTCTCATTAAACCTGGTGAAGCAAAA
AGGGCCSA-GCACC-ACAGACCATGAAACAAGAGCTTCTCATTAAACCTAGTGAAACAAAA

* **
GCCTGAACTGACAGAGGAGCAGCTGGCGGCTGTCACTGC 1576
GCCTGAAATGACAGAGGAGCAGCACGCGGCTGTCACTGC 299

% = 85.845

P(219, 188) = .000E+00 E = .000



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Restriction sites for both human and rat are as follows:

HUMAN

MboII (GAAGA)	108	261	328	1066	1069	1230		
MboII (TCTTC)	881	916	1361	1449				
MnII (CCTC)	273	574	1190	1200	1219	1245	1439	
MnII (GAGG)	44	47	358	449	653	887	1070	1162
	1250	1274	1378	1402	1436	1544		

RAT

MboII (GAAGA)	435							
MboII (TCTTC)	168							
MnII (CCTC)	100	159	323					
MnII (GAGG)	39	98	122	267	357	384	412	



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- It can be calculated that fragments containing a portion of the conserved sequence, 24, 34, 104 and 108 bp describe the human DNA. Fragments of 24, 59, 90 and 145 describe the rat DNA. While both sequences contain the
5. 24 bp fragment, it is the set of fragments (taxonomic characters) that is of significance.

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CLAIMS

1. A method of characterizing an unknown organism species which comprises:

- determining the position of part or whole of evolutionarily conserved sequences in genetic material of said
5. organism relative to a known position in said genetic material (other than by determining the chromatographic pattern of restriction endonuclease digested DNA from said known organism, which digested DNA has been hybridized or reassociated with ribosomal RNA information-containing
10. nucleic acid from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of said unknown organism, and

- comparing said characterization with information from at least two sets of identifying genetic character-
15. izations derived from the same conserved sequences; each of said sets defining a known organism species.

2. A method as claimed in Claim 1, wherein the known position is defined by one or more restriction endonuclease cleavage sites.

20. 3. A method as claimed in Claim 1, wherein the genetic material is DNA.

4. A method as claimed in Claim 1, which comprises comparing the chromatographic pattern of restriction endonuclease digested DNA from said unknown organism, which
25. digested DNA has been hybridized or reassociated with

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conserved DNA sequence information-containing nucleic acid from or derived from a probe organism or from a consensus sequence, with equivalent chromatographic patterns of at least two known different organism species.

5. 5. A method as claimed in Claim 4, wherein said conserved DNA information-containing nucleic acid is detectably labelled.

6. A method as claimed in Claim 5, wherein said conserved DNA information-containing nucleic acid is radiolabelled or metal labelled.

7. A method as claimed in Claim 4, 5 or 6, wherein said conserved DNA information-containing nucleic acid probe is an RNA probe.

8. A method as claimed in Claim 4, 5 or 6, wherein said conserved DNA information-containing nucleic acid probe is DNA complementary to RNA.

9. A method as claimed in Claim 4, 5 or 6, wherein said conserved DNA information-containing nucleic acid probe is DNA obtained by nick-translating or cloning DNA complementary to RNA.

10. A method as claimed in any one of Claims 1 to 4, wherein said unknown organism being characterized is a cell or cells of a strain in an in vitro culture.

11. A method as claimed in any one of claims 1 to 4, wherein said unknown organism being characterized and said probe organism are both from the same kingdom, subkingdom,



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division, subdivision, phylum, subphylum, class, subclass, order, family, tribe or genus.

12. A method as claimed in Claim 4, wherein said unknown organism being characterized and said probe organism are both eukaryotic.
- 5.

13. A method as claimed in Claim 4, wherein said unknown organism being characterized and said probe organism are both prokaryotic.

14. A method as claimed in Claim 4, wherein said unknown organism being characterized is eukaryotic and said probe organism is prokaryotic.
- 10.

15. A method as claimed in Claim 12 or 14, which further comprises detecting for the presence of a nucleic acid sequence or sequences creating a taxon below the rank of species or an infrasubspecific subdivision.

16. A method as claimed in Claim 4, wherein said unknown organism being characterized is prokaryotic and said probe organism is eukaryotic.

17. A method as claimed in Claim 13 or 16, wherein said prokaryotic organism being characterized is selectively being detected while in the presence of a eukaryotic organism.
- 20.

18. A method as claimed in Claim 17, wherein said prokaryotic organism is a bacterium.

19. A method as claimed in Claim 12, wherein the DNA from said eukaryotic organism being characterized is
- 25.



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nuclear DNA, and the conserved DNA information-containing nucleic acid from said eukaryotic probe organism is not derived from mitochondria or chloroplasts.

20. A method as claimed in Claim 12, wherein the DNA from said eukaryotic organism being characterized is mitochondrial DNA and the conserved DNA information-containing nucleic acid from said eukaryotic probe organism is derived from mitochondria or chloroplasts.

21. A method as claimed in Claim 12, wherein DNA from said eukaryotic organism being characterized is chloroplast DNA and the conserved DNA information-containing nucleic acid from said eukaryotic probe organism is derived from mitochondria or chloroplasts.

22. A method as claimed in Claim 14, wherein said DNA from said eukaryotic organism being characterized is derived from mitochondrial DNA.

23. A method as claimed in Claim 14, wherein said DNA from said eukaryotic organism being characterized is derived from chloroplast DNA.

20. 24. A method as claimed in Claim 16, wherein said conserved DNA information-containing nucleic acid probe is derived from mitochondria or from chloroplasts.

25. 25. A method as claimed in Claim 12 or 14, which further comprises identifying in said unknown eukaryotic organism being characterized a virus, or a virus-derived DNA creating a taxon below the rank of species.



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26. A method of identifying a bacterium of an unknown bacterial strain present in a sample which comprises:
- determining the position of part or whole of evolutionarily conserved sequences in DNA of said bacterium,
5. relative to a known position in said DNA (other than by determining the chromatographic pattern of restriction endonuclease digested DNA from said unknown organism, which digested DNA has been hybridized or reassociated with ribosomal RNA information-containing nucleic acid
10. from or derived from a known probe organism), thereby to obtain an identifying genetic characterization of said unknown bacterium, and

- comparing said characterization with information from at least two sets of identifying genetic character-
15. izations derived from the same conserved sequences, each of said sets defining a known bacterial species.

27. A method as claimed in Claim 26, wherein the known position is defined by one or more restriction endonuclease cleavage sites.

20. 28. A method as claimed in Claim 26, which comprises comparing the chromatographic pattern of restriction-endonuclease digested DNA from said unknown bacterium, which digested DNA has been hybridized or reassociated with conserved DNA sequence information-containing nucleic acid
25. from or derived from a probe bacterium or from a consensus sequence, with equivalent chromatographic patterns of known bacteria.



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29. A method as claimed in Claim 26, wherein said unknown bacterium is present in a fermentation medium or in a secretion or excretion product.

30. A method as claimed in Claim 26, wherein said
5. unknown bacterium is present in or associated with eukaryotic tissue.

31. A method as claimed in Claim 30, wherein said bacterium is present in or associated with animal or plant cells.

10. 32. A method as claimed in Claim 30, wherein said bacterium is present in or associated with human cells, or associated with plant root cells.

33. A method as claimed in any one of Claims 28 to 32, wherein said conserved DNA information-containing
15. nucleic acid from said probe bacterium is detectably labelled.

34. A method as claimed in Claim 33, wherein said label is a radiolabel or a metal label.

35. A method as claimed in Claim 33, wherein said
20. nucleic acid from said probe bacterium is RNA.

36. A method as claimed in Claim 33, wherein said nucleic acid from said probe bacterium is complementary DNA to RNA.

37. A method as claimed in any one of Claims 28 to
25. 32, wherein said unknown bacterium is pathogenic towards plants or animals.



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38. A method as claimed in any one of Claims 28 to 32, which further comprises detecting for the presence of a nucleic acid sequence or sequences creating a taxon below the rank of species, or an infrasubspecific sub-division.
- 5.

39. A method as claimed in Claim 38, wherein said nucleic acid sequence or sequences are all or part of a bacteriophage genome.

40. A method as claimed in Claim 38, wherein said nucleic acid sequence or sequences are all or part of an extrachromosomal genetic element, a plasmid, or an episome.
- 10.

41. A method as claimed in Claim 38, wherein said sequence or sequences code for an R-factor or for an anti-biotic resistance factor.

42. A method as claimed in Claim 28, wherein said chromatographic patterns of known bacteria are present in a catalog containing patterns for at least two different bacteria.
- 15.

43. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, wherein a first container means contains conserved genetic material sequence information-containing nucleic acid (other than ribosomal RNA information-containing nucleic acid) from or derived from a probe organism or from a consensus sequence; and wherein said kit also contains a catalog having hybridized or
- 20.
- 25.



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reassociated chromatographic band patterns for at least two known different organism species, or said kit contains at least two known organism species, or genetic material derived therefrom.

5. 44. A kit as claimed in Claim 43, wherein the genetic material is DNA.

45. A kit as claimed in Claim 43 or 44, which also comprises a second container means containing one or more restriction endonuclease enzymes.

10. 46. A kit as claimed in Claim 44, wherein the conserved DNA sequence information-containing nucleic acid probe is detectably labelled.

15. 47. A kit comprising a carrier being compartmentalized to receive in close confinement therein one or more container means, wherein a first container means contains conserved DNA sequence information-containing nucleic acid (other than ribosomal RNA information-containing nucleic acid) from or derived from a probe organism or from a consensus sequence, said nucleic acid being in detectably
20. labelled form; and

wherein said kit also comprises a second container means containing one or more restriction endonuclease enzymes.

25. 48. A kit as claimed in Claim 43, 45 or 47, wherein said conserved DNA sequence information-containing nucleic acid probe is RNA.

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49. A kit as claimed in Claim 43, 45 or 47, wherein said conserved DNA sequence information-containing nucleic acid probe is DNA complementary to RNA.

50. A kit as claimed in Claim 48, which also comprises a container means containing one or more detectably labelled deoxynucleoside triphosphates.

51. A kit as claimed in Claim 43, 45 or 47, wherein said probe organism is a prokaryote.

52. A kit as claimed in Claim 43, 45 or 47, wherein said probe organism is a eukaryote.

53. A kit as claimed in Claim 51, wherein said prokaryote is a bacterium.

54. A kit as claimed in Claim 52, wherein said nucleic acid probe of said eukaryote is derived from an organelle thereof.

55. A kit as claimed in Claim 49, wherein said cDNA is labelled with ^{32}P , ^{14}C or ^3H .

56. A kit as claimed in Claim 49, wherein said cDNA is a faithful copy of the RNA it is derived from.

57. A kit as claimed in Claim 43, 45 or 47, which also comprises one or more container means containing viral nucleic acid probes.

58. A kit as claimed in Claim 43, 45 or 47 wherein said catalog is a book, a computer tape, a computer disk, or a computer memory.

59. A kit as claimed in Claim 43, 45 or 47, wherein

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said catalogue also includes viral chromatographic patterns.

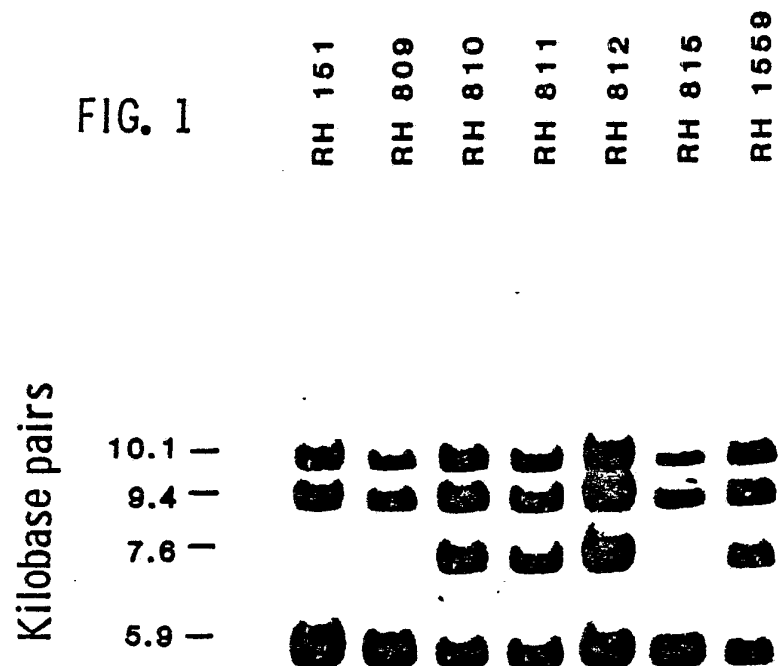
60. A kit as claimed in Claim 43, 45 or 47 wherein said catalogue also includes patterns for infrasubspecific
5. taxons.



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EcoR I digested DNA
from *P. aeruginosa* strains

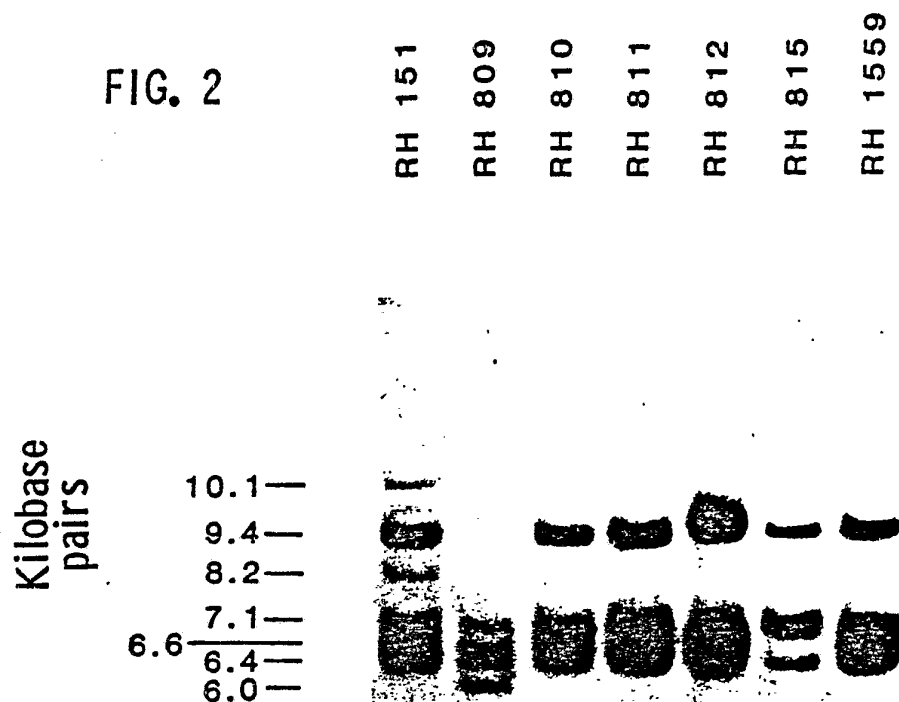
FIG. 1



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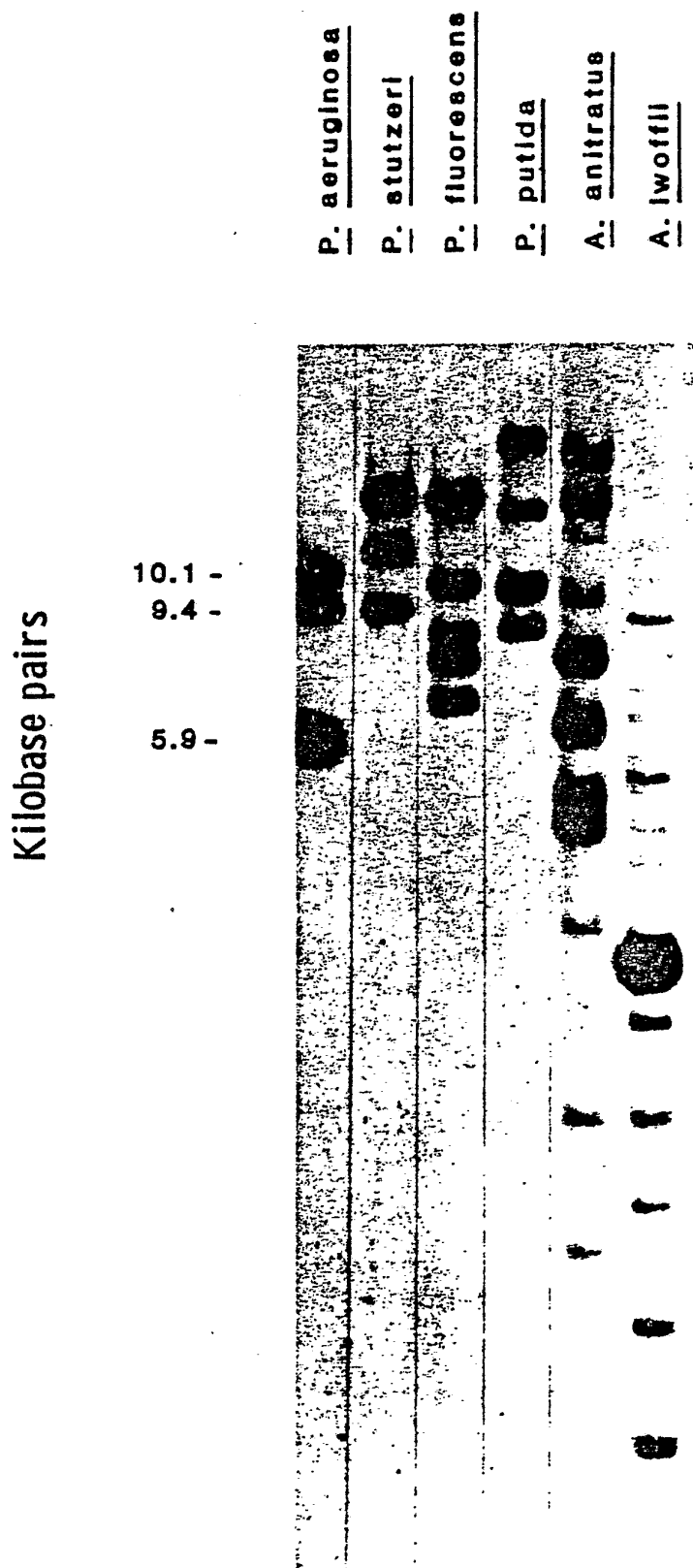
PST I digested DNA
from P. aeruginosa strains

FIG. 2

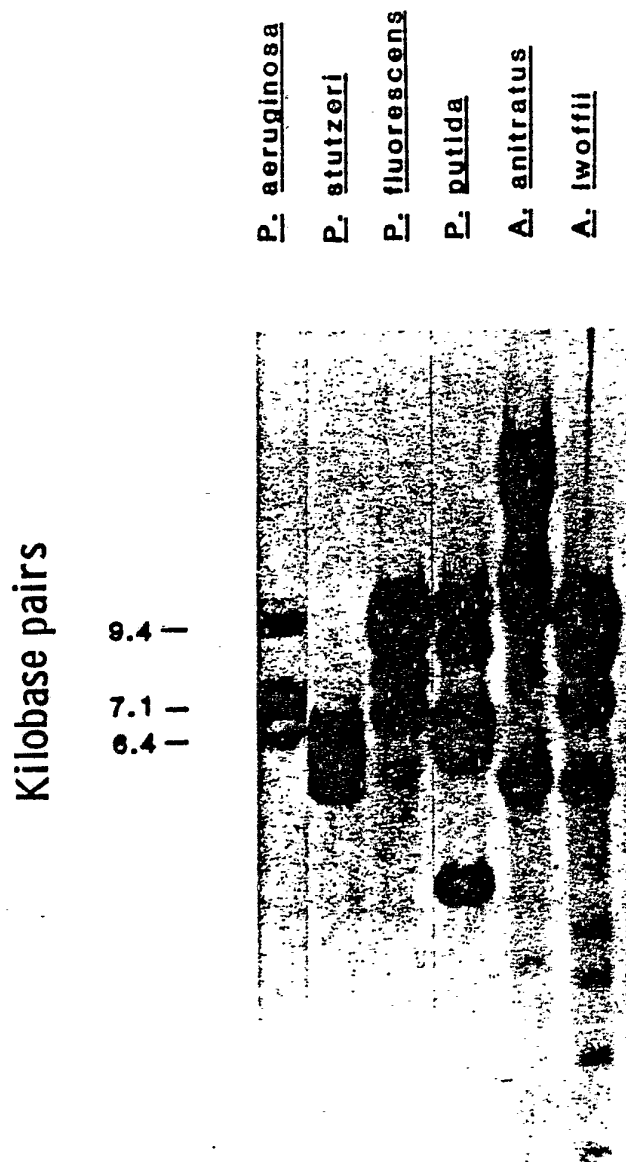


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FIG. 3 EcoR I digested DNA from Pseudomonas and Acinetobacter species



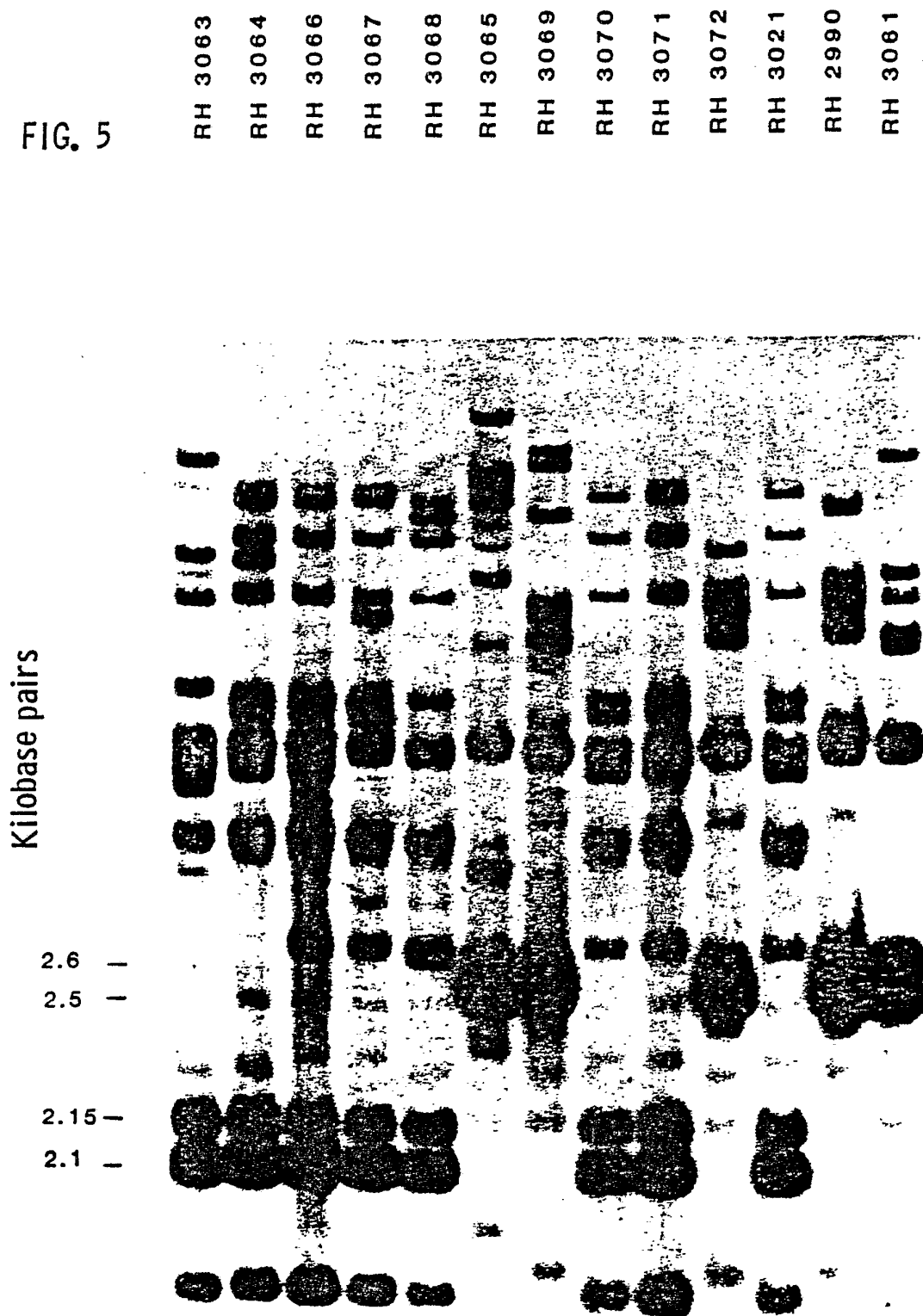
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FIG. 4 PST I digested DNA from Pseudomonas
and Acinetobacter species

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EcoR I digested DNA from Bacillus subtilis strains

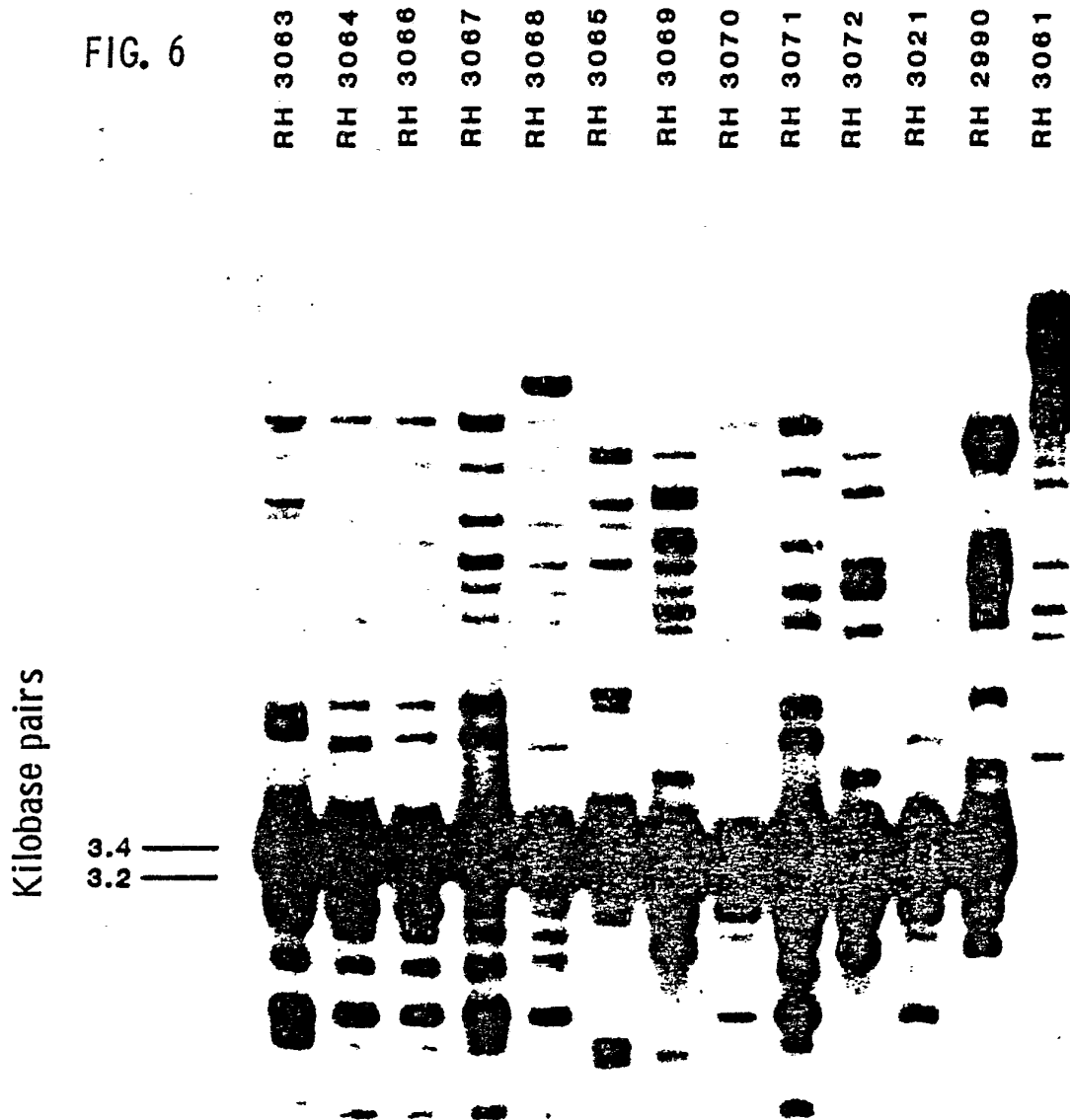
FIG. 5



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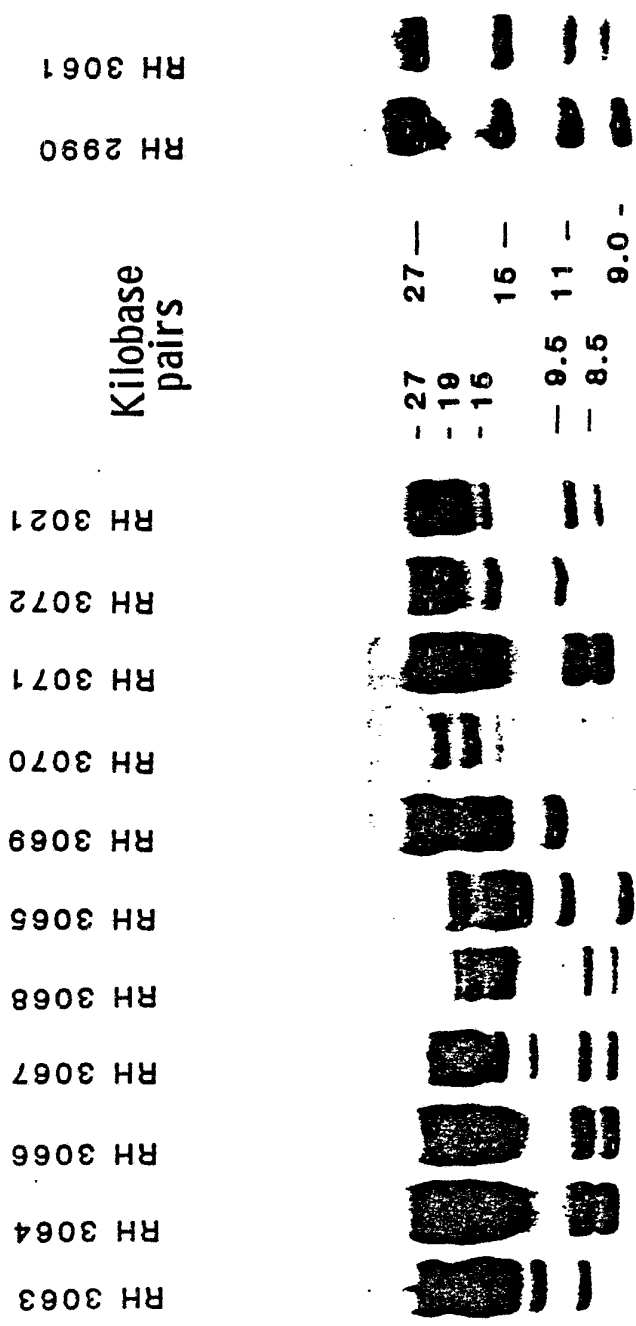
PST I digested DNA from Bacillus subtilis strains

FIG. 6



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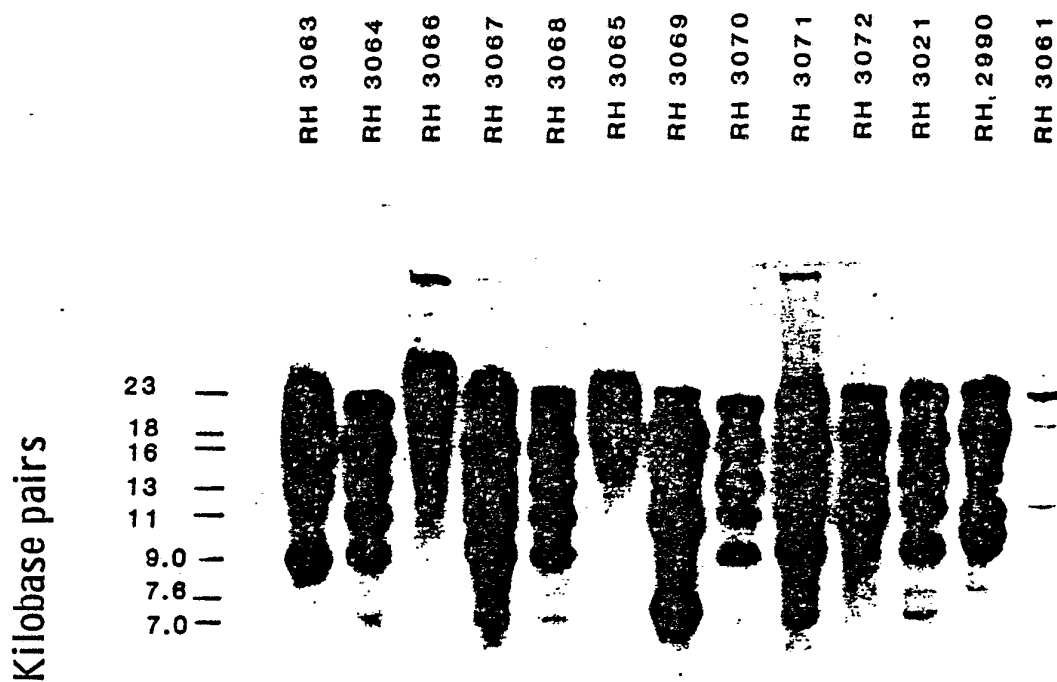
FIG. 7 Bgl II digested DNA from Bacillus subtilis strains



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Sac I digested DNA from Bacillus subtilis strains

FIG. 8

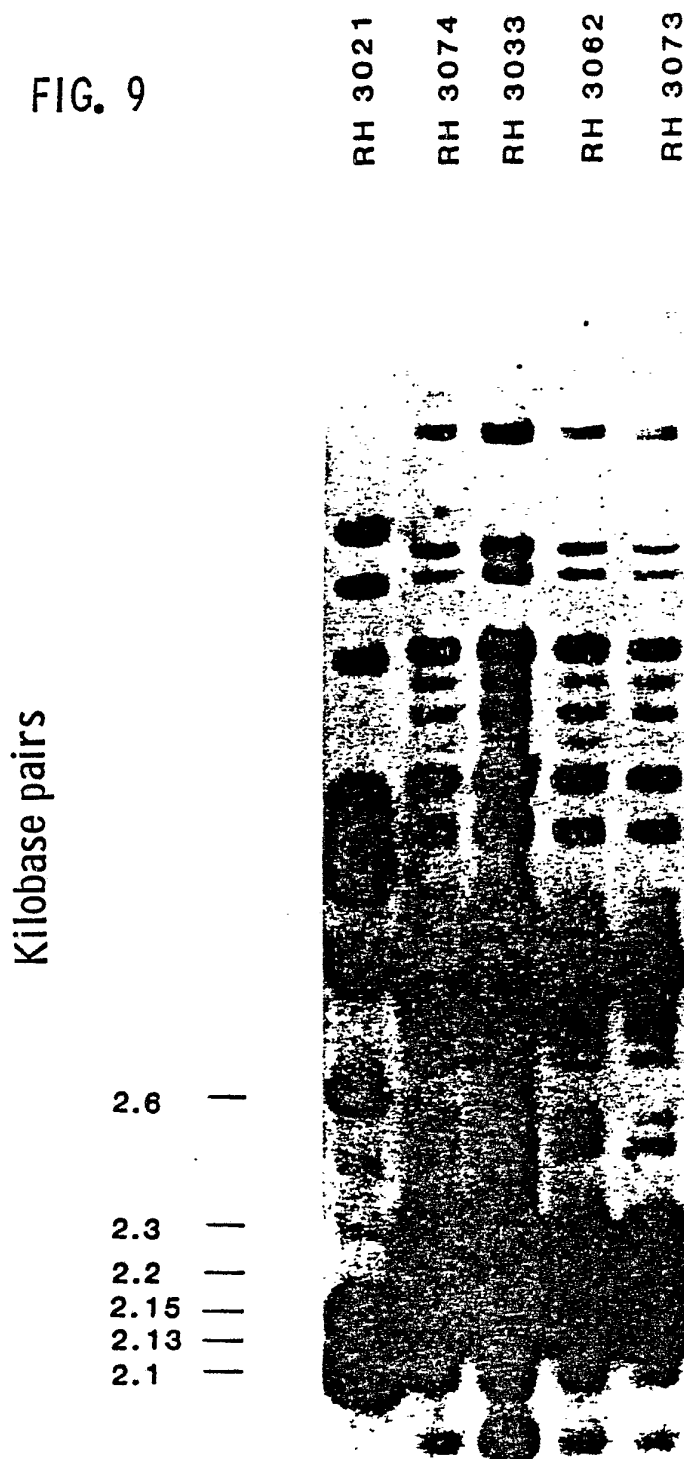


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EcoR I digested DNA from B. subtilisRH 3021 and B. polymyxa RH 3074,

RH 3033, RH 3062, RH 3073

FIG. 9



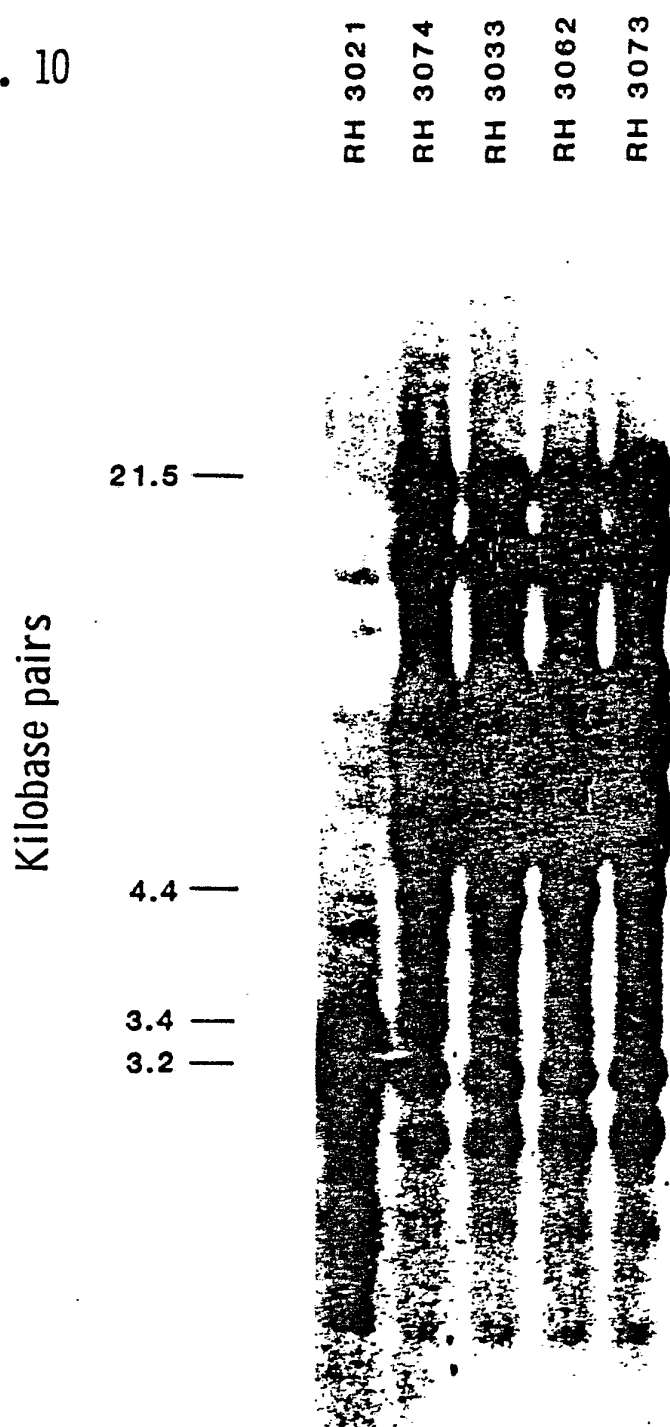
SUBSTITUTE SHEET



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PST I digested DNA from B. subtilis
RH 3021 and B. polymyxa RH 3074,
RH 3033, RH 3062, RH 3073

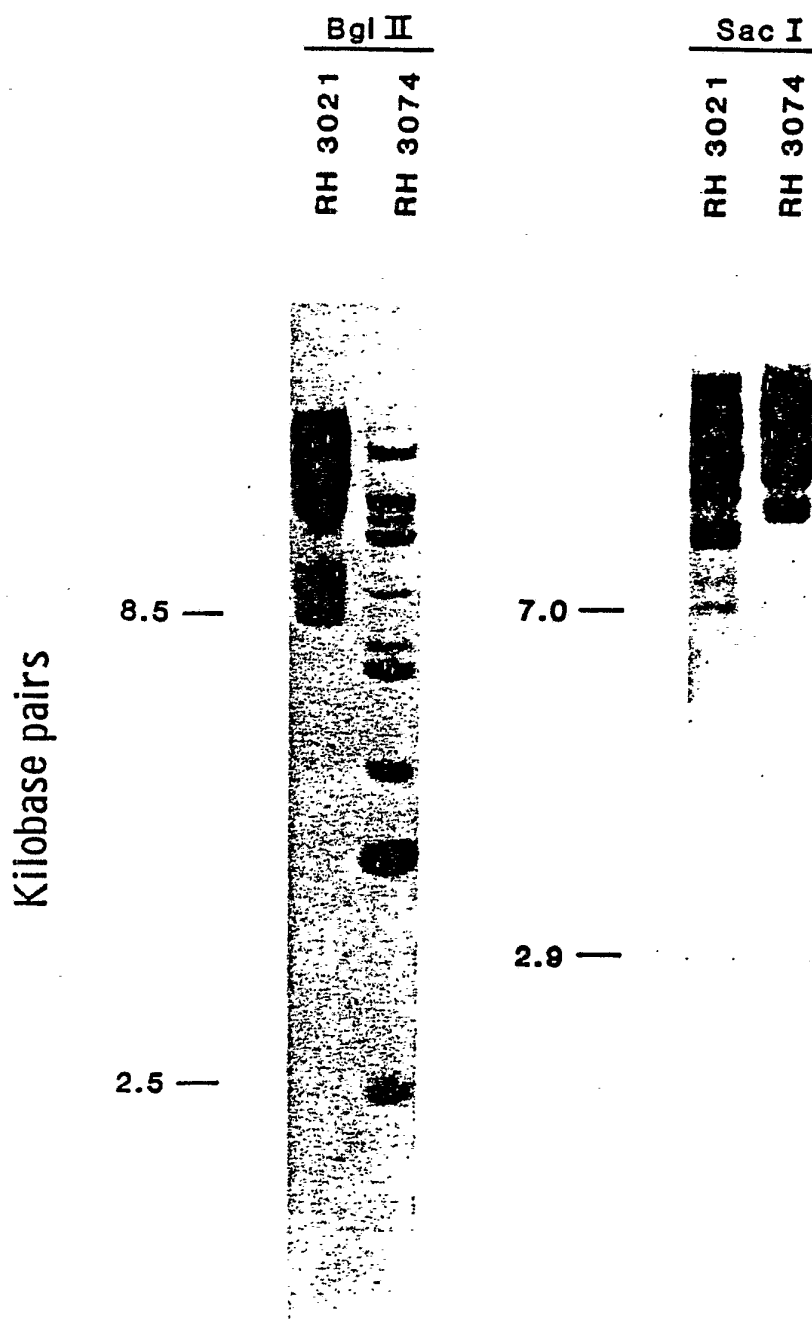
FIG. 10



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FIG. 11

Bgl II and Sac I digested DNA from
B. subtilis RH 3021 and B. polymyxa RH 3074



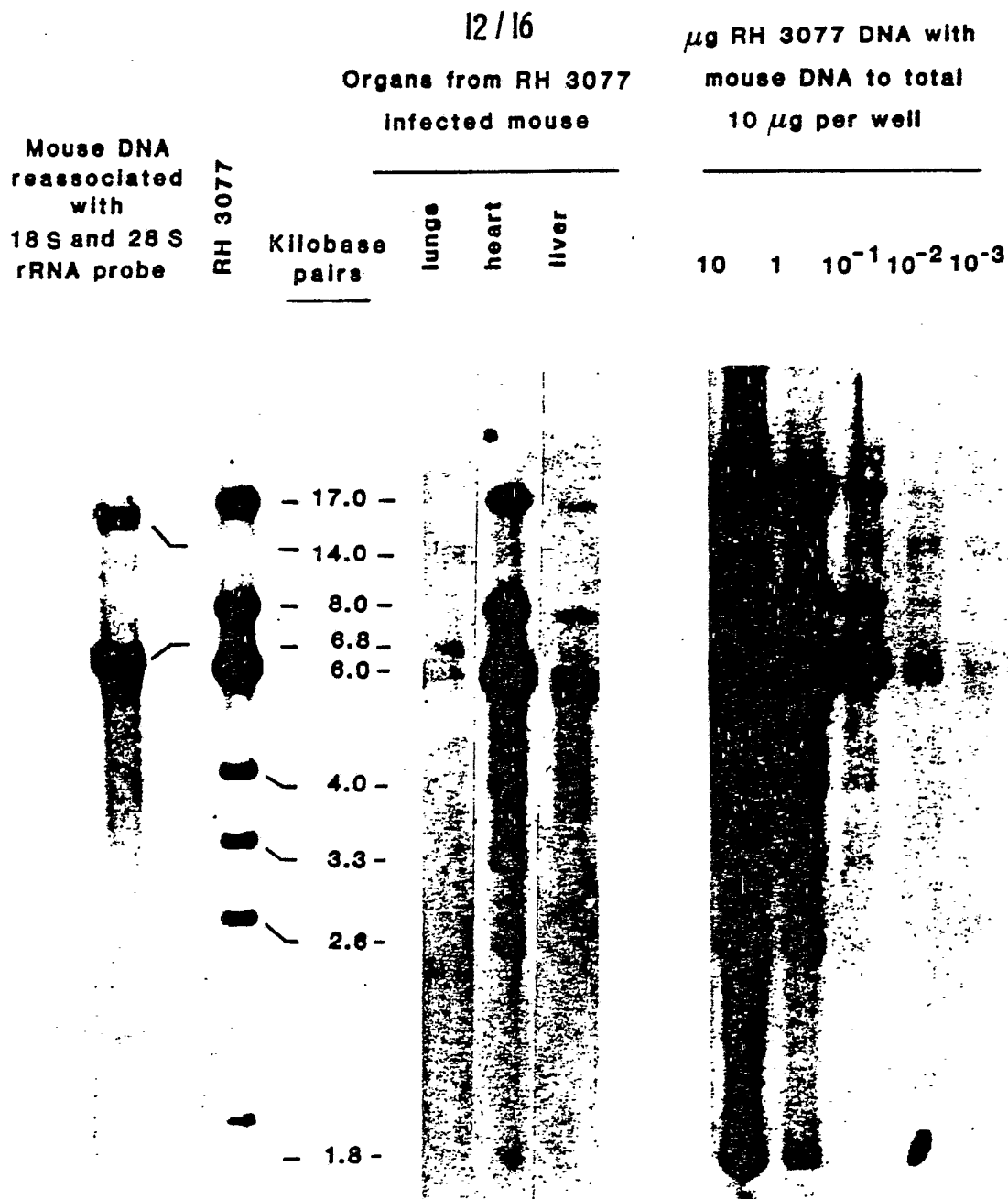


FIG. 12

Detection of *S. pneumoniae* RH 3077
ribosomal gene sequences in EcoR I
digested DNA from infected mouse tissues

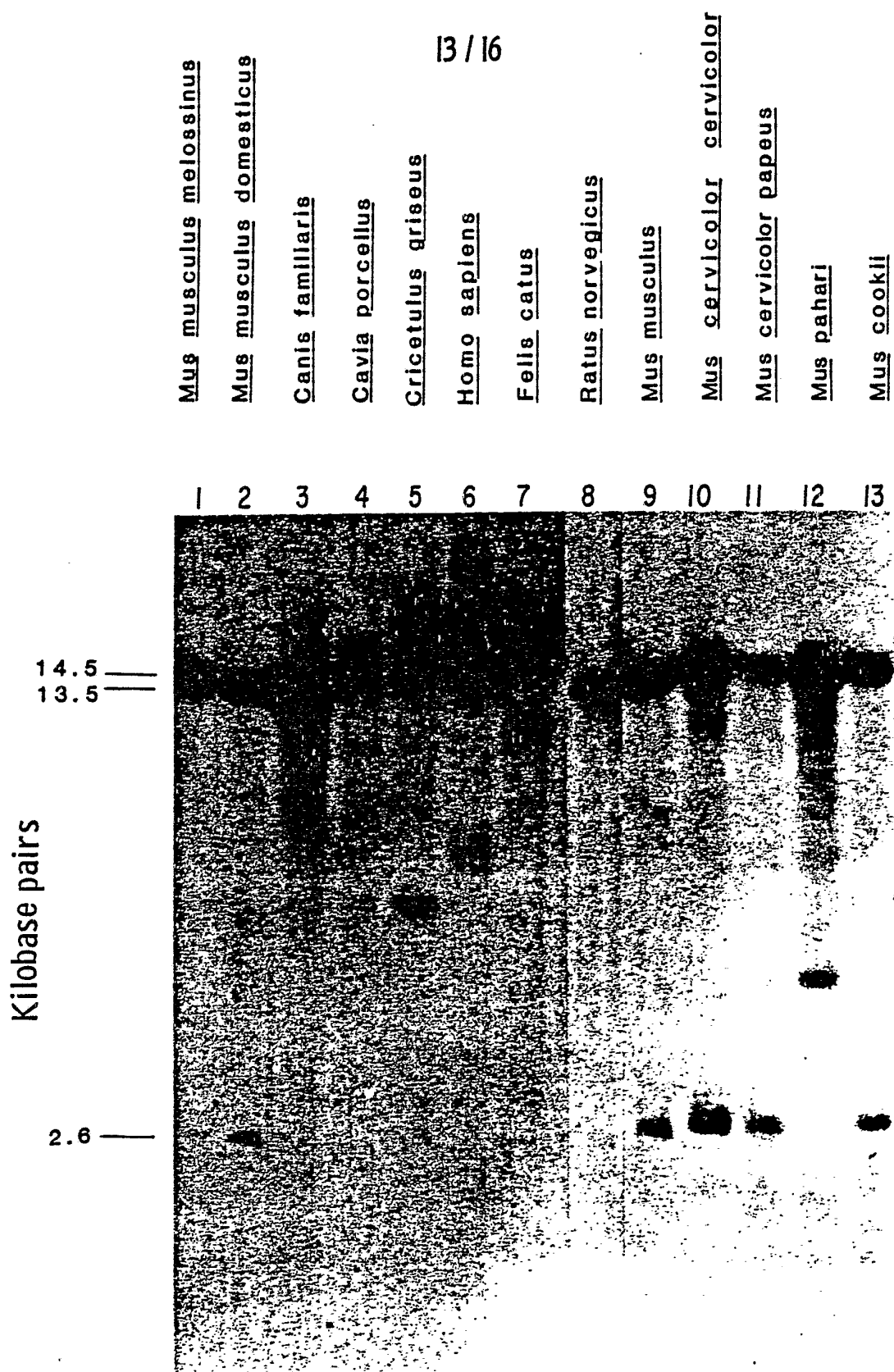


FIG. 13

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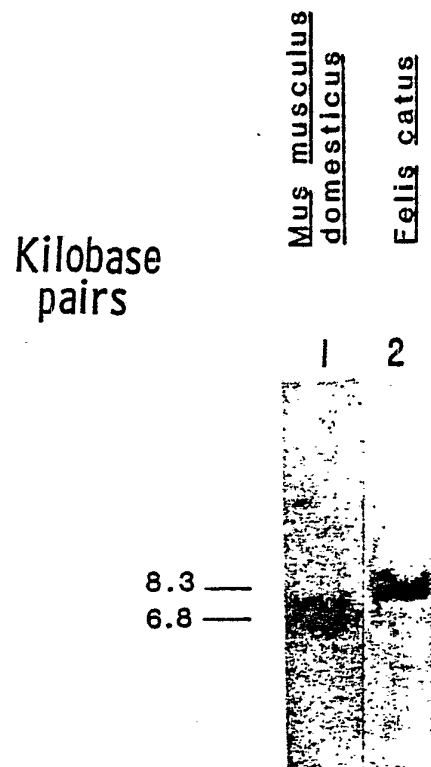


FIG. 14

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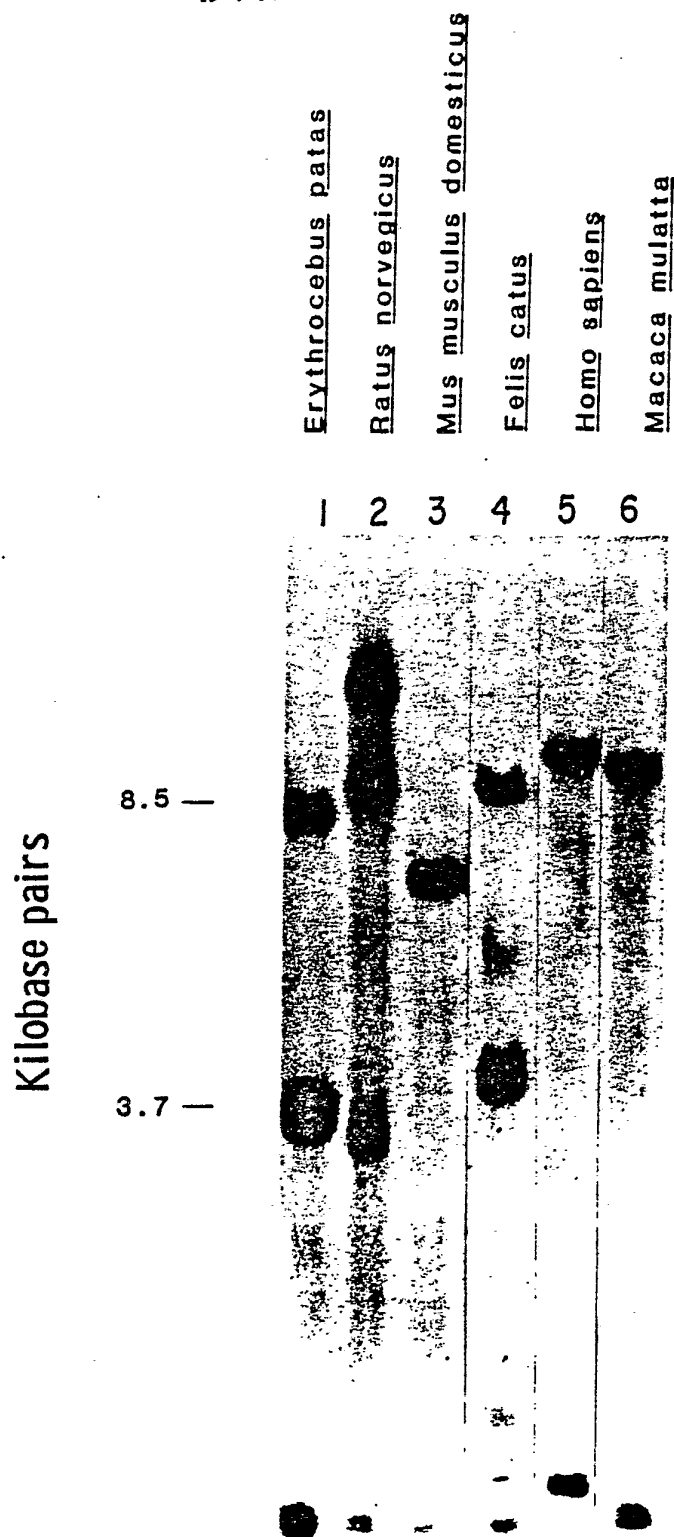


FIG. 15

SUBSTITUTE SHEET



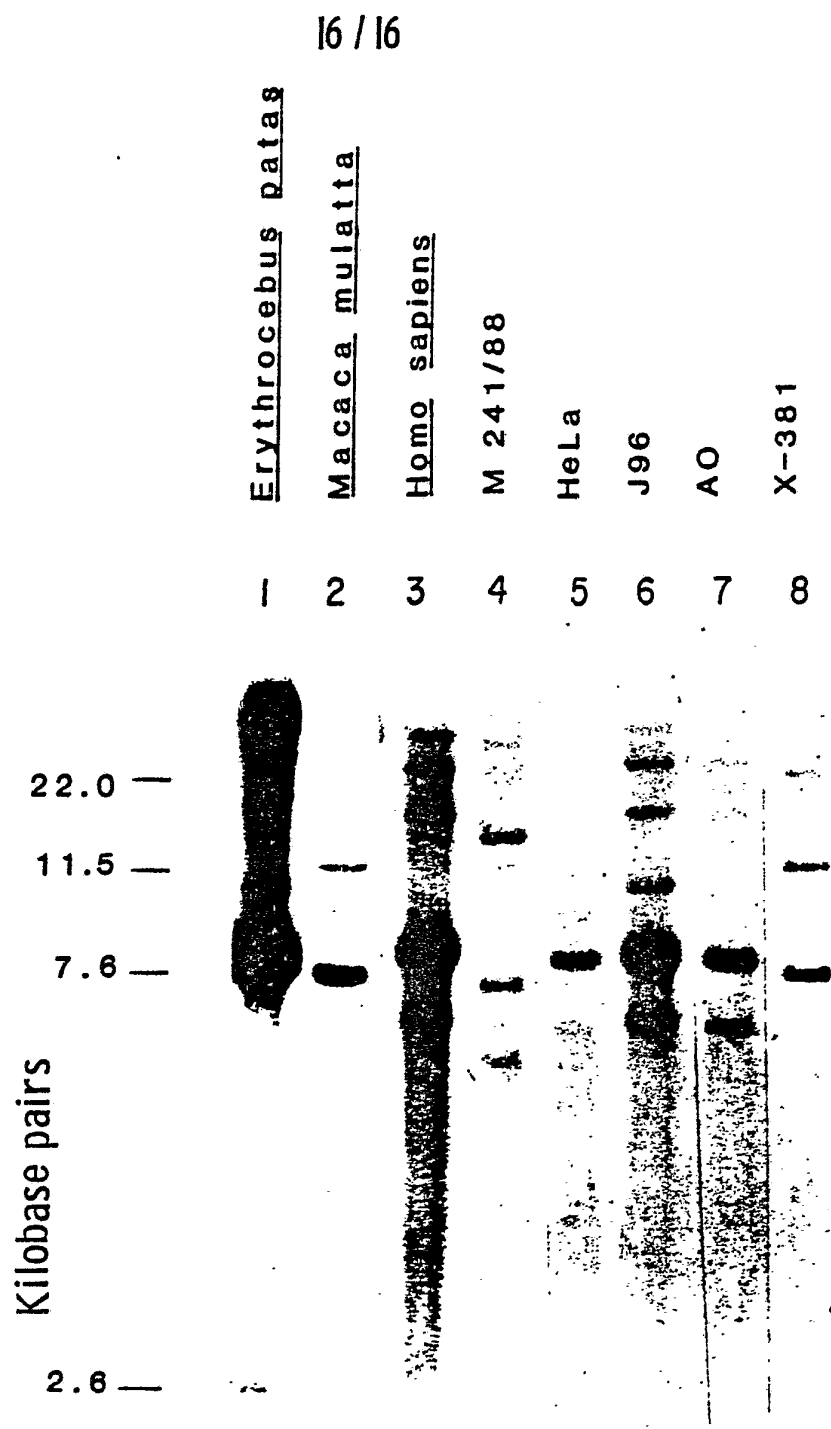
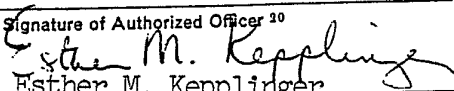


FIG. 16

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US84/00382

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³		
According to International Patent Classification (IPC) or to both National Classification and IPC US - 435/5, 6, 29, 810; 436/94 IPC C12Q 1/04, 68, 70		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/4,5,6,29,34,35,39,41,172.3,803,810 436/64,73,81,94,501,504,543,545,804 536/27,28	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁵		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	N, Am. J. Hum. Genet., 32, issued 1980, R.D. Schmickel et al., "Hela Cell Identification by Analysis of Ribosomal DNA Segment Patterns Generated by Endonuclease Restriction", 890-897.	1-42
Y,P	WO,A, 83/01073, Published 31 March 1983, Webster	1-60
Y	N, Molec. gen. Genet., 180, issued 1980, Philomena Ostapchuk, "Conserved Genes in Enteric Bacteria Are Not Identical," 475-477.	1-60
Y	N, Molec. gen. Genet., 182, issued 1981, Gayle L. Lampa et al., "Fine Scale Interspersion of Conserved Sequences in the Pea and Corn Chloroplast Genomes", 310-320.	1-60
Y	N, Biochim. Biophys. Acta, 442, issued 1976, John M. Taylor et al., "Efficient Transcription of RNA into DNA By Avian Sarcoma Virus Polymerase", 324-330.	8,36,49,56,57
Y	N, Cell, 22, issued November 1980, Richard A. Van Etten et al., "Precise Localization and Nucleotide Sequence of the Two Mouse Mitochondrial rRNA Genes	43-60
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ²		Date of Mailing of this International Search Report ³
03 July 1984		12 JUL 1984
International Searching Authority ¹		Signature of Authorized Officer ²⁰
ISA/US		 Esther M. Kepplinger

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

and Three Immediately Adjacent Novel tRNA Genes,
157-170.

Y,P US, A, 4,396,713, published 02 August 1983, Simpon

1-60

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 10

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter¹² not required to be searched by this Authority, namely:
2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out¹³, specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the International application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the International application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
☐ No protest accompanied the payment of additional search fees.