STUDIES ON *Aeromonas* TAXONOMY

A thesis submitted to the University of Stirling in candidature for the degree of Doctor of Philosophy

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Abstract

A diverse collection of 400 *Aeromonas* strains from clinical, veterinary and environmental sources worldwide has been assembled. A number of molecular-based typing methods: Amplified-Fragment Length Polymorphism (AFLP), Repetitive Extragenic Palindromic sequence Polymerase Chain Reaction (REP-PCR) and Randomly amplified polymorphic DNA (RAPD), have been adapted for this genus and used to assess the relatedness of isolates from different sources.

AFLP proved to be the most discriminatory, RAPD was found to be of limited value for discriminating *Aeromonas* spp. Though less discriminatory, a novel rapid and inexpensive one-tube method of performing REP-PCR is described as well as adaptation of the technique for use with a DNA sequencer for the first time. A novel use of degenerate PCR primer antagonists for improving the specificity of REP-PCR that may be of more general applicability in PCR is also described.

The AFLP data contributes much to the debate surrounding *Aeromonas* taxonomy. The data supports the hypothesis that *A. popoffii* and *A.bestarium* are stable and genetically distinct taxa. Evidence is presented for the first time that *A.caviae* HG4 may comprise two genetically distinct sub-groups. Further evidence is presented for the existence within HG7, of a group phenotypically similar to HG10.

Though genetically distinct groups corresponding to *A.encheleia*, HG11 and *A.eucrenophila* were identified, they appeared to separate below the species level. *A.encheleia,* however, was found to be far more diverse than previously suspected. Strains implicated in diseases in different hosts were not strongly correlated with particular species. However the most apparently virulent strains appeared to be rather

specialised and host-specific. In particular a genetically distinct group of HG3 isolates, geographically widespread and stable over several years, was recovered only from porpoises.

The presence of Class 1 integron-specific sequences is reported for the first time in *Aeromonas* spp. Though these appeared to have spread widely between strains isolated from different hosts, they were found to be comparatively rare in environmental isolates. Evidence of such widespread horizontal transfer has important implications for the population structure of the genus.

Chapter 1: General Introduction

1.1: Overview

Members of the genus *Aeromonas* are autochthonous inhabitants of aquatic environments. In freshwaters they can be the predominant bacterial group, particularly during the warmer months of the year and where there is a high organic load (Araujo *et al.*, 1991; Fiorentini *et al.*, 1998)

Though aeromonads are part of the normal flora of fish and amphibia, several species are recognised as serious pathogens of fish and, in aquaculture, cause substantial economic loss (Trust, 1986)

Recently there has been an explosion of interest in the mesophilic *Aeromonas* species because of growing recognition that they may cause disease in a wide spectrum of animals including humans (Janda, 1991; Janda, 1998).

There is mounting evidence that different *Aeromonas* species differ both in their virulence and associated disease spectra (Janda, 1994; Kühn *et al.*, 1997a). There is therefore a pressing need in both medicine and aquaculture alike for a means to accurately identify *Aeromonas* isolates to the species and strain level in order to improve our understanding of the epidemiology of these organisms and for determination of appropriate treatment.

Moreover, in aquaculture, disease outbreaks have usually been controlled using antibiotics and, increasingly treatments have been failing as antibiotic resistant strains emerge (Inglis *et al.*, 1997; Angka, 1997). Since it is likely that aquaculture facilities also harbour *Aeromonas* strains capable of causing human disease, it is possible that treatment of disease outbreaks could encourage the development of

antibiotic resistance in medically important strains also. As the aquaculture industry continues to grow worldwide, this alarming possibility, however remote, demands thorough investigation.

Unfortunately, precise identification and differentiation of *Aeromonas* isolates is notoriously difficult by traditional means. Biochemical markers often correlate poorly with species and moreover test results may vary with external conditions, such as incubation temperature and composition of the test medium (Altwegg *et al.*, 1988). Consequently the taxonomy of the genus has evolved amidst some confusion and controversy (Carnahan, 1993).

1.2: Aims

This work was therefore undertaken with the following aims:

1: To adapt and develop a number of different techniques for bacterial typing and to assess their utility for the high-resolution discrimination of *Aeromonas* isolates.

2: To apply these methods to type a diverse collection of *Aeromonas* isolates in order compare strains associated with disease in a variety of different hosts and also environmental isolates not associated with any disease. In particular to clarify the relationship between strains implicated in disease in fish and humans.

3: To determine the prevalence of resistance to a number of antibiotics used in aquaculture amongst strains isolated in several different countries.

4: To determine whether antibiotic resistance determinants found in strains isolated from aquaculture facilities may be transferred to other *Aeromonas* strains or to other species likely to colonise humans e.g. *Escherichia coli.*

1.3: General description of the Genus *Aeromonas*

1.3.1: Taxonomy

The taxonomy of the genus *Aeromonas* has evolved amidst some confusion and controversy and has undergone several major revisions since the genus was first proposed by Kluyver and vanNiel (1936).

In part this confusion may be attributed to the genuinely heterogeneous structure of the genus. But confusion also arises because their ubiquitous nature means they are of interest to a wide variety of microbiologists: medical, veterinary and environmental, who may have very different priorities. In particular, the lack of a coherent approach to isolation of these organisms means that different laboratories almost certainly isolate a different subset of the strains present in any given sample. Despite this, and whilst it is almost certain that further species remain to be discovered, a coherent picture is beginning to appear.

The genus is composed of gram-negative, oxidase- and catalase-positive, facultatively anaerobic rods. Most species contain representatives that are motile by means of a single polar flagellum. They are capable of growth in the absence of NaCl, but high salt concentrations (6-7%) are inhibitory.

A characteristic trait is resistance to the vibriostatic agent 2,4-diamino-6,7-diisopropylpteridine (0/129). Nutritional requirements are generally simple. Species differ in their abilities to ferment a wide variety of common monosaccharides, disaccharides, glycosides and some alcoholic sugars (d-mannitol, glycerol). Acid is rarely produced from pentoses except L-arabinose (Carnahan and Joseph, 1993).

Many extracellular enzymes are produced including cytolytic toxins, proteases, nucleases, lipases, sulphatases, lecithinase, chitinase, amylase and stapholysin (Janda, 1999). The G + C content of DNA is 57-63 mol% (von Graevenitz and Altwegg, 1991). The type species is *A. hydrophila* (Popoff, 1984)

In *Bergey's Manual of Systematic Bacteriology,* Popoff, (1984) places the genus in the family *Vibrionaceae*, along with two other genera *Vibrio* and *Plesiomonas*. This was a convenient classification since the three genera share a number of phenotypic features and ecosystems. Subsequently 16S rRNA sequence data indicated that *Aeromonas* were not closely related to *Vibrio* and would be best placed in a family of their own (Colwell, MacDonell and DeLey, 1986) and subsequent work has confirmed this (Ruimy *et al.*, 1994). Since 1992 the genus has resided in a new family: *Aeromonadaceae* (Holmes, 1992: International Committee on Systematic Bacteriology), within the γ subclass of the *Proteobacteria* (Woese *et al.*, 1985)

In the most recent edition of Bergey's Manual Popoff, (1984) the genus is separated into two groups based upon optimal temperatures for growth disease spectrum and biochemical characteristics. Psychrophilic non-motile aeromonads are placed in a single species, *A. salmonicida,* which includes strains which are primary pathogens of fish, particularly salmonids. The taxonomy of the psychrophilic group has remained relatively stable, though a number of sub-species have been proposed: *salmonicida; smithia; masoucida;* and most recently *pectinolytica* (Pavan *et al.*, 2000)

The second group was composed of mesophilic strains that may infect a wide range of vertebrate species, both warm and cold-blooded. Members of this group are

mostly motile, indole-positive and do not produce the brown pigment. Three species: *A.hydrophila, A. sobria* and *A. caviae* were described. However the manual was out of date even as it went to press as DNA-DNA hybridisation assays (Popoff *et al.,* 1981) had shown that within each of these species, more than one Hybridisation Group (HG) existed. This suggested that these taxa were in reality, phenospecies, i.e. genetically dissimilar groups of organisms that could not be separated by phenotypic means alone.

Research at the Centers for Disease Control in Atlanta, Georgia during the early 1980's, expanded the work of Popoff's group and at the first International Workshop on *Aeromonas/Plesiomonas* Farmer *et al.* (1986) presented an emended taxonomic scheme with the type strains of named species assigned to specific HG's for the first time. These were *A.hydrophila* (HG 1), *A. salmonicida* (HG 3), *A. caviae* (HG 4), *A.media* (HG 5) and *A. sobria* (HG 7). Thereafter a steady trickle of publications appeared, describing new species and assigning them to a hybridisation group. These include *A. veronii* (HG 10) (Hickman-Brenner *et al.,* 1987), *A. schubertii* (HG 12) and *Aeromonas* group 501 (HG13) (Hickman- Brenner *et al.*, 1988), *A. eucrenophila* (HG 6) (Schubert and Hegazi, 1988), *A. jandaei* (HG 9), (Carnahan, Fanning and Joseph, 1991), *A. trota* (HG 14) (Carnahan *et al.,* 1991b), *A. bestiarum* (HG 2), (Ali *et al.*, 1996), and *A. popoffii* (Huys *et al.*, 1997c).

HG11, which contains a small number of strains of clinical origin is currently unnamed and is the subject of some controversy. Whilst there is evidence based on polyphasic taxonomy (Huys *et al.,* 1997b), that it is related to *A. encheleia* proposed by Esteve Gutiérrez and Ventosa (1995b), it has been argued on the evidence of 16S rDNA sequences (Martínez-Murcia, 1999), that *A.encheleia* belongs in a separate

species. However *A.encheleia* was proposed in order to accommodate four atypical *Aeromonas* strains isolated from European eels and the value of establishing a new species on the basis of so few strains has to be questioned.

Similarly, the species *A.allosaccharophila* was proposed on the basis of phenotypic studies, and the 16S rDNA sequences of only three atypical *Aeromonas* strains (Martinez-Murcia *et al.*, 1992). Moreover no phenotypic property has so far been identified that could reliably distinguish *A.allosaccharophila* from other *Aeromonas* species. Though DNA-DNA hybridisation data has since been produced (Esteve Gutiérrez and Ventosa, 1995a) which supports the proposal to place *A.allosaccharophila* in a separate species, the authors did not include all HG type strains in their analysis and so, until further data becomes available, the true taxonomic status of this species remains unclear.

The position of two other proposed species: *A. enteropelogenes* (Schubert *et al.*, 1990a) and *A. ichthiosmia* (Schubert *et al.*, 1990b) also remains to be fully resolved. Collins *et al.* (1993) have suggested on the basis of rRNA sequence analysis that these species are identical to *A.trota* and *A.veronii,* respectively.

Despite there being no compelling reason to suppose that these two groups are not each synonomous, references to both *A. enteropelogenes* and *A. ichthiosmia* continue to appear in the literature, (Carnahan and Altwegg, 1996).

Herein lies the source of much of the confusion that surrounds the taxonomy of the genus *Aeromonas.* Several species (including *A.hydrophila*) were named prior to the arrival of molecular systematics and since most laboratories are not able to perform DNA-DNA hybridisation assays many authors continue to use a typing scheme based

on phenotypic data alone. Unfortunately the groups delineated on the basis of phenotypic characteristics and those identified using DNA-DNA hybridisation are not totally congruent. Furthermore many publications refer to isolates as, for example *A.hydrophila*, without specifying whether this means *A.hydrophila*, HG1, or the phenospecies *A.hydrophila*, which could include HG1, motile mesophilic strains from HG3 and some strains from HG2.

Janda and Abbott (1998) have argued that the practice of assigning new isolates to Hybridisation Group is outdated and needs to be abolished. Whilst there is much merit in this argument, specifying Hybridisation Group, does, at least provide some clarity as to which taxonomic scheme an identification has been based on.

The finer details of *Aeromonas* systematics continue to engage microbial taxonomists in a vigorous debate and even though the broader picture is much clearer now than even five years ago, there is still much uncertainty. It is important that these areas of uncertainty are resolved since an understanding of the ecology (see section 1.3.2) and pathology (section 1.3.3) of these organisms is only possible within the framework of a stable taxonomic system.

1.3.2: Habitat

Members of the genus *Aeromonas* are autochthonous inhabitants of aquatic environments. They can be isolated from virtually any freshwater source excluding hot springs (Holmes *et al.* 1996). They are also found in marine environments, though numbers are highest in estuarine and coastal regions where salinity is lower (Fiorentini *et al.*, 1998). Whilst they are usually recovered in low numbers from

unpolluted freshwater environments during colder seasons in temperate climates (typically1-10 CFU.ml^{-1}: Burke *et al.*, 1984), there is evidence that far higher numbers exist in a viable but non-cultivatable (VBNC) state (Montfort and Baleux, 1991; Rahman *et al.*, 2001). In tropical waters and during the warmer months of the year in temperate regions their relative numbers increase, to typically 20-4000 CFU.ml^{-1}, (Pettibone, 1998). During these periods aeromonads can be isolated from potable water sources including chlorinated supplies (Ørmen and Østenvik, 2001; Kühn *et al.* 1997b).

Frequent isolation of *Aeromonas* species from soil (Brandi *et al.*, 1999) and numerous foods including dairy products, meats, shellfish and fresh produce (Rahim and Aziz, 1994; Neyts *et al.*, 2000), indicates that the natural habitats of these organisms are not limited to aquatic environments.

The ubiquitous nature of *Aeromonas* spp., coupled with the taxonomic confusion discussed in section 1.3.1 confounds our understanding of the ecology of these organisms.

For example an association between faecal coliform bacteria and levels of *Aeromonas* spp. in fresh lotic waters has been reported (Araujo *et al.*, 1991) and in water polluted by sewage, numbers can exceed total coliforms (Ashbolt *et al.*, 1995). However *Aeromonas* densities are generally high in all waters with a high organic load regardless of whether there is contamination by sewage (Stecchini and Domenis, 1994). The origin of Aeromonads found in faecally contaminated water is therefore not clear. Undoubtedly *Aeromonas* spp. are frequently recovered from human stool specimens (Krovacek *et al.*, 1994; Altwegg *et al.*, 1990). However the relative contributions to total *Aeromonas* numbers made by human-associated

organisms introduced by the sewage on the one hand, and that made by rapid growth of autochthonous organisms as a result of influx of nutrients on the other, has not been evaluated.

Similarly soil, particularly agricultural soil is known to support growth of *Aeromonas* spp. (Brandi *et al.,* 1999) and an increase in aeromonad numbers is often observed in freshwaters following heavy rainfall (Pettibone, 1998). Again, this increase may be attributed to growth of autochthonous inhabitants following nutrient run-off, or to introduction of new organisms flushed from the soil. The relative contributions made by each is not known.

Regrettably much of the work which might have been expected to throw some light on these issues, often fails to do so because isolates are inadequately typed to species and strain level. Joseph and Carnahan (1994) argued very convincingly that much of the published work up to that date was based on typing schemes that were "at best questionable and at worst meaningless". Yet published work on *Aeromonas* ecology still appears in which isolates are identified only to genus level. It might be suggested that this work represents a missed opportunity.

The current poor understanding of the ecology of these organisms hampers epidemiological investigations attempting to link cases of *Aeromonas*-associated disease with particular foods or water supplies. Furthermore most clinical and veterinary laboratories do not routinely type *Aeromonas* isolates to the species level : (see section 1.3.3) Of the currently defined *Aeromonas* species, only *A.sobria, A.eucrenophila* and *A.encheleia* have not been recovered from clinical material (Janda and Abbot, 1998). However it is likely that the widespread distribution of

Aeromonas spp in the environment and in particular in drinking water means that their recovery from stool samples does not necessarily indicate clinical significance.

1.3.3: *Aeromonas* infections

Though part of the normal external and intestinal flora of aquatic animals, *Aeromonas* spp. have long been recognised as primary extra-intestinal pathogens of poikilothermic vertebrates. While their potential as opportunistic pathogens of warm-blooded animals also, was noted over a century ago, only over the past two decades their status as primary human pathogens has become clear, (Janda and Abbot, 1998). Interestingly, the explosion of interest in these organisms that followed this realisation coincided with a period of very rapid growth in aquaculture worldwide, and hence an expanding interest in fish diseases. Accordingly, as with the taxonomy of this group, research into their pathogenic potential has been driven by a number of very different priorities.

Until recently most attention was focused on the non-motile *Aeromonas salmonicida* the widely distributed caustive agent of furunculosis and septicaemia in fish, which causes substantial loss of farmed salmonids annually (Austin and Austin, 1999). Partly because of the taxonomic confusion surrounding this genus, diseases attributed to other *Aeromonas* species in aquaculture were usually described and recorded in general terms such as 'bacterial haemorrhagic septicaemia' or 'motile aeromonad septicamia' and the implicated agent described only as '*A.hydrophila*' or '*A.hydrophila* group' (Joseph and Carnahan, 1994).

As the diversity of the genus was revealed, the range of species that were implicated as fish pathogens expanded correspondingly. These now include *A.allosaccharophila* (Martinez-Murcia, 1992), *A.bestiarum*, (Ali, *et al.*,1996), *A.caviae*, (Paniagua *et al.*, 1990), *A.encheleia,* (Esteve, Gutierrez, and Ventosa., 1995b), *A. eucrenophila,* (Huys *et al.*, 1997b), *A.hydrophila,* (Santos *et al.*, 1988), *A.jandaei,*(Esteve *et al.*, 1994), *A.veronii* biovar sobria (Rahman *et al.*, 2001) and phenospecies *A sobria,* (Toranzo *et al.*, 1989). There is now strong evidence for the role of these *Aeromonas* species as etiological agents of a variety of systemic and localized diseases in a wide range of poikilothermic animals: fish, frogs, turtles, snakes and even snails (Kodjo *et al.*, 1996).

Intensively reared fish and shellfish are particularly at risk: in aquaculture stresses such as temperature change, handling, poor water quality, crowding, parasitic load and shipping can all increase susceptibility to disease. Awareness of this has led to improved husbandry practices which have considerably reduced the incidence of disease outbreaks generally (Dixon, 1994). The introduction of vaccines against *A.salmonicida* has reduced the incidence of furunculosis enormously. Despite this, opportunist *Aeromonas* infections are common in aquaculture and though they are often treatable with antibacterials, these organisms continue to cause substantial economic loss annually.

As awareness of the role of *Aeromonas* species as fish pathogens has grown, the range of species being recovered from clinical material has also expanded. In particular *A.hydrophila, A.caviae, A.veronii* bv. sobria and with less frequency *A.veronii* bv. veronii, *A.jandaeii,* and *A.schubertii* (Janda and Abbot, 1998). These have been implicated in a wide array of extra-intestinal and systemic infections,

including septicaemia, wound infections, meningitis, peritonitis, burn associated sepsis, respiratory diseases and hepatobiliary disease (Janda and Abbot, 1998). Septicaemia and peritonitis are particularly serious infections that are frequently fatal even in previously healthy patients (Wang *et al.,* 1993; Chopra and Houston, 1999). Hemolytic-uremic syndrome, a life-threatening condition normally associated with infections due to *Escherichia coli* O157: H7, has also been associated with *A.hydrophila* infections (Wang *et al.,* 2001); and a cytotoxin with homology to Shiga toxin 1 has been identified in both *A. hydrophila* and *A. caviae* (Haque *et. al.,* 1996). Wound infections are an especially common source of clinical specimens yielding aeromonads and commonly follow a trauma or penetrating injury that has been exposed to freshwater. The infections can range from mild, uncomplicated processes primarily involving cutaneous surfaces, to more complex illnesses where muscle, joints and bone may become infected, which can require amputations, (Janda and Abbot, 1998).

By far the most common human infection in which aeromonads are implicated is gastroenteritis, ranging from relatively trivial 'travellers diarrhea' (Hänninen, 1995) to more serious invasive infections that can become systemic leading to renal failure which can prove fatal (Filler, 2000). It has been suggested that *Aeromonas* spp. may cause up to 13% of the reported cases of gastroenteritis in the United States each year (Kingombe *et al.,* 1999)

Yet all of the mesophilic species can be recovered from the feces of asymptomatic humans, an inevitable consequence of their proliferation and persistence in drinking water. This, and the fact that Koch's postulates have never been fulfilled cast some doubt, for many years, over whether these organisms were indeed capable of causing

intestinal infections in humans, with the result that most clinical microbiology laboratories do not routinely screen for them (Sloane, 1996).

Despite this, the evidence for the involvement of *Aeromonas* species in human gastrointestinal infections is compelling. There are numerous documented examples of previously healthy adults succumbing to severe gastroenteritis coincident with the isolation of *Aeromonas* species in pure culture, or as predominant flora, in fecal and biopsy specimens, (Janda and Abbot, 1998). In many cases it has been possible to demonstrate the presence of neutralising antibodies to implicated cytotoxins and a rise in titre of immunoreactive antibodies to the whole cell proteins of the suspected infecting strain followed by a concomitant decline during the recovery period. There are also reports of the resolution of abnormal pathology revealed through colonoscopy, being accompanied by the disappearance of *Aeromonas* strains from stool specimens as the only likely enteropathogens, (Block *et al.,* 1994)

A feature common to all these reports is that the implicated organism was either identified only as '*Aeromonas* species' or else the identification to species level was based on a typing scheme that could not reliably differentiate all *Aeromonas* species. Yet many of these infections would not have been diagnosed until a very late stage, or not at all, by the majority of clinical microbiology laboratories that do not routinely screen for *Aeromonas* infections in patients presenting with gastroenteritis. This points to a pressing need for a greater understanding of the taxonomy of this genus, both for more accurate early diagnosis, and to improve our understanding of the correlation of particular diseases with different *Aeromonas* species.

This holds equally true for the extensive work aimed at clarifying the nature of the virulence characteristics of *Aeromonas* species and their association with particular

diseases. Whilst tremendous progress has been made in identifying specific virulence determinants such as hemolysins, cytotoxins and enterotoxins, much of this work has yielded confusing or misleading results because the strains investigated have been inadequately typed to species level. (Kingombe *et al.*, 1999)

Chapter 2: Collection and Isolation of *Aeromonas* strains

Chapter 2: Collection and Isolation of *Aeromonas* strains

2.1: Isolation of *Aeromonas* strains

An enormous range of media have been used for isolation of *Aeromonas* strains: Moyer (1996) lists 36. The majority have been developed for the clinical laboratory and are highly selective and thus not suitable for detecting low numbers of injured organisms in foods and drinking water.

The utility of *Aeromonas* Medium (Ryan) and its superiority over some other formulae for detection of *Aeromonas* species in tap water, bottled water and foods including meat, poultry, fish and seafoods has been reported (Holmes and Sartory, 1993; Pin *et al.*, 1994; Warburton *et al.*, 1995)

Aeromonas Medium (Ryan) is also specified by the MAFF/DHS Steering Group on the Microbiological Safety of Food for detection and enumeration of *Aeromonas hydrophila* in clinical specimens.

The medium is a modified formulation of XLD Medium that will support the growth of *Aeromonas* spp and *Plesiomonas* spp as well as the usual Enterobacteriaceae. To improve its performance in the isolation of aeromonads, the addition of ampicillin at 5mg/l is recommended by the manufacturer (Oxoid UK, Basingstoke, Hampshire, UK). The effectiveness of ampicillin as a selective agent for *Aeromonas* spp has been frequently reported (Want and Millership, 1990). This concentration is lower than in other *Aeromonas* selective media, but use of higher levels risks selecting against *A.trota* and other ampicillin sensitive groups (Rahim *et al.*, 1984)

Prepared medium is heated gently to 100°C, and not autolaved. On cooling to 50°C one 2.5mg ampicillin selective supplement vial (Oxoid, Basingstoke, Hampshire, UK) is added per 500ml.

Table 2-1: Formulation of *Aeromonas* agar (Ryan)

Formula	gm/litre
Proteose peptone	5
Yeast extract	3
L. Lysine monohydrochloride	3.5
L. Arginine monohydrochloride	2
Sorbitol	3
Inositol	2.5
Lactose	1.5
Xylose	3.75
Bile Salts No.3	3
Sodium thiosulphate	10.67
Sodium chloride	5
Ferric ammonium citrate	0.8
Bromothymol blue	0.04
Thymol blue	0.04
Agar	12.5

Final pH 8.0 + 0.1

Plates were inoculated with a suspension of food, faeces etc., diluted with sterile 0.85% saline to form single colonies. For isolation of strains from water 10-200µl water sample was spread onto the plate. For isolation of strains from chlorinated

water up to100ml water sample was passed through a 0.2μm membrane filter and the membrane placed directly on the surface of the plate.

Plates were incubated for 24-48 hrs at 30°C, then examined for the presence of dark green, opaque colonies with darker centre, diameter 0.5-1.5mm.

Suspected *Aeromonas* isolates were tested for catalase and oxidase activity and for resistanct to the vibriostatic agent 0/129, as follows:

2.1.1: Resistance to 0/129

Resistance to 0/129 (2,4-diamino-6,7-diisopropylpteridine) was determined by resuspending part of the colony in 2ml 0.85% saline and spreading 200μl of the suspension onto the surface of a Tryptone soya agar plate (Oxoid) and placing 0/129 impregnated discs (10μg, and 150μg, Oxoid) onto the surface.

2.1.2: Oxidase activity

The presence of cytochrome oxidase was determined by transferring a streak of a well separated colony to the end of an Oxoid oxidase identification stick, using a nichrome wire.

The tip of each stick is impregnated with a solution of N,N-dimethyl-p-phenylenediamine oxalate, ascorbic acid and α - naphthol.

In a positive reaction the enzyme cytochrome oxidase combines with N,N-dimethyl-p-phenylenediamine oxalate and α - naphthol to form the dye indophenol blue within 2 minutes.

2.1.3: Catalase activity

Catalase activity was determined by resuspending a portion of a colony in 3% hydrogen peroxide in an eppendorf tube and observing gas production.

2.2: Storage of isolates and type strains

Strains found to be Gram-negative, catalase-positive, oxidase positive and sensitive to the vibriostatic agent O/129 were considered to be putative *Aeromonas* isolates and were retained for further analysis.

Suspected *Aeromonas* strains were streaked onto Tryptone Soya Agar plates (TSA: Oxoid UK, Basingstoke Hampshire, UK) and a single colony was maintained for further analysis on TSA slope cultures in 5ml bijoux bottles, kept at room temperature for up to 6 months. Type strain cultures were also maintained in this way.

For longer term storage, a single colony was transferred to a 1.7ml screwtop microtube containing 30-40 1.5mm polystryrene beads and 800µl cryofluid: 60% (v/v) Tryptone Soya Broth (TSB: Oxoid UK, Basingstoke Hampshire, UK), 40% (v/v) glycerol, 10% (w.v) sucrose. After vigourous shaking to disperse the colony and coat the beads, excess cryofluid was removed and the beads stored at –70°C indefinitely.

To revive both slope and cryopreserved cultures from storage, a single bead or slope inoculum was transferred to a 7ml bijoux containing 4ml Tryptone Soya Broth (TSB: Oxoid UK, Basingstoke Hampshire, UK) and incubated at 30°C for 16 hours with

gentle agitation (50rpm) in an orbital shaker, then streaked onto a TSA plate to obtain single colonies.

2.3: Isolates used in this work

2.3.1: Previously Uncharacterised Isolates

Table 2.1 lists previously uncharacterised strains used in this work.

Strains prefixed "AA" were obtained from the Aquatic Animal Health Research Institute, Thailand (AAHRI)

Strains prefixed "RK" were the gift of Dr. Lothar Beutin, Robert Koch Institute, Berlin (RKI)

Strains prefixed "V" were the gift of Dr. Lewis Levay, School of Ocean Sciences, University of North Wales, Bangor, Menai Bridge UK (SOS)

Strains prefixed "F" were the gift of Dr. Marianne Pearson, Institute of Aquaculture (IOA)

Strains prefixed "RR" were the gift of Dr Rodolfo del RioRodriguez, Institute of Aquaculture (IOA)

Strains prefixed "C" were the gift of Dr.G. Foster, SAC Veterinary Laboratory, Inverness

Strains prefixed "H" were the gift of Dr. D. Reid, Aberdeen Royal Infirmary (ARI)

Type strains prefixed "T" were obtained from the collection held at the Institute of Aquaculture. (IOA)

Stains prefixed "AH" were isolated in the UK during the course of this study

Table 2-2: *Aeromonas* strains used in this study

Lab reference no	isolated from	location	source	original reference
AA001	catfish	Phillipines	AAHRI	$4MF_3K$
AA002	unknown fish	Malaysia	AAHRI	M1
AA003	catfish	Thailand	AAHRI	III/11
AA006	unknown fish	Phillipines	AAHRI	$7MF_4L$
AA007	unknown fish	Malaysia	AAHRI	M3
AA009	catfish	Thailand	AAHRI	III/23
AA010	turtle	Thailand	AAHRI	95126
AA011	unknown fish	Malaysia	AAHRI	M6
AA013	catfish	Phillipines	AAHRI	$10PF_2SP$
AA014	unknown fish	Bangladesh	AAHRI	FDR692
AA015	turtle	Thailand	AAHRI	94151
AA016	catfish	Thailand	AAHRI	III/27
AA018	catfish	Phillipines	AAHRI	$20UF_2SP$
AA019	unknown fish	Bangladesh	AAHRI	95014
AA020	turtle	Thailand	AAHRI	95191
AA021	catfish	Thailand	AAHRI	III/36
AA022	catfish	Phillipines	AAHRI	$21FF_1L$
AA023	catfish	Phillipines	AAHRI	$22SF_3E$
AA024	catfish	Thailand	AAHRI	III/37
AA025	catfish	Phillipines	AAHRI	$28F_9F_1K_2$
AA028	catfish	Thailand	AAHRI	III/40
AA029	turtle	Thailand	AAHRI	94163
AA030	catfish	Thailand	AAHRI	III/41
AA035	catfish	Malaysia	AAHRI	M15
AA038	unknown fish	Bangladesh	AAHRI	FDR042
AA039	catfish	Thailand	AAHRI	III/42
AA040	snake head fish	Thailand	AAHRI	III/17
AA041	catfish	Phillipines	AAHRI	$42AF_1K$
AA042	unknown fish	Malaysia	AAHRI	M38
AA043	unknown fish	Bangladesh	AAHRI	FDR126
AA044	common silver barb	Thailand	AAHRI	95255
AA045	snake head fish	Thailand	AAHRI	III/22
AA047	catfish	Phillipines	AAHRI	1A
AA050	unknown fish	Bangladesh	AAHRI	FDR621
AA051	unknown fish	Bangladesh	AAHRI	FDR622
AA052	catfish	Phillipines	AAHRI	5F
AA053	common silver barb	Thailand	AAHRI	96060
AA054	unknown fish	Bangladesh	AAHRI	FDR625
AA055	unknown fish	Bangladesh	AAHRI	FDR626
AA056	turtle	Thailand	AAHRI	unknown
AA057	catfish	Phillipines	AAHRI	6A
AA059	unknown fish	Bangladesh	AAHRI	FDR631
AA060	unknown fish	Bangladesh	AAHRI	FDR632

AA061	unknown fish	Bangladesh	AAHRI	FDR635
AA062	catfish	Phillipines	AAHRI	7C
AA065	catfish	Phillipines	AAHRI	7C (g)
AA068	unknown fish	Bangladesh	AAHRI	R117
AA069	unknown fish	Phillipines	AAHRI	11C
AA070	unknown fish	Phillipines	AAHRI	24E
AA071	unknown fish	Bangladesh	AAHRI	R119
AA072	catfish	Phillipines	AAHRI	44B
AA073	catfish	Phillipines	AAHRI	47B
AA074	unknown fish	Bangladesh	AAHRI	R842
AA075	catfish	Thailand	AAHRI	II/35
AA079	catfish	Thailand	AAHRI	III/51
AA081	unknown fish	Malaysia	AAHRI	M67
AA083	catfish	Thailand	AAHRI	III/49
AA085	unknown fish	Bangladesh	AAHRI	R849
AA087	turtle	Thailand	AAHRI	III/45
AA089	unknown fish	Bangladesh	AAHRI	unknown
AA091	catfish	Thailand	AAHRI	94036
AA092	catfish	Phillipines	AAHRI	48C
AA093	unknown fish	Bangladesh	AAHRI	R204/7
AA094	catfish	Thailand	AAHRI	96112
AA095	unknown fish	Bangladesh	AAHRI	R204/9
AA097	unknown fish	Malaysia	AAHRI	M64
AA098	catfish	Thailand	AAHRI	96127
F01	frog	Thailand	IOA	1D4
F02	frog	Thailand	IOA	1D12
F03	frog	Thailand	IOA	1D33
F04	frog	Thailand	IOA	1D54
F05	frog	Thailand	IOA	2D1
F06	frog	Thailand	IOA	2D8
F07	frog	Thailand	IOA	2D16
F08	frog	Thailand	IOA	2D25
F09	frog	Thailand	IOA	3D29
F10	frog	Thailand	IOA	4D5
RR002	ornamental fish	Brazil	IOA	n/a
RR005	ornamental fish	Brazil	IOA	n/a
RR006	ornamental fish	Singapore	IOA	n/a
RR007	ornamental fish	Singapore	IOA	n/a
RR008	ornamental fish	Brazil	IOA	n/a
RR009	ornamental fish	Singapore	IOA	n/a
RR010	ornamental fish	Brazil	IOA	n/a
RR011	ornamental fish	Brazil	IOA	n/a
RR012	ornamental fish	Brazil	IOA	n/a
RR013	ornamental fish	Singapore	IOA	n/a
RR014	ornamental fish	Brazil	IOA	n/a
RR015	ornamental fish	Singapore	IOA	n/a

RR016	ornamental fish	Singapore	IOA	n/a
RR018	ornamental fish	Brazil	IOA	n/a
RR019	ornamental fish	Brazil	IOA	n/a
RR020	ornamental fish	Brazil	IOA	n/a
RR021	ornamental fish	Singapore	IOA	n/a
RR022	ornamental fish	Brazil	IOA	n/a
RR024	ornamental fish	Brazil	IOA	n/a
RR028	ornamental fish	Brazil	IOA	n/a
RR031	ornamental fish	Brazil	IOA	n/a
RR032	ornamental fish	Singapore	IOA	n/a
RR034	ornamental fish	Brazil	IOA	n/a
RR035	ornamental fish	Brazil	IOA	n/a
RR037	ornamental fish	Singapore	IOA	n/a
RR040	ornamental fish	Brazil	IOA	n/a
RR041	ornamental fish	Brazil	IOA	n/a
RR042	ornamental fish	Singapore	IOA	n/a
RR043	ornamental fish	Brazil	IOA	n/a
RR044	ornamental fish	Brazil	IOA	n/a
RR045	ornamental fish	Singapore	IOA	n/a
RR046	ornamental fish	Singapore	IOA	n/a
RR047	ornamental fish	Brazil	IOA	n/a
RR048	ornamental fish	Singapore	IOA	n/a
RR049	ornamental fish	Brazil	IOA	n/a
RR050	ornamental fish	Brazil	IOA	n/a
RR051	ornamental fish	Brazil	IOA	n/a
RR052	ornamental fish	Brazil	IOA	n/a
RR053	ornamental fish	Brazil	IOA	n/a
RR054	ornamental fish	Singapore	IOA	n/a
RR056	ornamental fish	Singapore	IOA	n/a
RR057	ornamental fish	Brazil	IOA	n/a
RR059	ornamental fish	Brazil	IOA	n/a
RR060	ornamental fish	Brazil	IOA	n/a
RR061	ornamental fish	Brazil	IOA	n/a
RR062	ornamental fish	Singapore	IOA	n/a
RR063	ornamental fish	Brazil	IOA	n/a
RR064	ornamental fish	Brazil	IOA	n/a
RR065	ornamental fish	Brazil	IOA	n/a
RR066	ornamental fish	Brazil	IOA	n/a
RR067	ornamental fish	Singapore	IOA	n/a
RR068	ornamental fish	Singapore	IOA	n/a
C001	porpoise	Scotland	SAC	M1415/ 91/ 1
C003	porpoise	Scotland	SAC	M1908/ 92/ 1
C004	porpoise	Scotland	SAC	M2004/ 92/ 1
C005	porpoise	Scotland	SAC	M2195/ 92/ 1
C006	striped dolphin	Scotland	SAC	M2547/ 92/ 1
C007	otter	Scotland	SAC	M1969/ 92/ 2

C008	porpoise	Scotland	SAC	M2637/ 92/ 3
C010	common seal	Scotland	SAC	M1106/ 93/ 1
C011	porpoise	Scotland	SAC	M1227/ 93/ 1
C012	dolphin	Scotland	SAC	M2122/ 93/ 1
C014	unknown fish	Scotland	SAC	M1186/ 93/ 2
C015	sheep	Scotland	SAC	M1499/ 93/ 2
C016	dolphin	Scotland	SAC	M2051/ 93/ 2
C017	sheep	Scotland	SAC	S238/ 95/ 1
C018	porpoise	Scotland	SAC	M2627/ 93/ 3
C019	striped dolphin	Scotland	SAC	M700/ 93/ 4
C020	porpoise	Scotland	SAC	M576/ 93/ 4
C021	porpoise	Scotland	SAC	M906/ 93/ 6
C022	porpoise lung	Scotland	SAC	M2753/ 93/ 6
C023	porpoise	Scotland	SAC	M906 / 93/ 8
C024	sea mammal	Scotland	SAC	M137/ 94/ 1
C025	dolphin	Scotland	SAC	M1336/ 94/ 1
C026	porpoise	Scotland	SAC	M1710/ 94/ 1
C027	common seal	Scotland	SAC	M1959/ 94/ 1
C028	porpoise	Scotland	SAC	M2268/ 94/ 1
C029	sea trout	Scotland	SAC	M1167/ 94/ 2
C030	porpoise	Scotland	SAC	M1336/ 94/ 2
C031	porpoise	Scotland	SAC	M1951/ 94/ 2
C033	porpoise	Scotland	SAC	M1952/ 94/ 2
C038	grey seal	Scotland	SAC	M1959/ 94/ 2
C039	sheep	Scotland	SAC	M2179/ 94/ 3
C040	Atlantic white-sided dolphin	Scotland	SAC	M431/ 94/ 5
C043	beaver	Scotland	SAC	M2194/ 94/ 5
C044	common seal	Scotland	SAC	M1516/ 93/1
C045	dolphin	Scotland	SAC	M644/ 95/ 1
C049	porpoise	Scotland	SAC	M644/ 95/ 2
C050	porpoise	Scotland	SAC	M204/ 95/ 3
C055	porpoise	Scotland	SAC	M1575/ 95/ 4
C056	porpoise	Scotland	SAC	M2000/ 95/ 4
C058	porpoise	Scotland	SAC	M23/ 95/ 5
C059	otter	Scotland	SAC	B2/ 96/ 1
C060	porpoise	Scotland	SAC	M89/ 96/ 1
C061	beaver	Scotland	SAC	M891/ 96/ 1
C062	sheep	Scotland	SAC	M900/ 96/ 1
C063	porpoise	Scotland	SAC	M75/ 96/ 2
C064	porpoise	Scotland	SAC	M89/ 96/ 2
C065	porpoise	Scotland	SAC	M18/ 96/ 3
C066	sheep	Scotland	SAC	S217. 96/2
C069	grey seal	Scotland	SAC	M846/ 96/ 5
C071	common seal	Scotland	SAC	M843/ 97/ 1
C074	porpoise	Scotland	SAC	M821/ 97/ 2
C076	porpoise	Scotland	SAC	M990/ 97/ 2

C077	grey seal	Scotland	SAC	M181/ 93/ 3
C078	grey seal	Scotland	SAC	M836/ 97/ 3
C079	porpoise	Scotland	SAC	M181/ 97/ 4
C080	porpoise	Scotland	SAC	M843/ 97/ 2
C081	porpoise	Scotland	SAC	M821/ 97/ 1
C082	common seal	Scotland	SAC	B55/ 96/ 1
C083	porpoise	Scotland	SAC	M990/ 97/ 1
C084	porpoise	Scotland	SAC	M2475/ 96/ 4
C085	grey seal	Scotland	SAC	B1/ 97/ 2
C086	grey seal	Scotland	SAC	M2475/ 96/ 5
C087	porpoise	Scotland	SAC	M1675/ 96/ 3
C088	sea trout	Scotland	SAC	M1675/ 96/ 3
C089	sheep	Scotland	SAC	S178/ 98/ 1
C091	beaver	Scotland	SAC	M1049/ 96/ 5
C092	dolphin	Scotland	SAC	M1049/ 96/ 4
C093	porpoise	Scotland	SAC	M2445/ 97/ 1
C094	porpoise	Scotland	SAC	M1675/ 96/ 4
C095	grey seal	Scotland	SAC	M682/ 97/ 1
C097	porpoise	Scotland	SAC	M2936/ 97/ 4
C098	common seal	Scotland	SAC	M2900/ 97/ 1
C099	grey seal	Scotland	SAC	M2900/ 97/ 3
C102	common seal	Scotland	SAC	M474/ 98/ 2
C103	porpoise	Scotland	SAC	M1049/ 96/ 7
C104	porpoise	Scotland	SAC	M564/ 98/ 2
C105	porpoise lung	Scotland	SAC	M564/ 98/ 3
C106	dolphin	Scotland	SAC	M910/ 98/ 2
C107	striped dolphin	Scotland	SAC	M1591/ 98/ 3
C108	sheep	Scotland	SAC	M1591/ 98/ 4
C109	Atlantic white-sided dolphin	Scotland	SAC	M1747/ 98/ 2
C110	porpoise	Scotland	SAC	M2075/ 98/ 2
C111	grey seal	Scotland	SAC	M2660/ 98/ 2
C112	porpoise	Scotland	SAC	M2075/ 98/ 3
C114	sea mammal	Scotland	SAC	M2075/ 98/ 4
C115	porpoise	Scotland	SAC	M843/97/5
C116	porpoise	Scotland	SAC	M843/97/6
C117	grey seal	Scotland	SAC	M2122/ 98/ 1
C118	common seal	Scotland	SAC	M2122/ 98/ 2
C119	dolphin	Scotland	SAC	M846/ 96/ 3
C120	porpoise	Scotland	SAC	M846/ 96/ 4
C122	striped dolphin	Scotland	SAC	M846/ 96/ 5
C123	porpoise	Scotland	SAC	M1516/ 98/1
C124	porpoise	Scotland	SAC	M506 / 99/
C125	porpoise	Scotland	SAC	M1186/ 99/ 2
C126	sea trout	Scotland	SAC	F2075/991
AH001	salmon	Scotland	IOA	n/a
AH002	seagull feces	Scotland	IOA	n/a

AH003	seagull feces	Scotland	IOA	n/a
AH004	ice bucket	Scotland	IOA	n/a
AH005	salmon	Scotland	IOA	n/a
AH007	raw chicken	Scotland	IOA	n/a
AH009	reservoir	Scotland	IOA	n/a
AH010	reservoir	Scotland	IOA	n/a
AH011	raw chicken	Scotland	IOA	n/a
AH012	salmon	Scotland	IOA	n/a
AH014	salmon	Scotland	IOA	n/a
AH016	salmon	Scotland	IOA	n/a
AH018	salmon	Scotland	IOA	n/a
AH019	river water	Scotland	IOA	n/a
AH020	salmon	Scotland	IOA	n/a
AH021	salmon	Scotland	IOA	n/a
AH022	raw chicken	Scotland	IOA	n/a
AH023	ice bucket	Scotland	IOA	n/a
AH024	seagull feces	Scotland	IOA	n/a
AH025	salmon	Scotland	IOA	n/a
AH026	seagull feces	Scotland	IOA	n/a
AH027	seagull feces	Scotland	IOA	n/a
AH028	seagull feces	Scotland	IOA	n/a
AH029	reservoir	Scotland	IOA	n/a
AH030	salmon	Scotland	IOA	n/a
AH031	tilapia	Scotland	IOA	n/a
AH032	salmon	Scotland	IOA	n/a
AH033	seagull feces	Scotland	IOA	n/a
AH034	salmon	Scotland	IOA	n/a
AH035	seagull feces	Scotland	IOA	n/a
AH037	reservoir	Scotland	IOA	n/a
AH038	seagull feces	Scotland	IOA	n/a
AH039	seagull feces	Scotland	IOA	n/a
AH040	reservoir	Scotland	IOA	n/a
AH041	seagull feces	Scotland	IOA	n/a
AH042	seagull feces	Scotland	IOA	n/a
AH043	raw chicken	Scotland	IOA	n/a
AH044	seagull feces	Scotland	IOA	n/a
AH045	river water	Scotland	IOA	n/a
AH046	seagull feces	Scotland	IOA	n/a
AH047	river water	Scotland	IOA	n/a
AH048	salmon	Scotland	IOA	n/a
AH049	river water	Scotland	IOA	n/a
AH050	tilapia	Scotland	IOA	n/a
AH052	raw chicken	Scotland	IOA	n/a
AH053	raw chicken	Scotland	IOA	n/a
AH055	river water	Scotland	IOA	n/a
AH056	raw chicken	Scotland	IOA	n/a

AH059	river water	Scotland	IOA	n/a
AH060	seagull feces	Scotland	IOA	n/a
AH061	reservoir	Scotland	IOA	n/a
AH062	seagull feces	Scotland	IOA	n/a
AH063	salmon	Scotland	IOA	n/a
AH051	raw chicken	Scotland	IOA	n/a
AH064	seagull feces	Scotland	IOA	n/a
AH065	seagull feces	Scotland	IOA	n/a
AH066	salmon	Scotland	IOA	n/a
AH067	seagull feces	Scotland	IOA	n/a
AH068	river water	Scotland	IOA	n/a
AH069	salmon	Scotland	IOA	n/a
AH070	ice bucket	Scotland	IOA	n/a
AH071	salmon	Scotland	IOA	n/a
AH072	raw chicken	Scotland	IOA	n/a
AH073	salmon	Scotland	IOA	n/a
AH074	salmon	Scotland	IOA	n/a
AH075	river water	Scotland	IOA	n/a
AH076	salmon	Scotland	IOA	n/a
AH077	seagull feces	Scotland	IOA	n/a
AH080	river water	Wales	IOA	n/a
AH081	river water	Wales	IOA	n/a
AH082	river water	Wales	IOA	n/a
AH083	river water	Wales	IOA	n/a
AH084	river water	Wales	IOA	n/a
AH085	river water	Wales	IOA	n/a
AH086	river water	Wales	IOA	n/a
AH087	river water	Wales	IOA	n/a
AH088	river water	Wales	IOA	n/a
AH089	river water	Wales	IOA	n/a
AH090	river water	Wales	IOA	n/a
AH091	river water	Wales	IOA	n/a
AH092	river water	Wales	IOA	n/a
AH093	river water	Wales	IOA	n/a
AH094	river water	Wales	IOA	n/a
AH095	ostrich	Wales	IOA	n/a
AH096	ostrich	Wales	IOA	n/a
AH097	chlorinated tap water	Wales	IOA	n/a
AH098	chlorinated tap water	Wales	IOA	n/a
H09	human stool	Scotland	ARI	965274
H07	human stool	Scotland	ARI	966601
H02	human stool	Scotland	ARI	965915
H17	human stool	Scotland	ARI	966334
H01	human stool	Scotland	ARI	966669
H21	human stool	Scotland	ARI	421490
H14	human wound	Scotland	ARI	969640

H20	human stool	Scotland	ARI	971724
H18	human stool	Scotland	ARI	971765
H06	human stool	Scotland	ARI	972046
H08	human stool	Scotland	ARI	953490
H22	human stool	Scotland	ARI	972680
H23	human stool	Scotland	ARI	605777
H03	human stool	Scotland	ARI	605779
H16	human wound	Scotland	ARI	972171
H10	human stool	Scotland	ARI	972178
H11	human stool	Scotland	ARI	605609
H15	human stool	Scotland	ARI	605631
H19	human stool	Scotland	ARI	605632
H12	human stool	Scotland	ARI	605633
H13	human stool	Scotland	ARI	605634
H04	human stool	Scotland	ARI	605639
H05	human stool	Scotland	ARI	605640
V01	groundwater	Vietnam	SOS	V01
V02	unknown fish	Vietnam	SOS	V02
V03	unknown fish	Vietnam	SOS	V03
V04	groundwater	Vietnam	SOS	V04
V05	groundwater	Vietnam	SOS	V05
V06	groundwater	Vietnam	SOS	V06
V07	groundwater	Vietnam	SOS	V07
V08	groundwater	Vietnam	SOS	V08
V09	groundwater	Vietnam	SOS	V09
V10	groundwater	Vietnam	SOS	V10
V11	groundwater	Vietnam	SOS	V11
V12	groundwater	Vietnam	SOS	V12
V13	groundwater	Vietnam	SOS	V13
V14	groundwater	Vietnam	SOS	V14
V15	groundwater	Vietnam	SOS	V15
V16	groundwater	Vietnam	SOS	V16
V17	groundwater	Vietnam	SOS	V17
V18	groundwater	Vietnam	SOS	V18
V19	groundwater	Vietnam	SOS	V19
V20	groundwater	Vietnam	SOS	V20
V21	groundwater	Vietnam	SOS	V21
V22	groundwater	Vietnam	SOS	V22
RK1	aquarium water	Germany	RKI	4/10021/211/98
RK2	human blood	Germany	RKI	7/10021/211/98
RK3	human blood	Germany	RKI	9/10021/211/98

2.3.2: Type strains

Table 2.3 lists type strains used in this work

Key to Accession numbers in Table 2.3:

NCIMB: National Collections Of Industrial, Food and Marine Bacteria, Aberdeen, UK

ATCC: American Type Culture Collection, LGC Promochem, Teddington, UK

CECT: Coleccion Espanola de Cultivos Tipo, Universidad de Valencia, Spain

Type strain	**Strain source**
A. hydrophila HG1	NCIMB 9240
A. salmonicida subsp. *salmonicida* HG3	NCIMB 1102
A. caviae HG4	NCIMB 13016
A. media HG5	NCIMB 2237
A. eucrenophila HG6	NCIMB 74
A. sobria HG7	NCIMB 12065
A. veronii HG8/10	NCIMB13015
A. jandaei HG9	ATCC 49568
A. encheleia HG11	CECT 4342 T
A. schubertii HG12	NCIMB 13161
A. trota HG13	ATCC 49657
A. ichthiosmia	NCIMB 13205
A. veronii biovar sobria	NCIMB 37
A. allosaccharophila	ATCC 43946
A. enteropelogenes	NCIMB 13209

Table 2-3: Type strains used in this work

Chapter 3:AFLP Analysis

Chapter 3: AFLP Analysis

3.1: Introduction

3.1.1: Description of AFLP Analysis

Amplified-Fragment Length Polymorphism (AFLP) analysis (Vos *et al.,* 1995), is one of the newest and most promising methods for microbial typing and systematics. The technique was originally devised for the construction of very high density DNA marker maps in plant genome research, but has proved equally suitable for applications that require more modest marker densities. When deployed for microbial typing, the method has been found to combine high powers of discrimination and reproducibility with universal applicability, (Lin *et al.,* 1996). Over the last four years several major phylogenetic studies have been based primarily on AFLP analysis (Huys *et al.*, 1996; Janssen *et al.*, 1997; Clerc *et al.,* 1998), and whilst the technique is not suitable for routine use in most microbiology laboratories, epidemiologists have not been slow to recognise the potential of AFLP for monitoring the progress of epidemics and identifying the source of outbreaks (Van Eldere *et al.,* 1999; Jiang *et al.,* 2000b).

The great strength of the AFLP technique for DNA fingerprinting is that it is able to combine the sensitivity of PCR-based approaches, with the precision and reproducibility of restriction-enzyme based techniques, whilst avoiding the shortcomings of either.

AFLP is based on the detection, by PCR amplification of restriction fragments produced by digestion of genomic DNA. To accomplish this, double-stranded adaptor sequences, designed with cohesive ends complementary to the restriction site

are first ligated to each end of the restriction fragments, so that all fragments have a known sequence at each end which can serve as primer annealing sites in PCR.

As in restriction fragment length polymorphism (RFLP) analysis, the number of fragments generated by restriction digestion is far too great to be easily resolved by gel electrophoresis and it is therefore necessary that only a specific subset of restriction fragments is detected. In RFLP this is achieved following electrophoresis by means of the cumbersome and time-consuming transfer of restriction fragments from the gel to a nylon membrane, followed by hybridisation with labelled probes. These steps are unnecessary in AFLP, as during the PCR step of only a fraction of the restriction fragments are amplified. This is achieved by designing PCR primers that are complementary to the adaptor/restriction halfsite, but additionally have further 'selective' bases at their 3' ends. By using stringent PCR annealing conditions it can be ensured that amplification will only occur where fragments have bases complementary to the primer's selective bases adjacent to the restriction site (fig 3.1). Thus, using primers, one of which has a single selective base, one in four restriction fragments will be amplified, whereas if both primers have three selective bases, then, (assuming no nucleotide bias) only one fragment in 4096 are amplified (Savelkoul *et al.,* 1999).

This aspect of the technology also confers a tremendous degree of flexibility since the actual number of fragments amplified using any given template can be optimised by altering the number of selective bases in the PCR primers used.

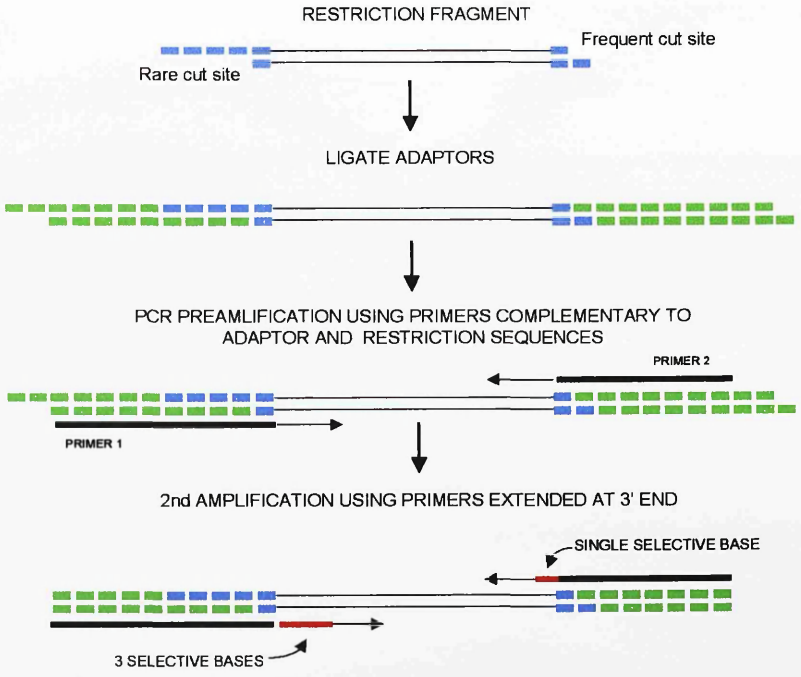


Figure 3-1: Schematic depiction of AFLP principle

It is important to recognise that the selective amplification of a small subset of restriction fragments implies that AFLP generates polymorphic markers that are quite different from RFLP, with which AFLP has inevitably been compared. RFLP markers are truly length polymorphisms, whereas AFLP fingerprints rest primarily on the presence or absence of possible fragments. In this sense the name Amplified fragment length polymorphism may be a little misleading, indeed Professor Vos advised against its use as a true acronym in the original 1995 paper.

Since the original publication a number of variations have been suggested, in particular a simplified version in which the PCR template is prepared using only one restriction enzyme and one adaptor, (Gibson *et al.,* 1999), and analysis by agarose gel electrophoresis, (Valsangiacomo *et al.,* 1995).

However the overwhelming majority of published papers (c200 in 2001), describe work that adheres more closely to the original procedures described by Vos *et al.* (1995). A consensus description of AFLP would involve digestion by two restriction enzymes, a rare cutter and a frequent cutter. The frequent cutter is used to generate smaller fragments which amplify well by PCR and are in the optimal size range for separation on sequencing gels. Since the PCR step appears to favour amplification of fragments with two different ends, (Vos *et al.*, 1995), use of the rare cutter effectively limits the number of fragments which can be amplified. Ligation of two suitable adaptors is followed by one or two rounds of PCR amplification and resolution of fragments using denaturing polyacrylamide gel electrophoresis (fig 2.1). One primer (usually the rare adaptor primer) is labelled in some way so that only one DNA strand from each fragment is visualised on a denaturing gel. Originally the primers were 5'-endlabelled using γ-^{33}P-ATP and fragments were

visualised by autoradiography and sized with reference to molecular weight markers in adjacent lanes. An elegant development has been the use of fluorescent labels with different emission spectra that can be detected separately by Automated DNA sequencers such as the Pharmacia ALFexpress (Van Elder *et al.*, 1999); the Amersham Vistra, (Koeleman *et al.*, 1998); or the Perkin-Elmer ABI (Duim *et al.*, 1999). The advantage of all these technologies is that a better correction for variation in migration rates and gel distortion is obtained by co-electrophoresis in each lane of both sample and marker. Moreover sample fragments labelled with different fluorescent labels may be multiplexed within the same lane, increasing three-fold the amount of information that can be obtained from a single gel.

3.1.2: Applications in Microbiology

The potential of AFLP for the high-resolution discrimination of bacteria was first investigated by Janssen *et al.*, (1996). In this comprehensive evaluation of the technique, the effects of different restriction enzymes and selective primers on the discriminatory power of AFLP was assessed in several different bacterial species. Optimised experimental parameters for 147 strains of nine different species were presented.

Working in the same laboratory, Huys *et al.* (1996) were the first to use AFLP analysis for a major taxonomic survey. A comprehensive reference collection of 98 *Aeromonas* strains, representing all 14 known hybridisation groups as well as four phenospecies not yet allocated to a HG was analysed. A strong correlation was observed between clusters revealed by AFLP analysis and HG, which is still considered by many to be the 'gold standard' for species definition (Wayne *et al.*, 1987; Wayne *et al.*, 1996).

Similarly Janssen *et al.*, (1997) were able to clearly distinguish closely related groups of *Acinetobacter,* while allocating all of 151 strains to a hybridisation group. Intraspecific similarity levels were mostly about 45%, but ranged from 29 to 74%. Focusing on a taxon with far greater intraspecific similarity levels, a taxonomic study of the honeybee pathogen *Paenibacillus,* by Heyndrickx *et al.,* (1996) found that despite more than 90% DNA relatedness, AFLP analysis could distinguish the strains at the subspecies level.

These and other taxonomic studies show that AFLP analysis is a powerful method for delineation of genomic groups within genera. This is very often the level of discrimination required for ecological studies and AFLP has been used very successfully to determine and compare levels of genetic diversity between different habitats or over time. For example Jiang *et al.* (2000a) were able to identify shifts in the population structure of *Vibrio cholerae* that was correlated with seasonal temperatures.

However it is important that any study of bacterial population structure in natural environments is backed up by a sound taxonomic understanding as AFLP is not suited for analysis beyond the genus level. This is because below approximately 40% similarity, very few bands are shared between strains (Rademaker *et al.,* 2000), because of the danger of homoplasy, comparisons based on very few bands may be misleading.

For epidemiological purposes, the level of divergence of interest is usually narrower than would be normal in a taxonomic study. Since AFLP is most discriminating at the strain and subspecies level (Savelkoul *et al.,* 1999), the technique is becoming increasingly popular in this area.

Simmoons-Smit *et al.* (2000), for example were able to demonstrate that clonal transmission of *Staphylococcus aureus* strains between members of a family and their pet cat and dog, was the cause of a recurring infection. On the basis of the AFLP fingerprints, the animals were identified as the reservoir.

Duim *et al.*, (2000) have recently published the results of an AFLP-based study that has greatly clarified the relationship between *Campylobacter jejuni* strains isolated from chickens and from humans with gastroenteritis or Guillain-Barré syndrome. Elsewhere Dijkshoorn *et al.*, (1996) have been able to distinguish between strains of *Acinetobacter baumannii* linked to a nosocomial outbreak, and other background strains. Keim *et al.*, (1997) were able to show that the ongoing anthrax epidemic in Canada and the northern United States is due to the introduction of a single strain that has remained stable for 30 years. And Valsangiacomo *et al.*, (1996) were able to identify, using AFLP, the water source that was the origin of *Legionella pneumophila* strains implicated in a particularly severe nosocomial outbreak.

Starting with a handful of papers published in 1996, the use of AFLP in medical and veterinary microbiology has mushroomed and several dozen studies were described in the first half of 2001 alone. It is likely that this trend will continue in the near future. It remains to be seen whether the highly reproducible nature of AFLP will encourage the compilation of AFLP databases which could be shared amongst laboratories as sequence data is. Certainly this would be a welcome development that could encourage collaborations that might uncover global trends. Unfortunately, the very versatility of the AFLP technique has been a disincentive to the adoption of standardised protocols and it is likely that this will hamper comparison between databases for the time being.

Aims of this study

This part of the present work was undertaken with three broad aims:

1: To develop an AFLP protocol suitable for use with the collection of *Aeromonas* strains that has been assembled for this study.

2: To further adapt the protocol for use with a PE-Applied Biosystems ABI377 automated DNA sequencer.

3: To exploit the high resolving power of AFLP that has been reported elsewhere to search for geographic, habitat and host associated clusters within the assembled collection. In particular to determine the degree of genetic similarity or otherwise between aquaculture-associated isolates and those implicated in human disease.

3.2: Methods and Materials

3.2.1: DNA Isolation

Chromosomal DNA was isolated using a modification of the method described by Pitcher *et al.* (1989).

Each strain was grown to late log phase in Tryptone Soya Broth (Oxoid) at 30°C in an orbital shaker and 1.5ml broth was harvested by centrifugation at 1000g for 1min. Cells were resuspended in 1ml resuspension buffer (0.15M NaCl, 0.01M EDTA pH8). Cells were pelleted again by centrifugation at 1000g for 1 min, the supernatant was removed with an aspirator and this washing step was repeated. Following the second wash the pellet was resuspended in 100µl TE buffer and lysed by the addition of 0.5ml GES reagent (5M guanidium thiocyanate, 100mM EDTA, 0.5% v/v sarkosyl, pH8).

Suspensions were mixed gently by inversion and held at room temperature for 10 minutes. Lysates were cooled on ice and 0.25ml chilled (-20°C) 7.5M ammonium acetate added. Samples were held on ice, with occasional gentle mixing for 10 minutes and 0.5ml chloroform/2-pentanol (24:1) was added. After thorough mixing the phases were separated by centrifugation at 10 000g for 10 min. Using a wide-bore pipette tip, 700µl of the upper aqueous phase was removed and transferred to a fresh eppendorf tube containing 0.54 volumes (378µl) chilled isopropanol. Tubes were gently inverted for approximately 1minute until a cloud of precipitated DNA was visible and the precipitated DNA deposited by centrifugation at 7000g for 1 min. DNA pellets were washed three times in 70% ethanol and dried for 2 hr at room

temperature. DNA was resuspended overnight at 50°C in 100µl sterile $TE_{0.1}$ buffer (pH8).

RNA was removed by the addition of 5µl of a 1mg.ml^{-1} stock solution of RNAaseA (Sigma) followed by incubation at 37°C for 1hr.

DNA integrity was checked by electrophoresis of a 2µl aliquot through a 0.8% agarose gel containing 5µg.ml^{-1} ethidium bromide for 1hr at 5V.cm^{-1} in 0.5X TBE buffer. As a concentration standard, 500ng, 250ng, 125ng, 62ng and 32ng of λ-DNA (Pharmacia) were loaded into the first five lanes on each gel.

Gels were examined under UV light on a transilluminator and an image recorded using a CCD camera. DNA concentrations were estimated by comparing the intensity of fluorescence relative to the concentration standard using Gelworks 1.1 (UVP) or ScionImage (Scion Corporation) software.

All samples were diluted to 5ng.µl^{-1} with sterile $TE_{0.1}$ and stored at 4°C.

3.2.2: AFLP Template Preparation

AFLP templates were prepared according to the protocols of Vos *et al.* (1995), Janssen *et al.* (1996) and G. Huys, Universitiet Gent, Belgium (Pers. com.) with modifications.

3.2.2.1: Preparation of Adaptors

Adaptors were prepared by mixing, in equimolar amounts, pairs of partially complementary single-stranded oligonucleotides in nanopure water. Perfect annealing was ensured by heating each pair to 94°C in a thermal cycler, which was

then switched off and allowed to cool slowly to room temperature. The *Apa*1 adaptor was prepared using 2μM (each) 5'-TCG TAG ACT GCG TAC AGG CC-3' and 5'-TGT ACG CAG TCT AC-3'. The *Taq*1 adaptor was prepared using 20μM (each) 5'-GAC GAT GAG TCC TGA C-3' and 5'-CGG TCA GGA CTC AT-3'.

3.2.2.2: *Taq*1 restriction

Digestion was carried out in a total volume of 30μl containing:

50ng purified genomic DNA;

1X One-Phor-All buffer (Pharmacia, Uppsala, Sweden) (=10mM Tris-acetate (pH7.5); 10mM magnesium acetate; 50mM potassium acetate);

5mM dithiothreitol (DTT) (Sigma);

100ng.μl^{-1} acetylated Bovine Serum Albumin (BSA) (Promega)

15Units *Taq*1 (Pharmacia).

Restriction was allowed to proceed for 90minutes at 65°C.

3.2.2.3: *Apa*1 restriction

Immediately following *Taq*1 restriction, tubes were allowed to cool to room temperature and 15Units *Apa*1, in 2μl 1X One-Phor-All buffer was added to each.

Restriction was continued for 90minutes at 37°C.

3.2.2.4: Ligation

Immediately following *Apa*1 restriction, 8μl ligation mix was added to each tube.

Ligation mix contained:

1X One-Phor-All buffer;

5mM DTT;

100ng.μl^{-1} BSA;

1mM ATP (Sigma);

2μM *Taq*1 adaptor;

0.2μM *Apa*1 adaptor

0.125U.$\mu l.^{-1}$ T4 DNA-ligase (Promega).

After incubation for a further 3hours at 37°C templates were diluted five-fold by addition of 160μl $TE_{0.1}$ and stored at –20°C.

3.2.3: Type strain Reference Template

For validation of the fingerprints of *Aeromonas* type strains held at the IOA, additional AFLP templates prepared from strains held in the type culture collection at the Universitied Gent, Belgium, were obtained (Table 3.1). These templates were the kind gift of Dr. Geert Huys, Laboratorium voor Microbiologie, Universitiet Gent.

Key to Accession numbers in Table 3.1:

LMG: Laboratorium voor Microbiologie, Universitiet Gent

NCIMB: National Collections of Industrial, Food and Marine Bacteria, Aberdeen, UK

ATCC: American Type Culture Collection, LGC Promochem, Teddington, UK

CECT: Coleccion Espanola de Cultivos Tipo, Universidad de Valencia, Spain

LMG designation	Strain type	IOA Type strain equivalent
LMG 2844T	(*A. hydrophila* HG1)	NCIMB 9240
LMG 13444T	(*A. bestiarum* HG2)	N/A
LMG 3780T	(*A. salmonicida* subsp. *salmonicida* HG3)	NCIMB 1102
LMG 3775T	(*A. caviae* HG4)	NCIMB 13016
LMG 9073T	(*A. media* HG5)	NCIMB 2237
LMG 3774T	(*A. eucrenophila* HG6)	NCIMB 74
LMG 3783T	(*A. sobria* HG7)	NCIMB 12065
LMG 9075T	(*A. veronii* HG8/10)	NCIMB13015
LMG 12221T	(*A. jandaei* HG9)	ATCC 49568
LMG 16330T	(*A. encheleia* HG11)	CECT 4342 T
LMG 9074T	(*A. schubertii* HG12)	NCIMB 13161
LMG 12223T	(*A. trota* HG13)	ATCC 49657
LMG 14059T	(*A. allosaccharophila*)	N/A
LMG 17541T	(*A. popoffii*)	N/A

Table 3-1: Type strain AFLP template obtained from Universitiet Gent

3.2.4: PCR Primers

Preselective primers was designed according to Vos (1995). The sequence of the *Apa*1 preselective primer was 5'-GACTGCGTACAGGCCC-3' and the sequence of the *Taq*1 preselective primer was 5'-CGATGAGTCCTGACCGA-3'. Stock solutions were diluted to 10μM before use.

Selective primers were designed according to Huys *et al.* (1996). The sequence of the *Taq*1 selective primer PT01 was 5'-CGATGAGTCCTGACCGAA-3'. The sequence of the selective *Apa*1 primer A01 (Huys *et al.* 1996) was 5'-GACTGCGTACAGGCCCA-3'. Two further selective *Apa*1 primers were designed, these were A02: 5'-GACTGCGTACAGGCCCT-3' and A03: 5'-GACTGCGTACAGGCCCG-3' (selective bases are underlined).

Unlabelled primers were purchased from MWG Biotech. Fluorescently labelled primers for use with the automated DNA sequencer were purchased from PE Applied Biosystems.

Primers A01, A02, and A03 were 5'-endlabelled with TET, 6-FAM and HEX respectively.

For detection of AFLP fragments by autoradiography primers were 5'-endlabelled immediately prior to use.

3.2.5: 5'-Endlabelling of primer A01

To label sufficient primer for 100 PCR reactions, 500ng primer A01 was incubated at 37°C for 90 minutes with 20Units T4 polynucleotide kinase (PNK) (Pharmacia) and

1μCi γ^{32}P-ATP or 1.5 1μCi γ^{33}P-ATP (ICN Biomedical, Oxon, UK) in 50μl of 1X PNK buffer. The PNK was then inactivated by heating to 65°C for 15minutes. Primers labelled with γ^{32}P were used immediately whereas primers labelled with γ^{33}P may be stored for several weeks.

3.2.6: Preselective amplification

Where appropriate, preselective amplification was performed according to the protocol that accompanies the PE Applied Biosystems AFLP microbial fingerprinting kit.

Reactions were performed in a final volume of 20μl containing:

1X Promega PCR reaction buffer (=10 mM Tris-HCI (pH 9 at 25 ^{0}C); 50 mM KCl; 0.1 % Triton X-100);

200μM each dNTP (Promega, MA., USA);

O.25μM (each) preselective primer;

1.5mM $MgCl_2$;

0.5 Units *Taq* DNA polymerase (Promega);

and 200pg AFLP template

PCR amplification reactions were performed in thin walled 200μl dome topped microcentrifuge tubes (PE Applied Biosystems), using a Perkin Elmer 9700 96-well thermal cycler.

The PCR profile was as follows:

1): 72°C for 2 minutes

2): 94°C for 20seconds (denaturation)

3): 56°C for 30seconds (annealing)

4): 72°C for 2minutes (elongation)

Ramp rates were set to 90%

Steps (2) to (4) were repeated for 20 cycles:

Preselective amplification products were diluted 20-fold with $TE_{0.1}$ buffer and stored at 4°C.

3.2.7: Selective amplification

Selective amplification was performed according to the protocols of Vos *et al.* (1995), Janssen *et al.* (1996) and the PE Applied Biosystems AFLP microbial fingerprinting kit protocol with modifications.

In order to compare fingerprint patterns generated using a preselective amplification step (as recommended by PE Applied Biosystems and without (as recommended by Vos *et al.* (1995), the selective amplification step was performed in two ways, using either the original AFLP templates or the preselective PCR products as template for the selective step.

All amplification reactions were performed in thin walled 200µl dome topped microcentrifuge tubes (PE Applied Biosystems), using a Perkin Elmer 9700 96-well thermal cycler.

Each batch of PCR reactions included a negative control containing $TE_{0.1}$ buffer in place of template.

Selective PCR was carried out in 20µl volumes, containing:

1X Promega PCR reaction buffer (=10 mM Tris-HCI (pH 9 at 25 ^{0}C); 50 mM KCl; 0.1 % Triton X-100);

200μM each dNTP (Promega);

30ng unlabelled primer, PT01;

5ng labelled primer, PA01, PA02, or PA03

1.5mM $MgCl_2$;

0.4 Units *Taq* DNA polymerase (Promega);

either 5μl (c1.25ng) AFLP template or 1.25μl diluted preselective PCR product.

The PCR profile was as follows:

Cycle 1 1): Slow ramp ($1°C.s^{-1}$) up to 72°C

2): 30seconds at 94°C

3): 30seconds at 65°C

4): 1minute at 72°C

Cycles 2-13 1): 30seconds at 94°C

2): 30seconds at 65°C, decreasing 0.7°C per cycle

3): 1minute at 72°C

Cycles 14-40 1): 30seconds at 94°C

2): 30seconds at 56°C

3): 1minute at 72°C

NB: At all stages other than step 1, the ramp rate was set to maximum.

3.2.8: Cycle-sequencing M13 size standard

SS-M13 DNA template was used to prepare a single-base resolution size ladder using a Gibco M13 cycle sequencing kit according to the manufacturers instructions.

Briefly, 3µl of each ddNTP was placed in one of four separate tubes and held on ice.

A cycle sequencing master mix was prepared, consisting of:

50fmoles M13 template;

4µl 10X buffer;

1µl *Taq* DNA polymerase;

1µl M13 –20 Universal primer

0.5-1µl α^{33}P-ATP (3000Ci.mmol.$^{-1}$) (ICN Biomedical, Oxon, UK)

nanopure water to 30µl.

A 7µl aliquot of master mix was then added to each of the four ddNTP's and tubes transferred swiftly to a thermal cycler pre-chilled to 4°C with the lid pre-heated.

The PCR profile was as follows:

1): 95°C for 30seconds

2): 95°C for 30seconds

3): 60°C for 30 seconds

4): 72°C for 30 seconds; Steps 2-4 repeated for 30 cycles.

5): Hold at 4°C

As soon as the PCR was complete, 5µl stop solution was added to each tube.

3.2.9: P.A.G.E. of radio-labelled AFLP fingerprints

Radioactively-labelled AFLP products were resolved in a 38x50x0.04cm denaturing 5% polyacrylamide gel using a BioRad SequiGen electrophoresis unit.

Prior to casting both glass plates were cleaned thoroughly and rinsed with ethanol. The larger (back) plate was treated with repel silane (Pharmacia), allowed to dry, rinsed first with ethanol, then deionised water.

The plates were assembled according to the manufacturer's instructions, using 0.4mm spacers and the casting unit attached to the clamped plates. The assembly was levelled precisely with the aid of a spirit level.

Approximately 70ml deionised water and 10ml 10X TBE and added to 40.45g urea (Sigma), this was warmed for 20seconds in a microwave oven and the urea dissolved with the aid of a magnetic stirrer. The solution was transferred to a closed measuring flask and 12.5ml 40% 19:1 acrylamide/methylene bisacrylamide solution (BioRad) was added. The mix was made up to 100ml with deionised water and, stirring rapidly, 250µl 10% Ammonium persulphate (Sigma) and 100µl N,N,N',N'-tetramethylethylenediamine (TEMED; Sigma) was added. Avoiding air bubbles, the mix was immediately transferred to a 150ml syringe and the gel cast at once. The flat edge of a 97 well sharkstooth comb was inserted between the plates to a depth of 4-5mm and the plates were clamped using bulldog clips.

The gel was left to polymerise for 2hours, or longer provided precautions were taken to prevent the gel drying out.

Following polymerisation, the top of the gel was flooded with 1X TBE and the comb eased out, then reinserted so that the teeth made contact with, but did not penetrate, the surface of the gel.

Immediately prior to electrophoresis, 2.5µl aliquots of each AFLP product were mixed with an equal volume of loading dye and denatured by heating to 94°C for 4 minutes then quickly cooled on ice.

Using 1X TBE as the running buffer the gel was pre-run for approximately 30 minutes at 110W until it had reached 55°C. Following the pre-run, cooling whilst loading was lessened by means of home-made foam heat shields.

Prior to loading wells were flushed out using a Pasteur pipette. Depending on the depth of the well, between 1.5 and 2µl sample was loaded into alternate wells. M13 sequence ladder was loaded at each side and in the middle of each gel.

After the first gel, one fingerprint (C78) with a well-spaced set of clear fragments was chosen for inclusion on subsequent gels, for comparison between gels. Eventually this fingerprint was substituted for the M13 as a size marker.

The gel was run at 110W and all samples were resolved using two gels: one was run for 90minutes and the second for 3hours for better resolution of fragments larger than 300bp.

Following the run, the gel was allowed to cool and the repel-silane treated plate was prized away leaving the gel adhering to the front plate.

The comb and spacers were removed and a piece of Whatman 3MM paper cut to size was placed on top of the gel. The paper was lifted away with the gel attached. The gel and paper were covered with Clingfilm.

The gel was dried using a Flowgen vacuum slab gel dryer for 1 hour at 80°C, then placed in a cassette with a sheet of Kodak Biomax MS film and exposed for 5hours to one week depending on the age of the isotope.

3.2.10: Electrophoresis using an ABI377 automated DNA sequencer

Fluorescently labelled AFLP products were resolved using 0.2mm denaturing 5% polyacrylamide gels using a PE Applied Biosystems 377 automated DNA sequencer.

Preparation and casting the gel followed essentially the same procedures described above with minor differences to allow for the slightly different casting apparatus. The main difference was in the additional precautions necessary to avoid contamination of the gel by any material that might cause fluorescence. A non-fluorescent detergent (Alconox) was used to clean the plates which were thoroughly rinsed with nanopure water and allowed to dry naturally or using a hair-drier. Lint-free tissue was used to dry all other components.

Urea (18g) was dissolved in 27.5ml nanopure water and 5.2ml 40% 19:1 acrylamide/methylene bisacrylamide (Biorad) added.

The urea/acrylamide solution was deionised by addition of 0.5g mixed-bed resin, then filtered using a 0.2µ filter, 5ml 10X TBE was then added to the filter and the mix was de-gassed under vacuum for 2 minutes prior to addition of 250µl 10% Ammonium persulphate and 25µl TEMED.

The mix was introduced into a 50ml syringe and the gel cast immediately between 36cm plates and allowed to polymerise for up to four hours.

Following a plate check and assembly of the buffer reservoirs and heat-plate, the gel was pre-run using module PR-GS2400-36C for approximately 30 minutes.

The following were assembled in a 200µl PCR tube;

1.25µl deionised formamide

0.25µl blue dextran/50mM EDTA loading solution (PE Applied Biosystems)

0.25µl GeneScan-500 [TAMRA] internal size standard (PE Applied Biosystems)

0.3µl TET-labelled sample

0.4µl 6-FAM-labelled sample

0.6µl HEX-labelled sample

Tubes were heated to 95°C for 3minutes and quickly cooled on ice.

The pre-run was paused, 1.4µl sample was loaded into alternate wells and the pre-run resumed for 2 minutes, then paused again whilst the remaining wells were loaded.

Using run module R-GS2400-36C, the gel was run for 3.2hours.

3.2.11: Data Processing and Image Analysis

Using a flatbed scanner an image of each autoradiograph was recorded at a resolution of 300dpi. Using the M13 sequence as a guide, images were cropped to include only fragments sized between 50 and 300bp (gels run for 90minutes) and 301-500bp (gels run for 180minutes) and stored as tif files. These images were imported into GelCompar version 2.0 (Applied Maths BVBA, Kortrijk, Belgium). After normalisation and background subtraction using the rolling disc principle, recorded fragments were sized using the M13-G fragments as a size marker. The accuracy of size calling was confirmed by comparing predicted sizes of the other M13 fragments, with their known values.

After installation of the matrix file, ABI gel files were tracked and extracted using GeneScan analysis software (PE Applied Biosystems) and each chromatogram was saved as an individual sample file. These were then imported into ABICONV 2.0 (Applied Maths BVBA, Kortrijk, Belgium).

This program converts the ABI sample files into the GelCompar 2.0 curve format by splitting the multichannel sample files into separate virtual gel files for each available colour. Logically each of these virtual gels contains the same lanes at the

same positions. One contains the internal reference patterns, whereas the others contain the real data to be normalised according to the reference pattern. Having imported the ABICONV files into GelCompar 2.0, the reference gel was normalised and the GeneScan-500 fragment sizes assigned to bands. The normalisation pattern from the reference gel was then superimposed onto each of the other three virtual gels.

Comparative analysis of both ABI and autoradiography data was performed using the Pearson product-moment correlation coefficient algorithm applied to all points falling in the active zones of the band patterns. Cluster analysis was performed using the unweighted pair-group method, using arithmetic averages (UPGMA) algorithm (Sneath and Sokal (1973). Optimisation and position tolerance settings were determined by applying the maximised group contrast method to the fingerprints obtained from Hybridisation Group type strains.

Similarity matrices based on the Pearson product-moment correlation coefficient, generated by Gelcompar 2.0 were exported as text files and imported into Microsoft Excel for further editing. Edited matrices were compared using a Mantel Test (Mantel, 1967; Smouse, Long and Sokal, 1986) using 'Mantel' software, which was the kind gift of Professor Roger Thorpe, Dept of Biological Sciences, University of North Wales, Bangor.

Principal co-ordinate analysis was performed using Multivariate Statistical Package (MVSP; Kovach Computing Services, Pentraeth, Angelsey, Wales, UK. (http//www.kovcom.com).

3.3: Results

3.3.1: Radiolabelled AFLP Fingerprints

Initially AFLP fingerprints were produced using template prepared using the method of Janssen *et al.* (1996). Fig 3.2 shows an autoradiograph obtained in this way. This was clearly an unsatisfactory result: AFLP bands vary considerably in intensity and are not sharply delineated, in addition there is much 'smearing' of bands caused by non-specific PCR amplification.

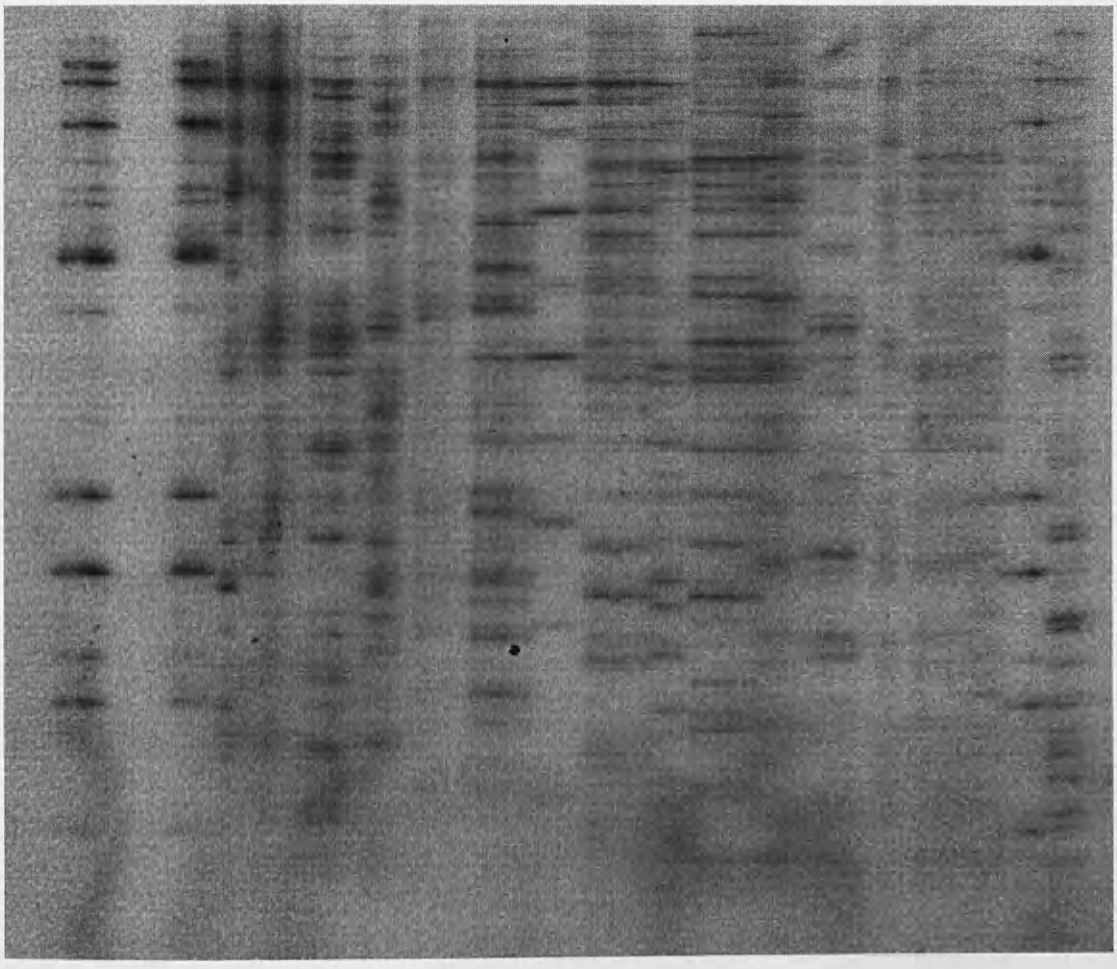


Figure 3-2: Autoradiograph of AFLP1

Note the poor band definition, subsequently attributed to polysaccharide co-purified with template DNA

Closer examination of early autoradiographs revealed that the least satisfactory fingerprints were obtained from those *Aeromonas* strains which produce visible amounts of extracellular polysaccharide. These strains are easily identifiable by the translucent appearance of colonies on agar and wet texture when picked up using a culture loop.

Large amounts of polysaccharides can interfere with restriction digestion (Milligan, 1998) and since complete restriction is crucial for AFLP reproducibility measures were introduced to minimise the influence of excess polysaccharides. These were:

1: Growing bacteria for DNA extraction in broth rather than on agar.

2: Ensuring that cultures harvested for DNA extraction were in mid, not late log phase.

3: Addition of two wash steps, in isotonic buffer, prior to cell lysis and DNA extraction.

4: Use of a much smaller amount of DNA for restriction digestion than suggested by Janssen *et al.* (1996): 50ng instead of 1µg.

5: Use of a much smaller amount of AFLP template for PCR amplification than suggested by Janssen *et al.* (1996): (1.25ng instead of 20ng)

6: Use of more PCR cycles than suggested by Janssen *et al.* (1996): (30 instead of 24) to compensate for the smaller amount of template used.

Using this modified protocol, non-specific PCR amplification was much reduced and AFLP bands were more sharply delineated. An example of an autoradiograph produced this way is shown in Fig 3.3.

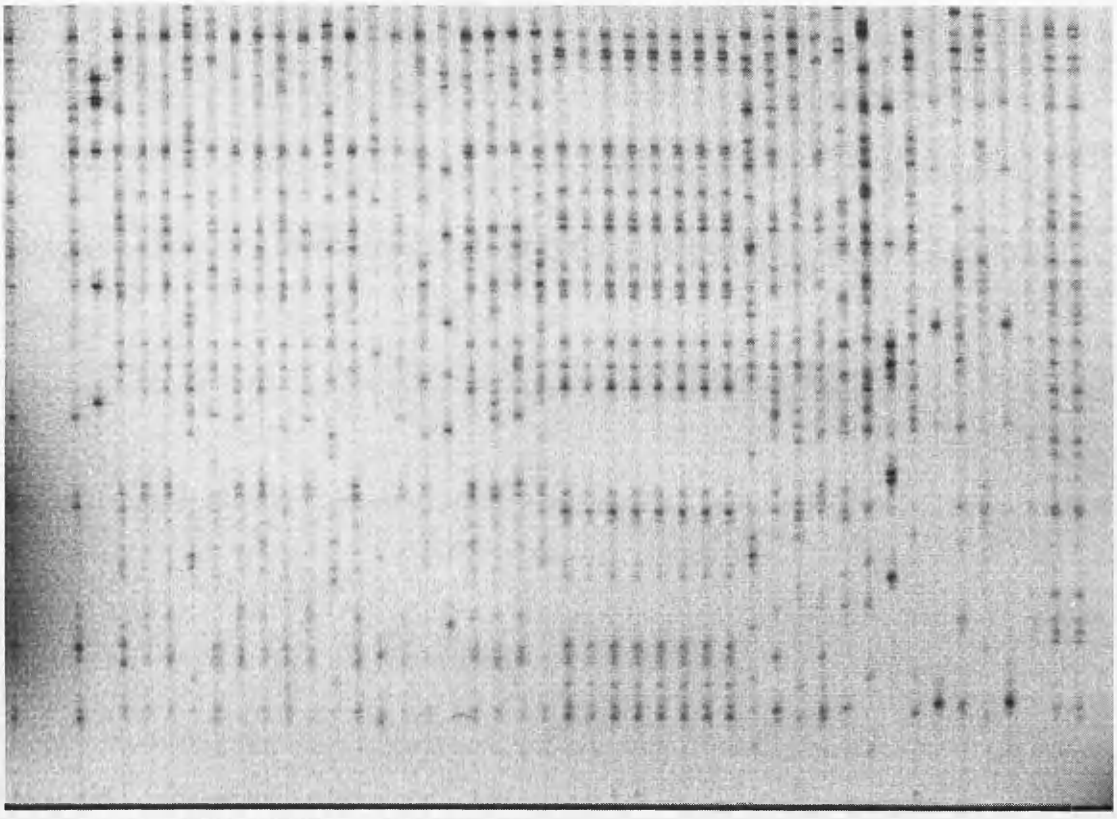


Figure 3-3: Autoradiograph of AFLP4 showing improved fragment resolution
Note the improved band resolution resulting from measures to reduce polysaccharide contamination of restricted template.

Using PA01 as the labelled primer in AFLP reactions, between 50 and 80 PCR products were visualised per lane, from each *Aeromonas* genome. Generally fragment sizes were evenly distributed so that, for each sample, a roughly equal number of fragments was observed on each of the two autoradiographs (90 minute and 180minute run time).

In order to assess the reproducibility of the method, the AFLP fingerprints generated from *Aeromonas hydrophila* type strain NCIMB9240, run on four different gels were compared pairwise using GelCompar, with each other and with the fingerprints generated using AFLP template independently prepared from DNA separately isolated. Following normalisation and subtraction of background values it was found

that the correlation between samples run on different gels and in separate lanes of the same gel was between 97.6 and 98.9%. The correlations between fingerprints generated using independently prepared template also fell within this range suggesting that most of the variability in AFLP data produced using this method, arises during the electrophoresis step.

3.3.2: Fluorescently labelled AFLP fingerprints

Effect of Including a Preselective PCR step

The AFLP fingerprints of 9 *Aeromonas* type strains, produced with and and without inclusion of the preselective PCR step, and resolved on a single gel, were compared. There were significant differences in the fingerprints obtained via these two methods. Pairwise correlations, ranging from 81.7% to 91.1% are presented in table 2.1.

Strain	HG	Pearson correlation
A.hydrophila NCIMB 9240	1	86.2%
A.caviae NCIMB 13016	5A	87.9%
A.media NCIMB 2237	5B	91.1%
A.eucrenophila NCIMB 74T8	6	88.4%
A.sobria NCIMB 12065	7	84.5%
A.veronii bv. sobria NCIMB 37	8	89.3%
A.jandaei ATCC 49568	9	90.4%
A.schubertii NCIMB 13161	12	81.7%
A.trota ATCC 49657	13	87.9%

Table 3-2:pairwise Pearson product moment correlations between AFLP fingerprints obtained with and without inclusion of a pre-selective PCR step

Although a small number of bands visible when the preselective step was omitted, were not seen when the preselective step was included, the majority of fragments produced were of comparable sizes. Most of the disagreement appeared to arise as a result of differences in band intensity. Peak heights were far more even when the preselective step was not included.

Since it appeared that inclusion of the preselective step offered no particular advantage, it was not included in subsequent work.

Fig 3.4 shows a representation of a gel image, generated as pict file via ABI collection software.

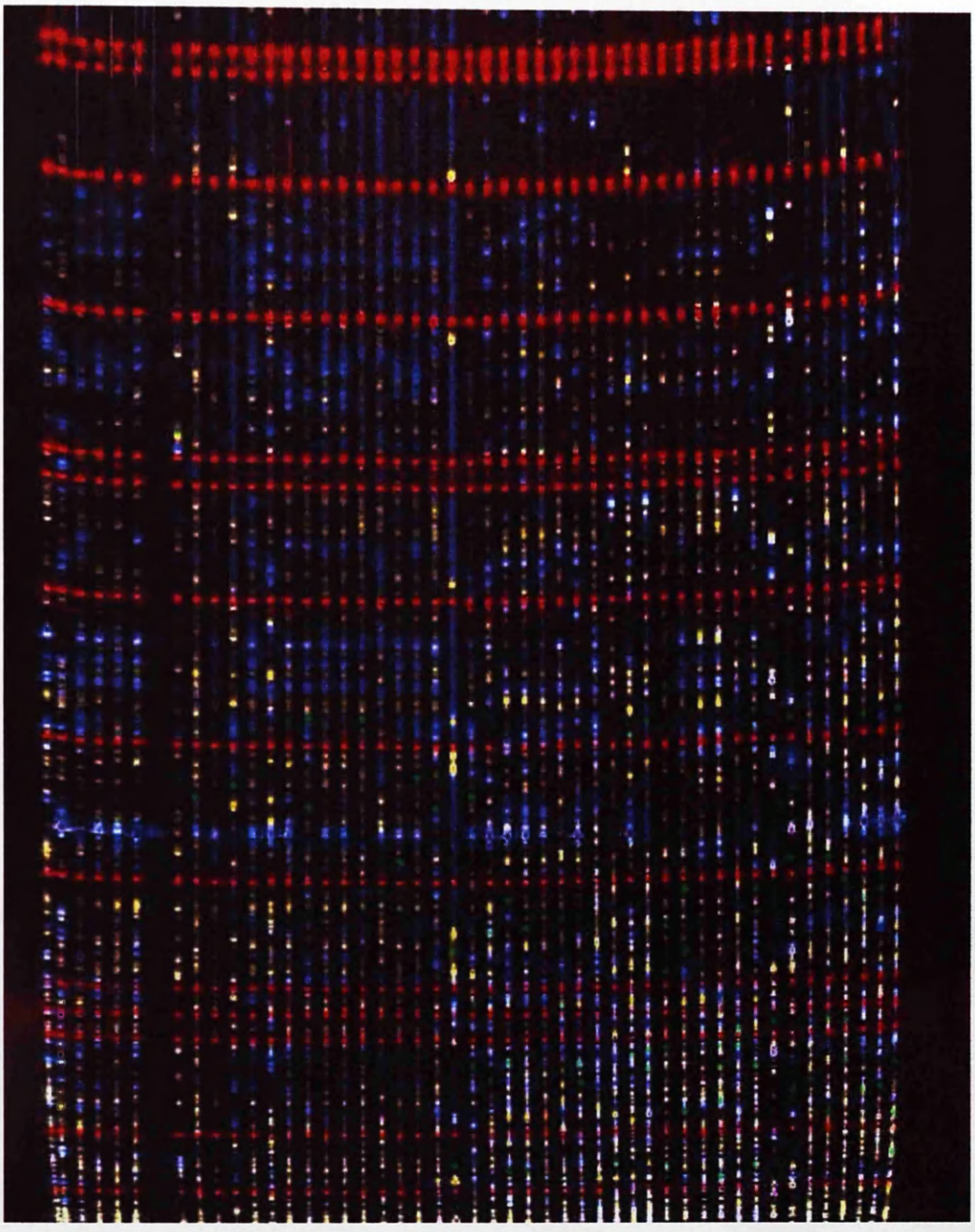


Figure 3-4: ABI Gel image

3.3.3: Reproducibility

To assess reproducibility and to compare the reproducibility of this method with the radioactive method, the AFLP fingerprints generated from *Aeromonas hydrophila* type strain NCIMB9240, run on four different gels were compared pairwise, as above.

The correlation between equivalent samples run on different gels ranged from 97.3% to 98.5%, significantly lower than the correlation between fingerprints resolved using autoradiography.

3.3.4: Comparability of the two methods

AFLP fingerprints using radioactively labelled primer was performed using primer PA01 only. Therefore comparison between the two methods is limited to comparison of the results obtained using using TET- labelled PA01 and radiolabelled PA01.

Direct visual comparison between the two methods is not possible: the gel image in Fig 3.4 gives a rather poor representation of the bands present. A more accurate visual comparison is possible by comparing the autoradiograph in Fig 3.3 with Fig 3.5 which is the virtual image of the green channel generated by Gelcompar 2.0, after normalisation and background subtraction and therefore shows only the bands generated by TET- labelled primer, PA01.

It is apparent that the fluorescently labelled fragments vary far more in intensity than do the radioactively labelled fragments. The signal to noise ratio of autoradiograph images ranged from 200 to 1050 whilst the ABI images generally had lower signal to noise ratios, ranging from 140 to 600.

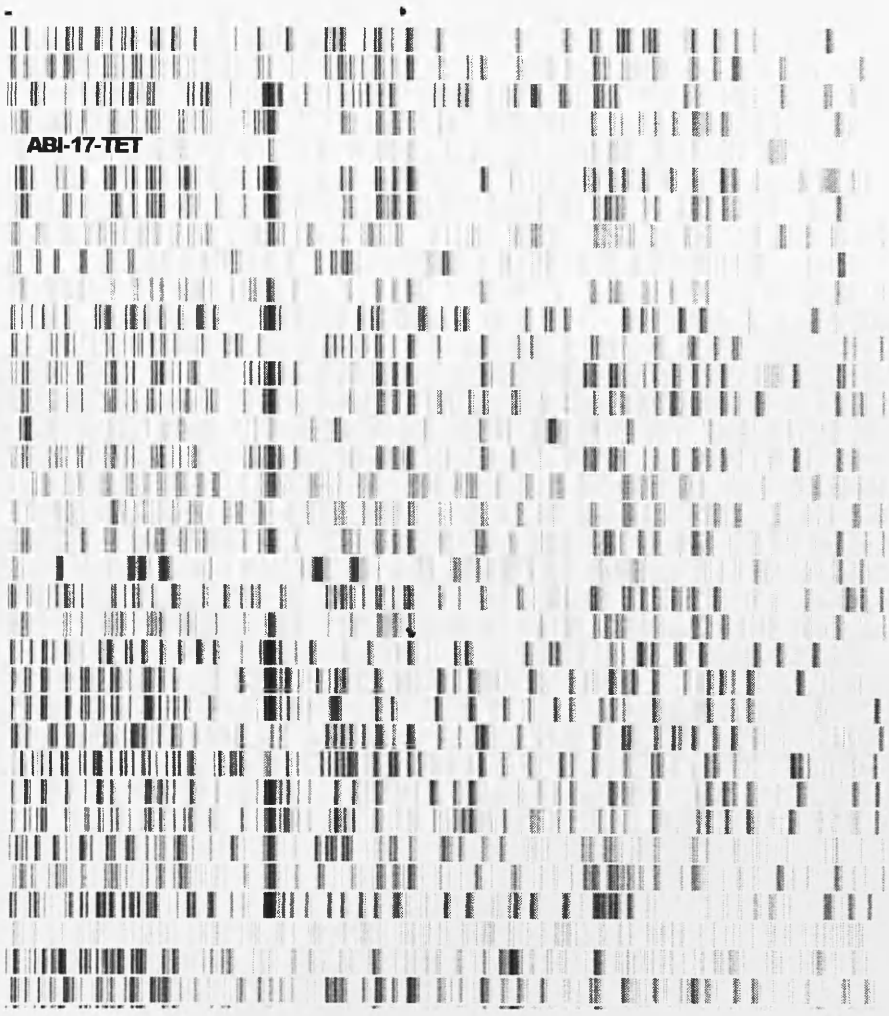


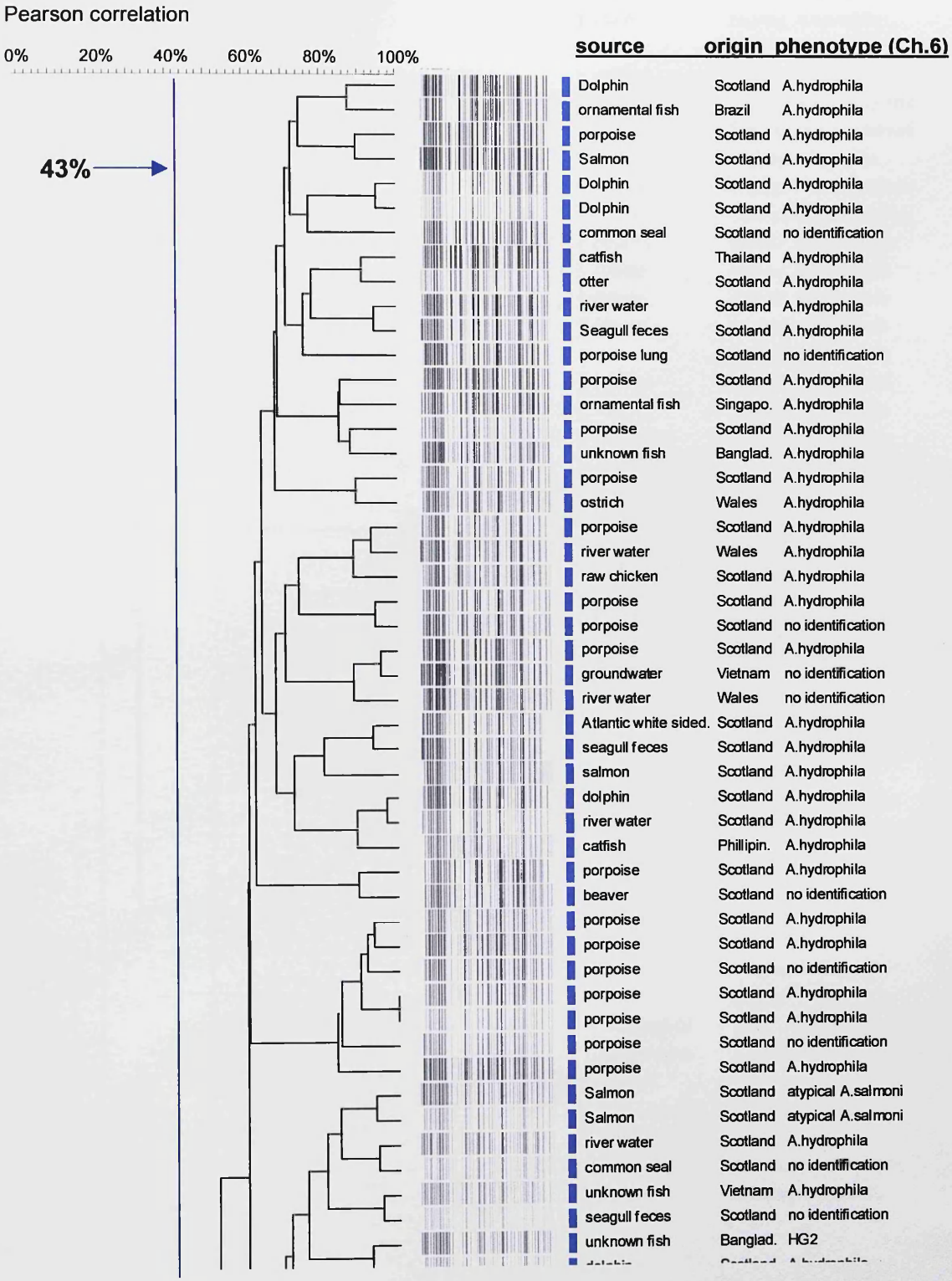
Figure 3-5: Section of virtual Gelcompar image showing TET-labelled bands

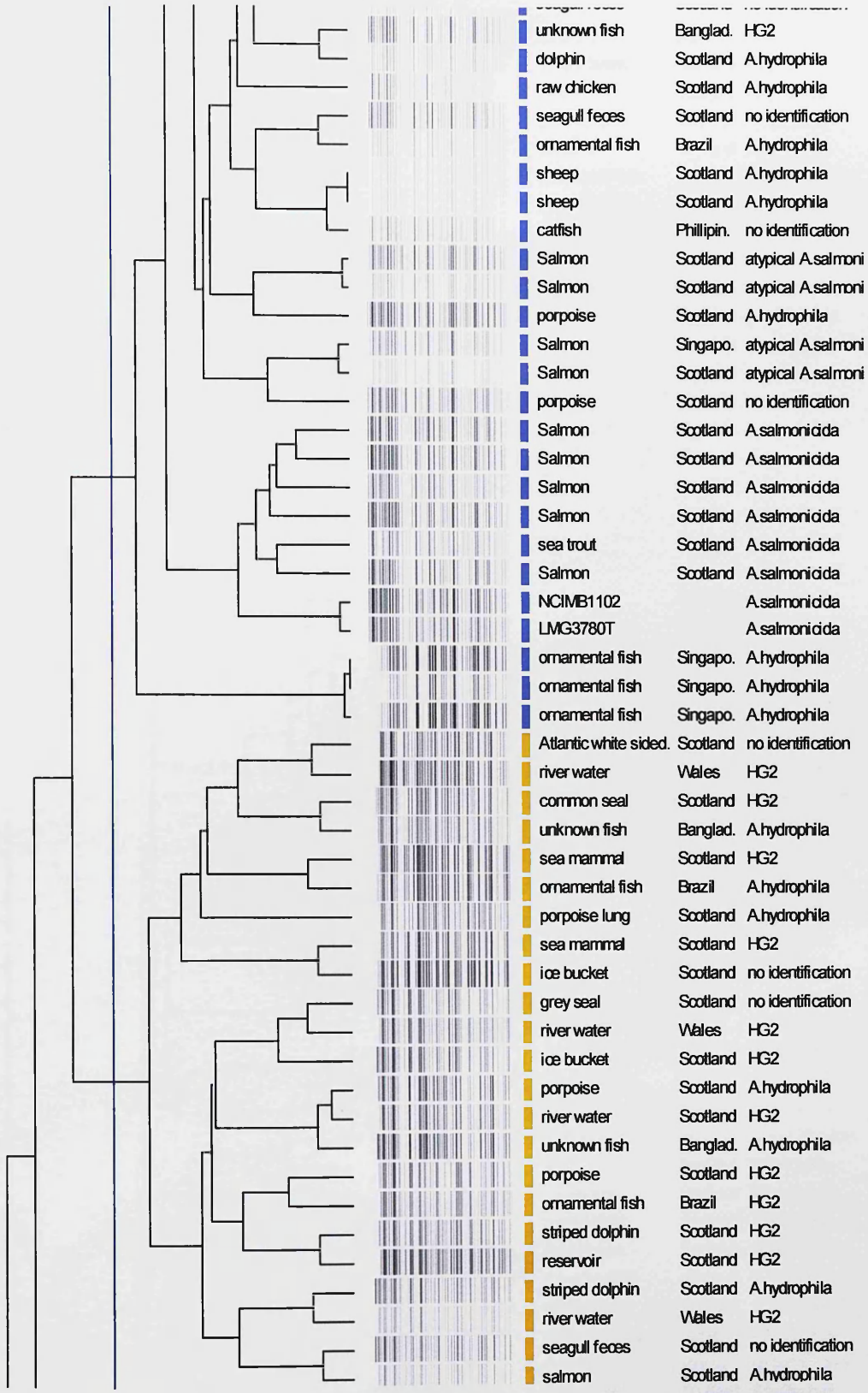
The AFLP fingerprints of 96 strains were analysed using both methods. Similarity matrices were generated for each. These were compared using via a Mantel test and found to be not significantly different at 95% significance level.

Although band assignment was not used for cluster analysis, the band autosearch facility in Gelcompar 2.0 was used to compare the fingerprints generated using the two methods. This comparison revealed that approximately 10% fewer bands are visualised when fragments are fluorescently labelled, than when radioactively labelled.

3.3.5: Cluster analysis of *Aeromonas* strains

Figure 3-6: Gelcompar generated dendrogram showing AFLP clustering of all isolates

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dolphin Scotland A.hydrophila
raw chicken Scotland A.hydrophila
seagull feces Scotland no identification
ornamental fish Brazil A.hydrophila
sheep Scotland A.hydrophila
sheep Scotland A.hydrophila
catfish Phillipin. no identification
Salmon Scotland atypical A.salmoni
Salmon Scotland atypical A.salmoni
porpoise Scotland A.hydrophila
Salmon Singapo. atypical A.salmoni
Salmon Scotland atypical A.salmoni
porpoise Scotland no identification
Salmon Scotland A.salmonicida
Salmon Scotland A.salmonicida
Salmon Scotland A.salmonicida
Salmon Scotland A.salmonicida
sea trout Scotland A.salmonicida
Salmon Scotland A.salmonicida
NCIMB1102 A.salmonicida
LMG3780T A.salmonicida
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ornamental fish Singapo. A.hydrophila
Atlantic white sided. Scotland no identification
river water Wales HG2
common seal Scotland HG2
unknown fish Banglad. A.hydrophila
sea mammal Scotland HG2
ornamental fish Brazil A.hydrophila
porpoise lung Scotland A.hydrophila
sea mammal Scotland HG2
ice bucket Scotland no identification
grey seal Scotland no identification
river water Wales HG2
ice bucket Scotland HG2
porpoise Scotland A.hydrophila
river water Scotland HG2
unknown fish Banglad. A.hydrophila
porpoise Scotland HG2
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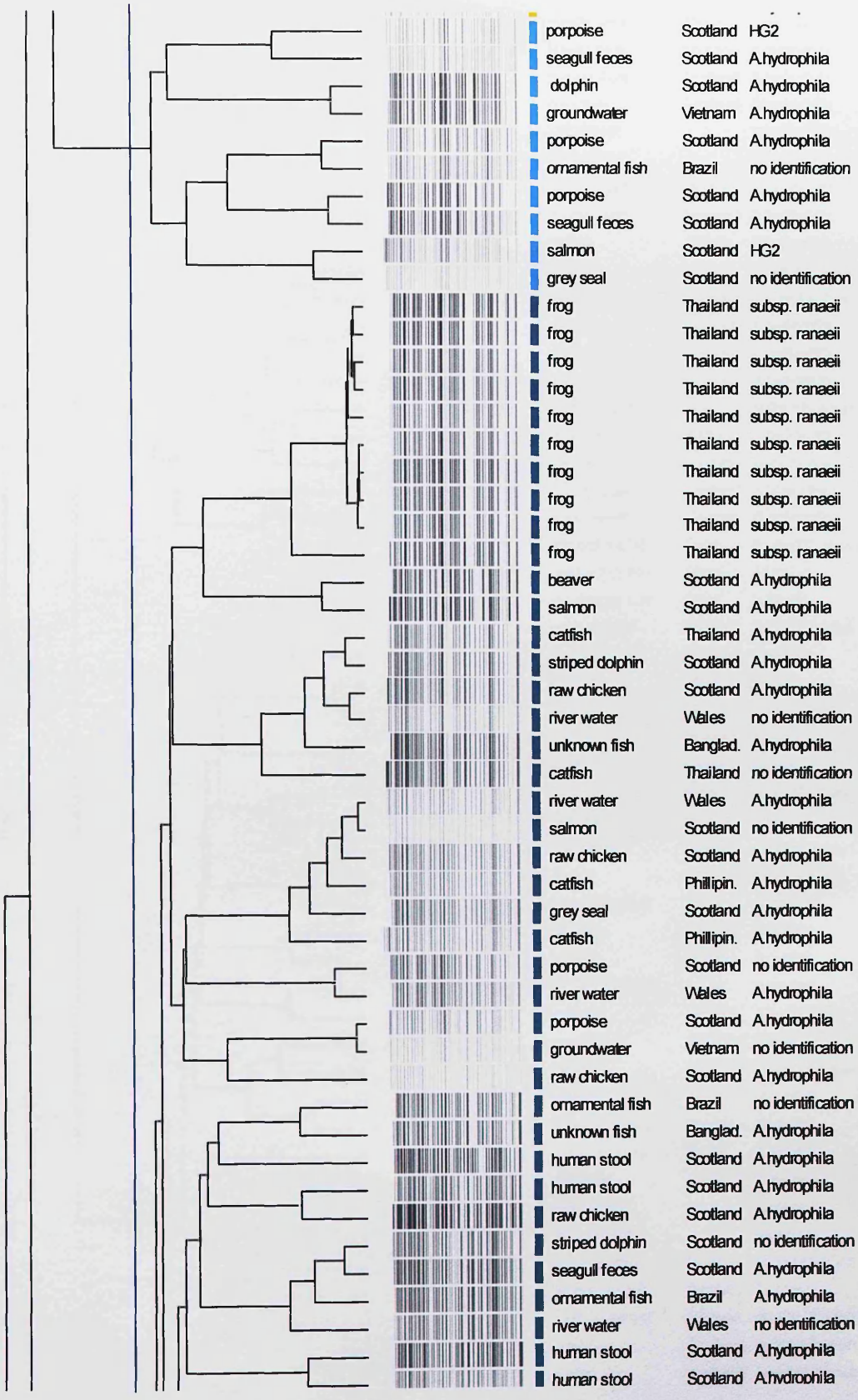
salmon Scotland A.hydrophila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seagull feces Scotland A.hydrophila
salmon Scotland HG2
grey seal Scotland no identification
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
frog Thailand subsp. ranaeii
beaver Scotland A.hydrophila
salmon Scotland A.hydrophila
catfish Thailand A.hydrophila
striped dolphin Scotland A.hydrophila
raw chicken Scotland A.hydrophila
river water Wales no identification
unknown fish Banglad. A.hydrophila
catfish Thailand no identification
river water Wales A.hydrophila
salmon Scotland no identification
raw chicken Scotland A.hydrophila
catfish Phillipin. A.hydrophila
grey seal Scotland A.hydrophila
catfish Phillipin. A.hydrophila
porpoise Scotland no identification
river water Wales A.hydrophila
porpoise Scotland A.hydrophila
groundwater Vietnam no identification
raw chicken Scotland A.hydrophila
ornamental fish Brazil no identification
unknown fish Banglad. A.hydrophila
human stool Scotland A.hydrophila
human stool Scotland A.hydrophila
raw chicken Scotland A.hydrophila
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ornamental fish Brazil A.hydrophila
river water Wales no identification
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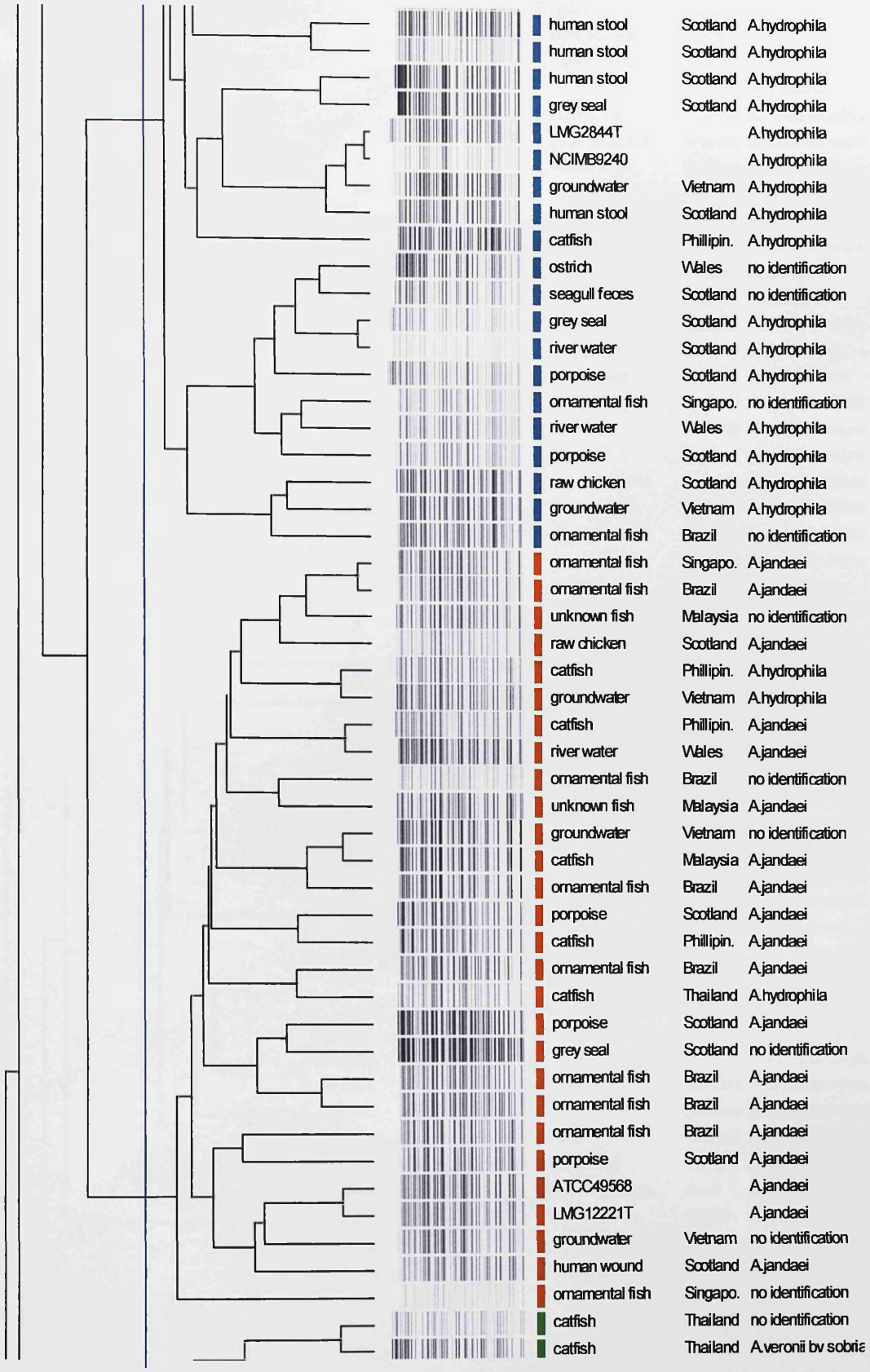
human stool Scotland A.hydrophila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human stool Scotland A.hydrophila
catfish Phillipin. A.hydrophila
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grey seal Scotland A.hydrophila
river water Scotland A.hydrophila
porpoise Scotland A.hydrophila
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raw chicken Scotland A.hydrophila
groundwater Vietnam A.hydrophila
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ornamental fish Brazil A.jandaei
unknown fish Malaysia no identification
raw chicken Scotland A.jandaei
catfish Phillipin. A.hydrophila
groundwater Vietnam A.hydrophila
catfish Phillipin. A.jandaei
river water Wales A.jandaei
ornamental fish Brazil no identification
unknown fish Malaysia A.jandaei
groundwater Vietnam no identification
catfish Malaysia A.jandaei
ornamental fish Brazil A.jandaei
porpoise Scotland A.jandaei
catfish Phillipin. A.jandaei
ornamental fish Brazil A.jandaei
catfish Thailand A.hydrophila
porpoise Scotland A.jandaei
grey seal Scotland no identification
ornamental fish Brazil A.jandaei
ornamental fish Brazil A.jandaei
ornamental fish Brazil A.jandaei
porpoise Scotland A.jandaei
ATCC49568 A.jandaei
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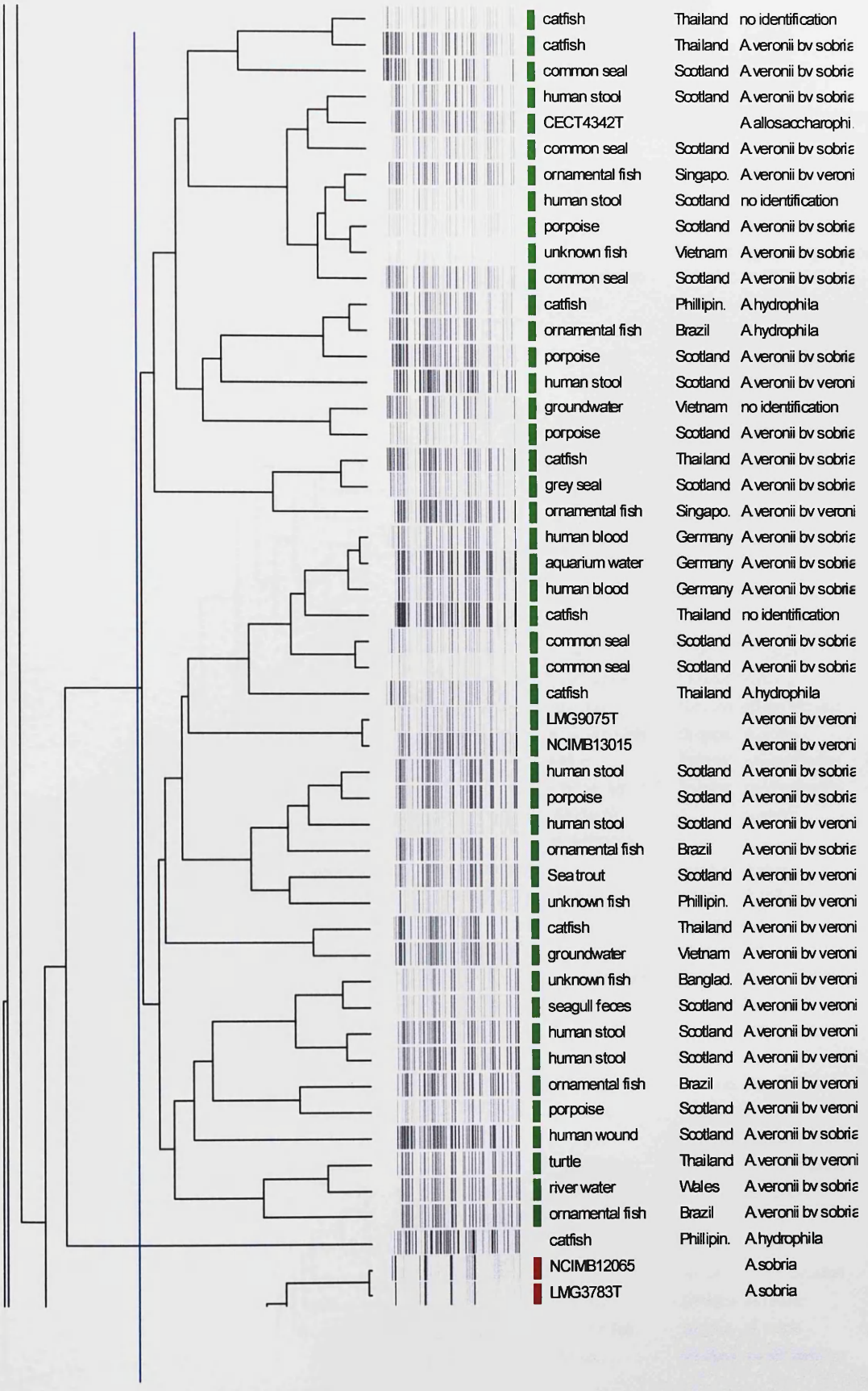
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common seal Scotland A.veronii bv sobria
ornamental fish Singapo. A.veronii bv veroni
human stool Scotland no identification
porpoise Scotland A.veronii bv sobria
unknown fish Vietnam A.veronii bv sobria
common seal Scotland A.veronii bv sobria
catfish Phillipin. A.hydrophila
ornamental fish Brazil A.hydrophila
porpoise Scotland A.veronii bv sobria
human stool Scotland A.veronii bv veroni
groundwater Vietnam no identification
porpoise Scotland A.veronii bv sobria
catfish Thailand A.veronii bv sobria
grey seal Scotland A.veronii bv sobria
ornamental fish Singapo. A.veronii bv veroni
human blood Germany A.veronii bv sobria
aquarium water Germany A.veronii bv sobria
human blood Germany A.veronii bv sobria
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common seal Scotland A.veronii bv sobria
common seal Scotland A.veronii bv sobria
catfish Thailand A.hydrophila
LMG9075T A.veronii bv veroni
NCIMB13015 A.veronii bv veroni
human stool Scotland A.veronii bv sobria
porpoise Scotland A.veronii bv sobria
human stool Scotland A.veronii bv veroni
ornamental fish Brazil A.veronii bv sobria
Sea trout Scotland A.veronii bv veroni
unknown fish Phillipin. A.veronii bv veroni
catfish Thailand A.veronii bv veroni
groundwater Vietnam A.veronii bv veroni
unknown fish Banglad. A.veronii bv veroni
seagull feces Scotland A.veronii bv veroni
human stool Scotland A.veronii bv veroni
human stool Scotland A.veronii bv veroni
ornamental fish Brazil A.veronii bv veroni
porpoise Scotland A.veronii bv veroni
human wound Scotland A.veronii bv sobria
turtle Thailand A.veronii bv veroni
river water Wales A.veronii bv sobria
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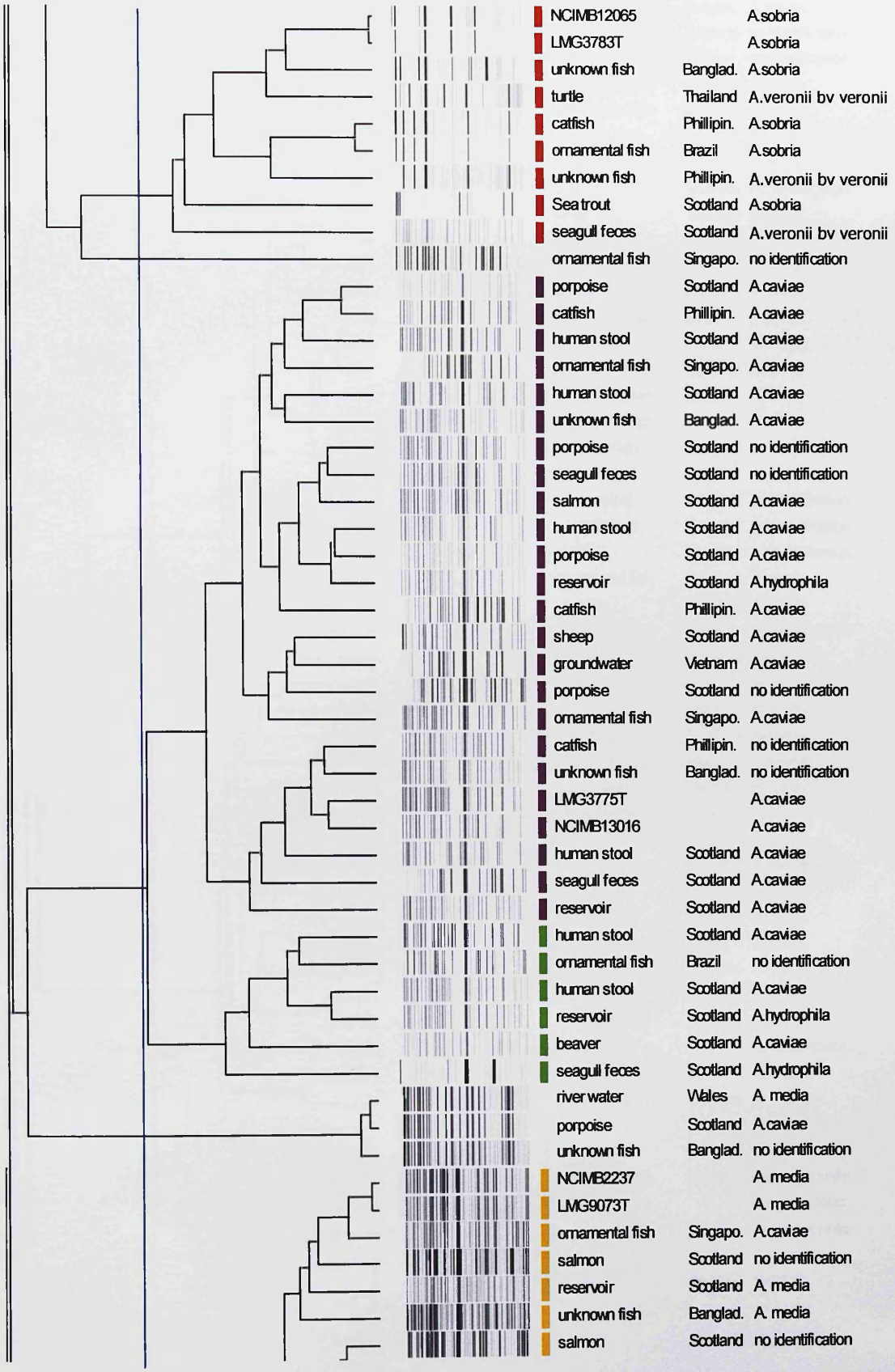
NCIMB12065 A.sobria
LMG3783T A.sobria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catfish Phillipin. A.sobria
ornamental fish Brazil A.sobria
unknown fish Phillipin. A.veronii bv veronii
Sea trout Scotland A.sobria
seagull feces Scotland A.veronii bv veronii
ornamental fish Singapo. no identification
porpoise Scotland A.caviae
catfish Phillipin. A.caviae
human stool Scotland A.caviae
ornamental fish Singapo. A.caviae
human stool Scotland A.caviae
unknown fish Banglad. A.caviae
porpoise Scotland no identification
seagull feces Scotland no identification
salmon Scotland A.caviae
human stool Scotland A.caviae
porpoise Scotland A.caviae
reservoir Scotland A.hydrophila
catfish Phillipin. A.caviae
sheep Scotland A.caviae
groundwater Vietnam A.caviae
porpoise Scotland no identification
ornamental fish Singapo. A.caviae
catfish Phillipin. no identification
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LMG3775T A.caviae
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human stool Scotland A.caviae
ornamental fish Brazil no identification
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beaver Scotland A.caviae
seagull feces Scotland A.hydrophila
river water Wales A. media
porpoise Scotland A.caviae
unknown fish Banglad. no identification
NCIMB2237 A. media
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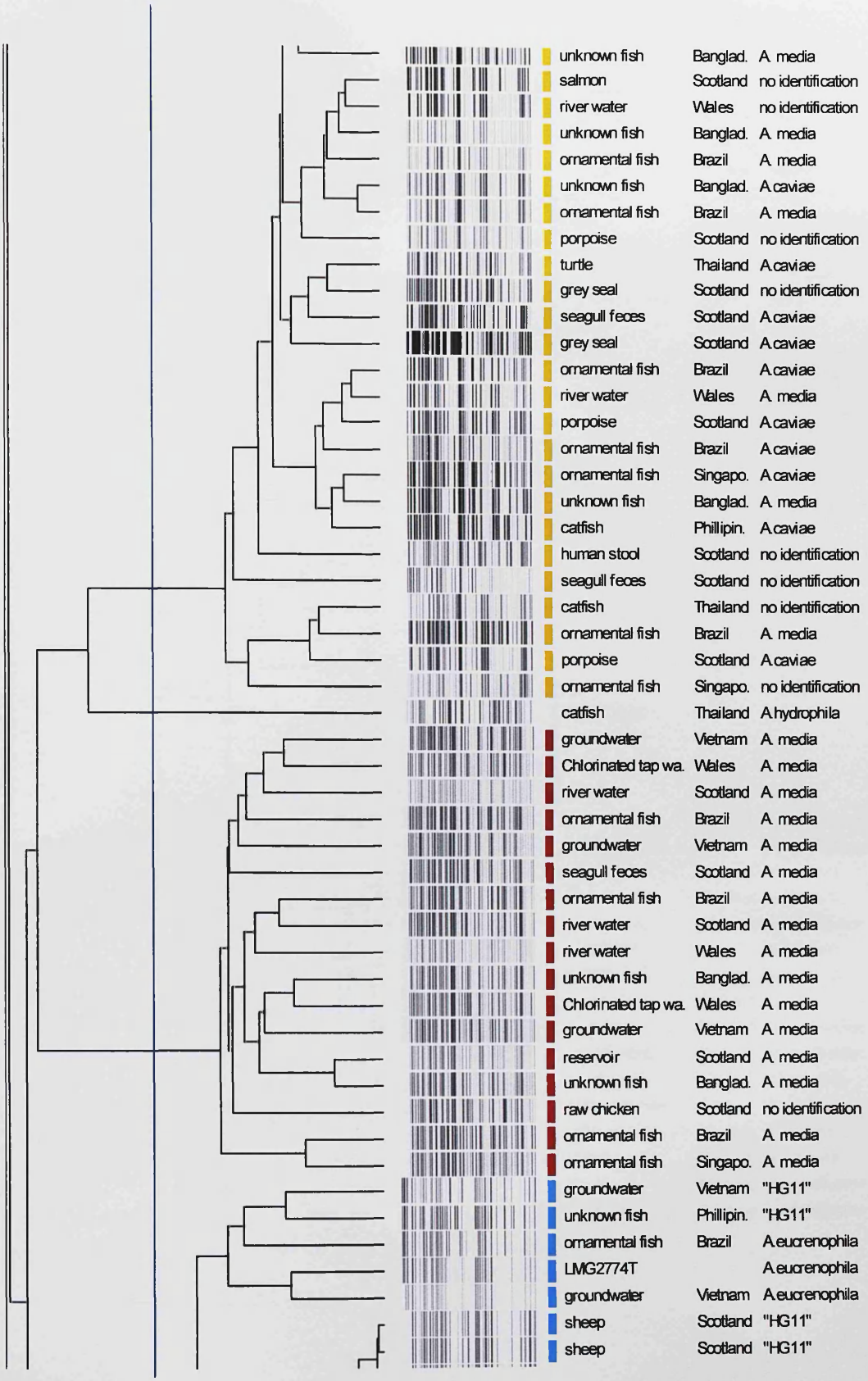
salmon Scotland no identification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salmon Scotland no identification
river water Wales no identification
unknown fish Banglad. A. media
ornamental fish Brazil A. media
unknown fish Banglad. A.caviae
ornamental fish Brazil A. media
porpoise Scotland no identification
turtle Thailand A.caviae
grey seal Scotland no identification
seagull feces Scotland A.caviae
grey seal Scotland A.caviae
ornamental fish Brazil A.caviae
river water Wales A. media
porpoise Scotland A.caviae
ornamental fish Brazil A.caviae
ornamental fish Singapo. A.caviae
unknown fish Banglad. A. media
catfish Phillipin. A.caviae
human stool Scotland no identification
seagull feces Scotland no identification
catfish Thailand no identification
ornamental fish Brazil A. media
porpoise Scotland A.caviae
ornamental fish Singapo. no identification
catfish Thailand A.hydrophila
groundwater Vietnam A. media
Chlorinated tap wa. Wales A. media
river water Scotland A. media
ornamental fish Brazil A. media
groundwater Vietnam A. media
seagull feces Scotland A. media
ornamental fish Brazil A. media
river water Scotland A. media
river water Wales A. media
unknown fish Banglad. A. media
Chlorinated tap wa. Wales A. media
groundwater Vietnam A. media
reservoir Scotland A. media
unknown fish Banglad. A. media
raw chicken Scotland no identification
ornamental fish Brazil A. media
ornamental fish Singapo. A. media
groundwater Vietnam "HG11"
unknown fish Phillipin. "HG11"
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LMG2774T A.eucrenophila
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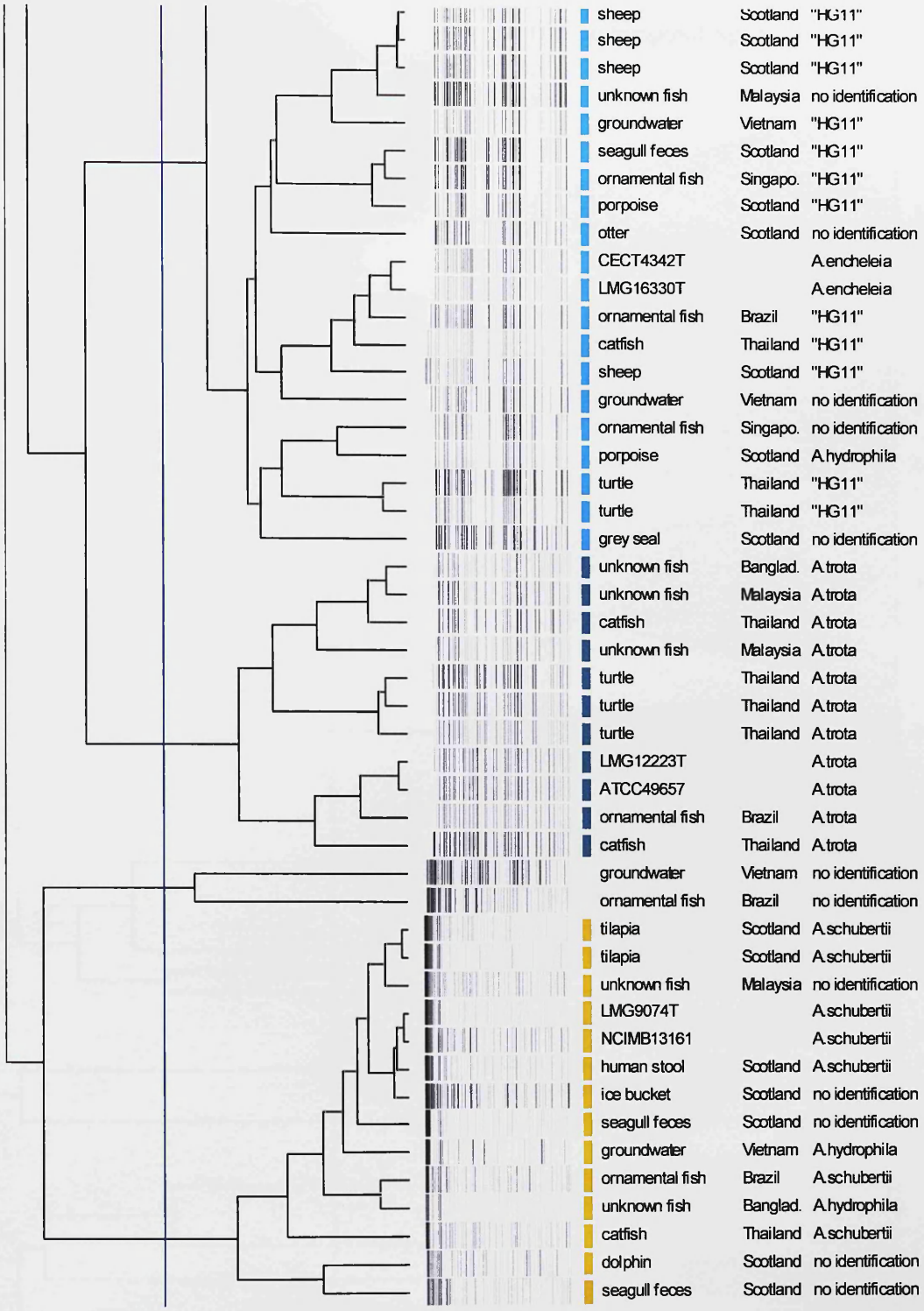
sheep Scotland "HG11"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sheep Scotland "HG11"
sheep Scotland "HG11"
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groundwater Vietnam "HG11"
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porpoise Scotland "HG11"
otter Scotland no identification
CECT4342T A.encheleia
LMG16330T A.encheleia
ornamental fish Brazil "HG11"
catfish Thailand "HG11"
sheep Scotland "HG11"
groundwater Vietnam no identification
ornamental fish Singapo. no identification
porpoise Scotland A.hydrophila
turtle Thailand "HG11"
turtle Thailand "HG11"
grey seal Scotland no identification
unknown fish Banglad. A.trota
unknown fish Malaysia A.trota
catfish Thailand A.trota
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turtle Thailand A.trota
turtle Thailand A.trota
turtle Thailand A.trota
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ATCC49657 A.trota
ornamental fish Brazil A.trota
catfish Thailand A.trota
groundwater Vietnam no identification
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tilapia Scotland A.schubertii
unknown fish Malaysia no identification
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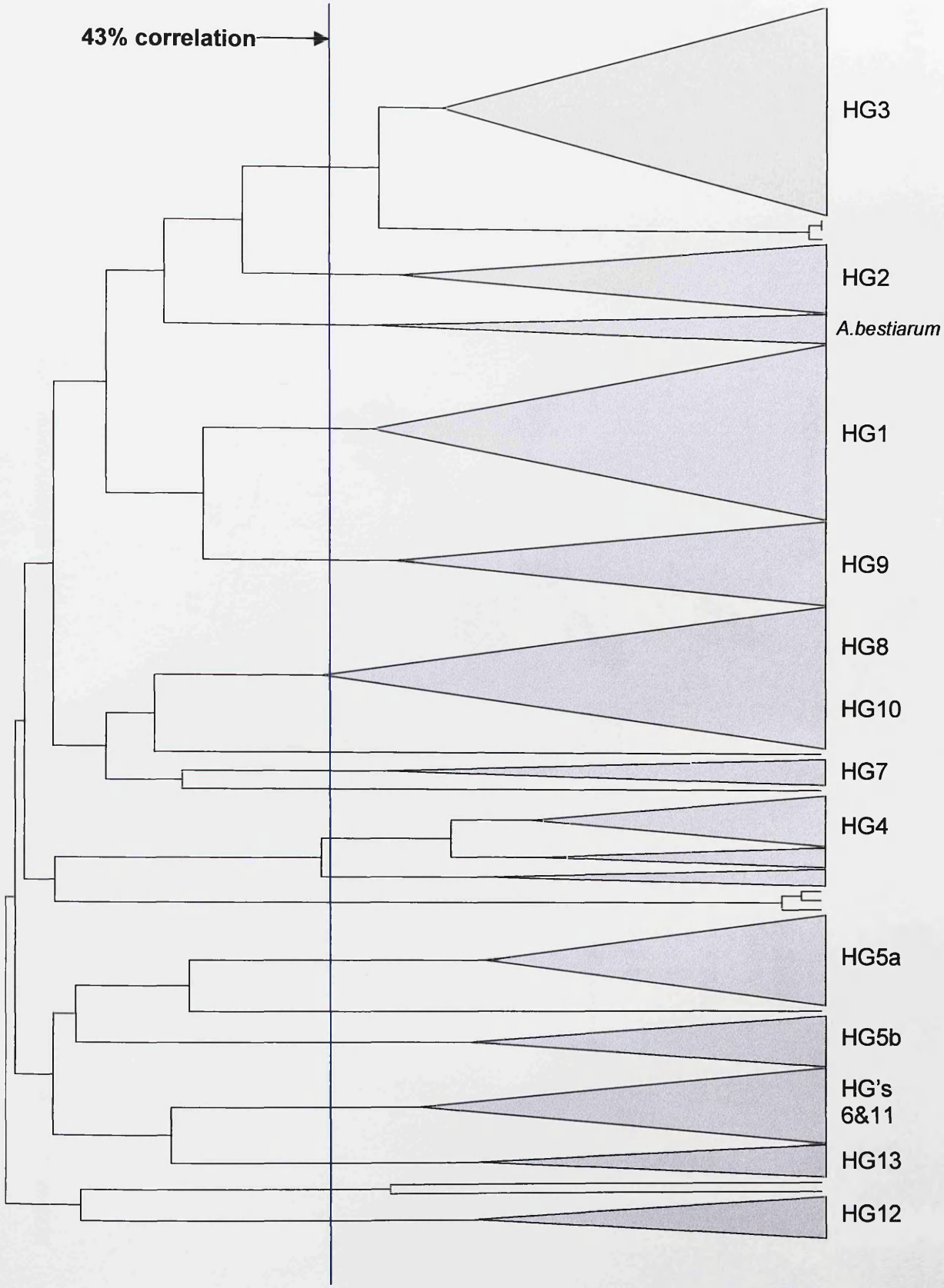
NCIMB13161 A.schubertii
human stool Scotland A.schubertii
ice bucket Scotland no identification
seagull feces Scotland no identification
groundwater Vietnam A.hydrophila
ornamental fish Brazil A.schubertii
unknown fish Banglad. A.hydrophila
catfish Thailand A.schubertii
dolphin Scotland no identification
seagull feces Scotland no identification

Figure 3-7: collapsed dendrogram showing clusters corresponding to hybridisation groups



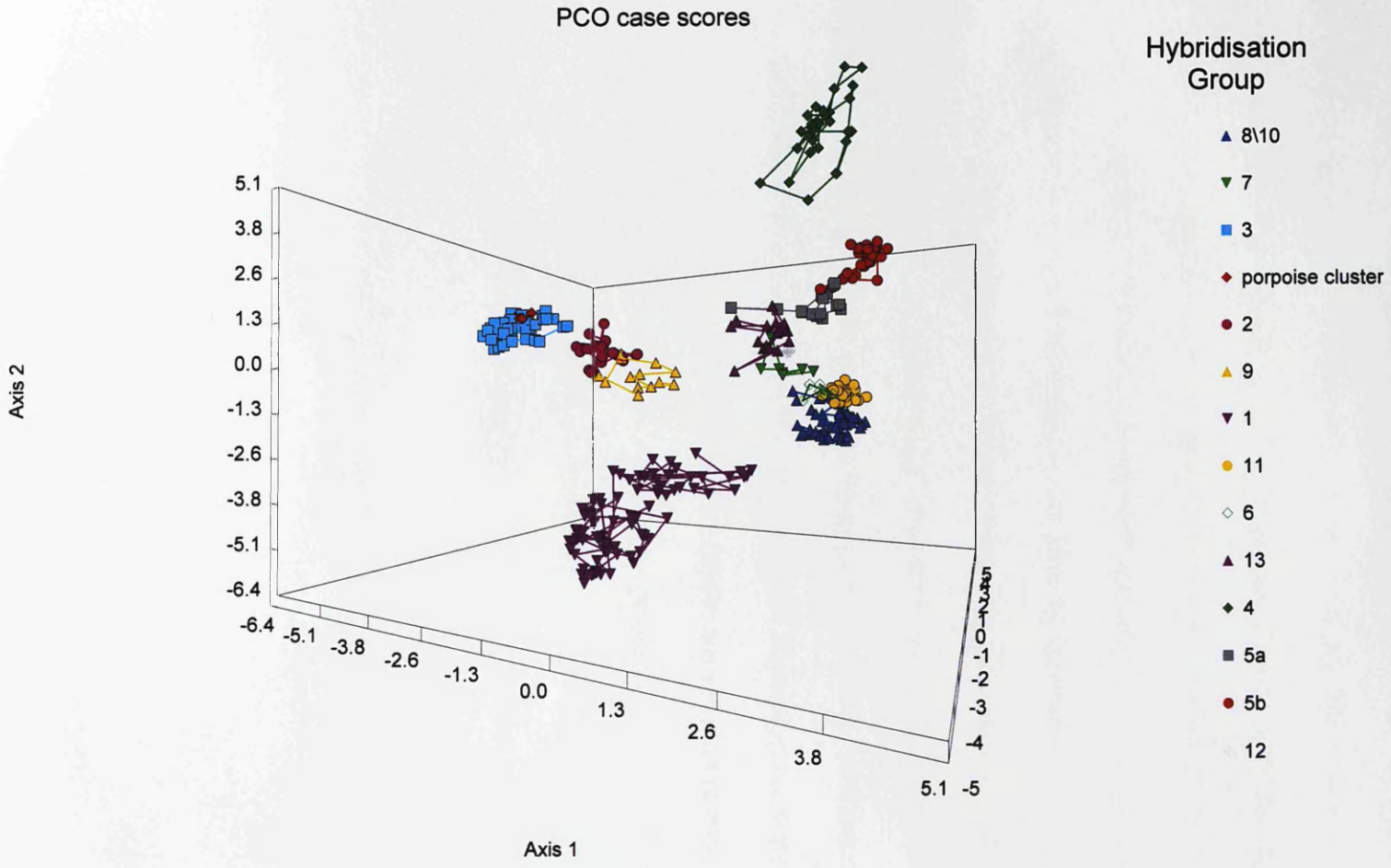


Figure 3-8: Clustering revealed by Principle Coordinates Analysis of Pearson product-moment correlation coefficient similarity matrix

3.4: Discussion

3.4.1: Optimisation

After some adaptation and optimisation a protocol for performing AFLP analysis of a diverse collection of *Aeromonas* strains has been developed. The technique generates extremely complex fingerprints that are reproducible and highly discriminatory at the strain and species level.

During the initial optimisation work it was revealed that the quality of the fingerprints is highly dependent on the quality of the genomic DNA and that extremely poor results are obtained using contaminated or degraded DNA. When steps were taken to improve the quality of genomic DNA: using younger cultures grown in broth and introducing multiple wash steps prior to cell lysis, improved results were obtained. Beginning with DNA at a much lower concentration than had been recommend previously (Janssen *et al.*, 1996; Vos *et al.,* 1995) during the restriction digestion step of AFLP template preparation was also found to improve fingerprint quality, presumably because any contaminating inhibitory material is diluted. It is likely that this alteration is possible when working with bacterial DNA, as the original AFLP protocol was developed for use with much larger genomes. Using smaller amounts of template DNA does have the disadvantage that airborne contaminants, which can be common in laboratories where PCR products are routinely handled (Kwok and Higuchi, 1989), may present more of a danger.

On the other hand, using an excessive amount of PCR template, particularly during multiple amplicon PCR has been shown to encourage non- specific product formation (Roux, 1995). The protocol on which this work was initially based

(Janssen *et al.,* 1996) recommended including 20ng of template DNA in PCR reactions. However it could be argued that when primers carrying only a single selective base are used, a greater proportion of the restriction fragments present in the initial template are amplified than is the case when more selective bases are included. Consequently much less total template should be required for the PCR. In this work 1.25ng template DNA was found to produce clear, sharp fingerprints.

Precise consistency in the amount of template used per reaction is not essential since the unlabelled primer is present in great excess. Therefore the factor that determines the intensity of bands will be the amount of labelled primer, which is completely consumed during the PCR (Vos and Kuiper, 1997).

Working with smaller bacterial genomes, complex fingerprints were obtained using PCR primers carrying only a single 3'-selective base. All primers tested generated fingerprints containing between 50 and 80 bands, fairly evenly spaced between 50 and 500bp. This is important since it has been reported that even band distribution is crucial for accurate discrimination in cluster analysis (Janssen *et al.*, 1996).

All the primers tested generated similar numbers of fragments. This is as expected when only a single selective base is used and may be taken as evidence that all primers amplify with equivalent efficiency. This robustness must be attributed to the touchdown cycles at the start of the PCR, since the three primers tested, PA01, PA02, and PA03, had different calculated T_m values: 51.7°C; 51.6°C; and 54.6°C respectively.

3.4.2: Reproducibility

Comparison of the fingerprints obtained by running the same PCR products in different lanes on the same gel revealed that correlation levels within the same gel were between 95.6 and 98.7% using autoradiography and between 92.5% and 94.6%, using the automated sequencer. Surprisingly the correlations between different lanes on the same gel were no higher than the correlations between different gels and this was found to be the case using both techniques.

Using autoradiography it was found that there was no greater difference between fingerprints generated using separate PCR products obtained using independently prepared template, than between identical PCR products run on different gels. This indicates that most of the variability in AFLP data arises during the electrophoresis step and that any endeavour to improve the accuracy of the method should begin here.

The observation that both inter- and intra- gel correlations were lower using the ABI than had been obtained using autoradiography was surprising and a little disappointing. However this is something that has been reported elsewhere. Huys and Swings (1999) for example reported even poorer intra-gel correlations, using an ABI 377 than were observed in this work. In previous work, they had used a well defined AFLP product as the reference standard on autoradiographs. They attributed the poorer performance of the ABI to the smaller number of reference bands in the Genescan-500 size standard, than had been present in their autoradiography reference standard and found that intra-gel correlations were much improved when a mix of GS-500 and GS-2500 reference standard was used. The finding that correlations between autoradiographs observed during this work were significantly higher than

Huys and Swings (1999) report is consistent with this theory since the reference standard used here was the single base resolution M13 ladder.

Work is under way to develop an improved reference marker more suitable for AFLP work than GS-500. Brondani and Grattapaglia (2001) have described a method to prepare just such a high density size standard. Their method relies on synthesis of defined PCR products of known size from conserved regions of plant choroplast DNA template. The danger of this approach, however is the inherent inconsistency of PCR reactions: it is likely that factors such as inconsistent non-template A-addition between reactions could introduce unacceptable batch to batch variations.

A potentially more reliable approach is being pursued which involves cloning a series of evenly spaced AFLP fragments. The size of the fragment carried by an individual clone is easily determined using the non-selective AFLP primers. The cloned AFLP fragment is then freed from the vector and a singly ROX-labelled adaptor ligated onto one end only. Since each fragment then carries only one label, the fragment is visualised as a single peak on a denaturing gel.

Once a complete set of clones carrying a range of suitable fragments has been assembled, it is anticipated that this size standard will replace GS500 in future work.

3.4.3: Effect of Including a Preselective Step

The fingerprints generated from equivalent templates differed considerably according to whether a preselective PCR amplification was included as recommended by Applied Biosystems. The fingerprints generated with and without a preselective step were of equivalent intensity overall, but the intensities of individual bands were far more even when the preselective step was omitted. The

sizes of bands in equivalent fingerprints appeared to agree well. In contrast Huys and Swings (1999) found that fewer bands were observed when a preselective step was included. These two findings may not be inconsistent since the greater range of band intensities might result in some bands falling below the threshold intensity for detection by the band autosearch algorithm.

The greater difference in band intensities probably results from uneven preamplification of some of the fragments that are subsequently selected during the second PCR, so that the selective amplification step begins with unequal amounts of each fragment to be amplified.

The preselective step was recommended in the original AFLP protocol (Vos *et al.* 1995) when complex genomes were to be analysed. In these cases a tiny minority of the total number of restriction fragments need to amplify and if there were no preselective step sufficiently selective primers would need four or more selective bases. It was found that selectivity was lost if more than three selective bases were included in any one primer. Therefore a preselective step in which one, or both primers carried a single selective base was recommended, followed by a selective PCR in which both primers carried three selective bases.

Since the preselective step is necessary only to avoid the need for more than three selective bases, its inclusion when working with bacterial DNA seems hard to justify on theoretical grounds.

Moreover there are several practical objections to its use in terms of the additional use of reagents, additional time taken, and greater scope for sample cross-contamination.

Finally it should be pointed out that the preselective step recommended in the protocol accompanying the Applied Biosystems microbial fingerprinting kit is not, in fact a preselective step, but rather a nonselective step since there are no selective bases included in the primers.

Since the "preselective" step did not appear to contribute anything of value, it was not included in subsequent work. A clear advantage of omitting this step is that PCR amplification and electrophoresis can be completed in a single day.

3.4.4: Comparison between the two methods

The differences in inter- and intra- gel reproducibility between the two methods has been discussed above, and work is under way aimed at improving the reproducibility of gels run using the ABI 377.

The signal to noise ratio of autoradiograph images ranged from 200 to 1050 whilst the ABI images generally had lower signal to noise ratios, ranging from 140 to 600. The difference is probably of no significant consequence since Applied Maths recommend that for optimum resolution, gel images should have a signal to noise ratio of greater than 50.

It was clear that bands visualised fluorescently varied in intensity far more than bands visualised radioactively. However, pairwise comparison of equivalent samples produced in independent PCR reactions revealed very little difference when assessed visually *ie.* there was consistency between which particular bands amplified strongly and which less so, in equivalent fingerprints. In contrast, Kotovic and On (1999) reported considerable variability between peak heights in profiles of repeated

Campylobacter samples. They did not find, however that this affected the outcome of cluster analysis.

In this study, it was found that when fragments are fluorescently labelled, approximately 10% fewer bands are visualised per profile than when radioactively labelled. Interestingly Huys and Swings (1999), also working with *Aeromonas* DNA identified, approximately 35% fewer bands using fluorescently labelled fragments, whereas Koeleman *et al.* (1998) observed no difference at all between the two methods in their studies on *Acinetobacter.*

It is possible that the apparent differences arise because of the narrower 'window' of acceptable signal strength when detecting the fluorescent bands. Using an ABI 377, signals stronger than the preset maximum appear as flat-topped peaks, which are impossible to compare. The need to allow for this means that the very faintest bands may not be detected. This is not the case when fragments are detected using autoradiography since, particularly when using ^{33}P, the signal does not travel far through the gel, so that signals that over-saturate the film still produce scorable bands. Accordingly, autoradiography is far more forgiving of a wider variation in signal strength.

However this explanation is merely a speculation and it appears that there has thus far been no research aimed at reconciling these differences, with most researchers content that they do not alter the outcome of cluster analysis. (Applied Biosystems technical support, pers comm.)

This seems a rather complacent stance, since one of the great strengths of the AFLP technique is that there is believed to be extremely close agreement between observed fragments and those predicted theoretically. In the original Vos *et al.* (1995) paper

the fingerprint of phage λ detected by autoradiography was found to agree 100% with the profile predicted from its known sequence. In contrast, Arnold *et al.* (1999a; 1999b) observed only 92% agreement between fragments predicted from the published sequence of the *E.coli* K12 genome and those actually visualised using fluorescent AFLP. Although they did not observe any fragments that were not theoretically expected, the failure to visualise almost one in ten expected fragments, is a discrepancy that should not be ignored.

Cluster analysis of fingerprints generated using the two different methods produced distance matrices which though not identical, were found to be not significantly different at a probability of 0.95 using a Mantel test. The comparability of the results obtained using such different methodologies has profound implications for the comparability of data generated in different laboratories. Indeed, in a survey of the reproducibility of AFLP and other techniques across a network of European laboratories, Jones *et al.*, (1997) were able to show that AFLP data was virtually free of artefacts introduced by minor variations in methodologies and equipment.

In this work curve-based cluster analysis has been found to be greatly superior to band-based assignments. Traditionally analysis has been band-based whereby gels are converted to a matrix of binary variables: band present = 1; band absent = 0. The mathematics involved in analysis of such binary matrices is simple, however a great deal of the data available within a fingerprint is lost by reducing it to a series of binary variables since a fingerprint is generally more complex than a series of bands present or absent: some bands are intense, some barely visible. In order to convert a

fingerprint into a matrix of bands present or absent an arbitrary threshold must be set, any bands less intense than that threshold are ignored.

Since PCR-based fingerprints are inherently variable between repeats, it is inevitable that borderline bands will fall below this threshold on some occasions while exceeding it on others. This means that a band based analysis is not only wasteful of available data, it inevitably introduces errors as well. Fig 3.6 illustrates this point: superimposed on two repeated Rep-PCR fingerprints are the bands assigned using the band calling algorithm in GelCompar 2.0. Two faint bands are visible in the lower fingerprint that have been scored as absent, though only marginally more intense in the upper lane they were scored as present

Figure 3-9: GelCompar image of REP fingerprints of identical samples with band calling super-imposed: two marginal peaks are scored as present (upper lane) and absent (lower lane)

Over the last decade image analysis software packages have become available that are able to convert the fingerprint to an array of densitometric values and so capture the full complexity of the fingerprint. These arrays may be compared using the Pearson product moment correlation, which compares every point on a densitometric trace. This method is therefore more robust since subjective band-scoring is eliminated and is also relatively insensitive to differences in background. More

importantly it is not affected by differences in relative concentrations of fingerprints which is of particular value when comparing complex fingerprints.

3.4.5: Cluster Analysis

Using the unweighted pair group method with arithmetic averages, the collection of strains assembled for this study separate into 15 distinct clusters when delineated at a correlation level of 43% (figs 3.6 and 3.7).The threshold value of 43% was the level at which Huys *et al.* (1996) found separated their collection of 96 strains into clusters that corresponded to Hybridisation Group. Although the AFLP database at their laboratory has since been expanded considerably, the 43% threshold has been retained (Geert Huys, pers. comm.) and has been used here for the purpose of comparing the two datasets.

Each of the 15 clusters contains the type strain of only one Hybridisation Group and all hybridisation groups so far described appear to be represented. Moreover where type strains have been obtained from more than one collection (LMG, Universitiet Gent and IOA) the fingerprints were either indistinguishable or very nearly so.

A particular aim of this section of the present study was to identify distinct genotypic clusters in order to see whether apparently pathogenic strains could be reliably associated with certain genetic groups. In this context the distinct clustering represented in fig.3.6 is most encouraging.

Many of the observations that follow regarding the structure of these clusters are relevant to the controversial (and at times quite fractious) ongoing debate regarding the taxonomy of the genus *Aeromonas*. It should be stressed however, that resolving taxonomic issues was not a primary aim of this project and therefore this topic is approached with caution. The status of AFLP data relative to DNA-DNA hybridisations, hitherto regarded as the 'gold standard' for bacterial taxonomy

(Wayne *et al.,* 1987; Wayne *et al.,* 1996) has previously sparked a lively debate in the context of *Aeromonas* taxonomy (Esteve, 1997; Huys *et al.,*1997). Moreover it should be noted that the phenotypic identification scheme used in this work comprises only 24 tests which is extremely crude relative to those used in recently described taxonomic investigations. For example the scheme used by Kaznowski (1998) uses 136 tests, Valera and Esteve (2002) included 121 separate tests in a recent survey, whilst Huys *et al.* (1997) used 132 tests to define the type strain of *A.popoffii* sp. nov.

The most striking feature of the clusters represented in fig. 3.6 is the small number (8) of 'outliers' that are not included in any of the clusters and show very low correlation to adjacent clusters. Of these, two are phenotypically similar to strains in adjacent clusters, two are phenotypically similar to distant clusters, while four isolates could not be identified at all using the phenotypic tests used here. Without performing DNA-DNA hybridisations and a more comprehensive battery of phenotypic tests on these isolates their status must remain unclear. No such outliers were identified in the previously published AFLP survey of *Aeromonas* strains (Huys *et al.,* 1996), though it should be noted that not only did their work examine a much smaller number of strains, but more importantly the strains included in the study were chosen to form a balanced collection of previously well-characterised isolates and no wholly atypical isolates were included. The strains included in this study were obtained from a far more diverse range of sources and so it is possible that

the anomalous strains are representatives of further *Aeromonas* taxa that have never been included in any genetic analyses. This is an intriguing possibility that merits further investigation.

Though the possibility that these fingerprints represent genotyping artefacts cannot be ruled out completely, this seems unlikely given that the data is elsewhere so reproducible and consistent.

It is generally accepted that further *Aeromonas* taxa remain undescribed and in a recent comprehensive numerical phenotypic study Valera and Esteve (2002) found compelling evidence for the existence of new biotypes.

If these anomalous strains are indeed representatives of previously unseen taxa, the relatively small numbers recovered might reflect their genuine rarity but it is equally possible that they are representatives of abundant types that do not survive well on media commonly used for the isolation of *Aeromonas spp.* In this context it is particularly interesting to note that most of these strains are sensitive to ampicillin, which is routinely used as a selective agent in media used to screen for *Aeromonas*. The diverse range of media and selective agents used for this purpose in different laboratories described in a review by (Joseph and Carnahan, 1994) would suggest that different laboratories are likely to recover different sub-sets of the full range of *Aeromonas* types.

A second unexpected finding is that the cluster containing the HG4 type strain clearly separates into two sub-groups that share 55% correlation. Both clusters comprise strains that are phenotypically *A.caviae* or *A.caviae*-like. The *A.caviae* type strain resides in the smaller of the two clusters. This suggests that HG4 is not a

homogenous taxon but in fact comprises two genetically distinct sub-groups. Furthermore a third cluster, also containing mainly isolates that are phenotypically most similar to *A.caviae* is closely associated with these two clusters, but on the basis of the AFLP data presented here would appear to be genetically quite distinct. This raises the possibility that these isolates, all but one of which was isolated from temperate freshwater, could be representatives of an as yet un-described hybridisation group, closely related to HG4 and which cannot be distinguished using the limited range of phenotypic tests so far used to identify these isolates. Most of the strains in this cluster failed to reach the probability threshold of 0.95 using the PIB software (see Chapter 6) and could be identified as *A.caviae* only at the 0.8 threshold. This was because several isolates produced gas from glucose and were Lysine decarboxylase positive which meant that those isolates could not be separated from *A.hydrophila*. It is particularly interesting to note that Kodjo *et al.* (1997) associated a number of atypical *Aeromonas* strains with a new summer disease of temperate snails which they designated as *A.hydrophila/A.caviiae* on the grounds that the phenotypic properties examined were not able to rule them out of either group.

The type strains *A.sobria* HG7, *A.allosaccharophila* now regarded as a synonym of *A.veronii* bv sobria, HG8 (Huys *et al.*, 2001) and *A.veronii* bv veronii HG10, all fall into separate clusters. The HG7 cluster contains a relatively small number of isolates, which is consistent with previously published observations that this is a relatively rare *Aeromonas* species (Carnahan and Altwegg, 1996; Carson *et al.*, 2001). Almost half of the strains in this cluster were identified as *A.veronii* bv veronii, on the basis of a positive aesculin test. It is interesting to compare this

finding with Huys *et al.* (1996) who included in their study only two strains previously identified as *A.sobria* on phenotypic grounds, including the type strain, but found that these clustered together with three other strains that had previously been classified as *A.veronii* HG10 by Kämpfer and Altwegg (1992). The aesculin positive strains described here lend support to the suggestion by Huys *et al.*(1996) that there exists an undefined *Aeromonas* taxon that is phenotypically similar to HG10 but genotypically more closely related to HG7.

Two clusters containing roughly equal numbers each, of strains phenotypically identified as *A.veronii* bv sobria, HG8 and *A.veronii* bv veronii HG10, separate from each other at just below the 43% correlation threshold. Although this agrees well with the findings of Huys *et al.* (1996) it is also a little disappointing since it was anticipated that the larger numbers of isolates included in this study would allow the two groups to be separated more distinctly.

A relatively large proportion of strains fall into HG's 1, 2, and 3 and the structure and composition of these clusters, together with the clusters containing the type strains of *A.trota,* HG13, *A.encheleia* and *A.veronii* –like, HG11 – smaller in number but nevertheless a far larger number than have previously been analysed using AFLP are particularly interesting as the taxonomy of both these groups is controversial. For many years the striking lack of congruence between groups delineated on the basis of phenotypic characteristics and on the basis of DNA-DNA hybridisations, confounded by the different reference strain collections in use in different laboratories meant that *Aeromonas* taxonomy harboured two unnamed HG's: HG2 and HG11. Though there was ample genotypic evidence that these two taxa were distinct homogenous DNA hybridisation groups, the lack of stable phenotypic

markers that would distinguish them from their respective taxonomic neighbours meant that recognition as named species could not be justified (Huys *et al.*, 1997).

Ali *et al.* (1996) were able to show that nine HG2 strains, mainly of animal origin, could be distinguished from HG1 and HG3 via three biochemical tests: utilisation of DL-lactate, acid production from D-sorbitol, and utilisation of urocanic acid and on this basis proposed that a new species named *A.bestiarum* be established.

A survey of genotypic diversity among 168 *Aeromonas* strains isolated from Flemish drinking water production plants, (Huys *et al.*, 1996c) found that a group of 24 strains clustered separately in an AFLP-based identification library in which all *Aeromonas* taxa then recognised were represented.

Closer numerical analysis of the AFLP fingerprints revealed that the unidentified strains constituted a separate genotypic cluster that was most closely related to, but clearly distinct from, the recently recognised *A.bestiarum*, formerly HG2.

A subsequent study of *Aeromonas* strains isolated from Scottish drinking water supplies (Huys and Lamb, unpublished data) also identified a number of mesophilic *A.bestiarum*- like strains.

DNA-DNA hybridisation later showed that these isolates were in fact quite distinct from *A.bestiarum* and a new species *A.popoffii* was proposed (Huys *et al.*, 1997c). Phenotypically closely related to *A.bestiarum*, the new species could be distinguished on the basis of utilisation of DL-lactate and failure to ferment D-sucrose or produce Lysine Decarboxylase.

The AFLP data presented here is powerful evidence that these two taxa are indeed stable and widespread species. The cluster containing the type strain *A.bestiarum* HG2 consists of 25 sorbitol-positive strains, some isolated from freshwater fish, but

mostly from marine mammals. The cluster is most closely related to the extremely diverse HG3 cluster. Adjacent to this cluster, but apparently genetically quite distinct is a smaller cluster containing 10 strains, including the *A.popoffii* type strain. Here the phenotypic data is less consistent in that only half were sucrose-minus, though, with only eight strains previously tested, it is too early to say whether failure to utilise sucrose is a consistent diagnostic feature of the species. These strains were, without exception isolated from freshwater, which is that habitat from which all previously identified *A.popoffii* strains have been isolated, suggesting that *A.popoffii* is a more specialised aquatic species than other *Aeromonas* spp.

By far the most variable cluster genetically and phenotypically contains the HG3 type strain *A.salmonicida* NCIMB1102, a number of typical and atypical *A.salmonicida* strains and a larger number of *A.hydrophila* – like mesophilic strains. Whilst the atypical *A.salmonicida* seem to be genetically quite diverse, the typical *A.salmonicida* strains form a very tight AFLP cluster. It is surprising to find that within the psychrophilic atypical *A.salmonicida* there is almost as much genetic variability as in the cluster as a whole including the mesophilic strains. Indeed one of the reasons why so few psychrophilic strains were included in this study is because when this work began it was generally believed that *A.salmonicida* HG3 was a distinct species quite different from the highly variable mesophilic species (Inglis *et al.*,1995; Boyd *et al.*, 1994). It should, however be noted that Pavan *et al.* (2000) concluded on the basis of 16S rDNA sequences and DNA-DNA hybridisations, that the three *A.salmonicida* sub-species, *salmonicida, masoucida, and achromogenes* together with a new subspecies they designated *pectinolytica*, together formed an

extremely tight cluster when compared with other *Aeromonas* spp. Unfortunately they did not include any mesophilic representatives of HG3 in their analysis.
Using I-*Ceu*I PFGE fingerprints for the construction of a physical map of the *A.salmonicida* chromosome, Umelo and Trust (1998) also found evidence for genetic homogeneity within geographically diverse *A.salmonicida* strains. They also commented on the surprising variation in the I-*Ceu*I digestion fingerprints of the atypical *A.salmonicida* and *A.hydrophila* commenting that such variation has not been reported for any other bacterial species.
These observations raise the interesting possibility that, though widely thought of as a species, typical *A.salmonicida* are in fact representatives of a very small number of specialised and highly successful clonal lines that arose out of a much more heterogeneous background population.

An AFLP-based investigation into the status of *A. eucrenophila*, (Huys *et al.,* 1996a) showed that this species divides into distinct genotypic groups.
In a later more extensive polyphasic taxonomic study (Huys, 1996b) the same group were able to show that the original *A. eucrenophila* type strain resides in one AFLP cluster which they designated subgroup 1 whilst a second *A. eucrenophila* subgroup appears to form part of a genospecies that includes *A. encheleia* and the unnamed *A.veronii*-like HG11 strains. In the original description of *A.encheleia*, based on three isolates from European eels, Esteve (1995) had proposed that *A.encheleia* be considered as a separate species and the suggestion that it be included in HG11 re-ignited a vigorous debate over the status of AFLP data over DNA-DNA hybridisations (Esteve, 1997). In a further study (Huys *et al.*, 1997b) published

DNA-DNA hybridisation data that supported their taxonomic scheme and it was proposed that *A.eucrenophila,* subgroup 1 be regarded as the true *A.eucrenophila* while *A.eucrenophila* subgroup2, *A.encheleia,* and the previously unnamed HG11 isolates be classified together as *A.encheleia.* Though these two species are phenotypically very similar, it was shown that they could be distinguished on the basis of assimilation of D-cellobiose and production of acid from lactose and D-cellobiose.

The debate continued with Martínez-Murcia (1999) adding 16S rDNA sequence data in support of the claim that HG11 and *A.encheleia* are distinct taxa, but suggesting that decisions be deferred pending an investigation into the implications of the very different techniques used for DNA-DNA hybridisations in the two laboratories. Most recently, Valera and Esteve (2002) also produced further phenotypic evidence that *A.encheleia* should be regarded as a separate species.

The data presented here involves a far larger number of isolates from a far wider range of sources than has previously been examined by all of these groups combined. Though the validity of AFLP analysis for taxonomic purposes has been questioned by Dr. Esteve's group (Esteve, 1997; Valera and Esteve, 2002), what cannot be denied that it makes comparison of such numbers possible, instead of confining the debate to consideration of the status of single, or at most two or three strains.

The contribution of this AFLP data would be to suggest that close to *A.trota* HG13 is a heterogeneous cluster that divides at a correlation level of 62% into two sub-groups, the smaller of which contains the *A.eucrenophila* HG6 type-strain. The larger group further subdivides into three clusters, one of which contains the HG11 type strain, another the *A.encheleia* type-strain. Whilst the evidence presented here

does not support the idea that *A.encheleia* is a separate species, neither does it suggest that *A.eucrenophila* is sufficiently different genetically from the HG11/ *A.encheleia* cluster to place any of them in separate species. Perhaps far more importantly, the surprising finding that within the *A.encheleia* cluster are strains apparently pathogenic to sheep (see below) suggests that this taxon is far more diverse than previously suspected and holding a debate over three strains isolated from eels in one location is premature in that neither group has yet examined a large enough sample on which to base a meaningful discussion.

Within the some of the species clusters, a number of small genetically related groups are of interest. The first consists of three *A.veronii* bv. sobria isolates which were the kind gift of Dr. Lothar Beutin of the Robert Koch Institute in Berlin, two were recovered in pure culture from the blood of an infant admitted to hospital with acute renal failure. The infection was an extremely severe one which later necessitated a kidney transplant and this prompted an investigation into the likely source. (Filler *et al.*, 2000). A suspected source was via a bathtub in which an aquarium had been washed and the third isolate in this cluster was recovered from the aquarium water. Although other isolates from the aquarium water do not form part of this cluster, the strain that does has an AFLP fingerprint sufficiently similar to one of the patient's isolates to confirm the original suspicion that the aquarium was indeed the source of the infection. This is an extremely thought provoking finding and is one of the most compelling pieces of evidence yet that even while the status of *Aeromonas* spp. as frequent human pathogens continues to be debated, the potential dangers of these organisms should not be underestimated.

Within the *Aeromonas hydrophila* HG1 is a cluster of closely related strains isolated from farmed frogs throughout Thailand in 1994, (Pearson *et al.,* 1997). The isolates had unusual phenotypic properties for *A.hydrophila* and were originally designated Au – unspeciated Aeromonad. Subsequent work showed them to be highly pathogenic to frogs (Pearson *et al.,* 2000) and further investigation of their taxonomic status using AFLP, repPCR, DNA-DNA hybridisations and extensive phenotypic tests, led to the proposal to name them as a new sub-species: *Aeromonas hydrophila* subsp. *rannaei* subsp. nov. (Huys *et al.,* 2002).

Recovery of an organism from dead or moribund animals, even from intact internal organs is circumstantial evidence and cannot be taken as compelling evidence for pathogenicity. This is especially true when the organism is common in the aquatic environment and is isolated from an aquatic animal. However in a survey of this kind it is not realistic to expect to satisfy Koch's postulates and it may be that the recovery of a particular type at a disproportionately high frequency in association with apparently diseased individuals coupled with the failure to find representatives in the absence of disease is the most compelling evidence that could be expected. In this context an interesting find is a small cluster of 9 genetically and phenotypically very similar strains, all recovered from the internal organs of recently dead or moribund porpoises. They were isolated from several sites throughout the UK and over a period of several years, suggesting that they are representatives of a persistent and stable clonal lineage. This biotype was only ever isolated from porpoises even though numerous other marine mammals were sampled at the same locations and over the same time period. It is particularly interesting to discover an

apparently highly specialised cluster against the background of such diversity of other strains isolated from porpoises and other marine mammals.

AFLP template prepared from these strains was sent to LMG, Universitiet Gent for comparison with their AFLP reference library and it was confirmed that these strains form an unusually closely related cluster within HG3. Previously the only other report of recovery of such closely related strains over several years was the repeated isolation of a single Fatty Acid Methyl Ester (FAME) phenotype over a four- year period from a single drinking water well (Kühn *et al.,* 1996).

The closely related *A.hydrophila* subsp. *ranaei* cluster, isolated from tropical farmed frogs, mentioned above were all isolated within a few months of each other. It would seem therefore, that the recovery of this number of genetically and phenotypically similar strains, from a single host species, from a number of distant sites and over a lengthy time period, is without precedent.

Although only six strains recovered from sheep were included in this study, three *A.veronii*- like HG11 strains isolated over a two year period also appear to be closely related genetically and may indicate another persistant and stable genotype. Though smaller in number than the porpoise cluster it represents a far higher proportion of sheep-associated strains examined raising the possibility that this is an extremely common strain. All were isolated from post mortem sheep spleen or blood under sterile conditions and in each case no other potential pathogens were identified. This raises the possibility that a significant pathogen of sheep has been uncovered here and this certainly warrants further investigation.

A further strain isolated from a sheep is also of interest in that it resides in the same cluster as the *A.encheleia* type strain. This is an important observation not just

because of the taxonomic implications mentioned above but also because *A.encheleia* is one of the few *Aeromonas* species that has never been recovered from clinical material (Janda and Abbot, 1998). To find it implicated in an extra-intestinal infection in a land mammal so closely associated with humans raises the possibility that this species could also be a potential human pathogen. In fact it is perfectly feasible that *A.encheleia* has been frequently recovered from human clinical samples but has never been identified as such. Of the few clinical microbiology laboratories that do type *Aeromonas* isolates to species level, very few would discriminate between *A.encheleia* and *A.veronii* (Abbot *et al.,* 1998). Furthermore several of the strains in the *A.encheleia* cluster were sensitive to the vibriostatic agent O/129 which is consistent with the observation of Carson *et al.* (2001) that *A.encheleia* type-strain ATCC 35941 was O/129 sensitive. Since resistance to O/129 is normally considered to be a characteristic of the genus *Aeromonas* as a whole, it is likely that *A.encheleia* strains recovered in some previous surveys would have been eliminated from further analysis on that basis.

Generally the relationships between closely related species clusters agrees well with other published AFLP data (Huys *et al.,* 1996) and the close relationship between strain groups shown by other means to be very similar is also very consistent: such as the porpoise and the *A.hydrophila* subsp. *ranaeii* subsp. nov. clusters described above, both of which appear to be also highly related phenotypically and using RAPD and REP genotyping. The frog isolates also share common pathogenicity determinants (Pearson *et al.,* 2000)

The relationship between more distantly related clusters is not so clear and so comparison is difficult. This is probably a good indication of the 'window' of similarity for which AFLP is suitable for discriminating.

In bacteriology discrimination to the species level is generally referred to as identification, whilst typing denotes differentiation to the species level, (Savelkoul, 1999). It would appear that AFLP is suitable for both identification and typing but the breakdown of a clear pattern of relationships between the apparently more distantly related species indicates that taxonomic inferences beyond the species level should be drawn with great care. The underlying assumption when comparing fingerprints of this type is that bands of the same size are homologous (Krause, 2000), and indeed there is evidence to show that comigration of non homologous fragments is rare (Rouppe Van Der Voort *et al.,* 1997).

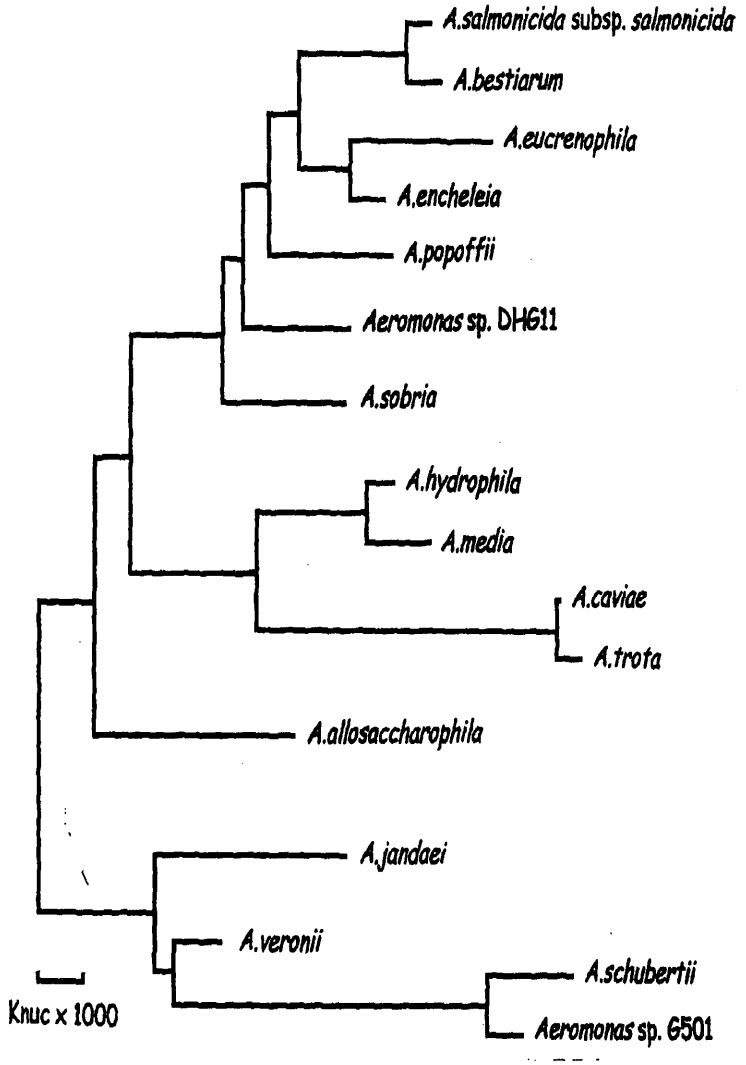
However at similarity levels of below 40% very few AFLP bands are shared, which means that not only is cluster analysis based on fewer comparisons, but the danger of homoplasy – where non homologous bands are coincidentally the same length – increases, (Savelkoul, 1999; Rademaker *et al.*,2000).

Traditionally the relationships between bacterial genera and beyond has been explored using 16S rDNA sequencing, indeed, much of our current understanding of bacterial systematics is based on this technique, (Woese, 1987).

However use of 16S rDNA sequencing to explore relationships closer than the genus level can be problematic as ribosomal sequences may vary very little within a genus. Between *Aeromonas* species 16S rDNA sequences may be as much as 98.7% identical (Martinez- Murcia *et al.*,1992; Collins *et al.,* 1993).

There is a striking lack of congruence between *Aeromonas* species relationships suggested by 16s ribosomal DNA sequencing and the results of DNA-DNA hybridisations (Martinez- Murcia *et al.*,1992).

In this context, the profound disagreement between the apparent relationship between *Aeromonas* species suggested here using AFLP data, and the relationship reported elsewhere (fig 3.10: Martinez-Murcia, 1999) is significant and warrants further investigation.



Phylogenetic relationships of described *Aeromonas* species (or DNA Homology Groups) as determined by a continuous 1502-nucleotide 16S rDNA sequence comparison using the neighbour-joining method. Strains and sequence accession numbers (in parentheses) are: *Aeromonas hydrophila* ATCC 7966^T, (X60404); *A. bestiarum* CIP 7430^T, (X60406); *A. salmonicida* NCIMB 1102^T, (X60405); *A. caviae* NCIMB 13016^T, (X60408); *A. media* ATCC 33907^T, (X60410); *A. eucrenophila* NCIMB 74^T, (X60411); *A. sobria* NCIMB 12065^T, (X60412); *A. veronii* ATCC 35624^T, (X60414); *A. jandaei* ATCC 49568^T, (X60413); *Aeromonas* sp. DHG11 ATCC 35941^T, (X604417); *A. schubertii* ATCC 43700^T, (X60416); *A. trota* ATCC 49657^T, (X60415); *A. allosaccharophila* CECT 4199^T, (S39232); *A. encheleia* CECT 4342^T, (AJ224309); *A. popoffii* LMG 17541^T, (AJ224308); *Aeromonas* Group 501 CECT 4254, (U88663).

Figure 3-10: *Aeromonas* relationships deduced from 16S rDNA sequences: Data from Marinez-Murcia (1999)

Comparison of the relative proportions of each species identified here with the numbers identified in other work is not easy as most published work does not identify isolates to species level (Pettibone 1998) and even those that do generally discriminate only between '*A.hydrophila* ', '*A.caviae'*, and *'A.sobria'* and do not specify exactly what is meant by these labels (Fiorentini *et al.,* 1998; Legnani *et al.,* 1998). The exception tends to be work published by medical microbiology laboratories but even in these papers, identification to HG level is rare and besides is focused on a much narrower range of species and sources (Janda and Abbot, 1998).

The extent to which this kind of work can ever produce an accurate assessment of the true numbers of different microbes in different environments is questionable. Obviously assembling a collection from such diverse sources inevitably involves surrendering some control over sampling strategies and isolation methods. However even when these can be kept consistent, choosing any single method immediately introduces a bias. Moreover estimates of the proportion of viable environmental bacteria that are recovered by cultivation on laboratory media vary between fractions of 1% to at most a few per cent. Most aquatic bacteria exist under near starvation conditions and so only a minority of strains will be recovered and furthermore the subset of strains recovered will be profoundly affected by the method of isolation (Holmes *et al.*, 1996). There is much evidence to suggest that many, possibly most environmental aeromonads exist in a VBNC state (Morgan *et al.,* 1993; Rahman *et al.,* 2001). It is likely also that only a subset of pathogens implicated in an infection will be recovered by conventional means.

Molecular techniques to circumvent the need to cultivate organisms of interest such as PCR amplification of rDNA (Head *et al.*, 1998; Hugenholtz *et al.*, 1998) would enhance the value of surveys such as this and should be routinely included in future work of this type

CHAPTER 4:

Optimisation and Application of Rep-PCR

Chapter 4: Optimisation and use of REP-PCR

4.1: Introduction

Non-transcribed and apparently non-coding transcribed sequences located primarily in extragenic regions of the genome are a common feature of genome organisation in eukaryotes (Cox and Mirkin, 1997), and account for a substantial proportion of the genome in most, if not all eukaryotes, (van Belkum *et al.*, 1998). Because these sequences are often not subject to the same selective constraints as coding sequences and may accumulate mutations at a higher rate than in regular sequences they have been much studied by evolutionary biologists: tandem repeat sequences in particular are unusually prone to mutations which add to or reduce the number of repeats, and hence are highly polymorphic. These polymorphisms have been widely exploited for DNA fingerprinting and phylogenetic analysis in eukaryotes.

Until recently it was generally believed that the contrasting absence of non-transcribed sequences in prokaryotic genomes was one of the fundamental differences that separate the two domains. It was suggested that selective pressure in favour of rapid DNA replication and cell division in smaller more streamlined prokaryotic genomes would ensure that non-coding DNA is kept to a minimum. Similarly it was hypothesised that multiple copies of coding sequences would only be tolerated where this would facilitate rapid growth: for example those encoding ribosomal RNAs, (Doolittle, 1988). Indeed, as sequence data became available it became clear that a very high proportion of prokaryotic DNA was indeed, transcribed coding sequences (Versalovic, Koeth and Lupski, 1991).

Field and Wills (1998) conducted the first systematic computerised search for repeat arrays within published prokaryotic genome sequences. Their findings indicated that

in prokaryotic genomes these repeat units are being generated by mutational pressures but that most were shorter than expected statistically, suggesting that there is indeed strong selective pressure against repeat expansion in prokaryotes. As yet there has been no experimental evidence to support this model but as the GenBank sequence database continues to expand at an accelerating rate, it will be interesting to see if further searches reveal a similar trend.

Such searches may yet uncover markers useful for the very high resolution discrimination of microbial strains. The promising potential of this approach was demonstrated by the work of Marshall *et al.* (1996) who found that probing the genomic DNA of different *Helicobacter pylori* strains with short, microsatellite-type DNA probes revealed clear and epidemiologically informative DNA polymorphisms. Such markers could prove to be particularly valuable in view of the growing evidence that variation in tandem repeat number may be involved in the expression of virulence factors by previously commensal microbes (Moxon and Thraler, 1997; deBolle *et al.* 2000).

A particular class of non-transcribed sequences in eukaryotes are interspersed repetitive sequence elements, the best known of which is the *Alu* family of sequences identified in mammalian species. The function of these sequences is not known but they frequently contain highly conserved domains to which it has been possible to design complementary PCR primers in order to amplify the polymorphic regions between them in a technique known as *Alu*-PCR (Nelson *et al.,* 1989).

Despite the selective pressures constraining genome size in bacteria, analogous sequences have been identified in many species. From the late 1980's onwards of a

steadily expanding class of repeated elements dispersed throughout bacterial genomes began to be identified (Lupski and Weinstock, 1992).

Several dozen have now been identified, most are shorter than 200 bp, noncoding, intercistronically located, and distributed evenly throughout the genome (Versalovic and Lupski, 1998)

The best characterised are the REP (Repetetive Extragenic Palindromic), the ERIC (Enterobacterial Repetitive Intergenic Consensus) and the BOX (Martin *et al.,* 1992) sequences.

ERIC, REP, and BOX motifs constitute different classes of prokaryotic dispersed repeat elements that may occupy as much as 1% of bacterial genomes (Tyler *et al.,* 1997). Although little is known about their function, their ubiquity suggests that they are involved in important aspects of microbial life (van Belkum *et al.*, 1998)

A common feature of these elements is a highly conserved central inverted repeat sequence and these conserved sequences have been exploited as primer binding sites to develop a technique analogous to *Alu*-PCR, collectively known as rep-PCR.

Where these primer-binding sites occur within a few kb of each other, on opposite DNA strands and in opposite orientation, the sequences between them are amplified. Insertions, deletions and other chromosomal re-organisation within the intervening sequences will alter the length and number of amplicons so that a strain-specific DNA fingerprint is generated. Unlike RAPD fingerprints, polymorphisms are not dependent on subtle alterations in primer binding sequences and therefore should be more reproducible (Johnson and O'Bryan, 2000) and the PCR can be conducted under stringent conditions (Johnson and Clabots, 2000).

Whilst it is true that better knowledge of the function of these elements and an understanding of the mechanisms controlling their evolution, would assist in the design and interpretation of experiments, even while these are little understood, this need not be an obstacle to the use of rep-elements as anonymous markers in the meantime.

Although the dispersed repeats were initially deemed to be rather species specific, closely related sequences have also been detected in other microbial species (Versalovic *et al.,* 1998) and rep-PCR has seen increasing use for microbial typing over the past 4 years. In one major study Dombek *et al.* (2000) were able to differentiate *Escherichia coli* isolates from human and animal sources using the BOX primer; whilst Jeršek *et al.,*(1999) were able to discriminate between all tested serotypes of *Listeria monocytogenes.* Gomez-de-Leon *et al.,* (2000) were able to distinguish between the fingerprints generated by *Haemophilus influenzae* isolates obtained from healthy carriers and those obtained from sick children and van der Zee *et al.,*(1999) found Rep-PCR the fastest and easiest way to trace nosocomial methicillin-resistant *Staphylococcus aureus* (MRSA) outbreaks. In two comparative studies, Marshall *et al.,* (1999) found ERIC-PCR to be more informative than PFGE for tracing epidemiological relationships between *Vibrio parahaemolyticus* isolates; whilst Sechi *et al.* (1998) found the level of differentiation obtained using ERIC-PCR for typing *Mycobacterium tuberculosis* strains greater than using IS*6110* fingerprinting.

Rep-PCR has not previously been used for the discrimination of *Aeromonas* strains. Therefore this work was undertaken with the aims of adapting the technique for use

with *Aeromonas* spp. and assessing its value for differentiating the diverse collection of *Aeromonas* strains that had already been assembled.

4.2: Methods and Materials

4.2.1: ERIC Magnesium and temperature titration

Little consensus exists in the literature as to PCR protocols so initial parameters were chosen that would span most published PCR conditions.

All PCR reactions took place in a total volume of 20µl, in thin walled 200µl dome topped microcentrifuge tubes (Abgene), using an Eppendorf Gradient Mastercycler 96-well thermal cycler.

Initially all reactions contained 50ng template DNA, 0.25mM (each) dNTP's and 200nM primers, 1X dynazyme buffer 1X Dynazyme reaction buffer (Flowgen, Staffs., UK), (=10 mM Tris-HCI (pH 8.8 at 25 ^{0}C); 50 mM KCl; 0.1 % Triton X-100) and 1U DynazymeII (Flowgen, Staffs., UK).

All primers were obtained from MWG Biotech.

Primers used were ERIC1R (Rademaker and de Bruijn, 1997):

5'- TGT AAG CTC CTG GGG ATT CAC – 3'

and ERIC2 (Rademaker and de Bruijn, 1997):

5' – AAG TAA GTG ACT GGG GTG AGC G – 3'

Initial magnesium and temperature titrations were performed using one template: the *Aeromonas hydrophila* NCIMB9240 HG1 typestrain.

12.5X reaction mixes were prepared incorporating $MgCl_2$ concentrations of 1.0mM; 1.2mM; 1.5mM; 2.0mM and 2.5mM.

Master mixes without template were prepared on ice and each was aliquoted into one of 12 reaction tubes.

Template DNA was pipetted into the lid of the reaction tubes, which were held on ice. When all components had been assembled the template was added to the rest of the PCR mix by centrifugation for 10s at 1000g.

Reactions were placed quickly into the thermal cycler previously set to hold at 4°C with the lid pre-heated.

The thermal profile of the cycler was set to:

96°C for 2 minutes (initial denaturation)

94°C for 30seconds (denaturation)

X°C for 30seconds (annealing)

72°C for 1minute (elongation)

Steps (2) to (4) were repeated for 30 cycles.

The annealing temperature gradient was set to 56°C +/- 10°C. Actual annealing temperatures in each column were:

column	1	2	3	4	5	6	7	8	9	10	11	12	
temp	46°C	46.3°C	47.4°C	49.2°C	51.5°C	54.1°C	56.8°C	59.5°C	62°C	64.1°C	65.7°C	66.5°C	

Table 4-1: Annealing temperature gradient in Rep-PCR optimisation experiments

This experiment was repeated using 5ng template DNA.

4.2.2: BOX Magnesium and temperature titration

A single primer was used: BOX-A1 (Rademaker and de Bruijn, 1997):

5' – CTA CGG CAA GGC GAC GCT GAC G – 5'

The single box primer was used at a concentration of 200nMol.

All other reaction conditions for the BOX Magnesium and temperature titration were as for the ERIC titration, except that 10ng template DNA was used throughout and the 2.5mM $MgCl_2$ treatment was omitted.

4.2.3: Further Temperature Titrations

After initial temperature and Magnesium titrations, using ERIC and BOX primers further temperature titrations were carried out using five different templates:

A.hydrophila	NCIMB 9240
A.caviae	NCIMB 13016
A.media	NCIMB 2237
A.veronii bv. sobria	NCIMB 37
A.jandaei	ATCC 49568

All reactions using the BOX primer contained 1.3mM $MgCl_2$, whereas all ERIC-PCR reactions incorporated 1.5mM $MgCl_2$.

Reactions were carried out using the thermal profile described above, using annealing temperatures of 53°C; 54.4°C, 55.8°C 57.1°C and 58.2°C.

In all cases 10ng template DNA was used.

4.2.4: Final Reaction Conditions

Reactions were carried out in 10µl volumes in 96-well low profile Thermofast plates (Abgene). Each reaction contained 10ng template DNA, 0.25mM (each) dNTP's and 200nM primers, 1X Dynazyme reaction buffer (Flowgen, Staffs., UK), (=10 mM Tris-HCI (pH 8.8 at 25 ^{0}C); 50 mM KCl; 0.1 % Triton X-100) and 1U DynazymeII (Flowgen, Staffs., UK).

ERIC PCR reactions contained 1.5mM $MgCl_2$ and BOX-PCR reactions contained 1.3mM $MgCl_2$.

The thermal profile of the cycler was set to:

96°C for 2 minutes (initial denaturation)

94°C for 30seconds (denaturation)

X°C for 30seconds (annealing)

72°C for 1minute (elongation)

Steps (2) to (4) were repeated for 30 cycles.

ERIC-PCR used an annealing temperature of 57.5°C whereas for BOX-PCR it was 56°C.

4.2.5: Electrophoresis and visualisation of fingerprints

Following PCR, 6µl 5X loading buffer (0.2% bromophenol blue, 20% sucrose, 50mM EDTA (Sigma)) was added to each reaction tube and 14µl was loaded onto a horizontal agarose gel (1.4% in 0.5X TBE buffer), containing 0.5µg.ml.$^{-1}$ ethidium

bromide. Two lanes containing 5µl PCR marker (Promega) were included at either side of each gel.

During optimisation work electrophoresis was carried out using a homemade10cm submerged minigel electrophoresis tank at $6V.cm^{-1}$ for approximately 1 hour. For analysis of fingerprints electrophoresis was carried out using a Flowgen15cm submerged gel electrophoresis tank at $4V.cm^{-1}$ for approximately 2.5 hours.

The gel was destained for 5 minutes in running water and examined under UV on a transilluminator. An image was recorded using a CCD camera and stored as a tif file.

4.2.6: Image Analysis

Images were imported into GelCompar image analysis software version 2.0 (Applied Maths BVBA, Kortrijk, Belgium).and lanes tracked at a resolution of 600. After background subtraction using the rolling disc principle, and normalisation to the size standard markers, the fingerprints in each lane were compared using the Pearson product-moment correlation coefficient algorithm applied to all points falling in the active zones of the band patterns. Cluster analysis was performed using the unweighted pair-group method, using arithmetic averages (UPGMA) algorithm (Sneath and Sokal, 1973). Optimisation and position tolerance settings were determined by applying the maximised group contrast method to the fingerprints obtained from Hybridisation Group type strains.

Similarity matrices based on the Pearson product-moment correlation coefficient, generated by Gelcompar 2.0 were exported as text files and imported into Microsoft Excel for further editing. Edited matrices were compared using a Mantel Test (Mantel, 1967; Smouse, Long and Sokal, 1986) using 'Mantel' software, which was

the kind gift of Professor Roger Thorpe, Dept of Biological Sciences, University of North Wales, Bangor.

Principal co-ordinate analysis was performed using Multivariate Statistical Package (MVSP; Kovach Computing Services, Pentraeth, Angelsey, Wales, UK. (http//www.kovcom.com).

4.2.7: PCR Using Whole Cells

Twenty-five PCR mixes were prepared as described in section. For each of the five typestrains listed in section, one PCR was prepared using 10ng purified template, and for each typestrain a further reaction mix was prepared as follows.

1: A whole inoculation loopful taken from a single colony growing on a 2-day old agar plate was added to the PCR mix and resuspended.

2: Aproximately 0.5mm diameter spherical mass of cells obtained by inserting the tip of a sterile cocktail stick into a single colony, was added to the PCR mix and resuspended.

3: A suspension was prepared by resuspending an inoculation loopful of cells in 500µl sterile 0.85% saline, 1µl of this was added to the PCR mix

4: Using the same suspension as above, 0.1µl plus 0.9µl 0.85% saline was added to the PCR mix.

4.2.8: 'PCR 'n Go' – A one-tube Protocol for Rep-PCR

The effect of the presence of high concentrations of sucrose on the PCR was compared by preparing replicate PCR reactions using each of the five type-strain templates. One was prepared as normal and one was prepared substituting 2µl 60% sucrose for 2µl of mQ. The 60% sucrose was filtered through a 0.1µ membrane filter and held at 95°C for one hour before use to eliminate nucleases.

The effect of various dyes on the PCR was assessed by comparing the fingerprints generated using a standard PCR mix with those generated including in the PCR mix 0.1mM bromophenol blue; 0.1mM bromocresol purple; 0.1mM neutral red; 0.1mM cresol red; and 1µl of a 1:500 dilution of red (E123) and yellow (E110) food colouring (Tescos).

Thereafter 0.1µl of a suspension prepared by resuspending a single inoculation loopful of bacterial cells in 1ml 0.85% saline was added to each PCR instead of purified template and 2µl of a 60% sucrose: 1mM cresol red was added to each PCR mix in place of 2µl mQ.

4.2.9: Use of 3'-degenerate primer antagonists to inhibit non-specific primer binding

Primers were prepared (MWG Biotech) incorporating a modified amine base at the 3'-end. Each primer was degenerate at the 5 bases at the 5'-end ie. towards the 5'end of each primer antagonist was each possible base except the base in the original primer.

ERIC1R-am

5'- VHV BBG CTC CTG GGG ATT CAC- – 3'

ERIC2-am

5' – BBH VBA GTG ACT GGG GTG AGC G – 3'

BOXA1-am

5' – DVB DHG CAA GGC GAC GCT GAC G – 5'

Where V= G, A or C;D= G,A, or T B= G,T, or C and H= A,T, or C.

Using the five type-strain templates described above, PCR mixes were prepared using the standard PCR mix described in section (0-am) and by adding 100nM primer antagonists (0.5X-am); 200nM primer antagonists (1X-am); 400nM primer antagonists (2X-am); and 1μM primer antagonists (5X-am).

All reactions using the BOX primers contained 1.3mM $MgCl_2$, whereas all ERIC-PCR reactions incorporated 1.5mM $MgCl_2$.
Reactions were carried out using the thermal profile described above, using annealing temperatures of 53°C; 54.4°C, 55.8°C 57.1°C and 58.2°C.
In all cases 10ng template DNA was used.

4.2.10: High resolution analysis of Rep-PCR products using an automated DNA sequencer

For visualisation of Rep PCR products at higher resolution than achievable using agarose-gel electrophoresis, forward PCR primers were synthesised 5'fluorescently labelled. Primer ERIC2 was 5'labelled with JOE and BOXA1 with FAM (MWG Biotech). Additionally the primers REP1R and REP2I (Versalovic *et al.,* 1994) were obtained (MWG Biotech) with REP2I labelled with 5' TAMRA.

REP1R: 5' - III ICG ICG ICA TCI GGC – 5'

REP2I: 5' – ICG ICT TAT CIG GCC TAC – 3'

PCR amplification of the REP locus was performed according to the protocol of Rademaker and deBruijn, (1997) with minor modifications.

The PCR mix was prepared as described for ERIC-PCR, substituting the REP primers. The $MgCl_2$ concentration was 1.5mM.

Thermal cycling parameters were as follows:

96°C for 2 minutes (initial denaturation)

94°C for 30seconds (denaturation)

44°C for 30seconds (annealing)

65°C for 8minute (elongation)

Steps (2) to (4) were repeated for 30 cycles.

A final elongation step of 16 minutes at 65°C was included.

Loading cocktail was prepared as follows:

16µl blue dextran +

16µl Genescan 2500 size standard (Applied Biosystems)

0.6µl was aliquoted into alternate rows of a 96 well PCR plate.

Following PCR samples were aliquoted: for each lane

0.4µl JOE labelled ERIC product;

0.25µl FAM labelled BOX product;

0.6µl TAMRA labelled REP product

was added to the loading cocktail.

A 4% non-denaturing 19:1 acrylamide: bis-acrylamide gel was prepared using:

4mls 10X TBE

4mls 40% 19:1 acrylamide solution (Biorad)

This was made up to 40ml using mQ water.

The gel mix was not de-gassed.

Stirring in a beaker, 200µl freshly thawed 10% Ammonium Persulphate (Sigma) was added.

Then 40µl TEMED (Sigma) was added and the gel cast immediately using 36cm glass ABI 377 sequencing plates (Applied Biosystems) using a stepped front plate and two hours allowed for polymerisation.

Following a plate check 48 1.2µl samples were loaded into alternate wells of a 96 lane sharkstooth comb and the samples run into the gel for 2 minutes at 1500V. At

this point the comb was removed and the run continued at 750V for 3hrs, using filter set A.

Although no chiller module was available, care was taken to run all non-denaturing gels on cold evenings with the laboratory well ventilated and the target run temperature was set to 24°C.

After installation of the matrix file, ABI gel files were tracked and extracted using GeneScan analysis software (PE Applied Biosystems) and each chromatogram was saved as an individual sample file. These were then imported into ABICONV 2.0 (Applied Maths BVBA, Kortrijk, Belgium).

This program converts the ABI sample files into the GelCompar 2.0 curve format by splitting the multichannel sample files into separate virtual gel files for each available colour. Logically each of these virtual gels contains the same lanes at the same positions. One contains the internal reference patterns, whereas the others contain the real data to be normalised according to the reference pattern. Having imported the ABICONV files into GelCompar 2.0, the reference gel was normalised and the GeneScan-500 fragment sizes assigned to bands. The normalisation pattern from the reference gel was then superimposed onto each of the other three virtual gels.

Comparative analysis was performed using the Pearson product-moment correlation coefficient algorithm applied to all points falling in the active zones of the band patterns. Cluster analysis was performed using the unweighted pair-group method, using arithmetic averages (UPGMA) algorithm (Sneath and Sokal,1973).

Optimisation and position tolerance settings were determined by applying the

maximised group contrast method to the fingerprints obtained from Hybridisation Group type strains.

Similarity matrices based on the Pearson product-moment correlation coefficient, generated by Gelcompar 2.0 were exported as text files and imported into Microsoft Excel for further editing. Edited matrices were compared using a Mantel Test (Mantel, 1967; Smouse, Long and Sokal, 1986) using 'Mantel' software, which was the kind gift of Professor Roger Thorpe, Dept of Biological Sciences, University of North Wales, Bangor.

4.3: Results

4.3.1: Magnesium and annealing temperature optimisation

Initially ERIC- PCR mixes containing $MgCl_2$ concentrations of 1.2mM, 1.5mM, 2.0mM and 2.5mM were tested, the higher concentrations were included as several published protocols recommend this level.

Both 2.0mM and 2.5mM were found to be too high for this application – Figs 4.1& 4.2 as there was much non-specific amplification at all annealing temperatures.

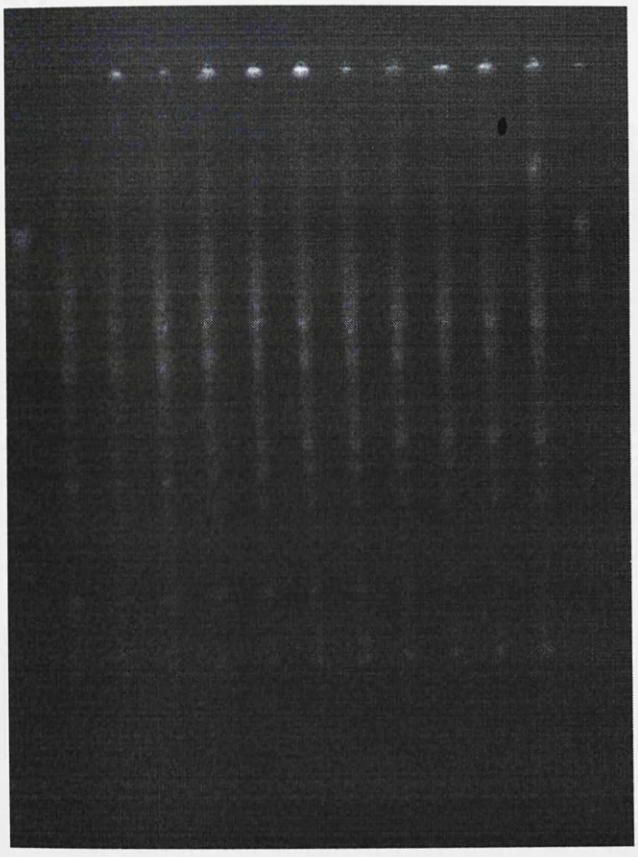


Figure 4-1: ERIC temperature titration: 2.0 mM $MgCl_2$

Lanes 1 & 13; size marker.
Lanes 2-12: annealing temperatures (left to right):
46.3°C, 47.4°C, 49.2°C, 51.5°C, 54.1°C, 56.8°C,
59.5°C, 62°C, 64.1°C, 65.7°C, 66.5°C

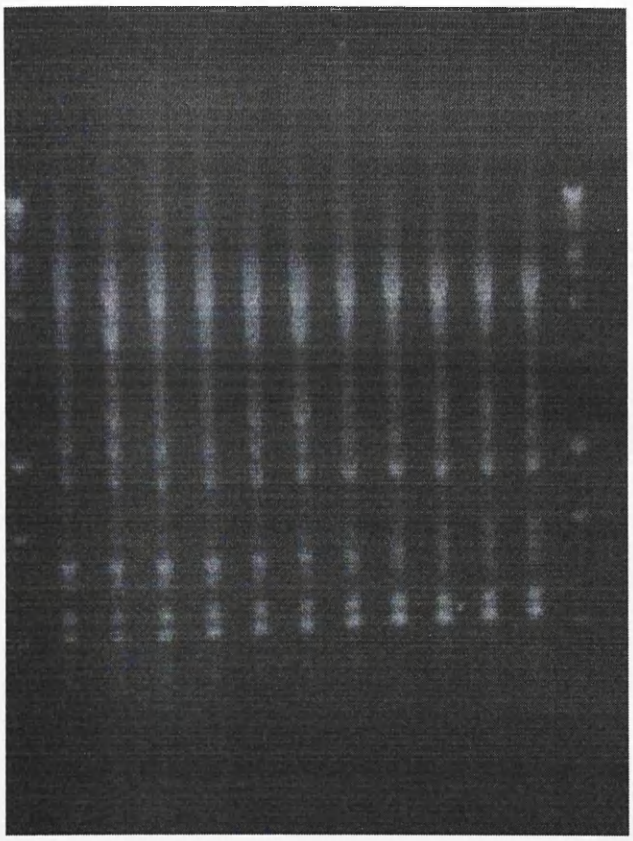


Figure 4-2: ERIC temperature titration, 2.5mM $MgCl_2$

Lanes 1 & 13; size marker.
Lanes 2-12: annealing temperatures (left to right):
46.3°C, 47.4°C, 49.2°C, 51.5°C, 54.1°C, 56.8°C,
59.5°C, 62°C, 64.1°C, 65.7°C, 66.5°C

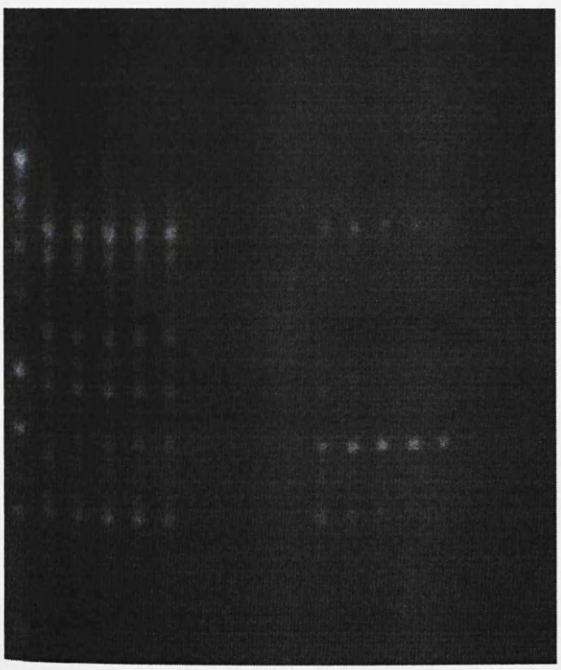


Fig 4.3 lane order:

Lane 1: size marker

Lanes 2-6: 1.5mM $MgCl_2$

Lanes 11-16: 1.2mM $MgCl_2$

Annealing temperatures in both cases were (left to right) 53°C, 54.4°C, 55.8°C, 57.1°C, and 58.2°C.

Figure 4-3: ERIC temperature titrations, 1.5 & 1.2 mM $MgCl_2$

Figure 4-3: ERIC temperature titrations, 1.5 & 1.2 mM $MgCl_2$

Below 1.5mM amplification was poor at all temperatures: Fig 4.3

4.3.2: Fingerprints using optimised conditions

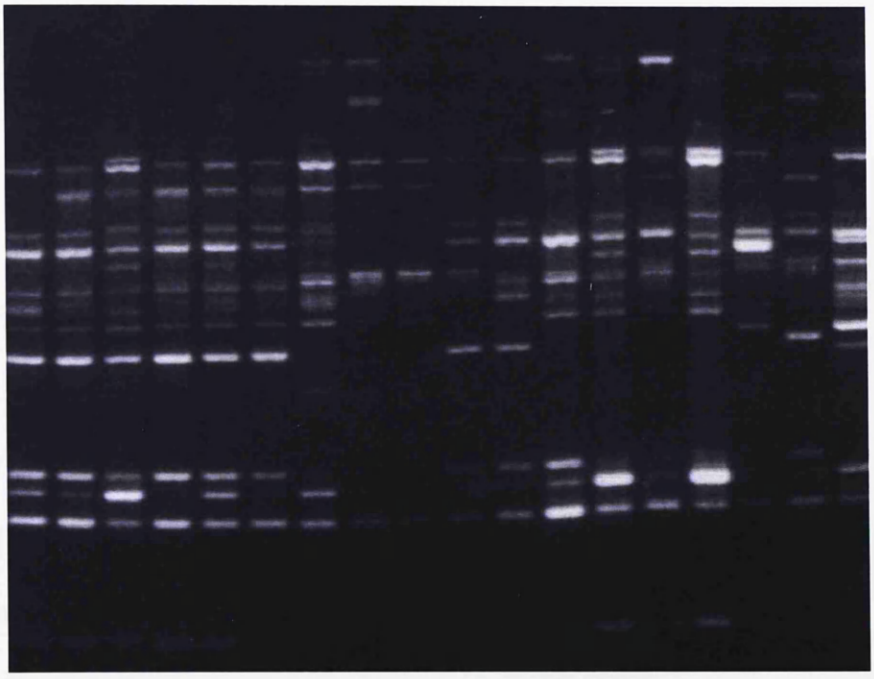


Figure 4-4: ERIC-PCR fingerprints produced using 1.5mM $MgCl_2$ and an annealing temperature of 57.5°C

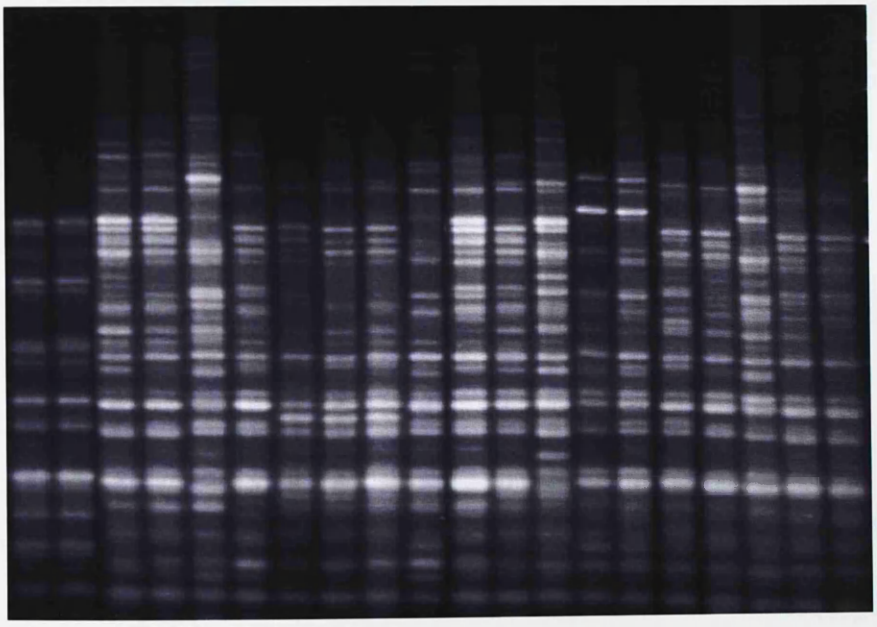


Figure 4-5: BOX-PCR fingerprints using 1.3mM $MgCl_2$ and 56°C annealing temperature

4.3.3: PCR using whole cells

Introduction of cell directly to the PCR mix – both using an inoculation loopful and a smaller mass of cells using a sterile toothpick – resulted in much non-specific amplification, obscuring the fingerprint.

Both 1µl and 0.1µl cell suspension however produced fingerprints that were almost identical to fingerprints prepared using purified DNA. Use of the lower amount appeared to produce marginally clearer fingerprints and this amount was used thereafter.

This would indicate that the non-specific amplification using the larger mass of cells was due to excessive amounts of template.

4.3.4: PCR n GO additive

PCR in the presence of 6% sucrose had no discernable effect on the resulting fingerprints.

Both bromophenol blue and bromocresol purple were found to inhibit PCR completely. Neutral red did not inhibit PCR, but was almost colourless in the PCR product. Red food colouring also inhibited PCR to an extent, interestingly inhibiting the amplification of larger fragments preferentially.

Both yellow food colouring and cresol red were found to have no apparent effect on the fingerprints. Since cresol red is far more visible when loading agarose gels, this dye was chosen for subsequent use.

4.3.5: Use of 3'-degenerate primer antagonists to inhibit non-specific primer binding

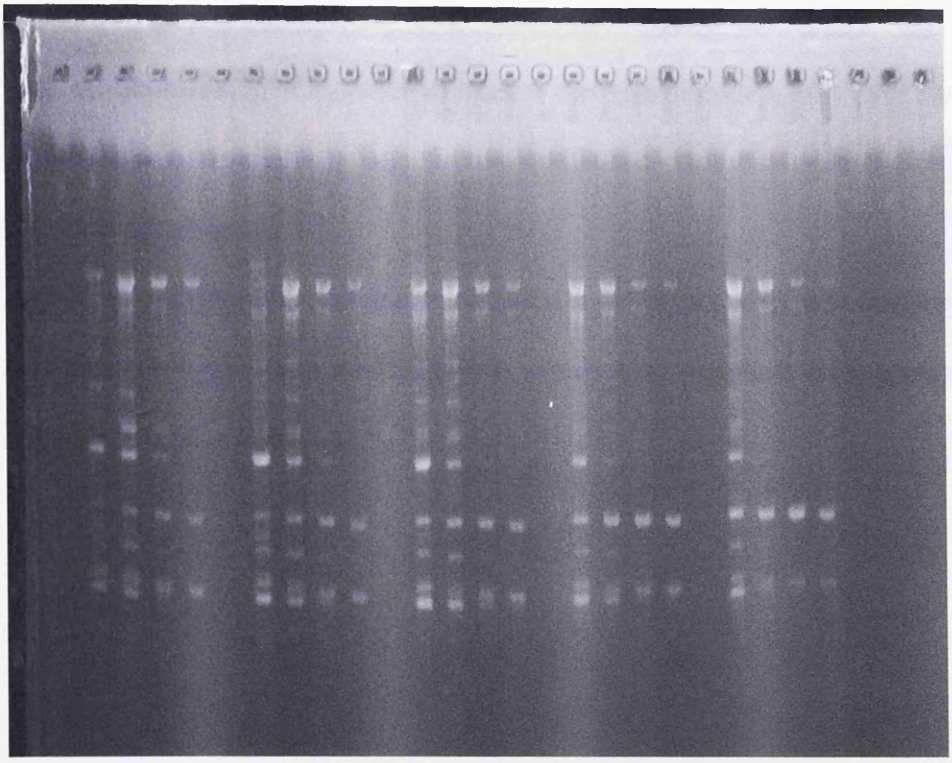


Figure 4-6: Effect of degenerate primer antagonists on ERIC-PCR amplification
Each block of 4 represents corresponds to a different annealing temperature. Temperatures were (left to right) 53°C, 54.4°C, 55.8°C, 57.1°C, and 58.2°C. Within each block consecutive lanes correspond to increasing amounts of primer antagonist relative to the regular primers: 1: standard PCR; 2: 0.25X ERIC-AM; 3: 0.5X ERIC-AM; 4: 1X ERIC-AM.

4.3.6: High Resolution Electrophoresis of REP-PCR products using an ABI377 DNA Sequencer and non-denaturing gel

The largest Genescan 2500 fragment of interest was visualised in a little over 2hrs. 750V proved to be a suitable voltage for this application since at no point did the gel temperature rise above 27°C.

A considerable improvement in resolution and size calling accuracy was achieved. It proved possible to resolve several fragments, which when separated using agarose had appeared to be a single band.

Figure 4-7: comparison of two equivalent ERIC-PCR fingerprints resolved using 15cm 1.4% agarose (bottom) and 36cm 4% acrylamide (top).

4.3.7: Cluster analysis of REP-PCR data

Cluster analysis using data produced using ERIC-PCR fragments resolved on agarose broadly agreed with the AFLP data, though there were inconsistencies: of 188 fingerprints analysed 16 (8.5%) did not assign to the same hybridisation group. Cluster analysis of BOX-PCR fragments was inconclusive and there was no apparent correlation between the BOX and the AFLP data.

Similarity indices between fingerprints produced from all isolates typed with both AFLP and REP-PCR were calculated within GelCompar using the Pearson product-moment correlation coefficient algorithm applied to all points falling in the active zones of the fingerprints.

The combined exported similarity matrices are shown in Table 4.2: with the AFLP data recorded in the upper diagonal and the ERIC-PCR data in the lower diagonal. The Pearson correlation between corresponding cells was r=0.83265.

Figure 4.8 shows a scatterplot illustrating the correlation between the two matrices.

Table 4-2: pairwise comparisons of AFLP and REP similarity indices

AFLP data

REP data

	001	002	003	004	005	006	007	008	009	010	011	012	013	014	015	016	017	018	019
001	100	13.79	22.46	11.89	12.33	56.35	55.41	57.32	54.67	48.42	55.07	56.54	55.28	53.44	63.01	27.35	20.93	18.93	19.47
002	10.81	100	87.6	57.21	7.41	15.97	17.88	16.33	18.25	12.65	17.73	15.66	16.5	17.08	10.89	18.99	10.89	9.91	9.15
003	14.18	89.83	100	54.01	5.33	20.35	20.55	21.07	20.59	17.6	20.59	20.28	19.33	19.56	16.23	20.38	12.86	11.41	10.34
004	13.16	59.05	57.49	100	43.87	15.45	17.89	15.71	19.33	11.52	17.58	14.77	16.4	17.75	12.75	16.45	9.69	12.79	10.91
005	24.05	11.25	9.87	44.38	100	12.83	13.85	11.68	14.89	9.87	13.25	10.87	13.9	14.67	12.18	36.49	34.9	37.94	33.17
006	53.01	8.67	9.33	13.33	18.82	100	96.69	98.33	94.83	86.41	95.97	98.35	95.89	95.75	60.65	28.91	26.13	20.85	23.77
007	54.26	10.7	10.67	17.02	21.82	97.42	100	95.65	96.81	82.16	97.37	95.55	97.08	96.09	58.97	28.95	24.71	20.35	22.03
008	54.72	9.17	10.22	13.04	18.14	98.25	96.21	100	94.17	88.27	95.45	98.39	94.5	94.01	61.67	27.93	24.05	20.03	21.9
009	53.93	11.89	11.27	19.21	23.2	94.99	97.05	93.59	100	82.01	98.12	93.38	96.01	95.65	58.65	28.93	24.22	19.65	20.4
010	53.21	9.53	10.25	14.09	18.37	98.79	97.15	98.95	95.08	100	82.26	87.53	83.77	80.54	55.39	24.55	22.09	16.67	19.46
011	53.55	12.69	12.48	17.74	21.37	95.77	97.23	95.01	97.91	96.05	100	95.35	97.01	96.29	58.68	28.33	24.21	19.54	20.47
012	53.39	8.97	9.99	12.97	18.06	98.51	96.92	98.77	94.41	98.79	95.71	100	94.7	94.59	62.1	28.14	25.21	19.73	22.11
013	53.36	10.83	10.47	16.4	22.33	95.91	97.22	93.97	96.87	95.69	97.57	94.59	100	94.41	58.2	27.11	25.39	19.05	20.79
014	53.01	10.31	10.08	16.9	20.27	97.02	96.61	94.42	95.62	96.05	96.06	95.96	95.74	100	57.77	29.45	25.31	21.01	21.23
015	61.33	9.18	9.3	17.17	19.57	56.89	58.44	58.87	58.89	56.14	57.7	57.95	54.69	57.37	100	29.37	24.17	25.75	21.12
016	34.25	27.55	27.89	26.11	15.06	33.19	36.15	34.24	36.61	33.99	35.92	34.66	33.34	34.95	36.89	100	72.59	72.51	68.09
017	23.89	16.7	17.73	17.89	14.93	29.21	30.91	28.74	30.27	29.28	30.23	30.27	31.58	30.49	30.02	59.35	100	68.77	72.73
018	21.21	13.33	13.95	21.62	15.5	21.11	23.08	21.91	22.39	21.26	21.79	21.2	20.22	20.91	31.73	56.79	53.44	100	70.21
019	21.69	11.71	11.9	16.9	15.57	27.43	29.36	27.07	27.96	26.57	26.98	27.24	26.45	27.43	25.2	55.25	64.05	62.58	100
020	30.75	14.34	15.41	14.24	9.71	33.88	37.53	33.74	37.39	34.13	36.15	34.71	35.15	34.35	35.28	56.19	61.06	57.75	65.99
021	25.3	16.77	18.58	21.95	21.61	32.39	35.4	32.19	34.37	31.97	33.54	33.5	32.41	33.37	33.15	59.07	59.68	56.77	60
022	32.31	25.76	24.88	23.28	20.53	30.25	32.01	30.79	31.33	30.25	30.31	30.73	29.52	30.5	38.25	69.2	66.83	69.08	62.13
023	50.76	14.11	10.35	22.17	19.83	51.39	53.1	52.57	55.17	52.15	52.39	51.87	50.87	50.85	59.49	33.88	29.51	22.08	28.83
024	22.65	18.41	16.4	19.65	16.43	30.12	32.27	28.94	31.79	28.69	30.03	29.39	29.65	30.21	28.19	58.41	63.33	60.99	91.96
025	31.72	20.82	20.57	16.73	14.93	28.61	29.24	29.29	28.06	28.35	27.05	29.01	26.69	27.79	36.77	65.81	66.75	64.52	61.89
026	55.66	10.54	11.36	20.51	22.91	54.52	57.67	54.97	58.62	55.42	58.77	55.15	56.43	55.76	57.67	40.25	33.47	28.4	29.46
027	31.99	21.08	22.66	18.65	12.68	32.44	35.29	32.71	34.17	32.63	34.23	33.83	31.71	33.53	34.47	86.37	58.66	52.01	59.53
028	16.98	8.97	10.74	6.15	7.96	23.73	24.84	23.57	24.22	22.99	24.11	24.14	24.07	23.93	20.87	43.69	50.83	44.15	54.96
029	57.45	15.68	13.51	15.1	21.07	51.53	54.65	51.52	57.06	51.39	53.46	51.11	53.39	54.39	62.99	31.11	20.79	21.36	23.49
030	21.4	31.45	26.98	21.37	13.21	21.49	22.05	24.11	20.49	22.57	21.08	21.68	21.5	19.29	15.39	15.4	15.61	13	6.73
031	22.85	11.37	13.93	10.22	54.71	17.77	19.65	19.27	17.35	18.25	17.69	19.16	16.79	17.12	16.47	20.81	16.17	5.81	14.81
032	19.71	17.63	16.93	13.67	28.93	18.33	19.81	19.66	19.68	18.79	20.78	18.75	20.25	18.26	13.08	13.23	19.2	16.35	13.03
033	27.04	5.76	5.51	2.34	47.45	23.73	24.93	24.66	23.78	23.43	25.03	24.19	25.41	22.75	22.67	14.27	12.32	10.13	10.37
034	21.71	19.05	16.3	17.84	30.87	20.75	22.12	21.77	22.26	20.56	22.33	20.17	22.3	20.13	20.69	11.95	10.13	11.32	6.66
035	46.43	16.09	10.37	30.4	27.8	47.72	52.65	48.71	57.67	49.11	53.57	48.57	52.53	50.73	57.59	31.31	24.87	22.31	23.01
036	22.41	33.45	27.41	24.75	11.46	21.18	23.21	23.75	22.37	22.35	22.29	21.22	21.94	19.67	17.14	15.45	13.38	13.19	9.13
037	18.31	30.1	28.6	23.11	12.2	16.38	15.61	18.11	14.19	16.66	15.1	17.13	14.66	14.74	9.69	13.23	16.36	6.8	8.64
038	12.77	73.11	73.63	45.5	8.59	11.21	11.24	11.75	10.49	12.31	11.35	12.09	9.89	10.51	15.25	28.27	15.61	22.28	13.28
039	11.39	58.78	60.49	36.71	8.75	5.7	6.17	6.19	6.6	6.39	6.97	6.31	5.63	6.15	11.08	25.43	15.49	15.77	15.11
040	16.82	32.79	28.53	26.91	12.27	13.42	14.39	15.42	14.25	14.43	15.23	14.1	14.64	13.16	9	16.67	17.48	11.07	10.18
041	21.71	29.37	26.43	19.99	6.41	17.26	19.46	19.77	20.04	18.25	20.66	18.27	20.03	16.89	15.72	15.59	13.71	6.38	6.59
042	18.5	38.37	32.55	23.77	6.52	12.57	14.48	13.86	13.53	13.67	14.43	13.84	13.77	13	10.96	20.76	22.84	14.24	12.16
043	25.8	26.21	24.24	18.46	10.01	23.83	25.53	26.6	24.34	25.13	24.73	24.48	24.15	21.25	20.62	17.04	19.89	13.51	13.85
044	58.38	8.16	7.33	15.95	28.87	59.83	62.59	60.61	59.5	58.41	60.71	61.27	58.75	59.43	58.67	37.18	25.77	24.84	27.87
045	23.41	32.47	31.34	22.69	11.55	23.19	23.94	25.4	23.33	23.54	23.23	23.01	23.04	21.05	19.95	15.91	13.17	11.45	10.13
046	16.49	59.31	59.95	37.71	11.77	11.91	13.23	12.2	12.95	12.86	13.22	12.54	12.21	12.99	12.42	28.43	17.43	20.05	13.41
047	63.47	4.79	5.27	10.86	15.41	53.48	55.52	53.93	54.81	52.57	53.95	53.95	52.94	54.71	60.74	30.94	21.25	22.51	27.84
048	37.39	17.91	14.95	22.87	16.17	33.27	36.59	32.93	41.79	33.33	41.45	32.87	39.24	35.5	43.95	23.54	15.22	20.93	17.62
049	53.89	6.42	6.35	10.66	13.8	38.15	41.85	36.64	42.27	37.29	40.67	38.34	39.7	40.77	53.43	33.79	23.89	23.83	27.23
050	50.71	13.07	10.73	15.83	18.27	39.01	43.81	37.95	47.29	38.83	45.95	39.31	43.35	42.55	54.35	32.95	23.43	19.17	21.57
051	62.01	11.89	10.39	24.29	26.91	48.6	52.31	48.77	54.86	48.34	52.51	48.57	51.74	51.17	65.57	35.38	32.45	26.55	27.35
052	14.69	22.71	21.43	23.75	30.12	16.02	17.61	16.92	16.52	15.7	17.47	16.23	18.12	16.31	10.74	7.7	14.02	11.06	11.16
053	59.31	8.96	8.97	12.52	19.75	52.51	54.23	53.12	52.74	52.59	53.03	53.21	53.59	53.01	52.8	33.69	36.08	21.99	28.03
054	25.29	15.87	17.31	22.64	20.31	28.55	31.21	28.45	31.59	28.3	30.19	28.83	28.61	29.2	35.15	62.65	61.99	68.67	67.8
055	52.19	14.7	11.61	21.71	21.97	56.8	59.79	56.16	61.21	56.6	59.89	56.19	57.55	58.94	61.91	30.42	24.51	23.74	23.4
056	11.81	61.23	58.03	41.71	14.77	15.09	16.27	14.75	16.11	15.07	16.61	15.85	15.73	15.31	14.98	26.15	26.63	18.51	16.8
057	57.93	16.3	15.07	20.89	22.96	54.29	56.93	55.73	60.33	56.44	57.63	55.1	55.91	54.37	56.75	37.86	30.55	22.72	28.57
058	14.64	58.82	54.97	34.99	11.15	12.81	15.21	13.32	17.11	13.59	16.99	13.25	14.8	13.54	15.09	24.7	18.39	14.74	13.2
059	15.61	62.53	58.9	40.18	14.52	13.41	15.47	13.61	17.65	13.71	17.27	14.25	15.13	13.89	16.94	29.42	20.47	18.97	16.97
060	21.18	15.83	14.55	9.99	27.3	20.32	22.19	21.71	20.41	21.01	21.43	20.88	20.69	19.03	16.7	9.53	10.68	4.87	6.85
061	31.88	19.77	19.86	22.97	21.31	32.12	33.85	32.26	32.61	31.7	32.52	33.17	31.72	32.41	35.29	59.06	61.19	59.24	62.91
062	60.22	16.97	16.07	12.43	16.36	55.31	57.13	55.41	58.19	54.7	58.55	55.13	58.93	56.25	58.85	34.96	31.56	23.63	28.63
063	18.26	35.51	29.89	28.61	11.43	11.81	13.97	12.83	12.26	12.67	14.07	13.48	12.97	12.31	11.29	18.25	19.3	10.37	9.53
064	20.59	32.45	28.75	21.28	8.31	18.9	19.91	21.91	18.47	20.53	20.25	20.23	18.28	17.59	15.66	19.65	14.89	12.1	7.91
065	24.1	32.48	29.01	20.43	9.05	23.05	23.44	26.06	22.67	24.44	24.83	24.21	23.88	21.83	16.49	18.48	17.28	15.71	9.94
066	15.94	39.02	34.05	24.16	7.97	17.09	17.89	19.77	18.1	18.22	19.08	18.21	18.06	15.72	11.69	13.65	14.75	12.51	9.08
067	27.87	40.5	38.52	26.37	17.13	23.13	24.18	25.94	23.4	24.23	24.35	23.58	23.57	21.01	18.56	22.41	20.85	18.15	13.33
068	21.79	39.53	33.88	24.21	8.69	18.65	18.29	20.38	17.27	19.07	19.2	18.91	18.45	17.13	13.1	18.66	18.29	9.75	10.57
069	29.15	22.01	19.21	21.44	38.53	24.57	26.63	24.9	24.59	23.98	26.32	24.91	27.09	26.21	16.93	15.61	18.49	8.5	13.31
070	13.87	75.93	73.49	49.39	12.47	7.89	8.69	7.53	9.92	8.07	10.13	8.3	9.43	8.91	10.56	23.25	18.75	16.31	15.17
071	54.53	8.37	8.77	11.27	17.92	45.43	45.42	46.21	44.32	45.46	44.09	45.64	43.79	45.43	51.39	25.55	26.57	15.39	23.79
072	16.33	30.69	26.21	26.81	35.61	19.41	19.79	21.15	19.03	19.79	20.89	19.91	22.03	18.2	12.62	14.85	19.71	11.61	10.05
073	15.25	58.73	54.31	35.47	13.39	14.81	15.78	15.49	18.19	15.37	16.78	15.6	14.84	13.91	21.95	33.76	25.05	23.83	23.11
074	28.31	39.47	35.22	24.79	10.68	23.14	24.24	25.23	23.62	24.09	25.17	23.61	23.71	22.31	18.19	24.99	19.46	15.19	13.42
075	25.71	28.22	27.42	18.38	8.93	25.05	25.2	28.58	23.53	26.38	25.18	26.35	24.42	22.1	16.72	16.3	18.09	7.35	8.75
076	14.58	32.45	27.4	22.81	10.53	11.25	13.43	13.12	11.7	12.53	13.93	13.25	11.99	11.61	8.93	16.79	20.77	9.53	9.16
077	20.51	16.53	14.17	12.43	24.87	16.89	18.21	18.65	18.38	17.73	19.45	17.62	19.47	16.05	12.69	13.35	23.63	15.36	12.2
078	24.81	37.52	31.96	21.47	9.55	25.53	25.85	29.14	23.35	26.89	25.06	26.34	25	23.05	19.51	18.3	16.23	14.88	9.23
079	13.63	20.43	19.39	55.17	33.36	9.97	13.17	9.82	14.46	9.59	14.31	9.55	12.47	12.26	14.93	13.32	10.13	17.15	11.05
080	61.5	16.93	15.55	17.77	18.73	51.91	52.91	52.15	55.69	51.96	52.37	51.51	52.53	52.47	59.04	33.01	21.93	18.35	24.17
081	88.65	9.3	12.19	5.1	17.69	48.77	47.4	51.01	46.48	48.88	47.27	49.24	48.13	46.1	52.41	29.79	18.37	14.27	16.16
082	19.58	23.41	23.44	13.04	11.71	13.03	13.31	15.41	12.01	13.59	13.13	13.49	13.64	10.31	8.83	14.6	12.27	7.74	7.2
083	28.15	15.77	15.3	8.01	56.11	24.88	23.93	26.65	22.44	24.95	23.41	25.31	24.73	21	16.56	11.05	12.8	8.69	13.65
084	19.95	22.7	22.39	31.47	24.31	22.41	23.62	23.16	23.08	21.85	23.68	22.94	22.77	22.35	24.37	54.29	53.05	62.85	63.67
085	52.99	11.38	8.13	20.57	20.97	56.59	58.92	55.81	59.16	55.63	58.37	55.65	56.27	57.17	59.87	27.11	22.54	20.56	21.65
086	18.83	25.43	21.44	30.97	39.89	20.16	22.36	20.59	21.35	19.53	23.09	20.13	22.15	21.14	20.07	14.42	11.99	12.73	6.57
087	24.39	42.63	36.37	27.35	11.9	21.19	22.81	22.93	22.28	21.99	23.6	21.58	22.62	20.48	17.78	24.37	22.35	19.43	14.67
088	29.85	31.49	28.25	32.57	19.67	23.67	25.67	27.17	25.67	25.23	26.33	24.52	23.73	22.09	25.25	23.7	15.23	16.57	14.11
089	24.81	34	31.84	21.17	11.71	22.61	22.43	24.62	21.21	23.05	22.88	22.9	22.65	20.37	15.94	15.77	19.79	10.55	11.7
090	42.71	10.87	8.29	15.81	20.49	44.55	47.23	46.83	49.43	46.27	47.3	46.47	45.55	45.33	56.14	28.85	19.91	19.05	21.36
091	59.67	9.36	9.32	15.45	16.55	56.09	57.75	58.57	59.21	58.22	58.43	57.51	57.94	55.73	60.42	35.23	23.36	22.81	23.14
092	9.31	37.93	23.67	21	7.76	8.37	10.27	8.52	11.89	8.89	10.93	8.42	9.96	10.07	13.81	16.64	12.59	11.48	4.85
093	89.52	8.7	12.15	5.83	19.54	50.81	50.19	53.71	49.87	51.57	50.32	51.56	50.07	48.31	56.91	30.29	19.11	15.31	15.41
094	18.84	25.77	25.33	14.73	12.1	12.75	12.99	15.28	11.83	13.38	12.92	13.31	13.03	10.13	9.18	16.09	13.69	8.11	8.14
095	25.9	14.76	13.87	7.92	64.06	25.48	24.88	26.85	23.71	25.58	24.43	25.67	25.73	22.08	15.31	12.39	13.07	9.51	14.33
096	60.71	16.71	15.43	19.46	20.24	58.95	59.4	58.87	60.44	56.51	60.96	57.08	61.83	58.05	57.55	35.83	31.27	24.26	28.93
097	14.77	38.3	30.55	37.89	12.34	11.04	13.63	11.31	12.6	11.84	13.99	12.27	12.63	12.77	13.11	24.85	22.55	16.06	16.37
098	20.69	35.17	28.76	21.05	9.28	15.28	16.01	16.57	15.45	15.93	16.76	15.81	15.57	14.73	11.59	14.33	13.37	9.15	6.87
099	55.51	14.5	14.56	14.14	19.21	48.23	50.12	48.95	49.37	48.17	49.03	48.91	49.39	48.45	52.33	30.07	34.25	17.46	23.54
100	17.28	53.55	49.89	46.27	18.49	10.57	12.89	10.57	13.43	10.9	13.06	10.5	11.71	12.17	17.57	26.61	20.69	16.33	15.84
101	19.04	32.71	31.15	25.06	11.73	17.61	18.58	19.47	18.04	17.91	18.99	17.75	18.63	15.99	12.31	12.14	10.71	10.56	6.66
102	30.09	33.54	29.24	28.85	18.73	27.21	27.62	30.65	26.49	28.49	28.36	27.78	28.12	23.97	18.33	23.37	24.83	22.11	15.11
103	57.01	11.89	11.8	18.27	20.57	50.91	53.02	52.27	53.22	51.81	52.64	52.48	52.12	51.9	54.29	33.93	32.03	19.91	26.51
104	26.93	37.75	32.49	35.92	18.29	21.73	22.95	24.23	22.54	22.8	23.75	21.89	22.77	20.06	17.85	22.45	18.34	14.81	14.71
105	20.23	53.31	48.57	45.48	18.1	14.21	16.17	14.31	17.09	14.93	16.27	14.79	14.74	15.17	20.11	32.53	17.79	18.83	16.23
106	19.9	37.34	33.21	22.01	9.33	16.93	17.23	19.04	16.04	17.87	17.81	17.85	17.18	15.58	12.54	18.19	19.56	8.99	10.56
107	50.35	15.34	11.99	29.74	26.93	40.99	44.45	41.58	47.08	41.43	43.98	41.47	43.17	42	53.68	34.31	27.62	25.61	30.35
108	24.11	38.73	32.41	39.29	20.03	21.01	22.75	24.11	22.19	22.2	23.34	21.32	22.17	19.38	16.54	20.08	15.39	15.43	10.67
109	24.11	35.46	27.69	32.16	15.83	23	24.53	25.81	24.22	24.16	24.67	23.48	23.58	21.25	17.81	17.81	13.72	13.94	9.79
110	14.97	36.83	31.75	36.91	11.36	11.92	14.32	12.44	12.83	12.73	14.25	13.19	12.95	13.18	13.01	25.63	23.87	14.14	16.9
111	27.66	33.21	25.03	45.46	22.31	25.04	27.57	27.97	28.02	26.2	28.69	25.29	27.53	24.31	23.41	20.78	15.47	18.8	11.33
112	21.72	49.37	41.11	45.51	24.65	17.21	20.89	17.47	23.89	17.93	22.87	18.04	20.85	19.93	24.65	34.63	24.29	25.23	19.43
113	19.65	17.5	16.53	14.91	26.89	22.2	21.37	23.09	19.32	21.51	20.65	22.17	21.35	19.61	15.72	11.45	18.19	15.73	10.92

Table 4.2 continued

	020	021	022	023	024	025	026	027	028	029	030	031	032	033	034	035	036	037	038
001	25.77	22.67	22.85	52.72	21.56	26.86	48.57	28.91	21.76	56.38	11.93	12.53	15.09	15.55	14.69	47.33	14.91	11.02	14.19
002	11.45	10.28	15.32	14.41	13.11	14.15	13.21	17.75	8.04	15.71	21.85	6.41	12.49	5.54	10.83	18.41	22.23	20.15	66.94
003	13.24	12.5	14.99	17.1	13.07	15.75	18.13	19.35	11.29	21.39	18.89	7.93	11.63	4.57	9.81	19.35	20.03	20.02	63.91
004	9.92	12.27	12.9	16.95	12.82	10.43	15.5	12.69	4.57	12.55	13.96	6.43	8.39	1.59	10.3	21.79	15.4	14.73	41.03
005	30.23	38.17	39.07	14.24	31.92	31.47	14.19	30.29	24.61	11.69	34.03	49.39	39.14	57.51	47.05	17.01	33.35	31.17	5.49
006	28.54	28.27	24.59	55.98	26.66	28.03	54.09	30.5	26.89	57.65	16.12	12.07	13.99	17.95	16.61	51.91	15.86	14.51	17.91
007	28.33	27.63	24.56	55.83	25.38	25.82	53.93	30.14	24.37	58.98	15.69	11.2	14.75	17.31	15.71	55.85	16.12	12.62	17.85
008	27.26	26.67	23.69	56.41	24.36	26.83	53.67	29.18	25.11	57.58	16.56	10.99	13.73	17.1	15.56	52.33	15.95	13.89	18.23
009	27.96	27.11	23.75	55.81	24.05	24.42	52.52	28.93	22.98	58.99	14.71	10.22	14.97	16.81	16.1	58.53	16.1	11.71	16.9
010	24.29	23.67	20.51	46.69	20.87	23.3	48.54	25.53	21.75	48.91	14.06	11.66	12.14	14.74	14.53	44.23	13.93	10.53	13.56
011	27.43	26.57	23.37	55.35	23.86	24.53	53.55	28.9	23.78	58.49	14.81	10.12	14.83	17.33	15.93	56.1	15.26	12.29	17.07
012	27.85	27.46	23.51	56.02	24.83	26.83	55.11	29.78	26.1	57.51	15.23	10.65	12.86	16.55	14.39	51.36	14.31	13.04	17.92
013	27.01	26.11	22.88	54.59	24.25	24.29	54.72	28.07	24.12	58.93	15.79	9.89	14.8	17.61	15.86	55.45	14.81	12.26	15.91
014	27.87	28.32	24.87	53.9	24.33	25.41	53.01	29.43	24.22	58	15.69	9.97	16.93	17.96	15.11	53.99	15.88	13.05	17.64
015	29.12	28.85	27.04	60.19	24.29	31.39	54.16	30.63	27.19	61.88	9.19	9.84	11.89	17.11	15.25	58.09	12.51	6.66	15.2
016	69.97	72.19	75.29	30.21	68.7	71.44	30.71	68.47	59.83	22.15	41.46	31.65	35.03	40.33	42.04	25.91	43.25	40.98	18.35
017	72.01	71.91	72.7	27.73	69.63	72.87	25.1	69.33	64.65	19.15	39.84	31.39	37.27	37.93	41.84	21.3	41.69	41.54	8.8
018	71.07	71.77	76.89	24.09	68.55	71.07	22.97	65.07	57.75	17.66	42.03	24.28	39.04	39.77	42.99	20.36	43.29	37.15	14.31
019	71.5	69.27	68.45	25.66	93.43	70.37	22.83	68.48	66.63	17.31	32.16	29.31	29.51	34.1	36.82	17.01	36.74	35.45	9.05
020	100	69.63	71.72	33.69	73.11	70.55	27.31	68.38	63.89	25.17	39.54	26.31	32.47	34.81	38.26	26.55	40.09	38.51	14.49
021	56.78	100	72.78	30.02	70.1	71.35	27.97	70.91	61.95	20.45	37.89	31.04	33.85	38.71	38.79	23.26	40.06	37.61	12.45
022	62.61	66.55	100	26.85	69.35	91.31	25.91	69.33	62.7	21.11	41.38	29.78	38.87	37.91	41.51	19.93	41.24	40.49	17.7
023	35.96	33.41	31.13	100	28.07	29.97	53.17	30.45	26.65	56.37	15.09	11.35	14.04	16.04	19.85	79.76	16.89	15.11	13.89
024	71.31	64.77	65.87	31.63	100	69.97	25.21	69.63	65.54	20.15	32.36	27.07	29.05	32.18	34.95	20.11	35.89	35.75	12.08
025	60.09	63.41	96.24	30.01	63.64	100	28.03	69.27	67.45	24.57	35.08	27.53	30.71	33.37	36.89	20.29	35.56	37.19	17.93
026	36.09	35.11	35.07	54.43	31.1	32.17	100	28.65	24.3	54.83	11.13	9.77	13.71	13.3	14.1	53.31	13.81	14.19	17.03
027	59.35	61.29	66.18	31.37	62.47	64.27	37.11	100	62.64	19.09	34.71	28.13	30.51	34.07	36.65	23.45	37	36.53	15.37
028	52.37	51.03	55.34	21.56	54.77	56.63	23.66	48.95	100	21.38	29.66	25.25	25.18	30.69	33.35	16.25	31.77	32.73	8.78
029	31.73	24.75	28.67	51.09	25.97	27.19	53.33	22.69	19.58	100	8.66	10.36	9.3	12.67	12.15	80.49	10.8	7.55	15.67
030	16.75	13.25	16.38	15.65	10.28	15.07	16.68	11.63	11.75	14.53	100	26.83	42.02	38.51	42.67	11.09	79.86	69.85	19.35
031	13.95	22.5	16.75	15.51	14.29	16.23	13.76	19.82	11.31	14.86	14.54	100	32.85	56.04	40.92	9.13	28.59	30.92	6.17
032	13.73	12.61	14.27	16.63	12.69	14.71	17.59	12.52	10.51	12.77	20.98	31.33	100	48.08	52.07	13.81	42.87	34.53	8.68
033	8.54	15.19	9.2	15.97	8.45	9.05	15.52	10.31	7.89	16.49	15.2	55.95	35.77	100	52.87	14.66	38.75	34.86	4.97
034	9.17	9.5	10.61	21.02	6.72	8.79	17.89	8.61	5.03	16.76	21.35	28.9	48.05	31.55	100	15.66	44.21	40.23	6.98
035	32.89	28.87	26.25	80.24	26.63	22.29	59.45	23.99	13.23	59.85	15.87	12.36	17.29	16.63	20.23	100	15.55	10.57	13.63
036	15.89	13.65	15.06	17.17	11.21	12.97	16.4	10.83	9.57	17.49	76.89	13.82	22.99	13.02	20.05	21.88	100	73.38	17.35
037	15.68	14.67	15.63	15.33	13.7	16.72	13.6	13.3	13.51	11.97	66.41	20.39	19.52	11.92	13.57	14.33	70.55	100	19.15
038	18.33	21.75	30.5	11.42	18.45	25.47	14.49	23.51	11.58	14.76	26.48	12.33	14.72	5.89	13.27	10.58	25.79	25.68	100
039	23.44	12.01	23.28	11.13	20.97	20.23	10.63	22.48	8.94	16.45	27.04	12.13	12.39	1.25	10.02	8.45	26.27	27.78	59.99
040	17.79	20.65	19.42	14.65	14.05	18.5	12.09	18.67	18	12.8	71.07	15.01	21.19	9.95	17.72	14.79	69.81	73.15	26.11
041	23.02	14.88	14.55	23.65	9.41	15.43	16.49	15.76	10.79	14.44	73.1	16.15	22.23	12.03	19.07	22.62	77.59	70.45	25.77
042	21.31	22.37	23.17	17.11	17.29	24.73	14.73	19.83	18.31	12.36	68.25	16.57	17.6	8.72	13.25	15.15	72.79	75.89	35.24
043	23.43	18.84	19.11	22.8	17.6	19.58	20.44	17.75	15.1	16.67	76.07	16.66	22.28	14.67	18.37	20.79	74.96	68.59	24.21
044	29.27	30.8	27.26	50.07	28.77	24.93	53.73	38.01	20.83	50.99	17.64	26.85	17.33	32.05	21.35	51.21	19.89	16.03	6.37
045	21.48	14.81	17.19	17.77	13.63	15.08	20.28	14.24	11.94	17.19	71.28	14.3	22.11	12.1	19.59	18.51	77.9	66.94	34.18
046	17.02	19.58	28.39	9.27	17.57	24.05	15	22.74	9.73	15.86	23.9	11.43	8.47	1.14	8.37	9.47	20.1	24.67	57.99
047	35.3	27.05	29.63	51.99	26.57	28.76	48.38	31.39	21.37	60.46	14.35	12.16	16.98	10.91	14.12	48.3	14.43	7.01	8.34
048	26.36	14.52	23.63	35.83	18.32	18.62	33.49	19.83	12.01	43.86	11.25	6.49	13.25	12.47	10.75	42.5	14.85	8.31	11.59
049	35.7	26.74	30.83	48.45	29.53	31.11	51.03	35.21	21.67	55.17	7.7	10.16	10.11	7.09	11.11	48.74	11.3	10.03	6.52
050	34.29	24.19	28.72	46.53	24.97	27.27	48.53	31.67	20.84	59.01	8.11	10.51	10.13	11.55	10.34	52.49	12.97	11.17	9.61
051	34.41	29.98	33.97	56.29	28.16	31.09	48.09	32.63	18.89	63.21	13.28	16.68	23.55	21.37	20.14	59.23	21.3	10.17	10.33
052	7.48	8.52	7.45	13.65	10.38	5.31	13.56	5.87	7.65	13.59	19.51	25.4	40.82	25.69	49.16	14.53	20.37	14.03	17.67
053	29.46	30.56	34.5	50.85	25.1	34.99	48.64	36.62	20.38	46.24	17.95	17.09	28.07	19.48	25.42	44.64	20.57	14.13	10.46
054	59.27	65.69	72.15	28.61	68.91	68.17	37.74	60.55	48.57	24.15	13.6	14.13	12.59	9.87	6.32	34.23	16.21	13.05	20.59
055	29.14	30.47	30.56	50.31	29.06	25.13	46.72	27.24	14.82	58.73	15.67	12.13	14.79	19.75	18.07	52.23	16.41	10.39	12.45
056	26.39	22.92	27.35	15.03	22.2	23.24	15.47	22.43	13.39	17.44	34.77	20.25	15.22	4.67	13.8	17.73	41.57	37.07	52.31
057	37.68	30.6	33	60.73	31.43	28.94	57.71	30.71	15.48	56.02	9.77	17.3	13.42	16.99	18.07	59.13	11.83	8.56	15.55
058	20.77	19.45	22.9	15.66	17.42	19.2	13.68	17.19	13.23	18.66	31.93	17.09	8.49	5.89	11.84	21.16	33.09	27.64	47.88
059	28.51	19.58	27.27	15.12	24.01	21.23	14.64	22.93	11.64	21.99	27.99	14.06	11	6.63	13.36	20.45	32.89	24.37	56.13
060	12.69	9.57	9.46	17.05	6.28	8.76	14.65	10.27	6.97	14.45	23.99	38.81	43.44	38.1	53.44	13.41	28.01	26.97	15
061	63.85	65.73	73.06	27.18	65.51	69.89	32.21	57.67	46.72	25.85	19.32	16.71	18.53	10.01	12.53	25.54	18.67	20.45	26.02
062	29.73	32.04	29.87	54.37	30.4	27.6	48.98	32.18	21.05	56.24	22.98	14.97	14.55	23.11	15.67	50.63	19.87	16.81	11.99
063	18.85	17.33	19.83	12.98	13.61	19.5	11.69	16.02	13.71	9.67	60.74	24.9	18.58	6.86	13.97	15.05	67.77	66.89	31.49
064	19.3	17.43	16.07	15.48	9.63	18.02	12.59	17.13	13.59	15.56	65.7	19.6	21.24	13.77	16.6	15.66	70.45	62.17	29.45
065	22.09	19.72	19.31	17.42	13.45	17.72	18.49	16.6	13.7	19.01	71.39	21.49	28.79	17.01	24.47	18.99	70.81	64.87	31.11
066	18.09	16.81	17.37	15.61	13.52	17.28	13.71	14.34	17.06	12.93	67.17	11.57	26.3	8.11	22.83	16.67	75.15	67.99	29.21
067	21.93	23.95	27.77	15.09	16.35	25.17	18.35	20.79	20.39	18.37	72.48	16.42	19.8	16.77	19.48	17.61	77.36	69.6	37.37
068	19.19	19.83	19.89	16.06	15.63	19.24	16.65	18.8	14.5	16.83	62.59	16.43	23.29	14.08	18.41	17.24	67.71	67.61	30.04
069	11.94	13.7	11.71	22.31	11.29	13.35	20.16	15.32	9.23	20.57	24.43	44.69	43.38	40.11	49.02	21.96	29.05	33.73	14.53
070	18.07	15.45	23.35	13.29	19.37	19.51	12.59	16.99	7.24	20.89	31.74	12.95	16.6	3.08	12.13	16.78	33.61	32.59	64.39
071	26.03	23.06	24.44	45.29	20.94	25.45	48.04	27.35	13.93	46.04	15.09	16.59	18.28	14.96	23.21	43.48	17.99	14.37	9.29
072	7.71	10.97	11.55	12.04	9.45	9.39	17.27	9.31	9.81	15.31	31.99	38.44	50.92	43.03	55.53	17.25	33.71	29.21	23.29
073	32.08	20.65	34.77	21.87	30.43	30.29	15.92	24.38	16.27	21.76	29.39	18.2	16.38	7.42	13.46	24.77	33.15	31.41	52.55
074	23.63	20.71	23.65	22.69	18	22.89	21.63	23.03	16.15	19.88	69.11	18.65	23.31	16.74	23.02	21.62	70.53	68.71	34.24
075	20.48	17.8	16.17	17.85	10.81	17.19	17.55	15.59	13.19	18.18	59.21	21.04	20.29	18.27	19.39	16.91	62.35	62.27	24.73
076	18.82	17.25	18.26	11.81	12.77	19.33	11.4	16.44	14.05	10.14	63.61	27.11	18.55	3.49	13.83	12.85	68.64	68.17	29.35
077	14.7	13.17	15.35	14.8	12.09	16.01	16.3	11.61	8.64	9.27	29.2	36.5	82.93	30.17	47.57	15.97	26.53	22.76	12.25
078	19.38	14.07	19.84	17.28	12.79	18.67	16.49	13.85	13.15	18.91	89.39	18.99	24.49	20.22	21.45	16.91	75.89	64.39	31.43
079	8.73	13.59	14.22	13.85	11.8	7.75	12.72	10.25	10.07	11.53	9.88	4.6	7.14	7.88	9.07	24.45	12.43	9.41	14.94
080	32.13	22.61	30.49	51.4	26.23	29.35	54.33	24.79	18.89	90.12	20.41	16.75	13.45	16.62	18.71	57.43	22.57	15.01	13.83
081	24.85	18.54	26.49	44.41	16.15	28	45.89	28.94	12.15	44.73	19.75	22.85	18.59	25.85	21.24	38.35	19.75	16.13	12.45
082	13.65	17.77	19.23	15.01	10.29	19.25	5.78	16.45	11.63	7.6	47.46	17.61	14.73	9.83	18.57	7.7	45.81	54.12	24.82
083	10.37	18.18	15.19	16.93	12.31	15.27	13.57	11.27	11.53	13.71	19.91	56.72	31.88	49.96	35.53	13.59	17.69	23.97	12.15
084	52.03	54.86	64.25	21.4	62.66	60.33	18.54	51.2	40.87	12.62	13.71	11.65	20.94	8.49	10.52	20.15	12.86	8.73	24.38
085	26.15	29.93	29.51	48.15	27.16	25.51	44.05	26.83	16.01	54.1	17.1	12.89	15.21	19.59	17.5	46.95	16.79	13.04	8.51
086	8.67	10.68	9.65	20.8	7.99	5.95	18.47	8.53	4.09	17.56	27.24	33.65	43.59	37.14	55.26	26.23	28.17	28.11	16.71
087	24.12	21.82	28.51	20.75	19.51	25.97	16.25	21.21	14.09	17.92	70.08	13.41	26.51	12.84	22.24	22.59	76.29	64	34.91
088	28.46	18.04	22.8	28.8	17.97	21.19	26.8	17.6	13.65	25.89	63.31	19.5	20.63	18.18	19.28	31.17	69.03	57.65	24.89
089	20.5	15.29	18.09	16.67	15.93	18.29	14.57	17.48	16.99	16.33	73.53	18.69	24.77	14	22.44	15.64	70.01	71.85	25.62
090	30.11	26.92	24.27	53.57	19.76	20.21	56.4	24.21	13.32	49.51	10.41	11.85	17.59	13.25	16.24	51.89	18.54	7.84	5.99
091	33.09	27.9	32.41	53.85	23.27	28.47	47.24	32.12	19.56	51.27	16.91	18.58	20.43	23.25	22.8	51.44	17.98	10.92	9.76
092	11.07	11.86	14.89	10.84	10.92	12.1	10.13	9.61	8.07	18.29	53.9	10.24	16.35	9.23	12.54	20.93	46.95	34.77	23.16
093	25.15	20.13	26.57	47.55	15.43	27.67	49.95	28.95	11.94	48.53	20.67	22.51	18.77	24.87	21.67	43.05	20.63	14.11	13.67
094	14.55	19.14	21.16	15.58	12.01	20.88	6.17	18.09	13.11	7.67	49.19	17.46	12.97	9.13	18.01	8.03	47.05	54.3	28.25
095	10.98	20.07	16.05	17.28	13.91	15.49	16.25	12.61	10.61	15.21	20.38	61.04	35.57	53.64	39.83	15.86	14.96	21.84	11.65
096	30.17	31.55	32.1	54.19	29.61	29.34	47.69	33.75	22.07	53.8	24.31	15.97	16.21	23.05	16.71	53.55	24.29	16.76	11.77
097	21.77	21.41	24.53	17.05	20.49	23.21	17.71	21.23	15.08	13.67	56.89	14.57	14.99	4.6	11.39	20.8	63.93	62.74	30.97
098	16.94	14.67	14.44	14.37	10.75	13.85	14.25	12.65	10.15	15.18	72.15	18.31	22.97	12.57	20.42	18.33	73.79	70.29	24.55
099	30.98	29.93	31.87	46.75	22.75	30.98	46.91	30.07	15.87	44.87	24.94	19.2	27.21	15.97	24.23	43.2	26.41	19.38	13.43
100	19.53	18.24	27.05	19.68	19.19	23.2	13.4	18.87	8.74	19.83	33.15	22.71	17.42	7	14.68	22.68	39.06	37.62	43.03
101	14.95	14.75	15.02	12.76	12.29	14.43	14.59	12.72	11.73	13.89	67.35	13.59	21.68	10.64	23.21	14.84	68.97	67.95	24.99
102	24.23	23.77	28.59	17.11	18.52	26.37	19.29	21.33	15.85	16.54	71.01	14.9	25.67	19.41	21.93	19.21	74.19	71.03	31.01
103	31.18	31.11	34.45	52.1	24.65	32.13	50.96	33.07	17.24	48.44	15.05	17.73	29.52	17.19	20.77	50.58	20.13	13.81	9.53
104	23.36	19.99	23.78	19.53	18.73	22.43	16.44	19.46	13.87	16.37	65.91	16.3	23.21	13.86	21.63	22.23	76.99	70.49	31.28
105	29.95	19.36	32.25	19.36	22.8	26.84	16.21	25.14	14.87	22.42	33.97	20.46	15.08	6.06	11.12	23.13	32.69	35.84	48.07
106	20.17	16.03	19.76	17.49	15.6	19.28	11.34	17.41	16.59	15.05	62.85	15.7	22.39	11.98	18.87	18.42	66.67	73.16	28.35
107	36.67	32.03	31.99	60.19	32.4	28.92	54.64	29.71	17.03	50.05	14.61	14.69	14.59	14.82	19.46	62.12	21.08	15.89	7.47
108	17.78	16.7	22.23	16.72	14.53	19.01	15.9	15.17	10.15	14.88	67.93	13.63	22.9	14.05	21.96	25.96	75.34	63.13	29.84
109	18.01	15.83	17.38	19.89	12.93	14.96	18.53	14.85	9.73	18.24	71.15	13.77	27.46	13.39	24.37	25.86	69.65	68.34	28.84
110	21.85	22.73	23.64	17.37	20.47	23.41	17.38	21.85	16.15	12.41	57.25	16.83	14.45	5.13	10.74	19.37	64.75	66.14	30.7
111	15.47	13.73	19.1	21.91	13.83	14.11	21.33	13.73	6.59	19.05	57.04	9.64	25.64	16.51	26.22	33.89	65.63	46.87	24.57
112	29.29	24.11	36.09	22.89	26.53	27.15	22.49	24.37	13.58	24.36	24.94	14.25	11.1	6.44	5.73	32.83	24.55	21.83	38.92
113	12.25	14.74	13.12	16.65	10.11	12.28	13.71	8.32	11.27	11.74	25.37	37	44.61	33.28	44.39	14.27	24.89	22.89	14.95

Table 4.2 continued

	039	040	041	042	043	044	045	046	047	048	049	050	051	052	053	054	055	056	057
001	17.39	12.38	13.43	11.07	13.28	58.93	15.17	19.25	32.77	44.35	58.21	54.83	55.09	10.16	44.77	24.17	54.86	19.62	54.79
002	57.73	24.18	21.8	24.41	21.05	11.3	21.25	63.95	2.4	17.49	10.78	15.64	13.11	12.16	10.87	10.99	16.99	55.34	14.45
003	56.65	21.35	20.06	21.24	19.23	14.59	18.57	60.75	2.63	20.93	14.94	18.59	15.72	12.09	12.91	14.05	19.93	51.22	19.35
004	33.57	17.81	13.97	14.65	13.74	13.61	12.33	38.39	5.43	17.15	10.45	14.29	17	12.11	11.41	13.2	18.07	35.91	15.25
005	6.39	32.45	29.42	30.5	31.33	18.28	7.92	5.89	11.67	11.22	10.55	14.17	21.29	44.29	21.22	38.25	13.23	11.31	14.69
006	14.93	13.99	15.55	12.66	16.28	60.17	17.82	16.91	28.65	50.21	52.04	53.99	56.22	14.01	42.15	27.59	63.77	21.34	54.73
007	14.37	14.11	16.9	12.48	16.72	60.99	19.17	17.67	30.18	51.15	53.07	55.56	58	12.77	42.65	26.87	64.29	22.22	55.07
008	15.21	13.89	15.89	11.95	16.71	60.13	19.47	17.12	28.92	49.91	51.01	53.14	56.15	12.72	42.6	26.14	63.22	21.13	54.93
009	14.85	14.02	17.77	11.93	16.25	58.51	19.58	17.41	30.34	53.95	52.59	57.99	59.37	12.51	41.49	26.81	64.57	22.06	56.73
010	13.1	10.37	12.89	9.96	13.98	50.65	16.65	12.63	32.83	43.47	44.08	45.5	46.23	11.81	39.54	23.69	54.97	15.49	47.24
011	14.71	14.45	17.29	12.35	15.99	60.23	19.11	17.26	29.39	53.95	52.65	57.27	58.29	13.01	41.61	26.25	64.74	22.43	55.67
012	14.77	13.05	14.37	11.92	15.25	60.67	17.82	16.78	28.74	49.61	51.92	53.57	55.49	12.23	42.52	26.86	63.5	21.05	54.45
013	13.43	13.39	16.8	11.67	15.55	59.43	20.11	16.24	29.38	53.83	52.87	55.66	57.7	13.53	44.31	25.41	63.86	21.3	55.2
014	14.66	14.99	18.03	12.73	15.68	57.89	17.01	17.85	28.79	49.28	50.81	54.47	56.46	12.31	43.49	26.89	64.14	21.41	53.23
015	15.15	8.13	11.07	8.38	11.6	63.51	18.73	15.15	33.29	53.17	57.51	59.07	63.81	10.19	42.91	29.29	62.32	20.04	54.11
016	18.25	41.5	41.09	45.96	41.37	27.84	9.05	19.09	18.16	22.81	26.58	28.16	31.49	37.41	30.68	74.17	25.79	19.41	27.98
017	12.26	41.76	37.39	47.89	41.84	20.84	7.69	10.91	13.86	18	21.08	22.27	29.17	41.85	32.93	75.93	22.8	16.89	22
018	13.3	40.45	37.33	43.45	40.67	22.31	6.98	13.59	14.25	20.99	20.57	19.73	27.14	40.56	28.8	77.19	22.87	14.07	19.35
019	13.77	34.96	30.32	39.27	35.55	22.47	5.07	9.75	16.71	15.68	20.03	17.06	24.89	37.43	25.51	73.47	21.49	12.05	21.03
020	18.67	40.57	40.81	42.37	41.72	24.4	10.74	12.94	19.78	24.99	27.41	28.15	32.77	34.52	28.54	69.97	25	19.29	27.78
021	10.07	41.68	39.27	44.7	39.02	27.29	7.41	12.53	16.55	18.37	23.51	23.72	29.66	35.03	31.99	73.66	27.18	15.59	23.95
022	16.81	42.24	37.46	46.14	42.06	22.11	11.89	16.4	17.35	19.96	24.09	23.53	29.69	37.53	30.19	76.66	24.21	16.62	22.5
023	13.79	16.31	18.15	15.79	17.57	53.36	14.12	11.84	28.91	50.22	50.69	50.86	56.04	15.54	42.31	28.43	55.03	18.28	57.66
024	17.42	35.39	31.13	39.24	35.83	24.98	7.99	12.5	15.73	17.65	23.29	21.07	27.07	34.98	24.21	71.94	26.12	15.84	23.57
025	19.48	37.33	31.94	42.49	36.89	26.13	7.54	16.17	16.24	21.53	27.14	25.58	30.33	31.8	27.87	73.64	25.39	15.74	24.16
026	13.48	10.25	12.53	11.49	13.5	52.94	11.01	14.73	25.76	41.41	49.41	48.04	47.11	13.48	43.81	28.71	52.83	20.52	53.33
027	18.25	38.51	37.12	40.94	37.63	30.4	8.07	16.97	17.16	22.76	29.15	28.91	30.51	32.9	32.25	69.79	25.91	16.29	24.99
028	12.37	33.93	27.72	37.57	31.71	25.39	5.97	9.36	12.25	19.46	24.15	23.53	25.99	31.32	21.98	62.84	22.41	12.51	17.79
029	16.13	10.13	9.52	8.46	9.59	57.17	17.56	15.5	32.63	50.67	56.1	58.89	59.09	11.08	38.4	21.63	59.09	19.17	58.23
030	17.72	78.02	76.83	74.18	80.49	11.89	38.32	17.32	11.15	11.18	5.37	6.67	15.19	40.91	23.28	39.24	11.69	20.69	6.29
031	6.73	27.64	26.18	31.35	29.21	15.39	7.67	5.72	14.43	4.9	7.74	8.13	15.37	37.38	13.96	30.01	6.99	11.1	12.1
032	8.61	40.58	43.07	35.58	38.69	12.49	20.38	6.12	13.68	11.58	12.05	13.49	21.26	47.09	32.83	32.75	12.84	11.29	10.43
033	4.84	36.17	36.59	35.25	37.36	23.23	10.03	1.84	11.67	13.27	10.63	14.43	23.84	46.91	22.7	37.96	14.35	6.99	14.09
034	7.43	41.85	40.9	42.69	41.53	17.19	11.67	4.81	11.79	11.47	11.65	11.7	23	65.9	23.97	40.11	14.41	12.49	12.07
035	12.39	12.28	17.03	10.63	13.65	52.63	19.9	13.34	29.49	53.91	50.5	54.24	57.43	13.16	38.31	27.44	54.31	22.83	57.62
036	19.17	76.63	78.83	76.98	79.94	12.89	42.36	15.78	10.71	13.34	8.37	10.15	19.91	43.45	26.57	43.55	12.71	25.33	8.17
037	19.46	73.73	70.49	78.21	72.86	11.76	33.47	16.97	6.91	7.61	7.46	7.35	12.15	39.49	16.99	40.45	7.71	22.15	6.07
038	57.15	21.38	19.23	23.44	22.75	11.97	19.43	55.74	4.17	13.26	11.41	13.05	12.27	8.83	10.32	12.81	17.23	41.71	13.39
039	100	17.79	17.36	20.13	19.31	12.65	17.4	58.19	5.41	16.69	14.33	14.87	13.33	11.24	9.11	12.1	16	45.11	16.24
040	21.95	100	78.13	76.1	82.07	11.23	34.24	20.41	8.05	13.15	9.25	11.15	16.28	42.22	23.21	38.93	8.79	26.06	7.72
041	23.97	72.44	100	74.65	79.45	12.45	42.69	20.78	12.29	13.94	10.15	12.57	15.6	38.52	27.92	34.99	10.73	27.85	10.42
042	30.97	70.88	71.65	100	77.7	8.6	37.19	19.56	7.79	7.39	7.53	9.04	13.75	43.46	21.78	45.58	8.31	27.55	7.19
043	26.32	76.82	77.15	70.88	100	12.76	40.99	20.84	13.11	11.92	8.09	9.19	16.87	43.71	25.02	40.04	11.45	24.73	8.29
044	6.43	12.43	16.81	8.59	20.9	100	19.56	14.01	32.11	49.31	55.94	53.27	63.73	21.71	39.83	25.06	59.46	20.43	46.89
045	31.33	66.61	79.92	70.01	72.19	20.63	100	16.26	16.49	23.18	16.21	18.24	24.39	14.41	21.65	7.95	12.21	22.56	10.25
046	54.97	24.81	27.57	28.47	26.3	9.83	27.77	100	5.39	15.63	11.13	12.77	14.14	7.36	8.57	11.74	15.69	48.96	14.05
047	10.82	8.82	12.79	6.66	18.97	56.91	16.98	10.77	100	31.44	34.45	31.57	37.79	15.55	37.93	15.32	28.54	6.91	28.57
048	15.47	12.93	15.83	6.93	16.83	34.92	19.75	15.01	53.65	100	51.29	62.89	56.35	11.63	34.81	22.33	51.09	22.62	45.71
049	8.45	8.83	11.98	9.52	12.05	48.73	11.22	9.73	56.16	36.11	100	87.49	57	10.1	44.89	26.24	54.73	20.02	48.52
050	11.53	12.02	15.32	11.48	12.98	45.68	13.5	10.99	50.77	55.26	85.06	100	59.61	10.95	42.47	27.15	57.73	21.65	51.25
051	8.69	12.22	12.1	10.1	17.37	59.6	19.85	10.21	65.4	46.64	51.99	53.47	100	18.97	46.91	30.5	56.99	20.33	51.82
052	13.82	20.39	15.85	13.72	22.83	25.07	18.09	13.45	18.39	9.59	8.22	9.72	16.11	100	18.75	38.55	10.82	19.54	9.26
053	4.62	16.81	16.37	15.51	23.01	46.5	18.49	5.87	57.27	34.89	47.03	43.76	55.59	13.94	100	28.65	42.73	14.35	38.25
054	13.22	12.93	9.19	17.16	14.98	28.89	12.95	17.63	23.32	20.94	28.97	28.82	28.73	5.45	29.89	100	26.86	14.07	25.67
055	10.64	11.1	12.82	9.31	17.37	53.14	17.52	15.39	53.46	41.6	48.23	52.17	55.81	12.21	44.16	27.39	100	18.81	59.7
056	50.21	37.34	40.14	42.41	38.67	17.37	40.19	50.38	13.81	20.56	14.8	17.26	14.85	26.93	10.39	19.37	15.02	100	18.62
057	13.41	11.77	15.94	11.77	15.23	44.82	16.59	17.49	51.85	42.42	45.62	48.11	55.47	10.96	48.33	31.52	59.99	16.53	100
058	45.37	32.69	30.56	32.43	29.75	11.29	29.33	44.35	12.83	20.97	12.45	19.37	14.21	17.71	10.51	17.96	17.16	73.62	18.68
059	55.18	26.54	26.63	30.03	27.93	18.57	30.75	52.18	15	25.29	16.1	20.78	14.76	15.49	11.18	19.98	21.63	71.99	20.53
060	17.72	25.49	33.33	22.58	28.01	24.62	30.69	14.07	17.11	8.89	17.03	14.19	15.57	44.09	16.92	6.27	17.89	21.39	14.7
061	18.05	20.14	18.85	28.61	23.01	31.93	22.91	22.27	30.17	24.59	28.18	25.84	34.11	13.75	36.59	70.34	29.23	28.73	32.36
062	7.68	15.57	19.38	14.9	22.53	52.09	22.69	12.15	49.63	37.65	54.01	51.52	58.19	8.35	52.11	34.07	57.24	14.87	54.49
063	25.78	61	66.1	72.7	63.01	11.47	62.91	31.59	6.78	9.98	9.87	11.67	11.87	14.98	16.13	12.73	8.84	46.63	10.77
064	25.46	70.01	71.71	71.07	69.74	14.51	65.94	26.53	16.61	12.15	12.01	13.51	15.79	17.78	19.61	13.9	14.83	47.96	10.36
065	27.24	71.41	66.01	66.54	70.79	18.39	66.59	28.8	16.61	16.08	12.99	15.16	16.62	23.9	26.58	14.85	15.27	41.22	13.99
066	29.75	80.19	68.54	72.55	70.68	10.55	66.31	23.31	11.13	13.09	7.08	10.25	13.34	20.97	20.34	10.79	14.3	43.45	10.93
067	29.53	70.58	72.25	74.5	73.83	21.24	74.07	29.51	16.07	16.22	11.82	13.76	18.93	24.54	22.25	20.57	21.11	43.59	15.4
068	29.46	70.91	64.73	73.69	64.85	18.35	56.03	30.67	9.62	10.28	14.37	16.77	14.34	17.59	21.12	13.37	13.51	46.81	17.9
069	14.47	29.53	28.08	29.2	31.68	30.75	26.47	16.38	20.43	8.98	15.03	15.35	19.94	49.75	29.89	9.49	14.59	26.71	19.54
070	61.82	33.39	29.61	36.19	29.31	11.97	34.9	63.96	11.41	17.63	12.05	16.65	13.39	22.98	11.01	17.56	18.62	61.29	23.25
071	4.11	12.44	14.31	12.75	17.63	39.67	16.86	5.37	49.47	27.37	42.73	39.19	47.26	8.89	75.43	30.56	35.25	7.71	46.79
072	23.41	29.03	27.69	25.21	28.3	20.93	30.1	20.33	11.39	8.41	5.13	8.07	16.72	52.52	16.48	9.48	14.64	29.38	17.49
073	53.41	31.09	33.17	36.43	33.54	20.19	33.78	44.11	18.99	21.8	13.85	18.36	18.51	22.15	10.77	22.07	19.47	70.29	22.03
074	29.35	73.01	68.5	75.55	72.12	22.61	65.29	29.99	16.38	16.59	15.7	16.88	19.73	20.48	25.2	17.97	17.17	47.79	18.54
075	24.46	61.41	70.81	62.66	64.46	21.95	65.05	31.3	19.58	14.87	11.55	12.45	13.88	21.95	32.37	9.95	12.24	39.08	14.54
076	24.33	63.45	66.57	71.89	64.55	10.75	63.91	29.23	7.33	8.73	9.37	12.26	10.74	14.86	13.76	10.3	10.34	44.07	10.07
077	11.12	23.85	26.85	20.89	28.07	17.26	23	6.73	13.83	8.95	7.21	7.38	25.68	36.62	26.99	12.59	9.83	15.76	12.88
078	32.8	68.02	71.76	70.49	72.07	21.57	70.73	31.47	16.81	12.33	7.64	8.59	16.41	23.81	22.11	13.58	18.41	42.91	10.87
079	8.23	12.04	7.27	4.71	5.5	13.95	9.46	8.19	10.68	19.32	8	16.9	20.41	11.57	17.53	20.07	24.83	14.43	15.39
080	17.78	13.36	17.23	12.99	22.86	51.91	21.03	15.27	60.51	48.37	53.51	56.41	59.69	10.72	53.45	26.27	56.04	18.61	56.73
081	9.89	13.5	23.71	19.71	24.1	47.39	25.52	12.2	57.39	32.79	42.12	40.33	50.35	12.43	58.71	18.49	35.12	7.53	48.13
082	27.79	50.06	55.78	54.33	56.69	11.02	53.24	30.58	9.96	4.72	2.96	1.42	6.34	17.52	15.07	10.6	12.14	27.66	6.37
083	13.01	19.42	20.71	17.23	19.79	31.75	20.99	11.87	14.21	8.12	3.27	4.68	18.65	30.83	21.29	11.59	10.59	17.67	15.41
084	20.73	10.76	6.37	9.51	11.41	22.42	15.56	26.25	19.18	22.25	12.49	14.57	30.27	9.85	26.81	63.36	20.92	17.75	18.87
085	8.17	13.51	14.98	11.35	18.75	54.35	18.66	11.17	51.11	39.33	49.31	52.57	53.9	8.3	45.53	24.88	92.79	13.69	54.63
086	15.33	27.93	25.83	24.44	25.37	25.73	27.03	11.71	17	13.33	7.91	11.7	15.89	48.51	19.84	10.9	20.83	22.72	19.07
087	31.19	72.19	73.36	74.03	73.05	18.79	71.21	31.5	14.09	16.11	11.89	13.97	16.8	24.68	23.3	20.28	22.57	49.15	17.03
088	24	61.19	68.45	61.25	64.18	26.3	70.01	22.91	20.15	25.42	18.35	21.43	22.75	17.63	20.58	19.21	26.01	39.37	25.85
089	22.53	75.43	72.19	70.57	70.11	18.05	68.08	24.14	16.48	12.55	9.09	10.21	15.01	19.65	23.07	9.99	18.38	38.51	12.77
090	8.48	7.18	16.64	6.35	15.54	44.38	13.97	7.76	51.71	41.02	41.06	44.16	47.39	7.09	50.15	28.85	47.84	13.19	55.95
091	9.27	13.74	20.91	12.26	20.63	50.15	19.2	12.27	55.41	39.75	49.71	50.03	55.45	15.63	53.83	27.16	59.64	12.72	60.33
092	19.01	47.13	43.17	49.05	47.06	11.5	37.45	22.11	5.91	16.75	8.5	14.13	10.68	10.24	12.59	14.98	21.85	37.3	12.85
093	8.56	12.62	21.28	17.32	23.89	49.97	22.77	12.02	59.84	34.99	40.7	41.55	54.62	11.75	59	19.23	42.17	7.19	54.16
094	28.6	52.37	56.19	55.02	58.03	11.22	53.65	29.93	10.46	4.49	3.15	1.87	6.99	17.32	15.46	12.8	12.92	29.05	7.09
095	12.4	16.55	16.93	14.67	16.92	31.07	18.9	12.22	14.55	9.15	4.66	7.07	16.85	32.37	20.97	11.45	11.32	15.38	14.55
096	8.04	18.59	21.33	15.63	25.5	54.94	26.07	14.39	48.26	41.26	53.77	52.71	59.14	7.62	55.69	35.59	56.8	17.6	53.09
097	28.98	60.02	61.83	71.56	58.51	17.43	56.75	31.72	9.62	13.63	14.53	15.78	13.07	18.13	16.87	17.73	11.58	53.47	17.15
098	24.37	73.6	72.91	74.59	71.83	16.13	64.25	25.28	9.93	11.55	11.09	15.12	12.31	21.71	19.09	12.2	11.37	43.35	12.93
099	7.29	22.06	21.48	19.69	25.17	41.27	24.15	8.51	53.83	31.3	42.27	39.77	53.67	10.2	82.29	29.23	42.94	15.96	49.77
100	42.99	34.07	39.2	40.42	36.17	19.97	35.33	44.33	13.81	19.09	16.83	18.73	19.97	18.3	13.28	21.43	17.42	78.05	16.19
101	28.6	69.33	63.31	69.11	67.52	13.94	63.96	23.24	10.45	9.73	8.2	9.23	12.99	21.15	16.98	10.77	11.76	40.11	8.6
102	26.4	66.89	73.67	75.21	71.15	22.13	71.55	29.64	15.51	15.39	10.09	11.39	18.27	22.93	28.43	22.45	21.22	41.32	13.65
103	7.16	15.82	15.14	13.24	20.23	42.66	18.47	7.32	57.99	40.51	44.67	45.05	55.34	11.19	84.46	32.93	46.57	14.14	53.77
104	29.57	66.83	72.66	74.01	71.51	21.07	72.77	27.39	14.7	15.44	12.83	14.31	18.71	21.11	22.51	19.63	21.73	47.96	17.08
105	53.81	33.87	38.06	37.71	35.34	21.91	35.3	51.59	14.84	24.23	13.99	18.82	19.05	14.61	10.71	20.86	20.91	67.47	18.57
106	27.79	72.03	71.32	70.38	61.9	18.19	61.85	31.83	9.52	9.47	9.67	10.87	13.17	18.7	16.96	8.82	13.71	44.73	9.54
107	12.03	12.72	21.35	14.17	18.45	53.97	18.66	12.16	48.17	38.64	54.51	50.31	55.45	10.73	48.57	37.03	49.85	19.58	55.44
108	30.99	63.03	70.39	69.06	67.49	20.84	66.24	30.17	13.23	16.47	10.25	11.45	18.17	22.19	23.92	19.45	22.05	49.25	13.33
109	27.44	67.33	75.91	72.33	72.47	21.93	72.21	23.95	16.37	17.5	12.11	14.09	21.01	24.01	25.57	18.23	20.75	47.65	14.26
110	28.57	61.73	64.25	73.38	60.25	17.61	58.38	30.51	9.85	11.64	14.58	14.99	13.44	18.52	17.01	17.68	10.2	53.6	16.39
111	17.43	53.65	57.97	50.59	55.93	24.09	56.96	20.3	17.72	22.07	10.64	13.24	26.29	24.2	24.89	22.89	26.75	37.35	17.07
112	45.23	25.14	26.39	24.62	25.61	25.05	27.19	44.98	17.57	39.43	18.36	26.9	24.85	10.35	16.1	28.1	34.18	55.46	27.56
113	18.57	19.59	25.36	19.95	27.29	27.67	23.06	13.33	14.23	6.73	11.41	9.86	24.42	35.26	20.49	9.99	13.39	25.45	14.13

Table 4.2 continued

	058	059	060	061	062	063	064	065	066	067	068	069	070	071	072	073	074	075	076
001	19.25	17.5	15.69	24.98	60.18	10.7	11.65	15.17	9.29	15.63	12.21	18.17	14.37	55.21	10.64	14.79	15.56	14.61	8.94
002	50.19	54.76	9.87	13.39	18.04	22.38	21.16	23.17	26.38	26.26	22.62	14.19	71.51	12.88	18.35	50.65	26.61	19.35	22.49
003	47.6	50.77	9.43	14.59	23.14	19.43	18.39	20.92	22.86	25.35	19.26	12.23	66.22	17.03	15.7	45.99	24.63	19.15	19.72
004	31.31	34.28	6.53	13.8	13.36	15.95	12.76	14.13	15.44	16.53	13.83	12.85	44.69	12.4	15.73	30.79	16.18	12.06	14.09
005	6.81	10.85	38.59	38.19	10.56	32.29	31.72	28.68	32.35	33.67	34.6	52.49	8.45	10.99	49.89	12.45	33.79	31.79	32.33
006	16.66	20.71	17.09	25.14	59.49	10.37	14.11	17.29	14.65	16.97	13.62	18.55	15.01	53.33	15.6	19.44	17.23	17.4	10.69
007	18.21	20.85	16.93	24.46	60.01	11.1	14.24	17.12	14.81	16.92	13.17	18.47	15.88	52.59	14.85	20.67	17.31	17	11.63
008	17.53	20.58	16.5	24.03	59.56	9.93	14.57	17.61	14.87	17.22	13.15	17.36	14.49	53.69	14.99	19.45	17.24	17.95	10.63
009	19.75	21.72	15.49	23.23	59.99	10.56	13.17	16.97	14.91	16.75	12.91	17.79	16.59	51.47	14.88	21.35	17.03	16.41	11.11
010	13.24	16.26	13.53	21.19	48.67	9.13	12.28	14.43	11.55	13.41	10.78	14.69	9.7	43.61	12.19	14.72	14.47	16.14	9.69
011	19.99	21.85	16.23	23.63	60.84	10.77	13.92	17.86	15.07	17	13.51	18.5	16.24	52.38	15.63	20.76	17.25	16.67	11.29
012	16.91	20.38	16.12	24.84	59.5	9.96	13.71	16.48	13.63	15.93	12.3	17.21	14.93	53.93	13.97	19.31	16.11	16.49	10.31
013	17.05	20.45	15.79	23.05	60.2	9.82	12.5	17.1	13.71	16.29	12.38	18.39	14.57	52.01	15.63	20.01	16.17	15.78	9.9
014	17.34	20.3	15.37	24.11	58.78	11.43	13.53	16.84	13.99	16.33	14.1	19.03	16.91	52.33	15	19.7	17.11	16.15	12.03
015	16.61	20.48	14.06	27.86	60.43	7.21	9.43	10.85	7.45	11.05	7.99	13.89	11.54	54.88	11.42	16.87	11.29	10.14	6.39
016	18.61	22.11	34.91	70.55	26.6	43.89	46.68	40.74	42.84	44.65	47.45	45.09	18.23	22.75	45.03	23.32	48.39	43.21	43.58
017	12.75	15.14	35.25	70.08	24.11	43.57	44.71	39.85	42.83	43.67	46.9	47.15	14.07	22.81	47.53	18.83	45.64	42.52	44.03
018	11.39	14.41	33.54	71.79	21.68	40.99	43.67	39.73	42.15	42.59	43.77	43.03	12.45	17.45	45.11	19.06	44.97	39.53	40.66
019	9.81	14.11	30.85	70.71	20.83	35.29	37.27	33.25	36.39	36.02	38.89	40.07	11.33	17.73	38.99	16.19	38.04	34.85	35.21
020	15.13	21.21	33.93	72.87	25.57	40.76	43.33	40.43	41.19	40.93	43.6	40.15	16.11	22.07	38.79	24.28	44.65	41.82	40.55
021	13.39	14.79	32.71	72.08	27.73	40.57	42.42	37.93	39.81	41.22	44.31	40.28	11.11	22.03	39.37	16.21	42.71	40.91	40.55
022	13.69	16.75	35.74	77.47	23.17	44.26	44.61	40.54	43.97	46.33	46.95	43.68	16.46	19.82	43.95	22.19	47.03	42.31	43.23
023	16.87	19.18	19.39	26.9	56.52	13.63	14.4	16.49	15.43	13.65	14.17	19.11	14.5	52.56	14.31	20.47	19.04	15.31	12.85
024	12.63	17.8	29.44	70.82	23.89	35.53	35.63	33.63	36.56	35.8	39.17	36.99	14.13	18.56	36.56	19.79	38.11	33.76	35.13
025	13.59	16.75	31.97	74.25	26.53	38.02	39.27	35.92	38.48	40.69	41.07	38.9	15.59	24.16	37.63	19.29	40.57	37.31	38.24
026	17.77	17.34	16.63	25.23	50.8	8.37	9.78	23.58	11.43	21.61	10.69	15.49	13.17	51.81	14.29	19.64	14.11	11.57	8.53
027	13.57	18.3	31.94	66.65	27.07	38.84	40.19	35.03	37.95	38.59	42.35	39.12	16.03	26.08	36.75	18.25	42.12	38.73	38.75
028	12.81	13.55	28.67	60.41	24.57	32.87	34.83	30.94	35.87	35.84	35.9	35.06	8.13	20.39	35.99	12.76	35.34	32.41	33.15
029	17.09	19.73	14.27	19.59	58.65	5.41	9.39	13.58	8.79	12.27	9.03	14.42	17.23	50.51	12.05	18.66	12.69	9.85	5.51
030	19.11	17.85	37.97	41.85	13.48	68.57	75.03	71.14	74.61	72.2	73.23	47.03	21.12	9.64	49.92	21.69	76.51	68.51	72.19
031	9.2	8.49	40.25	28.81	8.27	31.89	32.53	29.94	28.55	28.55	32.37	48.02	6.48	8.91	44.11	10.73	30.95	32.87	32.95
032	5.09	7.89	44.22	33.78	11.3	36.75	37.65	36.89	39.38	32.97	41.59	51.11	11.34	15.35	54.97	15.29	39.97	36.55	38.6
033	3.65	7.91	50.38	37.91	18.41	34.55	38.79	37.16	36.55	37.69	40.53	58.91	4.67	11.83	59.39	10.2	41.24	40.23	33.25
034	10.37	12.09	64.6	44.59	14.01	42.83	44.72	44.2	48.32	43	45.87	67.26	9.05	17.05	70.33	15.21	48.51	46.13	41.47
035	21.49	23.26	13.48	20.57	52.79	9.92	9.68	15.32	12.13	12.65	11.28	15.97	17.64	48.97	14.49	24.23	14.96	11.51	9.21
036	19.63	19.61	41.58	43.11	13.77	73.24	75.67	71.35	78.41	76.05	75.57	50.37	21.45	12.15	51.96	22.92	77.23	70.87	75.72
037	16.39	15.99	40.53	43.21	9.47	69.37	69.87	67.94	73.71	70.89	72.57	51.01	22.39	9.19	48.54	23.71	73.47	67.7	70.44
038	39.15	44.41	9.65	16.33	16.37	20.03	19.42	20.47	21.47	24.91	18.83	9.8	59.15	13.44	13.87	41.25	22.95	17.42	20.42
039	38.56	46.56	10.43	15.61	16.02	17.29	16.27	18.58	19.69	20.59	17.07	11.58	57.17	10.35	15.79	44.75	19.95	16.57	18.18
040	21.35	17.94	39.64	41.89	11.47	68.46	77.75	72.52	82.79	70.81	76.95	49.69	24.43	9.57	48.85	24.95	78.39	71	71.58
041	20.37	19.15	42.07	39.42	12.59	70.39	73.23	65.8	72.15	68.83	72.23	46.4	20.95	10.83	45.69	26.59	74.2	72.74	72.56
042	21.96	21.67	41.95	50.59	9.85	78.3	78.29	70.3	79.09	75.3	80.35	51.5	23.45	9.75	48.91	28.76	81.07	73.03	77.68
043	17.78	17.81	41.53	44.13	12.89	71.21	77.57	72.82	78.36	74.13	74.58	50.13	22.42	10.86	48.03	24.59	78.89	73.29	73.22
044	14.61	22.47	19.87	27.77	59.68	8.62	10.73	13.45	9.71	14.04	12.47	22.19	14.55	50.9	18.01	19.37	15.34	13.91	8
045	17.93	16.07	16.95	11.45	15.4	33.77	34.66	35.23	34.04	37.64	29.95	15.53	20.67	13.97	17.91	18.19	36.16	33.52	34.63
046	42.68	45.8	8.72	15.51	16.6	21.09	18.5	19.23	17.69	20.11	17.74	11.15	64.65	9.58	13.53	38.57	22.33	20.45	21
047	6.43	7.5	14.26	18.03	24.81	6.97	11.86	11.59	10.86	12.78	8.78	14.38	5.71	26.18	9.53	9.5	12.45	12.89	7.42
048	20.15	23.14	10.65	22.4	50.97	9.67	10.13	15.19	11.69	13.41	8.04	10.38	17.5	43.23	11.29	22.48	14.37	11.84	9.39
049	14.14	17.14	16.26	22.73	60.47	7.58	7.33	11.02	6.3	9.05	8.51	13.61	13.71	52.47	7.97	18.17	10.95	8.95	7.11
050	18.02	20.81	14.79	21.56	58.81	8.31	8.69	12.35	8.77	10.24	10.15	15.19	17.97	53.55	11.15	21.06	11.85	9.06	8.41
051	16.8	21.13	19.2	30.67	62.41	13.65	16.16	19	16.79	18.61	14.75	22.63	14.11	51.95	20.72	19.8	18.93	14.67	13.01
052	13.23	13.63	59.6	44.35	8.31	45.06	44.88	43.79	47.14	45.53	44.53	65.56	14.67	10.53	66.89	19.73	47.31	46.7	42.17
053	10.65	12.08	18.23	31.46	40.87	23.05	20.88	24.12	19.37	20.73	21.85	23.84	11.85	60.28	17.43	16.19	25.41	27.21	20.97
054	14.12	15.59	34.82	77.09	29.54	41.21	44.22	39.73	41.71	45.09	45.27	43.74	14.65	27.18	43.3	16.69	45.47	40.7	39.73
055	18.15	23.33	13.73	25.11	65.25	7.4	9.97	11.89	10.45	14.21	8.85	11.65	17.98	53.37	11.61	19.57	12.17	9.13	8.14
056	70.59	70.18	17.05	20.01	21.33	25.61	27.19	29.57	27.15	28.91	27.78	21.42	49.69	17.47	23.64	63.72	29.35	25.49	24.69
057	17.75	20.85	15.89	24.38	56.69	6.1	5.88	12.39	6.82	11.64	9.93	13.35	17.68	48.04	12.36	19.53	9.86	8.81	5.61
058	100	63.59	10.33	17.57	17.91	20.8	21.29	23.52	23.68	23.95	23.35	13.62	44.07	12.68	15.09	59.13	24.19	20.81	19.15
059	68.05	100	14.57	19.45	18.97	18.81	19.56	20.02	20.85	19.6	25.43	14.75	49.43	15.95	20.47	62.49	22.51	17.07	17.69
060	13.35	17.43	100	41.12	13.69	43.64	43.57	42.92	46.11	43.29	41.24	59.38	12.44	13.56	63.01	16.95	45.49	44.02	39.02
061	23.9	26.3	12.72	100	25.92	50.85	47.1	44.11	45.69	49.25	47.83	44.79	15.81	21.89	44.94	24.99	50.78	46.43	46.43
062	13.31	14.26	12.64	29.99	100	9.46	12.06	17.76	11.78	16.61	11.85	17.87	17.23	59.77	11.02	17.15	18.03	15.55	8.83
063	36.53	33.85	21.41	24.12	14.03	100	73.65	66.45	72.78	72.14	70.68	50.11	24.47	8.65	47.92	27.64	76.13	72.07	94.07
064	38.86	35.03	26.18	20.7	21.21	65.59	100	79.35	80.56	75.93	78.86	54.91	22.16	12.3	53.31	23.77	82.18	78.72	74.87
065	33.67	32.31	28.79	22.91	25.67	62.34	78.16	100	78.31	80.77	72.16	51.91	23.97	14.75	50.56	26.19	76.59	74.02	66.82
066	40.39	32.62	28.32	19.79	17.43	63.64	71.93	77.13	100	76.26	79.3	54.72	26.3	11.32	55.86	23.9	82.93	73.88	74.23
067	34.3	30.95	26.89	30.13	24.75	70.03	72.48	71.65	72.79	100	72.97	51.05	24.79	12.98	50.67	28.48	77.36	73.41	72.56
068	38.13	43.21	21.69	22.25	21.69	61.5	68.66	70.01	72.64	66.85	100	55.53	24.53	11.57	55.27	27.55	82.31	72.87	72.61
069	16.32	15.42	46.79	14.73	22.76	30.01	31.99	35.02	30.99	29.84	31.03	100	15.75	19.34	80.65	15.79	55.65	54.12	51.08
070	57.01	63.07	16.85	20.55	15.07	36.01	33.28	34.25	37.35	36.67	41.5	23.67	100	12.95	18.48	47.35	27.52	21.89	23.79
071	3.65	7.34	12.71	26.34	53.49	13.29	17.83	21.35	15.32	18.6	17.43	28.52	9.24	100	11.21	16.23	13.19	14.83	8.2
072	19.37	25.93	54.27	12.85	11.77	25.81	29.13	33.63	35.09	31.21	31.65	68.21	26.8	11.41	100	22.84	53.97	49.89	46.81
073	65.91	70.67	19.01	30.51	14.39	38.75	35.71	31.8	35.03	38.28	41.83	16.95	58.97	6.28	28.67	100	29.27	22.43	23.34
074	39.55	37.37	28.4	27.6	28.43	65.69	75.57	76.15	76.66	75.29	78.95	33.44	40.08	19.83	31.08	40.95	100	76.43	75.79
075	33.57	26.55	25.22	19	26.44	61.3	68.31	69.89	61.83	69.39	60.83	35.34	32.06	24.51	27.08	28.53	67.39	100	71.66
076	32.51	30.21	19.55	21.62	12.03	93.75	66.51	62.63	64.59	69.21	61.93	31.29	34.39	11.16	24.06	35.5	64.26	61.05	100
077	8.99	10.19	43.34	19.01	12.06	30.53	22.33	31.95	28.83	23.77	26.64	41.99	15.07	17.54	48.89	17.23	25.84	24.66	31.47
078	40.5	32.95	27.58	24.13	25.41	63.65	72.31	74.99	69.35	73.11	65.02	31.61	36.9	16.96	36.25	36.27	73.49	66.99	63.59
079	23.57	14.37	4.84	11.69	18.56	12.16	10.22	7.59	15.8	8.34	9.13	8.11	13.95	15.07	11.49	10.78	12.41	4.39	11.74
080	21.77	21.24	13.56	30.43	58.47	12.41	20.06	21.63	16.13	23.01	17.93	20.82	21.17	53.25	14.63	24.68	23.12	22.57	10.71
081	9.74	11.37	18.02	28.93	48.63	15.51	21.23	22.93	13.93	25.58	19.69	31.37	10.32	54.76	15.19	11.76	26.97	27.27	13.45
082	20.25	18.14	30.81	21.27	14.23	48.5	46.14	41.73	47.33	57.4	41.29	25.81	27.59	11.28	20.33	24.75	51.65	52.17	46.28
083	10.92	12.59	42.24	22.87	15.05	17.18	16.3	18.59	19.46	22.49	15.13	43.13	14.48	19.18	41.49	17.31	21.58	22.66	14.94
084	16.01	20.44	8.19	62.47	23.34	11.95	7.28	13.03	11.75	18.52	10.83	11.33	21.43	20.91	14.53	21.55	15.74	9.83	10.29
085	16.23	18.27	17.91	26.55	56.46	10.27	16.49	16.14	14.99	20.56	12.61	12.16	15.15	35.91	12.11	18.34	17.98	12.72	11.18
086	16.18	17.77	51.17	12.22	14.03	20.72	27.51	32.7	34.14	27.08	28.59	65.71	22.1	14.49	70.49	18.57	31.51	26.13	21.13
087	38.54	40.53	29.96	30.29	22.33	67.2	73.06	68.89	72.83	77.01	69.55	32.7	40.75	16.65	35.3	43.88	73.13	62.79	65.81
088	37.06	38.79	21.58	24.34	26.94	62.11	64.99	61.07	62.15	67.63	59.49	23.56	30.53	20.68	28.51	39.25	65.11	63.27	59.35
089	30.77	23.01	26.7	20.01	24.22	63.43	67.63	70.91	75.49	77.05	65.54	30.28	31.75	22.32	29.72	32.15	75.57	64.67	64.94
090	16.77	18.81	12.9	26.05	44.2	7.94	12.92	16.97	9.84	11.27	12.61	15.33	11.16	54.88	13.3	22.3	16.05	14.23	7.28
091	19.92	19.6	19.6	32.9	55.61	14.21	18.07	22.05	13.02	17.78	13.51	18.27	12.97	48.71	18.67	18.31	22.47	21.47	13.35
092	41.86	43.44	14.46	12.55	15.22	43.44	51.23	51.55	53.21	44.71	52.61	14.66	35.17	6.69	24.02	37.37	46.87	32.43	41.07
093	9.89	11.86	17.98	28.52	50.71	13.91	18.83	21.22	11.85	23.27	18.05	28.42	10.13	55.05	15.13	12.3	26.15	24.72	11.05
094	22.09	19.95	32.09	22.06	13.82	48.83	47.52	42.57	49.39	58.73	42.03	25.75	28.79	11.49	21.33	26.33	52.13	51.18	46.49
095	8.93	11.33	43.15	23.28	14.2	14.92	13.49	17.32	16.39	20.41	13.4	45.26	13.41	20.05	45.29	15.65	17.96	21.11	14.18
096	17.87	18.47	12.54	32.97	93.77	17.37	23.73	28.37	20.01	28.34	22.18	22.86	16.83	52.45	12.83	15.89	29.74	27.72	14.36
097	39.75	42.98	21.34	28.26	14.57	77.28	66.08	57.3	61.99	66.48	65.45	25.75	39.75	14.29	28.23	42.92	67.59	56.65	72.67
098	34.33	31.12	29.38	18.24	17.74	68.45	73.58	75.39	75.96	71.76	74.27	32.53	35.91	19.53	32.1	35.09	78.67	64.94	66.89
099	18.63	15.2	13.92	35.15	53.16	23.24	24.86	30.75	25.97	27.05	22.28	25.23	15.89	75.15	16.6	13.84	28.2	30.25	20.85
100	64.3	67.45	20.06	23.37	15.95	44.29	41.99	34.45	36.31	37.02	48.71	23.55	55.93	10.16	27.31	68.01	45.25	33.25	39.17
101	36.33	25.89	23.87	14.89	20.09	61.71	63.92	66.31	79.47	69.21	65.09	28.06	33.27	16.11	31.36	29.25	73.89	61.59	59.22
102	33.13	29.59	29.98	34.71	25.37	71.65	71.03	68.75	70.71	86.29	64.07	34.95	31.89	20.35	36.46	33.81	73.19	72.52	68.32
103	14.05	15.03	15.47	36.99	55.81	16.22	19.09	26.69	21.09	21.22	17.49	25.08	13.85	77.11	14.49	14.99	23.27	23.94	13.65
104	37.96	37.79	31.39	29.1	22.06	74.49	72.49	67.17	71.75	77.4	66.19	34.9	39.34	18.85	35.46	43.94	73.75	63.41	70.93
105	86.44	88.38	19.16	28.86	13.17	39.63	41.47	33.68	33.73	38.18	42.23	16.19	55.35	7.63	25.83	69.53	41.24	34.93	36.15
106	34.89	34.69	29.07	23.28	17.33	65.59	63.29	65.11	73.92	68.62	72.74	29.79	36.97	12.97	33.57	40.43	72.25	61.33	63.17
107	20.99	24.8	12.94	30.61	53.8	14.33	14.34	16.63	12.58	16.53	15.73	20.69	18.21	47.07	12.27	28.68	20.92	15.8	10.89
108	38.31	39.66	28.21	24.18	22.17	69.67	69.6	63.17	67.99	73.93	65.13	32.53	38.2	19.5	36.03	40.65	70.75	65.69	65.74
109	36.99	37.89	32.68	21.5	21.74	73.7	73.03	69.59	76.16	75.58	69.43	31.57	36.69	22.48	38.72	38.27	72.3	63.9	69.67
110	38.19	39.93	22.53	28.29	14.18	77.15	68.35	58.15	62.57	67.6	65.59	26.57	38.27	14.97	28.02	41.68	69.33	59.76	73.73
111	31.35	30.57	26.87	21.28	25.62	60.09	60.66	58.59	61.12	65.27	46.13	31.91	29.02	23.6	39.09	30.59	58.49	50.15	54.05
112	59.27	63.15	10.03	36.28	18.16	32.42	28.79	26.3	25.33	28.68	33.15	10.11	50.05	7.21	21.79	58.6	31.17	22.26	27.62
113	15.13	15.74	53.57	22.68	16.13	22.05	18.27	23.46	24.37	21.35	18.11	44.03	23.33	11.79	45.97	20.55	23.29	24.44	20.28

Table 4.2 continued

	077	078	079	080	081	082	083	084	085	086	087	088	089	090	091	092	093	094	095
001	12.66	13.81	11.89	71.21	90.85	15.11	17.69	16.79	54.84	12.65	17.9	20.16	14.74	54.24	55.21	12.04	92.31	14.63	16.05
002	9.63	24.24	16.25	16.46	12.56	17.23	7.89	12.61	14.89	14.95	29.55	24.97	24.14	13.03	12.54	28.89	11.93	19.04	7.38
003	8.01	21.17	15.99	20.47	19.43	17.97	7.65	14.25	17.31	12.87	27.31	23.56	22.41	16.71	15.37	21.64	19.43	19.61	6.94
004	6.59	13.11	30.91	16.85	11.16	10.03	4.01	16.37	16.71	16.78	18.83	21.35	14.08	13.45	12.66	15.93	10.84	11.31	3.96
005	44.61	32.68	28.56	10.96	10.71	29.17	57.56	41.01	10.85	50.94	32.81	36.86	33.78	15.15	10.13	24.45	11.48	28.54	64.54
006	13.47	18.53	12.35	48.93	50.15	14.66	17.25	18.6	63.46	16.41	20.18	21.48	17.66	57.03	54.09	13.81	51.59	15.02	17.07
007	13.18	17.47	15.07	48.15	47.53	13.79	15.77	17.41	63.51	16.25	19.81	21.71	16.81	57.31	55.51	16.14	49.57	14	15.97
008	12.91	18.81	11.79	49.43	51.55	15.17	17.07	17.45	62.9	14.74	19.65	21.89	17.12	57.77	54.87	12.71	53.27	15.55	16.89
009	13.15	15.92	15.51	49.32	46.38	12.5	14.36	17.16	62.11	16.08	19.74	21.61	16.22	58.73	56.01	17.65	48.96	12.82	14.85
010	11.01	16.13	11.11	41.63	42.27	10.71	13.61	15.23	54.5	12.69	17.34	18.29	14.54	50.21	48.52	10.55	44.35	11.11	14.29
011	13.67	16.61	15.36	47.68	47.25	13.29	15.18	17.23	62.91	16.65	19.88	21.54	16.94	57.69	55.91	16.22	49.63	13.61	15.44
012	12.06	17.42	11.68	48.59	50.67	14.69	16.35	17.17	63.47	13.97	18.94	20.39	16.17	57.11	54.96	11.69	52.11	14.99	16.25
013	13.41	17.01	14.91	47	47.83	13.33	15.32	16.52	62.68	15.91	19.01	19.54	16.03	57.54	55.98	14.56	49.5	13.28	15.95
014	13.22	17.17	14.09	47.33	45.95	13.18	15.1	17.61	61.88	16.05	18.83	20.07	15.88	55.46	53.87	15.7	47.67	13.22	15.51
015	9.84	11.49	16.19	55.02	56.06	12.25	13.53	21.03	61.55	14.37	14.16	18.39	10.78	60.83	57.15	11.75	58.33	12.47	12.91
016	43.94	44.67	19.15	24.61	23.56	39.21	35.41	69.75	23.48	43.71	47.95	47.64	43.07	25.38	27.01	34.39	23.85	39.15	38.61
017	49.05	43.5	18.56	16.95	16.83	37.92	35.5	68.74	21.6	43.38	48.24	44.18	46.18	19.12	22.19	30.33	17.16	38.73	38.76
018	46.69	44.26	21.25	15.53	14.51	37.83	35.6	73.88	20.53	42.93	45.1	44.02	40.45	19.33	20.78	28.34	14.91	36.59	38.88
019	39.96	36.67	15.99	18.47	15.37	32.31	34.47	70.79	20.51	36.79	40.76	40.03	38.39	19.55	19.05	22.48	14.8	32.88	37.19
020	41.66	42.53	16.35	24.83	21.81	36.8	33.02	65.49	23.33	37.09	44.91	46.23	41.92	24.8	27.5	25.99	21.73	36.32	35.53
021	41.32	40.53	17.92	18.87	17.61	39.62	38.31	68.58	26.13	38.98	43.29	41.53	39.25	24.2	24.51	26.74	18.48	39.53	41.39
022	45.67	44.64	19.44	20.91	18.93	41.2	39.04	73.05	22.87	41.17	48.27	46.47	43.49	20.43	23.87	29.64	18.6	41.73	42.99
023	14.34	16.09	11.85	47.25	47.05	17.59	15.53	22.2	54.01	19	21.74	24.27	15.73	55.66	50.12	12.86	49.15	17.91	14.73
024	37.82	36.33	15.51	20.83	16.66	31.89	32.27	67.92	24.99	35.32	40.72	39.76	37.93	20.68	20.92	24.85	16.02	32.29	34.13
025	40.38	39.18	14.5	23.65	23.71	36.13	35.48	69.92	25.04	34.87	43.86	41.64	39.49	20.98	25.66	25.1	23	37.21	37.79
026	12.07	11.69	12.56	40.15	39.31	9	10.69	16.95	52.24	14.26	14.05	19.21	11.33	53.06	44.58	8.92	42.02	9.54	12.67
027	38.36	38.51	14.69	22.05	25.64	36.06	32.41	64.91	25.63	36.84	42.47	41.48	39.77	25.31	27.73	27.87	25.65	36.11	33.79
028	33.73	33.21	13.55	19.61	18.3	31.89	30.83	57.97	23.56	31.26	36.19	36.37	36.31	19.17	23.43	21.59	17.61	32.88	31.17
029	8.13	10.36	14.92	66.47	46.67	8.97	10.32	11.45	56.77	13.26	13.95	17.55	10.23	55.06	50.69	14.32	49.01	9.19	10.45
030	51.96	92.08	18.1	10.48	10.31	58.49	36.22	40.53	11.62	47.65	73.61	70.77	77.23	8.52	10.64	56.59	10.73	58.19	39.69
031	43.65	31.41	10.97	8.97	11.87	26.14	48.74	29.75	6.45	43.38	29.67	31.93	33.13	10.24	9.29	21.31	11.57	26.2	52.45
032	75.37	43.12	14.09	10.83	13.62	30.39	37.81	38.21	11.33	50.97	38.68	36.37	39.09	14.44	14.93	27.33	13.91	27.84	42.35
033	53.03	42.27	16.69	9.28	13.63	32.67	58.69	38.01	12.95	56.07	37.47	40.9	38.75	15.25	15.9	27.1	13.29	31.23	62.9
034	66.53	46.39	19.43	12.31	12.97	40.52	52.62	45.1	13.92	71.35	48.25	46.42	48.45	14.49	16.11	32.6	13.45	39.94	56.98
035	12.83	11.36	20.57	45.88	38.31	8.27	8.58	17.36	50.32	19.64	18.13	21.61	11.17	60.2	49.81	21.19	42.15	8.51	9.51
036	49.59	79.88	17.49	14.41	12.53	59.73	36.64	42.49	11.99	49.35	79.11	74.32	76.01	12.07	13.09	51.31	13.09	59.33	37.92
037	46.45	70.79	15.69	7.51	8.07	61.03	38.73	38.85	10.79	48.39	70.02	66.11	74.93	9.51	9.25	43.21	7.89	60.76	40.61
038	7.1	20.78	9.95	14.4	13.13	19.47	6.08	13.53	14.45	9.46	23.57	21.13	18.09	11.41	13.38	16.33	13.81	20.29	5.83
039	9.07	20.35	7.95	20.39	15.97	18.95	7.02	14.17	14.71	11.35	22.99	20.25	16.76	13.96	15.37	16.28	15	20.07	7.07
040	47.37	76.97	18.6	8.28	9.91	61.07	36.73	38.74	9.81	47.59	76.89	71.98	79.82	8.81	11.49	54.91	9.75	61.14	37.88
041	47.92	76.23	15.75	8.61	12.29	61.37	36.01	34.31	11.25	44.7	72.79	71.43	73.25	14	14.82	53.03	11.67	59.35	35.93
042	48.24	77.91	14.68	7.65	10.89	65.83	39.31	42.47	9.03	48.55	78.99	72.6	78.63	6.73	9.23	55.51	9.63	66.06	41.06
043	49.07	79.07	14.95	12.52	12.05	66.14	37.23	38.97	11.49	46.23	77.11	73.25	77.35	11.08	11.65	54.77	11.95	65.93	38.32
044	15.03	14.42	15.16	49.34	49.59	12.03	23.09	20.75	61.79	19.33	15.3	19.53	13.85	56.53	52.8	12.37	51.35	12.54	21.86
045	12.92	36.89	14.1	13.59	13.42	26.62	10.49	7.78	12.28	16.42	38.29	35.87	36.02	17.28	13.77	25.67	11.79	26.83	9.45
046	4.5	20.83	8.66	19.12	18.07	24.04	5.94	14.97	13.63	7.38	22.83	20.6	18.6	11.74	13.26	17.33	17.12	23.47	6.11
047	10.92	11.74	13.09	30.42	28.7	7.29	8.81	12.06	26.52	13.94	10.55	13.4	11.81	32.12	30.07	7.39	29.92	7.36	9.85
048	9.2	11.33	20.23	41.97	37.67	8.65	8.68	19.54	49.77	13.02	18.03	21.55	11.71	52.27	47.58	18.84	39.28	9.03	8.51
049	8.08	5.75	15	49.59	48.21	5.97	7.77	12.47	54.97	10.47	12.15	15.25	8.3	55.69	54.91	14.84	47.7	6.56	8.16
050	9.12	6.46	19.69	50.55	45.53	5.51	8.59	14.9	57.07	13	13.26	17.21	8.61	58.75	55.24	17.07	46.61	6.31	9.78
051	22.93	16.29	19.39	48.73	45.65	13.41	18.11	27.55	55.04	19.37	19.79	21.7	16.5	54.33	51.39	16.6	47.8	13.9	16.88
052	60.29	48.07	20.51	7.71	7.29	39.87	49.76	43.37	8.57	67.04	49.05	46.81	47.85	10.21	11.63	32.97	7.21	39.57	51.45
053	26.25	24.42	14.71	36.99	40.83	20.05	20.32	26.48	41.52	19.09	21.76	20.24	19.7	44.9	41.01	13.2	41.45	18.97	21
054	44.18	42.46	23.77	22.14	19.15	39.05	36.63	75.01	24.95	43.18	48.06	48.03	41.62	26.57	24.36	31.66	19.39	40.44	39.03
055	8.46	12.64	17.7	49.98	42.73	13.66	8.65	19.28	92.43	14.22	18.27	20.07	11.99	59.69	58.29	17.99	46.85	14.56	9.34
056	12.83	24.22	16.81	20.03	15.6	21.78	11.43	11.75	18.55	18.34	32.53	27.89	25.67	18.17	16.68	34.2	15.22	22.42	10.24
057	10.55	7.01	14.49	47.58	47	6.73	11.99	14.09	54.15	14.94	12.67	16.45	7.85	59.75	51.57	11.97	50.83	6.93	11.41
058	6.8	22.98	19.08	19.58	15.16	14.18	6.7	11.25	18.73	11.34	25.69	24.22	20.55	15.15	17.28	33.64	15.11	15.15	5.41
059	10.09	20.56	14.02	18.31	14.63	12.45	10.87	14.55	21.78	14.34	26.91	25.64	17.08	20.41	18.95	33.55	14.57	13.75	9.1
060	60.21	43.77	16.3	11.26	13.16	43.78	56.48	37.98	13.03	63.91	45.49	42.21	43.23	14.19	13.45	26.49	12.85	43.86	55.33
061	49.32	48.22	18.42	21.9	22.63	45.41	47.79	75.67	23.63	43.49	51.41	49.2	45.32	22.91	25.3	29.55	22.11	45.4	48.43
062	9.86	14.53	16.11	53.52	52.01	14.38	13.66	19.65	63.41	12.95	18.49	21	15.55	54.77	55.11	14.77	52.72	14.64	12.13
063	54.5	73.93	17.01	6.36	8.42	62.69	42.77	43.53	7.67	46.35	74.65	72.83	72.27	6.69	9.47	50.48	7.72	61.71	41.56
064	49.57	79.9	17.73	10.27	11.19	60.74	38.29	42.53	10.87	51.81	80.02	75.83	78.55	9.28	11.14	57.68	10.08	61.91	41.25
065	48.84	74.61	15.87	13.35	13.21	53.19	35.34	39.23	12.01	49.42	72.32	68.05	73.5	16.2	14.52	54.13	12.75	53.86	37.39
066	52.39	77.21	21.83	8.86	6.97	62.19	40.21	42.97	10.49	54.85	79.38	74.92	82.37	9.55	9.04	59.55	6.07	63.46	42.11
067	46.42	74.2	16.38	12.41	13.49	63.03	39.15	44.02	13.47	49.19	77.57	72.64	77.42	11.13	11.75	49.21	12.29	64.06	40.27
068	52.77	75.24	17.51	9.19	10.17	57.34	37.62	44.49	8.17	52.74	75.99	70.91	76.09	9.27	9.81	57.63	9.57	57.08	40.49
069	64.33	52.28	19.87	13.2	18.55	46.19	54.89	45.01	10.31	76.09	54.33	50.3	54.57	14.31	13.48	35.19	17.03	45.85	59.89
070	11.23	24.1	12.67	18.89	12.21	21.33	9.47	13.92	16.1	15.45	28.87	25.42	22.91	14.26	15.07	24.93	11.72	22.04	8.52
071	13.2	10.69	13.39	48.25	52.16	11.47	13.25	19.63	52.93	12.35	14.73	16.96	15.39	58.11	48.99	10.35	52.85	12.61	13.71
072	67.95	54.11	21.71	9.19	8.91	42.72	54.14	47.51	9.99	78.65	55.09	51.95	53.36	13.72	14.95	39.55	9.21	42.82	59.62
073	16.35	25.67	13.02	16.97	11.17	19.03	15.04	15.36	19.24	15.33	29.17	26.51	24.24	20.23	21.39	29.04	11.97	18.39	11.81
074	52.27	80.57	20.01	12.19	14.15	65.73	42.09	45.36	12.19	53.8	80.11	76.79	81.75	11.62	14.39	55.16	13.54	65.55	42.95
075	50.42	75.16	14.69	12.65	13.97	64.85	43.45	42.13	9.14	49.73	74	73.91	74.03	10.68	13.65	48.15	13.03	63.97	44.07
076	53.37	74.05	17.37	5.51	7.22	61.17	37.3	41.27	7.97	45.51	74.65	71.57	74.17	6.28	10.18	50.27	6.33	60.55	39.14
077	100	53.91	20.03	7.46	12.02	42.01	54.63	51.31	7.24	64.74	52.21	50.89	54.59	12.33	11.85	34.09	11.54	39.89	56.49
078	27.17	100	17.89	12.66	12.64	63.17	41.98	46.26	12.75	54.13	78.94	75.43	81.34	11.2	14.78	56.67	12.85	62.72	43.42
079	6.83	9.04	100	9.23	5.49	11.03	14.25	27.53	18.49	31.57	22.1	23.71	19.66	17.7	18.07	23.36	7.52	11.31	16.93
080	11.55	24.24	12.96	100	69.61	10.91	12.85	12.59	48.29	10.88	13.79	17.29	10.13	52.58	44.95	9.77	70.64	10.87	11.7
081	21.44	24.29	5.35	53.31	100	15.16	20.64	13.8	42.71	10.65	15.81	16.72	13.15	47.67	46.2	3.45	96.91	14.25	18.27
082	16.76	52.44	0	12.35	20.08	100	45.85	40.93	13.95	38.71	65.84	60.32	64.97	8.83	8.51	30.88	13.46	97.7	45.9
083	35.8	23.14	3.77	18.31	33.89	31.44	100	43.37	8.08	50.85	39.81	39.03	42.33	10.4	10.99	19.22	19.01	44.44	91.97
084	21.38	17.23	30.53	15.27	16.7	14.96	17.98	100	18.47	47.82	47.95	48.27	44.24	17.02	20.21	29.41	14.1	42.47	47.66
085	9.01	19.58	25.81	54.91	36.63	13.29	10.93	20.05	100	12.95	18.73	20.2	12.73	58.42	59.89	16.54	46.99	14.71	8.4
086	42.18	32.69	31.73	16.75	18.34	16.15	33.24	15.52	20.05	100	55.62	53	55.04	16.32	15.21	42.09	11.58	38.98	55.42
087	29.68	75.52	17.8	20.28	23.7	55.04	20.27	19.85	23.62	33.98	100	83.4	83.04	14.62	17.32	65.73	14.73	68.33	41.09
088	25.13	66.23	20.36	28.1	26.22	42.63	15.41	19.09	26.79	28.05	75.33	100	78.09	18.83	19.72	60.57	16.21	62.65	41.63
089	32.34	76.1	9.99	20.13	24.75	54.92	25.83	13.07	19.28	31.67	75.93	65.79	100	10.05	13.38	58.94	12.37	66.53	43.9
090	13.2	16.1	19.89	52.64	38.5	6.79	11.56	19.78	46.95	17.85	15.93	24.35	13.49	100	60.45	16.54	52.04	9.15	11.13
091	16.55	25.22	18.03	55	50.47	11.03	17.7	24.41	60.87	22.5	23.23	28.34	19.57	58.66	100	19.17	49.93	8.93	10.15
092	15.98	54.06	17.87	16.57	3.05	17.69	1.9	9.3	18.39	27.45	60.74	51.73	46.41	14.55	20.25	100	6.15	34.63	22.96
093	19.97	24.57	8.36	55.79	96.25	17.43	31.63	16.65	43.83	19.38	20.85	24.73	22.73	43.98	55.24	5.03	100	13.03	17.35
094	14.79	53.26	0	12.51	18.54	98.37	30.23	15.76	14.1	16.21	57.45	44.87	56.09	7.53	11.05	22.73	16.63	100	47.05
095	36.33	22	5.66	18.56	30.69	29.04	92.3	20.17	11.41	37.85	17.3	15.56	21.39	12.95	15.95	3.8	29.45	28.39	100
096	13.59	27.42	20.81	59.64	49.26	15.53	15.35	27.89	58.63	16.66	25.2	29.86	24.53	45.51	58.07	15.55	51.33	15.19	14.47
097	17.31	60.71	23.73	15.39	11.89	42.8	12.17	16.17	12.58	26.81	73.27	66.34	62.01	14.91	14.84	55.38	11.13	44.57	8.71
098	24.98	72.75	6.86	17.21	20.31	43.61	19.11	6.94	12.03	31.07	74.18	63.55	72.51	11.73	21.17	60.31	19.8	45.28	16.91
099	31.89	28.59	28.73	52.98	53.75	15.15	21.27	24.26	45.45	20.49	28.95	27.3	32.53	54.02	54.28	18.64	54.95	16.37	20.98
100	16.83	38.58	21.21	21.41	9.96	23.33	15.65	19.27	17.48	19.6	48.09	40.83	33.77	13.95	15.2	38.85	9.69	24.39	13.37
101	25.46	68.66	11.59	19.09	17.82	50.01	22.91	7.76	13.53	29.35	66.51	61.36	70.89	9.79	11.47	44.44	16.29	51.11	19.69
102	29.79	75.94	13.9	23.47	29.98	62.46	29.35	24.03	22.58	30.28	81.17	70.1	76.69	13.17	21.96	44.15	27.83	63.23	26.58
103	25.35	19.02	26.38	57.59	53.68	14.12	20.21	24.29	47.41	18.7	23.97	23.37	23.66	61.26	55.97	15.92	56.69	14.97	19.44
104	27.04	71.38	19.49	20.87	26.12	57.96	25.77	17.55	22.81	32.08	83.03	77.4	73.19	14.95	20.58	47.35	23.87	59.7	21.93
105	13.71	39.72	24.37	25.23	13.53	28.87	15.49	25.55	21.23	17.86	46.38	43.39	35.44	19.25	22.81	39.79	13.51	30.15	14.51
106	24.17	68.18	7.38	17.33	17.63	53.71	25.03	11.93	13.8	29.95	76.25	62.26	77.09	11.75	13.73	49.81	17.2	54.11	21.95
107	14.01	15.36	34.32	57.68	42.67	8.4	13.3	26.6	50.1	22.81	20.74	29.09	14.45	59.91	51.81	13.27	45.95	8.89	14.21
108	27.87	73.38	21.45	21.07	22.05	55.08	23.11	19.92	24.52	31.78	80.17	72.87	69.06	15.48	21.77	52.68	20.76	56.37	20.71
109	31.87	75.19	17.99	24.84	22.01	49.51	20.13	15.37	21.41	32.15	81.65	73.49	70.89	19.58	20.59	52.38	22.25	51.25	16.87
110	16.9	61.31	21.95	14.85	13.22	47.35	13.91	15.27	12.03	26.19	72.4	65.27	63.59	13.99	14.11	49.08	12.19	48.53	10.21
111	31.07	61.15	32.97	25.04	24.75	36.99	15.17	21.88	27.46	39.31	71.76	66.77	59.91	20.95	26.55	51.27	24.53	39.41	14.62
112	9.59	30.35	37.84	30.53	9.84	23.49	10.2	34.94	33.32	17.53	40.98	40.84	22.41	28.35	30.99	45.56	12.77	24.56	9.93
113	53.11	26.23	7.49	10.73	18.86	22.46	47.17	16.99	14.61	40.74	26.22	17.03	22.32	11.81	18.56	10.72	18.07	22.77	44.81

Table 4.2 continued

	096	097	098	099	100	101	102	103	104	105	106	107	108	109	110	111	112	113
001	58.44	8.62	11.59	57.87	17.43	10.92	15.05	53.83	16.81	16.56	11.79	54.51	14.4	16.21	8.55	14.65	17.31	13.39
002	18.43	22.59	25.51	15.89	49.31	22.58	20.95	13.58	23.76	46.41	24.18	16.23	25.29	23.92	22.03	22.51	40.29	9.99
003	23.38	18.95	22.64	20.03	46.31	21.59	18.29	17.62	21.61	40.46	21.45	19.73	22.4	21.28	19.67	17.35	34.23	9.75
004	17.01	19.76	15.09	13.4	36.21	16.09	16.42	15.45	20.45	35.88	14.05	20.75	22.94	19.96	19.43	25.53	36.05	8.13
005	12.1	33.49	28.91	11.42	11.15	31.81	37.35	12.45	34.71	13.25	33.33	13.95	34.77	33.39	33.15	38.33	19.65	37.39
006	58.11	10.78	13.66	52.25	14.99	13.47	16.55	53.71	16.5	15.38	12.71	49.59	16.09	17.49	11.13	16.98	16.73	17.59
007	59.03	11.47	13.65	52.36	17	13.58	16.33	54.31	16.42	16.89	12.47	50.64	16.91	17.75	11.83	17.87	19.97	15.35
008	58.09	9.88	13.26	52.45	14.69	13.16	17.21	54.55	16.39	15.15	12.69	49.75	16.69	17.94	10.36	17.29	16.37	16.21
009	58.63	11.09	14	51.14	17.07	13.55	16.13	53.89	16.29	17.09	12.34	51.13	16.99	18.33	11.21	18.05	22.01	14.3
010	48.22	10.3	11.15	43.96	9.64	10.69	15.11	45.83	14.14	11.49	10.77	39.92	14.01	15.73	10.4	14.21	12.59	14.43
011	59.49	11.19	13.58	51.61	16.77	13.55	16.44	54.1	16.32	16.68	12.53	49.97	16.86	17.43	11.27	18.19	21.15	14.97
012	58.27	10.17	12.39	52.46	14.27	11.84	15.51	54.75	15.15	14.99	11.54	49.61	14.89	16.43	10.49	15.67	15.67	15.51
013	59.46	10.33	12.21	52.67	15.55	12.71	16.06	54.8	15.36	15.93	11.68	49.8	15.55	16.13	10.33	17.25	20.08	15.12
014	57.05	11.97	13.43	50.06	16.53	12.84	15.41	53.17	15.3	15.97	11.84	48.24	15.58	16.61	11.99	17.25	19.03	14.59
015	58.62	8.33	7.51	54.83	17.59	7.26	9.39	53.59	12.95	14.93	8.47	58.31	10.73	12.45	8.31	13.54	18.33	11.59
016	25.98	48.76	38.89	24.28	18.25	40.45	47.21	27.09	46.25	18.16	45.52	26.33	43.15	42.41	49.98	44.67	20.91	34.95
017	23.57	46.67	39.15	26.85	16.5	38.82	47.64	25.74	44.69	10.96	46.37	22.84	40.32	40.49	47.57	41.8	15.03	39.34
018	21.36	45.09	36.96	18.22	11.49	39.01	47.26	19.09	43.05	12.27	41.27	21.87	40.43	40.83	44.43	44.95	15.54	36.47
019	20.11	41.03	31.67	18.24	10.14	33.55	38.73	20.35	39.17	8.85	37.67	21.61	34.3	35.55	41.44	36.45	10.49	32.65
020	25.24	43.79	37.71	25.45	15.79	37.69	43.77	25.28	42.91	16.98	42.94	29.31	38.23	39.73	44.26	39.17	16.7	31.69
021	26.13	44.59	36.54	26.18	10.52	38.13	44.37	26.17	42.27	9.73	40.43	25.27	37.97	38.35	45.11	38.95	12.68	34.85
022	23.17	47.66	38.62	23.64	15.38	40.8	49.34	23.97	46.4	16.13	46.11	22.71	43.63	41.95	47.83	43.81	18.83	34.67
023	55.18	14.3	13.58	51.71	18.42	13.27	13.57	53.82	17.91	15.07	15.36	80.21	15.07	18.35	14.63	18.16	18.55	15.35
024	22.29	40.51	32.12	19.41	12.03	34.23	38.07	21.71	38.95	12.59	37.77	23.91	34.33	34.87	41.01	35.63	14.37	30.85
025	26.28	41.41	33.95	27.09	15.11	35.15	42.13	26.23	40.77	13.61	40.35	25.54	36.55	35.72	42.02	35.83	13.57	31.97
026	49.47	11.96	10.19	47.71	15.76	12.95	11.55	51.14	11.57	17.44	9.01	53.15	12.54	13.77	11.62	14.28	19.61	12.47
027	26.99	43.23	34.41	28.23	13.63	36.64	41.77	28.44	41.02	13.93	39.92	27.13	36.52	37.24	44.28	38.04	14.68	31.27
028	25.02	34.47	29.05	21.21	8.48	30.76	33.72	22.98	34.39	8.95	35.98	20.74	29.75	31.15	35.49	29.14	7.21	30.84
029	57.12	6.84	8.18	47.75	18.11	8.84	8.27	50.01	11.46	16.73	8.37	54.02	9.55	11.5	6.21	10.79	19.57	9.99
030	14.05	66.57	74.05	14.12	18.45	70.72	75.87	9.43	68.64	17.62	71.96	9.76	70.89	73.09	67.37	65.74	14.77	37.05
031	8.52	27.32	29.19	9.6	11.36	27.51	29.23	8.87	29.19	10.43	31.28	9.54	26.1	28.94	29.11	25.22	10.28	39.99
032	11.76	35.41	34.58	20.74	10.58	35.15	40.44	20.65	35.02	8.45	38.09	9.85	35.35	38.9	35.06	39.96	9.28	44.79
033	18.33	33.87	34.59	12.36	5.93	34.93	41.71	12.62	36.5	5.39	38.31	11.09	35.14	36.11	34.66	39.77	9.82	47.45
034	14.3	41.79	43.49	17.94	11.61	47.88	47.89	16.69	45.92	8.06	47.03	13.69	44.59	46.83	41.72	49.76	9.63	58.89
035	53.88	12.45	11.85	47.95	23.91	11.02	11.27	50.95	15.91	21.02	11.87	80.59	16.02	16.42	11.77	20.53	31.71	11.89
036	15.87	71.94	74.59	15.73	22.71	72.18	76.93	12.93	76.43	18.61	74.11	12.7	75.84	88.04	72.75	70.85	14.21	39.83
037	10.81	67.92	72.05	13.05	22.53	72.52	73.35	11.29	70.55	21.64	76.31	11.55	67.66	71.55	70.24	60.18	13.72	38.17
038	16.37	18.63	20.11	12.73	36.54	18.51	19.77	11.77	20.41	37.58	19.67	14.05	19.27	18.11	18.85	18	26.07	7.81
039	17.75	17.93	19.17	12.55	40.84	18.88	17.58	10.86	21.04	44.94	18.08	16.28	20.19	20.65	18.01	13.91	33.75	11.21
040	13.14	67.63	75.59	14.6	21.41	73.01	71.86	10.79	69.17	19.85	76.81	10.41	68.55	71.59	68.87	65.11	17.88	34.94
041	13.67	68.51	72.19	13.57	26.35	67.99	73.77	12.63	70.03	24.02	72.77	13.97	70.15	73.09	69.76	66.13	20.39	37.21
042	10.45	78.33	78.26	11.81	24.49	76.57	79.67	10.47	78.02	21.85	79.15	9.3	75.57	77.6	79.2	67.03	15.52	38.75
043	14.13	68.7	76.38	13.12	22.55	74.03	75.46	11.84	73.71	20.62	74.19	11.02	72.76	76.77	70.16	67.08	15.67	39.44
044	61.33	11.81	10.65	52.41	18.21	11.12	13.27	49.77	14.41	20.15	12.65	57.81	14.71	14.92	12.15	16.07	20.95	21.49
045	16.93	30.74	34.75	18.71	21.71	33.59	36.73	16.23	38.42	19.15	32.75	15.09	35.57	39.57	31.47	29.71	23.9	13.95
046	18.53	20.14	20.38	10.79	40.2	17.32	19.71	9.22	19.01	43.7	21.89	15.55	22.39	19.57	19.87	15.33	33.14	6.67
047	24.34	8.62	7.72	30.41	11.77	10.93	12.75	31.86	10.62	7.42	8.64	26.63	11.79	11.92	8.92	13.93	15.24	11.52
048	52.01	11.24	12.61	44.27	19.32	9.59	10.99	47.93	12.96	20.85	8.69	50.05	13.64	16.06	10.6	15.53	34.51	10.25
049	60.15	9.29	7.85	54.36	18.21	6.55	5.89	53.55	8.87	13.99	7.77	55.61	8.05	9.91	9.24	8.95	20.54	12.97
050	58.29	10	10.4	52.2	18.75	7.68	6.99	55.35	9.83	17.29	8.54	53.38	9.15	11.36	9.65	10.69	25.17	12.17
051	61.07	13.57	13.98	54.19	19.9	14.81	15.9	53.66	16.91	16.77	15.13	54.9	17.09	19.64	13.89	20.71	20.89	22.62
052	8.4	46.51	43.89	11.25	15.36	48.21	49.44	11.39	47.09	13.54	47.43	9.58	46.36	47.69	46.97	49.61	13.65	56.27
053	41.68	24.66	19.39	67.29	14.82	19.97	25.6	67.2	20.83	10.97	17.65	40.17	22.27	25.03	22.23	28.01	14.98	19.37
054	29.51	45.23	39.05	25.62	14.25	40.23	47.42	27.79	46.81	11.97	41.91	29.77	43.23	44.07	45.81	46.06	15.64	35.62
055	61.48	9.45	8.53	51.6	19.27	9.31	11.71	55.76	16.06	17.69	9.23	55.65	13.75	15.17	8.47	16.71	24.45	9.83
056	23.33	27.86	26.59	20.99	70.59	24.81	23.93	19.73	30.29	63.29	26.33	22.65	33.19	28.87	27.95	23.55	54.27	18.22
057	53.49	9.02	6.99	47.53	16.34	5.68	7.48	48.53	12.13	17.21	6.45	56.63	8.12	9.54	8.77	9.82	23.36	10.79
058	21.48	20.4	21.08	18.73	60.4	22.58	19.23	17.18	22.45	56.55	20.31	21.28	23.45	23.01	19.66	17.63	50.53	11.89
059	21.67	22.27	19.08	19.74	64.2	16.05	18.19	18.81	23.16	60.45	20.02	24.17	25.28	22.25	20.87	19	49.55	15.23
060	12.84	42.79	43.33	14.19	13.4	46.5	48.71	15.91	48.24	10.85	47.47	13.61	44.52	47.67	43.97	47.36	8.15	64.74
061	27.04	53.36	43.34	26.49	15.69	45.69	56.01	26.26	52.55	16.31	49.95	24.52	47.81	48	53.93	49.35	20.13	44.91
062	93.91	8.97	12.2	55.89	17.15	12.66	13.69	56.44	15.05	13.59	11.54	57.87	13.65	15.63	8.78	14.67	17.31	15.1
063	10.97	85.39	75.33	12.31	24.46	74.39	79.91	10.81	79.04	19.86	76.13	8.76	76.07	80.28	85.49	74.11	16.51	42.95
064	13.04	74.46	78.22	14.58	21.99	73.89	78.07	11.87	77.29	21.27	77.25	8.82	76.63	77.89	76.24	70.03	15.53	37.53
065	19.11	63.24	73.81	19.43	22.56	71	70.37	17.3	68.11	23.01	71.6	13.08	66.62	71.15	64.37	64.12	21.87	38.38
066	12.69	71.33	79.26	15.63	23.67	82.87	76.94	14.41	75.85	19.42	82.14	8.72	74.61	79.57	72.54	71.14	16.73	40.65
067	18.8	69.85	73.63	15.61	23.75	73.11	83.9	13.58	77.1	23.93	74.54	11.03	74.35	75.93	71.56	69.13	20.13	38.67
068	12.11	73.39	75.39	12.41	27.01	71.53	73.56	10.7	73.55	22.43	79.45	9.53	72.47	74.05	74.31	61.53	18.63	36.83
069	18.12	48.24	49.71	17.16	17.33	49.13	54.42	17.09	53.41	13.05	54.63	13.77	50.23	50.31	49.43	51.47	11.85	55.61
070	18.69	24.63	26.43	15.46	48.82	24.47	21.76	13.95	26.32	44.01	24.77	18.14	26.27	24.9	24.33	22.52	37.53	14.81
071	56.9	10.37	12.13	77.47	14.87	11.31	11.69	79.98	14.19	12.41	9.43	55.65	13.45	15.3	10.34	15.22	9.96	10.63
072	12.08	49.94	49.03	13.66	19.54	50.9	55.23	12.95	53.51	19.19	56.01	10.75	52.49	53.79	50.41	55.38	19.11	56.05
073	19.09	28.55	24.61	18.33	61.45	24.67	22.5	19.39	26.58	64.74	25.15	23.92	27.79	25.35	26.49	24.57	51.75	17.33
074	19.11	76.95	82.35	16.81	27.73	79.69	79.75	14.84	77.85	23.88	81.71	13.49	76.31	79.35	78.59	71.69	20.21	41.33
075	15.76	69.71	74	17.65	20.62	74.01	78.84	13.53	72.87	19.87	73.78	10.24	73.97	73.85	71.94	66.65	15.48	41.71
076	9.77	81.68	73.65	11.6	22.71	69.81	76.58	10.46	76.03	19.02	74.29	7.08	72.52	77.01	82.83	68.49	14.73	37.75
077	10.55	48.37	47.13	20.57	10.85	50.49	55.7	17.1	50.25	9.33	51.85	9.79	49.18	51.8	48.39	55.09	9.45	64.27
078	15.49	72.36	77.29	15.87	20.56	75.83	81.76	11.3	74.99	20.2	77.49	9.75	77.01	78.34	73.01	71.89	16.69	43.15
079	17.75	23.32	16.23	21.89	20.74	18.67	19.73	20.85	21.48	20.66	17.33	23.33	23.11	20.95	22.94	30.3	34.31	17.17
080	51.8	7.83	8.61	49.53	16.8	10.37	11.74	46.95	13.71	18.29	9.35	51.63	11.63	16.18	7.89	12.52	18.75	8.48
081	50.23	6.19	10.16	53.93	11.9	9.01	14.99	48.15	15.41	12.63	9.43	48.11	11.51	14.25	6.7	12.38	8.77	12.1
082	15.19	59.71	59.01	11.59	15.87	62.65	69.63	11.59	68.89	17.16	66.15	10.29	64.39	64.06	63.09	57.01	11.75	36.37
083	13.31	40.19	39.38	14.27	7.83	45.15	49.8	13.07	44.53	7.75	45.69	8.79	42.01	41.97	41.19	43.25	5.1	57.89
084	21.91	47.67	38.53	21.23	10.34	42.08	51.1	20.68	47.15	12.77	45.91	21.16	46.19	44.96	47.37	48.46	17.47	42.41
085	64.13	9.34	8.34	54.29	20.01	9.55	11.79	57.34	16.49	18.71	8.89	57.82	14.63	14.88	8.91	16.09	24.33	9.63
086	13.87	50.16	49.58	15.33	13.43	51.61	54.8	14.05	53.69	12.29	54.31	15.57	51.55	51.94	50.33	56.85	16.43	58.9
087	20.18	77.55	79.71	19.98	29.49	74.58	82.65	17.48	84.05	26.47	82.63	17.41	80.58	84.49	77.97	75.44	26.18	42.81
088	22.43	74.89	74.82	18.42	25.93	72.69	77.4	16.75	81.45	24.83	75.95	20.83	77.2	79.26	74.99	74.39	26.41	39.64
089	15.71	72.15	78.48	18.32	21.71	77.1	80.21	15.13	77.61	21.65	84.83	9.25	76.08	77.43	73.65	70.19	17.46	41.29
090	53.59	9.81	9.78	59.39	15.05	9.63	8.15	60.71	11.83	17.19	9.3	61.43	11.59	14.09	9.33	14.87	25.74	11.71
091	57.81	9.99	14.57	54.18	17.23	8.03	12.93	57.08	14.33	17.58	9.01	52.06	15.52	15.67	9.73	16.49	24.35	12.11
092	15.59	55.9	61.87	16.32	33.69	54.69	50.29	16.2	53.49	29.03	58.26	13.97	58.65	56.44	53.11	53.01	43.21	25.98
093	51.2	6.01	9.9	55.43	11.95	8.52	13.92	50.57	14.35	12.67	9.61	50.19	11.3	14.3	8.41	12.27	11.07	11.63
094	15.31	60.75	61.03	13.22	16.41	63.57	70.23	13.11	70.54	17.91	67.42	10.49	65.85	65.97	63.45	57.8	12.43	35.74
095	11.73	40.92	40.67	14.53	6.79	44.97	50.13	12.83	44.41	7.26	47.85	8.68	43.59	42.68	41.2	43.49	5.23	53.49
096	100	9.33	13.22	58.27	21.28	12.45	15.39	58.47	17.42	17.09	12.69	61.87	15.71	17.27	9.59	17.66	22.42	14.53
097	16.14	100	77.87	13.22	27.99	74.27	80.09	12.78	80.9	23.89	77.2	10.71	79.2	81.19	98.29	76.07	21.31	39.31
098	19.27	73.14	100	14.01	25.69	80.71	78.99	10.85	77.99	22.63	81.45	9.21	77.23	82.99	78.18	72.15	18.24	39.46
099	56.33	21.57	24.31	100	21.52	13.77	15.77	83.03	16.73	16.88	10.73	58.18	15.4	19.85	12.15	19.15	20.59	14.56
100	20.58	52.63	42.64	19.59	100	23.3	21.47	17.66	28.97	68.48	26.2	24.63	30.9	29.19	27.91	24.09	55.57	14.39
101	19.85	62.45	74.41	22.16	37.48	100	79.87	10.73	77.16	20.56	79.13	8.33	79.34	81.11	74.73	73.14	18.25	44.02
102	28.94	71.42	72.39	31.31	37.61	72.09	100	12.92	86.68	20.07	81.01	9.39	84.99	85.18	81.2	80.88	17.07	45.67
103	59.43	18.31	17.61	84.59	15.49	14.67	24.23	100	15.4	14.39	10.59	56.46	14.57	16.45	12.15	18.17	18.97	11.91
104	27.09	77.09	72.78	27.19	49.77	71.61	84.85	23.81	100	23.96	79.61	15.17	84.83	86.73	82.57	79.04	20.91	43.35
105	17.04	47.41	38.57	16.63	72.97	35.2	38.91	15.15	44.85	100	24.38	22.95	26.71	23.23	22.81	19.63	71.93	8.57
106	19.85	66.45	74.79	19.06	47.36	68.6	72.47	15.71	72.95	44.98	100	10.32	76.7	80.58	78.55	68.82	19.23	41.87
107	58.73	19.79	14.34	48.68	26.35	11.87	17.77	53.95	21.62	27.96	17.57	100	13.78	15.11	10.92	17.65	29.78	9.37
108	26.09	75.23	71.43	26.89	48.61	71.39	83.98	21.77	88.75	42.52	66.09	23.39	100	87.47	79.48	80.71	25.23	41.56
109	24.99	74.54	79.19	32.53	48.37	74.76	81.29	25.09	86.83	40.11	72.23	24.45	86.98	100	81.74	81.58	20.23	44.55
110	16.24	97.82	72.68	20.85	52.43	62.91	72.29	18.04	78.19	45.59	67.71	19.97	75.13	74.45	100	75.31	19.13	40.25
111	31.69	62.17	61.61	31.85	37.89	58	73.41	28.23	75.79	33.47	51.42	30.95	80.87	78.35	80.47	100	23.72	44.19
112	25.76	42.54	27.86	23.34	57.75	26.12	31.54	24.51	37.71	77.39	33.39	37.05	40.96	34.53	38.25	37.87	100	11.14
113	15.53	16.7	23.49	20.83	23	22.59	25.12	15.06	26.32	15.37	21.97	12.73	25.32	29.75	17.99	23.49	10.07	100

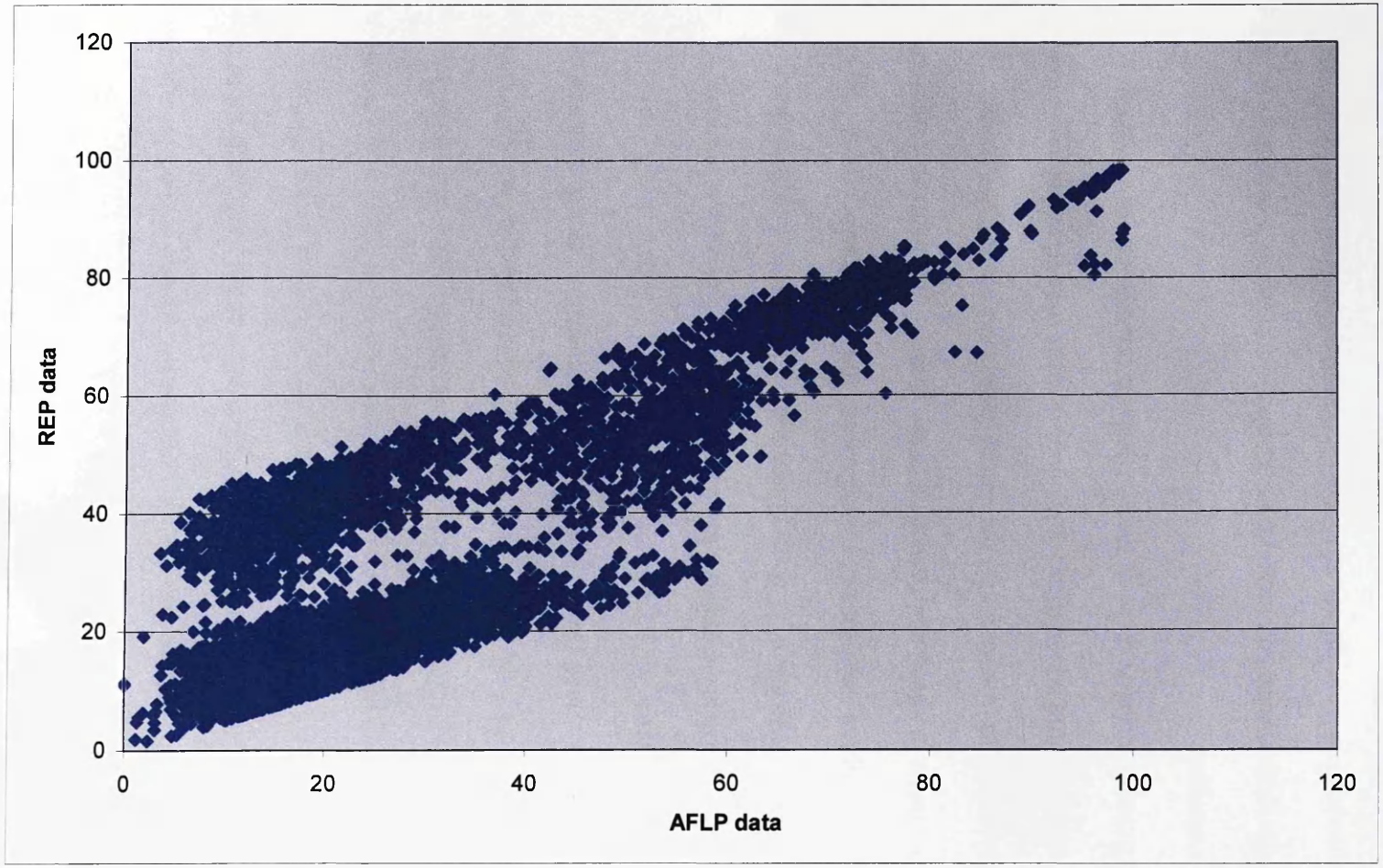


Figure 4-8: correlation between REP and AFLP similarity indices: r=0.83265

4.4: Discussion

ERIC and BOX-PCR have been successfully adapted for the analysis of *Aeromonas* isolates. Modifications to previously published protocols enable a fingerprint to be produced in two steps from a colony on an agar plate within four hours. These modifications have been possible because it has been shown that both amplifications are sufficiently robust to tolerate the presence during the PCR of cresol red and a high concentration of sucrose, so that PCR products may be loaded directly onto an agarose gel, without the need for the time consuming operation of adding loading dye to individual samples.

Furthermore the amplification appears to be sufficiently forgiving of variations in template concentration to allow PCR amplification to use untreated cell suspensions as template, omitting the need for prior purification and quantification of DNA. These improvements in speed and reduction in the costs of producing a fingerprint could have important implications for the use of these methods for diagnostic purposes in medicine and aquaculture alike since a strain identification could now be available sufficiently quickly to be of use in reaching decisions as to the most appropriate treatment.

After extensive temperature and magnesium titrations a protocol was arrived at that generated reproducible fingerprints of moderate complexity using both ERIC and BOX primers. PCR conditions chosen were far more stringent than in most published protocols at the expense of fingerprint complexity in the belief that this would confer greater reproducibility, (Johnson and O'Bryan, 2000; Johnson and Clabots, 2000).

ERIC-PCR fingerprints were generally of lower complexity than BOX-PCR in terms of apparent number of bands and range of intensities. However ERIC-PCR fingerprints were found to be more reproducible in that the relative intensity of bands within BOX-PCR fingerprints varied markedly between repeats. Although increasing the PCR annealing temperature when using either primer was found to increase reproducibility, as would be expected, this was at the expense of fingerprint complexity.

It was this dilemma that prompted the investigation into the use of the degenerate primer antagonists.

The concept has been proposed elsewhere (Atamas *et al.,* 1998) as a means of inhibiting the amplification of closely related sequences when trying to amplify a single locus. This is the first time the principle has been applied to improve specificity in a multiplex PCR.

This work was very much a speculative pilot experiment: only one type of degenerate primer antagonist was tested, and it is possible that antagonists with a different number of degenerate 3' bases – or indeed a combination of several antagonists - would produce a more profound effect.

Nevertheless even though the effect is marginal it was found to be quite consistent and this is most encouraging. Reproducing such marginal effects in photographs of agarose gels is notoriously difficult, however it is fairly clear from fig. 4.6 how it has been possible to lower the annealing temperature to allow the amplification of fragments that do not appear when higher annealing temperatures are used. The presence of low (0.25X) concentration of the primer antagonist has reduced the

amplification of non-specific products that appear at these annealing temperatures using a standard PCR recipe.

These initial findings show that this technique does have the potential to improve the fingerprint complexity and possibly the reproducibility of this type of PCR fingerprinting and this is an idea that is certainly worth pursuing further.

The reason why the results of REP-PCR cluster analysis should not be wholly consistent with the AFLP data is not clear. Tibayrenc (1996; 1999) has suggested that where the results of two different methods of molecular fingerprinting of microorganisms disagree, this may be taken as evidence for recombination. This may be the case here, however it is also likely that technical shortcomings have also played a big part. In particular the method used to record the gel images was unsatisfactory for part of this work. Recording a photographic image was not possible on all occasions and when this was not possible images were recorded with a digital camera that had low resolution. Moreover many of the recorded images were poorly in focus and therefore the quality of data that can be extracted from them is badly reduced.

4.4.1: High Resolution Electrophoresis of REP-PCR products

REP-PCR protocols have been successfully adapted for use with a fluorescent DNA sequencer for the first time. An anticipated limitation to the usefulness of this innovation was the run time that would be required to pass fragments up to 3Kb in size through an acrylamide gel, particularly as the need to maintain the gel at very little above room temperature meant that the running voltage was constrained.
In fact the 2hour run time means that this can be a rapid and cost-effective method of increasing the resolving power of REP-PCR fingerprinting. Using an acrylamide gel with an internal lane size-standard increased substantially the amount of information within each fingerprint, not only because of the greater accuracy of size-calling, but also because many of the bands within an agarose fingerprint turned out to be composed of more than one fragment when resolved through agarose. (fig 4.7)

Chapter 5:

Optimisation and use of RAPD analysis

Chapter 5: Optimisation and use of RAPD analysis

5.1: Introduction

The exquisite specificity of the polymerase chain reaction (PCR) rests on the unliklihood that a well-designed PCR primer 20-30 bases long, will find fortuitously, a perfectly complementary sequence even once in a genomic DNA template under optimal PCR conditions.

PCR-based DNA fingerprinting requires instead, that a number of DNA amplicons are produced, which must be reliably template specific. A number of techniques for DNA fingerprinting have been described that achieve this by using instead, a single non-specific primer and less stringent PCR conditions to amplify many arbitrary regions of a genome simultaneously. Amplification is conducted using low annealing temperatures, which allows for mismatches and thus permits arbitrary primer sequences to bind non-specifically as well as specifically to the DNA template. Amplified fragments are generated whenever two correctly oriented primers are able to anneal closely enough for the sequence between them to be amplified efficiently.

Several classes of mutations could prevent amplification of one fragment and/or promote the amplification of another. Point mutations in the annealing sites could prevent the primer from pairing with the target DNA at one or both sites, or could introduce a priming site where previously there was none. An inversion containing one annealing site would prevent amplification, as would an insertion that moved the annealing sites too far apart for efficient PCR amplification. Conversely, a deletion could bring two distant priming sites sufficiently close to each other for amplification to occur.

PCR reactions using template from different individuals will therefore generate a variable number of PCR products of a range of different sizes, generally between ~100 and 4000bp long (Power, 1996) some of which will be polymorphic. These fragments may be resolved by agarose or polyacrylamide gel electrophoresis (PAGE) and the resulting pattern of differently sized fragments, generated from different DNA templates compared.

Unlike PCR reactions that amplify specific, targeted regions of the genome, none of these techniques require sequence information for construction of primers. Furthermore, the single primer used amplifies multiple genomic regions that vary in their presence among individuals and species. Therefore, a single PCR reaction may detect genetic variation at a large number of loci.

There are a number of rather different approaches based on this general theme, which have been collectively described as multiple arbitrary amplicon profiling (MAAP), (Caetano-Anollés, 1993).

DNA Amplification Fingerprinting (DAF) (Caetano-Anollés, Bassam and Gresshoff, 1991) uses primers as short as 5 bases long, with very rapid PCR cycling: 10s annealing, 10s extension, to generate amplimers of a few hundred bp. which must be resolved using PAGE and visualised using silver staining.

Arbitrarily primed PCR (AP-PCR) developed by Welsh and McClelland (1990; 1991) uses a single long primer (e.g., the M13 universal sequencing primer). A series of low stringency PCR cycles initially generate the template for subsequent high stringency cycles. Generally amplimers are internally labelled radioactively by incorporation of α-[^{32}P]-dCTP, resolved using PAGE and visualised by autoradiography.

Randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) uses intermediate length (10-12 bases) primers and relatively low stringency PCR to amplify a range of fragments that may conveniently be resolved using agarose electrophoresis.

Because of the technical difficulties of optimising DAF on the one hand, and the need for radioactive labelling with AP-PCR on the other, neither technique has seen widespread use. RAPD analysis however, is technically simple to perform, requires no radioactivity or specialist equipment and can conveniently be undertaken in most laboratories. Because of this RAPD analysis has become one of the most widely used techniques for DNA fingerprinting. The method has also found widespread favour for use in genetic mapping, (Rafalsk and Tingey, 1993) and identification of qualitative trait loci (QTL's) particularly in crop species (Waugh and Powell, 1992). Over 1000 articles were published in 1999 alone, in which RAPD was the main technique used.

However, when any technology finds widespread acceptance, there is a danger that its limitations will be under-recognised and there are a number of grounds for applying RAPD analysis with care.

In particular, the underlying theory behind all MAAP techniques is that the differences in the number and sizes of amplified fragments generated with different templates is assumed to be indicative of meaningful genetic differences between the organisms under study. As will be discussed below, this is an assumption that must be made with great caution.

There are also a number of practical reservations that must be kept in mind when designing and interpreting MAAP-based experiments.

Firstly, the 'universal' nature of the primers used means that any DNA template from whatever source is likely to contain sequences that can be selected by the primers for amplification. Moreover, the consequences of any contamination will be extremely hard to identify. With great care, the danger of accidental contamination of PCR mixes by the operator may be minimised, (Kitchin *et al.*, 1990). It is much more difficult to prevent contamination by airborne DNA fragments, which can be common in laboratories where PCR products are routinely handled (Kwok and Higuchi, 1989).

Even the minute amounts of bacterial DNA that inevitably contaminate *Taq* and other thermostable polymerases, (Hughes, Beck and Skuce, 1994) will often generate amplified fragments in negative control reactions. Care must be taken to identify these amplimers and, if necessary eliminate them from analysed data (Tyler *et al.*, 1997).

Secondly, the products of MAAP are exquisitely sensitive to many different factors in the PCR reaction. The concentration of primers, (Caetano-Anollés, Bassam and Gresshoff, 1991); magnesium, (Ellsworth, Rittenhouse. and Honeycutt, 1993); and enzyme, (Penner *et al.*, 1993), can all affect results. Polymerases supplied by different manufacturers have also been reported to affect results (Schierwater and Ender, 1993), whether because of inherent differences in the enzymes or merely because of differences in enzyme activity remains unclear. Products can also vary due to the quality and amount of template DNA, (Micheli *et al.*, 1994). MAAP products are sensitive to the temperature and ramp times during the amplification process, (MacPherson *et al.*, 1993) so that the outcome of MAAP reactions have been shown to vary when performed using different thermal cyclers (Meunier and

Grimont, 1993) and even across blocks within the same cycler (He, Viljanen and Mertsola, 1994). These last two difficulties are of particular concern since it is difficult to see what measures could be taken overcome them sufficiently to make data generated in different laboratories comparable.

With careful optimisation of reaction parameters it has been possible to produce good quality, reproducible data using these techniques, (Rafalski, 1997; Vogt *et al.*, 1997). However it is clear that much published work has under-estimated the complexity of the technology and insufficient measures have been taken to allow for its limitations (Lamboy, 1994a; 1994b)

5.2: Methods and Materials

5.2.1: Optimisation of RAPD-PCR

All amplification reactions were performed in thin walled 200µl dome topped microcentrifuge tubes (Applied Biosystems), using a Perkin Elmer 9700 96-well thermal cycler.

Initial magnesium titration was performed using one primer:

5'-d[GTTTCCCTCC]-3' (primer 2 from RAPD Analysis Primer kit, Pharmacia, Uppsala, Sweden),

Reactions were performed in a final volume of 25µl containing:

1X Dynazyme reaction buffer (Flowgen, Staffs., UK), (=10 mM Tris-HCI (pH 8.8 at 25 ^{0}C); 50 mM KCl; 0.1 % Triton X-100)

100µM each dNTP (Promega, MA., USA)

O.4µM primer

0.5 Units DynazymeII (Flowgen, Staffs., UK)

Together with $MgCl_2$ concentrations varying from 1.2-2.0mM in 0.1mM increments

Master mixes without template were prepared on ice and aliquoted into reaction tubes.

For each magnesium concentration tested three reactions were prepared containing 5ng, 10ng and 25ng *Aeromonas hydrophila* type strain (NCIMB9240) DNA template.

Each batch of PCR included a negative control containing $TE_{0.1}$ buffer in place of template.

Template DNA was pipetted into the lid of the reaction tubes, which were held on ice. When all components had been assembled the template was added to the rest of the PCR mix by centrifugation for 10s at 1000g.

Reactions were placed quickly into the thermal cycler previously set to hold at 4°C with the lid pre-heated.

The PCR profile was as follows:

96°C for 2 minutes (initial denaturation)

94°C for 30seconds (denaturation)

42°C for 30seconds (annealing)

72°C for 1minute (elongation)

Steps (2) to (4) were repeated for 40 cycles:

This experiment was then repeated using 5ng, 10ng and 25ng *Aeromonas schubertii* type strain NCIMB13161 DNA.

5.2.2: Electrophoresis and visualisation of fingerprints

Following PCR, 6µl 5X loading buffer (0.2% bromophenol blue, 20% sucrose, 50mM EDTA (Sigma)) was added to each reaction tube and 10µl was loaded onto a horizontal agarose gel (1.4% in 0.5X TBE buffer), containing $0.5\mu g.ml.^{-1}$ ethidium bromide. Electrophoresis was carried out using an Anachem H2 submerged gel electrophoresis tank at $4V.cm^{-1}$ for approximately 2 hours.

The gel was destained for 5 minutes in running water and examined under UV on a transilluminator. An image was recorded using a CCD camera and stored as tif file.

5.2.3: Magnesium titration of subsequent primers

All primers optimised after the first were tested as above, but using 5ng template and $MgCl_2$ concentrations of 1.5-1.9mM only.

All six 10-mer RAPD primers supplied as the Pharmacia RAPD primer kit were tested:

RAPD Analysis Primer 1: 5'-d[GGTGCGGGAA]-3'

RAPD Analysis Primer 2: 5'-d[GTTTCCCTCC]-3'

RAPD Analysis Primer 3: 5'-d[GTAGACCCGT]-3'

RAPD Analysis Primer 4: 5'-d[AAGAGCCCGT]-3'

RAPD Analysis Primer 5: 5'-d[AACGCGCAAC]-3'

RAPD Analysis Primer 6: 5'-d[CCCGTCAGCA]-3'

Additionally, three 10-mer primers used by Oakey, Ellis and Gibson (1996):

OK-6: 5'-d[TGCTCTGCCC]-3'

OK-7: 5'-d[GGTGACGCAG]-3'

OK-10: 5'-d[CTGCTGGGAC]-3'

and two 12-mers used by Miyata *et al.* (1995):

AO5: 5'-d[AGCAGCGCCTCA]-3'

AO7: 5'-d[TGCCTCGCACCA]-3'

were supplied by Oswell DNA, Southampton, UK.

5.2.4: Polymerase titration

Using the PCR parameters described in section 1.1.1, but using only 5ng template per reaction PCR's were prepared including 0.25, 0.5, and 1unit of DynazymeII per reaction.

5.2.5: Final reaction conditions

PCR's were prepared to a total volume of 10µl in 96 well low profile PCR plates (Abgene). All primers were used at a concentration of 0.4pM. All PCR mixes contained 1.7mM $MgCl_2$ 100µM (each) dNTP's ,).5U DynazymeII and 10ng template per reaction.

PCR's were performed on an Applied Biosystems 9700 thermal cycler.

Thermal cycling parameters were as follows

96°C for 2 minutes (initial denaturation)

94°C for 30seconds (denaturation)

47°C for 30seconds (annealing)

72°C for 1minute (elongation)

Steps (2) to (4) were repeated for 40 cycles:

5.3: Results

5.3.1: Magnesium and Template titration

The effect of varying magnesium concentration and three different amounts of *Aeromonas hydrophila* NCIMB 9240 template are shown in figs 5.1, 5.2 & 5.3.

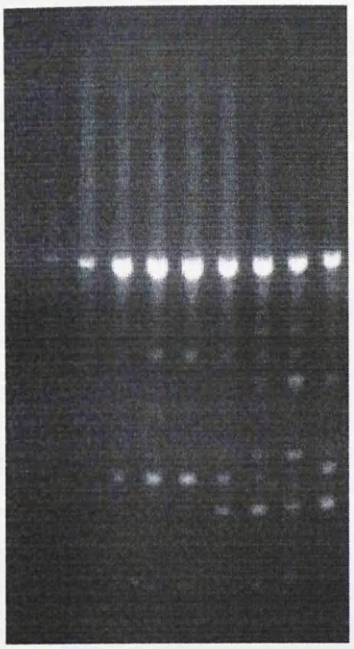


Figure 5-1: 5ng template template

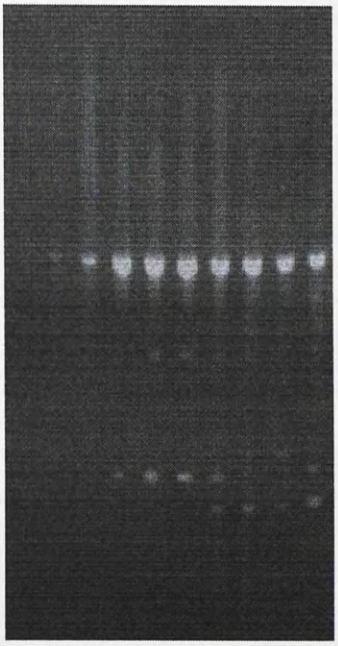


Figure 5-2: 10ng template

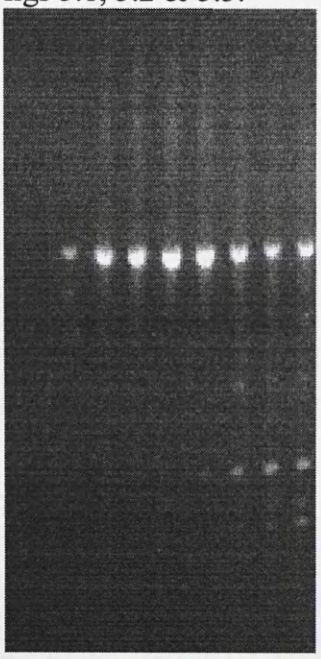


Figure 5-3: 25ng

In each lane $MgCl_2$ concentrations were (from left to right): 1.2; 1.3; 1.4; 1.5; 1.6; 1.7; 1.8; 1.9; and 2.0mM.

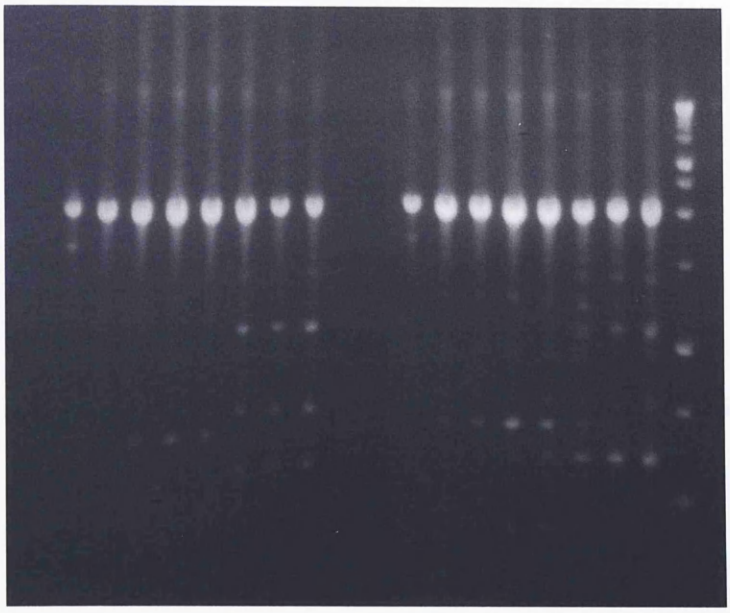
The effect of varying magnesium concentration and three different amounts of *Aeromonas schubertii* NCIMB13161template are shown in fig 5.4 below

template concentrations are (left to right): 5; 10; and 25ng

magnesium concentrations are (left to right): 1.6;1.7;1.8; and 1.9mM

Figure 5-4: *A. schubertii* template and Mg titration

5.3.2: Enzyme titration



Lanes 1-8: PCR using 0.25U enzyme per reaction with 1.3-2.0mM magnesium in 0.1mM increments.
Lanes 12--19: PCR using 1.0U enzyme per reaction with 1.3-2.0mM magnesium in 0.1mM increments.
Lane 20: 1kb marker

Figure 5-5:Effect of using 0.25U and 1U of DynazymeII per reaction

Fig. 5.6 below shows a typical RAPD fingerprint produced using primer2 using optimised conditions.

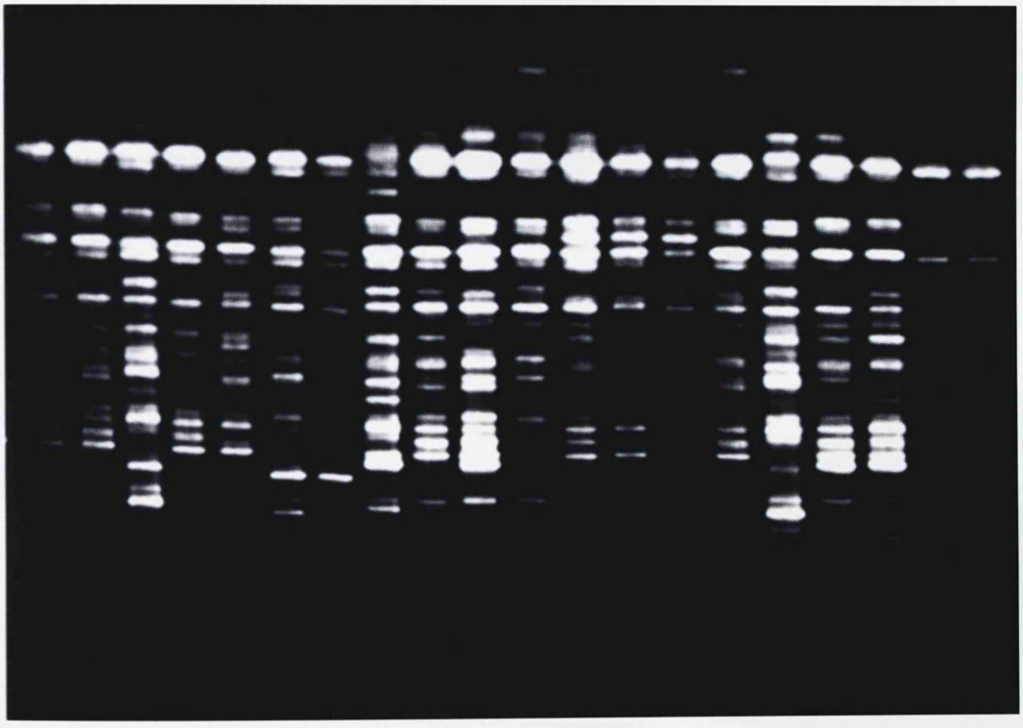


Figure 5-6: optimised RAPD fingerprint

5.4: Discussion

Cluster analysis of the RAPD fingerprints generated here initially yielded little useful information. Fingerprints apparently did not cluster into coherent groups and the arrangement of the first dendrogram appeared to have no correlation at all with the AFLP data.

This is consistent with the findings of previous work that has used RAPD to type *Aeromonas* spp.(Inglis *et al.,* 1995; Miyata *et al.,* 1995; Oakey *et al.,* 1996a). However strains that had been shown by other means to be closely related did generate fingerprints that were sufficiently similar to be recognised as such even by eye.

This probably indicates that the window of discrimination of which RAPD is capable is not sufficiently wide to span the great genetic variation within this genus. In fact when fingerprints from a single hybridisation group were compared in isolation, the revealed relationship between strains did bear a much better resemblance to the relationships within that group as suggested by the AFLP data: table 5.1 and fig 5.5.

RAPD Data

AFLP Data

	01	02	03	04	05	06	07	08	09	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33
01	100	71.5	77	82.3	84.5	83.3	83.7	82	92.6	83.1	84	83.7	80.5	90.2	90.4	81.1	89.5	76.1	89.2	77.8	86.6	87.9	83.4	83.4	86.6	75	80.9	82	78	84.2	74.3	86.7	84.6
02	46.4	100	46.5	56.4	63.4	65	56.4	59.6	66.7	64.6	60.5	57.1	54.8	70.7	69.4	57.5	64.1	56.7	65.9	53.7	62.6	63.5	63.9	59.5	59.3	44.1	59.3	55.7	51.7	62.7	62.9	62	62.5
03	56.1	22.7	100	76.9	76.8	76	77.2	81.4	80.7	75.3	80.7	78.4	76.2	79.2	80.4	74.6	76.3	65.2	77.5	69.4	72.1	79	76.8	74.6	72.2	97	67.2	71.6	67.2	69.9	62.5	77.1	73.8
04	62	55.4	44.6	100	82.6	86.1	88.8	84.7	88	83.2	87.8	98.8	90	81.8	83.4	93.5	82.8	69.2	80.7	73.2	81.3	86.3	82.8	90.7	84	76.6	76.3	80	73.1	75.2	75.2	85.1	78.9
05	72.5	60.3	45.3	73.1	100	87	85.6	83.2	88.1	83	86.8	83.7	82.7	85.2	86.1	82.2	82.9	72.2	82.6	75.5	76.5	86	83.1	80.6	81.8	74.4	76	75.4	73.8	77.6	71.5	81.9	80.9
06	70.9	44.4	51.1	62.5	74.4	100	87.6	82.7	89.7	87.3	87.5	86.6	88.3	82.7	84	87.1	83.9	75.7	86.3	77	78	85.7	86.4	80.4	83	75.3	74.1	75.4	77.1	76.7	72.7	84	80.5
07	76.7	44.1	63.2	71.4	72.4	72.1	100	88.5	89.6	86	89.1	90.1	88.4	84.1	84.7	88.2	85.1	72	83.2	81.5	83.1	86.3	85.2	84.8	87.6	76.8	80.7	79.7	75.7	76.8	73.9	84.1	79.8
08	73.2	47.4	59.7	77.1	72.8	71.6	84.9	100	86.3	82.9	86.6	86.9	82.3	85.1	85.5	83.6	82.1	69	80	76.8	80.9	82	81.9	81.1	78.6	79.8	71.4	75.9	70.6	71.5	67.4	82	75.9
09	77.1	49.8	54.1	66.5	74.8	68.6	72.5	73	100	87.3	88.9	89.4	86.2	89	89.6	86.7	91.2	78.1	90.4	80.5	86.2	91.2	86.2	85.4	86.8	78.6	81.1	81.6	79.5	81.6	74.2	87.9	86.5
10	69.1	52.7	56.4	75.2	71.4	71.4	84	86.8	66.1	100	88	83.8	80.6	81	81.5	82.5	83.5	70.1	81.2	74.8	79.8	81.9	82.2	79.3	80.6	73.7	73.8	76.3	72.2	74.1	69.9	82.1	78
11	70.9	52.4	51.3	74.5	79.2	74.8	81.3	86.8	72.2	87	100	89	84.8	87.3	85	86.9	82.7	72.7	83	76.3	78.7	86.4	83.8	84.4	81.5	78.6	75	86.4	74.8	75.8	70.3	82.9	81.1
12	63.6	49.1	48.5	97.8	72.7	62.9	72.3	78.2	67.7	75.1	74.5	100	90.1	83.5	84.7	93.8	84.1	70.6	82.5	75.5	83	87.9	84.1	91.9	84.7	78.8	77.5	80.7	74.3	76	75.3	85	80.1
13	59.9	51.3	39.4	62.2	61.6	58	73.4	75.8	51.4	80.9	78.4	60.5	100	79.1	82	88.1	79.4	69.6	81.2	73	76.9	84.9	83.1	82.9	82.6	77	74.4	76.1	73.5	76.6	74.3	83.5	78.2
14	75.9	60.7	57.5	73.3	74.2	66.5	81.2	83	76.3	80.2	81.7	72.4	71.8	100	91.5	82.1	87	75.8	85.9	78.1	82.4	87.1	85.2	83.5	82.4	76.6	77.2	81.9	76	81.6	72.2	84	81.2
15	65.8	51.7	44.9	66.3	63.6	61.4	70.1	77.4	62.3	72.9	73.5	65.3	66.8	75.3	100	83.6	86.7	75	87.7	77.7	82.3	88.5	84.6	83.8	84.6	78	78.2	79.6	74.6	82.8	74.4	84	82.3
16	63.4	43.4	48.8	77.3	68.5	61.7	71.7	74.5	65.6	69.7	73.7	77.2	60.1	67.2	62.1	100	81.7	70.6	81.9	74.3	79.9	86.6	82.8	94.4	84.2	76.9	76.4	78.7	71.9	75.9	74.7	83.9	79.4
17	67.6	51.2	47.5	66.1	73.6	63.9	71	72.5	63.3	69.8	73	68.4	60.7	73.1	65	65.6	100	80.6	89.2	79.4	89.1	88.8	89.1	83.2	87.5	75.3	84.4	80.9	77.6	85.5	74.8	85.5	85.4
18	70.9	51.6	42.6	57.3	75.4	66.3	68.8	67.2	65.1	63.2	69.6	58.2	58.6	68.9	61.1	62.3	78.2	100	79.5	89.9	74.3	77	78.2	71	74.2	64.6	70.9	71.9	71	73.6	65.6	74.1	74.9
19	75.5	53.2	49.4	62	76	79.5	70.7	71.8	73.9	68	76.2	62.6	61.1	72.8	62.2	63.6	71.9	77.1	100	79.7	86	89.2	85.5	83.9	85.1	77	82.1	81.7	79.4	85.4	75.8	85.6	86
20	77.1	44.7	58.7	66.5	71.8	69.2	86.3	77.4	68.6	73.9	75.6	67.6	65.3	77	67.6	70	72.5	71.7	72.8	100	79.1	79.4	77.4	75.9	75.3	68.7	71.9	74.7	72.2	71	65.4	76.1	74.4
21	65.5	52.6	42	65.5	74.3	65.1	64.1	66.2	72.7	65.1	69.4	65.6	46.1	69.6	59.5	61.5	68.7	70	72.6	66.9	100	85.7	84.9	83.3	85.5	73.4	83.9	83.4	77.5	83	79.7	87	84.3
22	75.6	46.9	52.1	67.6	78.7	73.7	73.2	73.8	72.3	70.8	72.3	69.3	58.5	73.1	65.1	65.7	75.6	76.2	76.7	75.3	79	100	85.5	87.3	87.6	79.8	83.9	83.9	78.2	83.8	79.9	86.6	85.7
23	64.7	32.4	51.2	56.7	64.9	61.6	72.5	63.4	61.3	65.7	63.9	59.8	50.2	62.8	63.3	61.3	68.3	69.9	61.8	69.4	60.8	67.4	100	82.3	83.3	77.5	77.8	79.4	73.1	80.6	74.7	83.4	82.1
24	64.9	41.1	46.5	72.7	66.7	59.2	68.3	70.9	63.2	65.7	69.7	73.7	54.1	65.8	59.4	93.8	66.5	62.6	64.6	69.2	61.9	64.3	61.1	100	84.5	76.1	80.8	82.8	72.7	79.7	78.6	83.5	81.9
25	76.1	54.1	53.3	60.7	72.8	68.7	75.9	71.4	68.2	73.4	75.2	61.3	61.2	75.5	66.2	63.7	72.3	75	69.4	73.1	65	73.5	67	63.6	100	73.9	94.8	83.9	77.2	85.9	80.7	85.3	86.1
26	54.9	17.7	98.4	42.8	43.6	50	62.5	58	53.7	55.1	49.5	47.4	37	55	42.6	48.5	46.1	41.7	47.3	57.4	41.3	51.7	52.2	46.3	52.4	100	69.5	73.7	67.9	72.1	67	77.3	75.6
27	73.5	53.9	49.2	56.9	72.2	67.4	71	65.9	62.9	67.9	71.2	57.3	57	70.1	63.3	60.7	65.7	71.4	67.2	72.5	62.6	69.1	59.2	63.6	89.4	47.5	100	82.8	73.3	85	80.6	80.1	84.9
28	70	47	47.1	63.9	73.8	69	74.2	77	66.7	75.3	89.7	64.8	65.6	76.3	69	67.8	70.5	70.8	75.2	77.4	67.7	70.5	62.4	68.6	75.9	45.8	76.9	100	76.2	83.5	80.1	81.2	84.9
29	71.9	34.8	54.3	62.7	70.3	68	71	70.5	73.2	63.1	68.3	66.1	46.9	64	57.7	66.9	62.2	64.9	68	69.6	67.6	68.7	62.3	68.2	64.4	54.1	66.4	70.6	100	74.3	70.5	80.5	77.1
30	75.4	47.1	52.4	60	73.6	69.3	66.9	66.8	72	63	67.3	61.7	53.7	72.2	61.2	61	70	71.4	80.2	70.6	70.9	73	61.4	63.5	68	50.1	71.1	69.8	73.2	100	83.8	81.3	87.3
31	72.2	43.2	56.2	61.8	72.9	63.3	73.7	72.7	71.3	70.4	75.9	64.3	58	73.4	68.5	66.1	71.7	66	68.5	72.3	64.7	68.5	70.8	66.6	71.8	55.8	73.1	77.6	70.5	72.4	100	77.7	81.7
32	70.6	49.1	55	71.6	74.6	69.1	75.2	74	70.4	69.1	72.3	73.4	50.6	74	61.3	72.7	71.1	66.5	72.6	74.5	73.7	75.6	62.7	71.9	70.5	54.3	68.3	72.8	75.9	70.9	71.7	100	84.5
33	70.1	47.1	58	58.5	71.8	67.5	71.2	71.5	61.9	67.5	72.5	60.3	55.9	73.1	64.18	63.0	69.7	70.8	70.7	73.8	64.9	72.1	64.5	64.6	72.1	56.7	76.1	75	68.6	76.8	77.2	70.9	100

Table 5-1: Relationship between RAPD (top diagonal) and AFLP (left diagonal) similarity indices (HG3 only)

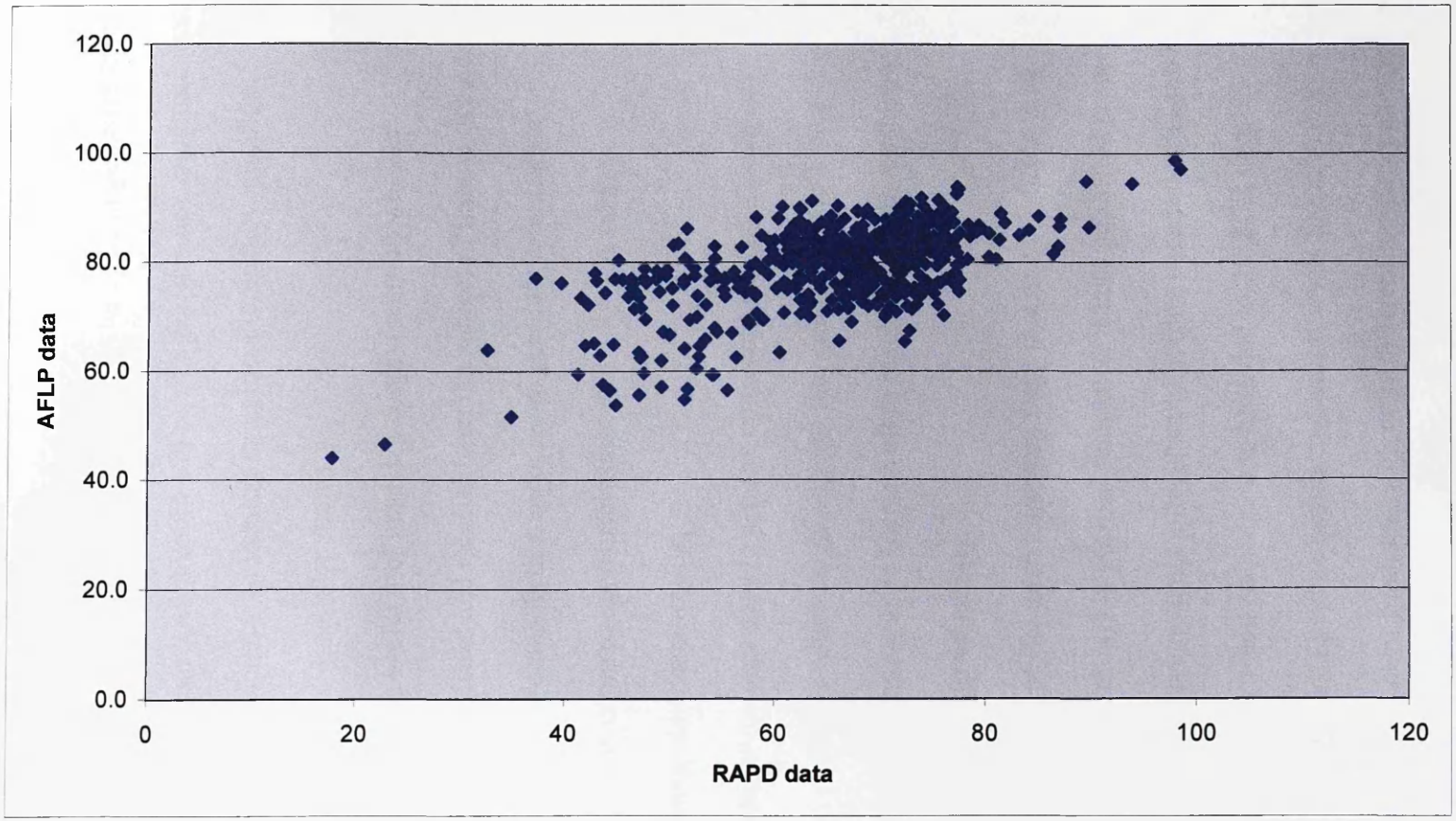


Figure 5-7: correlation between AFLP and RAPD similarity indices within Hybridisation Group 3

Though RAPD analysis does not appear to have been able to shed much light on the taxonomic structure of the genus *Aeromonas* the close similarity of fingerprints from related strains does indicate that RAPD could still be of value in some circumstances: for example when the task at hand is to identify an epidemiologically significant strain against a background of dissimilar strains.

Whether RAPD can ever be a useful tool in such a context rests on whether reproducible data can be produced and this has been the subject of intense debate. Having arrived at optimised protocols that appeared to generate informative fingerprints of acceptable complexity, considerable difficulty was indeed encountered generating consistent results.

The precautions found to be necessary to achieve consistent results often do not feature in published protocols which might suggest that much of the criticism that has been directed at the technique arise because often insufficient care is taken to eliminate sources of run to run variability.

The first and apparently one of the most important steps that must be taken is to ensure accurate quantitation of highly purified templates. Many published protocols describe crude DNA extraction methods, often simply boiling bacterial cells and then specify the volume of unquantified suspension to be added to the PCR mix.

During the course of this work template quality and quantity was found to have a profound effect on the fingerprint generated,with quite different banding patterns produced if template concentration was increased or decreased 2-fold.

A second extremely important factor is the thermal cycler used: it seems that many older generation thermal cyclers are not suitable for RAPD PCR since ramp rates and times are not sufficiently consistent between runs. This work began using an air-

cooled thermal cycler and the importance of the thermal cycling profile only became fully apparent when it was noticed that, during a cold period, fingerprints produced at the weekend, when the laboratory heating was off were quite different from those produced during the week.

Whilst a steady stream of work based on RAPD analysis continues to be published, it is likely that, at least for the purposes of bacterial typing, the technique will be increasingly superseded by more recently developed techniques. However it is important that previous work using RAPD analysis should not be dismissed for the wrong reasons.

It may be that previous work using air-cooled thermal cyclers, where the template was not adequately quantified and in particular where gels were scored by eye should be re-examined. However the conclusion here and elsewhere , (Rafalski, 1997; Vogt *et al.*, 1997) is that consistent and reliable RAPD fingerprints can be produced, providing sufficient precautions are taken and provided the technique is applied appropriately.

It is true, however that rarely has a technique seen such widespread use with such poor understanding of the underlying molecular mechanisms. Though there have been a small number of theoretical considerations of the processes involved in generating RAPD fragments (Caetano-Anollés , 1993), there has been very little investigation into the basis of multiple mismatch annealing on which RAPD depends. In one of the few studies, Venugopal *et al.* (1993), concluded that during amplification of a RAPD fragment, mismatch annealing occurred only at one primer binding site and that perfect annealing of at least one of the primers was required.

However their findings were based on work with mouse DNA template and it is possible that this rule does not hold good when working with smaller bacterial genomes: theoretically a RAPD primer should find a perfect annealing site only once in approximately once per 1.05 (4^{10}) Mbp.

A particular criticism that has been made of RAPD as a tool has been that not only are the sizes of fragments inconsistent but the suggestion has been made that the actual loci amplified are also inconsistent. There is some evidence for this: Oakey *et al.*, (1998) for example, excised a number of RAPD bands from gels, cloned then sequenced the amplified fragments. Their finding that more than non-homologous fragments could be recovered from a single band has raised the suspicion that this is a weakness of the technique. However this is to fundamentally misunderstand the basis on which RAPD generates a fingerprint: the principle is that some loci are preferentially amplified over others, not that some loci are amplified and all others not at all. It is likely that amplified fragments would be recovered throughout the length of a lane, even between the apparent bands. To recover more than one sequence within a single band does not invalidate the technique in any way.

Chapter 6: Phenotypic Analysis

Chapter 6: Phenotypic Analysis

6.1: Introduction

Though molecular methods play an increasing role in veterinary and clinical diagnostic laboratories it remains true that the majority of bacteriology laboratories continue to rely on biochemical tests for routine identification work.

In the past confusion over the taxonomic structure of the genus *Aeromonas* has hampered attempts to develop a comprehensive battery of biochemical tests capable of discriminating between the different *Aeromonas* groups. This was confounded by the fact that, as the diversity of the genus became apparent, different research groups were using different techniques to explore the taxonomic relationships between isolates and the results of DNA:DNA hybridisation work on the one hand and phenotypic studies on the other did not agree, (Carnahan and Altwegg, 1996).

During the early 1990's there existed two overlapping but quite systems of nomenclature with both 'phenospecies' and 'genospecies' described and the two often being confused.

Moreover most of the studies which did seek to correlate hybridisation grouping with biochemical analysis were focused primarily on clinical isolates and included relatively few veterinary and environmental isolates.

A particular difficulty hampering development of a phenotypic taxonomy in *Aeromonas* is that several tests are extremely sensistive to external conditions particularly incubation temperature. Slight variations in composition of the test medium can also affect the outcome of tests. Commercial tests such as API20 often

produce a different result to equivalent tests performed using traditional methods (Hanninen, 1994). This problem means that different laboratories are almost certainly producing conflicting data about the phenotypic properties of the different *Aeromonas* groups.

A series of numerical taxonomy studies have sought to reconcile phenotypic data with hybridisation group (Kämpfer and Altwegg, 1992; Carnahan and Joseph, 1993; Esteve, 1995; Noterdaeme *et al.*, 1996; Kaznowski, 1998; Valera and Esteve, 2002). While this work is improving our understanding of the taxonomy of the genus, the enormous range of biochemical tests used are impractical for routine work.

A more systematic approach to identification using an extensive but manageable range of tests and probability matrices has been made by Oakey *et al.*, (1996b) The probability matrix used included 40 biochemical tests and was constructed using the results of 16 previously published biochemical studies, which together included 1450 isolates identified to 13 hybridisation groups.

An obvious weakness of this approach is that the data from the different studies cannot be directly comparable, due to differences in the methodologies used. Also, the assumption was made that there was no duplication of strains between publications, which is almost certainly untrue, and duplication of strains must introduce a bias into the probability matrix. Moreover, not all the publications included data for all of the 40 tests.

Despite these limitations it proved possible to construct a probability matrix that was able to identify the type strains of hybridisation groups then known, with only ATCC 7966 HG1 being identified with a probability of less than 90%. The majority of strains in a collection of unknown strains were also identified to a high probability.

The appeal of this approach, despite the potential shortcomings of the initial probability matrix, is that once a set of standardised protocols is decided on, it should then be possible to improve the resolving power of the matrix, by an iterative process of progressively updating it, based on the results of further identifications of strains that have been assigned to hybridisation group by other means.

This work was undertaken with the aim of assessing the effectiveness of the battery of tests proposed, in identifying the diverse collection of *Aeromonas* strains assembled here, and to determine whether using the assignment of strains to hybridisation group using the AFLP data, is able to improve the discriminatory power of the probability matrix.

6.1.1: Probabalistic Identification of Bacteria (PIB) and Bayes Theorem

Probabalistic Identification of Bacteria (PIB) (Bryant, 1995) is a DOS-based programme written by Dr Trevor Bryant, Department of Medical Statistics, University of Southampton.

The programme is designed to identify unknown bacterial isolates using identification matrices supplied with the programme or created by the user.

The identification routine used in PIB is based on Willcox's implementation of Bayes theorem for use with bacteria (Willcox *et al.*, 1973; 1980).

$$P(t_i \mid R) = \frac{P(R \mid t_i)}{\Sigma P(R \mid t_i)}$$

where $P(t_i \mid R)$ is the probability that an unknown isolate, giving a pattern of test results R, is a member of taxon (group of bacteria) t_i. $P(R \mid t_i)$ is the probability that the unknown has a pattern R given that it is a member of taxon t_i.

To simplify the process for bacterial identification, the probability of isolation of all taxa is assumed to be equal and so the prior probabilities in the above equation can be dispensed with.

The equation can now be re-expressed as:

$$L_i^* = \frac{L_i}{\Sigma L_i}$$

The probabilities are now referred to as 'identification scores'. The identification scores for each taxon are 'normalised' values and L_i^* for all taxa adds up to one. Identification of an unknown isolate is achieved when L_i^* for one taxon exceeds a specified threshold value.

In the example below an unknown isolate is tested against a probability matrix consisting of three taxa for which there is data for four tests. The results for the unknown isolate in the first three tests are positive, negative and positive respectively.

		Tests			
		1	2	3	4
Taxa	a	0.01	0.30	0.99	0.90
	b	0.90	0.01	0.99	0.01
	c	0.99	0.10	0.85	0.99
Results of unknown		+	-	+	Missing

The likelihoods that the taxa a, b and c will give the pattern of results observed for the unknown is calculated by multiplying the probability of obtaining a positive result for test 1 by the probability of obtaining a negative result for test 2 by the probability of obtaining a positive result for test 3 for each taxon in turn. Note that original identification matrix only gives the probabilities for positive results and in order to use the probability for a negative result we must subtract the matrix entries for test 2 from 1. The likelihoods that the unknown is either taxon a, b, or c are calculated as follows:

				Likelihoods
	a	0.01 * (1-0.30) * 0.99	=	0.00693
Taxa	b	0.90 * (1-0.01) * 0.99	=	0.88209
	c	0.99 * (1-0.10) * 0.85	=	0.83385
			Sum	1.72287

Normalising these values gives:

				Identification Score
	a	0.00693 / 1.72287	=	0.004022
Taxa	b	0.88209 / 1.72287	=	0.511989
	c	0.83385 / 1.72287	=	0.483989
			Sum	1.000000

In this small example the unknown is not identified because no single taxon reaches a threshold value. A value of 0.999 was used by Lapage *et al.* (1973) but with other groups of bacteria lower values such as 0.95 are sometimes used. Taxa b and c are still both candidates for the identity of the unknown.

The probabilities used in a bacterial identification matrix range from 0.01 to 0.99. Entries of 0.0 and 1.0 are not employed since an isolate with an atypical or erroneous result would generate an identification score of zero for a strain similar to it. By allowing a 1% error for tests that are always positive or always negative an unknown with atypical results can still be identified.

6.1.2: BIOLOG identification

The biolog GN microplate system is a standardised method for the identification of Gram negative bacteria. The system has been used for the identification of *Vibrionaceae* (Noble and Gow, 1997) and bacterial pathogens associated with scallop *Pecten maximus* larval culture, (Nicolas *et al.,* 1996), but generally has seen little use for the identification of potential fish pathogens.

The system offers a standardised rapid method for simultaneously determining the ability of an isolate to utilise 95 different carbon sources.

Each Biolog plate consists of a standard 96-well microplate, each containing a redox dye, tetrazolium violet and one of 95 different carbon sources, the last, control well lacks any carbon source. Reagents are contained in a dried but soluble film so that addition of a cell suspension activates the identification process. Where the carbon source in a particular well can be used by the organism NADH is generated and electrons are donated to the electron transport chain, the redox dye traps a portion of this flow and in the process is irreversibly reduced to an insoluble purple formazan. After incubation the microtitre plate has a characteristic pattern of clear and purple wells. The pattern can be read automatically using an ELISA reader and the results compared with a computerised database. The Biolog gram negative database contains approximately 600 species and this is expected to expand (Bochner, 1989a; Bochner, 1989b).

Whilst the system is superficially similar to standard sugar fermentation tests, there are a number of theoretical advantages. Firstly use of a substrate will be detected even where there is no pH change and the redox colour change should happen far sooner than a pH. Moreover the range of substrates tested is far more

comprehensive: initially 500 carbon sources were surveyed for their usefulness in the battery of tests (B.Bochner, pers. com.) The 95 selected include 5 polymers, 28 carbohydrates, 24 carboxylic acids, 20 amino acids and a handful of amines, methyl esters, alcohols, aromatic and phosphorylated substrates. With 95 tests 2^{95} , about $4X10^{28}$ metabolic profiles are theoretically possible.

Interpretation of the results of a Biolog test requires the use of the Microlog software which compares the test pattern with those in its database and finds the closest match and calculates a 'similarity coefficient'. This is the score used in evaluating the identification. The closer it is to 1.00 the more confident the identification. It will be high if the entered data is very similar to the best choice and dissimilar to all other choices. It will be low if the entered data is either not similar to the best choice or is equally similar to more than one choice. The software will offer an identification if a similarity value of greater than 0.5 is calculated.

The aim of the present study was to evaluate the Biolog system for the identification of motile aeromonads and to compare the identifications with the molecular techniques.

6.2: Methods and Materials

Prior to all tests, the colony under test was transferred using a sterile loop to a bijoux bottle containing 2 ml autoclaved 0.85% saline and vortexed.

6.2.1: Motility, Indole production and Hydrogen Sulphide production

Motility, Indole production and Hydrogen Sulphide production were determined using SIM medium (Blazevic, 1968). (Appendix 1)

Autoclaved medium was dispensed into sterile 20ml universal bottles and when cool, inoculated once with a pure culture, by inserting a straight wire to about one third of the depth of the medium.

The inoculated medium was incubated at 30°C for 24 hours and examined for motility, hydrogen sulphide production and finally indole production from tryptophan.

Blackening of the line of inoculation was taken as a positive indication of hydrogen sulphide production.

Non-motile organisms grow only along the line of inoculation, whereas motile species show either a diffuse even growth spreading from the inoculum, turbidity of the whole medium, or more rarely, localised outgrowths.

To Test for Indole Production, 0.2ml of Kovac's Reagent (Harrigan and McCance,1966) (Appendix 1) was added to the tube and allow to stand for 10 minutes. A dark red colour in the reagent was interpreted as a positive indole test. No change in the original colour of the reagent was recorded as a negative test.

6.2.2: ONPG

The ONPG test (Lowe1962) was performed by placing a 4mm paper disc impregnated with ortho-nitrophenyl-β -D-galacto-pyranose (Oxoid, Basingstoke, UK) into a sterile bijoux bottle containing 2ml sterile 0.85% saline. This was inoculated with 200µl of the suspension under. The tube was incubated at 30°C and examined for a colour change after 6hours. A bright yellow colour was considered a positive result.

6.2.3: Decarboxylase reactions

Lysine decarboxylase, ornithine decarboxylase and arginine dihydrolase reactions were determined using the method of Taylor (1961) with the modification that the media were overlayed with paraffin.

Each amino acid was added to decarboxylase broth (Appendix1) to a concentration of 0.03M and the pH adjusted to 6.1+/-0.1 prior to autoclaving.

A control decarboxylase broth containing no amino acid was also prepared. The broths were dispensed into bijoux bottles and overlaid with paraffin.

100µl bacterial suspension was added to each and the tests incubated at 30°C for 24hrs. A colour change to yellow then back to purple was recorded as a positive test providing the control tube had turned yellow. Negative tests, where the control had turned yellow were re-examined after 72hrs.

6.2.4: Aesculin hydrolysis

Aesculin hydrolysis was determined using aesculin broth (Chan and Porchen, 1971) (Appendix1).

10µl bacterial suspension was added to 3ml aesculin broth in a bijoux bottle and a positive test was recorded if a colour change to black was observed after 48hrs at 30°C.

6.2.5: Gas from glucose

Gas production from glucose was determined by inoculating peptone water (Appendix1) containing 1% glucose and an inverted Durham tube.

Tests were incubated at 30°C for a maximum of 5 days and examined daily for production of acid and gas.

6.2.6: Fermentation of carbohydrates

Carbohydrate fermentation was determined by adding filter sterilised carbohydrate to peptone water (Cruickshank, 1968) (Appendix1) to a final concentration of 1%, before dispensing into bijoux bottles.

A 50µl inoculate of bacterial suspension was added and tests incubated at 30°C for up to4days, a colour change to yellow was recorded as a positive test.

This method was used to determine fermentation of arabinose, mannitol, sucrose, mannose, sorbitol, salicin, rhamnose, glycerol, cellobiose and lactose.

6.2.7: Citrate utilisation

Utilisation of citrate as a sole carbon source was determined according to the method recommended by the American Public Health Association (1981).

Simmons citrate agar (Appendix1) slopes were prepared in bijoux bottles. A loopful of bacterial suspension was streaked onto the surface and the butt of the slope was stabbed also.

Slopes were incubated at 30°C for up to one week, a colour change to bright blue was recorded as a positive test.

6.2.8: Pyrazinamidase activity

Pyrazinamidase activity was determined using the method described by Carnahan *et al.* (1990).

Slopes of pyrazine carboxamide agar (Appendix1) were inoculated with bacterial suspension and incubated at 36°C for 48h. The slopes were flooded with freshly prepared 1% w/v aqueous ferrous ammonium sulphate and development of a reddish-brown colour after 15 minutes, indicative of the presence of pyrazoic acid was recorded as a positive test.

6.2.9: Corn oil lipase

Corn oil lipase activity was determined according to the method of Oakey *et al.* (1996).

Bacterial suspensions were streaked onto plates of corn oil calcium chloride agar (Appendix 1), incubated at 25°C for 48h and examined for evidence of lipolysis around the colonies.

6.2.10: β-haemolysis

β-haemolysis was determined by streaking bacterial suspension onto 5% v/v sheeps blood Tryptone soya agar plate and incubating at 30°C for 24h.
Halos of haemolysis around colonies was recorded as a positive result.

6.2.11: Resistance to 0/129 and antibiotics

Resistance to Ampicillin, cephalothin, and 0/129 (2,4-diamino-6,7-diisopropylpteridine) was determined by spreading 200µl bacterial suspension onto the surface of a Tryptone soya agar plate (Oxoid) and placing antibiotic discs on the surface: 0/129 10µg, 0/129 150µg, ampicillin 10µg, ampicillin 25µg, and cephalothin 30µg.

6.2.12: Biolog

Microplates were prepared according to the manufacturer's instructions. Before inoculation, sterile 0.85% saline and microplates were prewarmed to 30°C to avoid temperature stress. Cells from an overnight Tryptone soya agar plate were suspended in saline and the suspension adjusted to a density of approximately $3X10^8$ cells/ml using the Biolog turbidimeter, callibrated using the standards supplied.

The cell suspension (150µl per well) was immediately dispensed into the microplate using a multi-channel pipette.

Plates were covered and incubated for 24h at 30°C and the colour density in each well compared using a microplate reader. Recorded values were normalised according to the density in the control well.

The data was analysed using the Micrologl software package.

6.2.13: Modification of Probability Matrix

The probability matrix used by Oakey *et al.,* (1996) was revised using the data produced during the following studies: Abbott *et al.* (1992), Ali *et al.* (1996), Borrell *et al.,* (1998); Carnahan *et al.* (1991b), Carnahan and Joseph, (1993), Esteve *et al.* (1995a), Huys *et al.* (1997b) Janda *et al.* (1996) Kämpfer and Altwegg, (1992); Kaznowski, (1998), and Martinez-Murcia *et al.* (1992).

Several tests with very poor species discrimination: fermentation of raffinose; resistance to O/129 at 10 µg, DNase, and gelatin hydrolysis were omitted from this study.

Data from the literature was used to add matrix probabilities for *Aeromonas trota,* HG13 to the list of species. Further, the matrix probilities for HG2, HG3 and HG11 were alterered to incorporate data published since the original matrix (Oakey *et al.,* 1996). The modified probability matrix is shown in table 6.1

	Hybridisation Group												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Motility	95	95	50	50	95	96	95	98	95	95	99	95	95
Indole	96	96	88	93	93	96	93	90	93	93	1	93	93
ONPG	97	97	97	97	97	97	97	97	97	97	97	97	97
Lysine_decarboxylase	95	85	2	7	1	99	94	99	99	50	82	99	99
Ornithine_decarboxylase	1	1	1	1	1	1	1	1	99	99	1	1	1
Arginine_dihydrolase	99	88	99	91	86	1	99	99	1	1	91	99	99
Aesculin	95	99	93	91	99	1	15	1	99	50	1	8	8
Gas_from_glucose	83	75	5	1	99	84	86	99	80	50	1	75	75
L-arabinose_acid	86	99	95	99	86	3	14	22	1	50	1	22	1
Mannitol_acid	95	99	95	99	99	99	99	91	99	99	47	75	75
Sucrose_acid	91	91	99	99	85	99	99	1	99	50	1	12	12
Mannose_acid	27	85	48	99	99	75	89	86	49	50	86	99	99
Sorbitol_acid	7	17	5	25	50	25	1	1	1	50	1	1	1
Salicin_acid	95	8	68	50	50	35	34	1	50	50	1	1	1
Rhamnose_acid	27	75	1	1	1	1	1	1	1	1	1	1	1
Glycerol_acid	99	99	83	91	1	93	50	93	50	50	29	50	50
Cellobiose_acid	43	43	86	50	50	38	50	1	50	50	1	99	99
Lactose_acid	23	12	48	50	50	50	50	50	50	50	1	50	50
Citrate_utilisation	44	29	90	55	1	23	53	99	50	50	93	99	93
β-haemolysis_sheep	55	55	14	30	86	54	44	50	50	50	50	77	77
H2S	50	50	1	1	71	50	50	50	50	50	1	62	62
Pyrazinamidase	50	50	80	18	99	50	50	50	50	50	50	50	50
Corn_oil_lipase	50	50	50	50	50	50	50	50	50	50	99	1	1
0/129_150ug_resist.	47	47	50	99	99	46	46	46	99	99	99	99	99
Cephalothin_30ug_sens.	20	20	20	20	50	50	80	90	80	50	73	1	99
Ampicillin_10ug_sens.	14	14	5	80	80	50	50	1	50	50	50	99	1
Growth_at_37C	99	99	70	99	99	99	99	99	99	99	99	99	99

Table 6-1: Modified probability matrix

6.3: Results

Table 6-2: PIBS identifications and corresponding AFLP cluster

strain ID	isolated from	location	PIBS Identification	AFLP HG cluster
AA001	catfish	Phillipines	no identification	3
AA002	unknown fish	Malaysia	no identification	12
AA003	catfish	Thailand	*A.veronii* bv.sobria	8
AA006	unknown fish	Phillipines	*A.veronii* bv.veronii	7
AA007	unknown fish	Malaysia	no identification	9
AA009	catfish	Thailand	*A.veronii* bv.sobria	8
AA010	turtle	Thailand	HG11	11b
AA011	unknown fish	Malaysia	*A.trota*	13
AA013	catfish	Phillipines	*A.hydrophila*	1
AA014	unknown fish	Bangladesh	*A.caviae*	4b
AA015	turtle	Thailand	HG11	11b
AA016	catfish	Thailand	*A.hydrophila*	9
AA018	catfish	Phillipines	*A.hydrophila*	3
AA019	unknown fish	Bangladesh	*A.hydrophila*	2
AA020	turtle	Thailand	*A.caviae*	5b
AA021	catfish	Thailand	*A.hydrophila*	10
AA022	catfish	Phillipines	*A.caviae*	5b
AA023	catfish	Phillipines	*A.hydrophila*	9
AA024	catfish	Thailand	*A.trota*	13
AA025	catfish	Phillipines	*A.sobria*	7
AA028	catfish	Thailand	no identification	8
AA029	turtle	Thailand	*A.veronii* bv.veronii	7
AA030	catfish	Thailand	no identification	5b
AA035	catfish	Malaysia	*A.jandaei*	9
AA038	unknown fish	Bangladesh	*A.media*	5b
AA039	catfish	Thailand	no identification	1
AA040	turtle	Thailand	*A.trota*	13
AA041	catfish	Phillipines	*A.veronii* bv.veronii	outlier
AA042	unknown fish	Malaysia	no identification	11b
AA043	unknown fish	Bangladesh	*A.caviae*	5b
AA044	catfish	Thailand	*A.schubertii*	12
AA045	turtle	Thailand	*A.trota*	13
AA047	catfish	Phillipines	*A.caviae*	4b
AA050	unknown fish	Bangladesh	*A.hydrophila*	1
AA051	unknown fish	Bangladesh	*A.hydrophila*	1
AA052	catfish	Phillipines	*A.jandaei*	9
AA053	catfish	Thailand	*A.hydrophila*	1
AA054	unknown fish	Bangladesh	*A.hydrophila*	3
AA055	unknown fish	Bangladesh	*A.trota*	13
AA056	turtle	Thailand	*A.trota*	13
AA057	catfish	Phillipines	no identification	4a
AA059	unknown fish	Bangladesh	no identification	outlier
AA060	unknown fish	Bangladesh	*A.veronii* bv.veronii	10

AA061	unknown fish	Bangladesh	no identification	4a
AA062	catfish	Phillipines	*A.hydrophila*	1
AA065	catfish	Phillipines	*A.hydrophila*	1
AA068	unknown fish	Bangladesh	*A.media*	5b
AA069	unknown fish	Phillipines	HG11	11a
AA070	unknown fish	Phillipines	*A.veronii* bv.veronii	10
AA071	unknown fish	Bangladesh	HG2	3
AA072	catfish	Phillipines	*A.jandaei*	9
AA073	catfish	Phillipines	*A.hydrophila*	8
AA074	unknown fish	Bangladesh	*A.media*	5a
AA075	catfish	Thailand	*A.veronii* bv.veronii	10
AA079	catfish	Thailand	*A.hydrophila*	5b
AA081	unknown fish	Malaysia	*A.trota*	13
AA083	catfish	Thailand	no identification	10
AA085	unknown fish	Bangladesh	*A.media*	5b
AA087	turtle	Thailand	*A.veronii* bv.veronii	10
AA089	unknown fish	Bangladesh	*A.media*	5a
AA091	catfish	Thailand	*A.trota*	13
AA092	catfish	Phillipines	*A.caviae*	4b
AA093	unknown fish	Bangladesh	*A.sobria*	7
AA094	catfish	Thailand	*A.hydrophila*	3
AA095	unknown fish	Bangladesh	*A.hydrophila*	12
AA097	unknown fish	Malaysia	*A.jandaei*	9
AA098	catfish	Thailand	HG11	11b
F01	frog	Thailand	*A.hydrophila*	1
F02	frog	Thailand	*A.hydrophila*	1
F03	frog	Thailand	*A.hydrophila*	1
F04	frog	Thailand	*A.hydrophila*	1
F05	frog	Thailand	*A.hydrophila*	1
F06	frog	Thailand	*A.hydrophila*	1
F07	frog	Thailand	*A.hydrophila*	1
F08	frog	Thailand	*A.hydrophila*	1
F09	frog	Thailand	*A.hydrophila*	1
F10	frog	Thailand	*A.hydrophila*	1
RR002	ornamental fish	Brazil	*A.hydrophila*	1
RR005	ornamental fish	Brazil	*A.veronii* bv.veronii	10
RR006	ornamental fish	Singapore	*A.veronii* bv. veronii	8
RR007	ornamental fish	Singapore	*A.hydrophila*	3
RR008	ornamental fish	Brazil	*A.jandaei*	9
RR009	ornamental fish	Singapore	*A.jandaei*	9
RR010	ornamental fish	Brazil	*A.jandaei*	9
RR011	ornamental fish	Brazil	no identification	9
RR012	ornamental fish	Brazil	*A.eucrenophila*	11a
RR013	ornamental fish	Singapore	*A.caviae*	5b
RR014	ornamental fish	Brazil	*A.hydrophila*	8
RR015	ornamental fish	Singapore	no identification	outlier
RR016	ornamental fish	Singapore	*A.caviae*	4b
RR018	ornamental fish	Brazil	*A.caviae*	5b
RR019	ornamental fish	Brazil	HG2	2
RR020	ornamental fish	Brazil	no identification	4
RR021	ornamental fish	Singapore	*A.veronii* bv. veronii	8

RR022	ornamental fish	Brazil	no identification	1
RR024	ornamental fish	Brazil	*A.veronii* bv.veronii	10
RR028	ornamental fish	Brazil	*A.media*	5a
RR031	ornamental fish	Brazil	*A.hydrophila*	3
RR032	ornamental fish	Singapore	no identification	1
RR034	ornamental fish	Brazil	*A.media*	5a
RR035	ornamental fish	Brazil	*A.jandaei*	9
RR037	ornamental fish	Singapore	*A.caviae*	4b
RR040	ornamental fish	Brazil	no identification	1
RR041	ornamental fish	Brazil	*A.media*	5b
RR042	ornamental fish	Singapore	*A.hydrophila*	3
RR043	ornamental fish	Brazil	*A.hydrophila*	3
RR044	ornamental fish	Brazil	*A.media*	5a
RR045	ornamental fish	Singapore	no identification	5b
RR046	ornamental fish	Singapore	*A.hydrophila*	3
RR047	ornamental fish	Brazil	*A.trota*	13
RR048	ornamental fish	Singapore	no identification	9
RR049	ornamental fish	Brazil	*A.schubertii*	12
RR050	ornamental fish	Brazil	*A.sobria*	7
RR051	ornamental fish	Brazil	*A.media*	5b
RR052	ornamental fish	Brazil	*A.jandaei*	9
RR053	ornamental fish	Brazil	*A.veronii* bv.sobria	10
RR054	ornamental fish	Singapore	no identification	11b
RR056	ornamental fish	Singapore	*A.hydrophila*	3
RR057	ornamental fish	Brazil	HG11	11b
RR059	ornamental fish	Brazil	*A.jandaei*	9
RR060	ornamental fish	Brazil	no identification	2
RR061	ornamental fish	Brazil	*A.media*	5b
RR062	ornamental fish	Singapore	*A.media*	5a
RR063	ornamental fish	Brazil	no identification	outlier
RR064	ornamental fish	Brazil	*A.caviae*	5b
RR065	ornamental fish	Brazil	*A.jandaei*	9
RR066	ornamental fish	Brazil	*A.hydrophila*	2
RR067	ornamental fish	Singapore	HG11	11b
RR068	ornamental fish	Singapore	*A.caviae*	5b
C001	porpoise	Scotland	*A.caviae*	outlier
C003	porpoise	Scotland	*A.hydrophila*	3
C004	porpoise	Scotland	*A.jandaei*	9
C005	porpoise	Scotland	no identification	4b
C006	striped dolphin	Scotland	HG2	2
C007	otter	Scotland	*A.hydrophila*	3
C008	porpoise	Scotland	HG2	2
C010	common seal	Scotland	no identification	3
C011	porpoise	Scotland	no identification	3
C012	dolphin	Scotland	*A.hydrophila*	3
C014	unknown fish	Scotland	*A.hydrophila*	2
C015	sheep	Scotland	HG11	11b
C016	dolphin	Scotland	*A.hydrophila*	2
C017	sheep	Scotland	HG11	11b
C018	porpoise	Scotland	*A.hydrophila*	3
C019	striped dolphin	Scotland	*A.hydrophila*	1

C020	porpoise	Scotland	*A.hydrophila*	3
C021	porpoise	Scotland	HG11	11b
C022	porpoise lung	Scotland	no identification	3
C023	porpoise	Scotland	*A.hydrophila*	1
C024	sea mammal	Scotland	HG2	2
C025	dolphin	Scotland	*A.hydrophila*	3
C026	porpoise	Scotland	no identification	1
C027	common seal	Scotland	*A.veronii* bv.sobria	10
C028	porpoise	Scotland	*A.veronii* bv.sobria	10
C029	sea trout	Scotland	*A.salmonicida*	3
C030	porpoise	Scotland	no identification	5b
C031	porpoise	Scotland	*A.hydrophila*	3
C033	porpoise	Scotland	*A.caviae*	4b
C038	grey seal	Scotland	*A.hydrophila*	1
C039	sheep	Scotland	HG11	11b
C040	Atlantic white-sided dolphin	Scotland	no identification	2
C043	beaver	Scotland	*A.hydrophila*	1
C044	common seal	Scotland	*A.veronii* bv.sobria	8
C045	dolphin	Scotland	no identification	12
C049	porpoise	Scotland	*A.hydrophila*	2
C050	porpoise	Scotland	*A.veronii* bv.sobria	8
C055	porpoise	Scotland	*A.hydrophila*	3
C056	porpoise	Scotland	*A.hydrophila*	3
C058	porpoise	Scotland	*A.hydrophila*	1
C059	otter	Scotland	no identification	11b
C060	porpoise	Scotland	A.hydrophila	3
C061	beaver	Scotland	no identification	3
C062	sheep	Scotland	*A.hydrophila*	3
C063	porpoise	Scotland	no identification	3
C064	porpoise	Scotland	*A.hydrophila*	2
C065	porpoise	Scotland	*A.hydrophila*	1
C066	sheep	Scotland	HG11	11b
C069	grey seal	Scotland	*A.caviae*	5b
C071	common seal	Scotland	*A.veronii* bv.sobria	8
C074	porpoise	Scotland	HG2	2
C076	porpoise	Scotland	*A.veronii* bv.veronii	10
C077	grey seal	Scotland	no identification	2
C078	grey seal	Scotland	no identification	2
C079	porpoise	Scotland	no identification	3
C080	porpoise	Scotland	*A.hydrophila*	3
C081	porpoise	Scotland	*A.jandaei*	9
C082	common seal	Scotland	no identification	3
C083	porpoise	Scotland	*A.veronii* bv.sobria	8
C084	porpoise	Scotland	*A.hydrophila*	3
C085	grey seal	Scotland	*A.hydrophila*	1
C086	grey seal	Scotland	no identification	9
C087	porpoise	Scotland	*A.hydrophila*	3
C088	sea trout	Scotland	*A.veronii* bv. veronii	10
C089	sheep	Scotland	*A.caviae*	4b
C091	beaver	Scotland	*A.caviae*	4
C092	dolphin	Scotland	*A.hydrophila*	3

C093	porpoise	Scotland	*A.jandaei*	9
C094	porpoise	Scotland	*A.hydrophila*	2
C095	grey seal	Scotland	no identification	11b
C097	porpoise	Scotland	*A.caviae*	4b
C098	common seal	Scotland	HG2	2
C099	grey seal	Scotland	no identification	5b
C102	common seal	Scotland	*A.veronii* bv.sobria	10
C103	porpoise	Scotland	*A.caviae*	5b
C104	porpoise	Scotland	*A.caviae*	5b
C105	porpoise lung	Scotland	*A.hydrophila*	2
C106	dolphin	Scotland	*A.hydrophila*	3
C107	striped dolphin	Scotland	no identification	1
C108	sheep	Scotland	*A.hydrophila*	3
C109	Atlantic white-sided dolphin	Scotland	*A.hydrophila*	3
C110	porpoise	Scotland	*A.hydrophila*	3
C111	grey seal	Scotland	*A.veronii* bv.sobria	8
C112	porpoise	Scotland	*A.veronii* bv.sobria	8
C114	sea mammal	Scotland	HG2	2
C115	porpoise	Scotland	*A.hydrophila*	3
C116	porpoise	Scotland	no identification	4b
C117	grey seal	Scotland	*A.hydrophila*	1
C118	common seal	Scotland	*A.veronii* bv.sobria	8
C119	dolphin	Scotland	*A.hydrophila*	3
C120	porpoise	Scotland	*A.hydrophila*	3
C122	striped dolphin	Scotland	*A.hydrophila*	2
C123	porpoise	Scotland	*A.hydrophila*	3
C124	porpoise	Scotland	*A.hydrophila*	11b
C125	porpoise	Scotland	no identification	3
C126	sea trout	Scotland	*A.sobria*	7
AH001	salmon	Scotland	*A.hydrophila*	1
AH002	seagull feces	Scotland	*A.veronii* bv.veronii	7
AH003	seagull feces	Scotland	no identification	3
AH004	ice bucket	Scotland	no identification	2
AH005	salmon	Scotland	atypical *A.salmonicida*	3
AH007	raw chicken	Scotland	*A.hydrophila*	1
AH009	reservoir	Scotland	*A.hydrophila*	4
AH010	reservoir	Scotland	HG2	
AH011	raw chicken	Scotland	no identification	5a
AH012	salmon	Scotland	atypical *A.salmonicida*	3
AH014	salmon	Scotland	atypical *A.salmonicida*	3
AH016	salmon	Scotland	*A.caviae*	4b
AH018	salmon	Scotland	atypical *A.salmonicida*	3
AH019	river water	Scotland	HG2	2
AH020	salmon	Scotland	no identification	5b
AH021	salmon	Scotland	*A.hydrophila*	3
AH022	raw chicken	Scotland	*A.hydrophila*	3
AH023	ice bucket	Scotland	HG2	2
AH024	seagull feces	Scotland	*A.veronii* bv.veronii	10
AH025	salmon	Scotland	atypical *A.salmonicida*	3
AH026	seagull feces	Scotland	*A.media*	5a
AH027	seagull feces	Scotland	no identification	1

AH028	seagull feces	Scotland	no identification	3
AH029	reservoir	Scotland	*A.hydrophila*	4b
AH030	salmon	Scotland	no identification	5b
AH031	tilapia	Scotland	*A.schubertii*	12
AH032	salmon	Scotland	*A.salmonicida*	3
AH033	seagull feces	Scotland	*A.hydrophila*	2
AH034	salmon	Scotland	atypical *A.salmonicida*	3
AH035	seagull feces	Scotland	*A.caviae*	4a
AH037	reservoir	Scotland	*A.media*	5a
AH038	seagull feces	Scotland	*A.hydrophila*	3
AH039	seagull feces	Scotland	*A.hydrophila*	2
AH040	reservoir	Scotland	*A.caviae*	4a
AH041	seagull feces	Scotland	*A.hydrophila*	3
AH042	seagull feces	Scotland	*A.hydrophila*	4
AH043	raw chicken	Scotland	*A.hydrophila*	1
AH044	seagull feces	Scotland	*A.hydrophila*	1
AH045	river water	Scotland	*A.hydrophila*	1
AH046	seagull feces	Scotland	*A.caviae*	5b
AH047	river water	Scotland	*A.media*	5a
AH048	salmon	Scotland	*A.salmonicida*	3
AH049	river water	Scotland	*A.hydrophila*	3
AH050	tilapia	Scotland	*A.schubertii*	12
AH052	raw chicken	Scotland	*A.hydrophila*	1
AH053	raw chicken	Scotland	*A.jandaei*	9
AH055	river water	Scotland	*A.hydrophila*	3
AH056	raw chicken	Scotland	*A.hydrophila*	1
AH059	river water	Scotland	*A.media*	5a
AH060	seagull feces	Scotland	HG11	11b
AH061	reservoir	Scotland	*A.media*	5b
AH062	seagull feces	Scotland	no identification	12
AH063	salmon	Scotland	*A.hydrophila*	3
AH051	raw chicken	Scotland	*A.hydrophila*	1
AH064	seagull feces	Scotland	no identification	12
AH065	seagull feces	Scotland	no identification	5b
AH066	salmon	Scotland	no identification	1
AH067	seagull feces	Scotland	no identification	2
AH068	river water	Scotland	*A.hydrophila*	3
AH069	salmon	Scotland	*A.salmonicida*	3
AH070	ice bucket	Scotland	no identification	12
AH071	salmon	Scotland	*A.salmonicida*	3
AH072	raw chicken	Scotland	*A.hydrophila*	3
AH073	salmon	Scotland	*A.hydrophila*	2
AH074	salmon	Scotland	*A.salmonicida*	3
AH075	river water	Scotland	*A.media*	5a
AH076	salmon	Scotland	HG2	2
AH077	seagull feces	Scotland	no identification	4b
AH080	river water	Wales	*A.media*	outlier
AH081	river water	Wales	*A.jandaei*	9
AH082	river water	Wales	HG2	2
AH083	river water	Wales	*A.veronii* bv.veronii	10
AH084	river water	Wales	*A.hydrophila*	3

AH085	river water	Wales	HG2	2
AH086	river water	Wales	*A.hydrophila*	1
AH087	river water	Wales	*A.media*	5b
AH088	river water	Wales	no identification	3
AH089	river water	Wales	*A.hydrophila*	1
AH090	river water	Wales	no identification	1
AH091	river water	Wales	HG2	2
AH092	river water	Wales	no identification	5b
AH093	river water	Wales	no identification	1
AH094	river water	Wales	*A.hydrophila*	1
AH095	ostrich	Wales	*A.hydrophila*	3
AH096	ostrich	Wales	no identification	1
AH097	chlorinated tap water	Wales	*A.media*	5a
AH098	chlorinated tap water	Wales	*A.media*	5a
H09	human stool	Scotland	*A.hydrophila*	1
H07	human stool	Scotland	*A.hydrophila*	1
H02	human stool	Scotland	*A.hydrophila*	1
H17	human stool	Scotland	*A.hydrophila*	1
H01	human stool	Scotland	*A.hydrophila*	1
H21	human stool	Scotland	*A.hydrophila*	1
H14	human wound	Scotland	*A.jandaei*	9
H20	human stool	Scotland	*A.veronii* bv.sobria	8
H18	human stool	Scotland	no identification	8
H06	human stool	Scotland	*A.veronii* bv. veronii	8
H08	human stool	Scotland	*A.veronii* bv.sobria	10
H22	human stool	Scotland	*A.veronii* bv. veronii	10
H23	human stool	Scotland	*A.veronii* bv.veronii	10
H03	human stool	Scotland	*A.veronii* bv.veronii	10
H16	human wound	Scotland	*A.veronii* bv.veronii	10
H10	human stool	Scotland	*A.caviae*	4b
H11	human stool	Scotland	*A.caviae*	4b
H15	human stool	Scotland	*A.caviae*	4b
H19	human stool	Scotland	*A.caviae*	4a
H12	human stool	Scotland	*A.caviae*	4
H13	human stool	Scotland	*A.caviae*	4
H04	human stool	Scotland	no identification	5b
H05	human stool	Scotland	*A.schubertii*	12
V01	groundwater	Vietnam	no identification	1
V02	unknown fish	Vietnam	*A.veronii* bv.sobria	8
V03	unknown fish	Vietnam	*A.hydrophila*	3
V04	groundwater	Vietnam	*A.caviae*	4b
V05	groundwater	Vietnam	*A.media*	5a
V06	groundwater	Vietnam	no identification	8
V07	groundwater	Vietnam	*A.eucrenophila*	11a
V08	groundwater	Vietnam	*A.hydrophila*	2
V09	groundwater	Vietnam	no identification	3
V10	groundwater	Vietnam	*A.hydrophila*	12
V11	groundwater	Vietnam	no identification	9
V12	groundwater	Vietnam	*A.hydrophila*	1
V13	groundwater	Vietnam	*A.media*	5a
V14	groundwater	Vietnam	no identification	outlier

V15	groundwater	Vietnam	HG11	11b
V16	groundwater	Vietnam	*A.hydrophila*	1
V17	groundwater	Vietnam	*A.media*	5a
V18	groundwater	Vietnam	no identification	11b
V19	groundwater	Vietnam	no identification	9
V20	groundwater	Vietnam	*A.hydrophila*	9
V21	groundwater	Vietnam	*A.veronii* bv.veronii	10
V22	groundwater	Vietnam	HG11	11a
RK1	aquarium water	Germany	*A.veronii* bv.sobria	10
RK2	human blood	Germany	*A.veronii* bv.sobria	10
RK3	human blood	Germany	*A.veronii* bv.sobria	10

Table 6-3: Proportion of strains identified to cluster above 0.95 threshold

AFLP HG cluster	number	no correct ID	%	no not ID	%	no wrong ID	%
1	56	45	80.4	11	19.6	0	0
2	33	14	42.4	6	18.2	13	39.4
3	70	56	80	13	18.6	1	1.4
4	28	21	75.0	2	7.1	3	10.7
5a	17	16	94.1	0	0.0	1	5.9
5b	29	8	27.6	9	31.0	12	41.4
6	5	4	80.0	0	0.0	1	20.0
7	7	4	71.4	0	14.3	3	14.3
8	19	14	73.7	3	15.8	2	10.5
9	26	20	76.9	6	23.1	0	0.0
10	25	16	64.0	1	4.0	8	32.0
11	18	13	72.2	2	11.1	1	5.6
12	12	9	75.0	2	16.7	1	8.3
13	9	9	100.0		0.0		0

Table 6-4: Biolog Identifications

Type strain	species	HG	BIOLOG identification	SIM clinical d/b	SIM environmental d/b
NCIMB9240	*A.hydrophila*	1	*A.hydrophila* group1	0.873	0.846
	A.bestiarum	2	*A.hydrophila* group1	0.833	0.800
NCIMB1102	*A.salmonicida*	3	*A.salmonicida*	0.938	0.938
NCIMB13016	*A.caviae*	4	*A.media*-like group5A	0.460	0.460
NCIMB2237	*A.media*	5	*A.media*-like group5A	0.407	0.406
NCIMB74	*A.eucrenophila*	6	*A.eucrenophila* group6	0.818	0.818
NCIMB12065	*A.sobria*	7	*A.sobria* group7	1.000	1.000
NCIMB37	*A.veronii.sobria*	8	*A.veronii*.sobria group8	0.573	0.566
ATCC49568	*A.jandaei*	9	*A.jandaei* group9	0.935	0.923
NCIMB13015	*A.veronii.veronii*	10	*A.veronii* group10	0.724	0.724
CECT4342T	*A.encheleia*		*Aeromonas* DNA group 11	0.694	0.684
NCIMB1329	*A.enteropelogenes*	11	*A.trota* DNA group13	0.938	0.938
NCIMB13161	*A.schubertii*	12	*A.schubertii* group12	0.997	0.997
ATCC49657	*A.trota*	13	*A.trota* DNA group13	1.000	1.000

6.4: Discussion

6.4.1: PIBS identifications

A number of phenotypic typing schemes for discriminating *Aeromonas* isolates have been proposed (Borrell *et al.,* 1998; Carnahan and Joseph, 1993; Kämpfer and Altwegg, 1992; Kaznowski, 1998; Wilcox *et al.,* 1992). These have been designed primarily to identify species of medical importance and evaluated using a strain collection in which veterinary and environmental isolates have been under-represented, where they have been included at all.

This work was undertaken with the aim of adapting a phenotypic testing scheme previously reported to have high discriminatory power for typing *Aeromonas* isolates from recovered from veterinary and environmental sources, as well as medical, (Oakey *et al.,* 1996)

Overall the proportion of strains accurately identified to the level of hybridisation group using the battery of tests described here was 73% with a confidence level of 0.95 or higher. This is comparable to the accuracy level reported to by Oakey *et al.,* (1996), but does not compare well with the accuracy levels reported for probability matrices using a similar number of tests that have been designed for other bacterial groups such as the 89.2% correct identifications recorded for Gram-negative fermentative bacteria by Holmes *et al.,* (1986).

In part this may be a result of the particularly variable nature of this genus and the fact that the collection of strains assembled here was specifically intended to be as heterogeneous a possible.

However the level of successful identification is not at all consistent across the different hybridisation groups with 100% of HG13strains and 94.1% of HG 5a, for example, being identified correctly with a confidence level of above 0.95.

In contrast, other hybridisation groups were much more prone to error, and it may be that identification of further discriminatory tests could improve the accuracy level for these species.

Thirteen strains that formed part of the AFLP HG2 cluster were identified as *A.hydrophila* HG1 by the PIBS probability matrix. None of these strains produced acid from mannose. Updating the matrix to reflect this prevented the incorrect identification, but lowered the probability score to below the 0.95 threshold. This is because mannose fermentation is one of the most discriminatory tests in distinguishing HG's 1 and 2. The mannose fermentation test was repeated for these strains, at a range of temperatures, from 22°C to 37°C, to determine whether they would ferment mannose at other temperatures. All thirteen strains produced the same result at all temperatures. Inclusion of an additional test will be necessary in any future work to improve the discrimination between HG1 and HG2.

Twelve strains that clustered with AFLP HG5b cluster were wrongly identified as *Aeromonas caviae* HG4. This is consistent with previous reports (Carnahan and Altwegg, 1996) that a large number of HG5b strains are phenotypically similar to *A.caviae*. For many years the phenospecies *A.media* included only the HG5a strains that could be reliably distinguished from *A.caviae* HG4., This is a significant observation: *A.media* is one of the few *Aeromonas* spp. that have not been implicated as a human pathogen, whilst *A.caviae* is the one of the species most frequently recovered from human clinical sources (Janda and Abbot, 1998). Since most clinical

microbiology laboratories continue to rely on phenotypic diagnosis alone, it is possible that an *A.media* HG5b strain that was isolated from a human patient, could be recorded as *A.caviae.*. None of the HG5b strains included in this study were recovered from human sources, however they were relatively few in number. It may therefore be premature to conclude that HG5 does not include potential human pathogens, particularly as a relatively high proportion of HG5 strains carry a haemolysin. Since two of the strains included in this study were recovered from chlorinated tapwater, this warrants further investigation.

In the light of the finding (Chapter 2) that the HG4 strains fell into two distinct AFLP clusters, this suggests that further work is needed to clarify the relationships between the HG4 and HG5 clusters and to determine with greater certainty how many and which clusters harbour potentially pathogenic strains.

Although few in number, (n=7) a relatively high proportion of *A.sobria* HG7 strains – almost half- were mis-identified as *A.veronii* bv. veronii. Paradoxically, Oakey *et al.,* (1996) found that the battery of tests described here, was able to accurately distinguish the two taxa with a high degree of probability. The mis-identified strains were all arginine dihydrolase negative, using the protocols adopted for this study. This test result has been reported as particularly discriminatory test for *A.veronii* bv. veronii.(Carson *et al.*, 2001) Interestingly the *A.sobria* HG7 type strain, CIP 7433 was reported to be arginine dihydrolase negative in the original definition of the species, (Popoff and Veron, 1976; Farmer *et al.,* 1986); elsewhere it has been reported (Bryant *et al.* 1986; Kuijper *et al.*, 1989) that *A.sobria* HG7 are arginine dihydrolase positive and that this is a characteristic of the species. Resolving this

discrepancy for the purposes of constructing an accurate probability matrix will be problematic, since *A.sobria* HG7 is believed to be a comparatively rare species and there are relatively few isolates available on which to base a dataset.

It has been reported previously that *A.sobria* HG7 may harbour a number of strains that are phenotypically similar to HG10 (Carnahan and Altwegg, 1996; Huys *et al.,* 1996). It may be that inclusion of further tests could improve the discrimination between HG7 and HG10 in future work, however it is questionable whether this would be of value in a typing scheme intended for routine use, given that *A.sobria* does appear to be genuinely rare and has never been isolated from clinical material (Janda and Abbot, 1998).

The failure of the tests to accurately discriminate between HG 8 and HG10 is more serious as there is evidence that the two species have a different disease spectrum (Janda and Abbot, 1998). Out of 25 *A.veronii* bv veronii HG10 strains tested, eight were mis-identified as *A.veronii* bv. sobria, HG8.

The AFLP data presented in Chapter 2 and reported by Huys *et al.,* (1996) suggests that these two species are genetically very closely related. They are also very similar phenotypically and only one test in the matrix – arginine dihydrolase – is definitive, with the literature recording HG8 as invariably arginine dihydrolase positive and HG10 as invariably negative. The eight mis-identified HG10 strains were all recorded as arginine dihydrolase positive in this work. Though it is quite possible that these are genuinely atypical arginine dihydrolase positive *A.veronii* bv veronii HG10 strains, the fact that a number of HG7 strains also produced an unexpected arginine dihydrolase test result, raises the suspicion that the arginine dihydrolase test as performed in this work was not working reliably. Further work will therefore be

required to establish whether the cause of the misidentifications lies with the probability matrix or in the way in which the test was performed.

6.4.2: Biolog Identifications

Using Biolog ™ GN plates and the Microlog ™ software, it proved possible to identify only nine out of fourteen type strains with a SIM value of greater than 0.75, see table 6.4 and Appendix2.

A.media HG5, *A.veronii* bv. sobria, HG8 and *A.veronii* bv. veronii HG10 were all correctly identified, but with lower SIM values.

A.bestiarum HG2 was incorrectly identified as *A.hydrophila* HG1 with a relatively high SIM value of 0.833. *A.enteropelogenes* was mis-identified as *A.trota* HG13 with an even higher SIM value of 0.938.

The *A.caviae* HG4 and *A.media* HG5 type strains were both identified as '*A.media*-like group 5A' but with low SIM values of 0.460 and 0.407 resepectively. Curiously the software seems to have mis-identified *A.caviae* with a greater degree of certainty than the correct identification of *A.media* HG5.

In most cases the correct identification was unequivocal in that the SIM values for the next most probable matches were extremely low. The exception being *A.veronii* bv. *sobria* HG8 which was correctly identified with a low SIM value of 0.573, but the next most probable match being *A.veronii* bv. veronii HG10, with a SIM value of 0.332.

When presented with strains other than the type strains the system performed very poorly. Of 60 strains tested, the identification corresponded to the AFLP cluster in only 7 cases (11.67%). Four of these were *A.hydrophila* HG1 and one each of

A.sobria, HG7, *A.schubertii* HG12 and *A.trota* HG13. The strains correctly identified had biochemical profiles identical to or very similar to the type strains, differing by up to three test results.

This would indicate that the system is not particularly robust in that it is unable to offer an identification if the strain in question differs by even a very small number of test results from the profile for that organism in the database. This is consistent with the findings of Knight *et al.,* (1993) who reported that it was possible to identify only 83% in identifying *Acinetobacter* isolates to genus level, and found the correlation between the Biolog assignment to genospecies to be extremely poor when compared to other phenotypic identification schemes.

This is a disappointing result, particularly as the tests were found to be highly reproducible, with identical biochemical profiles being obtained for repeated strains (n=5). However it may be that the poor performance of the Biolog system as a whole may be attributed to incomplete data in the Microlog database, rather than the underlying principle. The *Aeromonas* profiles in the database were arrived at using a collection of strains in which medical isolates were overwhelmingly over – represented (B.Bochner, pers. comm.). It may be that the failure to include environmental and veterinary isolates has biased the database, so that strains isolated from other sources are not recognised. A more advanced version of the Microlog software, Microlog2 does allow the user to progressively update the database to take account of newly analysed strains. Whilst it seems likely that this could improve the diagnostic capabilities of the system within one laboratory, it also has the disadvantage that different laboratories would then be working with ever diverging databases.

Another potential shortcoming with the Microlog software is that test results in the database are classified as positive or negative, depending on whether previously encountered examples of a species have been found to score positive on greater than 90%, or fewer than 10% of occasions. Any tests found to be more variable than these extremes are classified as borderline.

However it is likely that many of the tests ignored as borderline by the Microlog software, could, in fact be of diagnostic value if incorporated into a probability matrix and the identification based on Bayesian principles. The great strength of the Baysian approach is that by combining the probability scores for many tests, a, probabilistic identification can be made even though it is based on tests which, individually can have relatively low discriminatory power.

The great convenience with which 95 phenotypic tests can be performed simultaneously and reproducibly using the Biolog system, suggests that it has potential that is undermined by the current database and identification algorithms.

The tests are easy to perform and require no special equipment or training, which puts a potentially very powerful diagnostic tool within reach of the vast majority of diagnostic laboratories that are not equipped to perform advanced molecular-based testing.

Chapter 7: Antibiotic Resistance and Potential for Horizontal Transfer

Chapter 7: Antibiotic resistance and potential for horizontal transfer

7.1: Introduction

Deterioration and loss of stock due to infectious bacterial diseases is a considerable economic burden in both fish and shellfish aquaculture worldwide.

Generally it is considered that bacterial infections in aquaculture are a secondary consequence of the stresses imposed by intensive culture. Stresses such as temperature change, handling, poor water quality, crowding, parasitic load and shipping have all been correlated with disease outbreaks. Awareness of this has led to advances in breeding and nutrition together with improved husbandry practices which have considerably reduced the incidence of disease outbreaks (Dixon, 1994). The introduction of mineral oil-based vaccines has also improved the situation enormously. Despite this, periodic bacterial disease outbreaks remain a persistant feature of intensive aquaculture and these outbreaks are overwhelmingly treated using antibacterial agents.

Use of antibiotics in the rearing of food animals is a widespread practice and in the US, for example, accounts for approximately one half of all antibiotics produced (McPhearson *et al.,* 1991). Compared to this the amounts used in aquaculture are relatively small. There are a number of reasons, however, why the use of antibiotics in aquaculture may be grounds for particular concern.

Firstly antibiotic use in aquaculture was negligible until the 1980's (Weston, 1995) and grew enormously during that decade in parallel with the rapid growth in production worldwide. There is evidence that, because of the improvements in fish health mentioned above, antibiotic use in the developed world may now have peaked

(Burka *et al.*, 1997). In developing countries however antibiotic use continues to rise (World Health Organisation, 1997)

Secondly, in aquaculture systems there is often little or no effluent treatment and the fate of antimicrobials shed into the environment is poorly understood.

The growth of bacterial resistance to antibiotics since their introduction is not surprising from an evolutionary point of view. However the range and efficiency of the mechanisms by which bacteria are able to access the large selection of resistance genes scattered throughout the bacterial world is only now beginning to become apparent.

Mobilisation of DNA sequences from one cell to another can involve a number of different mechanisms including conjugation, transformation and transduction, all of which have been demonstrated to be important in the movement of resistance genes among clinically and agriculturally important bacteria. (Bennet, 1999)

More recently it has emerged that mechanisms have evolved to facilitate the movement of sequences from one molecule to another. The most familiar is transposition, which normally involves a discrete transposable element and requires no homology between the recombining sequences; many drug resistance transposons have been described in both Gram positive and Gram negative bacteria. Recently evidence has emerged that implicates Tn*1721* in dissemination of tetracycline resistance amongst aeromonads, (Rhodes *et al.*, 2000).

Frequently located on transposons are integrons: natural cloning and expression systems that capture and incorporate open reading frames (ORF's) and convert them to functional genes, (Rowe-Magnus and Mazel, 2001)

A functional integron consists of a site, *attI* at which additional DNA in the form of gene cassettes can be integrated by site-specific recombination, and a gene *(intI)* that codes for an integrase that mediates the recombination. Gene cassettes normally contain a single gene and a short sequence, *attC* (or 59-base element) that is the secondary target for the integrase. Following insertion of the cassette at the *attI* site the upstream integron promoter drives expression of the incorporated gene.

This process can occur sequentially leading to the stepwise accumulation of multiple gene cassettes.

Three classes of integron have been described, which though functionally very similar, differ in the sequences of the integrase genes. The degree of homology between the three classes (45-58%) suggests that evolutionary divergence has extended over a far longer period than the 50 years of the antibiotic era (Rowe-Magnus and Mazel, 1999).

Class 1integrons appear to be more numerous and have been more intensively studied, this is the only class for which gene cassette movement has been demonstrated experimentally, Bennett, (1999).

A key feature of class 1 integrons is the presence of highly conserved sequences but 5' and 3' to the *attI* site. This has enabled PCR primers to be devised that are able to screen for their presence and to indicate the number of inserted gene cassettes (Levesque *et al.,* 1995)

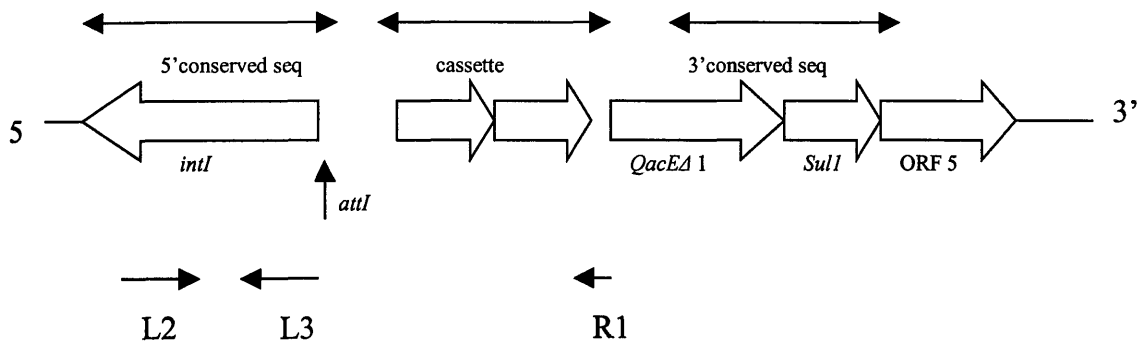
As evidence mounts for the spread of transposons amongst aeromonads (L'Abée-Lund and Sørum, 2000; Rhodes *et al.*, 2000) and that antibiotic resistance amongst fish pathogens associated with aquaculture is rising (Schmidt *et al.*,2000), it is important to determine how widespread class 1 integrons are becoming in this group.

7.2: Methods and materials

7.2.1: Oligonucleotide primers

Primers used were originally described by Levesque *et al.*,(1995).

Primers L2 and L3R were used to screen for the presence of class1 integrase (fig.1)



L2: 5'-GAC GAT GCG TGG AGA CC-3'

L3R: 5'-CTT GCT GCT TGG ATG CC-3'

Primers L2 - R1were used to confirm the presence of the integron.

R1: 5'AAG CAG ACT TGA CCT GA-3'

All primers were obtained from MWG Biotech.

7.2.2: PCR Reactions

All PCR reactions took place in a total volume of 20µl, in thin walled 200µl dome topped microcentrifuge tubes (Abgene), using an Eppendorf Gradient Mastercycler 96-well thermal cycler.

All reactions contained 50ng template DNA, 0.25mM (each) dNTP's and 200nM primers, 1X dynazyme buffer 1X Dynazyme reaction buffer (Flowgen, Staffs., UK), (=10 mM Tris-HCI (pH 8.8 at 25 ^{0}C); 50 mM KCl; 0.1 % Triton X-100) and 1U DynazymeII (Flowgen, Staffs., UK).

The thermal profile of the cycler was set to:

96°C for 2 minutes (initial denaturation)

94°C for 30seconds (denaturation)

X°C for 30seconds (annealing)

72°C for 1minute (elongation)

Steps (2) to (4) were repeated for 35 cycles.

A temperature titration was performed by setting the annealing temperature on the gradient cycler to 56°C +/-7°C.

After optimisation, an annealing temperature of 51°C was chosen for the L2-L3R primer pair and 59°C for the L2-R1 pair.

Amplification products were resolved by electrophoresis at 4V/cm for 2.5hrs on 15cm 2% agarose gels submerged in 0.5X TBE containing ethidium bromide, and visualised under UV.

The gel was destained for 5 minutes in running water and examined under UV on a transilluminator. An image was recorded using a CCD camera and stored as a tif file.

Images were imported into GelCompar image analysis software version 2.0 (Applied MathsBVBA, Kortrijk, Belgium).and lanes tracked at a resolution of 600. After background subtraction using the rolling disc principle, and normalisation to the size standard markers, the size in bp of each fragment visualised was estimated.

7.3: Results

The L2-L3R primer pair produced a product of the expected size in 7 out of 48 isolates tested (14.6%).

In all but one of these a product was observed using the L2-R1 primer pair also.

Size of product obtained for 40 isolates initially tested are shown in table 7.1.

strain	isolated from	location	phenospecies	L2-L3R	L2-R1
V02	unknown fish	Vietnam	*A.veronii* bv.sobria		
V03	unknown fish	Vietnam	*A.hydrophila*		
V04	groundwater	Vietnam	*A.caviae*		
V05	groundwater	Vietnam	*A.media*		
V07	groundwater	Vietnam	*A.eucrenophila*	297bp	1290bp
V08	groundwater	Vietnam	*A.hydrophila*		
RR010	ornamental fish	Brazil	*A.jandaei*		
RR011	ornamental fish	Brazil	no identification	294bp	857bp
RR012	ornamental fish	Brazil	*A.eucrenophila*		
RR013	ornamental fish	Singapore	*A.caviae*		
RR014	ornamental fish	Brazil	*A.hydrophila*		
RR015	ornamental fish	Singapore	no identification		
RR016	ornamental fish	Singapore	A.caviae		
RR018	ornamental fish	Brazil	A.caviae		
RR019	ornamental fish	Brazil	HG2	295bp	855bp
AA073	catfish	Phillipines	*A.hydrophila*		
AA074	unknown fish	Bangladesh	*A.media*		
AA075	catfish	Thailand	*A.veronii* bv.veronii		
AA079	catfish	Thailand	*A.hydrophila*		
AA081	unknown fish	Malaysia	*A.trota*		
AA083	catfish	Thailand	*no identification*		
AA085	unknown fish	Bangladesh	*A.media*		
AA087	turtle	Thailand	*A.veronii* bv.veronii		
AA089	unknown fish	Bangladesh	*A.media*		
AA091	catfish	Thailand	*A.trota*	295bp	
AA092	catfish	Phillipines	*A.caviae*		
AA093	unknown fish	Bangladesh	*A.sobria*	298bp	912bp
AA094	catfish	Thailand	*A.hydrophila*		
AA095	unknown fish	Bangladesh	*A.hydrophila*		
AA097	unknown fish	Malaysia	*A.jandaei*		
AA098	catfish	Thailand	HG11		
AH080	river water	Wales	*A.media*		
AH081	river water	Wales	*A.jandaei*		
AH082	river water	Wales	HG2		
AH083	river water	Wales	*A.veronii* bv.veronii		
AH084	river water	Wales	*A.hydrophila*		
AH085	river water	Wales	HG2		
AH086	river water	Wales	*A.hydrophila*		
AH087	river water	Wales	*A.media*		
AH034	salmon	Scotland	atypical *A.salmonicida*		
AH048	salmon	Scotland	*A.salmonicida*	298bp	1247bp
AH060	seagull feces	Scotland	HG11		
AH063	salmon	Scotland	*A.hydrophila*		
AH069	salmon	Scotland	*A.salmonicida*		
AH071	salmon	Scotland	*A.salmonicida*	298bp	906bp
AH073	salmon	Scotland	*A.hydrophila*		
AH074	salmon	Scotland	*A.salmonicida*		
AH076	salmon	Scotland	HG2		

Table 7-1: amplicon sizes obtained using integron-specific primer pairs

7.4: Discussion

The L2-L3R primer pair produced a product of the expected size in 7 out of 48 isolates tested (14.6%).

In all but one of these a product was observed using the L2-R1 primer pair also.

In the absence of corroborating sequence data, there must always be room for doubt as to the true identity of a PCR amplicon, even when a single, clear product of the expected size is amplified. However, the finding that in all but one case, amplification of a product using the L2-L3R primer pair, was accompanied by detection of a product amplified via the L2-R1 primer pair, is compelling evidence suggesting that the observed amplicons were indeed indicative of the presence of integron-specific sequences.

Moreover it would be expected that amplification of the integrase product would not, in every case, be associated with amplification of the integron product, since a cassette that accumulated a large number of inserted genes could become too large to amplify efficiently via the PCR, (Koeleman *et al.,* 2001).

This is the first report suggesting the presence of these genetic elements in *Aeromonas* spp. Class1 integron sequences were detected at a lower frequency among strains tested than has been reported in other studies (Koeleman *et al.,* 2001; Maguire *et al.*, 2001).

However these studies were confined to clinical isolates whereas twelve of the strains screened here were isolated from water. Significantly, only one of which was found to harbour an integron.

While the widespread distribution of these elements is evidence that their horizontal transfer has been commonplace amongst and between members of the genus *Aeromonas*, no inferences can be drawn as to the speed and frequency with which transfer occurs. Maguire *et al.* (2001), for example, noted that in cases where more than one bacterial species was isolated from the same patient, the presence of an integron was never observed in more than one of those isolates, which suggests that integron transfer between bacteria may not be as efficient as previously suggested. Furthermore, Dalsgaard *et al.*, (2000) recovered *Vibrio cholerae* strains in Thailand that harboured two integrons containing different cassettes.

Further work involving a larger number of isolates and including some isolates from the same and closely related sources is required to indicate how efficient horizontal transfer is in natural environments.

Further work would also be required to determine the extent to which acquisition of these elements is affected by the selective pressure of antimicrobial chemotherapy, both in the laboratory and in natural environments. In this work only one of twelve environmental isolates was found to harbour an integron which strongly suggests that there is little evolutionary imperative to acquire and maintain these elements in the absence of exposure to antibiotics.

In this work, presence of a Class1 integron does appear to be correlated with multiple antibiotic resistance, however the number of strains tested is not sufficiently large to make statistical inferences as to whether resistance to particular antibiotics is more closely associated with integron carriage.

A higher than expected proportion of integron positive strains were gentamicin resistant, however, and this agrees well with the observation by Martinez-Freijo *et al.*

(1998) and others, that aminoglycoside resistance genes are commonly associated with integrons.

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Appendix1: Media and Reagents

1: Sulphide Indole Motility medium (Blazevic, 1968).

Formula	gm/litre
Tryptone	20
Peptone	6.1
Ferrous ammonium sulphate	0.2
Sodium thiosulphate	0.2
Agar	3.5

pH adjusted to 7.3 ± 0.2 prior to autoclaving

2: Kovak's Reagent

para-dimethylaminobenzaldehyde.... 5g
amyl alcohol..... 75ml
concentrated hydrochloric acid..... 25ml

Dissolve the aldehyde in the alcohol by warming in water bath at 55°C. Cool and add the acid. Store in the dark at 4°C for up to 3 months.

3: Decarboxylase Broth (Taylor, 1961)

Formula	gm/litre
Yeast extract	3
Glucose	1
Bromocresol purple	0.016

pH adjusted to 6.1 ± 0.2 prior to autoclaving

4: Aesculin broth

Formula	gm/litre
Peptone	8
Ferric citrate 0.5	0.5
Aesculin 1.0	1

pH adjusted to 7.1 ± 0.2 prior to autoclaving

5: Peptone water (Cruickshank, 1968)

Formula	gm/litre
Peptone	10
Sodium chloride	5
Bromocresol purple	(0.002%)

pH adjusted to 7.2 ± 0.2 prior to autoclaving

6: Simmons citrate agar (American Public Health Association 1981)

Formula	**gm/litre**
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate, tribasic	2
Sodium chloride	5
Bromothymol blue	0.08
Agar	15

pH adjusted to 7.0 ± 0.2 prior to autoclaving

7: pyrazine carboxamide agar (Carnahan *et al.*, 1990)

Tris Maleate buffer (0.2M, pH 6.0)	500ml
Tryptone soya agar medium (Oxoid)	20g
Yeast extract (Oxoid)	1.5g
Pyrazine carboxamide	0.5g

8: Tris Maleate buffer pH 6.0 (Gomori, 1955)

Tris	3.025g
Malic acid	2.9g
To 125ml with mQ	
0.2M NaOH	65ml
to 500ml with mQ	

9: Corn oil calcium chloride agar

Peptone (Oxoid)	5g
Sodium chloride	2.5g
Calcium chloride	0.05g
Agar	10g
Distilled water	To 495ml
Corn oil	5ml

10: *Aeromonas* Agar (Ryan)

Formula	gm/litre
Proteose peptone	5
Yeast extract	3
L. Lysine monohydrochloride	3.5
L. Arginine monohydrochloride	2
Sorbitol	3
Inositol	2.5
Lactose	1.5
Xylose	3.75
Bile Salts No.3	3
Sodium thiosulphate	10.67
Sodium chloride	5
Ferric ammonium citrate	0.8
Bromothymol blue	0.04
Thymol blue	0.04
Agar	12.5

Appendix 2: BIOLOG IDENTIFICATIONS

MICROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.TROTA HG13
Strain # : ATCC49657
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	XX	XX	XX	XX	XX	XX	XX	..	..	..	XX
B	..	XX	XX	XX	XX	XX	XX	XX	..	XX	XX	XX
C	..	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX
D	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	..
E	..	..	XX	XX	..	XX	..	XX	XX	XX	..	XX
F	XX	XX	..	XX	XX	XX	XX	XX	XX	XX	XX	XX
G	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX	XX
H	XX	XX	XX	XX	..	XX	XX	XX	XX	XX	XX	XX

BIO-NUMBER : 3761-3767-3777-7776-1535-6777-7777-7577

SPECIES IDENTIFICATION : AEROMONAS TROTA DNA GROUP 13

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS TROTA DNA GROUP 13	0.633	5.657	0.229	4.353
	2)c SERRATIA MARCESCENS	0.000	10.980	1.781	5.031
	3)c SERRATIA LIQUEFACIENS/GRIMESII	0.000	11.032	0.844	4.069
	4)c ENTEROBACTER GERGOVIAE	0.000	11.547	0.979	4.494
	5)c BURKHOLDERIA CEPACIA	0.000	13.182	1.375	3.713
	6)c SALMONELLA SUBSPECIES 1 G	0.000	13.209	1.646	5.494
	7)c VIBRIO FURNISSII	0.000	13.451	0.844	3.444
	8)c ENTEROBACTER TAYLORAE	0.000	13.775	1.000	3.213
	9)c SERRATIA ODORIFERA	0.000	13.831	1.063	3.200
	10)c SALMONELLA SUBSPECIES 1 F	0.000	14.301	0.958	4.325
	other :	-----	-----	-----	-----

MICROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.TROTA HG13
Strain # : ATCC49657 RPT
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	XX	XX	XX	XX	XX	XX	XX	..	..	..	XX
B	..	XX	XX	XX	B	XX	B	B	..	XX	XX	XX
C	..	XX	XX	B	B	XX	B	XX	XX	B	XX	B
D	B	XX	XX	B	B	B	XX	B	B	XX	XX	..
E	..	..	XX	XX	..	XX	..	XX	B	B	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	B	B	B	XX	B	XX	B	XX	XX	XX	B	B
H	XX	XX	XX	XX	..	B	B	B	XX	XX	XX	XX

BIO-NUMBER : 3761-3767-3777-7776-1535-6777-7777-7577

SPECIES IDENTIFICATION : AEROMONAS TROTA DNA GROUP 13

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS TROTA DNA GROUP 13	1.000	0.000	0.229	4.353
	2)c VIBRIO FLUVIALIS II	0.000	3.112	0.708	5.525
	3)c VIBRIO FURNISSII	0.000	3.417	0.844	3.444
	4)c AEROMONAS VERONII DNA GROUP 10	0.000	3.714	0.813	3.081
	5)c VIBRIO METSCHNIKOVII	0.000	4.913	1.656	4.606
	6)c VIBRIO FLUVIALIS I	0.000	4.977	1.125	2.800
	7)c VIBRIO ALGINOLYTICUS	0.000	5.208	1.313	3.050
	8)c CITROBACTER AMALONATICUS	0.000	5.212	0.938	4.181
	9)c VIBRIO CARCHARIAE	0.000	5.642	1.188	7.931
	10)c CEDECEA LAPAGEI	0.000	5.839	1.031	6.119
	other :	-----	-----	-----	-----

MICROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.TROTA HG13
Strain # : ATCC49657 RPT
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	XX	XX	XX	XX	XX	XX	XX	..	..	..	XX
B	..	XX	XX	XX	B	XX	B	B	..	XX	XX	XX
C	..	XX	XX	B	B	XX	B	XX	XX	B	XX	B
D	B	XX	XX	B	B	B	XX	B	B	XX	XX	..
E	..	..	XX	XX	..	XX	..	XX	B	B	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	B	B	B	XX	B	XX	B	XX	XX	XX	B	B
H	XX	XX	XX	XX	..	B	B	B	XX	XX	XX	XX

BIO-NUMBER : 3761-3767-3777-7776-1535-6777-7777-7577

SPECIES IDENTIFICATION : AEROMONAS TROTA DNA GROUP 13

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS TROTA DNA GROUP 13	1.000	0.000	0.229	4.353
	2)c VIBRIO FLUVIALIS II	0.000	3.112	0.708	5.525
	3)c VIBRIO FURNISSII	0.000	3.417	0.844	3.444
	4)c AEROMONAS VERONII DNA GROUP 10	0.000	3.714	0.813	3.081
	5)c VIBRIO METSCHNIKOVII	0.000	4.913	1.656	4.606
	6)c VIBRIO FLUVIALIS I	0.000	4.977	1.125	2.800
	7)c VIBRIO ALGINOLYTICUS	0.000	5.208	1.313	3.050
	8)c CITROBACTER AMALONATICUS	0.000	5.212	0.938	4.181
	9)c VIBRIO CARCHARIAE	0.000	5.642	1.188	7.931
	10)c CEDECEA LAPAGEI	0.000	5.839	1.031	6.119
	other :	-----	-----	-----	-----

MICROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.CAVIAE HG4
Strain # : NCIMB13016
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	XX	..	XX
B	..	XX	..	XX	B	XX	..	B	B	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	XX
D	B	B	B	..	..	..	XX	..	..	B	B	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	XX	XX	..	..	..	..	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	..	..

BIO-NUMBER : 1725-2737-3173-7046-0101-6037-0134-7414

GENUS IDENTIFICATION : AEROMONAS

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.460	2.979	2.469	5.912
	2)c AEROMONAS CAVIAE DNA GROUP 4	0.256	3.173	1.500	4.872
	3)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.070	3.600	2.281	7.375
	4)c AEROMONAS MEDIA DNA GROUP 5B	0.005	4.454	0.771	3.331
	5)c AEROMONAS VERONII DNA GROUP 10	0.001	4.929	0.813	3.081
	6)c VIBRIO CHOLERAE	0.000	5.921	2.094	3.869
	7)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	6.037	1.771	4.213
	8)c VIBRIO METSCHNIKOVII	0.000	6.836	1.656	4.606
	9)c VIBRIO CINCINNATIENSIS	0.000	7.558	0.094	0.838
	10)c VIBRIO PARAHAEMOLYTICUS	0.000	7.578	1.719	4.191
	other :	-----	-----	-----	-----

ICROLOG (TM) 1 RELEASE 3.50

ate : 06/14/99
our : 24
late Type : GN
edia Type : TSA/BUGM
late # : 0
train Name : A.CAVIAE HG4
train # : NCIMB13016
ther Info : ENVIRONMENTAL D/B
nput Mode : Well-By-Well
ata Base : MicroLog GN

OSITIVE/NEGATIVE DATA
'XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	XX	..	XX
B	..	XX	..	XX	B	XX	..	B	B	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	XX
D	B	B	B	..	..	..	XX	..	..	B	B	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	XX	XX	..	..	..	..	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	..	..

BIO-NUMBER : 1725-2737-3173-7046-0101-6037-0134-7414

GENUS IDENTIFICATION : AEROMONAS

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.460	2.979	2.469	5.912
	2)c AEROMONAS CAVIAE DNA GROUP 4	0.256	3.173	1.500	4.872
	3)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.070	3.600	2.281	7.375
	4)c AEROMONAS MEDIA DNA GROUP 5B	0.005	4.454	0.771	3.331
	5)c AEROMONAS VERONII DNA GROUP 10	0.001	4.929	0.813	3.081
	6)e VIBRIO ANGUILLARUM	0.000	5.327	1.384	3.194
	7)c VIBRIO CHOLERAE	0.000	5.921	2.094	3.869
	8)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	6.037	1.771	4.213
	9)c VIBRIO METSCHNIKOVII	0.000	6.836	1.656	4.606
	10)e VIBRIO DIAZOTROPHICUS	0.000	7.462	0.688	3.194
	other :	-----	-----	-----	-----

MICROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.ENCHELIAE
Strain # : CECT4342T
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	B	B	B	B	..	B	..	..	..	..
B	..	B	..	..	..	XX	..	..	..	B	..	B
C	..	..	B	..	..	..	B	B	..	..	B	B
D	B	..	..	..	..	..	..	..	..	..	..	..
E	..	..	B	..	..	..	..	B	..	..	..	B
F	..	..	..	..	B	..	B	B	B	XX	B	B
G	..	..	..	..	..	..	..	B	B	..	..	..
H	B	XX	B	B	..	..	..	..	B	..	..	B

BIO-NUMBER : 1720-2105-1063-4000-1021-0277-0030-7411

SPECIES IDENTIFICATION : AEROMONAS DNA GROUP 11

	CLOSEST SPECIES :	SIM.	DIST.	AVG.	MAX
=>	1)c AEROMONAS DNA GROUP 11	0.684	0.000	0.813	3.819
	2)c PASTEURELLA ANATIPESTIFER	0.242	0.346	0.813	4.525
	3)c CDC GROUP EF-4	0.021	1.154	1.156	3.175
	4)c KINGELLA KINGAE	0.012	1.355	0.348	2.506
	5)c AEROMONAS SALMONICIDA SS SALMONICIDA	0.008	1.471	0.438	1.781
	6)e XANTHOMONAS ORYZAE PV ORYZAE E	0.008	1.481	0.500	1.356
	7)c ACINETOBACTER RADIORESISTENS/GENOSPCS 12	0.002	1.960	0.406	7.581
	8)e PHOTOBACTERIUM PHOSPHOREUM	0.002	2.000	0.900	5.786
	9)c CAPNOCYTOPHAGA GINGIVALIS	0.002	2.000	0.461	4.669
	10)e XANTHOMONAS ORYZAE PV ORYZICOLA A	0.002	2.000	0.979	4.931
	other :	-----	-----	-----	-----

ICROLOG (TM) 1 RELEASE 3.50

ate : 06/14/99
our : 24
late Type : GN
edia Type : TSA/BUGM
late # : 0
train Name : A.ENTEROPELOGENES
train # : NCIMB13209
ther Info : ENVIRONMENTAL D/B
nput Mode : Well-By-Well
ata Base : MicroLog GN

OSITIVE/NEGATIVE DATA
XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	XX	XX	XX	XX	XX	XX	XX	..	..	..	XX
B	..	XX	XX	XX	B	XX	..	B	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	B
D	B	XX	XX	..	..	..	XX	..	..	XX	XX	..
E	..	..	XX	XX	..	XX	..	XX	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	B	B	B	XX	B	XX	..	XX	XX	XX	B	B
H	XX	XX	XX	XX	..	..	..	B	XX	XX	XX	XX

BIO-NUMBER : 3761-3727-3173-7046-1521-6777-7737-7437

SPECIES IDENTIFICATION : AEROMONAS TROTA DNA GROUP 13

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS TROTA DNA GROUP 13	0.938	0.912	0.229	4.353
	2)c AEROMONAS VERONII DNA GROUP 10	0.000	3.714	0.813	3.081
	3)e VIBRIO ANGUILLARUM	0.000	4.992	1.384	3.194
	4)c VIBRIO ALGINOLYTICUS	0.000	5.249	1.313	3.050
	5)c VIBRIO FLUVIALIS II	0.000	5.420	0.708	5.525
	6)e VIBRIO MEDITERRANEI	0.000	5.463	0.781	5.819
	7)c VIBRIO METSCHNIKOVII	0.000	5.502	1.656	4.606
	8)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.000	6.152	2.469	5.912
	9)c AEROMONAS HYDROPHILA DNA GROUP 1	0.000	6.198	1.167	3.400
	10)e VIBRIO AESTUARIANUS	0.000	6.239	1.000	1.325
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.EUCRENOPHILA HG6
Strain # : NCIMB74
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	XX	..	XX
B	..	XX	..	XX	B	XX	..	B	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	XX
D	B	..	..	..	..	..	XX	..	..	..	B	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	XX	XX	..	..	..	..	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	XX	XX

ID-NUMBER : 1725-2727-3173-4042-0001-6037-0134-7417

SPECIES IDENTIFICATION : AEROMONAS EUCRENOPHILA DNA GROUP 6

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.818	0.854	2.281	7.375
	2)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.118	1.497	2.469	5.912
	3)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.001	3.207	1.771	4.213
	4)c AEROMONAS VERONII DNA GROUP 10	0.000	3.581	0.813	3.081
	5)c AEROMONAS MEDIA DNA GROUP 5B	0.000	3.875	0.771	3.331
	6)e VIBRIO ANGUILLARUM	0.000	5.040	1.384	3.194
	7)c AEROMONAS JANDAEI DNA GROUP 9	0.000	5.322	1.292	3.237
	8)c AEROMONAS CAVIAE DNA GROUP 4	0.000	6.175	1.500	4.872
	9)c VIBRIO CHOLERAE	0.000	6.376	2.094	3.869
	10)c VIBRIO PARAHAEMOLYTICUS	0.000	6.578	1.719	4.191
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.MEDIA HG5A
Strain # : NCIMB2237
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	XX	..	XX
B	..	XX	..	XX	B	XX	..	B	B	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	XX
D	B	B	B	..	..	..	XX	..	..	B	B	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	XX	XX	..	..	..	..	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	B	..

IO-NUMBER : 1725-2737-3173-7046-0101-6037-0134-7416

GENUS IDENTIFICATION : AEROMONAS

	CLOSEST SPECIES :	SIM.	DIST.	AVG.	MAX
=>	1)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.406	2.529	2.469	5.912
	2)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.328	2.600	2.281	7.375
	3)c AEROMONAS CAVIAE DNA GROUP 4	0.058	3.173	1.500	4.872
	4)c AEROMONAS MEDIA DNA GROUP 5B	0.025	3.454	0.771	3.331
	5)c AEROMONAS VERONII DNA GROUP 10	0.006	3.929	0.813	3.081
	6)e VIBRIO ANGUILLARUM	0.000	4.856	1.384	3.194
	7)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	5.037	1.771	4.213
	8)c VIBRIO CHOLERAE	0.000	5.617	2.094	3.869
	9)c VIBRIO METSCHNIKOVII	0.000	5.836	1.656	4.606
	10)c VIBRIO PARAHAEMOLYTICUS	0.000	6.578	1.719	4.191
	other : AER.SAL	0.000	14.062	0.500	2.037

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.MEDIA HG5A
Strain # : NCIMB2237
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
"..+ = negative, "=>" ID choice positive > 90% of time
"XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	XX	..	XX
B	..	XX	..	XX	B	XX	..	B	B	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	XX
D	B	B	B	..	..	..	XX	..	..	B	B	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	XX	XX	..	..	..	..	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	B	..

BIO-NUMBER : 1725-2737-3173-7046-0101-6037-0134-7416

GENUS IDENTIFICATION : AEROMONAS

CLOSEST SPECIES :	SIM	DIST	AVG	MAX
> 1)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.407	2.529	2.469	5.912
2)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.328	2.600	2.281	7.375
3)c AEROMONAS CAVIAE DNA GROUP 4	0.058	3.173	1.500	4.872
4)c AEROMONAS MEDIA DNA GROUP 5B	0.025	3.454	0.771	3.331
5)c AEROMONAS VERONII DNA GROUP 10	0.006	3.929	0.813	3.081
6)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	5.037	1.771	4.213
7)c VIBRIO CHOLERAE	0.000	5.617	2.094	3.869
8)c VIBRIO METSCHNIKOVII	0.000	5.836	1.656	4.606
9)c VIBRIO PARAHAEMOLYTICUS	0.000	6.578	1.719	4.191
10)c AEROMONAS JANDAEI DNA GROUP 9	0.000	6.985	1.292	3.237
other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.VERONII SOBRIA HG8
Strain # : NCIMB37
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	XX	XX	..	..	..	..
B	..	XX	..	XX	B	XX	..	..	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	B	..	XX	XX
D	B	B	..	..	..	..	XX	..	..	..	..	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	XX	..	..	B	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	XX	XX

IO-NUMBER : 3760-2707-3173-6040-0001-6777-4534-7417

SPECIES IDENTIFICATION : AEROMONAS VERONII/SOBRIA DNA GROUP 8

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.573	0.835	1.771	4.213
	2)c AEROMONAS VERONII DNA GROUP 10	0.332	1.016	0.813	3.081
	3)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.018	1.989	0.417	5.887
	4)c AEROMONAS HYDROPHILA DNA GROUP 1	0.008	2.271	1.167	3.400
	5)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.005	2.382	2.469	5.912
	6)c AEROMONAS JANDAEI DNA GROUP 9	0.001	2.977	1.292	3.237
	7)c VIBRIO METSCHNIKOVII	0.000	3.704	1.656	4.606
	8)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.000	3.763	2.281	7.375
	9)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 3	0.000	4.445	1.281	4.669
	10)c VIBRIO PARAHAEMOLYTICUS	0.000	4.463	1.719	4.191
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.VERONII SOBRIA HG8
Strain # : NCIMB37
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	XX	XX	..	..	..	..
B	..	XX	..	XX	B	XX	..	..	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	B	..	XX	XX
D	B	B	..	..	..	..	XX	..	..	..	..	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	XX	..	..	B	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	XX	XX

IO-NUMBER : 3760-2707-3173-6040-0001-6777-4534-7417

SPECIES IDENTIFICATION : AEROMONAS VERONII/SOBRIA DNA GROUP 8

	CLOSEST SPECIES :	SIM.	DIST.	AVG.	MAX
=>	1)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.566	0.835	1.771	4.213
	2)c AEROMONAS VERONII DNA GROUP 10	0.328	1.016	0.813	3.081
	3)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.017	1.989	0.417	5.887
	4)e VIBRIO ANGUILLARUM	0.009	2.192	1.384	3.194
	5)c AEROMONAS HYDROPHILA DNA GROUP 1	0.007	2.271	1.167	3.400
	6)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.005	2.382	2.469	5.912
	7)c AEROMONAS JANDAEI DNA GROUP 9	0.001	2.977	1.292	3.237
	8)e VIBRIO AESTUARIANUS	0.000	3.286	1.000	1.325
	9)c VIBRIO METSCHNIKOVII	0.000	3.704	1.656	4.606
	10)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.000	3.763	2.281	7.375
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.VERONII HG10
Strain # : NCIMB13015
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	XX	XX	..	..	..	..
B	..	XX	..	XX	B	XX	..	..	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	B	..	XX	XX
D	B	B	..	..	..	..	XX	..	..	..	B	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	XX	..	..	B	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	XX	XX

IO-NUMBER : 3760-2707-3173-6042-0101-6777-4534-7417

SPECIES IDENTIFICATION : AEROMONAS VERONII DNA GROUP 10

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS VERONII DNA GROUP 10	0.724	0.769	0.813	3.081
	2)c AEROMONAS HYDROPHILA DNA GROUP 1	0.159	1.271	1.167	3.400
	3)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.049	1.664	1.771	4.213
	4)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.002	2.699	0.417	5.887
	5)c VIBRIO METSCHNIKOVII	0.002	2.704	1.656	4.606
	6)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.002	2.738	2.469	5.912
	7)c VIBRIO PARAHAEMOLYTICUS	0.000	3.463	1.719	4.191
	8)c VIBRIO ALGINOLYTICUS	0.000	3.780	1.313	3.050
	9)c AEROMONAS JANDAEI DNA GROUP 9	0.000	3.977	1.292	3.237
	10)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.000	4.509	2.281	7.375
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.VERONII HG10
Strain # : NCIMB13015
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	XX	XX	..	..	..	..
B	..	XX	..	XX	B	XX	..	..	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	B	..	XX	XX
D	B	B	..	..	..	..	XX	..	..	..	B	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	XX	..	..	B	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	XX	XX

IO-NUMBER : 3760-2707-3173-6042-0101-6777-4534-7417

SPECIES IDENTIFICATION : AEROMONAS VERONII DNA GROUP 10

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS VERONII DNA GROUP 10	0.724	0.769	0.813	3.081
	2)c AEROMONAS HYDROPHILA DNA GROUP 1	0.159	1.271	1.167	3.400
	3)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.049	1.664	1.771	4.213
	4)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.002	2.699	0.417	5.887
	5)c VIBRIO METSCHNIKOVII	0.002	2.704	1.656	4.606
	6)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.002	2.738	2.469	5.912
	7)c VIBRIO PARAHAEMOLYTICUS	0.000	3.463	1.719	4.191
	8)c VIBRIO ALGINOLYTICUS	0.000	3.780	1.313	3.050
	9)c AEROMONAS JANDAEI DNA GROUP 9	0.000	3.977	1.292	3.237
	10)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.000	4.509	2.281	7.375
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.BESTARIUM HG2
Strain # : ?
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	XX	XX	..	XX	..	..
B	..	XX	..	XX	B	XX	..	..	..	XX	XX	XX
C	..	XX	XX	..	B	XX	XX	XX	B	..	XX	XX
D	B	B	..	B	..	..	XX	..	..	..	B	..
E	..	..	..	..	..	XX	..	B	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	XX	..	..	B	..	B	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	B	..	..	XX	XX	XX	XX

IO-NUMBER : 3764-2707-3373-6442-0121-6777-4534-7517

SPECIES IDENTIFICATION : AEROMONAS HYDROPHILA DNA GROUP 1

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS HYDROPHILA DNA GROUP 1	0.833	0.124	1.167	3.400
	2)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.142	0.710	0.417	5.887
	3)c AEROMONAS VERONII DNA GROUP 10	0.006	1.769	0.813	3.081
	4)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.002	2.141	2.469	5.912
	5)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.001	2.375	1.771	4.213
	6)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 3	0.000	2.755	1.281	4.669
	7)c VIBRIO PARAHAEMOLYTICUS	0.000	2.894	1.719	4.191
	8)c VIBRIO METSCHNIKOVII	0.000	3.115	1.656	4.606
	9)c AEROMONAS MEDIA DNA GROUP 5B	0.000	3.421	0.771	3.331
	10)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.000	3.441	2.281	7.375
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.BESTARIUM HG2
Strain # : ?
Other Info : ENVIRONMENTAL D\B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	XX	XX	..	XX	..	..
B	..	XX	..	XX	B	XX	..	..	..	XX	XX	XX
C	..	XX	XX	..	B	XX	XX	XX	B	..	XX	XX
D	B	B	..	B	..	..	XX	..	..	..	B	..
E	..	..	..	..	..	XX	..	B	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	XX	..	..	B	..	B	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	B	..	..	XX	XX	XX	XX

IO-NUMBER : 3764-2707-3373-6442-0121-6777-4534-7517

SPECIES IDENTIFICATION : AEROMONAS HYDROPHILA DNA GROUP 1

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS HYDROPHILA DNA GROUP 1	0.800	0.124	1.167	3.400
	2)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.137	0.710	0.417	5.887
	3)e VIBRIO ANGUILLARUM	0.036	1.151	1.384	3.194
	4)c AEROMONAS VERONII DNA GROUP 10	0.006	1.769	0.813	3.081
	5)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.002	2.141	2.469	5.912
	6)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.001	2.375	1.771	4.213
	7)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 3	0.000	2.755	1.281	4.669
	8)c VIBRIO PARAHAEMOLYTICUS	0.000	2.894	1.719	4.191
	9)e VIBRIO AESTUARIANUS	0.000	2.962	1.000	1.325
	10)c VIBRIO METSCHNIKOVII	0.000	3.115	1.656	4.606
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.HYDROPHILA HG1
Strain # : NCIMB9240
Other Info : ENVIRONMENTAL D\B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	XX	XX	..	XX	..	..
B	..	XX	..	XX	B	XX	..	B	B	XX	XX	XX
C	..	XX	XX	..	B	XX	XX	XX	B	..	XX	XX
D	B	B	..	B	..	..	XX	..	..	B	B	..
E	..	..	B	..	..	XX	..	B	..	..	..	XX
F	XX	XX	..	B	B	XX	XX	XX	XX	XX	XX	XX
G	XX	..	..	B	..	B	..	XX	XX	B	..	B
H	XX	XX	XX	XX	..	B	..	..	XX	XX	XX	XX

IO-NUMBER : 3764-2737-3373-6446-1121-6777-4535-7517

SPECIES IDENTIFICATION : AEROMONAS HYDROPHILA DNA GROUP 1

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS HYDROPHILA DNA GROUP 1	0.846	0.000	1.167	3.400
	2)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.100	0.710	0.417	5.887
	3)e VIBRIO ANGUILLARUM	0.027	1.137	1.384	3.194
	4)c AEROMONAS VERONII DNA GROUP 10	0.008	1.528	0.813	3.081
	5)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 3	0.002	1.999	1.281	4.669
	6)c AEROMONAS MEDIA DNA GROUP 5B	0.002	2.000	0.771	3.331
	7)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.002	2.071	1.771	4.213
	8)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.001	2.141	2.469	5.912
	9)e VIBRIO AESTUARIANUS	0.001	2.216	1.000	1.325
	10)c VIBRIO METSCHNIKOVII	0.000	2.471	1.656	4.606
	other :	-----	-----	-----	-----

MICROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.HYDROPHILA HG1
Strain # : NCIMB9240
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	XX	XX	..	XX	..	..
B	..	XX	..	XX	B	XX	..	B	B	XX	XX	XX
C	..	XX	XX	..	B	XX	XX	XX	B	..	XX	XX
D	B	B	..	B	..	..	XX	..	..	B	B	..
E	..	..	B	..	..	XX	..	B	..	..	..	XX
F	XX	XX	..	B	B	XX	XX	XX	XX	XX	XX	XX
G	XX	..	..	B	..	B	..	XX	XX	B	..	B
H	XX	XX	XX	XX	..	B	..	..	XX	XX	XX	XX

ID-NUMBER : 3764-2737-3373-6446-1121-6777-4535-7517

SPECIES IDENTIFICATION : AEROMONAS HYDROPHILA DNA GROUP 1

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS HYDROPHILA DNA GROUP 1	0.873	0.000	1.167	3.400
	2)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.103	0.710	0.417	5.887
	3)c AEROMONAS VERONII DNA GROUP 10	0.009	1.528	0.813	3.081
	4)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 3	0.002	1.999	1.281	4.669
	5)c AEROMONAS MEDIA DNA GROUP 5B	0.002	2.000	0.771	3.331
	6)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.002	2.071	1.771	4.213
	7)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.001	2.141	2.469	5.912
	8)c VIBRIO METSCHNIKOVII	0.001	2.471	1.656	4.606
	9)c AEROMONAS CAVIAE DNA GROUP 4	0.000	2.802	1.500	4.872
	10)c VIBRIO PARAHAEMOLYTICUS	0.000	2.894	1.719	4.191
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

```
)ate          : 06/14/99
lour          : 24
)late Type    : GN
ledia Type    : TSA/BUGM
)late #       : 0
;train Name   : A.ENCHELIAE HG
;train #      : CECT4342T
)ther Info    : CLINICAL DATABASE
[nput Mode    : Well-By-Well
)ata Base     : MicroLog GN
```

?OSITIVE/NEGATIVE DATA
'XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	B	B	B	B	..	B	..	..	..	..
B	..	B	..	..	..	XX	..	..	..	B	..	B
C	..	..	B	..	..	..	B	B	..	..	B	B
D	B	..	..	..	..	..	..	..	..	..	..	..
E	..	..	B	..	..	..	..	B	..	..	..	B
F	..	..	..	..	B	..	B	B	B	XX	B	B
G	..	..	..	..	..	..	..	B	B	..	..	..
H	B	XX	B	B	..	..	..	..	B	..	..	B

IO-NUMBER : 1720-2105-1063-4000-1021-0277-0030-7411

SPECIES IDENTIFICATION : AEROMONAS DNA GROUP 11

	CLOSEST SPECIES :	SIM.	DIST.	AVG.	MAX
=>	1)c AEROMONAS DNA GROUP 11	0.694	0.000	0.813	3.819
	2)c PASTEURELLA ANATIPESTIFER	0.245	0.346	0.813	4.525
	3)c CDC GROUP EF-4	0.021	1.154	1.156	3.175
	4)c KINGELLA KINGAE	0.012	1.355	0.348	2.506
	5)c AEROMONAS SALMONICIDA SS SALMONICIDA	0.008	1.471	0.438	1.781
	6)c ACINETOBACTER RADIORESISTENS/GENOSPCS 12	0.002	1.960	0.406	7.581
	7)c CAPNOCYTOPHAGA GINGIVALIS	0.002	2.000	0.461	4.669
	8)c SHEWANELLA PUTREFACIENS A	0.001	2.069	1.396	4.781
	9)c AEROMONAS SALMONICIDA	0.001	2.162	0.500	2.037
	10)c AEROMONAS SALMONICIDA SS ACHROMOGENES	0.001	2.241	0.094	0.606
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

ate : 06/14/99
our : 24
late Type : GN
edia Type : TSA/BUGM
late # : 0
train Name : A.TROTA HG13
train # : ATCC49657
ther Info : ENVIRONMENTAL D/B
nput Mode : Well-By-Well
ata Base : MicroLog GN

OSITIVE/NEGATIVE DATA
XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

1	2	3	4	5	6	7	8	9	10	11	12
..	XX	XX	XX	XX	XX	XX	XX	..	..	..	XX
..	XX	XX	XX	B	XX	B	B	..	XX	XX	XX
..	XX	XX	B	B	XX	B	XX	XX	B	XX	B
B	XX	XX	B	B	B	XX	B	B	XX	XX	..
..	..	XX	XX	..	XX	..	XX	B	B	..	XX
XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
B	B	B	XX	B	XX	B	XX	XX	XX	B	B
XX	XX	XX	XX	..	B	B	B	XX	XX	XX	XX

IO-NUMBER : 3761-3767-3777-7776-1535-6777-7777-7577

SPECIES IDENTIFICATION : AEROMONAS TROTA DNA GROUP 13

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS TROTA DNA GROUP 13	1.000	0.000	0.229	4.353
	2)c VIBRIO FLUVIALIS II	0.000	3.112	0.708	5.525
	3)c VIBRIO FURNISSII	0.000	3.417	0.844	3.444
	4)c AEROMONAS VERONII DNA GROUP 10	0.000	3.714	0.813	3.081
	5)e VIBRIO MEDITERRANEI	0.000	4.585	0.781	5.819
	6)c VIBRIO METSCHNIKOVII	0.000	4.913	1.656	4.606
	7)c VIBRIO FLUVIALIS I	0.000	4.977	1.125	2.800
	8)e VIBRIO ANGUILLARUM	0.000	4.992	1.384	3.194
	9)e VIBRIO PROTEOLYTICUS	0.000	5.000	0.094	1.944
	10)c VIBRIO ALGINOLYTICUS	0.000	5.208	1.313	3.050
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

te : 06/14/99
ur : 24
ate Type : GN
dia Type : TSA/BUGM
ate # : 0
rain Name : A.TROTA HG13
rain # : ATCC49657
her Info : CLINICAL DATABASE
put Mode : Well-By-Well
ta Base : MicroLog GN

SITIVE/NEGATIVE DATA
X" = positive, "B" = borderline, ".." = negative
.+ = negative, "=>" ID choice positive > 90% of time
X- = positive, "=>" ID choice positive < 10% of time

1	2	3	4	5	6	7	8	9	10	11	12
..	XX	XX	XX	XX	XX	XX	XX	..	..	..	XX
..	XX	XX	XX	B	XX	B	B	..	XX	XX	XX
..	XX	XX	B	B	XX	B	XX	XX	B	XX	B
B	XX	XX	B	B	B	XX	B	B	XX	XX	..
..	..	XX	XX	..	XX	..	XX	B	B	..	XX
XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
B	B	B	XX	B	XX	B	XX	XX	XX	B	B
XX	XX	XX	XX	..	B	B	B	XX	XX	XX	XX

IO-NUMBER : 3761-3767-3777-7776-1535-6777-7777-7577

SPECIES IDENTIFICATION : AEROMONAS TROTA DNA GROUP 13

CLOSEST SPECIES :	SIM.	DIST.	AVG.	MAX
1)c AEROMONAS TROTA DNA GROUP 13	1.000	0.000	0.229	4.353
2)c VIBRIO FLUVIALIS II	0.000	3.112	0.708	5.525
3)c VIBRIO FURNISSII	0.000	3.417	0.844	3.444
4)c AEROMONAS VERONII DNA GROUP 10	0.000	3.714	0.813	3.081
5)c VIBRIO METSCHNIKOVII	0.000	4.913	1.656	4.606
6)c VIBRIO FLUVIALIS I	0.000	4.977	1.125	2.800
7)c VIBRIO ALGINOLYTICUS	0.000	5.208	1.313	3.050
8)c CITROBACTER AMALONATICUS	0.000	5.212	0.938	4.181
9)c VIBRIO CARCHARIAE	0.000	5.642	1.188	7.931
10)c CEDECEA LAPAGEI	0.000	5.839	1.031	6.119
other :	-----	-----	-----	-----

_CROLOG (TM) 1 RELEASE 3.50

)ate : 06/14/99
Hour : 24
?late Type : GN
Media Type : TSA/BUGM
?late # : 0
Strain Name : A.SOBRIA HG7
Strain # : NCIMB12065
)ther Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
)ata Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	..	..	XX	XX	..	..	..	..
B	..	XX	..	..	XX	XX	..	..	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	B
D	..	..	..	..	..	..	XX	..	..	..	..	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	..	..	..	..	..	B	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	..	..	..	XX	..	..	..
H	..	XX	XX	..	..	..	..	..	XX	XX	XX	XX

_IO-NUMBER : 3460-2307-3173-0040-0001-0137-0010-3017

SPECIES IDENTIFICATION : AEROMONAS SOBRIA DNA GROUP 7

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS SOBRIA DNA GROUP 7	1.000	0.000	0.930	5.309
	2)c VIBRIO CHOLERAE	0.000	4.543	2.094	3.869
	3)c VIBRIO VULNIFICUS	0.000	5.358	1.750	5.375
	4)c VIBRIO MIMICUS	0.000	6.723	1.156	3.156
	5)c VIBRIO DAMSELA	0.000	7.250	1.125	3.775
	6)e VIBRIO SPLENDIDUS 2	0.000	7.309	0.896	3.969
	7)e ERWINIA AMYLOVORA A	0.000	7.533	1.063	5.166
	8)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	7.954	1.771	4.213
	9)c AEROMONAS JANDAEI DNA GROUP 9	0.000	8.300	1.292	3.237
	10)c AEROMONAS SCHUBERTII DNA GROUP 12	0.000	8.816	1.031	4.156
	other :				

_CROLOG (TM) 1 RELEASE 3.50

)ate : 06/14/99
Iour : 24
)late Type : GN
Iedia Type : TSA/BUGM
)late # : 0
Strain Name : A.ENTEROPELOGENES
Strain # : NCIMB13209
)ther Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
)ata Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	XX	XX	XX	XX	XX	XX	XX	..	..	..	XX
B	..	XX	XX	XX	B	XX	..	B	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	B
D	B	XX	XX	..	..	..	XX	..	..	XX	XX	..
E	..	..	XX	XX	..	XX	..	XX	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	B	B	B	XX	B	XX	..	XX	XX	XX	B	B
H	XX	XX	XX	XX	..	..	..	B	XX	XX	XX	XX

_IO-NUMBER : 3761-3727-3173-7046-1521-6777-7737-7437

SPECIES IDENTIFICATION : AEROMONAS TROTA DNA GROUP 13

	CLOSEST SPECIES :	SIM.	DIST.	AVG.	MAX
=>	1)c AEROMONAS TROTA DNA GROUP 13	0.938	0.912	0.229	4.353
	2)c AEROMONAS VERONII DNA GROUP 10	0.000	3.714	0.813	3.081
	3)e VIBRIO ANGUILLARUM	0.000	4.992	1.384	3.194
	4)c VIBRIO ALGINOLYTICUS	0.000	5.249	1.313	3.050
	5)c VIBRIO FLUVIALIS II	0.000	5.420	0.708	5.525
	6)e VIBRIO MEDITERRANEI	0.000	5.463	0.781	5.819
	7)c VIBRIO METSCHNIKOVII	0.000	5.502	1.656	4.606
	8)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.000	6.152	2.469	5.912
	9)c AEROMONAS HYDROPHILA DNA GROUP 1	0.000	6.198	1.167	3.400
	10)e VIBRIO AESTUARIANUS	0.000	6.239	1.000	1.325
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

ate : 06/14/99
lour : 24
late Type : GN
ledia Type : TSA/BUGM
late # : 0
train Name : A.ENTEROPELOGENES
train # : NCIMB13209
ther Info : CLINICAL DATABASE
nput Mode : Well-By-Well
ata Base : MicroLog GN

OSITIVE/NEGATIVE DATA
'XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	XX	XX	XX	XX	XX	XX	XX	..	..	..	XX
B	..	XX	XX	XX	B	XX	..	B	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	B
D	B	XX	XX	..	..	..	XX	..	..	XX	XX	..
E	..	..	XX	XX	..	XX	..	XX	..	..	..	XX
F	XX	XX	..	B	XX	XX	XX	XX	XX	XX	XX	XX
G	B	B	B	XX	B	XX	..	XX	XX	XX	B	B
H	XX	XX	XX	XX	..	..	..	B	XX	XX	XX	XX

IO-NUMBER : 3761-3727-3173-7046-1521-6777-7737-7437

SPECIES IDENTIFICATION : AEROMONAS TROTA DNA GROUP 13

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS TROTA DNA GROUP 13	0.938	0.912	0.229	4.353
	2)c AEROMONAS VERONII DNA GROUP 10	0.000	3.714	0.813	3.081
	3)c VIBRIO ALGINOLYTICUS	0.000	5.249	1.313	3.050
	4)c VIBRIO FLUVIALIS II	0.000	5.420	0.708	5.525
	5)c VIBRIO METSCHNIKOVII	0.000	5.502	1.656	4.606
	6)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.000	6.152	2.469	5.912
	7)c AEROMONAS HYDROPHILA DNA GROUP 1	0.000	6.198	1.167	3.400
	8)c VIBRIO FURNISSII	0.000	6.417	0.844	3.444
	9)c VIBRIO CARCHARIAE	0.000	6.642	1.188	7.931
	10)c AEROMONAS MEDIA DNA GROUP 5B	0.000	6.851	0.771	3.331
	other :	-----	-----	-----	-----

_CROLOG (TM) 1 RELEASE 3.50

ate : 06/14/99
our : 24
late Type : GN
edia Type : TSA/BUGM
late # : 0
train Name : A.SCHUBERTII HG12
train # : NCIMB13161
ther Info : ENVIRONMENTAL D/B
nput Mode : Well-By-Well
ata Base : MicroLog GN

OSITIVE/NEGATIVE DATA
'XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	B	XX	..	B	..	..
B	..	XX	..	B	..	XX	..	..	..	XX	B	XX
C	..	XX	XX	..	..	B	B	XX	..	..	B	B
D	..	..	..	..	..	..	B	..	..	B	..	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	B	..	..	B	B	XX	XX	B	B	B	B	B
G	..	..	..	..	..	..	..	B	XX	..	..	..
H	..	XX	B	..	..	B	..	..	B	B	XX	XX

_IO-NUMBER : 3764-2507-3163-0044-0101-4777-0030-3117

SPECIES IDENTIFICATION : AEROMONAS SCHUBERTII DNA GROUP 12

	CLOSEST SPECIES :	SIM.	DIST.	AVG.	MAX
=>	1)c AEROMONAS SCHUBERTII DNA GROUP 12	0.997	0.000	1.031	4.156
	2)c VIBRIO CHOLERAE	0.002	2.089	2.094	3.869
	3)c VIBRIO VULNIFICUS	0.000	2.577	1.750	5.375
	4)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	2.762	1.771	4.213
	5)c AEROMONAS JANDAEI DNA GROUP 9	0.000	2.827	1.292	3.237
	6)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.000	3.838	2.469	5.912
	7)e VIBRIO ANGUILLARUM	0.000	3.921	1.384	3.194
	8)e ERWINIA AMYLOVORA A	0.000	4.007	1.063	5.166
	9)c VIBRIO DAMSELA	0.000	4.454	1.125	3.775
	10)e JANTHINOBACTERIUM LIVIDUM A	0.000	5.105	0.625	4.137
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

ate : 06/14/99
our : 24
late Type : GN
edia Type : TSA/BUGM
late # : 0
train Name : A.SCHUBERTII HG12
train # : NCIMB13161
ther Info : CLINICAL DATABASE
nput Mode : Well-By-Well
ata Base : MicroLog GN

OSITIVE/NEGATIVE DATA
'XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	B	XX	..	B	..	..
B	..	XX	..	B	..	XX	..	..	..	XX	B	XX
C	..	XX	XX	..	..	B	B	XX	..	..	B	B
D	..	..	..	..	..	..	B	..	..	B	..	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	B	..	..	B	B	XX	XX	B	B	B	B	B
G	..	..	..	..	..	..	..	B	XX	..	..	..
H	..	XX	B	..	..	B	..	..	B	B	XX	XX

IO-NUMBER : 3764-2507-3163-0044-0101-4777-0030-3117

SPECIES IDENTIFICATION : AEROMONAS SCHUBERTII DNA GROUP 12

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS SCHUBERTII DNA GROUP 12	0.997	0.000	1.031	4.156
	2)c VIBRIO CHOLERAE	0.002	2.089	2.094	3.869
	3)c VIBRIO VULNIFICUS	0.000	2.577	1.750	5.375
	4)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	2.762	1.771	4.213
	5)c AEROMONAS JANDAEI DNA GROUP 9	0.000	2.827	1.292	3.237
	6)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.000	3.838	2.469	5.912
	7)c VIBRIO DAMSELA	0.000	4.454	1.125	3.775
	8)c AEROMONAS VERONII DNA GROUP 10	0.000	5.265	0.813	3.081
	9)c VIBRIO PARAHAEMOLYTICUS	0.000	5.267	1.719	4.191
	10)c SHIGELLA DYSENTERIAE	0.000	5.397	1.600	4.813
	other :				

CROLOG (TM) 1 RELEASE 3.50

ate : 06/14/99
our : 24
ate Type : GN
edia Type : TSA/BUGM
late # : 0
train Name : A.CAVIAE HG4
train # : NCIMB13016
ther Info : CLINICAL DATABASE
nput Mode : Well-By-Well
ta Base : MicroLog GN

OSITIVE/NEGATIVE DATA
XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

1	2	3	4	5	6	7	8	9	10	11	12
..	..	XX	XX	XX	XX	..	XX	..	XX	..	XX
..	XX	..	XX	B	XX	..	B	B	XX	XX	XX
..	XX	XX	..	..	XX	XX	XX	XX	..	XX	XX
B	B	B	..	..	..	XX	..	..	B	B	..
..	..	..	..	..	XX	..	..	..	..	..	XX
XX	XX	..	..	..	..	..	XX	XX	XX	XX	XX
..	..	..	..	..	XX	..	XX	XX	XX	..	..
XX	XX	XX	XX	..	..	..	..	XX	XX	..	..

IO-NUMBER : 1725-2737-3173-7046-0101-6037-0134-7414

GENUS IDENTIFICATION : AEROMONAS

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.460	2.979	2.469	5.912
	2)c AEROMONAS CAVIAE DNA GROUP 4	0.256	3.173	1.500	4.872
	3)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.070	3.600	2.281	7.375
	4)c AEROMONAS MEDIA DNA GROUP 5B	0.005	4.454	0.771	3.331
	5)c AEROMONAS VERONII DNA GROUP 10	0.001	4.929	0.813	3.081
	6)c VIBRIO CHOLERAE	0.000	5.921	2.094	3.869
	7)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	6.037	1.771	4.213
	8)c VIBRIO METSCHNIKOVII	0.000	6.836	1.656	4.606
	9)c VIBRIO CINCINNATIENSIS	0.000	7.558	0.094	0.838
	10)c VIBRIO PARAHAEMOLYTICUS	0.000	7.578	1.719	4.191
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

ate : 06/14/99
lour : 24
'late Type : GN
'edia Type : TSA/BUGM
late # : 0
train Name : A.JANDAEI HG9
train # : ATCC49568
)ther Info : CLINICAL DATABASE
nput Mode : Well-By-Well
)ata Base : MicroLog GN

?OSITIVE/NEGATIVE DATA
'XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	B	XX	..	..	..	..
B	..	XX	..	XX	B	XX	..	..	..	XX	XX	XX
C	B	XX	XX	..	..	B	..	XX	B	..	XX	B
D	B	B	B	B	..	..	XX	..	..	..	B	..
E	..	..	..	..	..	..	..	..	..	..	..	B
F	B	B	..	B	B	B	B	XX	XX	B	XX	XX
G	B	..	B	B	..	B	..	XX	XX	B	..	..
H	XX	XX	XX	B	..	..	..	..	XX	XX	XX	XX

IO-NUMBER : 3760-2707-7133-7442-0001-6777-5534-7417

SPECIES IDENTIFICATION : AEROMONAS JANDAEI DNA GROUP 9

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS JANDAEI DNA GROUP 9	0.935	0.000	1.292	3.237
	2)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.046	1.000	1.771	4.213
	3)c AEROMONAS VERONII DNA GROUP 10	0.006	1.652	0.813	3.081
	4)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.005	1.746	2.281	7.375
	5)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.001	2.342	2.469	5.912
	6)c YERSINIA ALDOVAE	0.001	2.488	1.063	6.881
	7)c VIBRIO PARAHAEMOLYTICUS	0.000	2.577	1.719	4.191
	8)c VIBRIO METSCHNIKOVII	0.000	2.794	1.656	4.606
	9)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.000	2.837	0.417	5.887
	10)c AEROMONAS HYDROPHILA DNA GROUP 1	0.000	3.105	1.167	3.400
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.EUCRENOPHILA HG6
Strain # : NCIMB74
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	XX	..	XX
B	..	XX	..	XX	B	XX	..	B	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	XX
D	B	..	..	..	..	..	XX	..	..	..	B	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	XX	XX	..	..	..	..	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	XX	XX

IO-NUMBER : 1725-2727-3173-4042-0001-6037-0134-7417

SPECIES IDENTIFICATION : AEROMONAS EUCRENOPHILA DNA GROUP 6

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.818	0.854	2.281	7.375
	2)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.118	1.497	2.469	5.912
	3)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.001	3.207	1.771	4.213
	4)c AEROMONAS VERONII DNA GROUP 10	0.000	3.581	0.813	3.081
	5)c AEROMONAS MEDIA DNA GROUP 5B	0.000	3.875	0.771	3.331
	6)c AEROMONAS JANDAEI DNA GROUP 9	0.000	5.322	1.292	3.237
	7)c AEROMONAS CAVIAE DNA GROUP 4	0.000	6.175	1.500	4.872
	8)c VIBRIO CHOLERAE	0.000	6.376	2.094	3.869
	9)c VIBRIO PARAHAEMOLYTICUS	0.000	6.578	1.719	4.191
	10)c VIBRIO VULNIFICUS	0.000	6.975	1.750	5.375
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.SOBRIA HG7
Strain # : NCIMB12065
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	..	..	XX	XX	..	..	..	..
B	..	XX	..	..	XX	XX	..	..	..	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	B
D	..	..	..	..	..	..	XX	..	..	..	..	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	..	..	..	..	..	B	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	..	..	..	XX	..	..	..
H	..	XX	XX	..	..	..	..	..	XX	XX	XX	XX

BIO-NUMBER : 3460-2307-3173-0040-0001-0137-0010-3017

SPECIES IDENTIFICATION : AEROMONAS SOBRIA DNA GROUP 7

	CLOSEST SPECIES :	SIM.	DIST.	AVG.	MAX
=>	1)c AEROMONAS SOBRIA DNA GROUP 7	1.000	0.000	0.930	5.309
	2)c VIBRIO CHOLERAE	0.000	4.543	2.094	3.869
	3)c VIBRIO VULNIFICUS	0.000	5.358	1.750	5.375
	4)c VIBRIO MIMICUS	0.000	6.723	1.156	3.156
	5)c VIBRIO DAMSELA	0.000	7.250	1.125	3.775
	6)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	7.954	1.771	4.213
	7)c AEROMONAS JANDAEI DNA GROUP 9	0.000	8.300	1.292	3.237
	8)c AEROMONAS SCHUBERTII DNA GROUP 12	0.000	8.816	1.031	4.156
	9)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.000	9.404	2.469	5.912
	10)c AEROMONAS VERONII DNA GROUP 10	0.000	9.816	0.813	3.081
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.SALMONICIDA HG3
Strain # : ?
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	..	..	..
B	..	XX	..	XX	..	XX	..	..	..	XX	XX	XX
C	..	..	XX	..	..	XX	..	..	..	..	XX	B
D	..	..	..	..	..	..	..	..	..	..	..	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	..	B-	..	B	B-	XX	XX	XX	B-	XX	XX	XX
G	..	..	B	..	..	..	..	XX	XX	..	..	B-
H	XX	XX	XX	XX	..	..	..	..	XX	..	..	..

IO-NUMBER : 1720-2507-1103-0000-0001-2777-1031-7410

SPECIES IDENTIFICATION : AEROMONAS SALMONICIDA

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
	1)c AEROMONAS SALMONICIDA	0.938	0.096	0.500	2.037
=>	2)c AEROMONAS SALMONICIDA SS SALMONICIDA	0.052	1.054	0.438	1.781
	3)c AEROMONAS DNA GROUP 11	0.000	6.845	0.813	3.819
	4)c VIBRIO CHOLERAE	0.000	7.559	2.094	3.869
	5)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	7.912	1.771	4.213
	6)c AEROMONAS SALMONICIDA SS MASOUCIDA	0.000	7.938	1.406	3.066
	7)c AEROMONAS JANDAEI DNA GROUP 9	0.000	8.429	1.292	3.237
	8)c VIBRIO VULNIFICUS	0.000	8.642	1.750	5.375
	9)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.000	8.987	2.469	5.912
	10)c AEROMONAS SCHUBERTII DNA GROUP 12	0.000	9.093	1.031	4.156
	other : AER.SAL	0.938	0.096	0.500	2.037

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.SALMONICIDA HG3
Strain # : ?
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
XX" = positive, "B" = borderline, ".." = negative
.+ = negative, "=>" ID choice positive > 90% of time
X- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	..	..	..
B	..	XX	..	XX	..	XX	..	..	..	XX	XX	XX
C	..	..	XX	..	..	XX	..	..	..	..	XX	B
D	..	..	..	..	..	..	..	..	..	..	..	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	..	B-	..	B	B-	XX	XX	XX	B-	XX	XX	XX
G	..	..	B	..	..	..	..	XX	XX	..	..	B-
H	XX	XX	XX	XX	..	..	..	..	XX	..	..	..

BIO-NUMBER : 1720-2507-1103-0000-0001-2777-1031-7410

SPECIES IDENTIFICATION : AEROMONAS SALMONICIDA

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
	1)c AEROMONAS SALMONICIDA	0.938	0.096	0.500	2.037
=>	2)c AEROMONAS SALMONICIDA SS SALMONICIDA	0.052	1.054	0.438	1.781
	3)e VIBRIO SPLENDIDUS 2	0.000	5.114	0.896	3.969
	4)e VIBRIO PELAGIUS I	0.000	6.581	0.667	7.250
	5)c AEROMONAS DNA GROUP 11	0.000	6.845	0.813	3.819
	6)e VIBRIO CAMPBELLI	0.000	6.962	0.500	2.213
	7)c VIBRIO CHOLERAE	0.000	7.559	2.094	3.869
	8)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	7.912	1.771	4.213
	9)c AEROMONAS SALMONICIDA SS MASOUCIDA	0.000	7.938	1.406	3.066
	10)c AEROMONAS JANDAEI DNA GROUP 9	0.000	8.429	1.292	3.237
	other : AER.SAL	0.938	0.096	0.500	2.037

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.CAVIAE HG4
Strain # : NCIMB13016
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	XX	..	XX
B	..	XX	..	XX	B	XX	..	B	B	XX	XX	XX
C	..	XX	XX	..	..	XX	XX	XX	XX	..	XX	XX
D	B	B	B	..	..	..	XX	..	..	B	B	..
E	..	..	..	..	..	XX	..	..	..	..	..	XX
F	XX	XX	..	..	..	..	..	XX	XX	XX	XX	XX
G	..	..	..	..	..	XX	..	XX	XX	XX	..	..
H	XX	XX	XX	XX	..	..	..	..	XX	XX	..	..

IO-NUMBER : 1725-2737-3173-7046-0101-6037-0134-7414

GENUS IDENTIFICATION : AEROMONAS

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.460	2.979	2.469	5.912
	2)c AEROMONAS CAVIAE DNA GROUP 4	0.256	3.173	1.500	4.872
	3)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.070	3.600	2.281	7.375
	4)c AEROMONAS MEDIA DNA GROUP 5B	0.005	4.454	0.771	3.331
	5)c AEROMONAS VERONII DNA GROUP 10	0.001	4.929	0.813	3.081
	6)e VIBRIO ANGUILLARUM	0.000	5.327	1.384	3.194
	7)c VIBRIO CHOLERAE	0.000	5.921	2.094	3.869
	8)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	6.037	1.771	4.213
	9)c VIBRIO METSCHNIKOVII	0.000	6.836	1.656	4.606
	10)e VIBRIO DIAZOTROPHICUS	0.000	7.462	0.688	3.194
	other :	-----	-----	-----	-----

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.ENCHELIAE HG
Strain # : CECT4342T
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	B	B	B	B	..	B	..	..	..	..
B	..	B	..	..	..	XX	..	..	..	B	..	B
C	..	..	B	..	..	..	B	B	..	..	B	B
D	B	..	..	..	..	..	..	..	..	..	..	..
E	..	..	B	..	..	..	..	B	..	..	..	B
F	..	..	..	..	B	..	B	B	B	XX	B	B
G	..	..	..	..	..	..	..	B	B	..	..	..
H	B	XX	B	B	..	..	..	..	B	..	..	B

IO-NUMBER : 1720-2105-1063-4000-1021-0277-0030-7411

SPECIES IDENTIFICATION : AEROMONAS DNA GROUP 11

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
>	1)c AEROMONAS DNA GROUP 11	0.684	0.000	0.813	3.819
	2)c PASTEURELLA ANATIPESTIFER	0.242	0.346	0.813	4.525
	3)c CDC GROUP EF-4	0.021	1.154	1.156	3.175
	4)c KINGELLA KINGAE	0.012	1.355	0.348	2.506
	5)c AEROMONAS SALMONICIDA SS SALMONICIDA	0.008	1.471	0.438	1.781
	6)e XANTHOMONAS ORYZAE PV ORYZAE E	0.008	1.481	0.500	1.356
	7)c ACINETOBACTER RADIORESISTENS/GENOSPCS 12	0.002	1.960	0.406	7.581
	8)e PHOTOBACTERIUM PHOSPHOREUM	0.002	2.000	0.900	5.786
	9)c CAPNOCYTOPHAGA GINGIVALIS	0.002	2.000	0.461	4.669
	10)e XANTHOMONAS ORYZAE PV ORYZICOLA A	0.002	2.000	0.979	4.931
	other :				

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.JANDAEI HG9
Strain # : ATCC49568
Other Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	B	XX	XX	XX	XX	B	XX	..	..	..	..
B	..	XX	..	XX	B	XX	..	..	..	XX	XX	XX
C	B	XX	XX	..	..	B	..	XX	B	..	XX	B
D	B	B	B	B	..	..	XX	..	..	..	B	..
E	..	..	..	..	..	..	..	..	..	..	..	B
F	B	B	..	B	B	B	B	XX	XX	B	XX	XX
G	B	..	B	B	..	B	..	XX	XX	B	..	..
H	XX	XX	XX	B	..	..	..	..	XX	XX	XX	XX

IO-NUMBER : 3760-2707-7133-7442-0001-6777-5534-7417

SPECIES IDENTIFICATION : AEROMONAS JANDAEI DNA GROUP 9

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS JANDAEI DNA GROUP 9	0.923	0.000	1.292	3.237
	2)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.045	1.000	1.771	4.213
	3)e VIBRIO ANGUILLARUM	0.012	1.432	1.384	3.194
	4)c AEROMONAS VERONII DNA GROUP 10	0.006	1.652	0.813	3.081
	5)c AEROMONAS EUCRENOPHILA DNA GROUP 6	0.005	1.746	2.281	7.375
	6)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.001	2.342	2.469	5.912
	7)c YERSINIA ALDOVAE	0.001	2.488	1.063	6.881
	8)c VIBRIO PARAHAEMOLYTICUS	0.000	2.577	1.719	4.191
	9)c VIBRIO METSCHNIKOVII	0.000	2.794	1.656	4.606
	10)c AEROMONAS HYDROPHILA-LIKE DNA GROUP 2	0.000	2.837	0.417	5.887
	other :				

_CROLOG (TM) 1 RELEASE 3.50

)ate : 06/14/99
Hour : 24
?late Type : GN
Media Type : TSA/BUGM
?late # : 0
Strain Name : A.SALMONICIDA HG3
Strain # : ?
)ther Info : ENVIRONMENTAL D/B
Input Mode : Well-By-Well
)ata Base : MicroLog GN

?OSITIVE/NEGATIVE DATA
'XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	..	..	..
B	..	XX	..	XX	..	XX	..	..	..	XX	XX	XX
C	..	..	XX	..	..	XX	..	..	..	..	XX	B
D	..	..	..	..	..	..	..	..	..	..	..	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	..	B	..	B	B	XX	XX	XX	B	XX	XX	XX
G	..	..	B	..	..	..	..	XX	XX	..	..	B
H	XX	XX	XX	XX	..	..	..	..	XX	..	..	..

_IO-NUMBER : 1720-2507-1103-0000-0001-2777-1031-7410

SPECIES IDENTIFICATION : AEROMONAS SALMONICIDA

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS SALMONICIDA	0.938	0.096	0.500	2.037
	2)c AEROMONAS SALMONICIDA SS SALMONICIDA	0.052	1.054	0.438	1.781
	3)e VIBRIO SPLENDIDUS 2	0.000	5.114	0.896	3.969
	4)e VIBRIO PELAGIUS I	0.000	6.581	0.667	7.250
	5)c AEROMONAS DNA GROUP 11	0.000	6.845	0.813	3.819
	6)e VIBRIO CAMPBELLI	0.000	6.962	0.500	2.213
	7)c VIBRIO CHOLERAE	0.000	7.559	2.094	3.869
	8)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	7.912	1.771	4.213
	9)c AEROMONAS SALMONICIDA SS MASOUCIDA	0.000	7.938	1.406	3.066
	10)c AEROMONAS JANDAEI DNA GROUP 9	0.000	8.429	1.292	3.237
	other : AER.SAL	0.938	0.096	0.500	2.037

CROLOG (TM) 1 RELEASE 3.50

Date : 06/14/99
Hour : 24
Plate Type : GN
Media Type : TSA/BUGM
Plate # : 0
Strain Name : A.SALMONICIDA HG3
Strain # : ?
Other Info : CLINICAL DATABASE
Input Mode : Well-By-Well
Data Base : MicroLog GN

POSITIVE/NEGATIVE DATA
"XX" = positive, "B" = borderline, ".." = negative
..+ = negative, "=>" ID choice positive > 90% of time
XX- = positive, "=>" ID choice positive < 10% of time

	1	2	3	4	5	6	7	8	9	10	11	12
A	..	..	XX	XX	XX	XX	..	XX	..	..	..	..
B	..	XX	..	XX	..	XX	..	..	..	XX	XX	XX
C	..	..	XX	..	..	XX	..	..	..	..	XX	B
D	..	..	..	..	..	..	..	..	..	..	..	..
E	..	..	..	..	..	..	..	..	..	..	..	XX
F	..	B	..	B	B	XX	XX	XX	B	XX	XX	XX
G	..	..	B	..	..	..	..	XX	XX	..	..	B
H	XX	XX	XX	XX	..	..	..	..	XX	..	..	..

IO-NUMBER : 1720-2507-1103-0000-0001-2777-1031-7410

SPECIES IDENTIFICATION : AEROMONAS SALMONICIDA

	CLOSEST SPECIES :	SIM	DIST	AVG	MAX
=>	1)c AEROMONAS SALMONICIDA	0.938	0.096	0.500	2.037
	2)c AEROMONAS SALMONICIDA SS SALMONICIDA	0.052	1.054	0.438	1.781
	3)c AEROMONAS DNA GROUP 11	0.000	6.845	0.813	3.819
	4)c VIBRIO CHOLERAE	0.000	7.559	2.094	3.869
	5)c AEROMONAS VERONII/SOBRIA DNA GROUP 8	0.000	7.912	1.771	4.213
	6)c AEROMONAS SALMONICIDA SS MASOUCIDA	0.000	7.938	1.406	3.066
	7)c AEROMONAS JANDAEI DNA GROUP 9	0.000	8.429	1.292	3.237
	8)c VIBRIO VULNIFICUS	0.000	8.642	1.750	5.375
	9)c AEROMONAS MEDIA-LIKE DNA GROUP 5A	0.000	8.987	2.469	5.912
	10)c AEROMONAS SCHUBERTII DNA GROUP 12	0.000	9.093	1.031	4.156
	other : AER.SAL	0.938	0.096	0.500	2.037