Conservation Genetics of Handfishes: Family Brachionichthyidae

Miles Lawler BSc (Tas)

A thesis submitted to the School of Zoology, University of Tasmania in partial fulfillment of the degree of Bachelor of Science with Honours in Marine, Freshwater and Antarctic Biology

June 1999, Hobart

Abstract:

This study investigated the conservation genetics of the handfishes, Family Brachionichthyidae. Two separate genetic investigations were undertaken. The first examined the population genetics of the spotted handfish, *Brachionichthys hirsutus* and the second examined the molecular phylogeny of the members of the Family Brachionichthyidae.

Population genetics:

A PCR based RFLP (restriction fragment length polymorphism) method was used to investigate the population genetics of the spotted handfish. An approximately 800 base-pair fragment of the mitochondrial D-loop was amplified, via a PCR based reaction, for 82 spotted handfish from four sites across the entire species' range. This fragment was digested with six restriction endonucleases, *HaeIII*, *HinfI*, *AflII*, *BslI*, *BstuI* and *RsaI*. The restriction profiles were compared across the four sites. Genetic distance, sequence divergence and population subdivision were all calculated. Low genetic variation was found with sequence divergence of around 0.06%. A suggested population structure based on the six restriction endonucleases grouped the two lower Derwent River sites together as one population, while the remainder of the sites appeared to represent the ancestral population.

Molecular Phylogeny:

The molecular phylogenetic relationships in the Family Brachionichthyidae were investigated by direct sequencing of the 16S rRNA and cytochrome oxidase I mitochondrial DNA genes. PCR was used to amplify a 614 base-pair fragment of the 16S rRNA and a 543 base-pair fragment of the cytochrome oxidase I genes. Five species were examined, with three of these having alternative species morphs. Parsimony analysis, maximum likelihood and distance analysis were used to infer phylogenetic relationships. The resultant molecular phylogeny supported the status of two genera, *Brachionichthys* and *Sympterichthys*. Based on morphological studies in progress, the molecular phylogeny supports the morphological taxonomy of this family. The species morphs of the Australian handfish, the warty handfish and the red handfish, all appeared to be sub-specific relationships. Further investigation of these species morphs is required.

Acknowledgments:

I wish to thank my supervisor Rob White for his continued advice and support throughout the year. I also wish to thank the other School of Zoology staff, especially Adam Smolenski for his assistance in keeping the lab work on track. Thanks also goes to those who helped with diving for samples.

I am grateful to all the staff at CSIRO who supported me throughout the year. Especially, Mark Green and Barry Bruce for helping with tissue collection, providing handfish pictures and general support. Peter Last for help with taxonomy, supplying some of the harder to get handfishes and more handfish pictures. The genetics crew for advice and Sequencing.

I would like to thank Chris B for his advice on primers and techniques, all in the office who made sitting in front of a computer fun, and the rest of the honours crew, especially the footy boys (and footy girls), the window still stands!

Thanks Doiv and the Woodbridge Marine Discovery Center for the D'Entrecasteaux anglerfish.

Thankyou to all who looked over drafts, especially mum, Lena, Tam and Alex.

Finally I'd like to thank my family for all their support throughout the year.

Abstract

Acknowledgements

Contents

1. General Introduction:

1.1 Conservation genetics:

Extinction is a naturally occurring event, part of the evolutionary process. Only about one in every thousand species are still extant since life first appeared around 3 billion years ago. The vast majority of species become extinct within 10 million years of their appearance (Newman & Roberts 1994). Yet in man's recorded history there is no case of a marine fish ever becoming extinct. There is fossil evidence that shows extinction in the marine environment is not only possible but has occurred. However, until recently there has been the view that the marine environment was so large and homogenous that it would require a large, widespread, destructive event to cause the extinction of a marine fish. Over the past few centuries the continued degradation of our waterways and oceans has proved this conception wrong.

It has been estimated that as high as one-third of fish species, mostly freshwater, are threatened with extinction (IUCN Press Release 1996). In 1996 the spotted handfish, *Brachionichthys hirsutus*, was listed as critically endangered under the IUCN Red List, thus becoming Australia's first recognised endangered marine fish. The IUCN classes species in one of three endangered categories based on changes in population numbers, critically endangered being the worst. Critically endangered species are defined as those which have experienced a minimum of 80% decline in population size. From a genetic view point such a decrease can have serious implications.

A reduction of greater than 80% can cause a serious population bottleneck. This can result in increased demographic stochasticity, rate of inbreeding, loss of genetic variation, and fixation of deleterious alleles and, thereby, reduce adaptive potential and increase the probability of population extinction (Luikart and Cornuet 1998). The effect of inbreeding has been well documented to be detrimental to small populations (Bryant *et al.* 1986). Genetic variation can also be lost through genetic drift. The mechanisms of these will be briefly discussed, then their relevance to the management of small populations will be outlined.

In natural populations there are often small amounts of inbreeding and assortive mating (Crow 1986). The effects become significant when the majority of the population is inbreeding (such as in small populations). Inbreeding leads to two major problems: potential increase of the influence of deleterious recessive genes, which are masked in a large population, surface, and decreased ability of the population to adapt, due to loss of allelic variation. It is commonly accepted that inbreeding leads to increased susceptibility to disease, decreased fecundity, and shortened life span (Ayala 1982, Leberg 1990). However, there is an alternative view that inbreeding can act to eliminate recessive genes, but there is little published evidence for this hypothesis.

The other major process affecting small populations is genetic drift. Genetic drift occurs in all heterozygous populations (Ayala 1982). It is the change in allele frequency over successive generations. In very small populations this can lead to rare alleles being lost relatively rapidly from the population. In extreme cases genetic drift can result in a single allele becoming fixed for a whole population, with results similar to inbreeding (Ayala 1982). It has been suggested that genetic drift has played a major role in the genetic divergence of both anadromous and non-anadromous Atlantic salmon, *Salmo salar*, populations in Newfoundland (Gosling 1994).

Genetic drift and inbreeding both have a disproportional effect on small populations. Therefore it is in the best interests of management to maintain population numbers at such a level where these processes do not greatly affect a population. However, before a population can be properly managed some basic data on its genetic structure and the variation it contains are required. It is therefore important to examine the population structure of endangered species and manage them in a manner that does not affect their genetic makeup, which can have serious consequences.

1.2 Population and stock assessment

Significant, temporally persistent, genetic differences between animals from different locations indicate that a species is subdivided into discrete, reproductively isolated units (non-interbreeding groups) (Ovenden 1990). A major part of any management of a species is to determine the basic population parameters defining it. Genetic studies of

fish population dynamics are becoming increasingly important in the conservation of fisheries resources and endangered fish species. Commercially important and endangered fish species are the major focus of such studies. Considerable effort has been applied to the study of the population structure of native salmonids in the Northern Hemisphere. This group is commercially and recreationally important, and in many of its native regions is considered vulnerable or endangered.

Studies on population genetic structure have greatly increased our knowledge of how populations interact, mechanisms that control stock integrity and migration rates and abilities. Conservation genetic studies aim to compare the amount of variation ofb the species to be conserved (both within and between populations) as well between this and other similar species (similar in habit and biology). The Atlantic cod *Gadus morhua* and the Greenland halibut *Reinhardtius hippoglossoides* are two commercially important species in the north-west Atlantic. The Atlantic cod shows little polymorphism which is put down to a bottleneck during the last glaciation (Carr *et al.* 1995; Carr and Marshall 1991a; Carr and Marshall 1991b), while the halibut exhibits extensive polymorphism and is believed to have maintained large populations during the last glaciation because it can reside in deep waters due to its bathypelagic lifestyle (Vis *et al.* 1997).

1.3 Mitochondrial DNA as a genetic marker

A large number of different techniques have been used in population genetic studies. Early studies focused on allozyme variation to separate populations (Ferguson *et al.* 1995). Recently mitochondrial DNA or microsatellites have become more favoured for population differentiation. As mitochondrial DNA was chosen for this study it will be examined in more detail.

Mitochondrial DNA (mtDNA) is a small closed loop of double stranded DNA which is approximately 16-20 kilobases long in vertebrates (Park and Moran 1995). There are several features of mitochondrial DNA that make it useful for phylogenetic and population level investigations. Firstly it is inherited through the cytoplasm and is non-

recombining. This means in the vast majority of species it is maternally (uniparentally) inherited. This makes it possible to determine parentage.

It is generally accepted that MtDNA has a higher rate of mutation than nuclear DNA (Avise *et al.* 1987*a*). This mutation rate is 4-10 times faster than nuclear DNA (depending on the gene). This higher mutation rate facilitates both the differentiation of closely related species, and the separation of populations. Mitochondrial DNA has an effective population size one quarter that of nuclear DNA. Therefore mitochondrial DNA is more sensitive to genetic drift than nuclear DNA (Park and Moran 1995).

The mitochondrial DNA is tightly packed with genes. There are genes for 13 messenger RNA's (protein genes), 2 ribosomal RNA's, and 22 transfer RNA's. A replication control region is also present which lacks structural genes but contains sequences that initiate replication and transcription. In vertebrates this contains a displacement loop (D-loop), roughly 0.8 kilobases long, with functions in replication (Moritz *et al.* 1987). Unlike nuclear DNA, the mtDNA lacks introns, repetitive DNA, pseudo genes, and even sizeable spacer sequences between genes (Moritz *et al.* 1987). Gene arrangement appears very stable, at least within a taxonomic class or phylum (Meyer 1994).

Most genetic changes in mitochondrial DNA are simple base substitutions; some are small additions or deletions, of one or a few nucleotides; and fewer still involve large length differences, of up to several hundred nucleotides (Moritz *et al.* 1987). The size differences are usually confined to the replication control region of the molecule, which is one of the faster evolving regions. Further, mtDNA mutations arising in different individuals are not recombined during sexual reproduction. Therefore all mutations will be passed onto the next generation. As a result of its fast mutation rate and uniparental mode of inheritance the mitochondrial DNA is excellent for population level work.

1.4 Captive breeding

The International Union for the Conservation of Nature (IUCN) recommends that vertebrate taxa numbering less than 1000 individuals in the wild should be considered for captive breeding programs (Ebenhard 1995). If an animal is able to be reared and induced to breed in artificial environments then it is easy to maintain the population number over the short-term. However, artificial breeding programs must never be considered an effective means for the long-term safeguard of most species (Philippart 1995). Captive breeding is not a substitute for protecting the habitats, even though it can be used to save species threatened with immediate extinction due to habitat degradation (Senanayake and Moyle 1982). Captive breeding is generally used as a last resort or to provide some breathing space.

There are several problems when attempting to set-up a captive breeding program. These include inducing fish to breed in captivity, maintaining their health, and the genetic consequences of their release into the wild. The first two must be addressed if such a program is to succeed (see Reid 1990). However, it would be easy to neglect the genetic consequences of such a program in the rush to boost population numbers. In the set-up of a captive breeding program the development of appropriate methods for genetic management are required (Philippart 1995). These include where the broodstock is to be collected from and released to, the number of individuals in the broodstock, as well as the monitoring of inbreeding and outbreeding.

For an endangered population with only a small number of individuals, removing a large number from the wild for a captive breeding program might be more damaging than not interfering at all. Ideally, the broodstock must maintain enough genetic diversity to provide for adaptive evolutionary changes when the captive individuals are returned to natural conditions (Meffe 1990, Philippart 1995). A minimum breeding population size of 50 individuals has been suggested (Nyman and Ring 1989, Nyman 1993). It should be noted that this is an effective genetic number of individuals and not an absolute one. The effective genetic number refers to only those breeding age individuals that pass genetic information onto the next generation. It assumes equal sex ratio and equal fecundity (Ryman *et al.* 1993). In many cases it may not be

possible to remove such a number of fish as to maintain an effective population size of this order. However, many endangered fish have such low levels of genetic variation that this variation can be maintained with a relatively small effective breeding population.

If the broodstock is too small, genetic drift and inbreeding depression will cause genetic changes compared to the native population. Gharrett and Smoker (1994) found that domestication of culture species may lead to loss of genetic variability, either by purposeful (selective breeding) or inadvertent selection (inbreeding). Therefore a sufficient number of individuals should be used to contribute to the captive breeding gene pool. Populations founded with a small effective population will also show the effects of genetic drift (Ashbaugh *et al.* 1994). The genetic problems associated with captive breeding are compounded if the target species has distinct population structuring. This is especially the case for many freshwater fishes (Ward *et al.* 1994). In this case, knowledge of the genetic structure of the natural population is essential for effective management (Frankel 1974).

If fish of different genetic makeup are released into a wild population and hybridise, outbreeding will result. Outbreeding depression is a process by which natural genetic adaptations can be broken down by the introduction of foreign genes. Outbreeding can cause a decrease in average survival and fitness (Gharrett and Smoker 1991). Outbreeding depression can be facilitated when small distinct populations are supplemented from external breeding sources (such as central captive breeding programs). Another problem with releasing genetically different individuals into a wild population is that these may compete against and replace the local fish. For example in the Baltic Sea, cultivated Atlantic salmon, *Salmo salar*, now comprise 90% of the total Atlantic salmon population (Petersson 1996).

If genetic concerns are considered from the outset, captive breeding can provide an effective short-term solution for management of endangered species. This will ensure that the chance of survival of the target species is greatly increased.

1.5 Study animal:

1.5a Taxonomy:

The Family Brachionichthyidae contains the handfishes or warty-anglers. The handfishes are endemic to southeast Australia. They occur in estuarine and coastal to deep shelf waters. All members of this family exhibit a benthic lifestyle. There are currently two genera supported in this family, *Brachionichthys* and *Sympterichthys*. The handfishes are closely related to the anglerfishes, Family Antennariidae. A full taxonomic classification for the handfishes is as follows.

Phylum	Chordata
Subphylum	Vertebrata
Superclass	Gnathostomata
Grade	Pisces
Class	Osteichthyes
Subclass	Actinopterygii
Infraclass	Neopterygii
Division	Halecostomi
Subdivision	Teleostei
Infradivision	Euteleostei
Superorder	Paracanthopterygii
Order	Lophiiformes
Suborder	Antennarioidei
Family	Brachionichthyidae
Genus	*Brachionichthys*
Genus	*Sympterichthys*

Based on Nelson (1984).

There is much taxonomic confusion surrounding the handfishes. There are currently 8 recognised species in the family of which 6 are endemic to Tasmania (Last *et al.* 1983). However, only three handfishes have been scientifically described: the spotted handfish, *Brachionichthys hirsutus* (Lacépède 1803); the red handfish, *Brachionichthys politus* (Richardson 1848); and the warty handfish, *S. verrucosus* (McCulloch and Waite 1918). The red handifsh was originally classified in the genus *Bachionichthys* (Last *et al.* 1983), however morphological studies in progress closely associate it with the warty handfish (Last pers. comm.). Current thoughts are that it is part of the *Sympterichthys* genus, and will therefore be refered to as *Sympterichthys politus* in this thesis. The other two species examined in this study were the Australian

handfish (voucher number H 3749-01 P) and *Brachionichthys* sp. D (voucher number H4995-01 P); specimens corresponding to these voucher numbers are held in the CSIRO fish collection in Hobart. Apart from the majority of handfish species being undescribed, further taxonomic confusion is caused by colour variation exhibited in several of the species.

Colour morphs have been reported in many species of handfish. The spotted handfish exhibits a diversity of markings from spots to stripes, and also light and dark background colours (Mark Green pers. comm.). The red handfish also shows light and dark colour morphs. The lighter forms are found in the more sheltered waters of Frederick Henry Bay while the darker forms are found in the large bays of coastal southeastern Tasmaia.

Along the Tasmanian southeast coast there are several undescribed handfish species. These include Ziebell's handfish, the Waterfall Bay handfish and Loney's handfish. Ziebell's handfish is pale with varying degrees of yellow on its fins, while the other two species are purple. These three species may all be colour morphs of a single species as there is some circumstantial evidence that they can interbreed (Karen Gowlett-Holmes pers. comm.). All these species are rarely seen due to their low population densities and cryptic colouration. Recently a new species of handfish was discovered off the Tasman Peninsula. This appeared as a red form of the Ziebell's handfish. It is currently known as *Brachionichthys* species D.

In Bass Strait the warty handfish, *Sympterichthys verrucosus*, exhibits two distinct morphs. It has both a reticulate and a non-reticulate form. It has been suggested that these should be classed as different species *S. verrucosus* and *S. "unipennus"*, however their taxonomy is still under review (Last pers. comm.). Likewise, the Australian handfish, *B. "australis"*, has at least two morphs. Two of these were looked at in this study and will be referred to as *B. "australis"* morph 1, which is the typical Australian handfish form, and *B. "australis"* morph 2, which is a slightly different morph. The handfish species examined in this study are shown in Figures 1.1-1.7.

Fig. 1.1 Red handfish- dark morph
Sympterichthys politus

Fig. 1.2 Red handfish- light morph
Sympterichthys politus

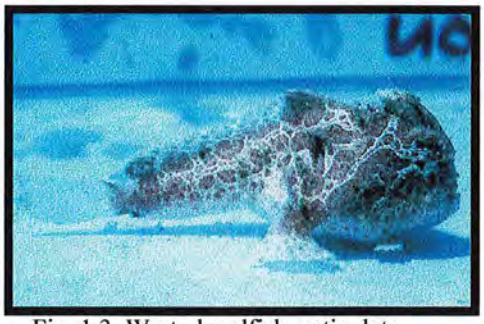


Fig. 1.3 Warty handfish- reticulate
Sympterichthys verrucosus

Fig. 1.4 Warty handfish- non-reticulate
Sympterichthys verrucosus

Fig. 1.5 Spotted handfish
Brachionichthys hirsutus

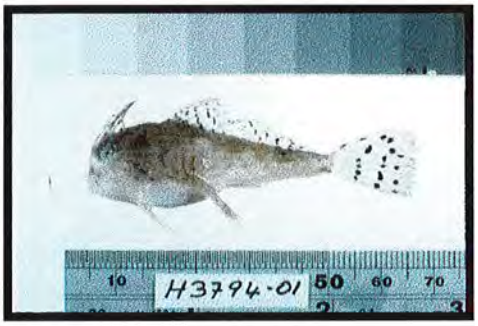


Fig. 1.6 Australian handfish
Voucher No-H3794-01
Brachionichthys "australis"

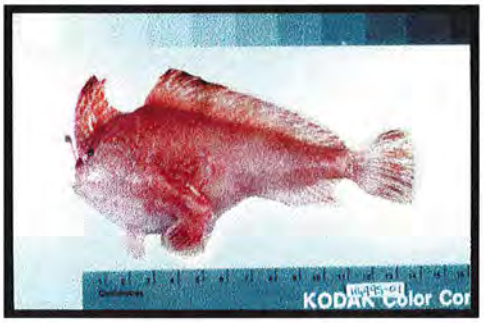


Fig. 1.7 New Species
Voucher No- H4995-01
Brachionichthys sp. D

Figures 1.1 – 1.5 courtesy of Mark Green and Barry Bruce, CSIRO
Figures 1.6 – 1.7 courtesy of Peter Last, CSIRO

1.5b Biology and ecology:

Handfishes are small benthic fishes. Their pectoral fins are modified into hand-like structures which they use to crawl around the bottom. They are distinguished from the closely related angler fishes (Family Antennariidae) by having the second and third dorsal fin spines joined by a membrane (Last *et al.* 1983, Last and Bruce 1996). The first dorsal fin spine is modified into an illicium as in the angler fish; however it does not appear to be used for fishing as in the former group. Handfishes are not covered in true scales, instead they may either be smooth skinned or covered in minute dermal spinules.

The handfishes have limited distributions and low abundances. They are usually encountered by diving and occasionally drawn in scallop dredges. Little is known of their biology, with only the spotted handfish having being recently studied to any degree. It is not known how long any of the handfishes live.

The spotted handfish is the best studied of handfishes. This has only been in the last two decades since its rapid population decline. They occur in 2-30m water but more usually 5-10m, in the deep sheltered bays of the Derwent River estuary, and also the D'Entrecasteaux Channel, Frederick Henry Bay and northern reaches of Storm Bay (Last *et al.* 1983). They grow to around 12 cm and prefers soft sediment, and usually found in close association to structures such as rocks or paper oysters. Spotted handfish primarily feed on polychaete worms, small crustacea and bivalve molluscs (Bruce *et al.* 1998).

Spawning occurs in late winter-early spring, with females laying their egg masses directly on to the substrate in clumps of 80-250 eggs. These eggs are wound around vertical structures, predominantly the stalked ascidian *Sycozoa* sp. in the Derwent River estuary, while seagrasses and sponges are also used throughout the remainder of their range (Bruce *et al.* 1998). Spotted handfish young hatch as fully formed juveniles, with the species having no pelagic larval phase. This, coupled with the low mobility of the adults, means that the spotted handfish has a limited dispersal capability. To a large

extent, the reproductive biology of handfishes may be responsible for their limited distribution.

The spotted handfish was once common throughout the Derwent Estuary (Last *et al.* 1983). During the 1980s they underwent a substantial population decline (Bruce *et al.* 1998). They are currently listed as endangered under Schedule 1 of the Commonwealth Endangered Species Protection Act 1996. This followed a similar listing by the Australian Society for Fish Biology in 1994. They are also protected under Tasmanian Fisheries Legislation 1995. In 1996 the spotted handfish (*Brachionichthys hirsutus*) was listed as critically endangered under the IUCN Red List, giving it the dubious honour of being the first Australian marine fish to be listed as endangered.

The reasons for the decline of the spotted handfish are not known. Coinciding with the decline in the late 1980's was the introduction (presumably via ballast water from Japan) of the North Pacific seastar, *Asterias amurensis* (Last and Bruce 1996). This seastar was initially thought to be preying directly on the handfish or their eggs. However current thinking is that the seastar may be removing the spotted handfish breeding substrate, the stalked ascidian, *Sycozoa* sp. There is no proof of this seastar being the major factor apart from the coincidental timing of its arrival. Sediment toxicity (due to urban development, agriculture and industry) and general river quality have been undergoing a gradual decline since European settlement. The spotted handfish, being a benthic dwelling fish, would be especially affected by sediment toxicity due to its close association with the substrate. Finally natural fluctuations and extinction cycles may be responsible for the demise of the handfish, although if this is the case other factors are probably helping this population decline.

As part of the management and recovery efforts for the spotted handfish a captive breeding program has been initiated with a view to supplementing native populations. Captive breeding has been used for many different fish species, such as cichlids (Reid 1990), cyprinodontids (Pister 1990), and salmonids (Fleming 1994; Philippart 1995). For successful captive breeding the introduced individuals should have minimal effect

on the wild population. Disease, genetic and competition concerns must all be addressed.

For the genetic concerns to be addressed basic data on the amount of genetic variation within spotted handfish needs to be collected. This variation needs to be looked at in terms of within and between population variation to see if there is genetic structuring. The genetic concerns include either reducing genetic variation or introducing new genes into the population. Both these mechanisms can lead to the reduction in fitness and breeding success of the natural population. Reduced genetic variation leads to inbreeding (Gharrett and Smoker 1994), which has detrimental effects on survival, fecundity and general fitness. The introduction of foreign genes into the population can also lead to outbreeding depression. This is where locally adapted allele complexes are broken down resulting in the decreased fitness of an individual and thus a population.

Information on the genetic variation within and between populations as well as population structuring is needed by managers to best maintain the genetic integrity of natural populations. A population with high genetic variation or a large number of rare alleles will need a larger captive breeding population than one with low variation. If there is significant population differentiation then several captive breeding populations may need to be used. Genetic information can also be used to determine which areas are most suitable for re-introductions.

1.6 Summary of problems relating to the Family Brachionichthyidae:

1.6a Population genetics:

1. Quantify the levels of genetic variation in the spotted handfish.
2. Establish if there is population structuring across the spotted handfish's range.

1.6b Systematics:

1. Confirm the status of the two genera, *Sympterichthys* and *Brachionichthys*.
2. Look at the species morphs for the warty handfish, *S. verrucosus*, the red handfish, *S. politus*, and the Australian handfish, *B. "australis"*.
3. Construct a handfish phylogeny

2 Materials and Methods

2.1 Tissues for genetic analysis:

2.1a Tissue collection and preservation:

Spotted handfish tissue was collected from September to November 1998. Tissue was collected by fin-clipping fish during dives on four known colonies in the Derwent Estuary and Frederick Henry Bay. All dives were conducted under the University of Tasmania Diving code (Talbot and Johnson 1998) using DCEIM dive tables. Fin-clipping was trialed on captive handfish during July/August of 1998 and found to have no apparent adverse effects on the fish.

Tissue samples approximately 2 x 2 mm were removed from the posterior margin of the dorsal fin. Fins were clipped using small scissors which were wiped after each clip to help reduce the chances of cross contamination. Clips were placed in small vials under water, and immediately transferred to 100% ethanol upon reaching the surface. These preserved fin-clips were stored at room temperature and out of direct sunlight. It has been highlighted by Dessauer *et al.* (1990) that DNA degradation is reduced if tissues are preserved immediately and not subject to DNA degrading heat or ultraviolet light.

2.1b Specimens:

Tissues for the phylogenetic component of this study came from a variety of sources. Red handfish finclips (*Sympterichthys politus*) were collected during diving for spotted handfish. Whole specimens of Australian handfish (*B. "australis"*) and the warty handfish (*Sympterichthys verrucosus*) collected during CSIRO fisheries surveys during 1996 were also made available for this study. These specimens had been stored frozen at -80° C. Further tissue samples from Australian handfish, warty handfish and several unidentified species were also provided by CSIRO for this project. These samples were also frozen at -80°C but were transferred to 100% ethanol prior to extraction. Tissue was also made available from an unidentified species of handfish (*Brachionichthys* sp. D) that was pulled up in a crayfish pot off the south tip of the Tasman Peninsula.

The D'Entrecasteaux anglerfish, *Trichophryne furcipilis* and Mitchell's angler fish *T. mitchelli,* were used as outgroups. The tissue from the D'Entrecasteaux anglerfish was collected from a specimen at the Woodbridge Marine Discovery Centre, while the Mitchell's anglerfish tissue was collected by CSIRO. Information on collection and storage methods of specimens is given in Table 2.1.

2.2 DNA extraction:

DNA was extracted using a modified CTAB protocol (Hillis *et al.* 1990). This method provides good yields but is generally more demanding than other methods such as chelex extraction (Walsh *et al.* 1991). As spotted handfish are endangered, whole individuals could not be removed from the wild for genetic analysis, therefore tissue samples had to be collected from the animals *in situ.* Due to the size of handfish, only small tissue samples were able to be collected to minimise the risk to the animals. CTAB extraction was used to maximise the yield of DNA obtained from each sample.

Prior to extraction, ethanol preserved material was washed in distilled water to remove excess ethanol. Up to 100 g of tissue was homogenised in 600 μl of CTAB Buffer (0.1M Tris-HCL pH 8.0, 0.02M EDTA, 1.4M NaCl, 55mM hexadecyltrimethyl-ammonium bromide). The tissue was ground in 1.5 ml microcentrifuge tubes using plastic pestles, and the homogenate was briefly vortexed. Five microlitres of proteinase K [20 $mg.ml^{-1}$ in dH_2O] were then added and the homogenate incubated at 65° C. During incubation the samples were reground and vortexed as needed. Samples were incubated a minimum of 60 min.

The homogenate was initially extracted with 1 equivalent volume (600 μl) of chloroform-isoamyl alcohol (24:1). This was mixed well and centrifuged at 13 000 rpm for 20 min. The upper aqueous layer was then removed, extracted with one volume of phenol/chloroform isoamyl (25:24:1), mixed well and centrifuged at the same speed for 10 min. This step was repeated until the upper aqueous layer was totally clear (2-3 extractions). This clear upper aqueous layer was then added to one volume of chloroform-isoamyl alcohol, mixed well and centrifuged for 30 sec at 13 000 rpm. This step removed excess phenol.

Table 2.1. Handfish samples for population and phylogenetic analysis including numbers, collection location, storage details, and details of morph variation if present for that species.

Species	Common Name	Date	Location and type	No	Storage	Collector
Brachionichthys hirsutus	Spotted handfish- Site 1	11/98	Mid Derwent	19	ethanol	M. Lawler
Brachionichthys hirsutus	Spotted handfish- Site 2	11/98	Lower Derwent	21	ethanol	M. Lawler
Brachionichthys hirsutus	Spotted handfish- Site 3	11/98	Lower Derwent	21	ethanol	CSIRO
Brachionichthys hirsutus	Spotted handfish- Site 4	12/98	Frederick Henry Bay	21	ethanol	M. Lawler
Brachioninchthys "australis"	Australian handfish- morph 1	5/96	Disaster Bay NSW	6	-80° C	CSIRO
Brachionichthys "australis"	Australian handfish- morph 2	5/96	Bermagui NSW	20	-80° C	CSIRO
Brachionichthys sp. D	New Species	3/99	Tasman Peninsula	1	ethanol	CSIRO
Sympterichthys politus	Red handfish- light	12/98	Frederick Henry Bay	4	ethanol	M. Lawler
Sympterichthys politus	Red handfish- dark	5/99	Tasman Peninsula	1	ethanol	M. Lawler
Sympterichthys verrucosus	Warty handfish- reticulate	5/96	Bass Strait	2	-80° C	CSIRO
Sympterichthys verrucosus	Warty handfish- non-reticulate	5/96	Bass Strait	2	-80° C	CSIRO
*Trichophryne furcipilis**	D'Entrecasteaux anglerfish	3/99	D'Entrecasteaux Channel	1	ethanol	Woodbridge Marine Discovery Centre
*Trichophryne mitchelli**	Mitchell's anglerfish	5/96	Disaster Bay NSW	1	-80° C	CSIRO

* = outgroup

The DNA was precipitated out in approximately 1.5 volumes of cold (-20° C) isopropanol. The DNA was left to precipitate for several hours at -20° C. This isopropanol mix was then centrifuged at 13 000 rpm for 30 min, allowing the precipitated DNA to form a pellet at the bottom of the tube. The supernatant was removed and 500 μl of cold 70% ETOH was added to the DNA pellet (to remove any excess salt). Again this was centrifuged at 13 000 rpm for 10 min. Finally the DNA pellet was dried under a vacuum and re-suspended in 100 μl distilled H_2O and allowed to rehydrate for several hours at 4° C. The extracted DNA was then stored at -18° C until needed in a PCR reaction.

2.3 Polymerase chain reaction:

2.3a PCR cleanliness and precautions:

All DNA extraction and PCR preparation was carried out in a separate room designated for such activities. This room remained free of amplified PCR products which would otherwise preferentially amplify. Further precautions to stop contamination included the use of autoclaved millicurie water, pipetting tips and the wiping down of surfaces with a diluted bleach solution. Gloves were worn at all times to reduce the risk of human DNA contamination and hair nets were also worn during PCR preparation.

Procedures were carried out in a fume hood to reduce the risk of airborne contamination entering preparations. New aliquots of dinucleotidetriphosphates (dNTPs) and primers were regularly used to prevent the risk of contamination and also because these products have the capacity to become inefficient with time. Magnesium chloride, BSA and 10x buffer were all exposed to short-wave radiation prior to use. This is generally enough to degrade DNA and RNA present in these reagents and therefore reduce the risk of contamination.

All PCR reactions included a positive and negative control. The positive control, orange roughy (*Hoplostethus atlanticus*) DNA, was used to ensure that the PCR reagents were working properly. This species was known to amplify using most primer combinations. A negative control, with no template DNA, was also used to highlight

any DNA contamination of the reagents. If the negative control showed up with amplified PCR product then the PCR experiment would be rerun with fresh reagents.

2.3b PCR amplification:

Target genes were amplified using a two-way PCR reaction (using both light and heavy strand primers). Three gene regions were amplified. The D-loop or replication control region was chosen for the spotted handfish population differentiation. This gene is highly variable and useful for population level work. Two genes were used in the phylogenetic study: 16S rRNA and cytochrome oxidase I. The oligonucleotide primer pairs used are given in Table 2.2.

Table 2.2. Oligonucleotide primer combinations used for the amplification of target handfish gene regions.

Primer	Sequence	Gene
L15995	5' AA CTC TCA CCC CTA RCT CCC AAA G 3'	D-loop
H16498	5' GGC CCT GAA RTA GGA ACC ARA TG 3'	D-loop
16SarL	5' CGC CTG TTT ATC AAA AAC AT 3'	16s rRNA
16SbrH	5' CCG GTC TGA ACT CAG ATC ACG T 3'	16s rRNA
Co1f-L	5' CCT GCA GGA GGA GGA GAY CC 3'	CO I
CO1e-H	5' CCA GCG ATT AGA GGG AAT CAG TG 3'	CO I

PCR was carried out using either a CR FTS 320 Thermal Cycler or more commonly a MJ Research PCT-200 Thermal Cycler. The thermal cycle consisted of a high denaturing phase (~94° C), followed by a low annealing phase (~50° C) allowing the primers to bind, and finally an extension phase (~72° C) where the DNA strand is extended. This cycle was run ~35 times to allow production of adequate DNA template for subsequent processing. As each new strand can be used as a template in subsequent cycles this process allows the exponential amplification of target DNA. The cytochrome oxidase I primers were run at 50° C for 10 cycles then 56° C for the remainder. Temperatures of 55° C and 56° C were used for the D-loop and 16S rRNA primer pairs respectively.

2.3c Gel electrophoresis:

The PCR products were visualised on agarose mini-gels (25 ml of a 1.5% gel in 1xTBE Buffer) using the BRL 'Horizon 58' apparatus. Between 3 and 5 μl of PRC product was mixed with 2 μl of loading buffer (dH_2O, sucrose, 0.25% bromophenol blue) and loaded onto the gel. All samples were run along side a 100 bp size standard to allow estimation of fragment sizes. Gels were run at 80 V for approximately 30 min. The finished gels were stained with approximately 6 μl of 10 $mg.ml^{-1}$ ethidium bromide for 20 min in approximately 100 ml of 1xTBE buffer de-stained for a further 5 min in tap water and viewed under ultra violet light (403 nm). Digital images were recorded for all gels using a Pulnix TM-6CN digital video camera and Mitsubishi video copy processor.

2.4 RFLP- restriction fragment length polymorphism:

The D-loop was the target of the restriction enzyme work. A 800 bp fragment of the D-loop was sequenced from 2 fish from each site (see section 2.5 for sequencing protocol). Potential four-base restriction endonucleases were selected using searches of the *DNAstar* computer program and the *WebGene* restriction enzyme analysis on-line search (http://darwin.bio.geneseo.edu/~yin/WebGene/RE.html). A total of twelve restriction endonucleases were selected that would cut the D-loop. These endonucleases were initially run on 10 handfish from the lower Derwent River site and 10 handfish from Frederick Henry Bay. Only two enzymes detected variation in this initial trial: these were used to digest the remaining handfish. A further four enzymes were selected to digest the remaining samples based on having clearly distinguishable haplotype banding patterns.

Restriction digests were run in 96 well polycarbonate microtitre plates. The enzyme buffer solution was mixed in bulk and added to the wells, an aliquot of DNA was then added to each of the wells. Each digest consisted of 10 μl of DNA template, 0.5 μl of enzyme, 2 μl of buffer and dH2O to make up a volume 20 μl. Bovine serum albumen (BSA), an enzyme stabiliser, was added when specified by the enzyme manufacturers. Digests were incubated for a minimum of four hours at the enzymes specified temperature (given in Table 2.3). Digests were visualised on large format 20 x 20 cm

2% agarose gels, using gel electrophoresis. Gels were run for 60 - 70 min at 100 V. Staining was done using ethidium bromide and visualised under ultraviolet light (403 nm).

Table 2.3 restriction enzymes used to digest spotted handfish 800 base-pair D-loop fragment.

enzyme	target sequence	BSA	temp
*Aci*I	5' C$^{\nabla}$CGC 3'	No	37° C
*Afl*II*	5' C$^{\nabla}$TTAAG	No	37° C
*Bsaj*I	5' C$^{\nabla}$CNNGG 3'	No	60° C
*Bsl*I*	5' CCNNNNN$^{\nabla}$NNGG 3'	No	60° C
*Bstu*I*	5' CG$^{\nabla}$CG 3'	No	60° C
*Dde*I	5' C$^{\nabla}$TNAG 3'	No	37° C
*Dpn*II	5' $^{\nabla}$GATC 3'	No	37° C
*Hae*III*	5' GG$^{\nabla}$CC 3'	No	37° C
*Hinf*I*	5' G$^{\nabla}$ANTC 3'	0.1 mg.ml^{-1}	37° C
*Hinp*I	5' G$^{\nabla}$CGC 3'	No	37° C
*Rsa*I*	5' GT$^{\nabla}$AC 3'	No	37° C
*Spe*I	5' A$^{\nabla}$CTAGT 3'	0.1 mg.ml^{-1}	37° C

$^{\nabla}$ indicates cleavage point *chosen for population work

2.5 Sequencing preliminaries:

2.5a PCR product purification:

Direct gene sequencing was used for the phylogenetic component of this study. PCR products were purified prior to sequencing. Two methods were employed for purification. For strong products, with no non-specific bands, a *QIAquick* column purification kit was used (QIAGEN 1997*a*). For products where non-specific amplification occurred, a *QIAquick* gel purification kit was used (QIAGEN 1997*b*). This provided up to 80% recovery of the target DNA band. Both procedures made use of special spin columns and a table top microcentrifuge.

2.5b Spin column

For strong products with no non-specific banding, the entire PCR reaction volume (~45 μl) was mixed with 5 volumes of PB buffer. This mix was added to a spin column and centrifuged at 13 000 rpm for 1 min to bind the DNA to the spin column filter. This was rinsed with 750 μl of PE buffer and again centrifuged at 13 000 rpm for 2 min. The DNA was eluted with 32 μl of elution buffer (EB), let stand for 1 min and

finally centrifuged at 14 000 rpm for 1 min. While 50 μl of elution buffer is recommended to maximise the amount of DNA eluted, a smaller volume was used to increase final concentration at the expense of total DNA recovery. This was to ensure a minimum concentration of DNA required for sequencing reactions.

2.5c Gel extraction:

The gel extraction protocol required the PCR product reaction be run out on fresh 1% agarose and stained with ethidium bromide as for PCR product visualisation. The time exposed to UV light was minimised as this can quickly degrade the DNA. The target bands were cut out of the gel using sterile scalpel blades and placed in clean 1.5 μl microcentrifuge tubes. 3 volumes of buffer QG was added to 1 volume of gel. Typical gel weights were around 100 mg, therefore 300 μl of buffer QG was added. The tubes were then incubated for 10 min at 50° C, with constant mixing until the gel was dissolved. One volume (100 μl) of isopropanol was added to the dissolved gel. The mix was then transferred to a spin column. An initial flush of 0.5 ml of buffer QG, to remove any trace of agarose, was performed. The protocol for spin column purification (as above) was then performed.

2.5d Fluorometry:

Prior to the mixing of sequencing reactions the DNA concentration was quantified using fluorometry against a calf thymus standard (100 ng.μl^{-1}, approximately 50% GC content). Fluorometry was performed in a *BIO-RAD Versafluor*™ fluorometer. A clean cuvette was loaded with 2 ml of room temp 1XTNE Buffer solution with H 33258 dye stock solution added. This was run as a blank to zero the machine. The 100 range was set with 2 μl of calf thymus DNA in 2 ml of this dye buffer solution. Once the range was set, 2 μl of DNA samples were assayed in 2 ml of buffer. All fluorometry readings were taken after a minimum of 10 sec to allow the reading to stabilise. The zero was checked after each assay. H 33258 dye binds to the minor groove of the DNA strand and will fluoresce under 365 nm light in this state. Typically 10 to 20 ng.μl^{-1} of DNA was present in each sample.

2.6 Sequencing:

Sequencing was based on end-termination reactions. Template DNA is amplified using PCR in a single direction. Fluorescently labelled dye terminators are randomly incorporated during this cycle and cause the termination of DNA extension. The reaction is then run on a gel and the order of incorporated dye terminators detected by a laser (Hillis *et al.* 1990). Reactions were carried out in both forward (light strand primer) and reverse (heavy strand primer) directions to allow verification of sequences against each other.

Reactions were run as half reactions according to the ABI big dye terminator sequencing reaction protocol. The following reagents were added to a 200 μl tube: 1 μl of primer, 4 μl dye terminator and 3-5 μl of template DNA (~50 ng.μl^{-1}). The reaction volume was made up to 10 μl with dH2O. This reaction was run in a Perkin Elmer GeneAmp PCR System Cycler 9600 using the built in ABI big dye terminator program. After the cycle was complete the reaction volumes were purified.

Unincorporated dye terminators were removed from sequencing reactions using an ethanol precipitation protocol. The product of each reaction was added to a 1.5 ml microcentrifuge tube containing 30 μl 95% ethanol and 1 μl 3M sodium acetate, pH 4.6. These were mixed and the DNA allowed to precipitate on ice for 10 min. Following precipitation the tubes were centrifuged at 13 500 rpm for 20 min. The ethanol solution was removed and the DNA pellet rinsed with 250 μl of 70% ethanol. Finally the DNA pellet was dried in a vacuum centrifuge.

All sequencing was done at CSIRO Marine Laboratories (Castray Esplanade, Hobart) on a *Perkin Elmer ABI 377* autosequencer. Forward and reverse sequences were aligned and verified using *Perkin Elmer ABI Prism Sequence Navigator*™ software.

2.7 Data analysis:

2.7a RFLP data analysis:

The restriction enzyme data collected was analysed using several methods. Both within and between population variation was assessed. For enzymes that showed variable

haplotypes, a chi-squared (χ^2) value was calculated to see if this variation was spread evenly across the four sites. The significance of these values was checked against one thousand Monte-Carlo estimates (Roff and Bentzen 1989). This method randomly re-samples an equivalent data set from the original data set with replacement, and calculates a chi-squared value from this new data set. Significance of the observed chi squared value is assessed by dividing the number of Monte-Carlo values greater than or equal to the observed value by the total number of random estimates. This method is useful when there is a low number of replicates (~20 per site in this case).

To determine if there was sub-population differentiation, a number of approaches were used. The G-statistic (G_{ST}) is a measure of genetic subpopulation differentiation. The G_{ST} is equal to the amount of genetic variation in the whole data set that is due to the division of the population into a series of interbreeding populations (Smolenski *et al.* 1993). The equations of Takahata and Palumbi (1985) were used to calculate the within site and between site identity probability using restriction site presence or absence data. The significance of the G_{ST} was evaluated using 1000 bootstrap replicates (Palumbi and Wilson 1990). To support the G_{ST} analysis an Ananlysis of MOlecular Variance, AMOVA, was used. This was used to show the levels of variation within and between sites. This test then compares the within site variations to asses if it is significantly different across the sites. The AMOVA was conducted using the computer program *Arlequin ver. 1.1* (Schneider *et al.* 1997).

Pair-wise Fst values of Wright (1943, 1951) were calculated from the composite haplotype data (cut site presence or absence). F_{ST} values were calculated using the computer program *Arlequin ver. 1.1* (Schneider *et al.* 1997). This is a measure of the differentiation of the among population variation. It varies from 0 (absence of differentiation) to 1 (complete differentiation) (Raymond and Rousset 1995). Probabilities were again calculated with 1000 bootstrap replicates.

Finally, sequence divergence was calculated from the restriction site presence or absence data. This was done using the delta (δ) value of Nei and Jin (1989). A pair-wise comparison was made of sequence divergence for each pair of sites. Sites are

deemed significantly different when the observed diversity was an order of magnitude greater than the standard error. Due to the low genetic variance, some standard errors were calculated as negative numbers; these were taken to be zero. Finally, sequence divergence estimates were used to infer a population structure tree using UPGMA algorithms.

2.7b Phylogenetic reconstruction:

Sequence data was collected for both 16S rRNA and cytochrome oxidase I genes. The two data sets produced similar phylogenetic reconstructions. A partition homogeneity test was used to test the null hypothesis that there was no difference between the two data sets (Farris *et al.* 1995). This test calculates the tree length for the combined data sets and then compares it to the randomly generated trees formed from sub-sampling an equivalent data set from the original combined data. If the initial combined tree length is within one standard deviation of the mean of the replicate tree lengths then the two data sets can be combined for phylogenetic analysis.

Phylogenetic trees were constructed using three principal methods. Firstly parsimony analysis was used to construct a phylogenetic tree. This method takes explicit notice of the character values observed for each species, rather than working with the distances between sequences that summarise differences between character values (Weir 1990). It attempts to reconstruct an evolutionary tree that requires the fewest nucleotide changes (Meyer 1994). Heuristic searches were used, as more thorough search options require excessive computational time. The heuristic approach does not guarantee the optimal tree will be found. Instead it starts with an initial tree and seeks to improve on this. Therefore this approach will find the optimum tree from a random starting point (Swofford and Begle 1993), which is not necessarily the global optimum tree.

Parsimony analysis was conducted in the software package *PAUP* 4.0* (Swofford 1998). For the 16S rRNA sequences, which had length mutations, the computer program *Clustalx* was used to align all the sequences prior to importation into *PAUP* 4.0*. Alignment was done using the slow-accurate alignment option, which starts by aligning the most distant sequences and proceeds to add sequences in a pair-wise fashion until all have been aligned. Five character states were recognised for the

fifth corresponded to gaps due to length polymorphisms. By doing this, insertion and deletion events were deemed to convey important phylogenetic information (Swofford and Begle 1993).

Maximum likelihood analysis and pair-wise distance analysis were also used to infer phylogenetic trees. Maximum likelihood method first assumes a model for the data and a form of the tree and then fits probabilities to the branches (Hueslsenbeck and Crandall 1997). The likelihood is then calculated assuming a multinomial distribution. The difference in the log-likelihood of the data represents the costs associated with the assumed phylogenetic tree and model. The higher the log-likelihood value the more reliable the tree. Distance analysis examines the taxa in a pair-wise fashion, by initially constructing a distance matrix (Weir 1990). Distance methods use clustering to group taxa that share more common features, based on these distances. For both distance analysis and maximum likelihood there are a number of different algorithms that can be used to link the clusters.

To construct maximum likelihood and distance trees both neighbour-joining and UPGMA algorithms were used (Sneath and Sokal 1973). The basis of the UPGMA algorithm is well covered in Weir (1990). Each separate taxonomic unit is considered initially as an independent cluster. A phylogenetic tree is constructed by linking the least distant pairs of clusters, followed by successively more distant clusters. When clusters are linked, they lose their individual identities and form a single cluster and distances are recalculated using the average of this cluster. At each stage as two clusters are merged the total number of clusters is reduced by one. Once the last two clusters are merged into a single cluster the process is complete (Swofford and Olsen 1990).

Neighbour-joining is an algorithm for inferring an additive tree (Saitou and Nei 1987). It is conceptually related to cluster analysis (e.g. UPGMA), but does not assume that all lineages have diverged equal amounts (Swofford and Olsen 1990). Trees are constructed using a modified distance matrix. In contrast to cluster analysis, which deals with taxa or clusters of taxa, neighbour-joining keeps track of nodes on the tree (Swofford and Olsen 1990). The tree is constructed by linking the least distant pair of

nodes and adding their common ancestor node to the tree. The tree is then pruned of the respective branches and terminal nodes. This process continues until two nodes remain, separated by a single branch.

The Kimura two-parameter model of sequence evolution was used in both the maximum likelihood and pair-wise distance analysis. This distance measure takes into account the nature of DNA (Kimura 1980). It makes the assumption that all four nucleotides are equally frequent and that there are independent rates for transition substitutions and for transversion substitutions. The general model allows each nucleotide to be present at a different frequency so long as the substitution rates are balanced to maintain the equilibrium abundance of the nucleotides (Swofford and Olsen 1990).

Bootstrapping was used to establish the probabilities that each of the branches being observed were not due to chance. Original branch values were compared to those of 1000 bootstrap replicate trees for the parsimony and distance trees, and 500 for the maximum likelihood analysis tree due to the excessive computing time this method requires. In all cases bootstrap trees were created by re-sampling an equivalent sized data set from the original data set with replacement. Significance was determined by the number of bootstraps trees that resolved each of the branches on the original tree. The widely used statistical rule that a group is supported significantly if it is supported in at least 95% of bootstrap trees is considered far too conservative in this type of analysis (Brown 1994). Generally any branch supported greater than 50% was considered to be a significant branch.

The effect of enforced monophyly within each genus was tested by comparing the single most parsimonious tree with the most parsimonious enforced monophyly tree. This was done using a Templeton (Wilcoxon signed-ranks) test (Templeton 1983). This is a non-parametric two tailed test. The same trees were also tested under maximum likelihood principles using a parametric Kishino-Hasegawa test (two-tailed test) (Kishino and Hasegawa 1989). Both these tests compare tree scores between the two trees and test the null hypothesis that there is no difference between the two.

3 Spotted Handfish Conservation Genetics:

3.1 Introduction:

The management of endangered fishes is becoming of increasing importance. It is estimated that up to one third of fish species are threatened with extinction (IUCN Press Release 1996). Many of these fish species are of commercial importance, and are threatened due to over fishing. Others are endangered by environmental change and habitat loss. Information needs to be collected on these species to aid in their management and recovery. The collection of genetic information forms a key part of this process. To properly manage an endangered species data on the genetic variation and population structure is needed.

Genetic variation can be detected by many different methods, each with their own advantages. One of the first methods employed was allozyme electrophoresis. Allozyme electrophoresis allows changes in the proteins expressed by different genes to be detected and used as a measure of genetic variation. The major drawback with this method is that it does not reflect all sequence variation, as many nucleotide changes will not affect the protein expressed. Its main advantages lie in its speed and relatively low cost (Ward and Grewe 1995). This technique is increasingly going out of favour.

The major techniques now used for the detection of genetic variation directly detect changes at the DNA level. There are a number of methods for the examination of both mitochondrial DNA (mtDNA) and nuclear DNA (nDNA). These methods include random amplified polymorphic DNA (RAPDs) and microsatellite techniques for the nuclear DNA, and direct sequencing and restriction fragment length polymorphisms (RFLP) for the mitochondrial DNA.

An inexpensive and rapid method for detecting variation in nuclear DNA uses RAPD's. RAPD (random amplified polymorphic DNA) techniques use random primers combinations to amplify fragments of nuclear DNA. These fragments are then visualised on 2-4% agarose gels. The primers used generally have a 10 base pair recognition sequence (Meyer 1994). Mutations in the nDNA nucleotide sequence will result in the loss or occasional gain of a primer binding site in the target nDNA. Thus

fragment pattern polymorphisms can be detected for different primer combinations. This method has some problems with repeatability, mainly that small changes in PCR conditions can affect primer site binding specificity. Regardless, this technique has been used successfully in addressing population level questions (Bardakci and Skibinski 1994, Meyer 1994, Mamuris *et al.* 1998). Another drawback is that, although it requires less target DNA than many RFLP, it does require information on the target DNA-sequences for designing specific primers (Dinesh *et al.* 1993).

Recently, the development of microsatellite techniques, which detect tandem repeats of one or a few nucleotides in nDNA, have been used to address population level questions. Microsatellites are highly sensitive compared to the other techniques (Ward and Grewe 1995). However, their application is lengthy and costly. There are other methods to detect DNA level variation, such as RAPDs and mitochondrial DNA techniques which, while not as sensitive, are much cheaper and quicker to run.

Examination of the mitochondrial DNA is currently the most popular tool for the assessment of genetic variation in fish (Meyer 1994). Two common methods exist to determine the variation of the mtDNA. These are direct sequencing and RFLP (restriction fragment length polymorphisms). These methods has their own advantages and disadvantages. Sequencing is a PCR (polymerase chain reaction) based process whereby the nucleotide composition of a target fragment is obtained. This allows easy comparison at both the intraspecific and interspecific levels. Sequencing detects all the variation in the target region and also allows transitions and transversions to be identified as well as insertions and deletions. The main drawback of this method is its expense. Generally only a small number of individuals can be sequenced due to cost and it is, therefore, more suited to phylogenetic studies.

Restriciton fragment length polymorphism (RFLP) is cheaper and allow large numbers of individuals to be examined. This technique uses restriction endonucleases to cut targeted DNA sequences, and the resulting fragment patterns or haplotypes, are compared. Generally the degree of population subdivision at the nucleotide level is comparable to that at the haplotype level (Lynch and Crease 1990). Restriction endonucleases are enzymes that recognise and cut DNA at specific nucleotide

sequences. Usually endonucleases of six base-pair sequence are used for phylogenetic studies of distantly related species while for more closely related species and population level work the more informative four base-pair endonucleases are used (Meyer 1994). This method, like RAPDs, sub-samples the target DNA.

By using a number of restriction endonucleases, a restriction profile or composite haplotype, can be built up which allows for the comparison of individuals. RFLP requires large amounts of relatively pure target DNA (Dinesh *et al.* 1993). For population level work, PCR and RFLP can be combined. By using restriction endonucleases to digest a target gene region, a larger relative proportion of nucleotides can be sampled than when targeting the entire mitochondrial genome. RFLP is a common method for population differentiation and stock separation (Graves *et al.* 1984, Graves and Dizon 1989, Graves and McDowell 1994; Grewe *et al.* 1994, Tringali and Bert 1996, Chow *et al.* 1996, Nedbal and Philipp 1994, Heist *et al.* 1996, Tabata *et al.* 1997).

A PCR-RFLP approach was used in this study to enable a large number of individuals to be examined. The D-loop gene region was chosen as the target DNA as this is generally considered one of the most variable regions of the mtDNA genome in vertebrates. An indication of the genetic variation present in a population can be obtained by sub-sampling a rapidly mutating gene region. Unless the entire mitochondrial genome is sequenced it is impossible to say that no variation is present if none is found through sub-sampling. However, using a highly variable region and a number of endonucleases will increase the confidence that the level of variation detected gives an indication of the relative level of variation in the population.

Tissue samples were collected from spotted handfish from four sites representing the known species range. Three of these sites were in the Derwent River. Site 1 was in the mid Derwent River, while Sites 2 and 3 were in the lower Derwent River. The fourth, site 4 was in the adjoining Frederick Henry Bay. The region of these sites is indicated in Figure 3.1. Tissue from 19 individuals was collected from site 1, and 21 individuals from each of the remaining sites. For each individual an approximately 800 base-pair fragment of the mitochondrial D-loop was amplified using PCR. A restriction profile

or composite haplotype was established for each fish using six restriction endonucleases. This allowed estimates of total genetic variation, within and between site variation and finally population structure to be made. To avoid confusion the fish of a particular site will be referred to as a colony, in that they are a group of organisms living together in close proximity. This is not to imply that they form an integrated society in which its members may be specialised sub-units (Lincoln *et al.* 1982).

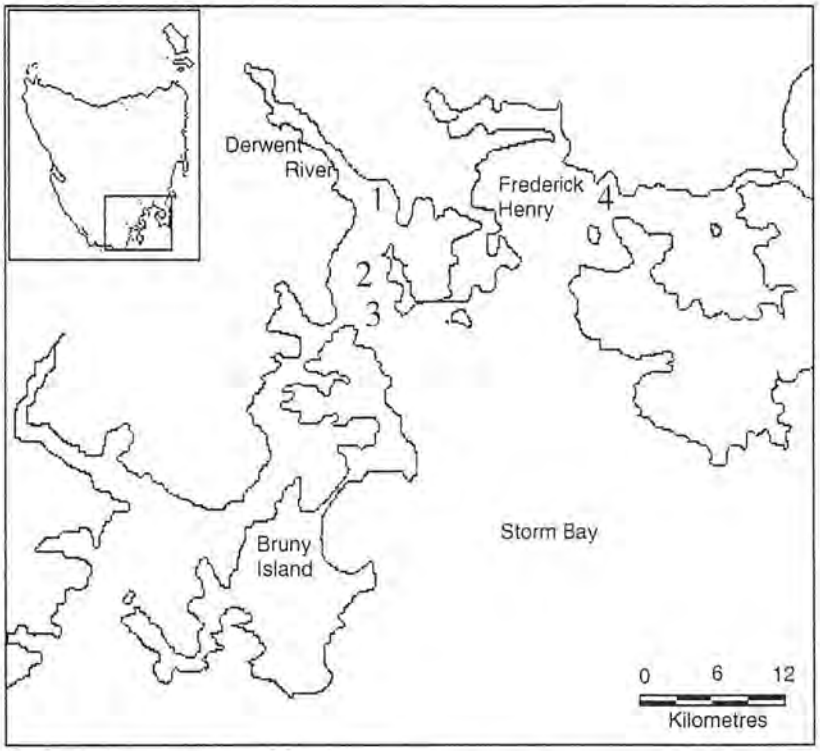


Figure 3.1 Spotted handfish tissue collection locations. Three sites were in the Derwent River (1 - 3), with the remaining site in Frederick Henry Bay (4). These sites encompass the known spotted handfish species range.

3.2 Results:

3.2a Haplotype information

An 800 base pair fragment of the mitochondrial D-loop was amplified using PCR (polymerase chain reaction). Polymorphisms were detected by digestion with a suite of restriction endonucleases. The 800 base pair fragment of the D-loop was sequenced and appropriate endonucleases were selected based on a WebGene (http://darwin.bio.geneseo.edu/~yin/WebGene/RE.html) search of possible restriction endonucleases. A total of 12 restriction endonucleases were initially trialed on ten individuals from sites 3 and 4. Six of these were then selected based on variable restriction profiles and/or clearly distinguishable fragment banding patterns. These were *Hae*III, *Hinf*I, *Afl*II, *Bsl*I, *Bstu*I and *Rsa*I. They were used to digest the 82 individuals collected from the four study locations.

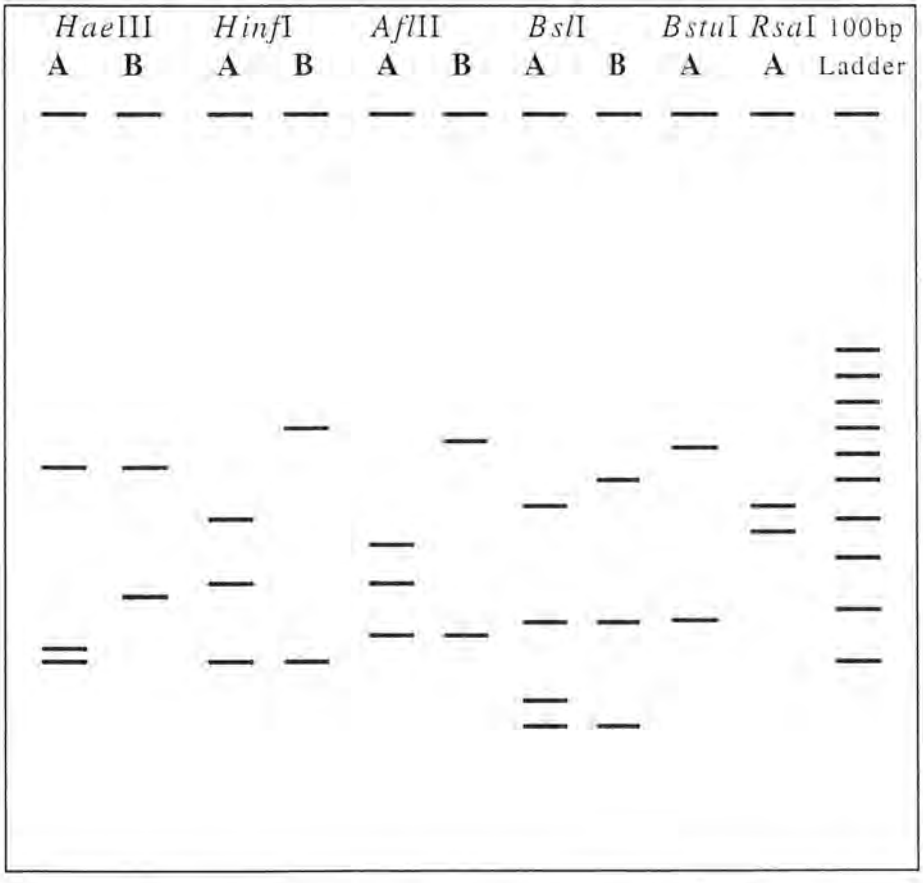


Figure 3.1. Restriction fragment profiles for spotted handfish 800 bp D-loop fragment for the endonucleases *Hae*III, *Hinf*I, *Afl*II, *Bsl*I, *Bstu*I and *Rsa*I.

Two of these endonucleases used (*BstuI* and *RsaI*) found no haplotype variation between colonies. The remaining four endonucleases displayed multiple haplotypes

between the colonies. A diagrammatic representation of the haplotype banding patterns is presented in Figure 3.1. The A haplotype for each endonuclease represents the most common haplotype; for all endonucleases the A haplotype was found from fish at all sites. The B haplotype was the less common alternative haplotype. For all endonucleases the B haplotype arose from the loss of a restriction site compared with the A haplotype. Fragments less than 100 base pairs were inferred from sequence data.

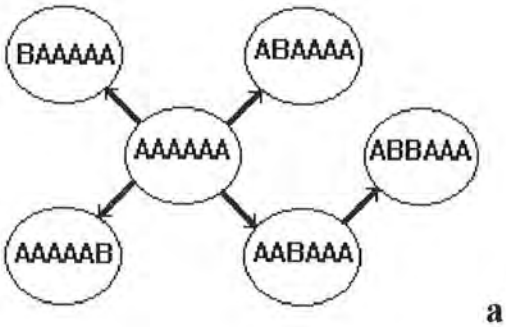
The distribution of composite haplotypes across the four colonies is presented in Table 3.1. For the two lower Derwent colonies (sites 2 and 3) three composite haplotypes were dominant; AAAAAA, ABAAAA and AABAAA. At the remaining two sites, AAAAAA was the dominant composite haplotype. Sites 1, 3 and 4 all had a unique rare composite haplotype present in only one individual. A parsimony network of composite haplotypes supports the AAAAAA as the ancestral haplotype (Figure 3.2). The chance of a mutation creating a new restriction site is much less than destroying a present site (Weir 1990). All the other haplotypes are formed by the loss of one or, in the case of ABBAAA, two restriction sites from the AAAAAA composite haplotype.

Table 3.1. Composite haplotypes frequency at the four handfish colonies for six restriction endonucleases (*Hae*III, *Hinf*I, *Afl*II, *Bsl*I, *Bstu*I and *Rsa*I).

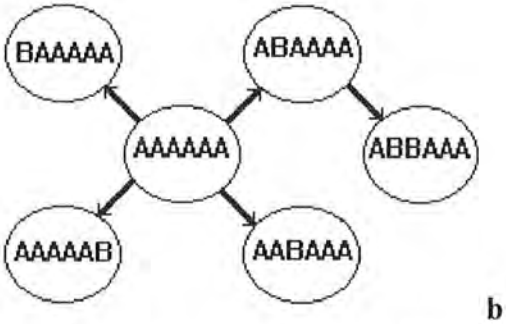
Composite Haplotype	Site 1	Site 2	Site 3	Site 4
AAAAAA	18	8	6	20
BAAAAA	0	0	0	1
ABAAAA	0	8	4	0
AABAAA	0	5	10	0
AAABAA	1	0	0	0
ABBAAA	0	0	1	0
Total	19	21	21	21

From the maximum number of fragments and the composite haplotype data cut site presence or absence was calculated for each individual. Presence or absence of restriction sites allows inferences to be made about the levels of sequence variation. There were a total of 11 restriction sites detected by the six restriction endonucleases.

All these endonucleases have four-base pair recognition sites except for the endonuclease *Afl*II. This endonuclease recognised and cut a six-base pair sequence. A total of 48 base pairs out of approximately 800, or 6% of the D-loop fragment was sampled.



a



b

Figure 3.2 The two most parsimonious networks (a and b) to explain the evolution of the composite haplotypes for the six endonucleases (*Hae*III, *Hinf*I, *Afl*II, *Bsl*I, *Bstu*I and *Rsa*I). The arrows indicate the loss of a restriction site to derive a new haplotype.

3.2b χ^2 analysis

To test if the variation detected by the four endonucleases *Hae*III, *Hinf*I, *Afl*II *and Bsl*I varied between the colonies a chi-squared (χ^2) test was used. This compared the observed frequency of haplotypes in the colonies with an expected frequency. The significance of these values was estimated against 1000 Monte-Carlo randomisations (Roff and Bentzen 1989). The endonucleases *Afl*II ($\chi^2 = 24.37$; $p < 0.0001$) and *Hinf*I ($\chi^2 = 16.32$; $p < 0.0001$) both demonstrated significant haplotype differences between colonies. The remaining endonucleases with variable restriction sites did not show significant differences between colonies: *Hae*III ($\chi^2 = 2.941$; $p > 0.99$) and *Bsl*I ($\chi^2 = 3.357$; $p > 0.99$). Graphs of observed chi-squared values versus 1000 bootstrap replicates are presented as Figures 3.3 - 3.6.

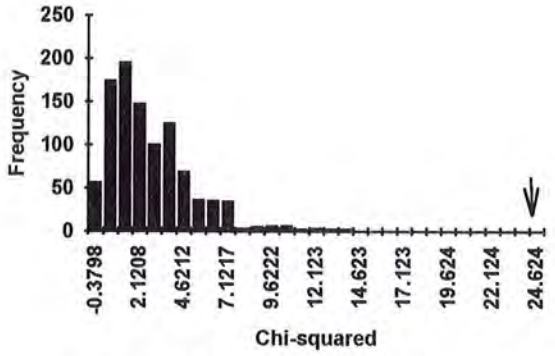


Figure 3.3 *Afl*II $\chi^2 = 24.39$

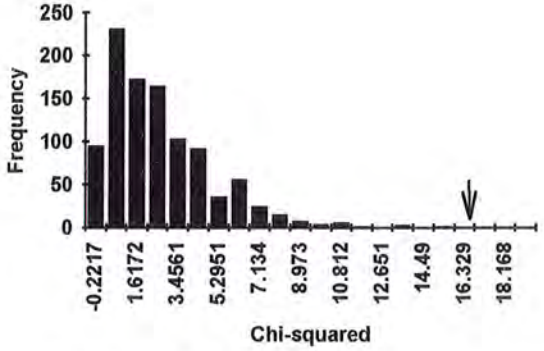


Figure 3.4 *Hinf*I $\chi^2 = 16.32$

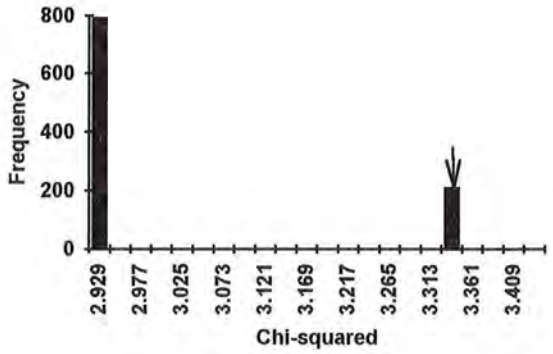


Figure 3.5 *Bsl*I $\chi^2 = 3.357$

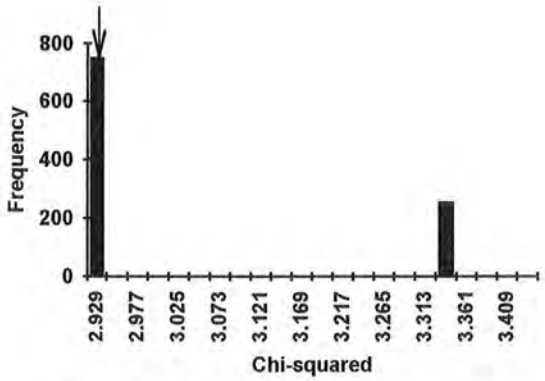


Figure 3.6 *Hae*III $\chi^2 = 2.941$

Figures 3.3-3.6. Chi-squared (χ^2) estimates for the endonucleases *Afl*II, *Hinf*I, *Bsl*I and *Hae*III that found variable haplotypes in the four spotted handfish colonies examined. Arrows indicate the observed χ^2 values for each endonuclease.

3.2c Population differentiation

Wright's (1951) F_{ST} statistic and Nei's (1973) G_{ST} statistic were calculated from the site presence/absence data to examine population differentiation between the colonies. G_{ST} is a measure of genetic sub-population differentiation (Ward *et al.* 1994). The G_{ST} statistic was based on the modified formulae of Takahata and Palumbi (1985). G_{ST} is a measure of genetic sub-population differentiation (Ward *et al.* 1994). The observed value for spotted handfish sites was $G_{ST} = 0.09522$; $p < 0.001$. Significance was obtained by comparison to 1000 Monte-Carlo randomisations. As for chi-squared estimates, a histogram of observed G_{ST} compared to Monte-Carlo replicates is presented (Figure 3.7). As the G_{ST} was significant this suggested that there was some degree of population structuring.

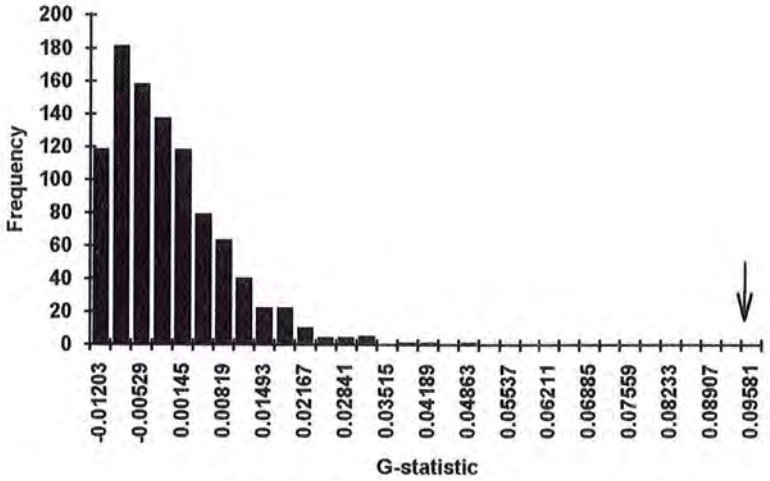


Figure 3.7 Genetic sub-population differentiation as indicated by the observed G_{ST} value (arrow) compared to 1000 Monte-Carlo bootstrap replications. The G-statistic was calculated on restriction site presence or absence data. $G_{ST} = 0.09522$; $p < 0.001$.

An Analysis of MOlecular VARiance (AMOVA) was carried out to determine the relative proportions of within and between site variation. This showed that 74.47% of variation was attributed to within colony variation while 25.53% of variation was due to between colony variation. This test found that the within colony variation was significantly different for between the colonies. ($F_{ST} = 0.25534$; d.f. = 81; $p < 0.001$).

To determine which of the colonies differed significantly a comparison of pair-wise distance was used. Distance was calculated based on Wright's 1951 F-statistic (F_{ST}). Probabilities were calculated using 1000 Monte-Carlo simulations (Table 3.2). All the pair-wise comparisons of colonies exhibited significant differences ($p < 0.001$), except for the colonies at sites 2 & 3 ($F_{ST} = 0.27143$; $p = 0.08157$), and sites 1 & 4 ($F_{ST} = 0.38235$; $p = 0.20443$). The colonies as sites 2 and 3 were the geographically closest colonies, less than 4 km apart, so it is possible there are low levels of gene flow between the two. The colonies at sites 1 and 4, however, are the most distant and represented extremes of this species' range. It is unlikely that continued gene flow is homogenising these two colonies. Possible explanations for this population structure will be suggested in the discussion.

Table 3.2. Pair-wise comparison of F_{ST} values (below diagonal) for the four spotted handfish colonies. Probabilities are given above the diagonal. Those in bold are significantly different at the 0.05% level.

	Site 1	Site 2	Site 3	Site 4
Site 1	---	**0.00000 ± 0.0000**	**0.00000 ± 0.0000**	0.20443 ± 0.0112
Site 2	0.025938	---	0.08157 ± 0.0088	**0.00000 ± 0.0000**
Site 3	0.36888	0.06277	---	**0.00000 ± 0.0000**
Site 4	0.00026	0.27143	0.38235	---

3.2d Population structure

An estimation of sequence divergence was made using a delta (δ) analysis (Nei and Jin 1989). Delta values (% sequence divergence) were made pair-wise between each of the four colonies and for all colonies combined. The combined delta estimate was around 0.06% sequence variation (δ = 0.005890 ± 0.001766). Pair-wise estimates (Table 3.3) showed again that colonies at sites 2 & 3 and sites 1 & 4 were not significantly different. This supported the findings of the pair-wise distance analysis (F_{ST}). These pair-wise sequence divergence estimates were used to used to construct an UPGMA dendrogram of population structure (Figure 3.8).

Table 3.3. Pair-wise comparisons of sequence divergence (δ), given below the diagonal, are based on site presence absence data, standard errors are given above the diagonal. The values in bold indicate a significant difference between two sites based on divergence >10x standard error.

	Site 1	Site 2	Site 3	Site 4
Site 1	---	-0.000642	-0.000926	0.000033
Site 2	**0.001530**	---	0.000268	-0.000610
Site 3	**0.002719**	0.000598	---	-0.000857
Site 4	0.000004	**0.001525**	**0.002713**	---

NB: negative SE was taken as zero.

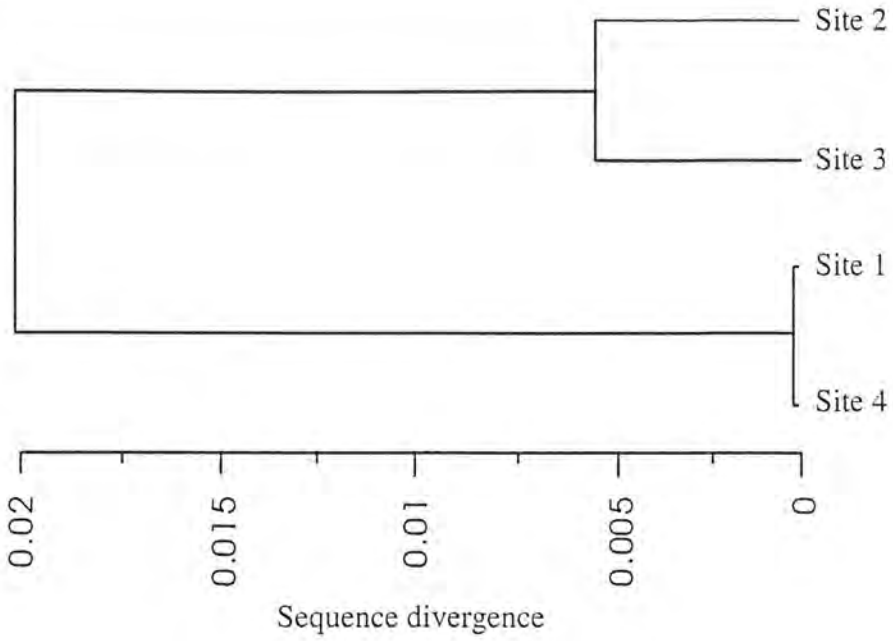


Figure 3.8 UPGMA dendrogram of spotted handfish population structure based on pair-wise comparison of sequence divergence (δ).

3.3 Discussion:

3.3a Low genetic variation:

The spotted handfish exhibited low genetic variation across its entire range. The calculated genetic diversity value of the spotted handfish was 0.006% sequence divergence. This is far less than equivalent estimates of 0.13 - 0.19% for orange roughy, *Hoplostethus atlanticus* (Ovenden *et al.* 1989, Smolenski *et al.* 1993), and of 0.14% for jack mackerel, *Trachurus declivis* (Smolenski *et al.* 1994), and slightly above estimate of 0.004% for the striped bass, *Morone saxatalis,* (Wirgin *et al.* 1988), which is one of the lowest published divergence estimates for a marine fish. Typically the majority of divergence estimates for marine fish range from 0.5% to 1.6%. The low variation of the spotted handfish may be a reflection of its life history, which is characterised by low dispersal abilities and a limited geographical distribution (Bruce *et al.* 1998).

Most marine fish are typically quite mobile as adults, inhabit a realm relatively free from firm geographic barriers and have pelagic eggs or larvae (Avise *et al.* 1987b). The spotted handfish lacks all these features in its life history. This suggest that there may be little genetic exchange between fish of two discrete colonies. Genetic drift would therefore be a major influence in causing population differentiation. This is a process that occurs in all heterozygous populations (Ayala 1982). Genetic drift is the random changes in allele frequency over time. In small populations these frequency shifts can be proportionally greater than in large populations, resulting in the loss of rare alleles. As the spotted handfish colonies are relatively small, genetic drift is likely to have caused the loss of alleles from many of them. These are not likely to be replaced by genetic exchange between colonies.

Intraspecific mtDNA diversity in some marine species could be low because these species have experienced recent bottleneck events (Nei *et al.* 1975). In the last 20 years the spotted handfish numbers have undergone a substantial decline (Last and Bruce 1996). Current estimates of spotted handfish abundance put the number of breeding individuals as low as 100 in some of the colonies (Mark Green pers. comm.). While there is no direct evidence, one explanation for the at low variation seen across

the spotted handfish's range the would be similar scale population bottleneck events during the last glaciation which finished around 10 000 years ago.

The two lower Derwent River handfish colonies at sites 2 and 3, both had greater variation compared to the colonies at sites 1 and 4 based on the six restriction endonucleases used in this study. These were generally an order of magnitude greater than the remainder of the population. The site 2 and 3 colonies could not be separated based on haplotype composition, or haplotype frequency differences. It appears that they are either a single population or there are sufficient levels of mixing between the two to homogenise them. It has been estimated that as less than 5% per year migration can genetically homogenise two populations (Gold *et al.* 1994). With the current population size estimates this would correspond to around 5 fish per year. Sites 2 and 3 are the closest two sites looked at (around 4 km apart). The required level of genetic exchange may well be possible over such a distance, especially if there was a continuous distribution of handfish in low abundances and suitable habitat between the two sites.

The site 1 and 4 handfish exhibit low genetic diversity. These sites had less than 0.001% within site sequence divergence. These two sites were on the edges of the species' range. Site 4 was separated by at least 30 km from the nearest known colony, while site 1 is at least 10 km from the nearest known colony in the Derwent. This means these colonies would be less likely to have lost haplotypes replaced by migration than would the nearby colonies at sites 2 and 3. The lack of variation at site 1 and 4 may represent historical factors or may reflect recent occurrences, but is likely to be a result of both.

The handfish colonies at sites 1 and 4 only differed by a single rare haplotype at each site. Both these sites were dominated by the common AAAAAA composite haplotype. It is suggested that either this is the ancestral haplotype or that through genetic drift both colonies have been left with the same common haplotype (random chance), This second option is highly unlikely as it would require the loss of exactly the same restriction sites from both colonies.

3.3b Population structuring:

From this study it is suggested that the site 2 and 3 handfish colonies of the lower Derwent Estuary form a single interbreeding population. The site 1 and 4 handfish of the mid-Derwent Estuary and Frederick Henry Bay also seem to be from the same population. It is impractical that these two colonies are homogenised by interbreeding, as they are at opposite ends of the species' range, instead they appear to represent the historical haplotype.

Of the six composite haplotypes identified in this study, three were found in single specimens. The three remaining haplotypes were present in more than one specimen and at more than one site. The haplotype AAAAAA was seen at each of the four sites. The other two common haplotypes, ABAAAA and AABAAA were present only at the two lower Derwent sites. These last two haplotypes were defined by variation of the restriction patterns of the enzymes *Hinf*I and *Afl*II respectively. These were the two enzymes that were found to have significant difference between sites in their restriction patterns from the chi-squared analysis. There are two plausible explanations for the current population genetic structure of the spotted handfish: multiple recruitment events or genetic drift.

The last period of glaciation, the Pleistocene, ended around 10 000 years ago. Linsley (1996) suggested that the sea level during this interglacial was 40-50 m below present levels, based on oxygen-isotope records. Given the current known species' range of the spotted handfish, the deeper waters of Storm Bay would form the logical glacial refuge. If this glaciation resulted in a bottle-neck then processes such as genetic drift and inbreeding would result in reduced genetic variation. Under this hypothesis the haplotype AAAAAA would be come fixed or at least the dominant haplotype in the populations.

The current sea level was reached around 6 000 years ago. Since then the sea levels have been stable around Australia only fluctuating a few meters (Lambeck and Nakada 1990). The radiation of the spotted handfish from their glacial refuge to their current

range would have occurred during this period. Recolonisation of the Derwent River estuary and Frederick Henry Bay would have occurred at around the same time. If the glacial refuge population was dominated by the AAAAAA haplotype this would explain why it is the common haplotype across the species range.

The ABAAAA and AABAAA haplotype are only present in the lower Derwent Estuary. This suggests that either mutations occurred in these two colonies that resulted in the ABAAAA and AABAAA haplotypes, or that they arrived into the colonies at a later stage to AAAAAA and have not had time to establish at the other sites. One mechanism for this secondary colonisation event is via the D'Entrecasteaux Channel, either a delayed recolonisation through the D'Entrecasteaux Channel from the Storm Bay glacial refuge population, or recolonisation from a separate glacial refuge population in the D'Entrecasteaux Channel.

There are some deep regions of the D'Entrecasteaux Channel, greater than 50 m, that may have formed an alternative glacial refuge of the handfishes. The ABAAAA and AABAAA haplotypes may have become dominant in the D'Entrecasteaux Channel through genetic drift (Figure 3.9). The delayed arrival into the lower Derwent River may have only been a recent event, which would explain why the ABAAAA and AABAAA haplotypes are only found in these colonies. Spotted handfish are known to occur in the D'Entrecasteaux Channel (Last *et al.* 1983), which adds support to this hypothesis. An alternate hypothesis of isolation in Ralphs Bay seems unlikely, as this bay is mostly less than 10 meters deep and much would disappear if sea levels dropped by even a few meters.

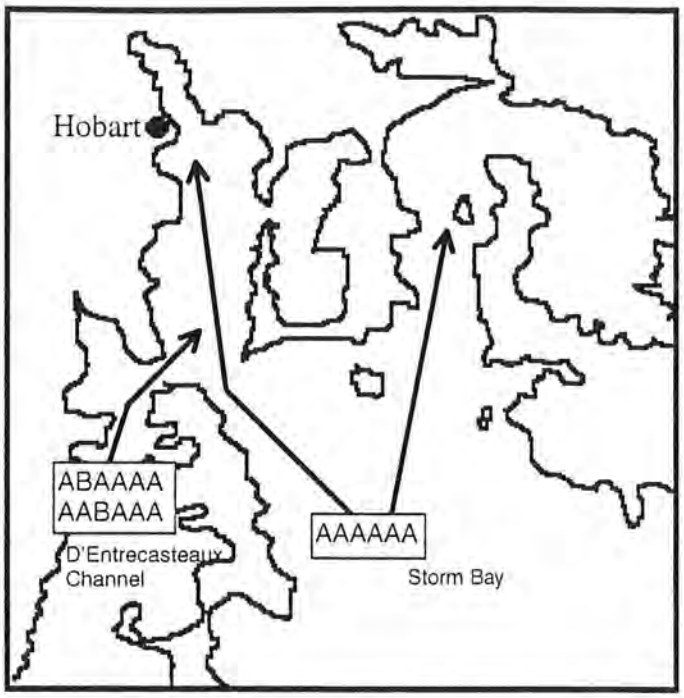


Figure 3.9. Suggested mechanism for colonisation of current spotted handfish species' range from a glacial refuge in the deep waters of Storm Bay. Primary colonisation of the AAAAAA haplotype across the entire species range followed by secondary colonisation of the ABAAAA and AABAAA haplotypes via the D'Entrecasteaux channel to the lower Derwent River sites only.

An alternative explanation for the current genetic population structuring is that genetic drift has caused the loss of variation at some of the sites. Assuming the entire spotted handfish population used to have all three of the common haplotypes seen at the lower Derwent River sites, the random loss of haplotypes, through genetic drift could explain why the mid-Derwent River and Frederick Henry Bay sites have lost two of these alternate haplotypes. This theory would require that the same haplotypes were lost in two widely separated populations.

Rapid loss of haplotypes is possible given the spotted handfishes' breeding biology and life history, but unlikely. As the spotted handfish lays its eggs directly onto the substrate, it is possible that an entire clutch could be destroyed either by predation or physical disturbance. In this way a certain haplotype could become rare and be lost if fish of a certain haplotype had successive poor recruitment years. According to Avise *et al.* (1984), the stochastic extinction of mtDNA lineages can be quite rapid. It is theoretically possible that a population founded by 1000 females will retain descendants from only one founding female after just a few thousand generations. The

spotted handfish colonies have currently been estimated as low as 100 breeding individuals at some of the sites. Therefore assuming an equal sex ration it could take a few hundred generations or less for the equivalent to occur in a spotted handfish population. As the spotted handfish has limited dispersal capabilities these haplotypes may not be re-introduced back into these colonies, especially if they are widely separated from other colonies.

Assuming this occurred in the mid-Derwent and the Frederick Henry Bay colonies it is theoretically possible that they both ended up with the AAAAAA haplotype as the dominant haplotype but probably unlikely. These two sites are at the edge of the species' range and are both separated from other known colonies. The two lower Derwent sites are in relatively close proximity so the loss of rare alleles may have been covered by migration between the two. This may explain why these sites maintain more variation than the other two sites looked at.

3.3c The mitochondrial D-loop for population level work:

In this study the replication control region or D-loop was examined. This is a non-coding gene region of the mitochondrial genome that is believed to be responsible for control of replication. D-loop shows extensive variation in higher vertebrates (Ferguson *et al.* 1995). However, for some fish species the D-loop has been found to have few polymorphisms. Bernatches *et al.* (1992) found no variation based on sequencing of the D-loop of Atlantic basin brown trout (*Salmo trutta*). Yet extensive variation among Atlantic brown trout has been found in the NADH1,5 and 6, and also 16S rRNA genes (Hall & Naweick 1995; Hynes *et al.* 1996) using restriction length polymorphisms. The fact that low variation was found in the spotted handfish may be because the D-loop in this species is not overly variable. Given the life history of the spotted handfish, low genetic variation is not an unexpected finding.

An attempt was made to look at other regions such as NADH5/6 and cytochrome *b*, however no amplification was achieved using combinations of recognised universal primers. Sequences of the 16S rRNA and cytochrome oxidase I regions were obtained, and although these were only for a four fish each from two sites (sites 3 and 4) they

also exhibited low variation. There were only 6 base differences out of a combined total of 1158 base pairs across the eight fish. These two genes are slower mutating than the D-loop. It is suggested that a more powerful technique rather than a different gene may be an appropriate option for further study.

A technique such as microsatellites may provide better resolution to determine levels of genetic variation. Microsatellites are segments of repeated copies of DNA that vary in length due to the number of repeats. This type of approach is more costly and time consuming than mitochondrial DNA but is more sensitive to detecting variation. It would require the development of specific microsatellite probes.

This study has provided some useful insights into the genetic population structure of the spotted handfish. It has also highlighted areas that require further investigation.

4 Handfish Molecular Phylogeny:

4.1 Introduction:

The relationship between species has long been the focus of scientific endeavour. The need to classify organisms forms a central part of most natural biology. Many techniques have been used to separate one group of organisms from another, morphology, behaviour, and more recently chemical and genetic makeup. Increasingly genetic information is being used to complement traditional taxonomy. The great levels of advancement in first protein (allozyme) and latter DNA (both nuclear and mitochondrial) analysis has provided further support for many existing phylogenies and helped clear up confusion.

As indicated in the general introduction, mitochondrial DNA (mtDNA) is an ideal molecular marker for both population work and systematic studies. MtDNA has been used in many past phylogenetic investigations of marine fish. There is a vast amount of literature on phylogenetic studies of fish. In these studies numerous mtDNA genes have been targeted. For closely related species and population level work, rapidly mutating genes such as the replication control region (Alvarado Bremer *et al.* 1997) or the sodium de-hydrogenase genes, NADH2-6 (Hogan *et al.* 1997, Ptacek and Breden 1998) have been used. For more distantly related species and higher order resolution slower mutating genes are targeted. These include the 12S and 16S rRNA genes (Caldara *et al.* 1996, Miya and Nishida 1996, Ritchie *et al.* 1997, Birstein and DeSalle 1998, Gilles *et al.* 1998, Miya and Nishida 1998 and Simons and Mayden 1998), cytochrome oxidase (Oohara and Okazaki 1996) and the cytochrome *b* gene (Cantatore *et al.* 1994, Orti *et al.* 1994, Apostilides *et al.* 1997, Dowling and Naylor 1997 and Ghedotti and Grose 1997).

Sequencing of mitochondrial genes is a popular method for obtaining phylogenetic information. This method does not have the problems of repeatability associated with many procedures, e.g. allozyme electrophoresis, and allows direct comparison and study of DNA sequences from different species that have been obtained in different laboratories (Meyer 1994). Sequences are generally taken from one, or a few individuals, from each species being compared. These are assumed to be characteristic

of the species. Sequence data are then transformed into similarity or distance data, by pair-wise comparisons.

Three major methods of phylogenetic reconstruction are currently used: distance, parsimony and likelihoods. Distance methods reconstruct phylogenies based on pair-wise distances. They attempt to fit a tree to a distance matrix, whilst minimising the discrepancy between observed differences and expected differences (based on the tree) (Meyer 1994). Parsimony methods aim to minimise the number of evolutionary steps to explain the variation seen (Swofford and Begle 1993). Likelihood methods assume the tree form and then fit lengths to the branches; likelihoods are then compared over trees to find the maximum likelihood (Weir 1990). All these three methods are used here to reconstructphylogenetic relationships of handfish species.

The relationships between eight distinct species or species morphs were investigated based on sequences of the mtDNA 16S rRNA and cytochrome oxidase I genes.

4.2 Results:

4.2a 16S rRNA sequence details:

A 614 base pair region of the mitochondrial 16S rRNA gene was amplified and sequenced using a PCR (polymerase chain reaction) based process. A total of 25 individuals were sequenced representing 5 species from the Family Brachionichthyidae; a further two individuals of the Family Antennariidae were used as an outgroup.

Sequences were validated by three methods. All animals were sequenced in the forward and reverse directions. These sequences were aligned and checked against each other for ambiguities using Perkin Elmer ABI Prism Sequence Navigator™. This ensured that all fish had been sequenced clearly. For all species (except species D) several individuals were sequenced; this allowed cross checking of ambiguities from closely related individuals. Finally all sequences were checked with existing sequences in *Genbank* using the National Centre for Biotechnology Information's Blast search option (http://www.ncbi.nlm.nih.gov). This was a final safety check to ensure that the correct gene region had been sequenced, and that the sequence was not that of contaminating tissue (i.e. human).

Consensus 16S rRNA sequence data for each of the major taxa are presented in Figure 4.4. The sequence from the spotted handfish (*Brachionichthys hirsutus*) from the lower Derwent River is used as the reference sequence. Three types of mutation are present: transitions, transversions and length mutations. Of the four nucleotides, A (170.63 ± 1.88) and C (169.89 ± 2.95) were the most abundant, while G (131.74 ± 1.56) and T (135.74 ± 2.30) were the least abundant (Figure 4.1). No significant difference was found between the base frequencies across the sequences, $\chi^2 = 3.244525$ (df = 3), P = 1. There was no bias towards either the purine or the pyrimidine bases.

Variation was observed at 102 nucleotide positions. Of these, 32 of the characters were parsimony-uninformative, only being present in comparisons against single species, and therefore could not be interpreted as a shared derived character from a common

ancestor. The remaining 70 characters were parsimony informative and were used to infer phylogenetic relationships. After alignment in *Clustalx* using the slow accurate search option there were 10 insertion deletion/points ranging from 1 to 3 bases across the 26 sequences. Transitions outweighed transversions with a ratio of 1.69 to 1.

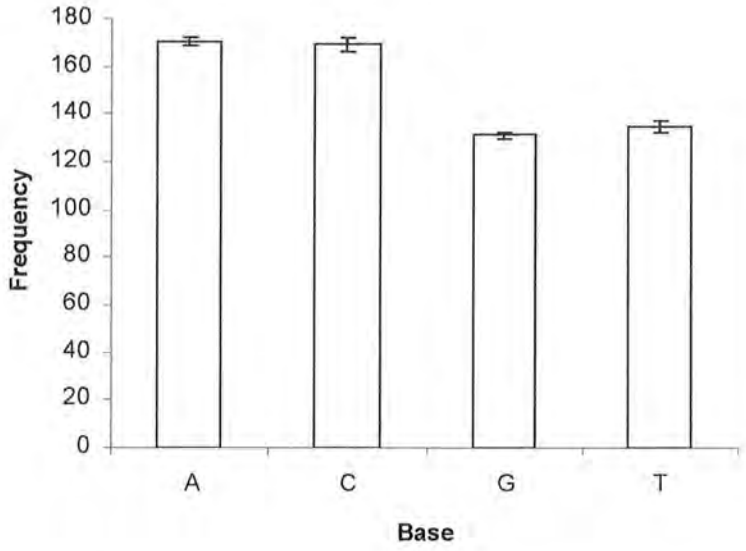


Figure 4.1. Frequency of the four nucleotide bases across a 614 base pair sequence of 16S rRNA mtDNA gene for 26 samples of handfishes. Vertical bars show standard error.

4.2b Cytochrome oxidase I sequence details:

A 543 base pair sequence of the cytochrome oxidase I mitochondrial DNA gene was amplified using PCR. The same 27 individuals used for the 16S rRNA gene were sequenced. Sequence details are presented in Figure 4.5. Again the lower Derwent River spotted handfish was used as the reference sequence. The cytochrome oxidase I gene is a protein coding gene. This means its length is highly conserved. Unlike the 16S rRNA gene no length mutations were seen in this gene. The corresponding amino acids are given for each triplet of nucleotides. An asterisk indicates that a mutation has resulted in a different amino acid to be encoded in at least one of the sequences.

There was no significant difference found between the base frequencies of the sequences, $\chi^2 = 33.975070$ (df = 3), p = 0.99999. The pyrimidine bases were the most common, C (157.63 ± 8.12) and T (150.56 ± 8.073). The purine bases were less

frequent, A (130.15 ± 6.68) and G (104.67 ± 4.583). The relative base frequencies are presented in Figure 4.2.

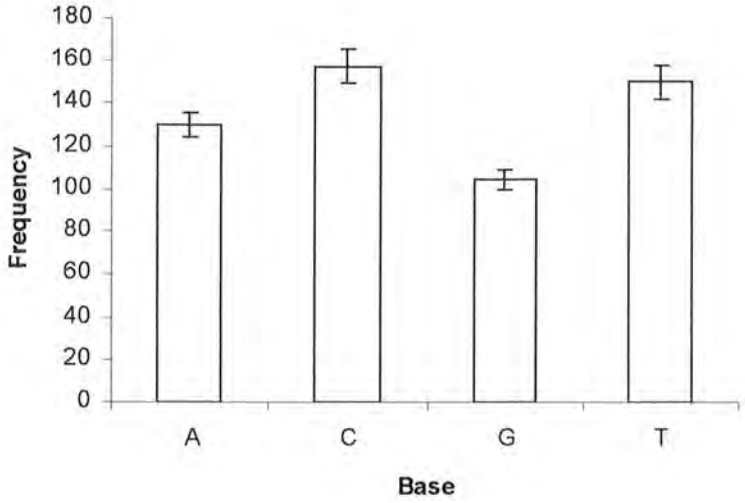


Figure 4.2 Frequency of the four nucleotide bases across a 543 base pair sequence of cytochrome oxidase I mtDNA gene for 26 samples of handfishes. Vertical bars show standard error.

Of the 543 characters, 13 of the variable characters were parsimony-uninformative with 122 being parsimony-informative. There were 4.86 times more transitions than transversions in this gene. Mutations were more common at the third codon position, with over 90% occurring at this codon position. The vast majority of these (96 vs. 17) were transitions (Figure 4.3). Transitions were also dominant at the first codon position (6 vs. 2), while at the second codon position two of each mutation type were seen. This order of mutations is commonly seen in most protein coding genes, with codon position 2 having the lowest rate of substitution and codon position 3 having the highest rate of substitution (Yang 1996).

Although the mutations at the third codon position outweighed those at codon positions one and two, these changes do not cause amino acid coding changes (Meyer 1994). Of the eight mutations at codon position one five resulted in amino acid coding changes. All of the mutations at codon position two resulted in amino acid coding changes. This reflects the nucleotide coding sequences of most amino acids, where variation at the third codon position does not lead to amino acid changes.

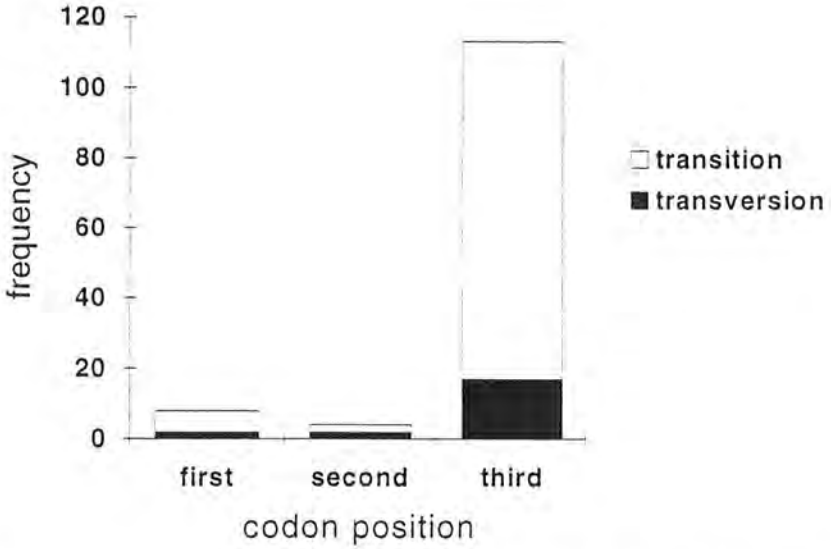


Figure 4.3. Frequency of transitions and transversions by codon position for partial sequence of cytochrome oxidase I mtDNA gene pooled for all handfish.

```
B. hirsutus 1    CGCCTGTTTT ATCAAAAAAC ATCGCCTCTT GCTTATCCTG AAATAAGAGG TCTCGCCTGC
B. hirsutus 2    .........A ...C....G. .......... ......A... .......... ..........
B. australis     .....TG... .........- .......... .......... ..-....... ..........
B. australis 3   .........- .........- .......... .......... ..-....... ..........
S. verrucosus 4  .........- .........- .......... .......... ..-....... ..........
S. verrucosus 5  .........- .........- .......... .......... ..-....... ..........
S. politus 6     .........- .........- .......... .......... ..-....... ..........
S. politus 7     .........- .........- .......... .......... ..-....... ..........
B. sp. D         .........- .........- .......... .......... ..-....... ..........
T. furcipilis    ...T.....- .........- .......... ...C.CTACA ..-....... ..C.......
T. mitchelli     .........- .........- .......... ...C.CTACA ..-....... ..C.......

B. hirsutus 1    CCCGTGACTT TATGTTTAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG CGCAATCACT
B. hirsutus 2    .......... .......... .......... .......... .......... ..........
B. australis     .......... .......... .......... .......... .......... ..........
B. australis 3   .......... .......... .......... .......... .......... ..........
S. verrucosus 4  .........A .......... .......... .......... .......... ..........
S. verrucosus 5  .........A .......... .......... .......... .......... ..........
S. politus 6     .........A .......... .......... .......... .......... ..........
S. politus 7     .........A .......... .......... .......... .......... ..........
B. sp. D         .........A ..C....... .......... .......... .......... ..........
T. furcipilis    .........-A .......... .......... .......... .......... ..........
T. mitchelli     .........-A .......... .......... .......... .......... ..........

B. hirsutus 1    TGTCTTTTAA ATAAAGACCC GTATGAATGG CTCAACGAGG GCTTAACTGT CTCCTTCCCC
B. hirsutus 2    .......... .......... .......... .......... .......... ..........
B. australis     .......... .......... .......... .......... .......... ..........
B. australis 3   .......... .......... .......... .......... .......... ..........
S. verrucosus 4  .......... .......... .......... ..T....... .......... ..........
S. verrucosus 5  .......... .......... .......... ..T....... .......... ..........
S. politus 6     .......... .......... .......... ..T....... .......... ..........
S. politus 7     .......... .......... .......... ..T....... .......... ..........
B. sp. D         .......... .......... .......... ..T....... .......... ..........
T. furcipilis    .......... .......... .......... .C......A. .......... ..........
T. mitchelli     .......... .......... .......... .C......A. A......... ..........

B. hirsutus 1    CAGTCAATGA AATTGATCTC CCCGTGCAGA AGCGGGGATA RACCCATAAG ACGAGAAGAC
B. hirsutus 2    .......... .......... .......... .......... .......... ..........
B. australis     .......... .......... .......... .......... .......... ..........
B. australis 3   .......... .......... .......... .......... .......... ..........
S. verrucosus 4  .G........ .......... .......... .......... .......... ..........
S. verrucosus 5  YG........ .......... .......... .......... .......... ..........
S. politus 6     .G........ .......... .......... .......... G......... ..........
S. politus 7     .G........ .......... .......... .......... G......... ..........
B. sp. D         .G........ .......... .......... .......... ..A....... ..........
T. furcipilis    .......... .......... .......... .......... ...T...... ..........
T. mitchelli     .......... .......... .......... .......... ...T...... ..........

B. hirsutus 1    CCTGTGGAGT TTTAGACACA AAACAGCCCT AAGCACACCC CCGCACTAAA GCAATGCCAC
B. hirsutus 2    .......... .......... .......... .......... .......... ..........
B. australis     .......... .......... .......... .......... .......... ..........
B. australis 3   .......... .......... .......... .......... .......... ..........
S. verrucosus 4  .......... .........C .......... ..--....G. .......... .....A....
S. verrucosus 5  .......... .........C .......... ..--....G. .......... .....A....
S. politus 6     .......... .........T .........C ..--...TA. .......... .....AT...
S. politus 7     .......... .........C .........C ..--...TA. .......... .....AT...
B. sp. D         .......... .........C .......... ..--....G. .......... .....A....
T. furcipilis    .......... .........A. G.CAGC..TA C.A..A..AA ..CACTC... CGGTAAT.G.
T. mitchelli     .......... .........A. G.CAGC..TA C.A..A..AA ..CACTC... CGGTAAT.G.

B. hirsutus 1    AGGCCCCTGT TCCCATGTCT TTGGTTGGGG CGACCACGGA GCATACAAAA CCTCCACGCG
B. hirsutus 2    .......... .......... .......... .......... .......... ..........
B. australis     .......... ....G..... .......... .......... .......... ..........
B. australis 3   .......... ....G..... .......... .......... .......... ..........
S. verrucosus 4  ....T..... .......... .......... ......T... .........C ......A...
S. verrucosus 5  ....T..... .......... .......... ......T... .........C ......A...
S. politus 6     .......... .......... .......... .......... .........C ......A...
S. politus 7     .......... .......... .......... .......... .........C ......A...
B. sp. D         ....T..... .......... .......... ......T... .....T...C ......A...
T. furcipilis    .......... ...T...... .......... ......T... ...C.....C ..........
T. mitchelli     .......... ...T...... .......... ......T... ...C.....C ..........
```

Figure 4.4. Partial 16s rRNA sequence aligned against the reference taxon *Brachionichthys hirsutus* from the lower Derwent River. Sequence consensus is represented by dots, gaps in alignment are represented as hyphens and correspond to insertion deletion points. (1 = lower Derwent River; 2 = Frederick Henry Bay; 3 = morph ; 4 = non-reticulate; 5 = reticulate; 6 = light; 7 = dark). The ambiguous bases Y (CT), R(AG) and M(AC) are used to indicate sequence variation within morphs.

```
B. hirsutus 1   GAATGGGAAT AA-CCCCCCT AAAACAAGGG CCCCCGCCCT AGTTACCAGA ATCTCTGACC
B. hirsutus 2   .......... .......... .......... .......... .......... ..........
B. australis    .......... ...A...... ........A. .......T.. .......... ..........
B. australis 3  .......... ...A...... ........A. .......T.. .......... ..........
S. verrucosus 4 .....A..G. .CGA.T.T.C .......... ....T..... .A........ ..........
S. verrucosus 5 .....A..G. .CGA.T.T.C .......... ....T..... .A........ ..........
S. politus 6    .....A..GC .CGA.T.T.C .......... ....T..... .A........ ..T.......
S. politus 7    .....A..GC .CGA.T.T.C .......... ....T..... .A........ ..T.......
B. sp. D        .....A..G. .CGA.T.TTC .......... ....T..... .A........ ..........
T. furcipilis   .....A..G. .CTA.T...C .......... .A..T..... .A...T.... .CT.......
T. mitchelli    .....A..G. .CTA.T...C .......... .A..T..... .A...T.... .CT.......

B. hirsutus 1   TCATGATCCG GCAAARCCGA TCAACGAACC AAATTACCCC AGGGATAACA GCGCAATCCC
B. hirsutus 2   .......... .......... .......... .......... .......... ..........
B. australis    .......... .......... .......... .......... .......... ..........
B. australis 3  .......... .......... .......... .......... .......... ..........
S. verrucosus 4 .......... .......... .......... .......... .......... ..........
S. verrucosus 5 .Y........ .......... .......... .......... .......... ..........
S. politus 6    .T........ .......... .......... .......... .......... ..........
S. politus 7    .T........ .......... .......... .......... .......... ..........
B. sp. D        .......... .......... .......... .......... .......... ..........
T. furcipilis   A..C...... ....-..... .T........ .......... .......... ..........
T. mitchelli    A..C...... ....-..... .T........ .......... .......... ..........

B. hirsutus 1   CTTCCCGAGA CCTTATCAAA AAGGGGGTTT ACGACCTCGA TGTTGGATCA GGACATCCTA
B. hirsutus 2   .......... .......... .......... .......... .......... ..........
B. australis    .......... .......... .......... .......... .......... ..........
B. australis 3  .......... .......... .......... .......... .......... ..........
S. verrucosus 4 .......... .......... .......... .......... .......... ..........
S. verrucosus 5 .......... .......... .......... .......... .......... ..........
S. politus 6    .......... .......... .......... .......... .......... ..........
S. politus 7    .......... .......... .......... .......... .......... ..........
B. sp. D        ...T...... ......T... ..........- ........T. .......... T..GGA...C
T. furcipilis   ...T.A.... .......... .......... .......... .......... ..........
T. mitchelli    ...T.A.... .......... .......... .......... .......... ..........

B. hirsutus 1   ATGGAGCAGC TACTA---TT AAGGGTTCGT TTGTTCAACG ATTAAAGTCC TACGTGATCT
B. hirsutus 2   .......... .......... .......... .......... .......... ..........
B. australis    .......... .......... .......... .......... .......... ..........
B. australis 3  .......... .......... .......... .......... .......... ..........
S. verrucosus 4 .......... .......... .......... .......... .......... ..........
S. verrucosus 5 .......... .......... .......... .......... .......... ..........
S. politus 6    .......... .......... .......... .......... .......... ..........
S. politus 7    .......... .......... .......... .......... .......... ..........
B. sp. D        C.A.T.G... AT...CTA.. .....G.TTG ....A....T ......C... ..........
T. furcipilis   ....T..... .......... .......... .......... .......... ..........
T. mitchelli    ....T..... .......... .......... .......... .......... ..........

B. hirsutus 1   GAGTTCAGAC CGGG
B. hirsutus 2   .......... ....
B. australis    .......... ....
B. australis 3  .......... ....
S. verrucosus 4 .......... ....
S. verrucosus 5 .......... ....
S. politus 6    .......... ....
S. politus 7    .......A.. ....
B. sp. D        .T.....C.. .C..
T. furcipilis   .......... ....
T. mitchelli    .......... ....
```

Figure 4.4. Continued.

	Pro	Ala*	Gly*	Gly	Gly*	Asp	Pro	Ile	Leu	Tyr	Gln	His	Leu	Phe	Trp	Phe	Phe	Gly	His	Pro
B. hirsutus 1	CCT	GCA	GGA	GGA	GGA	GAT	CCC	ATT	CTC	TAC	CAA	CAC	CTG	TTT	TGA	TTC	TTC	GGC	CAC	CCC
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	...	.AG	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis 3	...	.A.	.A.	...	.A.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S. verrucosus 4	..G	CA.	...	...	...	...	...	...	...	...	...	...	..A	..C	..G	...	...	...	...	...
S. verrucosus 5	..G	CA.	...	...	...	...	...	...	...	...	...	...	..A	..C	..G	...	...	...	...	...
S. politus 6	...	...	...	...	...	...	...	...	...	...	...	...	..A	..C	...	...	...	...	...	...
S. politus 7	...	...	...	...	...	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...
B. sp. D	..G	CA.	...	...	...	...	..G	...	...	...	...	...	...	..C	...	...	...	...	...	..T
T. furcipilis	...	.A.	...	...	...	...	..T	...	...	...	...	...	...	..C	...	..T	...	..T	..T	...
T. mitchelli	...	.A.	...	...	...	...	..T	...	...	...	...	...	..A	..C	...	..T	...	..T	..T	...

	Glu	Val	Tyr	Ile	Leu	Ile	Ile	Pro	Gly	Phe	Gly	Met	Ile	Ser	His	Ile	Val	Thr	Tyr	Tyr
B. hirsutus 1	GAG	GTC	TAT	ATC	TTA	ATC	ATC	CCC	GGG	TTC	GGC	ATA	ATC	TCA	CAC	ATT	GTG	ACC	TAT	TAC
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	..A	...	...	...	..G	...	...	...	..A	...	...	...	...	..M	...	...	...	...	...	...
B. australis 3	..A	...	...	...	..G	...	...	...	..A	...	...	...	...	..M	...	...	...	...	...	...
S. verrucosus 4	..A	...	...	...	C..	..T	..T	...	...	..T	..A	...	...	...	..T	..C	...	...	..C	...
S. verrucosus 5	..A	...	...	...	C..	..T	..T	...	...	..T	..A	...	...	...	..T	..C	...	...	..C	...
S. politus 6	..A	..T	...	...	C..	..T	..T	..T	..A	..T	..G	...	..T	...	...	..C	..A	...	..C	...
S. politus 7	..A	..T	...	...	C..	..T	..T	..T	..A	..T	..G	...	..T	...	...	..C	..A	...	..C	...
B. sp. D	...	...	...	...	C..	..T	..T	...	...	...	..G	...	...	...	..T	..C	..A	...	..C	...
T. furcipilis	..A	...	...	..T	..G	..T	...	...	..A	..T	..A	...	..T	..C	..T	..C	..A	..A	..C	...
T. mitchelli	..A	...	...	..T	..G	..T	...	...	..A	..T	..A	...	..T	..C	..T	..C	..A	..A	..C	...

	Ala*	Gly	Lys	Lys	Glu	Pro	Phe	Gly	Tyr	Met	Gly	Met	Val	Trp	Ala	Met	Met	Ala	Ile	Gly
B. hirsutus 1	GCA	GGT	AAA	AAA	GAG	CCR	TTC	GGA	TAC	ATG	GGC	ATA	GTT	TGA	GCC	ATA	ATA	GCC	ATC	GGA
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	...	...	...	...	..A	...	...	...	...	..A	...	...	...	...	...	...	...	...	...	...
B. australis 3	...	...	...	...	..A	...	...	...	...	..A	...	...	...	...	...	...	...	...	...	...
S. verrucosus 4	...	..C	..G	..G	..A	...	...	...	...	..A	...	...	...	..G	..A	...	...	...	..T	...
S. verrucosus 5	...	..C	..G	..G	..A	...	...	...	...	..A	...	...	...	..G	..A	...	...	...	..T	...
S. politus 6	...	..C	...	...	..A	...	...	..G	..T	..A	..T	...	...	...	..A	...	...	...	...	...
S. politus 7	...	..C	...	...	..A	...	...	..G	..T	..A	..T	...	...	...	..A	...	...	...	...	...
B. sp. D	...	...	..G	...	...	...	...	...	..T	...	..T	...	...	...	..A	...	...	...	...	..G
T. furcipilis	T..	..C	...	...	..A	...	...	...	...	...	..T	...	..C	...	..A	...	...	...	...	...
T. mitchelli	T..	..C	...	...	..A	...	...	...	...	...	..T	...	..C	...	..A	...	...	...	...	...

	Leu	Leu	Gly	Phe	Ile	Val	Trp	Ala	His	His	Met	Phe	Thr	Val	Gly	Met	Asp	Val	Asp	Thr
B. hirsutus 1	CTG	CTA	GGA	TTC	ATC	GTT	TGA	GCC	CAT	CAC	ATA	TTT	ACA	GTG	GGC	ATG	GAC	GTT	GAT	ACC
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	..A	...	...	...	...	...	..G	...	..C	...	...	...	...	...	...	...	...	...	..C	...
B. australis 3	..A	...	...	...	...	...	..G	...	..C	...	...	...	...	...	...	...	...	...	..C	...
S. verrucosus 4	T.A	T..	..G	..T	...	..C	...	..T	..C	...	...	...	...	..A	..A	...	..T	..C	..C	..T
S. verrucosus 5	T.A	T..	..G	..T	...	..C	...	..T	..C	...	...	...	...	..A	..A	...	..T	..C	..C	..T
S. politus 6	T.A	T..	...	..T	...	..C	...	...	..C	...	...	...	...	..A	..A	..A	...	...	...	...
S. politus 7	T.A	T..	...	..T	...	..C	...	...	..C	...	...	...	...	..A	..A	..A	...	...	...	...
B. sp. D	T.A	T..	...	..T	...	...	...	...	..C	...	...	...	...	..A	...	..A	..T	...	..C	...
T. furcipilis	..A	...	...	..T	...	...	...	..T	..C	...	...	..C	...	..A	...	..A	...	..C	...	...
T. mitchelli	..A	...	...	..T	...	...	...	..T	..C	...	...	..C	...	..A	...	..A	...	..C	...	...

	Arg	Ala	Tyr	Phe	Thr	Ser	Ala	Thr	Met	Ile	Ile	Ala	Ile	Pro	Thr	Gly	Val	Lys	Val	Phe
B. hirsutus 1	CGA	GCC	TAC	TTT	ACA	TCC	GCC	ACA	ATA	ATC	ATC	GCC	ATC	CCC	ACG	GGC	GTA	AAA	GTA	TTC
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...
B. australis 3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...
S. verrucosus 4	..G	...	...	...	...	...	..T	...	...	...	..T	...	..T	...	...	...	..T	..G	...	...
S. verrucosus 5	..G	...	...	...	...	...	..T	...	...	...	..T	...	..T	...	...	...	..T	..G	...	...
S. politus 6	..G	...	...	...	..G	...	..T	...	...	..T	..T	...	..T	..T	...	..A	..C	..G	..G	..T
S. politus 7	..G	...	...	...	..G	...	..T	...	...	..T	..T	...	..T	..T	...	..A	..C	..G	..G	..T
B. sp. D	...	...	...	...	..G	..T	..T	...	...	...	..T	...	..T	...	...	...	..C	..G	...	...
T. furcipilis	...	..A	...	...	...	...	...	...	...	...	...	...	...	...	...	..A	..A	..T	...	..T
T. mitchelli	...	..A	...	...	...	...	...	...	...	...	...	...	...	...	...	..A	..A	..T	...	..T

Figure 4.5. Partial cytochrome oxidase I sequence aligned against the reference taxon *Brachionichthys hirsutus* (1) from the lower Derwent River. Sequence consensus is represented by dots. Amino acid translation is given above the sequences with an asterisk corresponding to mutations changing the coded amino acid. (1 = lower Derwent River; 2 = Frederick Henry Bay; 3 = unidentified species morph; 4 = non-reticulate; 5 = reticulate; 6 = light; 7 = dark). The ambiguous bases Y (CT), R(AG) and M(AC) are used to indicate sequence variation within morphs.

	Ser	Trp	Leu	Ala	Thr	Leu	His	Gly	Gly	Thr	Ile	Lys	Trp	Glu	Ala*	Pro	Leu	Leu	Trp	Ala
B. hirsutus 1	AGC	TGA	CTT	GCA	ACC	CTG	CAC	GGA	GGC	ACA	ATC	AAA	TGA	GAA	GCC	CCC	CTT	CTC	TGG	GCC
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..Y	...	...	...	..A	...
B. australis 3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..Y	...	...	...	..A	...
S. verrucosus 4	...	..G	..C	...	..T	..A	..T	...	...	...	..T	..G	...	...	...	...	..C	..T	...	...
S. verrucosus 5	...	..G	..C	...	..T	..A	..T	...	...	...	..T	..G	...	...	...	...	..C	..T	...	...
S. politus 6	...	..G	..C	...	..T	..C	...	...	...	...	..T	..G	...	...	...	...	..C	..T	...	...
S. politus 7	...	..G	..C	...	..T	..C	...	...	...	...	..T	..G	...	...	...	...	..C	..T	..A	...
B. sp. D	...	...	..C	...	..T	...	..T	..C	...	...	..T	..G	...	...	...	...	...	..T	...	...
T. furcipilis	...	...	...	...	...	...	..T	...	..G	...	..T	...	...	...	A..	...	...	..T	..A	...
T. mitchelli	...	...	...	...	...	...	..T	...	..T	...	..T	...	...	...	A..	...	...	..T	..A	...

	Leu	Gly	Phe	Ile	Phe	Leu	Phe	Thr	Val	Gly	Gly	Leu	Thr	Gly	Ile	Val*	Leu	Ala	Asn	Ser
B. hirsutus 1	CTC	GGG	TTC	ATC	TTT	CTA	TTC	ACC	GTG	GGG	GGC	CTC	ACC	GGC	ATC	GTC	CTA	GCC	AAC	TCC
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	...	...	...	...	...	...	...	...	...	..A	...	...	...	...	...	A..	...	...	...	...
B. australis 3	...	...	...	...	...	...	...	...	...	..A	...	...	...	...	...	A..	...	...	...	...
S. verrucosus 4	..T	..C	...	...	...	..T	..T	...	..T	...	..T	...	..T	...	...	...	...	...	...	...
S. verrucosus 5	..T	..C	...	...	...	..T	..T	...	..T	...	..T	...	..T	...	...	...	...	...	...	...
S. politus 6	..T	..C	...	...	...	..C	..T	...	..T	...	..T	..T	..T	...	...	...	...	...	...	...
S. politus 7	..T	..C	...	...	...	..C	..T	...	..T	...	..T	..T	..T	...	...	...	...	...	...	...
B. sp. D	..T	..C	...	...	...	..C	..T	...	..T	..A	..T	...	...	...	...	..T	...	...	...	...
T. furcipilis	..T	..C	...	...	...	T..	..T	..A	..C	..A	..T	..T	...	...	...	A..	...	...	...	..T
T. mitchelli	..T	..C	...	...	...	T..	..T	..A	..C	..A	..T	..T	...	...	...	A..	...	...	...	..T

	Ser	Leu	Asp	Ile	Val*	Leu	His	Asp	Thr	Tyr	Tyr	Val	Val	Ala	His	Phe	His	Tyr	Val	Leu
B. hirsutus 1	TCC	CTA	GAC	ATT	GTC	CTT	CAT	GAC	ACC	TAT	TAT	GTA	GTA	GCC	CAC	TTC	CAC	TAT	GTA	CTT
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	...	...	...	...	...	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...
B. australis 3	...	...	...	...	...	...	...	...	...	..C	...	...	...	...	..Y	...	...	...	...	...
S. verrucosus 4	...	...	..T	...	...	...	...	...	..T	..C	...	..T	..G	...	...	...	...	...	..G	..C
S. verrucosus 5	...	...	..T	...	...	...	...	...	..T	..C	...	..T	...	...	...	...	...	...	..G	..C
S. politus 6	...	...	...	...	...	...	..C	...	..T	..C	...	..T	..G	...	...	...	...	...	..G	..C
S. politus 7	...	...	...	...	...	...	..C	...	..T	..C	...	..T	..G	...	...	...	...	...	..G	..C
B. sp. D	...	...	...	...	...	...	..C	...	...	..C	...	..T	...	...	...	...	...	...	..G	..C
T. furcipilis	..T	...	...	...	A..	...	..C	..T	..A	..C	...	...	...	..A	...	...	...	..C	...	..C
T. mitchelli	..T	...	...	...	A..	...	..C	..T	..A	..C	...	...	...	..A	...	...	...	..C	...	..C

	Ser	Met	Gly	Ala	Val	Phe	Ala	Ile	Met	Ala*	Ala	Phe	Ile	His	Trp	Phe	Pro	Leu	Ile	Ser
B. hirsutus 1	TCC	ATA	GGG	GCT	GTC	TTT	GCT	ATC	ATA	GCA	GCC	TTC	ATC	CAC	TGA	TTC	CCT	CTA	ATC	TCT
B. hirsutus 2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
B. australis 3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
S. verrucosus 4	...	...	..A	...	...	...	..C	...	...	...	...	..T	...	...	...	...	...	...	...	...
S. verrucosus 5	...	...	..A	...	...	...	..C	...	...	...	...	..T	...	...	...	...	...	...	...	...
S. politus 6	...	...	..A	...	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	...
S. politus 7	...	...	..A	...	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	...
B. sp. D	...	...	..A	...	...	...	...	...	..G	..G	...	..T	...	...	...	...	...	...	...	...
T. furcipilis	...	...	...	..C	...	...	..C	...	...	.G.	...	...	..T	...	...	...	...	...	...	...
T. mitchelli	...	...	...	..C	...	...	..C	...	...	.G.	...	...	..T	...	...	...	...	...	...	...

	Gly
B. hirsutus 1	GGG
B. hirsutus 2	...
B. australis	...
B. australis 3	...
S. verrucosus 4	...
S. verrucosus 5	...
S. politus 6	...
S. politus 7	..A
B. sp. D	...
T. furcipilis	...
T. mitchelli	...

Figure 4.5. Continued.

4.3 Phylogenetic reconstruction:

4.3a Preliminaries:

Initial analysis using both the 16S rRNA and cytochrome oxidase I data sets produced similar phylogenetic reconstructions. To test whether the two data sets could be combined a partition-homogeneity test was carried out in *PAUP* 4.0*. This test constructs a tree using both data sets, and then by re-sampling equivalent data sets from the original data, compares the tree lengths. If the original tree is within one standard deviation of the mean, the two data sets can be combined. One thousand replicate trees were calculated, with an average of 317.858 ± 1.103201 steps. The observed combined tree length of 318 falls within this range ($p = 0.67400$). This suggests that there is no significant difference between the two data sets and they were thus combined for further phylogenetic analysis. Figure 4.6 presents the observed combined tree length compared to 1000 bootstrap estimates.

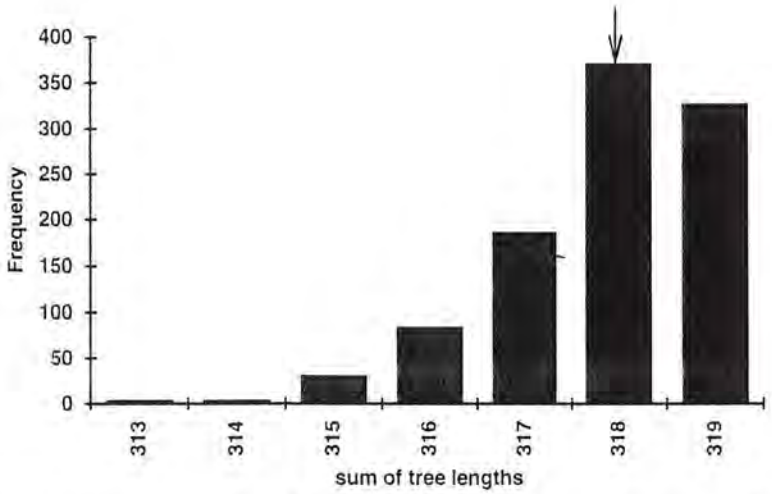


Figure 4.6. Partition-homogeneity test observed tree length (arrow) compared to 1000 bootstrap estimates. If observed value is within one standard deviation of mean then two data sets are not significantly different and can be combined.

Handfish phylogeny was inferred using a number of methods based on the pooled 16S rRNA and COI sequence data. Parsimony, pair-wise distance and maximum likelihood were the three methods used to construct trees. The most parsimonious phylogeny is one that requires the least evolutionary events to explain the differences seen. Maximum likelihood methods assume the form of a tree and then choose branch lengths to maximise the likelihood of the data given that tree. These likelihoods are then compared over different trees (Weir 1990). Finally, distance methods calculate the pair-wise distance and then proceed to create trees to minimise the overall distance.

4.3b Parsimony analysis:

Parsimony analysis was carried out using the combined 16S rRNA and COI DNA sequence data. For this analysis five character states were recognised: the four nucleotide bases, with the fifth being the gap state (insertions/deletions). Parsimony analysis was performed using *PAUP* 4.0* (Phylogenetic Analysis Using Parsimony* and Other Methods) (Swofford 1998). This program searches among alternate trees looking for those which minimise the number of evolutionary changes required to explain the observed character state differences.

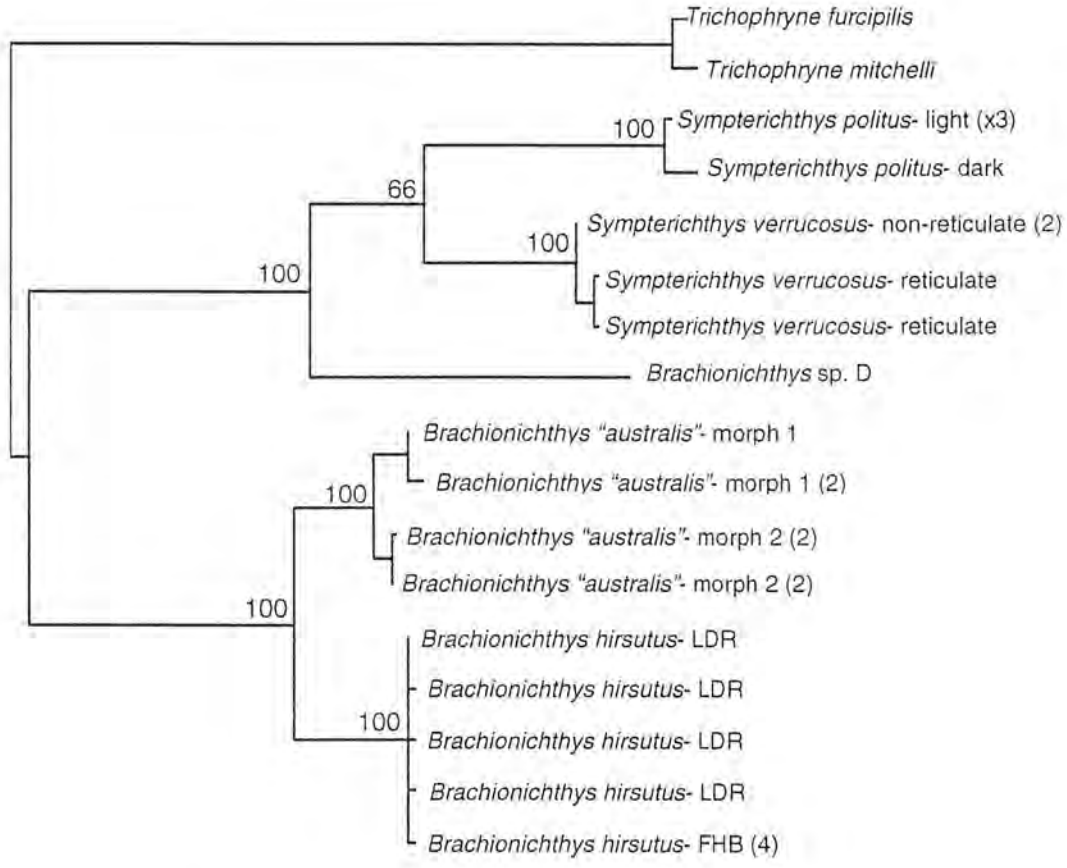


Figure 4.7 Single most-parsimonious cladogram rooted using anglerfish, *Trichophryne furcipilis* and *T. mitchelli* as sister taxa. Branch lengths are proportional to the number of character state changes, with tree length 305 steps. Values at each node represent bootstrap percentages based on 1000 replicates. Numbers in brackets correspond to the number of individuals sequenced with the same genotype. LDR = lower Derwent River and FHB = Frederick Henry Bay, referring to the origin of the spotted handfish sequenced.

The heuristic search option of PAUP was used in the maximum parsimony analysis. Heuristic searches randomly generate a starting seed and then proceed to build trees from this point. The single most parsimonious tree is presented in Figure 4.7. This tree is built from the sequence

data of the five species of handfish and two of anglerfish. The branch lengths are proportional to the number of nucleotide substitutions.

Bootstrapping was used to determine the probability of the branches being observed by chance. If a branch is resolved over 50% of the time, it is considered a significant branch. Bootstrap values are given above the branches at each node. The genus *Sympterichthys*, which contains the warty handfish (*S. verrucosus*) and the red handfish (*S. politus*), was resolved as a monophyletic group. This separation was resolved in 66% of the trials. The two forms of the warty handfish, the reticulate and non-reticulate, were separated in all trials. Unlike the *Sympterichthys*, the genus *Brachionichthys* does not appear to be monophyletic. While the Australian handfish (*B. "australis"*) and the spotted handfish (*B. hirsutus*) were grouped as a monophyletic group, the other member of the *Brachionichthys*, *B.* sp. D was grouped more closely to the *Sympterichthys*. Representatives of the two populations of spotted handfish sequenced, the lower Derwent River (DR) and Frederick Henry Bay (FHB), were separated by one or two base pairs difference. The Australian handfish and unidentified morphs of Australian handfish were separated in all bootstrap trials; this variation was only a few base pairs.

To test if the topology of the single most parsimonious tree was better than one with enforced monophyly of the *Brachionichthys* and *Sympterichthys*, a non-parametric Templeton (Wilcoxon signed-ranks) test was conducted (Templeton 1983). This compared the single most parsimonious tree to a tree of enforced monophyly. This tested the null hypothesis of no difference between the two trees (two-tailed test). This test showed a highly significant difference between the single most parsimonious tree and the best enforced monophyly tree ($p < 0.0001$), with the single most parsimonious tree being the most parsimonious of the two. This means that when monophyly is enforced under the parsimony criteria, its topology is inferior to that of the single most parsimonious tree.

4.3c Maximum likelihood analysis:

Maximum likelihood analysis was carried out using two approaches. Both neighbour-joining and UPGMA (unweighted pair-group method using arithmetic averages) were used to construct trees. UPGMA creates trees by calculating pair-wise distances and then joining the closest

pairs. The distances are then recalculated with this single pair as a group, and the next closest pair is joined. This continues until all individuals have been incorporated into a single tree. Neighbour-joining works on similar principles, using a modified distance matrix and joining nodes instead of branches. Both these trees had the same topology so only the neighbour-joining tree is presented. Figures 4.8. shows the best maximum likelihood tree using neighbour-joining. This tree is rooted with the two anglerfishes *Trichophryne furcipilis* and *T. mitchelli* as a sister taxa.

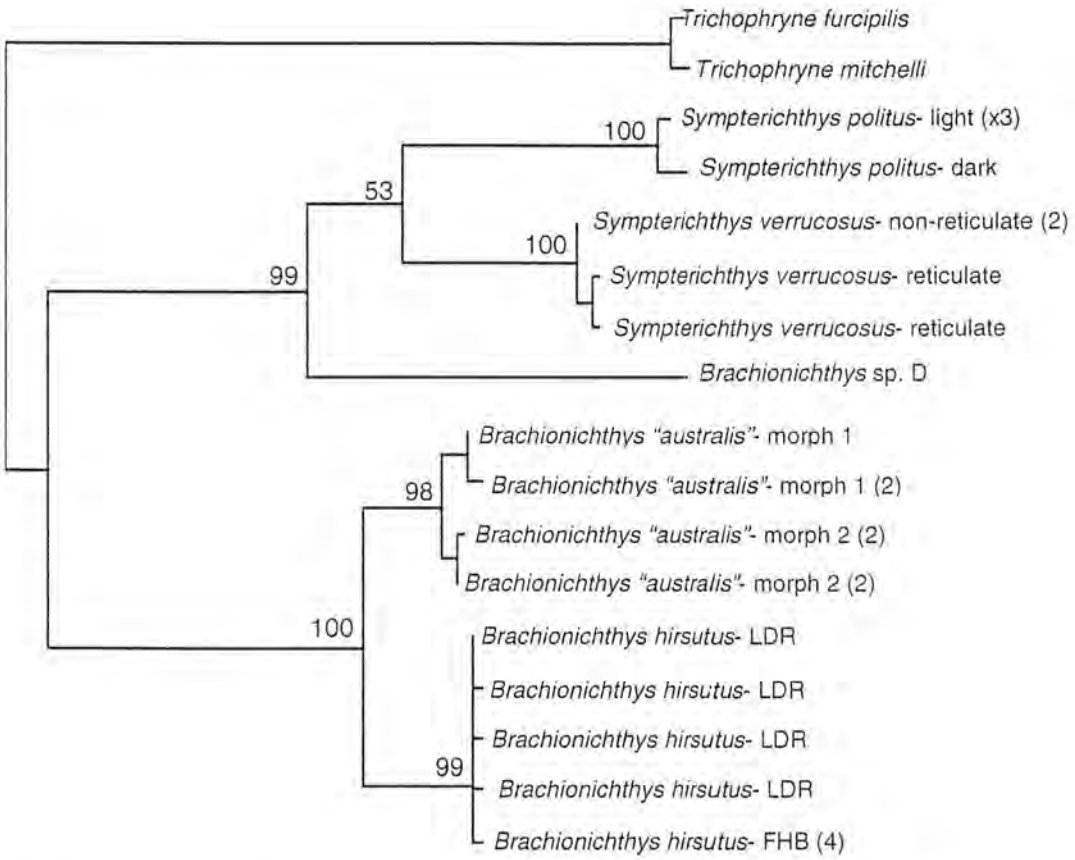


Figure 4.8. Maximum likelihood cladogram (likelihood = 3090.677), rooted with the anglerfish *Trichophryne furcipilis* and *T. mitchelli* as sister taxa. Branch lengths are proportional to the expected number of substitutions per site. Values at each node represent bootstrap percentages based on 500 replicates

This tree has the same topology as the maximum parsimony analysis. Bootstrap values based on 500 replicates are given at the nodes. All branches were resolved greater than 50% of the time. The log likelihood of this tree is -3090.677. Maximum likelihood values were calculated for the single most parsimonious tree. This had a log likelihood of -3090.677 (the same as the best maximum likelihood tree). This was compared to the likelihood of an enforced monophyly tree

using a parametric Kishino-Hasegawa test (two-tailed test) (Kishino and Hasegawa 1989). The enforced monophyly tree had a log likelihood of -3122.230, which was found to be significantly lower than the best maximum likelihood tree value ($T = 10.42228$, $p < 0.0025$). This means that when monophyly is enforced the topology is inferior to that of the best maximum likelihood tree.

4.3d Pair-wise distance analysis:

Pair-wise distances were calculated between each of the taxa. Distances are based on the Kimura 2-parameter model (Kimura 1980). This model makes the assumption that all four nucleotides are equally frequent and that there are independent rates for transition substitutions and for transversion substitutions. Again both neighbour-joining and UPGMA were used to construct trees. The trees were rooted with two anglerfishes *Trichophryne furcipilis* and *T. mitchelli* as a sister taxa. As for the maximum likelihood analysis, the UPGMA supported the neighbour-joining phylogenetic reconstruction in topology. Therefore only the neighbour-joining tree is presented here (Figure 4.9).

The topology of this tree is the same as that of the parsimony analysis and the maximum likelihood analysis. The genus *Sympterichthys* is supported as an distinct monophyletic group. This group was separated 53% of the time for 500 bootstrap replicates. The reticulate and non-reticulate morphs of the warty handfish were separated 99% of the time, again supporting the findings of the other two phylogenetic reconstructions. The genus *Brachionichthys* is not supported as a monophyletic group. *Brachionichthys* species D falls more closely with the *Sympterichthys* genus. The spotted handfish and the Australian handfish separate out into a single group, with the same internal relationships.

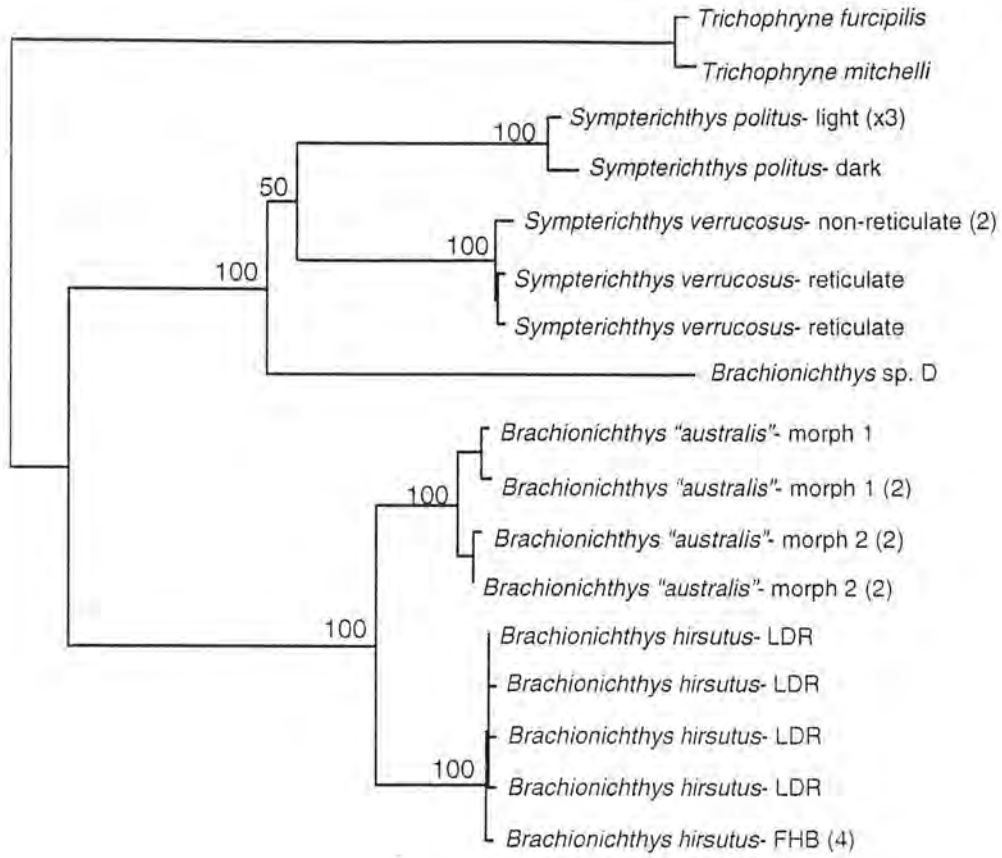


Figure 4.9. Neighbour-joining cladogram based on pair-wise distance using the Kimura 2-parameter model of sequence evolution. The tree is rooted using the anglerfish *Trichophryne furcipilis* and *T. mitchelli* as sister taxa. Percentage bootstrap support for branches is given at each node, and is based on 1000 replicates.

4.4 Discussion:

4.4a Consensus phylogeny:

There is general consensus between the three methods used to infer the phylogenetic relationships of the Family Brachionichthyidae. Currently there is no agreed position as to which of the methods is best for phylogenetic reconstruction (Meyer 1990). A conservative consensus phylogeny was therefore inferred from all of these three methods (Figure 4.9). The major clades were all resolved in the same groupings in each of the three phylogenetic reconstructions. Apart from the red handfish/warty handfish clade (53-70%) all branches were resolved greater than 95% of the time after bootstrapping. This suggests that the phylogenies are robust. The fact that the three methods of phylogenetic construction were all in concordance suggests that the inferred phylogenies may be an accurate reflection on the true molecular relationship.

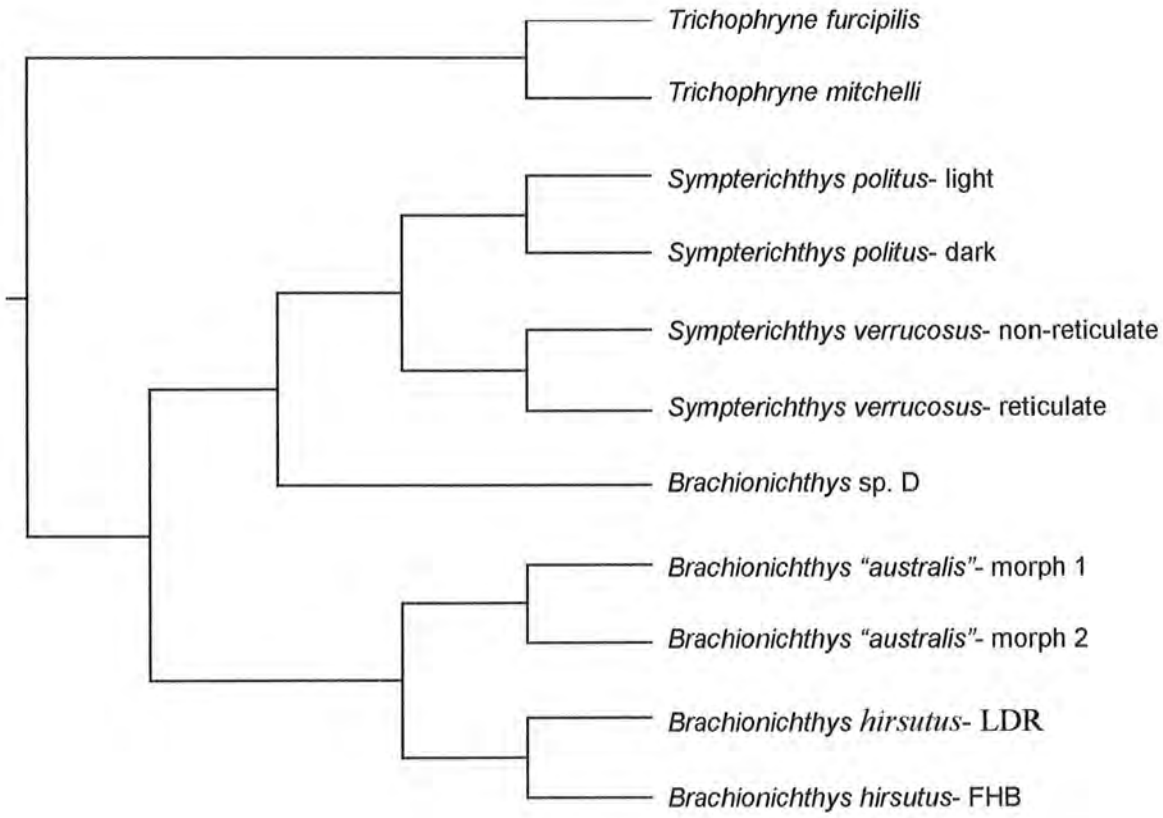


Figure 4.9 Conservative phylogeny, based on parsimony, maximum likelihood and distance methods of phylogenetic reconstruction. The tree was rooted using the anglerfishes *Trichophryne furcipilis* and *T. mitchelli* as sister taxa.

The two main clades in the conservative phylogeny represent *Brachionichthys* and *Sympterichthys*. *Sympterichthys* was resolved as a monophyletic clade in all reconstructions. This genus currently contains the warty handfish *S. verrucosus*, and with the taxonomic revision of the group it is likely that the red handfish *S. politus*, currently *Brachionichthys*, will also be included in this group (Peter Last pers. comm.). Under the current classification system the genus *Brachionichthys* does not appear to be a monophyletic group. However, this may be due to the fact that only the spotted handfish, *B. hirsutus*, has been described. Presumably, formal description of currently undescribed species may lead to their placement in other genera.

Of the remaining species classified in the genus *Brachionichthys*, samples were only available from the spotted handfish, *Brachionichthys hirsutus*, and the Australian handfish, *B. "australis"*. These were resolved as distinct species but in a single clade. The other species, *Brachionichthys* sp. D, appears to belong to a different genus than *Brachionichthys*. The reclassification of this species would make the genus *Brachionichthys* a monophyletic clade.

The new species of handfish *Brachionichthys* sp. D was closely associated with the *Sympterichthys* clade. This species may either be a member of the *Sympterichthys* genus, or part of a new closely related genus as it was divergent enough from the other members of the *Sympterichthys*. Based on morpholgical features it is similar to a Ziebell's or Loney's handfishes. These three species, all of which are currently undescribed, may be assigned to a new genus (Peter Last pers. comm.). Based on morphological studies in progress these species are likely to be removed from the *Brachionichthys* genus. These species are believed to be closely related and may even be colour morphs of a single species. There is some circumstantial evidence that Ziebell's and Loney's handfish can breed to form hybrids as intermediate colour morphs have been observed in the wild.

On the current molecular consensus phylogeny, moving of *B.* sp. D to either the *Sympterichthys* genus or a new genus is supported. *B.* sp. D grouped closer to the *Sympterichthys* than the *Brachionichthys*, but was still resolved from the *Sympterichthys* clade in 100% of bootstraps replications. This reflects the fact that *Brachionichthys* sp. D shares more sequence similarities with the *Sympterichthys* genus than *Brachionichthys* for the two gene regions looked at in this study.

4.4b Reticulate versus non-reticulate warty handfish:

The warty handfish, *Sympterichthys verrucosus,* exhibits two distinct morphs based on skin patterning. These are the non-reticulate form which is the described form, and the reticulate form (fish net patterning on skin). The reticulate form of the warty handfish has been suggested as a distinct but formally undescribed species, *S. "unipennus"*. This study found approximately 2-3 base pair difference between the two morphs over the 1157 base pairs sequenced for the 16S rRNA and cytochrome oxidase I genes. Within morphs there was a one base pair difference for the two reticulate form specimens, and no sequence difference between the two non-reticulate form specimens examined. A molecular clock calculation on the third codon position nucleotide variation of the cytochrome oxidase I gene (Bermingham *et al.* 1997) suggests that if these were two separate lines they would have diverged less than 200 000 years ago. While the two forms were resolved in over 95% of bootstraps, the low divergence between the two suggest that they do not warrant separate specific status.

Further investigation of these two morphs is clearly required. This would include using a large number of individuals of each morph from several known geographic locations. This would enable between morphs variation to be compared to the within morph variation. By comparing fish from known geographical locations this would show if differences between morphs was consistent over the species range. The low levels of variation observed between the two morphs of warty handfishes for both the cytochrome oxidase I and the 16S rRNA genes, suggests that a more sensitive technique may need to be applied to this problem. Options include a PCR-based RFLP technique such as that used for the spotted handfish population genetics investigation (chapter 3), or the use of nuclear microsatellites which is the most sensitive technique currently available.

4.4c Relationships within the genus *Brachionichthys*:

Two morphs of Australian handfish, *B. "australis"*, were examined. One morph was the recognised morph from Disaster Bay, NSW, while the other morph was an undescribed morph found from further up the NSW coast at Bermagui. These two morphs were resolved in each reconstruction over 95% of the time. As with the warty handfish, little sequence variation exists between the two morphs, with a 5 base pairs difference over the two genes. Within morph variation based on 3 and 4 specimens (recognised and unidentified morphs respectively) was

2 to 4 base pairs for each morph. As for the warty handfish this level of variation suggests that separate specific status is not warranted for the two morphs.

The Australian handfish, *B. "australis"*, and the spotted handfish, *B. hirsutus*, are clearly distinct species. Using the molecular clock calculation of Bremmer *et al.* (1997) on the cytochrome oxidase I third codon position mutations, these species probably diverged 2.5 - 3.0 x 10^6 years ago. As a comparison, the intra-specific divergence time between the two supposed morphs of Australian handfish would have been around 500 000 years. These measures are based on estimates of sequence divergence between groups of species separated when the Isthmus of Panama rose between 2 and 5 million years ago (Bremmer *et al.* 1997). These estimates have been used create a general model to predict the divergence time given a certain level of genetic variation between two taxa. This model assumes that the rate of mutation is stable over time, and that predictions of when species were cut off at the Isthmus are accurate.

The two populations of spotted handfish, lower Derwent River and Frederick Henry Bay, were distinguished by two nucleotide substitutions. This split was supported in over 95% of bootstrap replicates for all the methods of phylogenetic reconstruction. This supports the findings of the restriction enzyme population study (Chapter 3), which found the handfish of the lower Derwent River and those of Frederick Henry Bay constituted two distinct populations based on differences in genetic variation.

4.4d Explanation for the current distribution of handfishes:

The divergence of the *Sympterichthys* and the *Brachionichthys* appears to have occurred greater than 35 million years ago. The two genera share an overlapping distribution. Both range from around south-eastern Tasmania, to southern NSW and into the eastern regions of the Great Australian Bight. The majority of species are known from the south-east of Tasmania (Last *et al.* 1983). Each genus contains one widespread species, the Australian handfish in the *Brachionichthys*, and the warty handfish in the *Sympterichthys* (Scott *et al.* 1974, Last *et al.* 1983). There are two possible explanations for the current distribution of handfishes. Either handfishes as a group evolved in Tasmania and are currently radiating out from here, or handfishes were once widespread and have over time been confined to south-eastern Australia.

The number of handfishes endemic to Tasmania suggests that the family may have originated in this region. If this is the case, only two species, the Australian handfish and the warty handfish have been able to establish themselves across Bass Strait. These species are generally found in deep offshore waters (Hutchins and Swainston 1986, Edgar 1997). This largely explains why Bass Strait has proved less of a barrier to these two species than the remaining handfish, which are generally associated with shallow inshore waters. An alternative view is the handfishes were once wide spread, but are now confined to south-eastern Australia. There is little evidence of the handfishes ever being common outside their current range. However, a fossil, similar to the extant species, is known from the Eocene of Italy (Pietsch 1981).

6 General Discussion:

6.1 Spotted handfish conservation genetics:

The spotted handfish was found to have two genetically distinct populations. Population structuring is rare among most marine fish, which unlike the spotted handfish, have strong powers of dispersal and live in a large homogenous environment with few barriers to movement (Ward *et al.* 1994). The spotted handfish has poor dispersal powers, and being a benthic dwelling species, has specific habitat requirements in depth and sediment type (Last *et al.* 1983). Other marine fish that show population structuring include the striped bass, *Morone saxatalis* (Wirgin *et al.* 1989), and the toad fishes, *Opsanus tau* and *O. beta* (Avise *et al.* 1987*b*).

There appears to be little genetic exchange between the four sites looked at in this study with the exception of sites 2 and 3, in the lower Derwent River estuary. These sites formed a single population, which due to the presence of several haplotypes not found elsewhere in the population, and their close proximity, is likely to be due to continued genetic exchange. On the other hand, sites 1 and 4, which also formed an apparent genetic population, are unlikely to have any genetic exchange as they are at opposite ends of the species' range. Shared haplotypes between these two sites are likely to represent historic rather than present day events.

These findings have some important ramifications for the captive breeding and recovery of this species. The presence of low genetic variation throughout the entire spotted handfish species' range means that a smaller effective population can be used to maintain the quality and quantity of genetic variation (Philippart 1995). The two genetically distinct populations should ideally be treated as separate entities. This means that either two captive breeding populations could be used, or alternatively a single captive breeding population could be used to introduce handfish to selected locations. Maintaining two captive breeding populations is an expensive option. Therefore a single captive breeding colony targeting specific locations for release is the sensible option until the success of the program can be evaluated.

If introduced captive bred animals introduce disease, compete with wild fish or do not survive then the program may be harmful. It is better to trial captive breeding on a small scale initially prior to wide range supplementation. There are two viable options for the release of captive bred fish back into the wild. Both have the aim of re-establishing self-sustaining populations (Philippart 1995). The first is to release them into areas where handfish once lived but are now locally extinct (restocking). The second is to release them back into areas with low numbers due to poor recruitment (enhancement stocking) (Philippart 1995). A third option of introductions outside the historical range is not recommended as it can cause far greater ecological problems.

The first option has the advantage of not genetically "polluting" remaining populations with foreign genetic information. It does run a greater risk of failure in that unless the factors that initially caused the local extinction of the handfish from that area have been dealt with, they may also cause the introduced handfish to fail (Philippart 1995). The other option is to release handfish directly back into the area that the captive broodstock was taken from. This option is sound provided that little selection and genetic drift have occurred in the captive breeding population, and that there have been no great allele frequency shifts between the wild and the captive populations. Otherwise the introgression between the wild and captive reared fish may lead to a decrease in fitness and extinction of the wild population through outbreeding (Philippart 1995).

There is evidence that releasing fish into an environment that they are not genetically adapted to may decrease their ability to survive and reproduce. Jones *et al.* (1996) found failure of a hatchery reared strain of brook trout, *Salvelinus fontinalis*, to survive and reproduce when introduced into Fundy National Park, Canada. This highlights the problem facing captive breeding programs, the need to maintain levels of genetic variation close to those in the wild. Release of genetically different captive bred fish can also have a harmful effect on the wild populations. In Slovenia, the native marbled trout, *Salmo trutta marmorata,* populations are supplemented with captive bred fish (Budhina and Ocvirk 1990). Unfortunately, some of the restocking has involved a hybrid form between the marble trout and non-migratory brown trout,

Salmo trutta. Hybridisation between this hybrid form and wild marbled trout are now threatening the marbled trout with extinction in some streams.

The Desert pup fishes (Cyprinodontidae) of North America have been the focus of considerable conservation efforts. Due to their highly fragmented habitat, speciation has been quite prolific. Several distinct species now inhabit single isolated water bodies. Captive breeding has been successfully used to maintain entire species while their natural habitat is restored (Pister 1990). Captive breeding of the pup fish it undertaken at a hatchery devoted to the conservation of endangered species (Meffe 1990). Hatchery populations are monitored for genetic changes caused either due to small founder populations, continued small population size, and artificial selection. All these may alter or reduce genetic variation through genetic drift or natural selection (Meffe 1990).

A final problem facing the captive breeding program is whether it is better to use broodstock from the more genetically variable sites 2 and 3 or the less genetically variable sites 1 and 4. This to a degree depends on where the fish are going to be re-released. Broodstock should be chosen to be as similar as possible to the site of targeted release. Philippart (1995) suggests that where there is a choice between two populations the one having the greatest genetic diversity should be used for the breeding stock. This is supported by Meffe (1990) who states that higher heterozygosity (a measure of genetic variation at the allelic level) is positively associated with growth rates, survivorship, fecundity and developmental stability. All these features will increase the chances of successful reintroduction. Higher genetic variation will also allow the introduced population a chance to adapt to the environment and give natural selection a base on which to act (Meffe 1990).

This study found that the spotted handfish was characterised by low genetic variation. It also showed significant genetic population structuring between the lower Derwent River handfish and the remainder of the species. This information will allow the captive breeding and recovery of the handfish to proceed with a bit more certainty. However, caution should still be used in any decision. Only low genetic variation was found with restriction fragment length polymorphisms. Further study may want to focus on more

sensitive techniques such as microsatellites. This may show up higher levels of variation that were not apparent in the RFLP work. These may be used to support the suggested genetic population structure or may suggest different or more highly structured genetic populations. It is probable that the site 1 and site 4 handfish would be genetically different from each other considering their large geographical separation.

6.2 Phylogenetic relationships in the Family Brachionichthyidae:

The phylogenetic component of this study generally supported the traditional taxonomy of the handfish. Nelson (1984) recognised only a single genus, *Brachionichthys*. However, the *Sympterichthys* genus is currently recognised and contains at least two species, the warty handfish, *S. verrucosus* and the red handfish, *S. politus*. Based on molecular data the two genera *Brachionichthys* and *Sympterichthys* were both resolved as distinct clades. These genera appear to have diverged around 35 million years ago based on the molecular clock of Bermingham *et al.* (1997). Most of the species fell into their currently classified genera. The one exception was the newly discovered *Brachionichthys* sp. D. The classification of this species is only an interim classification and it, like many other handfish species, is still waiting to be described.

Brachionichthys sp. D is allied closely with the *Sympterichthys* genus, and it is suggested that it be moved from the *Brachionichthys* to this genus or a new closely related genus. Further study needs to be done to look at the relationship between this species and Ziebell's and Loney's handfishes, which are all believed to be closely related. Once samples of the latter two become available and analysed, it may become apparent whether these species are colour morphs of the same species, or separate species. It may also become clear whether these three species belong to the *Sympterichthys* genus, or perhaps a new genus of their own.

The status of some of the handfish species morphs were briefly examined. The reticulate and non-reticulate forms of the warty handfish, *S. verrucosus*, did not appear to warrant separate species status. The same was true for the morphs of the red handfish, *S. politus*, and the Australian handfish, *B. "australis"*. Molecular clock calculations based on models of the Isthmus of Panama (Bermingham *et al.* 1997)

using the cytochrome oxidase I gene, suggest that these species morphs have been separated less than five hundred thousand years. Further study needs to be done on the occurrence of morphs for these three species and also the Ziebell's handfish complex. Larger sample sizes from a variety of locations will be needed. This will allow the comparison of within morph and between morph genetic variation across the species range.

Molecular techniques have succesfully been used for the examination of species morphs. The tropical damselfish, *Acanthochromis polyacanthus*, has multiple colour morphs. This species lacks pelagic larvae and like the handfish lacks the capacity for broad scale dispersal (Planes and Doherty 1997). It was found that these colour morphs would interbreed and form hybrids when they occurred together. No clear genetic differences were seen between the two colour morphs based on allozyme data (Planes and Doherty 1997). There are also examples in the literature where two recognised species have been found to constitute only a single species based on molecular data. The rock lobster, *Jasus edwardsii,* was originally thought of as two species, *J edwardsii* in New Zealand and *J. novaehollandiae* in Australia. It is now considered a single species based on molecular data (Smith *et al.* 1980; Brasher *et al.* 1992).

Molecular techniques have also been successfully employed to detect distinct species where none are apparent based on morphology, ie. cryptic speciation. The tarakihi *Nemadactylus macropterus* from New Zealand waters was shown to have two distinct genetic forms (Smith *et al.* 1996). It was concluded that these were two species, the tarakihi and the previously undescribed king tarakihi, based on genetic distance between the two. Reproductive isolation is suggested as a key mechanism for the differentiation of such species (Knowlton 1993). There are also examples where presumed colour morphs of a single species have been shown to be quite genetically divergent. eg. The intertidal sea anemones of the genus *Actinia* in the Isle of Man (Solé-Cava and Thorpe 1992). Red and Green morphs exist which have been shown to be genetically different from both each other and between populations.

This study has provided insights into the molecular relationships within the Family Brachionichthyidae. The support of the three methods, used to infer phylogenetic relationships, for each other suggests a robust molecular phylogeny. This molecular phylogeny is also generally supported by the morphological relationships of the group, which suggests an accurate representation. Further investigations into some of the species morphs is still needed, and into the relationship of the Ziebell's and Loney's handfishes once specimens become available.

6 References:

Alvarado Bremmer, J.R., Naseri, I. and Ely, B. 1997. Orthodox phylogentic relationships among tunas revealed by the nucleotide sequence analysis of the mitochondrial DNA control region. *Journal of Fish Biology*. **50:** 540-554.

Apostilides, A.P., Triantaphyllidis, C., Kouvtsi, A. and Ecomonidis, P.S. 1997. Mitochondrial DNA sequence variation and pylogeography among *Salmo trutta* L. (Greek brown trout) populations. *Molecular Ecology*. **6:** 531-542.

Ashbaugh, N.A., Echelle, A.A. and Echelle, A.F. 1994. Genetic diversity in Red River pupfish *Cyprinodon rubrofluviatalis* (Atheriniformes: Cyprinodontidae) and its implications for the conservation genetics of the species. *Journal of Fish Biology*. **45:** 291-302.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A. and Saunders, N.C. 1987*a*. Intraspecific phylogeography: The mitochondrial DNA bridge between population genetics and systematics. *Annual Reviews in Ecology and Systematics*. **18:** 489-522.

Avise, J.C., Bermingham, E., Kessler, L.G. and Saunders, N.C. 1984. Characterisation of mitochondrial DNA variability in a hybrid swarm between subspecies of bluegill sunfish (*Lepomis Macrochirus*). *Evolution*. **38:** 931-941.

Avise, J.C., Reeb, C.A. and Saunders, N.C. 1987*b*. Geographic population structure and species differences in mitochondrial DNA of mouthbrooding marine catfish (Ariidae) and demersal spawning toadfishes (Batrachoididae). *Evolution*. **41:** 991-1002.

Ayala, F.J. 1982. *Population and Evolutionary Genetics: A Primer*. The Benjamin/Cummings Publishing Company, INC. California, USA.

Bardakci, F. and Skibinski, D.O.C. 1994. Application of the RAPD technique in tilapia fish: species and subspecies identification. *Heredity*. **73:** 117-123.

Bermingham, E., McCafferty, S.S. and Martin, A.P. 1997. Fish biogeography and molecular clocks: Perspectives from the Panama Isthmus. In *Molecular Systematics of Fishes*. Eds. Kocher, T.D. and Stepien, C.A. Academic Press. USA. pp 113-128.

Bernatches, L., Guyomard, R. and Bonhomme, P. 1992. DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. *Molecular Ecology*. **1:** 161-174.

Birstein, V.J. and DeSalle, R. 1998. Molecular phylogeny of Acipenserinae. *Molecular Phylogenetics and Evolution*. **9:** 141-155.

Brasher, D.J., Ovenden, J.R. and White, R.W.G. 1992. Mitochondrial DNA variation and phylogenetic relationships of *Jasus* spp. (Decapoda: Palinuridae). *Journal of Zoology, London*. **227:** 1-16.

Brown, J.K.M. 1994. Bootstrap hypothesis tests for evolutionary trees and other dendrograms. *Proceedings of the National Academy of Sciences, USA*. **91:** 12293-12297.

Bruce, B.D., Green, M.A. and Last P.R. 1998. Threatened fishes of the world: *Brachionichthys hirsutus* (Lacépède, 1804) (Brachionichthyidae). *Environmental Biology of Fishes*. **52:** 418.

Bryant, E.H., McCommas, S.A. and Combs, L.M. 1986. The effect of an experimental bottleneck upon quantitative genetic variation in the housefly. *Genetics*. **114:** 1191-1211.

Budhina, N. and Ocvirk, A. 1990. Breeding and restocking of salmonid fishes in Slovenia. *Journal of Fish Biology*. **37:**(Suppl.A). 239-240.

Caldara, F., Bargelloni, L., Ostellari, L., Penzo, E., Colombo, L. and Patarnello, T. 1996. Molecular phylogeny of grey mullets based on mitochondrial DNA sequence analysis: evidence of a differential rate of evolution at the infrafamily level. *Molecular Phylogenetics and Evolution*. **6:** 416-424.

Cantatore, P., Roberti, M., Pesole, G., Ludovico, A., Milella, F., Gadaleta, M.N. and Saccone, C. 1994. Evolutionary analysis of cytochrome *b* sequences in some Perciformes: evidence for a slower rate of evolution than in mammals. *Journal of Molecular Evolution*. **39:** 589-597.

Carr, S.M. and Marshall, H.D. 1991*a*. Detection of intraspecific DNA sequence variation in the mitochondrial cytochrome b gene of Atlantic cod (*Gadus morhua*) by the polymerase chain reaction. *Canadian Journal of Fisheries and Aquatic Sciences*. **48:** 48-52.

Carr, S.M. and Marshall, H.D. 1991*b*. A direct approach to the measurement of genetic variation in fish populations: Applications of the polymerase chain reaction to studies of Atlantic cod (*Gadus morhua*). *Journal of Fish Biology*. **39:**(Suppl.A) 101-107.

Carr, S.M., Snellen, A.J., Howse, K.A. and Wroblewski, J.S. 1995. Mitochondrial DNA sequence variation and genetic stock structure of Atlantic cod (*Gadus morhua*) from bay and offshore locations on the Newfoundland continental shelf. *Molecular Ecology*. **4:** 79-88.

Chow, S., Okamoto, H., Uozumi, Y., Takeuchi, Y. and Takeyama, H. 1997. Genetic stock structure of the swordfish (*Xiphias gladius*) inferred by PCR-RFLP analysis of the mitochondrial DNA control region. *Marine Biology*. **127:** 359-367.

Crow, J.F. 1986. *Basic Concepts in Population, Quantitative, and Evolutionary Genetics*. W.H. Freeman and Company. New York. USA.

Dessauer, H.C., Cole, C.J. and Hafner, M.S. 1990. Collection and storage of tissues. In *Molecular Systematics*. Eds. Hillis, D.M. and Moritz, C. Sinauer Associates, Inc. Publishers, USA. pp 25-42.

Dinesh, K.R., Lim, T.M., Chua, K.L., Chan, W.K. and Phang, V.P.E. 1993. RAPD analysis: An efficient method of DNA fingerprinting in fishes. *Zoological Science*. **10:** 849-854.

Dowling, T.E. and Naylor, G.J.P. 1997. Evolutionary relationships of minnows in the genus *Luxilus* (Teleostei: Cyprinidae) as determined from cytochrome *b* sequences. *Copeia*. **4:** 758-765.

Ebenhard, T. 1995. Conservation breeding as a tool for saving animal species from extinction. *Trends in Ecology and Evolution*. **10:** 438-443.

Edgar, G.J. 1997. *Australian Marine Life: The Plants and Animals of Temperate Waters*. Reed Books. Australia.

Farris, J.S., Källersjö, M., Kluge, A.G. and Bult, C. 1995. Testing significance of incongruence. *Cladistics*. **10:** 315-319.

Ferguson, A., Taggart, J.B., Prodohl, P.A., McMeel, O., Thompson, C., Stone, C., McGinnity, P. and Hynes, R.A. 1995. The application of molecular markers to the study and conservation of fish populations, with special reference to *Salmo*. *Journal of Fish Biology*. **47:**(Suppl.A). 103-126.

Fleming, I.A. 1994. Captive breeding and the conservation of wild salmon populations. *Conservation Biology*. **8:** 886-888.

Frankel, O.H. Genetic conservation: our evolutionary responsibility. *Genetics*. **78:** 53-65.

Gharrett, A.J. and Smoker, W.W. 1991. Two generations of hybrids between even- and odd-year pink salmon (*Oncorhynchus gorbuscha*): a test for outbreeding depression. *Canadian Journal of Fisheries and Aquatic Sciences*. **48:** 1744-1749.

Gharrett, A.J. and Smoker, W.W. 1994. Introduction to genetics of subarctic fish and shellfish. *Canadian Journal of Fisheries and Aquatic Sciences*. **51:**(Suppl.1). 1-3.

Ghedotti, M.J. and Grose, M.J. 1997. Phylogenetic relationships of the *Fundulus nottii* species group (Fundulidae, Cyprinodontiformes) as inferred from the cytochrome *b* gene. *Copeia*. **1997** 858-862.

Gilles, A., Lecointre, G., Faure, E., Chappaz, R. and Brun, G. 1998. Mithochondrial phylogeny of the European Cyprinids: Implications for their systematics, reticulate evolution, and colonisation time. *Molecular Phylogenetics and Evolution*. **10:**(1) 132-143.

Gold, J.R., Richardson, L.R., Furman, C. and Sun, F. 1994. Mitochondrial DNA diversity and population structure in marine fish species from the Gulf of Mexico. *Canadian journal of Fisheries and Aquatic Sciences*. **51:**(Suppl.1). 205-214.

Gosling, E.M. 1994. Speciation and wide-scale genetic differentiation. In *Genetics and Evolution of Aquatic Organisms*. Ed. Beaumont, A.R. Chapman and Hall, UK. pp 1-14.

Graves, J.E. and Dizon, A.E. 1989. Mithochondrial DNA sequence similarity of Atlantic and Pacific albacore tuna (*Thunnus alalunga*). *Canadian Journal of Fisheries and Aquatic Sciences*. **46:** 870-873.

Graves, J.E. and McDowell, J.R. 1994. Genetic analysis of striped marlin (*Tetrapturus audax*) population structure in the Pacific Ocean. *Canadian Journal of Fisheries and Aquatic Sciences*. **51:** 1762- 1768.

Graves, J.E., Ferris, S.D. and Dizon, A.E. 1984. Close genetic similarity of Atlantic and Pacific skipjack tuna (*Katsuwonus pelamis*) demonstrated with restriction endonuclease analysis of mitochondrial DNA. *Marine Biology* **79:** 315-319.

Grewe, P.M., Smolenski, A.J. and Ward, R.D. 1994. Mitochondrial DNA diversity in jackass morwong (*Nemadactylus macropterus*: Teleostei) from Australian and New Zealand waters. *Canadian Journal of Fisheries and Aquatic Sciences*. **51:** 1101-1109.

Hall, H.J. and Nawrocki, L.W. 1995. A rapid method for detecting mitochonndrial DNA variation in brown trout, *Salmo trutta*. *Journal of Fish Biology*. **46:** 360-364.

Heist, E.J., Musick, J.A. and Graves, J.E. 1996. Genetic population structure of the shortfin mako (*Isurus oxyrinchus*) inferred from restriction fragment length polymorphism analysis of mitochondrial DNA. *Canadian Journal of Fisheries and Aquatic Sciences*. **53:** 583-588.

Hillis, D.M., Larson, A. Davis, S.K, and Zimmer, E.A. 1990. Nucleic acids III: Sequencing. In *Molecular Systematics*. Eds. Hillis, D.M. and Mortiz, C. Sinauer Associates, Inc. Publishers. USA. pp 318-370.

Hogan, K.M., Davis, S.K. and Greenbaum, I.F. 1997. Mitochondrial-DNA analysis of the systematic relationships within the *Peromyscus maniculatus* species group. *Journal of Mammology*. **78:**(3) 733-743.

Huelsenbeck, J.P and Crandall, K.A. 1997. Phylogenetic estimation and hypothesis testing using maximum likelihood. *Annual Reviews in Ecology and Systematics*. **28:** 437-466.

Hutchins, B. and Swainston, R. 1986. *Sea Fishes of Southern Australia*. Swainston Publishing. Perth, Australia.

Hynes, R.A., Ferguson, A. and McCann, M.A. 1996. Variation in mitochondrial DNA and the patterns of post-glacial colonisation in north-west European populations of brown trout (*Salmo trutta*). *Journal of Fish Biology*. **48:**(**1**) 54-67

IUCN press release 1996 3 October. ANIMALS IN THE RED: Mounting Evidence of Jeopardy to World's Species. Washington, D.C.

Jones, M.W., Clay, D. and Danzamann, R.G. 1996. Conservation genetics of brook trout (*Salvelinus fontinalis*): Population structuring in Fundy National Park, New Brunswick, and eastern Canada. *Canadian Journal of Fisheries and Aquatic Sciences*. **53:** 2776-2791.

Kimura, M. 1980. A simple method for estimating evolutionary rates of base substitution through comparative studies of nucleotide sequences. *Journal of Molecular Evolution*. **16:** 111-120.

Kishino, H. and Hasegawa, M. 1989. Evaluation of the maximum likelihood estimate of the evolutionary tree topologies from DNA sequence data. and the branching order in Homonidea. *Journal of Molecular Evolution*. **29:**170-179.

Knowlton, N. 1993. Sibling species in the sea. *Annual Reviews in Ecology and Systematics*. **24:** 189-216.

Lambeck, K. and Nakada, M. 1990. Late Pleistocene and Holocene sea-level change along the Australian coast. *Palaeogeography, Palaeoclimatology and Palaeoecology*. **89:** 143-176.

Last, P.R. and Bruce, B. 1996. Spotted handfish. *Nature Australia, Summer Edition*. **25:**(7) 20-21.

Last, P.R., Scott, E.O.G. and Talbot, F.H. 1983. *Fishes of Tasmania*. Tasmanian Fisheries Development Authority, Hobart.

Leberg, P.L. 1990. Influence of genetic variability on population growth: implications for conservation. *Journal of Fish Biology*. **37:**(Suppl.A) 193-195.

Lincoln, R.J., Boxshall, G.A. and Clark, P.F. 1982. *A Dictionary of Ecology, Evolution and Systematics*. Cambridge University Press. UK.

Linsley, B.K. 1996. Oxygen-isotope record of sea level and climate variations in the Sulu Sea over the past 150,000 years. *Nature*. **380:** 234-237.

Luikart, G. and Cornuet, JM. 1998. Empirical evaluation of a test for identifying recently bottlenecked populations from allele frequency data. *Conservation Biology*. **12:** 228-237.

Lynch, M. and Crease, T.J. 1990. The analysis of population survey data on DNA sequence variation. *Molecular Biology and Evolution*. **7:** 377-394.

Mamuris, Z., Apostilidis, A.P., Theodorou, A.J. and Triantaphyllidis, C. 1998. Application of random amplified polymorphic DNA (RAPD) markers to evaluate intraspecific genetic variation in red mullet (*Mullus barbatus*). *Marine Biology*. **132:** 171-178.

Meffe, G.K. 1990. Genetic approaches to conservation of rare fish: examples from North American desert species. *Journal of Fish Biology*. **37:**(Suppl.A) 105-112.

Meyer, A. 1994. DNA technology and phylogeny of fish. In *Genetics and Evolution of Aquatic Organisms*. Ed. Beaumont, A.R. Chapman and Hall, UK. pp 219-248.

Miya, M. and Nishida, M. 1996. Molecular phylogenetic perspectives on the evolution of the deep-sea fish genus *Cyclothone* (Stomiiformes: Gonostomatidae). *Ichthyological Research*. **43:** 375-398.

Miya, M. and Nishida, M. 1998. Molecular phylogeny and evolution of the deep-sea fish genus *Sternoptyx*. *Molecular Pylogenetics and Evolution*. **10:** 11-22.

Moritz, C., Dowling, T.E. and Brown, W.M. 1987. Evolution of animal mitochondrial DNA: Relevance for population biology and systematics. *Annual Revues in Ecology and Systematics*. **18:** 269-292

Nedbal, M.A. and Philipp, D.P. 1994. Differentiation of mitochondrial DNA in largemouth bass. *Transactions of the American Fisheries Society*. **123:** 460-468.

Nei, M. 1973. Analysis of gene diversity in sub-divided populations. *Proceedings of the National Academy of Science, USA*. **20:** 3321-3323.

Nei, M. and Jin, L. 1989. Variances of the average numbers of nucleotide substitutions within and between populations. *Molecular Biology and Evolution*. **6:**(3) 290-300.

Nei, M., Maruyama, T. and Chakraborty, R. 1975. The bottleneck effect and genetic variability in populations. *Evolution*. **29:** 1-10.

Nelson, J.S. 1984. *Fishes of the World*, Second edition. John Wiley & Sons, Inc. NY USA.

Newman, M., & Roberts, B.W. 1994. *A Mathematical Model for Mass Extinction*. Laboratory of Atomic and Solid State Physics, Cornell University, Clark Hall Ithaca, NY 14853-2501.

Nyman, L. 1993. Reply to the comments of Ryman *et al. Journal of Fish Biology*. **42:** 481-483.

Nyman, L. and Ring, O. 1989. Effects of hatchery environment on three polymorphic loci in Arctic char (*Salvelinus alpinus* species complex). *Nordic Journal of Freshwater Research*. **65:** 34-43.

Oohara, I. and Okazaki, T. 1996. Genetic relationship among three subspecies of *Oncorhynchus masou* determined by mitochondrial DNA sequence analysis. *Zoological Science*. **13:** 189-198.

Ortí, G., Bell, M.A., Reichman, T.E. and Meyer, A. 1994. Global survey of mitochondrial DNA sequences in the three spine stickleback: evidence for recent migrations. *Evolution*. **48:** 608-622.

Ovenden, J.R. 1990. Mitochondrial DNA and marine stock assessment: a review. *Australian Journal of Marine and Freshwater Research*. **41:** 835-853.

Ovenden, J.R., Smolenski, A.J. and White, R.W.G. 1989. Mitochondrial DNA restriction site variation in Tasmanian populations of orange roughy (*Hoplostethus atlanticus*), a deep-water marine Teleost. *Australian Journal of Marine and Freshwater Research*. **40:** 1-9.

Park, L.K. and Moran, P. 1995. Developments in molecular genetic techniques in fisheries. In *Molecular Genetics in Fisheries*. Eds. Carvalho, G.R. and Pitcher, T.J. Chapman and Hall, UK. pp 1-28.

Petersson, E., Järvi, T., Steffner, N.G. and Ragnarsson, B. 1996. The effect of domestication on some life history traits of sea trout and Atlantic Salmon. *Journal of Fish Biology*. **48:** 776-791.

Philippart, J.C. 1995. Is captive breeding an effective solution for the preservation of endemic species? *Biological Conservation.* **72:** 281-295.

Pister, E.P. 1990. Desert fishes: an interdisciplinary approach to endangered species conservation in North America. *Journal of Fish Biology.* **37:**(Suppl.A). 183-187.

Planes, S. and Doherty, P.J. 1997. Genetic relationships of the colour morphs of *Acanthochromis polyacanthus* (Pomacentridae) on the northern Great Barrier Reef. *Marine Biology.* **130:** 109-117.

Ptacek, M.B. and Breden, F. 1998. Phylogenetic relationships among the mollies (Poeciliidae: *Poecilia: Mollienesia* group) base on mitochondrial DNA sequences. *Journal of Fish Biology.* **53:**(Suppl.A) 64-81.

QUIAGEN. 1997*a*. *QIAquick PCR Purification Kit.*

QUIAGEN. 1997*b*. *QIAquick Gel Extraction Kit.*

Raymond, M. and Rousset, F. 1995. An exact test for population differentiation. *Evolution.* **49:** 1280-1283.

Reid, G.M. 1990. Captive breeding for the conservation of cichlid fishes. *Journal of Fish Biology.* **37:**(Suppl.A) 157-166.

Ritchie, P.A., Lavoue, S. and Lecointre, G. 1997. Molecular phylogenetics and the evolution of Antarctic notothenioid fishes. *Comparative Biochemistry and Physiology.* **118A:** 1009-1025.

Roff, D.A. and Bentzen, P. 1989. The statistical analysis of mitochondrial DNA polymorphisms: chi-squared and the problem of small samples. *Molecular Biology and Evolution.* **6:** 539-545

Ryman, N. and Stahl, G. 1980. Genetic changes in hatchery stocks of brown trout (*Salmo trutta*). *Canadian Journal of Fisheries and Aquatic Sciences.* **37:** 82-87.

Ryman, N., Utter, F., Allendorf, F.W., Busack, C. and Shaklee, J. 1993. Genetic concerns about hatchery populations- a comment on the conclusions of Nyman and Ring. *Journal of Fish Biology.* **42:** 471-480.

Saitou, N. and Nei, M. 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* **4:** 406-425.

Schneider, S., Kueffer, J-M., Roessli, D. and Excoffier, L. 1997. *Arlequin ver. 1.1: A software for population genetic data analysis.* Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Scott, T.D., Glover, C.J.M. and Southcott, R.V. 1974. *The Marine and Freshwater Fishes of South Australia*, Second Edition. Government Printer, South Australia. pp 300.

Senanayake, F.R. and Moyle, P.B. 1982. Conservation of freshwater fishes of Sri Lanka. *Biological Conservation.* **22:** 181-195.

Simons, A.M. and Mayden, R.L. 1998. Phylogenetic relationships of the western North American phoxinins (Actinopterigii: Cyprinidae) as inferred from mitochondrial 12S and 16S ribosomal RNA sequences. *Molecular Pylogenetics and Evolution.* **9:** 308-329.

Smith, P.J., McKoy, J.L. and Machin, P.J. 1980. Genetic variation in the rock lobsters *Jasus edwardsii* and *Jasus novohollandiae*. *New Zealand Journal of Marine and Freshwater Research.* **14:** 55-63.

Smolenski, A.J., Ovenden, J.R. and White, R.W.G. 1993. Evidence of stock separation in southern hemisphere orange roughy (*Hoplostethus atlanticus*,

Trachichthyidae) from restriction-enzyme analysis of mitochondrial DNA. *Marine Biology*. **116:** 219-230.

Smolenski, A.J., Ovenden, J.R. and White, R.W.G. 1994. Preliminary investigation of mitochondrial DNA variation in jack mackerel (*Trachurus declivis*, Carangidae) from south-eastern Australian waters. *Australian Journal of Marine and Freshwater Research*. **45:** 495-505.

Sneath, P.H.A. and Sokal, R.R. 1973. *Numerical Taxonomy*. W.H. Freeman. San Fransisco, USA.

Solé-Cava, A.M. and Thorpe, J.P. 1992. Genetic divergence between colour morphs in populations of the common intertidal sea anemones *Actinia equina* and *A. prasina* (Anthozoa: Actiniaria) in the Isle of Man. *Marine Biology*. **112:** 243-252.

Swofford, D.L. 1998. *PAUP*: Phylogenetic Analysis Using Parsimony (*and other methods)*. Version 4.0. Sinauer Associates, Sunderland, Massachusetts. USA.

Swofford, D.L. and Begle, D.P. 1993. *PAUP: Phylogenetic analysis using parsimony*, Version 3.1. Users Manual. USA.

Swofford, D.L. and Olsen, G.J. 1990. Phylogenetic reconstruction. In *Molecular Systematics*. Eds. Hillis, D.M. and Moritz, C. Sinauer Associates, Inc. Publishers, USA. pp 411-501.

Tabata, K., Kishioka, H., Takagi, M., Mizuta, A. and Taniguchi, N. 1997. Genetic diversity of five strains of red sea bream *Pagrus major* by RFLP analysis of the mtDNA D-loop region. *Fisheries Sciences*. **63:** 344-348.

Takahata, N. and Palumbi, S.R. 1985. Extranuclear differentiation and gene flow in the finite island model. *Genetics*. **109:** 441-457.

Talbot, S. and Johnson, C. 1998. *University of Tasmania Diving Operations Manual*. University of Tasmania, Australia.

Templeton, A.R. 1983. Phylogenetic inference of restriction endonuclease cleavage site maps with particular reference to the evolution of humans and the apes. *Evolution*. **37:** 221-244.

Tringali, M.D. and Bert, T.M. 1996. The genetic stock structure of common snook (*Centropomus undecimalis*). *Canadian Journal of Fisheries and Aquatic Sciences*. **53:** 974-984.

Vis, M.L., Carr, S.M., Bowering, W.R. and Davidson, W.S. 1997. Greenland halibut (*Reinhardtius hippoglossoides*) in the North Atlantic are genetically homogenous. *Canadian Journal of Fisheries and Aquatic Sciences*. **54:** 1813-1821.

Walsh, P.S., Metzger, D.A. and Higuchi, R. 1991. Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *BioTech*. **10:** 506-513.

Ward, R.D and Grewe P.D. 1995. Appraisal of molecular genetic techniques in fisheries. In *Molecular Genetics in Fisheries*. Eds. Carvalho, G.R. and Pitcher, T.J. Chapman and Hall. UK. pp 29-54.

Ward, R.D., Woodwark, M. and Skibinski, D.O.F. 1994. A comparison of genetic diversity levels in marine, freshwater and anadromous fishes. *Journal of Fish Biology*. **44:** 213-232.

Weir, B.S. 1990. *Genetic Data Analysis*. Sinauer Associates, Inc. Publishers. Mass, USA.

Wirgin, I.I., Proenca, R. and Grossfied, J. Mitochondrial DNA diversity among populations of striped bass in the south eastern United States. *Canadian Journal of Zoology*. **67:** 891-907.

Wright, S. 1943. Isolation by distance. *Genetics*. **28:** 114-138.

Wright, S. 1951. The genetical structure of populations. *Annals of Eugenics*. **15:** 323-354.

Yang, Z. 1997. Among-site variation and its impact on phylogenetic analyses. *Trends in Ecology and Evolution*. **11:** 367-372.

Conservation Genetics of Fish

Miles Lawler (BSc.)

Supervisor: Rob White

A review of the literature submitted to the School of Zoology, University of Tasmania in partial fulfilment of the requirements for Honours in Marine, Freshwater and Antarctic Biology.

Contents:

1. Conservation Genetics Of Fishes:

1.1 Conservation Genetics:

Conservation can be dealt with by many different scientific fields. Ecologically, it involves the maintenance of ecosystems with all their interactions. Biologically, it involves monitoring of characters such as fecundity, vigour and fitness of an individual in a population. Conservation genetics, on the other hand, aims to maintain genetic variation, enabling a species to compete and adapt to its greatest potential. Without genetic variation, any population faced with a changing environment would struggle to survive. For this reason, findings from conservation genetics are increasingly being incorporated into the management of valuable or endangered populations.

The need to preserve genetic variation has long been known. Frankel (1974) studied agricultural crops and stated that urgent action is needed to collect and preserve irreplaceable genetic resources. Schonewald-Cox (1983) suggests that genetic variation can be preserved as

- species diversity,
- allele diversity (polymorphisms),
- allele frequency diversity (between individuals within populations and between populations), or
- the combination of species diversity with allelic diversity.

Each of these different aspects of diversity require different management strategies. It is desirable to conserve variation in all these forms if possible.

The conservation of single species ultimately leads to the conservation of species diversity. For a single species, it is important to determine the genetic structure of its populations. Populations of the same species are often genetically distinct. They are referred to as stocks and require separate management for the maintenance of genetic diversity. When considering the conservation of a single stock, it is important to have a measure of population size.

For conservation genetics, the number of individuals passing genetic information to the next generation is far more important than the total number of individuals in the population. The number of individuals contributing to the genetic makeup of subsequent generations is termed the effective population size. A small effective population size is more susceptible to a loss of genetic variation caused by genetic drift, inbreeding depression, outbreeding depression, and bottleneck events. These are discussed later in this review.

1.2 Molecular Techniques Used In Conservation Genetics:

There are numerous methods to obtain information on the molecular genetic makeup of a population. The basic principle of all molecular genetic methods is to employ inherited, discrete and stable markers to identify genotypes that characterise individuals, populations or species (Carvalho and Pitcher 1995). There are two types of molecules commonly analysed; proteins and DNA (Ferguson *et al.* 1995).

1.2.1 Protein Markers:

Early genetic studies used blood group or serology data as the basis of establishing distinct populations (Utter 1991). The development of starch gel electrophoresis (Smithies 1955), coupled with histochemical staining (Hunter and Markert 1957), allowed the detection of enzyme polymorphisms. This provided the first readily applicable, simple genetic markers for large scale studies on natural populations (Ferguson *et al.* 1995).

Protein molecular markers have been used in most studies of fish population genetics (Utter 1991; Ferguson *et al.* 1995). Protein electrophoresis has found particular application in the area of stock delineation of commercially important fishes (Ward *et al.* 1994).

There are several limitations of protein variants as molecular markers. Protein electrophoresis separates proteins by their charge (a function of their amino acid

sequence) at a given pH (Park and Moran 1995). The resolution for protein electrophoresis is not always adequate for detecting differences between populations and individuals (Park and Moran 1995). Small changes in protein coding DNA sequence will often not affect the amino acid sequence or electrophoretic mobility of corresponding protein.

Much of the DNA in an animal's genome does not encode protein sequences. For these reasons the majority of changes at the DNA level will remain unnoticed during protein electrophoresis studies (Park and Moran 1995). In some fish populations there is a low level of protein polymorphism, such that protein electrophoresis is unable to detect population differentiation (Ferguson *et al.* 1995).

1.2.2 DNA Markers:

Through the late 1970s and early 1980s the use of DNA differences to distinguish populations became possible. This was initially performed using mitochondrial DNA (mtDNA), then as molecular techniques developed, nuclear DNA (nDNA) (Ferguson *et al.* 1995). For population level studies mtDNA offers several advantages over nDNA. Due to its uniparental inheritance, the effective population size of mtDNA markers is one quarter that of biparental nDNA markers (except bits of the Y-chromosome). This means that any change in genetic diversity will be more apparent in mtDNA than nDNA markers. MtDNA also mutates more rapidly, regardless of uni/biparental inheritance (Avise *et al.* 1987), and therefore accumulates changes more rapidly than nDNA.

There are two fundamental procedures used in DNA sequencing: (1) separation of DNA fragments with the intent of isolating or visualising a particular fragment(s); and (2) manipulation of molecules through use of various enzymes (Park and Moran 1995). The first procedure allows the base pair sequence of a specific DNA fragment to be determined. This is less commonly employed than the second procedure but is a very powerful tool when used.

For both procedures, separations of different DNA fragments is an important step. Separation is usually accomplished by gel electrophoresis, although other techniques such as size fraction columns and magnetic bead separation can be used. DNA fragments are separated by charge, which is proportional to their size and shape (Park and Moran 1995). DNA is typically electrophoresed on agarose or polyacrylamide gels.

Staining of gels is commonly done by either ethidium bromide (fluoresces under ultraviolet light) or silver oxide (dark images in ambient light). These techniques however are non-specific and stain all DNA. For comparison of different DNA sequences, a labelled probe is used. Probes are usually labelled with radionuclear, or fluorescent tags.

Restriction fragment length polymorphisms (RFLPs) are the most commonly employed DNA markers. Restriction enzymes are used to cut or digest DNA at specific recognition sites, thus breaking them into smaller DNA fragments. These fragments can then be differentiated by gel electrophoresis. This allows detection of DNA variation between individuals and populations (Park and Moran 1995).

Another method being developed to look at nuclear DNA is analysis of random amplified polymorphic DNA (RAPD). This is similar to RFLP, but instead of cutting DNA, random sequence primers are used to direct the PCR process. There is a problem with repeatability using this technique. Careful control over experimental conditions is required (Ward and Grewe 1995).

Recently attention has turned to another type of DNA marker. Sections of nuclear DNA contain repeated sequences, the length of which can be compared (Ferguson *et al.* 1995). Depending on size these are minisatellites (9 to 100 base pairs, repeated two to several hundred times), and microsatellites (1 to 6 base pairs, repeated up to 100 times). Minisatellites and microsatellites are collectively known as variable number tandem repeats (VNTRs).

One of the major advantages of DNA markers is that they can be extracted from small tissue samples. A technique known as polymerase chain reaction (PCR) allows markers from small tissue samples to be amplified (Ferguson *et al.* 1995). This has enormous benefits for conservation studies. It allows non-destructive tissue samples to be taken from endangered animals. There is no need for tissue to be fresh or frozen. It also allows archived (museum) samples to be sequenced, which can be used to look at changes in genetic diversity over time.

Before PCR, large tissue samples had to be used in RFLP and VNTR studies. This meant that labelled probes had to be employed for detection of fragments rather than more simple staining/detection procedures such as ethidium bromide. The continued development of techniques such as PCR, and new DNA markers has allowed more and more genetic information to be collected on endangered populations. This has allowed better management plans to be developed, implemented and assesed

2. Factors Affecting The Genetics Of A Population

2.1 Introduction:

The basis of all genetic management is the genetic viability of the population. While there is no definitive measure of genetic viability that can be used in management, there are several important areas that can give indications of the genetic well-being of a species. These are genetic variation, stock composition, and effective population size.

Genetic variation in one form or another is a measure of the amount of heterozygosity both within a population and among populations. From a management point of view, individual interbreeding units are often more important than the population as a whole. Managers refer to these units as stocks. Normally the size of a population is taken as the absolute number of individuals. In conservation genetics it is much more appropriate to look at effective population size, which relates the number of breeding individuals to fecundity and sex ratio. The above factors are the basic information desired to manage and conserve a population.

2.2 Genetic Variation

As highlighted in the first chapter, genetic variation is present as species diversity, allele diversity, allele frequency differences, and the combination of species diversity with allelic variation. Genetic variation can be measured by allele frequencies at different protein coding loci, and direct nuclear or mitochondrial DNA sequencing. For management of a single species there are two distinct components to genetic variation: (1) intra-stock variability, among individuals within a stock; and (2) inter-stock variability, between stocks (Philipp *et al.* 1993). While intra-stock variation is the basic unit on which natural selection acts, inter-stock variation represents the evolutionary product of a population becoming adapted to a certain environment.

Migration rate, life history and historical events all affect genetic variation. Smith and Fujio (1982) correlated lifestyle of marine teleosts with genetic variability. High levels

of genetic variation were found in temperate pelagic, tropical, intertidal-sublittoral and wide-range species. Low levels of genetic variation were found in temperate demersal, polar and narrow-range species. Jones *et al.* (1996) found a significant effect of habitat type for brown trout (*Salmo trutta*). Stream brown trout were more heterozygous than lake brown trout because they have anadromous individuals, which will allows mixing with other streams.

There is a trend for marine fish species to be more heterozygous than freshwater species (Gyllensten 1985; Ward *et al.* 1994). The average heterozygosity of marine species falls between 5.9-6.3%, compared to 4.3-4.6% for freshwater species. The proportion of total genetic variation within a species due to population differentiation (G_{ST}) ranged from a mean of around 4.2% in marine fishes to 32.8% in freshwater fishes. In both cases anadromous fish species showed intermediate levels. This evidence suggests that in freshwater species much of the overall genetic variation occurs between relatively homozygous subpopulations. In marine species much of the overall genetic variation occurs within relatively heterozygous subpopulations.

Ward *et al* (1994) calculated that, for freshwater species, there were 5.5 ± 1.8 migrants between subpopulations per generation, while for marine fish this value was 22.8 ± 5.0. However, they suggested that migration rate between subpopulations of marine fish may actually be between 10 and 100 times greater than for freshwater fish. Due to the method of calculation this difference can be attributed to either large effective populations or high rates of migration in marine fish. A large population size can lead to large numbers of migrating individuals even when migration rates are low and vice versa.

Gold *et al.* (1994) studied population structures in three fish species in the Gulf of Mexico. They found that homogeneity is highest in the migratory red drum (*Sciaenops ocellatus*) and lowest in the non-migratory red snapper (*Lutjanus campechanus*), black drum (*Pogonias cromis*) have intermediate levels. This suggests that among migratory species, gene flow between localities is sufficient to preclude genetic divergence. Small amounts of gene flow (e.g. 5%) will homogenise populations genetically (Gold *et al.*

1994). In many cases observed homogeneity may reflect past rather than present-day events.

2.3 Stock Concept

The stock concept dominates much of marine fisheries management theory. The identification of discrete stocks is basic to the conservation and rational exploitation of fisheries resources (Lenfant and Planes 1996). Stock units are defined as being self-maintaining groups of fish that are temporally or spatially isolated from one another. They are thought to be genetically distinct because of their reproductive isolation (Grant, 1985). A stock boundary may be indicated by significant differentiation of morphological, physiological, or biochemical characters between fish from different sampling sites (Graves and Dizon 1989). They often correspond with physical or temporal barriers, but in some cases there may be no apparent barrier to gene flow.

Freshwater fishes generally have distinct populations based on geographic boundaries (either present day or historical). A good example of this is the chum salmon (*Oncorhynchus keta*), which has the widest natural distribution of all salmon species (Asia and North America). When comparing North American chum salmon with those from Russia, five major groups of populations were found. These were (1) lower Yukon River summer-run; (2) upper Yukon River autumn-run; (3) Bristol Bay area; (4) Alaska Peninsular; and (5) Russia (Wilmont *et al.* 1994). Similar population structuring has been found in most salmonids (Jones *et al.* 1996). Clearly the disconnected nature of freshwater habitats acts as a barrier to gene flow. On the other extreme are marine pelagic fish, which tend to have no apparent barriers to gene flow.

In a mtDNA study on the albacore tuna (*Thunnus alalunga*), Graves and Dizon (1989) could not distinguish North Atlantic from South Pacific albacore. For this species and skipjack tuna (*Katsuwonus pelamis*) migration between populations has been suggested as the reason for this genetic similarity (Graves and Dizon 1989; Graves *et al.* 1984). Migration levels of 1% can maintain the genetic similarity of two populations. Two supposed albacore stocks have been reported in the North Pacific,

based on modal sizes of year classes, growth rates, movements and areas of occurrence. These two stocks have yet to be distinguished by analysis of genetic characters (Graves and Dizon 1989).

In contrast several studies on billfishes (marlins, swordfishes, and sailfishes) have demonstrated that there are distinct genetic differences between stocks (Graves and McDowell 1994). Significant spatial partitioning of genetic variation within striped marlin (*Tetrapturus audax*) occurs between Mexico, Ecuador, Australia, and Hawaii. Similarly Chow *et al.* (1997) found distinct stocks in the swordfish (*Xiphias gladius*) both among and within the Atlantic and Pacific basins.

2.4 Effective Population Size

For management purposes the effective number of individuals passing their genetic information to the next generation is more important than total population size. This effective population size (N_e) is related to the number of breeding individuals in a population, and reflects the effects of inbreeding and genetic drift (Crow and Denniston 1988). It is difficult to evaluate directly, so is generally estimated from temporal changes in allele frequency.

Ryman and Stahl (1980) studied genetic changes in hatchery stocks of brown trout (*Salmo trutta*). They concluded that it would not be possible to found or perpetuate stocks using less than approximately 30 parents of the least numerous sex. As with most concepts in conservation genetics, effective population size assumes that the population is mating at random. This assumption is rarely if ever met in natural populations as there is always a small percentage of inbreeding. This results in N_e being underestimated for most natural populations.

Effective population sizes have been calculated for a large number of different fish stocks. These estimates vary considerably from close to the theoretical minimum to many thousands. Jorde and Ryman (1996) estimated the effective sizes of lake trout populations from four lakes in Sweden. Their estimates ranged from 52-480 breeding

individuals. For river trout N_e estimates have been made in the range of 120 to 38 601 breeding individuals (Kijima and Fujio 1984). In most natural populations, effective population size is less than consensus population size (Cambers 1983; Kijima and Fujio 1984). In captive populations, effective population size can be increased to twice the census population size through intensive management of breeding (Chesser 1983).

3. Factors Affecting Small Populations:

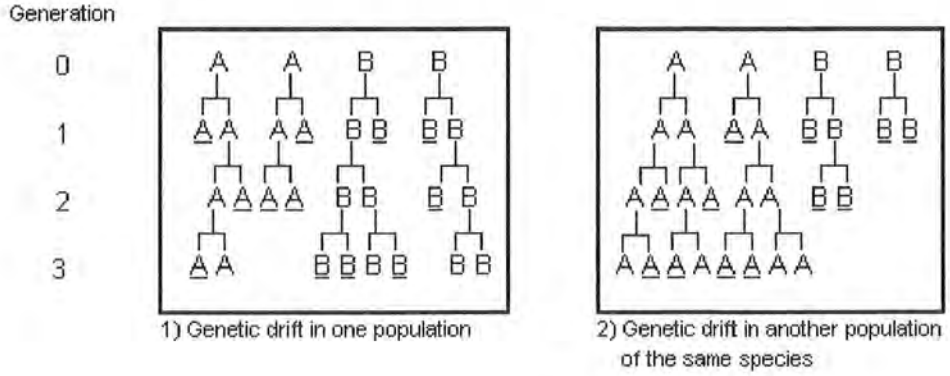
3.1 Introduction:

One of the major problems in conserving the genetic viability of a species is a small population size. Genetic drift, inbreeding depression and outbreeding depression all adversely affect small populations. Competition, introduced species, destruction of habitat, disease and pollution are some of the major factors causing populations to go through bottlenecks. During these periods genetic variation is generally decreased. This can have two major consequences. Firstly it may result in a population being less able to adapt to selection pressures. Secondly it can make populations less viable through increased susceptibility to disease, decreased fecundity, and shortened life span.

3.2 Genetic Drift:

Genetic drift occurs in all heterozygous populations. It is a process of pure chance and one of a group of phenomena known as sampling errors (Ayala 1982). It is one of the major factors causing temporal allele frequency shifts and contributes to genetic differentiation (Jorde and Ryman 1996; Jones *et al.* 1996). The smaller the number of breeding individuals in a population, the larger the allele frequency changes due to genetic drift are likely to be (Ayala 1982). This can produce consequences rather similar to those of inbreeding in small populations.

Figure 3.2.1 Genetic drift in two populations of the same species (After Starr and Taggart 1992).



The mechanism of genetic drift is relatively simple. It is best highlighted by a simple scenario involving two identical populations (Figure 3.2.1.) Where in both populations each individual has two identical offspring, and half the offspring of each generation die before breeding (underlined). Initially the proportion of alleles A and B are 0.5. After three generations the population in (1) has the proportions of B = 0.75 and A = 0.25. Population (2) has the proportions of B = 0 and A = 1.

Perdices *et al.* (1996) found elevated levels of an allele (rare in parental populations) in two re-introduced populations of the cyprinodontid, *Valencia hispanica*. They suggested that this difference from the parent population was due to genetic drift increasing the frequency of the rare allele in the post-bottleneck populations. Lenfant and Planes (1996) suggested genetic drift as one possibility for population differentiation in white sea bream (*Diplodus sargus*) within the Lion's Gulf and the Ligurian Sea (Mediterranean Sea).

Genetic drift can also cause alleles to become fixed. Seeb *et al.* (1987) found little genetic variation in northern pike (*Esox lucius*) populations in North America. Average heterozygosity was found to be 0.0001 and proportion of polymorphic loci 0.01. One explanation for this is that during glaciation many alleles were fixed by genetic drift in small remnant populations. Following the end of glaciation there has not been adequate time for allelic variation to be restored through mutation. A similar situation was found for northern pike across Europe.

3.3 Bottleneck Events and the Founder Effect:

Populations are dynamic. They fluctuate both temporally and spatially. Many have experienced periods where their numbers have been severely restricted. Theoretically these bottleneck periods lead to reduced genetic variation in a population (Nei 1975; Crow and Denniston 1988). A bottleneck where a small number of individuals establish a population is known as a founder event. Founder events have been investigated experimentally by Leberg (1992) in a study on eastern mosquitofish (*Gambusia holbrooki*). Artificial refugia were used to examine the effect of founder populations on

subsequent population structure. Where small founder populations were used the resulting population had reduced genetic variation.

Many studies have used bottleneck events as an explanation for low variability in large populations. Ashbaugh *et al.* (1994) suggested that the paucity of genetic variation in one stock of red river pupfish (*Cyprinodon rubrofluviatilis*) was due to population bottlenecking. Likewise, Apostolidis *et al.* (1997) suggested bottleneck and subsequent genetic drift phenomena as the major reasons for significant mtDNA sequence variation among the populations of Greek brown trout (*Salmo trutta*). During glaciation, populations were reduced in number and isolated for a long period. In such cases, common mtDNA genotypes among populations may have become rare or extinct through stochastic lineage loss (Apostolidis *et al.* 1997)

The founder effect is a historical explanation for low genetic variation in some populations (Lenfant and Planes 1996). Brykov *et al.* (1996) looked at geographical and temporal mitochondrial DNA variability in populations of pink salmon. They found that odd year generations had higher variation than even year. They suggested that the even year generations may have arisen from odd year generations about 1 Myr ago and further bottlenecks may have kept their genetic variation low.

The amount of genetic variation lost is dependent on the magnitude of and length of recovery from bottleneck events. Large losses of heterozygosity are most likely if the recovery of a bottlenecked population to a large size is slow or delayed for several generations (Leberg 1992). This is because genetic drift and inbreeding will have more generations to take effect.

3.4 Inbreeding Depression:

Inbreeding increases the frequency of homozygotes and decreases the frequency of heterozygotes (Ayala 1982). This generally leads to the decreased fitness of the population. In natural populations there are often small amounts of inbreeding and assortive mating (Crow 1986). The effects become significant when the majority of the

population is inbreeding (such as in small populations). Inbreeding leads to two major problems: (1) deleterious recessive genes, which are masked in a large population, surface, and (2) decreased ability of the population to adapt, due to loss of allelic variation. There is an alternative view that inbreeding can act to eliminate recessive genes, but there is little evidence for this hypothesis.

A study on the genetic variation in the cyprinodontid *Valencia hispanica* (Perdices *et al.* 1996) found low variability in some small populations, which they attributed to inbreeding. Petersson *et al.* (1996) looked at the restoration of anadromous salmonid populations in Europe and North America. They showed if the founder population is too small, inbreeding can be a problem. This has important implications for the restoration of endangered species.

Domestication of fish species for commercial purposes is another area where inbreeding can be a problem. Gharrett and Smoker (1984) found that domestication of culture species may lead to loss of genetic variability, either by purposeful (selective breeding) or inadvertent selection (inbreeding). Managers must be careful that selective breeding does not result inadvertently in inbreeding depression.

3.5 Outbreeding Depression:

Outbreeding can cause a decrease in average survival and fitness, which results from hybridising genetically different individuals (Gharrett and Smoker 1991). Through natural selection, individuals acquire adaptations that make them better suited to the specific environment they inhabit (Philipp *et al.* 1993). Hence, all individuals in a local population will share similar genetic adaptations to their environment (co-adapted allele complexes). Outbreeding depression results when these co-adapted allele complexes are disrupted.

Increased F_1 variation followed by low F_2 returns and increased bilateral asymmetry is a pattern to be expected when co-adapted allele complexes are disrupted (Gharrett and Smoker 1991). Behavioural patterns can also be disrupted by outbreeding. For

anadromous fish, their homing mechanism is generally thought to be linked with co-adapted allele complexes. Outbreeding may therefore result in diminished homing ability.

Stocking lakes and rivers with salmon and trout is a common practice in many countries. If the fish that are supposed to be used for breeding are caught near the river mouth, different subpopulations might be mixed causing outbreeding depression (Petersson *et al.* 1996). If ranched salmonids are allowed to breed with wild populations, the genetic changes in the wild populations will probably result in poorer adaptation to local environmental conditions. This will lead to lower productivity of wild stocks (Petersson *et al.* 1996). For this reason outbreeding can result in unique wild populations being wiped out.

4. Case Histories From Salmonid Management:

4.1 Introduction to Salmonids:

Salmonids are a major group of fishes throughout their natural range (temperate to sub arctic Northern Hemisphere), and have been widely introduced into similar regions in the Southern Hemisphere. Salmonids are one of the most studied groups of fishes (Ward *et al.* 1995). This reflects their commercial, cultural and recreational importance. In their natural range they present some unique problems for management. In many regions there is a conflict between maintaining a viable commercial stock and maintaining unique natural populations. Another problem results in recognising stocks of salmonids due to their variation in lifestyle.

There has been much work done on defining salmonid stocks throughout their natural range. Defining wild stocks is one of the primary objectives in formulating a management plan (Saunders 1983). In the past stocks have been based on phenotypic characteristics, life history and geographical range (Utter 1991). Current molecular genetic techniques have allowed the status of many of these stocks to be revised.

4.2 Case Histories

Pink salmon (*Oncorhynchus gorbuscha*) from the north-west of North America have been shown to have both geographical and temporal genetic variation. Through mtDNA analysis distinct odd and even year runs have been distinguished (Brykov *et al.* 1996). Simply based on phenotypic and lifehistory characteristics, these genetically distinct runs had been previously managed as a single stock. Skaala and Naevdal (1989) compared freshwater resident with anadromous life history forms of brown trout (*Salmo trutta*) from three watercourses in Norway. They demonstrated that there were significant genetic differences between the life history forms in all three watercourses. From a management point of view, this creates a problem when the two stocks are present in the same geographical area at the same time. Under these circumstances, it becomes difficult to exploit or control either stock independently.

Restoration of anadromous salmonid populations has developed in recent years in Europe and North America. This has lead to a situation where the numbers of the deliberately released cultured fish now approaches, or even exceeds, the numbers of the naturally produced individuals in certain places (Petersson *et al.* 1996). There is a concern that this trend will result in an evolutionary divergence of the cultured strain away from the wild phenotypic norm (Petersson *et al.* 1996).

Hatchery stocks have historically been used to compensate for decreased natural production in many important salmonid species. For example, 60% of smolt production in the Baltic Sea in the early 1980s was due to artificial propagation (Stahl 1983). Often the hatchery stocks used were either from different parental stocks, a mixture of many parental stocks, or had differentiated due to selection and genetic drift.

Stahl (1983) showed that for Atlantic salmon (*Salmo salar*), hatchery stocks in the Baltic Sea exhibited a significantly lower amount of genetic variation than natural populations. This was displayed as a reduced variation within hatchery stocks and a lower amount of genetic divergence between stocks. The lower variation in the hatchery stocks results from two causes. First, many hatchery stocks are founded and perpetuated using small numbers of parents. This results in genetic drift having a large influence on genetic variation (Cross and King 1983). Second, continued artificial selection over successive generations for certain traits (growth rates, disease resistance and high conversion rates) can result in loss of variation through inbreeding.

Supplementing natural stocks with genetically different stocks has two major consequences. The co-adapted allele complexes of the wild populations can be broken down should they interbreed with the introduced stocks (Gharrett and Smoker 1991). Ryman and Stahl (1981) found that hatchery fish hybridise and drastically alter the genetic composition of valuable remnant natural populations. Furthermore, the introduced stocks will not be adapted to their new environment, which can result in lower survival rates. Altukhov and Salmenkova (1990) showed that introduced chum

salmon (*Oncorhynchus keta*) had a lower coefficient of return to spawn than locally adapted populations.

4.3 Management Issues

The examples given above demonstrate some of the problems facing the management of salmonid populations. The massive amount of molecular genetic work done on salmonids has resulted in a gradual change in management strategies associated with this valuable resource. The practice of boosting numbers of a species in a depleted area by transplanting individuals from a different population or hatchery is now deplored (Altukhov and Salmenkova 1990). The transplanted population will rarely succeed and when it does it will generally be at the expense of the unique local population.

Current management strategies focus on the maintenance of unique stocks and their habitat. Artificial breeding programs, with the return of offspring to their parental waters, are being developed (Budihna and Ocvirk 1990). With any management strategy there must be a target, or means of assessing the effectiveness of any program. A target effective population size is one such target system. Jorde and Ryman (1996) calculated that for four populations of lake brown trout (*Salmo trutta*) in Sweden, the effective population size ranged from 52 to 480 breeding individuals.

Often a precise management target is not known for a particular stock. When a population has been altered either by reduction in genetic variation or changed genetic composition, managers have no measure of what it was like before the genetic disturbance. Therefore the ultimate aim of any management is to maintain the sustainability and evolutionary potential of a population.

5. Case Histories From Endangered Freshwater Fish Conservation:

5.1 Introduction to Endangered Freshwater Fish:

There are an increasing number of endangered freshwater fishes all over the world. Degradation of their natural environments, over exploitation and the impact of introduced species are major reasons for this trend. Freshwater fishes are more vulnerable than marine fishes to change and disturbance because they live in a more fragmented and variable environment. In this situation genetic drift can lead to isolated populations diverging and may result in localised loss of genetic variation. This leads to genetic drift greatly affecting individual populations. The freshwater fishes of North America provide a good example of the problems associated with the conservation of a species.

5.2 Case Histories

In 1990, North American fish fauna included 292 species of fish in the categories of endangered, vulnerable, rare, indeterminate and extinct. This represents 28% of the known freshwater fauna (Williams and Miller 1990). The desert areas of western USA and northern Mexico have the highest proportion of these endangered fish. In recent geological times these areas have been subject to volcanism, mountain building and increased aridity (Williams and Miller 1990). The major cause of decline in the fish populations of this area however, is the destruction and alteration of waterways by agriculture and development.

The desert fishes of North America are a well-studied group. These fish exist as relict populations isolated by harsh terrestrial environments (Echelle and Connor 1989). There is a common view that, historically, there has been little gene flow between populations (Meffe 1990). Echelle and Echelle (1993) looked at the mtDNA of death valley pupfishes (Cyprinodontidae: *Cyprinodon*). They suggested that during wetter times in the past there have been higher levels of introgressive hybridisation and

secondary contact between populations than are generally appreciated. Despite this, characteristic populations are often small with low heterozygosities.

Another endangered desert fish, the Sonoran topminnow (*Poeciliopsis occidentalis*) occurs in several drainages in southern Arizona and north-west Mexico. Genetic clusters of populations have been identified based on types and frequencies of alleles (Meffe1990). Several genetically similar populations have been isolated as a result of habitat destruction. It was predicted that they would lose genetic variation by genetic drift and thus diverge over time. Replicating low levels of gene flow through transfer of individuals has been suggested as one method to avoid loss of genetic variation (Meffe 1990).

5.3 Management Issues:

Long-term adaptability of populations, and hence species, is dependent upon a base of genetic variation. This is required to respond to environmental or biotic novelties, as expounded in Fisher's Fundamental Theorem of Natural Selection from 1930 (Meffe 1990). For this reason management should have the main objective of maintaining large population sizes, which will be less susceptible to forces such as genetic drift and inbreeding. Because the ultimate goal of all conservation efforts is to maintain biological lineages over evolutionary time, attention to genetic aspects at the outset is critical. (Meffe1990).

Endangered desert fishes present a unique set of problems for conservation. They live in a highly specialised and fragmented environment with little gene flow among populations. The management strategies of such populations should take this situation into account when considering appropriate action. Where fish have been historically isolated with no natural gene flow among them, artificial gene flow should be strictly avoided. Artificial gene flow could reduce or eliminate genetic divergence among colonies across the species range and substantially reduce overall genetic diversity of the species. Management should concentrate instead on maintaining large populations at each site to reduce problems associated with drift and inbreeding (Meffe1990).

Pister (1990) examined ways to prevent the extinction of the highly specialised Devil's Hole pupfish (*Cyprinodon diabolis*). This species was threatened by groundwater pumping. Management focused on two aspects, stopping the groundwater pumping, and aquarium culture. Captive breeding has been a major method used to guard against extinctions in small desert fishes. Typically the original captive stock contains about 30 to several hundred specimens from a single location (Echelle *et al.* 1987). The aim of captive breeding programs is to maintain a large effective population size, which will reduce the effects of genetic drift and inbreeding.

Recovery programmes for some endangered species cannot afford the luxury of genetic manipulations. Some are in too dire a condition to allow use of any valid genetic options and should be rescued in any way possible. However, populations in better condition should be dealt with at the outset with a distant time-frame in mind and with the knowledge that management decisions made today will have genetic impacts for millennia (Meffe1990). Long term prospects for conservation depend, in part, on the availability of genetic variation within populations as the raw material to respond to natural selection and allow continued evolutionary change (Meffe1990).

6. Case Histories From Marine Fisheries Management:

6.1 Introduction to Marine Fishes:

Many species of marine fish are exploited commercially. Most of the genetic studies on such fish have therefore revolved around the differentiation of stocks for management purposes. Genetic studies have shown population structuring where none is apparent by other methods, and also have shown perceived stocks to be interbreeding (Graves and Dizon 1989).

Marine fishes live in a relatively homogenous environment. They generally show a low level of intraspecific nuclear and mtDNA differentiation. The main reasons for this are their high dispersal ability and a lack of geographic isolating mechanisms in the pelagic environment (Graves and Dizon 1989). High dispersal ability results from either long lived larvae or, for several of the pelagic fishes, strong swimming ability (Graves and Dizon 1989).

Some species do show marked stock differentiation, such as many of the billfishes (Graves and McDowell 1994). Some degree of reproductive isolation may result from spawner homing behaviour or from some physical barrier to migration such as a sharp salinity or temperature gradient (Grant 1985). Because the marine environment allows greater gene flow than freshwater environments, stocks of marine fishes are generally less genetically distinct than freshwater fish.

6.2 Case histories:

Tunas are a group of large pelagic fishes. They are found in all ocean basins and support large commercially important fisheries. They are a well-studied group of fishes from all management perspectives. The major focus of many studies, both genetic and other, has been determination of stocks. In many cases stocks based on phenotypic, behavioural or other biological characteristics can not be differentiated with genetic

techniques. This is presumably due to gene flow homogenising populations (Graves *et al.* 1984).

Graves *et al.* (1984) found a surprisingly high level of genetic similarity between skipjack tuna (*Katsuwonus pelamis*) from the Atlantic and Pacific Oceans. Similarly Graves and Dizon (1989) could not distinguish albacore tuna (*Thunnus alalunga*) from the Pacific and Atlantic Oceans based on mtDNA analysis using 13 restriction endonucleases. In both cases continued genetic contact, presumably via the Southern Ocean, was given as the reason for the lack of genetic differentiation.

Billfishes (marlins, sailfishes and swordfishes) are similar to tuna in that they are large powerful swimmers. They have the ability to migrate over large distances and thus a similar population structure to that of tunas would be expected. Graves and McDowell (1994) demonstrated significant spatial partitioning of genetic variation within striped marlin (*Tetrapturus audax*) in the Pacific Ocean. They suggested spawning site fidelity as one mechanism that could promote genetic differentiation among striped marlin.

Another billfish, the swordfish (*Xiphias gladius*) was shown to have genetically structured populations not only among but also within ocean basins (Chow *et al.* 1997). Again spawning site fidelity was suggested as the major cause of this structuring. Another possibility that has not been touched on by the authors is water temperature limiting gene flow between ocean basins. Most species of billfish prefer temperate to tropical waters; therefore, the cold waters of the Southern Ocean may act as a partial barrier to migration between ocean basins. This would result in population differentiation between ocean basins.

Many coastal fishes are linked to certain habitat types for breeding. Jackass morwong (*Nemadactylus macropterus*) are commercially fished off the coasts of Australia and New Zealand. Grewe *et al.* (1994) found little stock differentiation across this range, with only the New Zealand samples showing a weak divergence from the Australian samples. While adult jackass morwong have been shown to migrate long distances, the species 8-10 months pelagic larval phase coupled with local currents appears to be a

major factor in homogenising the Australian populations. The Tasman Sea seems to act as a partial barrier to larval drift.

Rosenblatt and Waples (1986) highlighted the effect even small amounts of gene flow can have on maintaining population homogeneity. They examined 12 species of trans-Pacific fishes (Hawaii and North America) separated by 5000 km of ocean. They found that populations of trans-Pacific shore fishes were no more different genetically than local populations of other organisms. They suggested a mechanism of recent dispersal or more likely continued gene flow as the major cause of this homogeneity.

Smith *et al.* (1991) compared genetic diversity in three New Zealand stocks of orange roughy (*Hoplostethus atlanticus*) before and after heavy fishing pressure. Over a six year period (1982-1988) there was a 70% reduction in biomass and a significant reduction in genetic diversity. The orange roughy fishery is based on spawning aggregations. It is known, for many fish species that the more heterozygous fish live longer and attain greater size. These fish also spend more time at spawning sites. In a spawning ground based fishery, such as with the orange roughy, these older and more heterozygous individuals will suffer a higher mortality than younger and less heterozygous fish.

6.3 Management issues:

The above examples have highlighted some of the major problems facing marine fisheries management. The focus of current management is on identifying and managing discrete stocks. However, these genetic stocks are less distinct for marine fish than for freshwater fish. This is because the are fewer barriers to migration between populations, and the amount of gene flow between populations needed to maintain genetic homogeneity is very small (Grant 1985).

Even where populations have been isolated for many generations, stock differentiation of marine fishes may not be genetically apparent. Marine fishes that spawn *en masse*, or which have pelagic eggs or larvae have very large effective population sizes. At such

large population sizes the effects of genetic drift are minimal, and the rate of divergence between populations can be very slow. (Grant 1985).

The lack of clear genetic stock differentiation of many marine fish creates a problem for management. A common theme of genetic stock studies is to approach management with a conservative approach (Grewe *et al.* 1994). Where stocks have been differentiated, either by genetic or other factors, they should be managed as separate units (Graves and Dizon 1989; Graves and McDowell 1994). Finally fishing pressure can result in loss of genetic variation. This occurs either by reducing stocks to a level where genetic drift and inbreeding will significantly affect the population, or preferential removal of more heterozygous fish such as in the orange roughy fishery.

7. References:

Altukhov, Yu.P. and Salmenkova, E.A. 1990. Introduction of Distinct Stocks of Chum Salmon, *Oncorhynchus keta* (Walbaum), into Natural Populations of the Species. *Journal of Fish Biology*. **37:**(Suppl. A). 25-33.

Apostolidis, A.P., Triantaphyllidis, C., Kouvatsi, A. and Economides, P.S. 1997. Mitochondria DNA Sequence Variation and Phylogeography among *Salmo trutta* L. (Greek Brown Trout) Populations. *Molecular Ecology*. **6:** 531-542.

Ashbaugh, N.A., Echelle, A.A. and Echelle, A.F. 1994. Genic Diversity in Red River Pupfish *Cyprinodon rubrofluviatilis* (Atheriniformes: Cyprinodontidae) and its Implications for the Conservation Genetics of the Species. *Journal of Fish Biology*. **45:** 291-302.

Ayala, F.J. 1982. *Population and Evolutionary Genetics: A Primer*. The Benjamin/Cummings Publishing Company, INC. California. USA.

Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A. and Saunders, N.C. 1987. Intraspecific Phylogeography: The Mitochondrial DNA Bridge between Population Genetics and Systematics. *Annual Reviews in Ecology and Systematics*. **18:** 489-522.

Brykov, VL.A., Polyakova, N., Skurikhina, L.A. and Kukhlevsky, A.D. 1996. Geographical and Temporal Mitochondrial DNA Variability in Populations of Pink Salmon. *Journal of Fish Biology*. **48:** 899-909.

Budihna, N. and Ocvirk, A. 1990. Breeding and Restocking Salmonid Fishes in Slovenia. *Journal of Fish Biology*. **37:**(Suppl. A). 239-240.

Carvalho, G.R. and Pitcher, T.J (eds.). 1995. *Molecular Genetics in Fisheries*. Chapman & Hall. London. UK.

Chambers, S.M. 1983. Genetic Principles for Managers, pp. 15-46. *In* Schonewald-Cox, C.M., Chambers, S.M., MacBryde, B. and Thomas, L. (eds.), *Genetics and Conservation: A reference for Managing Wild Animal and Plant Populations*. The Benjamin/Cummings Publishing Company, INC. California. USA.

Chesser, R.K. 1983. Isolation by Distance: Relationship to the Management of Genetic Resources, pp. 66-77. *In* Schonewald-Cox, C.M., Chambers, S.M., MacBryde, B. and Thomas, L. (eds.), *Genetics and Conservation: A reference for Managing Wild Animal and Plant Populations*. The Benjamin/Cummings Publishing Company, INC. California. USA.

Chow, S., Okamoto, H., Uozumi, Y., Takeuchi, Y. and Takeyama, H. 1997. Genetic Stock Structure of the Swordfish (*Xiphias gladius*) Inferred by PCR-RFLP Analysis of the Mitochondrial DNA Control Region. *Marine Biology*. **127:** 359-367.

Cross, T.F. and King, J. 1983. Genetic Effects of Hatchery rearing in Atlantic Salmon. pp 33-40. *In* Wilkins, N.P. and Gosling, E.M. (eds.), *Genetics in Aquaculture: Developments in Aquaculture and Fisheries Science*, **Volume 12**. Elsevier Science Publishers B.V. The Netherlands.

Crow, J.F. 1986. *Basic Concepts in Population, Quantitative, and Evolutionary Genetics*. W.H. Freeman and Company. New York. USA.

Crow, J.F. and Denniston, C. 1988. Inbreeding and Variance Effective Population Numbers. *Evolution* **42:** 482-495.

Echelle, A.A. and Connor, P.J. 1989. Rapid, Geographically Extensive Genetic Introgression after Secondary Contact between Two Pupfish Species. *Evolution*. **43:** 717-727.

Echelle, A.A. and Echelle, A.F. 1993. An Allozyme Perspective on Mitochondrial DNA Variation and Evolution of the Death Valley Pupfishes (Cyprinodontidae: *Cyprinodon*). *Copeia* **1993:** 275-287.

Echelle, A.A., Echelle, A.F. and Edds, D.R. 1987. Population Structure of Four Pupfish Species (Cyprinodontidae: *Cyprinodon*) from the Chihuahuan Desert Region of New Mexico and Texas: Allozymic Variation. *Copeia*. **1987:** 668-681.

Ferguson, A., Taggart, J.B., Prodohl, P.A., McMeel, O., Thompson, C., Stone, C., McGinnity, P. and Hynes, R.A. 1995. The Application of Molecular Markers to the Study and Conservation of Fish Populations, with Special Reference to *Salmo*. *Journal of Fish Biology*. **47:**(Suppl. A). 103-126.

Frankel, O.H. 1974. Genetic Conservation: our evolutionary Responsibility. *Genetics* **78:** 53-65.

Gharrett, A.J. and Smoker, W.W. 1991. Two Generations of Hybrids between Even- and Odd-Year Pink Salmon (*Oncorhynchus gorbuscha*): A Test for Outbreeding Depression. *Canadian Journal of Fisheries and Aquatic Sciences*. **48:** 1744-1749.

Gharrett, A.J. and Smoker, W.W. 1994. Introduction to Genetics of Subantarctic Fish and Shellfish. *Canadian Journal of Fisheries and Aquatic Sciences*. **51:**(Suppl.1). 1-3.

Gold, J.R., Richardson, L.R., Furman, C. and Sun, F. 1994. Mitochondrial DNA Diversity and Population Structure in Marine Fish Species from the Gulf of Mexico. *Canadian journal of Fisheries and Aquatic Sciences*. **51:**(Suppl.1). 205-214.

Grant, W.S. 1985. Biochemical Genetic Stock Structure of the Southern African Anchovy, *Engraulis capensis* Gilchrist. *Journal of Fish Biology*. **27:** 23-29.

Graves, J.E. and Dizon, A.E. 1989. Mithochondrial DNA Sequence Similarity of Atlantic and Pacific Albacore Tuna (*Thunnus alalunga*). *Canadian Journal of Fisheries and Aquatic Sciences*. **46:** 870-873.

Graves, J.E., Ferris, S.D. and Dizon, A.E. 1984. Close Genetic Similarity of Atlantic and Pacific Skipjack Tuna (*Katsuwonus pelamis*) Demonstrated with Restriction Endonuclease Analysis of Mitochondrial DNA. *Marine Biology* **79:** 315-319.

Graves, J.E. and McDowell, J.R. 1994. Genetic Analysis of Striped Marlin (*Tetrapturus audax*) Population Structure in the Pacific Ocean. *Canadian Journal of Fisheries and Aquatic Sciences*. **51:** 1762- 1768.

Grewe, P.M., Smolenski, A.J. and Ward, R.D. 1994. Mitochondrial DNA Diversity in Jackass Morwong (*Nemadactylus macropterus*: Teleostei) from Australian and New Zealand Waters. *Canadian Journal of Fisheries and Aquatic Sciences*. **51:** 1101-1109.

Gyllensten, U. 1985. The Genetic Structure of Fishes: Differences in Intraspecific Distribution of Biochemical Genetic Variation between Marine, Anadromous, and Freshwater Species. *Journal of Fish Biology*. **26:** 691-699.

Hunter, R. and Markert, C. 1957. Histochemical Demonstration of Enzymes Separated by Zone Electrophoresis in Starch Gels. *Science* **125:** 1294.

Jones, M.W., Clay, D. and Danzmann, R.G. 1996. Conservation Genetics of Brook Trout (*Salvelinus fontinalis*): Population Structuring in Fundy National Park, New Brunswick, and Eastern Canada. *Canadian Journal of Fisheries and Aquatic Sciences*. **55:**(12). 2776-2787.

Jorde, P.E. and Ryman, N. 1996. Demographic Genetics of Brown Trout (*Salmo trutta*) and Estimation of Effective Population Size From Temporal Changes of Allele Frequencies. *Genetics*. **143:** 1369-1381.

Kijima, A. and Fujio, Y. 1984. Relationship Between Average Heterozygosity and River Population Size in Chum Salmon. *Bulletin of Japanese Society of Science and Fisheries*. **54:**(4). 603-608.

Leberg, P.L. 1992. Effects of Population Bottlenecks on Genetic Diversity as Measured by Allozyme Electrophoresis. *Evolution*. **46:**(2). 477-494.

Lenfant, P. and Planes, S. 1996. Genetic Differentiation of White Sea Bream within the Lion's Gulf and the Ligurian Sea (Mediterranean Sea). *Journal of Fish Biology* 1996. **49:** 613-621.

Meffe, G.K. 1990. Genetic Approaches to Conservation of Rare Fishes: Examples from North American Desert Species. *Journal of Fish Biology*. **37:**(Suppl. A). 105-112.

Nei, M. 1975. *Molecular Population Genetics and Evolution*. Elsevier Science Publishers. New York. USA.

Park, L.K. and Moran, P. 1995. Developments in Molecular Genetic Techniques in Fisheries. *In* Carvalho, G.R. and Pitcher, T.J (eds.). *Molecular Genetics in Fisheries*. Chapman & Hall. London. UK.

Perdices, A., Machordom, A. and Doadrio, I. 1996. Allozymic Variation and Relationships of the Endangered Cyprinodontid genus *Valencia* and its Implications for Conservation. *Journal of Fish Biology*. **49:** 1112-1127.

Petersson, E., Järvi, T., Steffner, N.G. and Ragnarsson, B. 1996. The Effect of Domestication on some Life History Traits of Sea Trout and Atlantic Salmon. *Journal of Fish Biology*. **48:** 776-791.

Philipp, D.P. Epifanio, J.M. and Jennings, M. 1993. Point/Counterpoint: Conservation Genetics and Current Stocking Practices- Are They Compatible. *Fisheries*. **18:**(12). 14-17.

Pister, E.P. 1990. Desert Fishes: an Interdisciplinary Approach to Endangered Species Conservation in North America. *Journal of Fish Biology*. **37:**(Suppl. A). 183-187.

Rosenblatt, R.H. and Waples, R.S. 1986. A Genetic Comparison of Shore Fish Species from the Eastern and Central Pacific Ocean: Dispersal or Vicariance? *Copeia* **1986:** 275-284.

Ryman, N. and Stahl, G. 1980. Genetic Changes in Hatchery Stocks of Brown Trout (*Salmo trutta*). *Canadian Journal of Fisheries and Aquatic Sciences*. **37:** 82-87.

Saunders, R.L. 1983. Atlantic Salmon (*Salmo salar*) Stocks and Management Implications in the Canadian Atlantic Provinces and New England, USA. *Canadian Journal of Fisheries and Aquatic Sciences*. **38:** 1612-1625.

Schonewald-Cox, C.M., Chambers, S.M., MacBryde, B. and Thomas, L. (eds.), *Genetics and Conservation: A reference for Managing Wild Animal and Plant Populations*. The Benjamin/Cummings Publishing Company, INC. California. USA.

Seeb, J.E., Seeb, L.W., Oats, D.W. and Utter, F.M. 1987. Genetic Variation and Postglacial Dispersal of Populations of Northern Pike (*Esox lucius*) in North America. *Canadian Journal of Fisheries and Aquatic Sciences*. **44:** 556-561.

Smith, P.J., Francis, R.I.C.C. and McVeagh, M. 1991. Loss of Genetic Diversity due to Fishing Pressure. *Fisheries Research*. **10:** 309-316.

Smith, P.J. and Fujio, Y. 1982. Genetic Variation in Marine Teleosts: High Variability in Habitat specialists and Low Variability in Habitat Generalists. *Marine Biology*. **69:** 7-20.

Smithies, O. 1955. Grouped Variation in the Occurrence of New Protein Components in Normal Human Serum. *Nature* **175:** 307-308.

Stahl, G. 1983. Differences in the Amount and Distribution of Genetic Variation between Natural Populations and Hatchery Stocks of Atlantic Salmon. pp 23-32. *In* Wilkins, N.P. and Gosling, E.M. (eds.), *Genetics in Aquaculture: Developments in Aquaculture and Fisheries Science*, **Volume 12**. Elsevier Science Publishers B.V. The Netherlands.

Starr, C. and Taggart, R. 1992. *Biology: The Unity and Diversity of Life*, Sixth Edition. Wadsworth Publishing Company. California, USA.

Utter, F.M. 1991. Biochemical Genetics and Fisheries Management: an Historical Perspective. *Journal of Fish Biology*. **39:**(Suppl. A). 1-20.

Ward, R.D. and Grewe, P.M. 1995. Appraisal of molecular genetic techniques in Fisheries. *In* Carvalho, G.R. and Pitcher, T.J (eds.). *Molecular Genetics in Fisheries*. Chapman & Hall. London. UK.

Ward, R.D., Woodward, M. and Skibinski, D.O.F. 1994. A Comparison of Genetic Diversity Levels in Marine, Freshwater, and Anadromous Fishes. *Journal of Fish Biology*. **44:** 213-232.

Williams, J.E. and Miller, R.R. 1990. Conservation Status of the North American Fish Fauna in Freshwater. *Journal of Fish Biology*. **37:** (Suppl. A). 79-85.

Wilmont, R.L., Everett, R.J., Spearman, W.J., Baccus, R., Varnavskaya, N.V. and Putivkin, S.V. 1994. Genetic Stock Structure of Western Alaska Chum Salmon and a Comparison with Russian Far East Stocks. *Canadian Journal of Fisheries and Aquatic Sciences*. **51:**(Suppl.1). 84-94.