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# Restriction endonuclease analysis of atypical *Aeromonas salmonicida* isolates from goldfish *Carassius auratus*, silver perch *Bidyanus bidyanus*, and greenback flounder *Rhombosolea tapirina* in Australia

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ABSTRACT: Total genomic DNA samples of atypical isolates of *Aeromonas salmonicida* from goldfish *Carassius auratus* L., silver perch *Bidyanus bidyanus* (Mitchell), and greenback flounder *Rhombosolea tapirina* Günther from Australia and isolates from goldfish from the USA and Singapore were digested with the restriction enzyme *Cfo*I, separated in 3.5% acrylamide gels and visualised by silver staining. Isolates from goldfish and silver perch comprised an homogenous group while those from greenback flounder formed a second group. Within the goldfish and silver perch isolates there were minor differences in restriction endonuclease fragment pattern. Isolates from Australian goldfish obtained between 1977 and 1989 were indistinguishable from isolates from goldfish from the USA but slightly different from an isolate from a Singaporean goldfish. These and other data suggest that *A. salmonicida* probably crossed a species barrier from goldfish to silver perch on the same farm but that farmed greenback flounder in Tasmania have acquired a distinct strain of *A. salmonicida* probably from wild-caught marine fish.

KEY WORDS: Atypical *Aeromonas salmonicida* · DNA analysis · Molecular epidemiology · *Carassius auratus* · *Bidyanus bidyanus* · *Rhombosolea tapirina* · Australia

## INTRODUCTION

*Aeromonas salmonicida* is a significant pathogen of many freshwater and marine species of fish and has been reported to occur in most countries around the world. The taxonomy of the bacterium is not fully resolved but currently 4 subspecies are described: *A. salmonicida* ssp. *salmonicida*, *A. salmonicida* ssp. *achromogenes*, *A. salmonicida* ssp. *masoucida*, and *A. salmonicida* ssp. *smithia* (Holt et al. 1994). In addition to the recognised subspecies there is a heterogenous collection of strains which have the essential characteristics of the species but have phenotypic characters unlike those of the named subspecies. *A. salmonicida* ssp. *salmonicida* is the cause of the disease furunculosis in salmonid fish, a septicaemic condition which varies in severity. This subspecies has been arbitrarily termed typical to distinguish this taxon from all other forms of *A. salmonicida*, including the subspecies *achromogenes*, *masoucida* and *smithia*, which together with all other unnamed strains are referred to as atypical members of the species (Austin & Austin 1993). Disease in non-salmonid fish with these atypical strains often is characterised by cutaneous ulceration.

In Australia, an atypical form of *Aeromonas salmonicida* is the cause of goldfish ulcer disease (GUD), an ulcerative dermatitis prevalent in fish in the aquarium trade. Humphrey & Ashburner (1993) documented the events associated with the establishment of atypical *A. salmonicida* in Australia. Following introduction in 1974 with broodstock goldfish *Carassius auratus* L. imported from Japan, *A. salmonicida* became enzootic

on a commercial goldfish farm in Victoria by 1975. It was disseminated with consignments of goldfish from this farm within Victoria and to 4 other Australian states by 1977. The occurrence of *A. salmonicida* in Australia was formally reported in 1980 (Trust et al. 1980) and by 1985 had caused GUD on 2 fish farms in New South Wales and become enzootic in wild goldfish populations (Whittington et al. 1987). In 1992, atypical *A. salmonicida* was isolated from cutaneous ulcers on silver perch *Bidyanus bidyanus* (Mitchell) with ulcerative dermatitis (R. Callinan, S. Rowland & R. Whittington unpubl. data). These fish were from a fish farm on which goldfish with GUD were detected in 1985 (Whittington et al. 1987). Despite apparent eradication of goldfish from the farm at that time, large numbers of clinically normal goldfish and carp *Cyprinus carpio* L. were known to be present on the farm between 1986 and 1992 (Callinan et al. unpubl. data). The farm draws water from a tributary of the Murray-Darling river system in which goldfish and carp are established. Also in 1992, atypical *A. salmonicida* was detected in roach *Rutilus rutilus* L. with ulcerative dermatitis from Lake Burrumbeet, Victoria (Humphrey & Ashburner 1993) by immunofluorescence, but the organism was not isolated (N. Gudkovs pers. comm. 1994). Goldfish with GUD were found in this lake in 1985, but roach examined at that time were unaffected (Whittington et al. 1987).

Isolates of atypical *Aeromonas salmonicida* from GUD lesions are highly pathogenic for Atlantic salmon in laboratory challenge (Carson & Handlinger 1988, Whittington & Cullis 1988), and for this reason goldfish entering Tasmania are subject to quarantine. GUD has been diagnosed in goldfish held in quarantine following importation from Singapore into Tasmania (J. Carson unpubl. data). During 1993, an atypical *A. salmonicida* was recovered from ulcerative dermal lesions and kidney of marine greenback flounder *Rhombosolea tapirina* in Tasmania (Carson unpubl. data). These fish consisted of juvenile hatchery reared and wild-caught fish held in shore-based tanks for experimental grow-out trials.

Genomic characterisation is being used increasingly in epidemiological studies of bacterial pathogens (Pitt 1994) and has also been applied to *Aeromonas salmonicida*. Plasmid profiling (Nielsen et al. 1993), ribotyping (Nielsen et al. 1994), DNA probing (Hennigan et al. 1989, Hiney et al. 1992), restriction endonuclease analysis (McCormick et al. 1990), DNA:DNA reassociation analysis (Belland & Trust 1988) and polymerase chain reaction (Gustafson et al. 1992) have been used to supplement the data available from phenotypic observations such as biochemical reactions and protein or enzyme profiles (e.g Whittington et al. 1987, Boyd et al. 1994). Knowledge of the true affinities of bacterial isolates from different sources enables meaningful assessment of the likely means of disease transmission and may assist in the formulation of disease control strategies.

The aim of this study was to evaluate the genomic relationships of atypical *Aeromonas salmonicida* isolates from different species of fish in Australia which have experienced clinical disease. Specifically, we were interested in determining the likelihood that *A. salmonicida* from goldfish may have crossed species barriers to infect silver perch and greenback flounder.

## MATERIALS AND METHODS

**Strains of *Aeromonas salmonicida*.** Atypical *A. salmonicida* isolates were obtained from reference collections and are detailed in Table 1. Cultural and biochemical characteristics of strains from silver perch and greenback flounder will be published separately with detailed clinical, pathological, and epidemiological data.

**Growth of bacteria.** Lyophilised bacteria were reconstituted in a small volume of tryptic soy broth (Difco), plated onto sheep blood agar (Oxoid), and incubated at 22°C for 6 d. Subcultures were made onto the same medium and incubated for 48 or 96 h (greenback flounder isolates). Bacteria were harvested from the plates using phosphate buffered saline pH 7.2, containing 0.1 mM phenyl methyl sulphonyl fluoride and were washed 3 times in the same buffer. Bacterial pellets were collected by centrifugation at 6500 × *g* and frozen at -20°C.

**DNA isolation.** DNA was isolated from *Aeromonas* spp. isolates using a protocol modified from that of McCormick et al. (1990). Bacterial pellets were resuspended in 8 ml of TEN buffer (10 mM Tris-HCl; 1 mM EDTA; 10 mM NaCl, pH 7.0) by vortexing. Cell lysis was induced by the addition of 50 µl of Proteinase K (10 mg $ml^{-1}$) (Boehringer Mannheim) and 1 ml of SDS 10% w/v (Rhone Poulenc). The cell lysate was incubated for 2 h at 37°C followed by a 55°C incubation overnight. Phenol chloroform extractions and a final chloroform extraction were performed in Becton-Dickinson (Rutherford, New Jersey, USA) serum separator tubes containing silica gel polymer (Tilzer et al. 1989). Solutions containing DNA were dialysed for 3 d against TEN buffer with a fresh change of buffer every 24 h. DNA was precipitated with 0.7 volumes of isopropanol, washed with 70% v/v ethanol followed by 100% v/v ethanol, vacuum dried, and resuspended in TEN buffer DNA purity was assessed by restriction-endonuclease digestion and agarose gel electrophoresis.

Table 1. *Aeromonas salmonicida*. Strains which were evaluated by bacterial restriction endonuclease analysis All locations and sources are in Australia unless otherwise indicated. NSW: New South Wales

| Isolate DNA | Strain | Accession no. | Isolated from: | Location (date) | Source | Reference |
|---|---|---|---|---|---|---|
| 18 | 133 | S8/AS11 | Goldfish skin | Tennessee, USA | N. Gudkovs, AFHRL Victoria. Originally supplied by Professor E. B. Shotts, University of Georgia, USA | |
| 17 | 130 | AS3 Trust 419 | Goldfish skin | USA | N. Gudkovs, AFHRL Victoria. Originally supplied by Professor E. B. Shotts, University of Georgia, USA | Trust et al. (1980) |
| 16 | 171 | 89/5017-3 | Goldfish skin | Singapore (1989) | J. Carson, DPIF Tasmania. Isolated from fish imported from Singapore during quarantine in Tasmania | This study |
| 4 | 168 | F364 1107-1B | Goldfish skin | Victoria (1977) | L. Eaves, DPI Queensland. Originally supplied by N. Gudkovs, AFHRL Victoria, to Dr P. Hanna, Deakin University, Victoria. Isolated from first affected farm in Victoria by Trust and Ashburner | Whittington et al. (1987) |
| 14 | 129 | S4 AS1 Trust 404 | Goldfish skin | Victoria | N. Gudkovs, AFHRL Victoria. Originally supplied by Professor E. B. Shotts, University of Georgia, USA. Isolated from first farm affected in Victoria | Trust et al. (1980) |
| 1 2 | 170 | F365 85/9370A | Goldfish skin | Victoria | L. Eaves, DPI Queensland. Originally supplied by N. Gudkovs, AFHRL, to Dr P. Hanna, Deakin University, Victoria. Probably isolated from first affected farm in Victoria | |
| 7 | 167 | F64 89-142515/5C | Goldfish skin | Queensland (1989) | L. Eaves, DPI Queensland. Isolated in Queensland from fish from a Victorian farm | |
| 19 | 72 | RN85/1923A | Goldfish skin | Narrandera, NSW (1985) | EMAI NSW | Whittington et al. (1987) |
| 3 | 169 | F366 86/674 3339 | Goldfish skin | Lake Burrumbeet, Victoria (1985) | L. Eaves, DPI Queensland. Originally supplied by N. Gudkovs, AFHRL Victoria, to Dr P. Hanna, Deakin University, Victoria | Whittington et al. (1987) |
| | 71 | ON85/1287 | Goldfish skin | Condobolin, NSW (1985) | EMAI NSW | Whittington et al. (1987) |
| 1 | 182 | WN92/2136-1 | Silver perch skin | Condobolin, NSW (1992) | RVL Wollongbar, NSW | This study |
| 10 | 183 | WN92/2136-3 | Silver perch skin | Condobolin, NSW (1992) | RVL Wollongbar, NSW | This study |
| 6 | 184 | WN92/2136-4 | Silver perch skin | Condobolin, NSW (1992) | RVL Wollongbar, NSW | This study |
| 8 | 185 | WN92/2136-5 | Silver perch skin | Condobolin, NSW (1992) | RVL Wollongbar, NSW | This study |
| 12 | 186 | WN92/2136-6 | Silver perch skin | Condobolin, NSW (1992) | RVL Wollongbar, NSW | This study |
| 21 | 192 | 93/1586 BF30 | Flounder skin | Tasmania (1993) | J. Carson, DPIF Tasmania | This study |
| 22 | 193 | 93/956-2 | Flounder skin | Tasmania (1993) | J. Carson, DPIF Tasmania | This study |
| 23 | 194 | 93/1061-3 | Flounder skin | Tasmania (1993) | J. Carson, DPIF Tasmania | This study |
| 24 | 195 | 93/1171-2 | Flounder skin | Tasmania (1993) | J. Carson, DPIF Tasmania | This study |
| 25 | 196 | 93/1061-1 | Flounder skin | Tasmania (1993) | J. Carson, DPIF Tasmania | This study |
| 9 | 149 | H9 *A. hydrophila* | Goldfish skin | NSW | EMAI NSW | This study |

**Restriction-endonuclease digestion of DNA and polyacrylamide gel electrophoresis.** *Aeromonas* spp. DNA was digested to completion by overnight incubation at 37°C with *Cfo*I (*Hha*I) (Boehringer Mannheim) according to the manufacturer's recommendations. Digested DNA (0.5 µg per lane) was loaded onto 14 × 16 cm 3.5% polyacrylamide (acrylamide:methylene bis-acrylamide of 29:1) (Boehringer Mannheim) in tris borate EDTA buffer gels and electrophoresed at 38 to 40 V per gel for up to 17 h using an SE 600 Vertical Slab Gel Unit (Hoefer). Acrylamide solutions were polymerised with an excess of ammonium persulphate (1.25 mg $ml^{-1}$) (Sigma) and TEMED (1 µl $ml^{-1}$) (Bio-Rad) as described by Djordjevic et al. (1994). Molecular weight markers used were a combination of DNA fragments of known size generated by the cleavage of *Bacillus subtilus* phage SPP-1 DNA (Progen) with *Eco*RI, lambda phage DNA (Boehringer) digested with *Hind*III, plasmid pbluescribe DNA (Stratagene) digested with *Pst*I and bromophenol blue which migrates at approximately 100 bp in 3.5% polyacrylamide gels. Polyacrylamide gels were stained with silver using a Rapid Silver Stain kit (ICN Radiochemicals) according to the manufacturer's instructions.

## RESULTS

Digestion of *Aeromonas salmonicida* total DNA with the restriction-endonuclease *Cfo*I followed by electrophoresis through 3.5% polyacrylamide under the conditions stated resolved DNA fragments ranging from approximately 3.5 to 0.1 kb depending on the duration of electrophoresis. Comparisons of *Cfo*I generated the restriction endonuclease fragment patterns (REFP) of *A. salmonicida* electrophoresed for 6 h (Fig. 1) or 14 to 17 h (Figs. 2 & 3) highlighted the following, which will be described in more detail below: (1) the REFP for

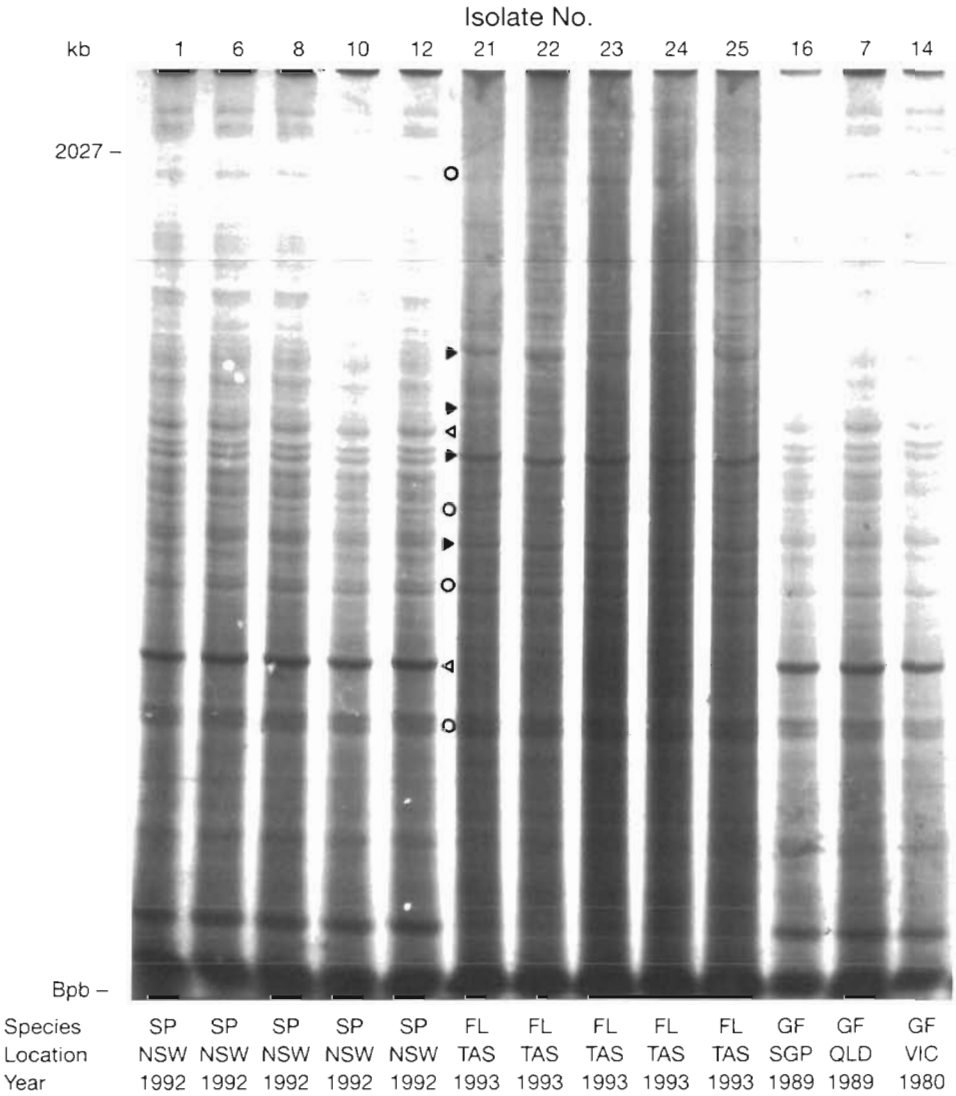


Fig. 1. *Aeromonas salmonicida*. *Cfo*I generated DNA restriction endonuclease fragment patterns of *A. salmonicida* isolates from silver perch (1, 6, 8, 10, 12), greenback flounder (21, 22, 23, 24, 25), and goldfish (16, 7, 14). Restriction endonuclease digested DNA fragments were resolved by electrophoresis through a 3.5% polyacrylamide gel matrix for 6 h at 40 V until the bromophenol blue dye reached the bottom of the gel. The gel was stained with silver. Molecular weight makers were lambda phage DNA and bromophenol blue (Bpb). (○) Bands common to all isolates; (►) and (◅) indicate band differences (see 'Results'). SP: silver perch; FL: greenback flounder; GF: goldfish; NSW: New South Wales; TAS: Tasmania; SGP: Singapore; QLD: Queensland; VIC: Victoria

silver perch and goldfish isolates were very similar; (2) the REFP for all 5 greenback flounder isolates were identical but were clearly different from the patterns of silver perch and goldfish isolates; (3) the REFP for silver perch isolates were indistinguishable from one another; (4) minor variations existed among the REFP of goldfish and silver perch isolates; and (5) the REFP of *A. hydrophila* showed no similarity with those of *A. salmonicida* isolates.

The REFP of 5 silver perch, 5 greenback flounder, and 3 goldfish isolates of *Aeromonas salmonicida* over the molecular weight range 2 to 0.1 kb are illustrated in Fig. 1. While the REFP for silver perch and goldfish were indistinguishable there were REFP differences between these isolates and greenback flounder isolates. There were at least 4 restriction endonuclease fragments which were unique to the greenback flounder isolates (filled triangle) and at least 2 restriction endonuclease fragments which were unique to the goldfish and silver perch isolates (open triangle). It is important to note that there were also restriction endonuclease fragments which were common to all *A. salmonicida* isolates (open circle).

The REFP profile in a higher molecular weight region (3.5 to 1.0 kb) is shown in Fig 2. At least 5 restriction endonuclease fragments unique to greenback flounder isolates (open triangle) and at least 6 restriction endonuclease fragments unique to silver perch and goldfish isolates (filled triangle) were identified. The pattern for Singaporean goldfish isolate 16 was slightly different from Victorian goldfish isolates 2 and 3, there being at least 3 band deletions from isolate 16 (closed circle).

Variations in REFP among goldfish and silver perch isolates are highlighted in Fig. 3. Minor variations were resolved between 3.5 and 1.0 kb. The REFP of USA goldfish isolates 18 and 17 were indistinguishable, but they were slightly different from that of Singaporean goldfish isolate 16 in which there were at least 3 band deletions (open triangle). Australian

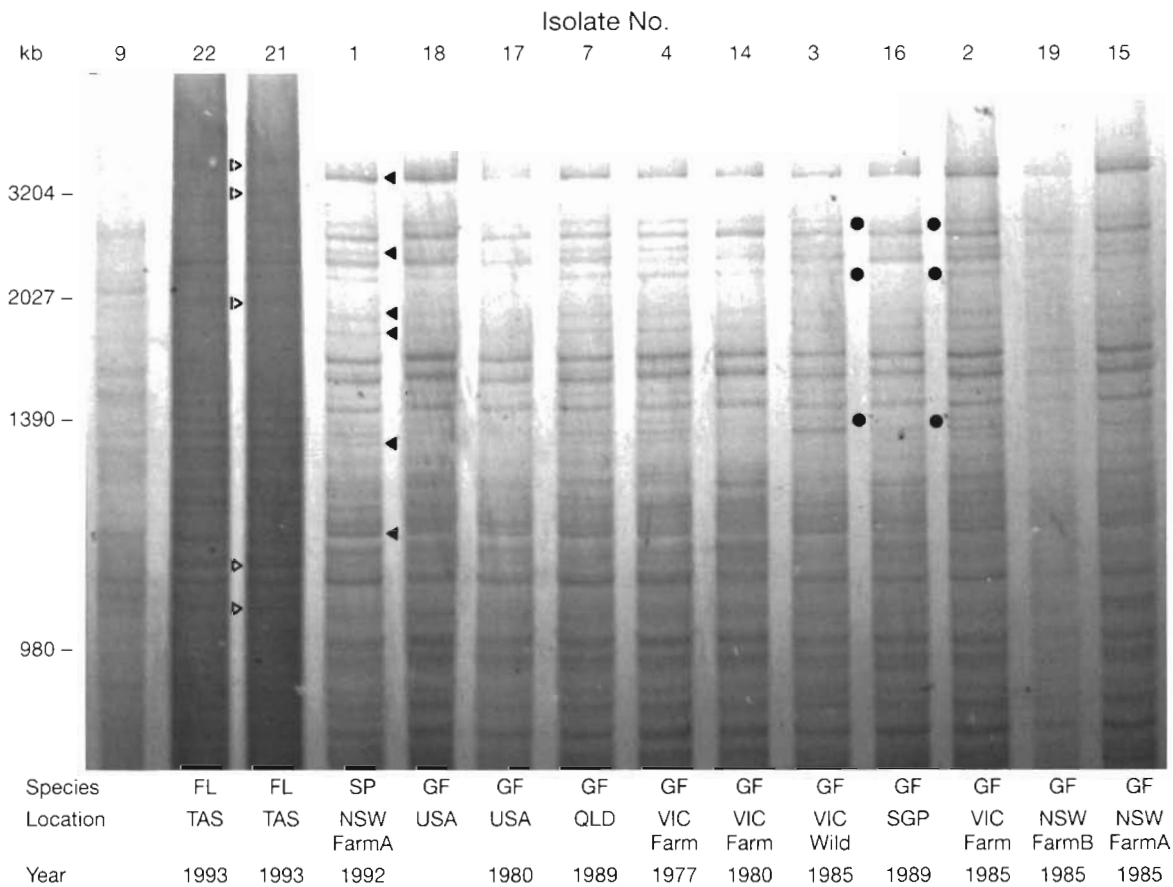


Fig. 2. *Aeromonas hydrophila* and *A. salmonicida*. *Cfo*I generated DNA restriction patterns of *A. hydrophila* (isolate 9) and *A. salmonicida* isolates 22, 21, 1, 18, 17, 7, 4, 14, 3, 16, 2, 19, and 15. Restriction endonuclease digested DNA was resolved by electrophoresis through 3.5% polyacrylamide gel matrix for 17 h at 38 V. Molecular weight markers were digested *Bacillus subtilus* phage SPP-1 DNA, lambda phage DNA and plasmid pbluescribe DNA. (▷), (◀) and (●) indicate band differences (see 'Results'). FL: greenback flounder; SP: silver perch; GF: goldfish; TAS: Tasmania; NSW: New South Wales; USA: United States of America; QLD: Queensland; VIC: Victoria; SGP: Singapore

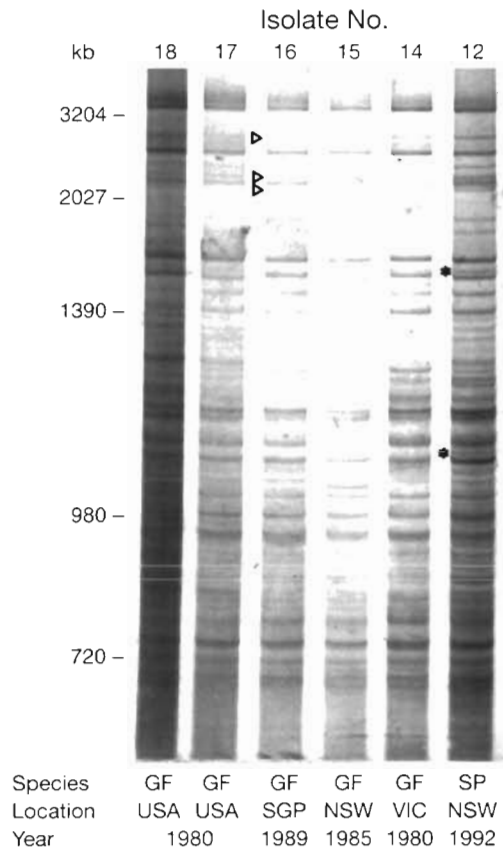


Fig. 3. *Aeromonas salmonicida*. *Cfo*I generated restriction patterns of *A. salmonicida* isolates 18, 17, 16, 15, 14, and 12. Restriction endonuclease digested DNA fragments were resolved through a 3.5% polyacrylamide gel for 14 h at 38 V. (▷) and (✱) indicate band differences (see 'Results'). Molecular weight markers as described for Fig. 2. GF: goldfish; SP: silver perch; USA: United States of America; SGP: Singapore; VIC: Victoria; NSW: New South Wales

goldfish isolates 15 and 14 were, therefore, more similar to the USA isolates than to the Singaporean isolate. Silver perch isolate 12 was also most similar to the USA isolates. The presence of 2 additional fragments (asterisk) was a feature which distinguished these isolates.

The *Cfo*I generated REFP of *Aeromonas hydrophila* was clearly very different from that of *A. salmonicida* isolates with very few fragments comigrating in 3.5% polyacrylamide gels (Fig 2).

## DISCUSSION

The results of this study indicate a high degree of REFP homology between *Aeromonas salmonicida* isolates from goldfish and silver perch consistent with a common genetic origin. In contrast, isolates from greenback flounder were clearly different and belonged to a different subgroup of *A. salmonicida*. Although formulae and criteria have been proposed for assessment of the degree of similarity between REFP (Orskov & Orskov 1983, Kristiansen et al. 1984, 1986), these do not appear to be widely accepted. The most important factor is that point mutations which occur during bacterial replication may change the restriction fragment pattern by deleting a restriction endonuclease recognition site (Binns 1993) and minor variation in REFP (say 1 or 2 bands different in a complex pattern) need not necessarily indicate that isolates are clonally different. In the present study, numerous REFP differences existed between greenback flounder and other isolates of *A. salmonicida* and these were clearly not due to a few point mutations.

Previous studies have confirmed that *Aeromonas salmonicida* isolates can be placed into subgroups based on genomic analysis. McCormick et al. (1990) found that *Eco*RI and *Hind*III REFP of *A. salmonicida* spp. *salmonicida* isolates were distinct from those of *A. salmonicida* spp. *masoucida* and *A. salmonicida* spp. *achromogenes* and found subgroups of possible epizootiological significance. Belland & Trust (1988), using DNA:DNA reassociation analysis, confirmed that atypical *A. salmonicida* were genotypically diverse and distinct from *A. salmonicida* spp. *salmonicida*. Isolates from goldfish from Australia and the USA formed 1 biotype but were distinct from atypical isolates from carp with erythrodermatitis from Europe. In contrast, certain gene probes and polymerase chain reaction procedures have not distinguished within or among atypical and typical isolates of *A. salmonicida* (Gustafson et al. 1992, Hiney et al. 1992).

In the present study minor REFP differences were observed among isolates of *Aeromonas salmonicida* from goldfish and silver perch, but it is not yet clear whether these represent true differences or merely point to mutations which arose during laboratory handling. The low level of REFP difference does not warrant separation of these *A. salmonicida* isolates into subgroups. Isolates from the USA appeared to be nearly identical to Australian isolates while an isolate from Singapore was slightly different, having 3 band deletions. Australian goldfish isolates were obtained over a long period of time (1977 to 1989) from different sources, have been subjected to repeated laboratory subculture with the possibility of genetic mutation, but have retained REFP homology. The identification of minor variation in the REFP of these goldfish isolates may be useful in the future in tracing the spread of a particular isolate of *A. salmonicida* during outbreaks of GUD, but additional studies will be required. While the procedure described in this report was relatively simple to perform and the results were repeatable, the final REFP were quite complex and could not be evaluated without detailed examination. Investigation of the use of other restriction enzymes and DNA probes

would be worthwhile in an attempt to simplify the fragment patterns to enable rapid evaluation of gels. Furthermore, we were unable to overcome dark background staining in lanes containing DNA from greenback flounder isolates of *A. salmonicida*.

The similarity of REFP in atypical strains of *Aeromonas salmonicida* from goldfish and silver perch is reflected in the uniformity of phenotypes of the isolates from both fish species (Whittington et al. 1987, Callinan et al. unpubl. data). This is in contrast to the REFP of the greenback flounder isolates which form a homogenous group but are distinct from the atypical strains from goldfish. This apparent genotypic difference is also seen in the greenback flounder phenotype, which was different from that of goldfish isolates (J. Carson, K. Pinkard, L. Schmidtke unpubl. data) and silver perch isolates.

The silver perch isolates were obtained from diseased fish on a farm on which goldfish with GUD had occurred 7 yr earlier. Isolates from the 2 species of fish were almost indistinguishable despite the interval of 7 yr between occurrence of ulcerative skin disease. This supports the hypothesis that *Aeromonas salmonicida* has crossed a species barrier from goldfish to silver perch. However, it is uncertain how or whether the infection was maintained on the farm during the 7 yr interval between an outbreak of GUD in goldfish and an outbreak of disease in silver perch.

As *Aeromonas salmonicida* isolates from greenback flounder in Tasmania were distinct from other isolates, these fish were most probably infected with *A. salmonicida* from the marine environment through introduction of wild-caught fish. The greenback flounder had no contact with freshwater fish and there is no evidence that *A. salmonicida* has ever occurred in goldfish or other species of freshwater fish outside quarantine facilities in Tasmania.

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