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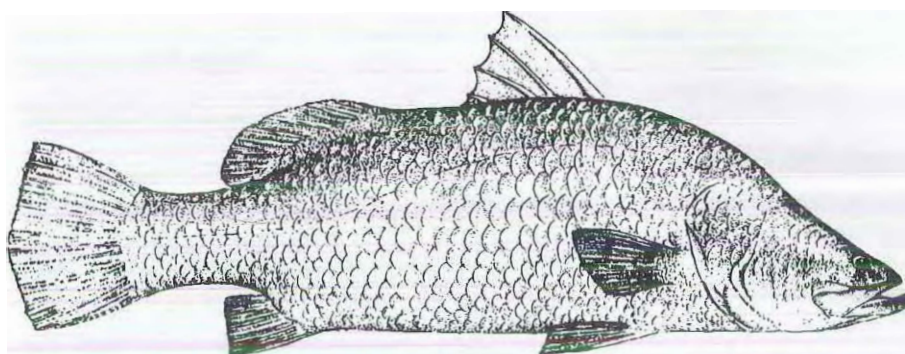
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**Genealogical studies of selected Australian barramundi
(*Lates calcarifer*) using mtDNA: Implications for stock transfer
to the Kimberley region of Western Australia.**



by

Robert G. Doupé

A Thesis Submitted in Partial Fulfilment of the Requirements for the Award of:
Bachelor of Science (Environmental Management) with Honours
at the Faculty of Science, Technology and Engineering, Edith Cowan University

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Submitted to the Department of Environmental Management at Edith Cowan University
April 11, 1997



The art, beauty and pleasure of fishing lies in the act itself.

For Allan and Maureen, and the far north

Amoris labor

Abstract

This study resulted from concerns for the present and proposed movement of barramundi (*Lates calcarifer*) across presumed population genetic boundaries into the Kimberley region of Western Australia for net-pen aquaculture and a recreational fishery development in dams no longer available to seasonal barramundi dispersal.

Direct DNA sequencing of the non-recombining, maternally inherited mitochondrial genome of barramundi thought to represent wild populations from a broad section of a still wider Australian range were used for phylogenetic reconstructions that support hypotheses for historic gene flow between Kimberley and other populations during Recent sea level fluctuations. Nil or low levels of genetic diversity in samples beyond the Kimberley were reflected in highly significant estimates of population genetic subdivision and low gene flow between the contemporary Kimberley population and elsewhere. The observed population genetic structure of western Australian barramundi is discussed with regard to the island and isolation by distance models, however limited sampling and an absence of demographic data leaves a conclusion problematic. Stochastic, but long distance gene flow is predicted within Kimberley barramundi, and is discussed in relation to a distinct east-trending environmental cline that is thought to influence habitat availability and subsequent juvenile dispersal.

The effects of hybridization due to stock enhancement or escapement are discussed in the context of the management objective, which is to maintain genetic diversity. Given this, there are clear implications for hatchery practices and wild fishery management in the Kimberley, which leaves the present translocation of barramundi a questionable practice that should not occur unless no contravention of the management objective can be assured.

Declaration

I certify that this thesis does not incorporate without acknowledgement any material previously submitted for a degree or diploma in any institution of higher education; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signature....

Date..... *April 11, 1997*

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Chapter 1

1.1 General Introduction

1.1.1 The Kimberley

Barramundi, *Lates calcarifer* (Bloch), is a native fish species of the Kimberley region in Western Australia. Such is the mystique surrounding the barramundi that many people [often otherwise non-fishers] engage in recreational activities for this species; it is a highly regarded Australian freshwater sportfish and tablefish. The Kimberley is renowned for its natural resources, and is subject to increasing demands by developers and tourists wishing to explore its many attributes. Such demands also place pressures upon barramundi, as an increasingly urbanized populace expects the "fullest" experience in these "wilderness" areas; these pressures may change the nature of barramundi populations. The challenge now is to develop the natural resources of the Kimberley only in a manner that ensures the long term sustainability of a barramundi fishery, and ultimately the conservation of an Australian icon so that future generations may also experience the natural wonderment of the Australian bush and its inhabitants.

The Kimberley is Western Australia's northernmost region (Figure 1). Located between latitudes 14° and 21° south, the Kimberley climate ranges from the semi-arid tropics of the interior, to a sub-equatorial coastal fringe that is very much influenced by the wet, north-westerly monsoon (McGregor & Chester, 1992). The area is embroidered with ephemeral watercourses, with the two major rivers being the Ord and Fitzroy Rivers. While comparatively short in length, at full flow the two carry the largest volume of water of Australia's rivers, and rank among the most potent in the world (Australian Water Resources Council, 1976). Both the Ord and Fitzroy

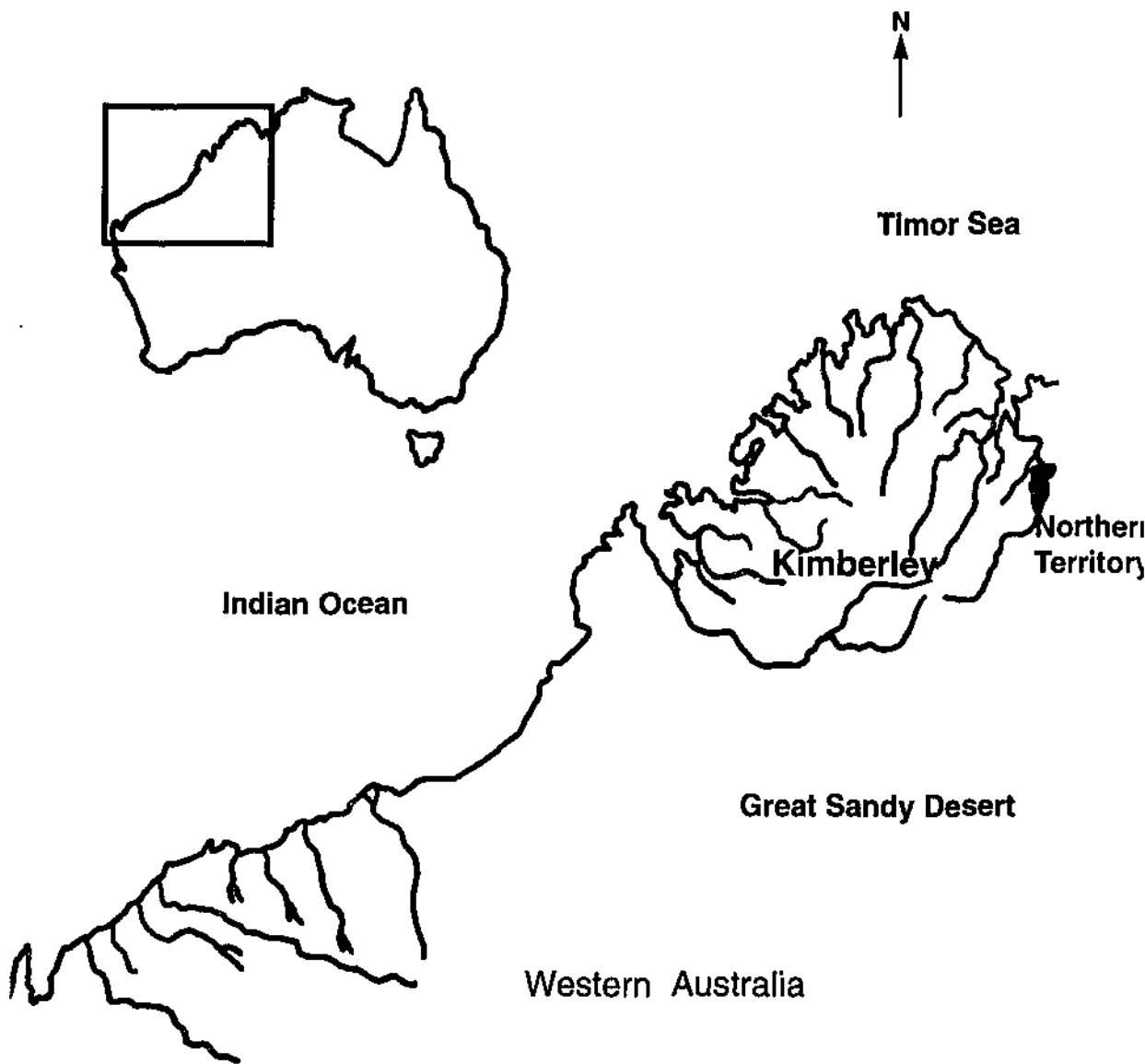


Figure 1. Geographic position of the Kimberley region in Western Australia.

rivers have been dammed for the purpose of irrigated agriculture, although the Fitzroy River project has long been abandoned. The barrage dam at Camballin (near Fitzroy Crossing; Figure 2) is thought to have had an insignificant impact upon river mechanics (Department of Commerce and Trade & Department of Resource Development, 1993). There are two dams situated on the Ord River near Kununurra (Figure 3). The installation of a diversion dam (Lake Kununurra) in the early 1960s established the Ord River Irrigation Area. About a decade later, the Ord River Dam at Lake Argyle was completed. When Lake Argyle exceeds bank-full stage, water enters Spillway Creek to rejoin Lake Kununurra a short distance downstream (Figure 3).

It is now widely recognized that the regulation of rivers can produce a myriad of ecological aberrations (see for example, Pressey & Middleton, 1982; Fernando, 1991). For instance, the barramundi has a catadromous life history, such that most juveniles swim upstream to mature in the fresh waters of tropical Australian watercourses (Dunstan, 1959). The diversion dam at Lake Kununurra appears to have reduced the distributional range of the barramundi, with the seasonal congregation of predominantly sub-adults at the dam wall lending the species to exploitation by recreational fishers (personal observation). Although the provision of fish ladders to allow upstream access has been considered at Kununurra and tested at both Camballin and in Queensland (Morrissy, 1983), the use of these constructions by juvenile barramundi is questionable (Morrissy, 1980) and remains largely unresolved (Mallen-Cooper, 1992).

1.1.2 Future scenarios for barramundi of the Kimberley: The recreational fishery

The East Kimberley Recreational Fishing Advisory Committee (EKRFAC), has expressed interest in restocking barramundi to Lake Kununurra to re-establish part of the species' distribution in the Ord River and develop the potential recreational fishery. In

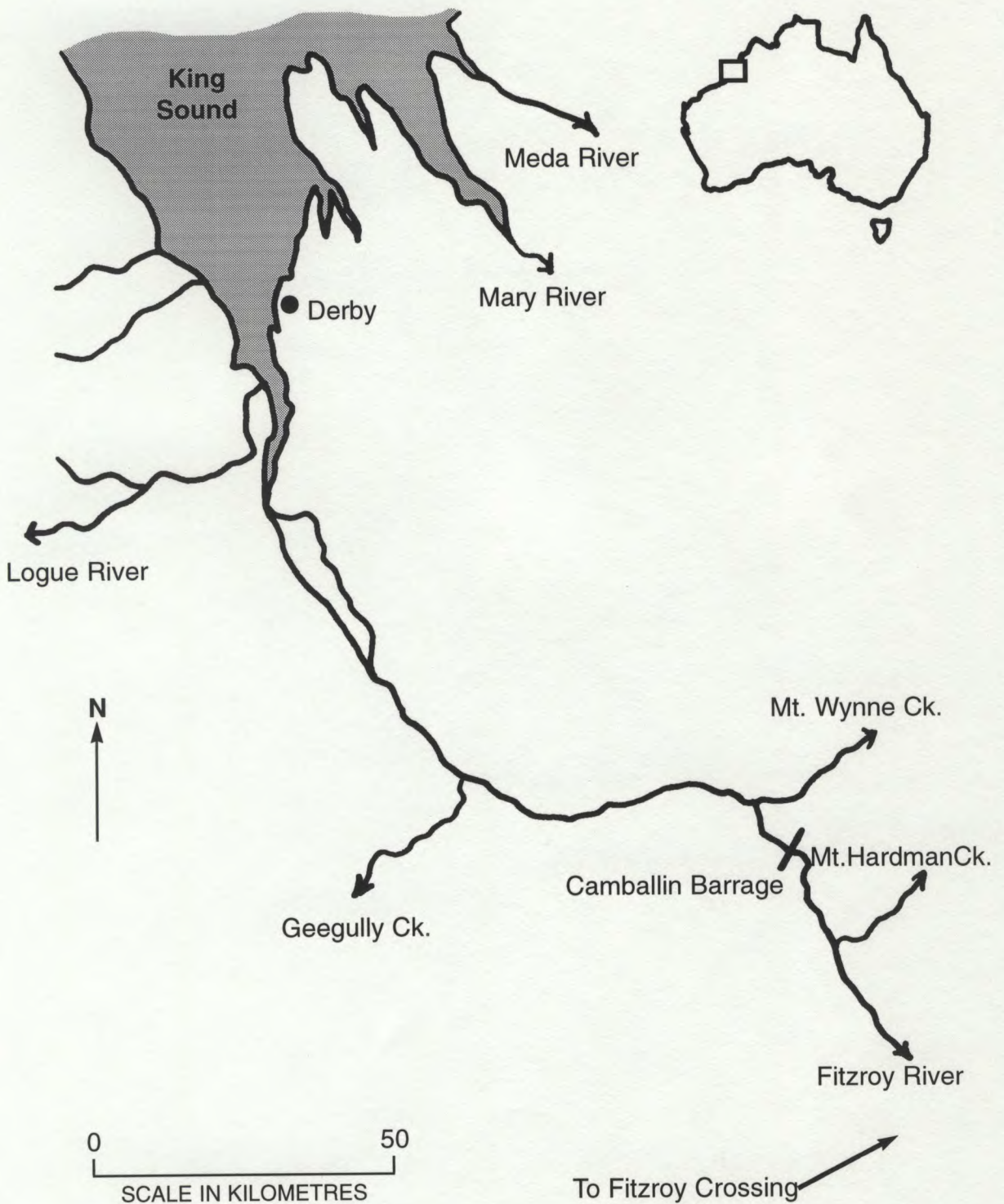


Figure 2. King Sound and the lower Fitzroy River showing major tributaries and dam point.

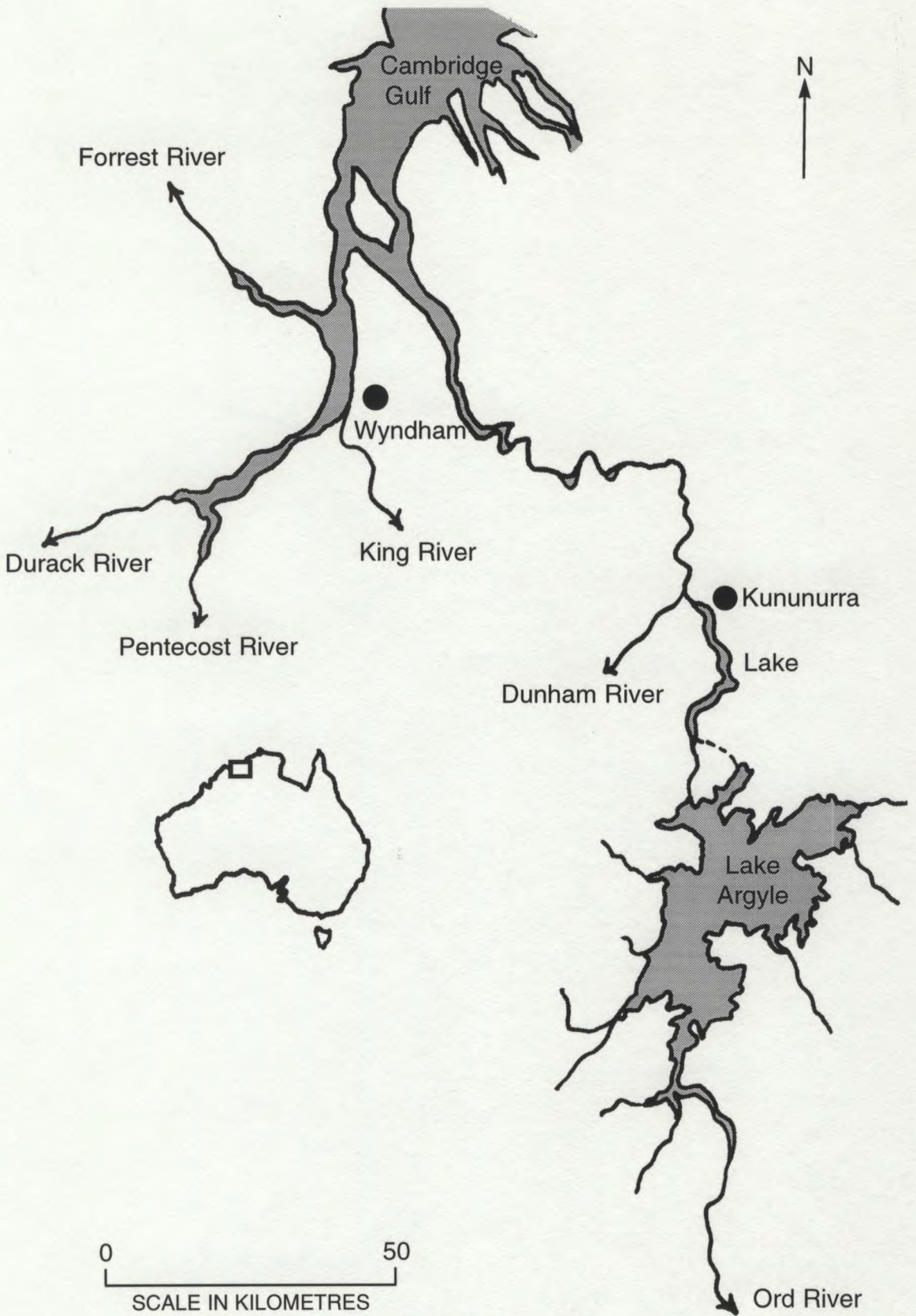


Figure 3. Position of major tributaries and damming points of the lower Ord River with primary drainage lines to Cambridge Gulf.

Queensland, hatchery-reared barramundi have been used to stock impoundments for recreational fishing (see MacKinnon & Cooper, 1987; Rutledge, 1990; Cadwallader & Kerby, 1995) with significant economic benefits (Rutledge et al. 1990), and many thousands have been released into estuaries in the Cairns region (Russell & Rimmer, in press). A previous attempt to reintroduce barramundi into Lake Kununurra (Bird, 1992) involved the release of 124 tagged barramundi close to the diversion dam wall. Only 8 fish (approximately 6%) were recaptured, and all were from below the dam wall. This scant evidence was interpreted by Bird (1992) as perhaps indicating that all the fish may have returned to the river proper, and that the release of trapped fish into the dam is expensive and possibly futile (Bird, 1992). In the Northern Territory, a barramundi introduction has occurred on the Adelaide River at Manton Dam (south of Darwin), where about 200,000 fingerlings have been released. Although no research has concluded the fate of these fish, it is believed that at least some passed through the dam wall (R. Griffin, Northern Territory Department of Primary Industry & Fisheries, personal communication, no date). The EKRFAC understands that the establishment of a recreational fishery would require constant replenishment, and that hatchery-reared fish could provide the basis for the fishery. The source of this material therefore requires careful consideration.

1.1.3 Aquaculture

The concept of fish-farming to meet food demands and support dwindling wild fish stocks (Pownall, 1969; Wells, 1969) was regarded by Smith (cited in Morrissy, 1980, p. 215) as "what must be one of the most exciting, challenging and potentially valuable areas of fisheries work". Cultural farming of barramundi has occurred in South-east Asia for many years (Wongsomnuk & Manevonk, 1973), and Australian facilities operate in Queensland, South Australia and the Northern Territory (Anderson et al. 1993; for a review of the Australian industry see Treadwell, McKelvie & Maguire, 1991). There are problems with barramundi husbandry; ectocommensal

parasites (Aquaculture WA, 1995) and viral infections (Awang, 1987; Mackinnon, 1987; Glazebrook & Campbell, 1987; Glazebrook, Heasman & De Beer, 1990; Renault, Haffner, Baudin Laurencin, Brevil & Bonami, 1991; Munday, Langdon, Hyatt & Humphrey, 1992; but see Anderson et al. 1993) have decreased hatchery success and stock viability.

The Western Australian Government and Fish Farms International Ltd. signed the Fish Farming (Lake Argyle) Development Agreement Act 1976 which proposed to "ranch" barramundi. Part of this proposition included the annual release of 200,000 fingerlings into the lake, and that 10% of the annual catch would be available to recreational fishing, although nothing eventuated (Morrissy, 1980). In more recent years net-pen aquaculture of barramundi has been achieved in Lake Argyle. Initially barramundi fingerlings produced from broodstock at the Northern Territory Government hatchery were supplied direct to the Lake Argyle farm. Larvae are now supplied from the Northern Territory to a Western Australian Government facility in Broome near Derby, Western Australia. After a short period, they are transported to Lake Argyle, and reared to market size (Baby Barra Boost, 1995).

1.1.4 A synopsis for the Kimberley barramundi fishery

In their discussion paper, the Regional Development Council and the Department of Commerce and Trade (1996) state:

The declining world wild caught fishery combined with economic and population growth in Asia will create opportunities for expansion of aquaculture. The Kimberley has comparative advantages for the development of aquaculture due to its quality water bodies and suitable species types. There is an emerging commitment to aquaculture research and development evidenced by the proposed Broome Tropical Aquaculture Park.

(p. 25)

Despite an 18% increase in the Kimberley fin fish industry in 1993/94 (Kimberley Development Commission, 1995), this statement implies that the concept of ecologically sustainable wild fisheries has a difficult future, especially perhaps, where comparatively small, isolated fisheries such as the Kimberley barramundi fishery (Morrissy, 1983, 1985, 1987; Aquaculture WA, 1995) are concerned. Indeed, the Kimberley Aquaculture Development Plan (Nel, 1996) promotes the perception that aquaculture will offset the depletion of wild stocks.

The popular perception of "quality water bodies and suitable species types" indicates a bountiful future for aquaculture in the Kimberley, with \$4.5 million budgeted for aquacultural development in Western Australia over the next 3 years (Kimberley Development Commission, 1995). Potential target species for the Broome aquaculture facility include crustaceans, molluscs and tropical fish species such as barramundi (Kimberley Development Commission, 1994).

The translocation of aquatic species into Western Australia is subject to regulation under the Fish Resources Management Act 1994. The Act is managed through a Memorandum of Agreement between the Fisheries Department and the Department of Environmental Protection, and the current aims of the policy include "...particular reference to maintenance of genetic and biodiversity" (Environmental Protection Authority & Fisheries Department, in review, p.3); presumably this refers to the genetic diversity of wild fish. Thorn (1995) reviews translocation policy and controls in Western Australia with an assurance that the appropriate decision-making process for assessing the suitability of the translocation or introduction of aquatic species into that state are secure. It is noteworthy that "...the term species can also be used to mean species group" (Thorn, 1995, p.20). The movement of barramundi within its natural distribution and over many watersheds is a "Type D" translocation proposal, which requires the establishment of

“whether or not the species...or genetically different strains in different watersheds or parts of its distribution” to be successful (Environmental Protection Authority & Fisheries Department, in review, p. 4). Guidelines for assessment of translocation proposals render the division of Western Australia into provincial geographic drainage basins, the Kimberley being classified in the Timor Sea Drainage Division. Analyses of risks, costs and benefits associated with the intended translocation to areas “in which they are not endemic”, include the source of stock for translocation, and the genetic characteristics of the stock (Thorn, 1995). Barramundi are acknowledged to comprise a number of genetically differentiated stocks for the remainder of its distribution eastwards from the Kimberley (Keenan, 1994). Although the extent of this separation along the Western Australian coastline is unknown, it is widely presumed.

1.1.5 The concept of fish stocks from the viewpoint of population genetics

The theoretical basis of population genetic studies is the Castle-Hardy-Weinberg law which describes the fate of genetic material in a panmictic population having no selection in a nil environment; where stable, predictable equilibrium of gene frequencies is quickly reached and maintained. Despite the problems associated with such ideal populations (e.g. Altukhov, 1981; Chakraborty & Leimar, 1987), the genetic structure of most natural populations is investigated from these first principles. Following this, the term “stock” has gained wide acceptance in fisheries management as being “...a panmictic population of related individuals within a single species that is genetically distinct from other such populations” (Shaklee, Phelps & Salini, 1991, p. 174), and as such, stocks will be considered the primary genetic and evolutionary units of fish species.

Wright (1931) first assumed a panmictic model of population structure in which the total population is divided into an infinite number of "islands", each randomly breeding within itself, except for a proportion of migrants drawn at random from the whole. Later, Wright (1943) acknowledged that inter-population estimates of migration under the island model were unlikely to be accurate under natural conditions, and proposed an "isolation by distance" model of population structure: where a population is distributed uniformly over a large territory, but the parents of any given individual are drawn from a small surrounding region. Kimura & Weiss (1964) extended the principle of isolation by distance, and proposed a "stepping-stone" model of population structure, which demonstrated that the decrease in genetic correlation with geographic distance was dependent upon the dimensionality of spatial migration. In one dimension, gene flow is confined to only adjacent populations in a linear array, whereas in two dimensional space gene flow occurs between many more populations, and as such, a two-dimensional stepping-stone structure is equivalent to the island model (Ward, Woodwark & Skibinski, 1994).

By studying the change in inbreeding coefficients of infinite island populations relative to the metapopulation, Wright (1951) proposed models to measure the properties of subdivided populations (F statistics, see Wright, 1965) that are an analogue of traditional analysis of variance (Weir, 1990; Barker, 1992). Advances in molecular technologies have led to the wide application of F statistics as further molecular variation within and between populations has been realized (e.g. Murayama, 1970; Nei, 1973; Nei & Tajima, 1981; Takahata & Nei, 1984; Nei & Jin, 1989; Nei & Miller, 1990; Lynch & Crease, 1990), yet the statistic used to measure gene flow relies on Wright's (1931) formula for estimating migration [i.e. Nm : the number of individuals replaced by migrants per population per generation] in an island model of population structure

(Cockerham & Weir, 1993). In an island model where $Nm > 1$, genetic drift causing population differentiation becomes ineffective (Slatkin, 1987; Trexler, 1988; Slatkin & Maddison, 1989), however other authors (e.g. Trexler, 1988; Slatkin & Barton, 1989; Keenan, 1994) have demonstrated that when populations approximate a stepping-stone model of population structure, Nm estimates can be much higher than one whilst maintaining significant population differentiation. A corollary of Wright's model is that populations will not diverge by the process of genetic drift through isolation if one or more individuals are exchanged per generation. Therefore, apart from complete geographic isolation, selection is often regarded as the most important force producing genetic structure within a species (Keenan, 1994). The premise of genetic variation and the consequence of natural selection is that the types and frequencies of alleles in populations gradually change to promote genotypic adaptation to the local environment (Hartl & Clark, 1989). Despite the restrictions of these models under natural conditions (Porter, 1990), the island model remains the yardstick by which the evolutionary consequences of gene flow are measured (Hellberg, 1994). The extent of gene flow determines the extent to which different populations of a species are independent evolutionary units (Slatkin, 1987; Ellstrand, 1992; Rannala & Hartigan, 1996), with the degree of isolation and the rates of genetic exchange among subpopulations being critical to understanding population dynamics.

Population genetics theory provides a methodological basis for investigating the effects of over-exploitation of wild populations, and the consequences of both planned and inadvertent mixing of stocks through the widespread practice of fishery supplementation programs and aquaculture (Ryman & Utter, 1987; Davidson et al. 1989; Pollard, 1990; Whitmore, 1991; Billington & Hebert, 1991; Dixon, 1992; and others). It is equally relevant where habitat

modifications have contributed to the decline of a great number of populations and species of fish (Martinez, Arias, Castro & Sanchez, 1993).

For some time ecologists have expressed anxiety for the consequences of increasing the spread of species by human agency, rather than by natural dispersal (Elton, 1958). Indeed, it is upon the assumption of local adaptation of discrete populations that there is much concern over the genetic interaction of wild stocks with conspecifics from elsewhere, or with fish bred in captivity (Altukhov & Salmenkova, 1987; Nelson & Soulé, 1987; Sattaur, 1989; Taylor, 1991; Keenan, 1994). Others have stressed the importance of maintaining or improving genetic diversity within native populations through gene flow from exogenous individuals (Moav, Brody & Hulata, 1978; Kapuscinski & Lannan, 1984, 1986). The apparent contrast between theories invoking selection and adaptation as the primary determinant of gene frequencies (see for example Dawkins, 1978, 1983) and population structure, against those promoting the variable associations of gene flow and its effects, has resulted in a plethora of mathematical models (e.g. Zhivotovsky et al. 1994). In summary, population structure depends on the balance of evolutionary forces; natural selection affects genetic differentiation where selective forces differ, genetic drift leads to genetic differentiation, and gene flow among subpopulations promotes genetic similarity.

The recognition of DNA-level polymorphism as a tool in fisheries science has been recognized for some time (Hallerman & Beckmann, 1988). As with many population surveys, fisheries surveys typically characterize the allele frequencies of a population at a given point, but fail to elucidate the historical evolutionary progression of the population. For example, if a single population becomes subdivided through a tectonic event, gene frequencies will diverge as a

result of drift and isolation. If these stocks were to experience secondary contact, gene frequencies at variable loci in the nuclear genome gradually erode until there is no evidence of past isolation (Billington & Hebert, 1991). Homogeneous populations are thought to be indicative of gene flow, irrespective of the geographic distributions of fish, and the fishery is managed as a single stock with the consequent movement of fish supposing no effect upon reproductive dynamics and population viability.

The historical relationships among lineages of stocks (phylogeny) and their comparison to geographic distributions (phylogeography, *sensu* Avise, 1992) are offered through the study of animal mitochondrial DNA (mtDNA). Mitochondrial DNA displays slow divergence erosion, is non-recombining and is predominantly maternally inherited in animals, including fish (Meyer, 1994). As such, mtDNA offers an insight to the maternal descent, or genealogy of populations (Hillis et al. 1996). For selectively equivalent genes like mtDNA, the ancestry of a sample of genes is followed using the "coalescent" or "genealogical" approach in population genetics (Tavaré, 1984; Slatkin & Maddison, 1989; Hudson, 1990). Subsequent estimates of gene flow differ from those previously described in that gene frequencies are ignored (Moritz, 1994), and historic genetic exchange can be inferred directly from the phylogenetic tree (Slatkin & Maddison, 1989; Barton & Wilson, 1995). Knowing the genealogy of a population offers fisheries managers an opportunity to understand how through evolutionary time, stocks within a species have accumulated genetic differences resulting from isolation, thus requiring management on a multi-stock, rather than single stock basis (e.g. Ovenden, 1990). Such rationales have been clearly demonstrated where studies of the mtDNA genome have been conducted in combination with historical geologic events (see Avise, 1994 and references therein).

1.1.6 Statement of objectives and research questions

This study aims to investigate how variation in the mitochondrial genome might provide evidence of the historic stock structure of selected Australian barramundi populations, and particularly in fish of the Kimberley region in Western Australia. If geographic isolation has promoted the genetic divergence of discriminate stocks of fish from distinct locations, then an argument against moving the species between disparate regions would be founded upon the presumption that temporal and spatial restrictions on gene flow might have promoted adaptation to local environments. Barramundi stock conservation and management in the Kimberley is discussed in the context of riverine habitat alteration and increased natural resource development of the region.

Specific questions are:

How does within-population genetic diversity compare between geographic locations, and is there evidence of genetic differentiation between barramundi populations?

What information can be obtained from mtDNA sequence analyses regarding the genetic divergence and geographic isolation of barramundi populations?

Is there a relationship between the genetic differences of populations and geographic distance?

How much gene flow is estimated to occur between contemporary barramundi populations separated by variable amounts of geographic distance?

What inferences can be made for gene flow between barramundi populations, and which model of population genetic structure best approximates contemporary barramundi populations?

What are the phylogenetic characteristics of selected Australian barramundi across a broad section of its geographic range, and what inferences can be drawn for the historic relationships of these populations?

Can an argument be made for managing barramundi populations as discrete evolutionary stocks, and what implications does this have for the translocation of the species to the Kimberley? On the basis of this study, what recommendations can be made for aquaculture and recreational fishery enhancement programs in the Kimberley to take into account the conservation of wild populations, and for achieving the management objective of maintaining genetic diversity?

Chapter 2

2.1 Literature Review

2.1.1 Biology and ecology of barramundi

Barramundi, *Lates calcarifer* (Bloch), is also known as pla-kapong (Yingthavorn, 1951), bhekti (Ghosh, 1973), sea bass (Wongsomnuk & Manevonk, 1973) and giant perch (Moore, 1979). It is a euryhaline species, known to inhabit freshwater ponds and rivers, tidal swamps and estuaries, and coastal reefs (Shaklee & Salini, 1985). The species is a member of the Centropomidae, a family of tropical estuarine, marine and freshwater percoid fish, represented by at least 18 species (Greenwood, 1976). The barramundi has an Indo-West Pacific distribution; ranging from the Persian Gulf through Asia to southern Japan, along the southern coast of New Guinea, and the northern coastline of Australia (Greenwood, 1976; Reynolds & Moore, 1982; Dunstan, 1959; Figure 4). In Australia (Figure 5), the species ranges from the Ashburton River in Western Australia, across the northern coastline, and southward along the east coast to the Mary River (Dunstan, 1959).

Early studies of barramundi life history concluded that the species was anadromous, that its adults spawned in fresh waters before returning to at least partially saline waters (Smith, 1945; Yingthavorn, 1951), however it has since been demonstrated that it is a catadromous species, with adults spawning in estuarine waters at optimal temperature and salinity regimes (Jones & Sujansingani, 1954; Dunstan 1959, 1962; Ghosh, 1973; Wongsomnuk & Manevonk, 1973; Moore, 1982; Keenan, 1994). Embryonic hatching is thought to occur within 24 hours of fertilization (Wongsumnok & Manevonk, 1973), with larvae foraging in estuaries for a short time before migrating upstream as maturing juveniles (Russell & Garrett, 1985; Shaklee, Salini & Garrett, 1993). Pender & Griffin (1996) concluded that many barramundi found in areas remote from freshwater parts of the Mary River in the Northern Territory probably had no freshwater phase. They thought this was due to spawning in coastal areas remote from the river and/or where late spawning prevented upstream juvenile migration prior to river subsidence.

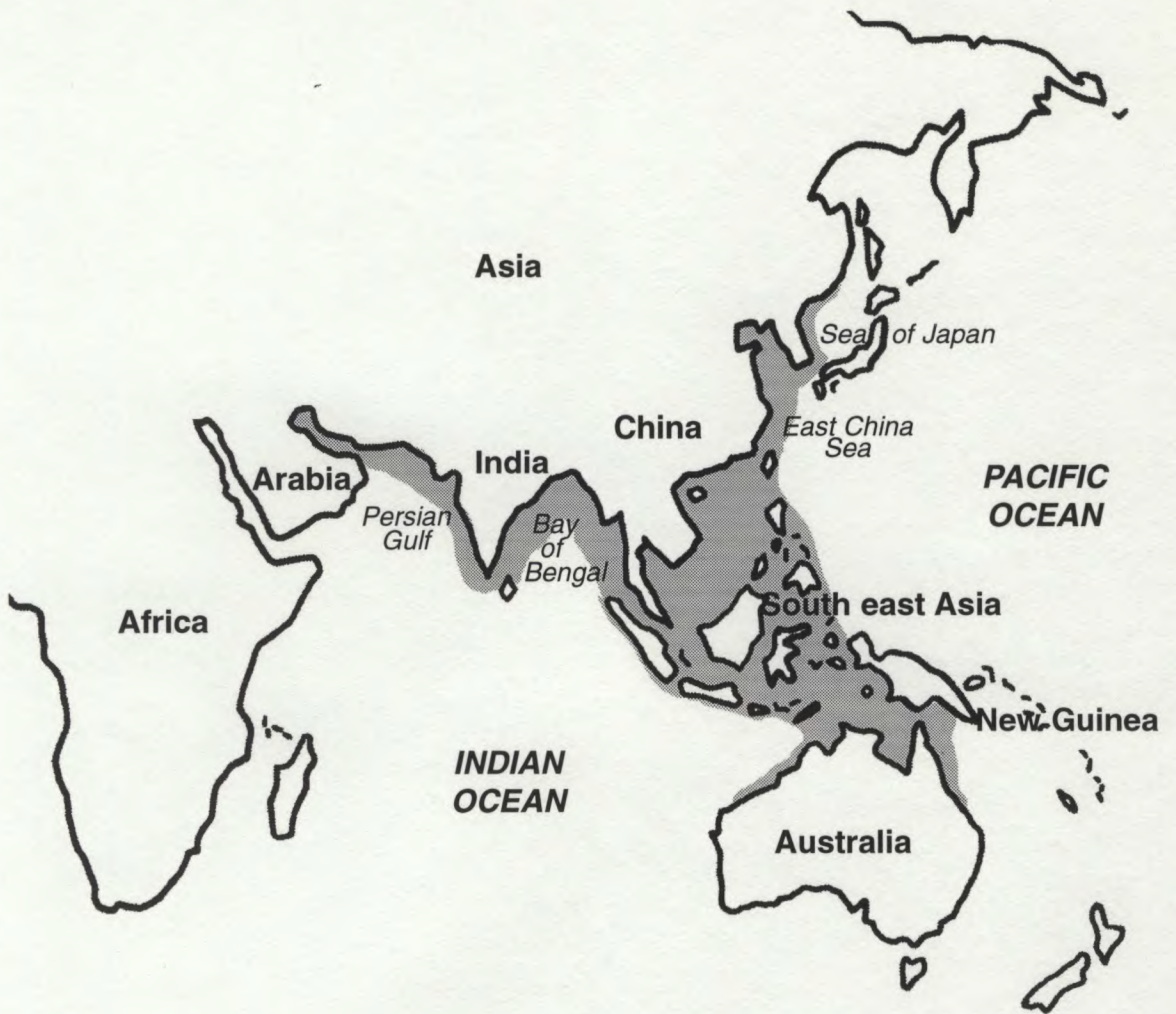


Figure 4. Indo-West Pacific distribution of barramundi.

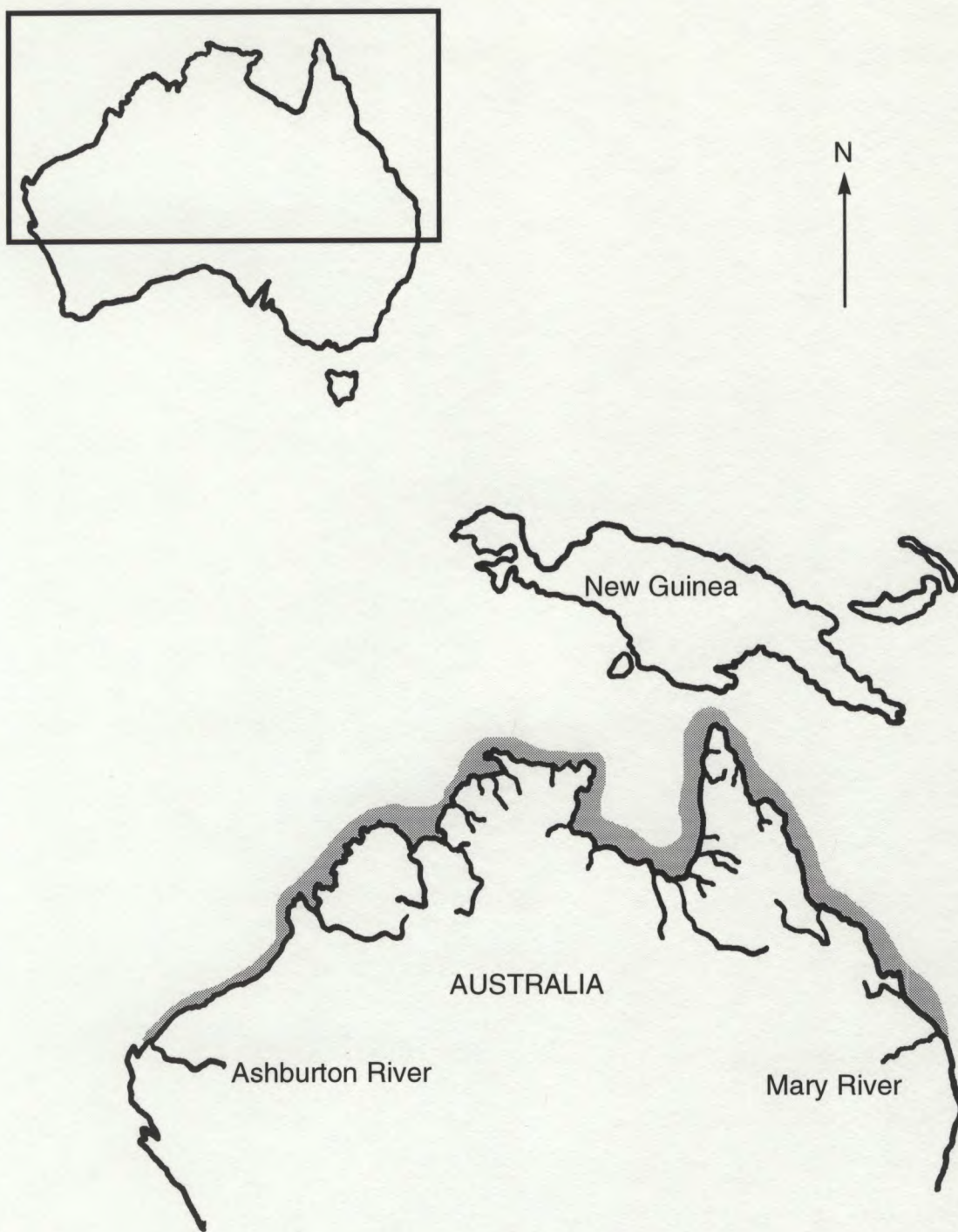


Figure 5. Distribution of barramundi across the Australian coastline.

Evidence from Australia and New Guinea has confirmed that barramundi is a protandrous hermaphrodite; individuals first become sexually mature males in their third and fourth years spawning at least once (Moore, 1979; Davis, 1982, 1984a; Shaklee & Salini, 1983), and subsequently become functional females, each producing 15-45 million eggs per year (Davis, 1984b). This being the case, there are regional, anomalous sexual aspects of barramundi life history. In New Guinea, Moore (1979) found that sex change occurs in inland waters, whereas Davis (1982) found the overwhelming majority of sex changes observed in Australian waters of the Northern Territory and Gulf of Carpentaria occurred in tidal waters. Additionally, Moore (1979) found a small proportion of primary females and the possibility that some males do not change to female. Similar findings have been recorded by Maneewong (1987) who has found primary males and females in Asian stocks. Davis (1982) collected one primary female from the Gulf of Carpentaria, Australia. Further work in that region (near Weipa, western Cape York Peninsula; Davis, 1984b) identified a discrete stock of sexually precocious barramundi that were maturing at 1-2 years of age with evidence of size stunting at maturity. A similar observation has been reported from King Sound, Western Australia, where professional catches early in 1996 revealed large quantities of "small" (600-700 mm), ripe females (F. Bergmann, professional fisherperson [Derby], personal communication, June 10, 1996).

The spawning of barramundi is typically prolonged, occurring just before or at the onset of the summer monsoonal period (Dunstan, 1959). Barramundi migration patterns prior to and following spawning are either poorly understood, or there is significant geographic variation. Dunstan (1962) concluded that a poor New Guinea wet season resulted in land-locking of fish, consequently reducing the number of spawners and recruitment. Similar results have been reported by Davis (1986) for fish in the Northern Territory and Gulf of Carpentaria. Moore & Reynolds (1982) disagree; arguing that adults in New Guinea do not become land-locked [compared to the situation in the Kimberley and western parts of the Northern Territory, personal observations], and that stimuli for downstream migration are due to either gonad maturity, and/or a response to changing water levels, with the number of spawners decreasing in an increased wet season.

Moore (1982) and Moore & Reynolds (1982) suggested that the substantial discharge of southern New Guinea rivers necessitates the significant movement of barramundi to more suitable salinities and spawning habitats up to 300km along the coast. Russell & Garrett (1985) speculated that the comparatively insignificant discharge of rivers and streams in north-eastern Queensland created conditions for localized spawning. On the basis of recorded movements of tagged fish, movements of Australian barramundi are believed to be essentially within river systems, with migration between adjacent river mouths more than 100km apart considered to be a rare event (Davis, 1986; Russell & Garrett, 1988; but see discussion in Keenan, 1994). Reynolds & Moore (1973) found New Guinea barramundi returned inland immediately following spawning, and subsequent studies (Moore & Reynolds, 1982) found fish continually return to the same general area from which they originally migrated. Davis (1985) found fish of the Northern Territory and Gulf of Carpentaria tended to remain within the tidal limit of rivers and did not partake in such migrations (but see Shaklee & Salini, 1983). If the movement of Australian barramundi is insignificant and seasonal spawning localized, then recruitment into major river systems would depend largely on the successful spawning of local populations. It is unknown whether the substantial flows of the Ord and Fitzroy Rivers result in a substantial movement of adults to optimal spawning locales, or in migrations between rivers within those major river basins.

The barramundi is long-lived (>10-20 years) and grows to a large size (>100 cm total length; >20-50 kg total weight, Reynolds & Moore, 1982; Shaklee et al. 1993). It has been found to grow faster in fresh water than salt water (Reynolds & Moore, 1982), and one might attribute this to a juvenile physiological requirement, however the species demonstrates geographic variation in age-specific growth rates and concomitant sex change. Comparisons of Northern Territory and Gulf of Carpentaria populations made by Davis (1982) showed length differences at which males first matured and changed sex, with Gulf fish being consistently smaller. Variable length/sex ratio relationships in barramundi populations have been observed in India (Patnaik & Jena, 1976), Thailand (Wongsomnuk & Manevonk, 1973), and New Guinea (Moore, 1979).

Further evidence for spatial variation in barramundi morphology is found in fish used for recreational stocking in Queensland. Barramundi from Weipa and Cairns have been introduced into Lake Tinnaroo, near Cairns for recreational fishing. Based on tag recaptures, 9 year old Weipa fish were equivalent in size to 5 year old Cairns fish (A. Hogan, Queensland Department of Primary Industries, personal communication, December 6, 1995). Whether this growth difference is a phenotypic indication of a very flexible biology (*sensu* Davis, 1987) or a genuine genetic difference is unknown, and is subject to continued debate.

Concerns for the conservation of wild barramundi stocks have been raised for some time (Shaklee & Salini, 1983). Those authors felt that the shift in size and sexual composition of the catch (60-90% males) would limit fishery recruitment. Davis (1982) feared for the vulnerability of barramundi stocks by even moderate fishing pressure if sex change was not adaptable. Presently, the commercial barramundi fishery in Western Australia is about 50 tonnes (Aquaculture WA, 1995); Northern Territory, 495 tonnes (Wild Stock Fisheries Summary, 1995); and Queensland, 423 tonnes (Australian Fisheries Statistics, 1995). The smaller Western Australian fishery is concentrated around the Ord and Fitzroy River basins, probably due to the relative absence of suitable juvenile habitat (Morrissy, 1983, 1987). Davis (1985) speculated that because adult barramundi tended to remain within the tidal limits of rivers, they were in danger of commercial exploitation. Whilst total commercial catches in Queensland and the Northern Territory have decreased since 1978 (Shaklee & Salini, 1985; also see Morrissy, 1987), there has been pressure from the recreational fishing fraternity for an increased catch quota (Griffin, 1979), or in the absence of this, to introduce an acceptable replacement species (see Williams, 1970, 1982; Pollard & Burchmore, 1986; Barlow & Rodgers, 1990).

In summary, the high fecundity of adults and the requirement for juvenile development in fresh water environments make barramundi an ideal species for inland aquaculture. Barramundi demonstrate what is thought to be a flexible biology over parts of their southern distribution, however this is relatively unknown in Western Australia. The prevalence of presumably locally-adapted traits within the species requires an understanding of the genetic structure of barramundi

populations across geographic distance, so that the relative roles of gene flow and local selective pressures in producing those differences might be better understood.

2.1.2 Population structure in Australian barramundi

Interest in the population structure of Australian barramundi was stimulated by the perceived decline of Queensland and Northern Territory commercial catches in the late 1970s (Salini & Shaklee, 1987a). Subsequent concern for the fishery led Shaklee & Salini (1983) to investigate the possibility of multiple barramundi stocks, and to substantiate an argument against the single stock management approach which largely prevails today. They (Shaklee & Salini, 1983) scored the differential mobility of certain esterase enzymes as allelic differences (allozymes), to discriminate three general populations; at the western side of the Northern Territory, the south-eastern area of the Gulf of Carpentaria, and the eastern side of Cape York Peninsula. Further allozyme studies appeared to confirm the existence of at least those three subpopulations or stocks (Shaklee & Salini, 1985).

Salini & Shaklee (1987a) analyzed allozyme data collected from Western Australia (Ord River), the Northern Territory, and the western side of the Gulf of Carpentaria (McArthur River) in an attempt to determine the coastal genotypes of barramundi stocks. They also collected data from the tip of Cape York Peninsula, Queensland, and from Papua New Guinea to determine the extent of exchange across Torres Strait. Allelic heterogeneity was such that seven discrete stocks were identified along the coastline between the Ord and McArthur Rivers. Further, it was concluded that there was little chance of genetic exchange between Australian and Papua New Guinean barramundi, leading to the belief that geographic distance ($\leq 100\text{km}$) was sufficient to inhibit significant gene flow between Australian stocks. In summarizing their findings for the seven Western Australian/Northern Territory stocks, Salini & Shaklee (1988) thought that since barramundi movements are most probably between adjacent populations, then the one-dimensional stepping-stone model of population genetic structure would be the most appropriate for the species, however they acknowledged that migration estimates for that model required more complex assumptions for estimating effective population sizes. Instead, Salini & Shaklee

(1988) used the island model to predict that between-population movements were less than 2.6 fish per year.

Salini & Shaklee (1988) argued that temporal stability in allele frequencies from one sample location implied such frequencies were characteristic of localities rather than simply of collections and that geographic isolation and/or regional barramundi behaviour was "sufficient to restrict gene flow to a level incapable of negating the effects of random genetic drift". Against this opinion, Stoddart & Trendall (1990) believed the genetic differences were attributable to the proportional differences of which genes were present between rivers, rather than differences in the genes themselves, and that the reported life history differences among barramundi were unlikely to have a genetic basis. Keenan & Salini (1990) found that in barramundi about 20% of loci are polymorphic, with some being highly variable. When fixed allelic differences in barramundi were compared to its congener, Nile perch, *Lates nilotica*, variation was observed at about 12% of loci. While that level of shared identity is small (assuming the electrophoretic data are representative), Keenan & Salini (1990) point to the spawning of Nile perch in fresh water as an example where small genetic variation can produce some important biological differences.

Keenan & Salini (1990) presented data suggesting that sufficient allelic polymorphism existed to identify fourteen discrete stocks, extending from Western Australia toward the southern Queensland limit of the barramundi. Shaklee et al. (1991) summarized this pattern of stock structure in Australian barramundi, substantiating their argument with the conformation of each locality to Hardy-Weinberg expectations; and in doing so presented evidence to suggest that the assumption of no migration between populations had not been violated.

An extensive review of allozyme frequencies within Queensland barramundi resulted in Shaklee et al. (1993) identifying 24 stocks over the range of the species. Shaklee et al. (1993) tested the hypothesis that barramundi population structure approximated a one-dimensional stepping-stone model. They used Queensland harvest rates to estimate effective population size, and estimated gene flow to be over 10 times more fish moving between populations per

generation than Salini & Shaklee (1988) estimated under the island model, despite substantial genetic differentiation. Whereas previous workers had refrained from discussing the role of local selection pressures in population genetic differentiation (e.g. Salini & Shaklee, 1988), Shaklee et al. (1993) cited discontinuous and inappropriate postlarval, juvenile and adult habitat, an apparent absence of extensive prespawning migration in Australia, and limited larval and juvenile dispersal (see Wongsumnok & Manevonk, 1973; Davis, 1985; and Russell & Garrett, 1985, 1988) as reasons for the reproductive isolation of geographically disjunct barramundi populations, and argued that managers should not ignore the role of local selection pressures. Shaklee et al. (1993) speculated that more stocks would be revealed if surveys were conducted in Western Australia, and concluded that the genetic differentiation observed among Australian barramundi was the result of long-term reproductive isolation.

Whereas previous studies had examined population differences on a regional basis, Keenan (1994) interpreted new and previously published data (Shaklee & Salini, 1983, 1985; Salini & Shaklee, 1987a, 1987b, 1988; Shaklee et al. 1993) from fish comprising collections from the Ord River, Western Australia to the Mary River, Queensland to address the genetic structure of Australian barramundi populations from an evolutionary perspective. Keenan (1994) found that duplicate samples over seven years in two areas confirmed Salini & Shaklee's (1988) theory of temporal allelic stability, and proposed sixteen discrete populations, all being confined to solitary rivers or to adjacent watercourses. For example, the Ord River population was found to be statistically homogeneous with the nearby Moyle River (Northern Territory). Keenan (1994) thought the generally low levels of heterozygosity found in Australian barramundi was due to the "founding" effects of populations that have rapidly recolonized many tropical Australian estuaries during the most Recent changes in sea levels. Heterozygosities have decreased proportional to migratory distance from the primary eastern and western "source" populations of barramundi split by the Australia/New Guinea land bridge, such that there are both marked effects of genetic drift toward the edges of those colonizing populations, and evidence of increased heterozygosity through the natural hybridization of those source populations (Keenan, 1994).

Keenan (1994) also tested the one-dimensional model of population structure for barramundi, and estimated effective population sizes by calculating the proportional loss of observed heterozygosity between selected locations to show that the migration of barramundi between adjacent rivers could be substantial [at least an order of magnitude higher than previous estimates], whilst maintaining population subdivision over geographic distance (*sensu* Wright, 1943). This model predicts that coastal gene flow acts as the primary determinant of population structure, and assumes there is substantial genetic exchange among barramundi. The implication to managers is that moving large amounts of fish about their geographic range is an acceptable management practice, despite population genetic boundaries. Such management regards as unimportant the selection pressures that derive from the requirement of fish for suitable habitat, of variable reproductive success in differing locations, and the local inheritance of particular traits in subsequent generations.

Genetic differences between Australian barramundi populations have been recognized for some time. The genetic structure of those populations has thus far been proposed to approximate an isolation by distance model, although estimates of gene flow are dependent upon which population genetic model is chosen, and how many assumptions the investigator chooses to make. Keenan (1994) has proposed that following Recent interglacial events, habitat recolonization and the hybridization of divergent populations have caused the genetic differences between locations. The next section reviews current understandings of northern Australia's Recent geologic history in the context of Keenan's (1994) hypothesis for the evolution of genetically differentiated stocks of Australian barramundi.

2.1.3 The role of Recent sea level change and the generation of differential stock hypotheses

Recent ice ages and associated changes in sea levels would have produced some dramatic changes in the biogeography of the northern Australian coastline (Galloway & Löffler, 1974). A history of unsteady continents and climates would be especially profound in the narrative of fish (Long, 1995), including barramundi living at a shifting land/sea interface.

Climates of the world have been characterized by a series of glacial-interglacial cycles for at least 2.5 million years (Berggren et al. 1980), and most probably well before this (Aplin, Baverstock & Donnellan, 1993). Data obtained from the coral staircase of the Huon Peninsula, New Guinea (Bloom, Broeker, Chappell, Matthews & Mesolella, 1974; Chappell, 1974, 1983; Chappell & Shackleton, 1986) have enabled global estimates of sea level change for the past 300,000 years. Lambeck & Nakada (1990) provided interpretations for sea level change in continental Australia for the past 18,000 years, and present understanding of the Pleistocene sea level history of Western Australia was summarized by Kendrick, Wyrwoll & Szabo (1991).

An interglacial peak approximately 125,000 years BP is thought to have reduced northern Australian sea levels to at least 150 metres below present levels (Chappell, 1983; Shackleton, 1987; Collins, Wyrwoll & France, 1991), thus forming a land bridge between Australia and New Guinea (Figure 6). There is no reason to presume this had not happened during interglacials prior to the aforementioned event. The Recent interglacial peak approximately 18,000 years BP is thought to have had similar effects (Chappell, 1983), with sea levels often 50 to 60 metres below present levels during the period 70,000-10,000 years BP (Torgersen, Hutchinson, Searle & Nix, 1983; Torgersen, Jones, Stephens, Searle & Ullman, 1985; Figure 6). Only during the peaks of the warmest periods, 7000 years BP to present and around 115,000 years BP, were sea levels sufficiently high to open the Torres Strait (Keenan, 1994). For the remainder of the time, the eastern population would have been isolated, however the western population possibly experienced genetic exchange with south-east Asian fish, particularly during times involving extremely low sea level and extensive flood pluming from north-western Australian rivers (Keenan, 1994).

The presence of a land bridge between Australia and New Guinea from about 115,000-7,000 years BP poses zoogeographic constraints to the distribution of Australian barramundi in the Recent period. Such a mechanism is thought responsible for significant population divergence in the prawn, *Penaeus monodon* (Benzie, Frusher & Ballment, 1992). Throughout this time the primary eastern (Pacific Ocean) and western (Indian Ocean) populations were

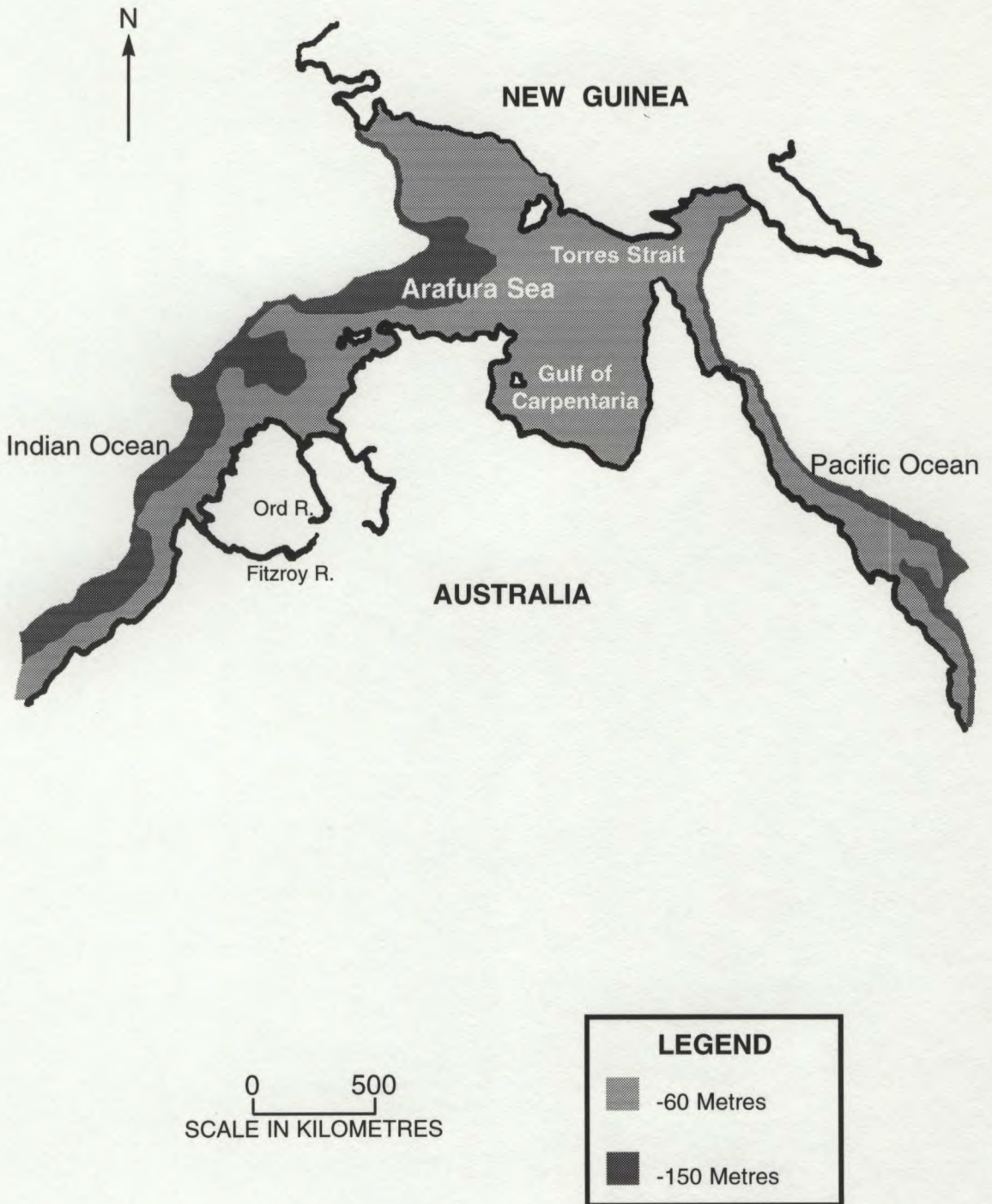


Figure 6. Regions of northern Australia and southern New Guinea showing two depth contours (adapted from Keenan, 1994).

separated for perhaps 108,000 years, allowing the components of genetic isolation and population subdivision to occur (Keenan, 1994). Again, the history of glacial-interglacial cycles significantly predates this relatively recent event, such that periodic genetic isolation could have a much longer history, and Keenan's usage of "ancestral" populations becomes very much a relative term. It is thought that marine water began to encroach upon the western edges of the Gulf of Carpentaria about 11,000 years BP (Jones & Torgersen, 1988), and Keenan (1994) supposed that this would be the boundary of the most easterly population from the western coastline.

From about 18,000 years BP, the barramundi of the western population would have colonized parts of what Galloway & Löffler (1974) describe as a rapidly rising land/sea interface. At sea levels of -60 metres, the most easterly population (i.e. from the west) might represent the eastern periphery of an established "central", Arafura Sea population, inhabiting prevailing estuarine conditions off the Northern Territory coastline (Figure 6). With similar conditions occurring in the ancient river basin to which the Ord and other rivers still flow (Figure 6), it raises the possibility of a barramundi population that has been isolated from the central population for perhaps 18,000 years. In itself, this hypothesis is problematic; the time taken for a sea level rise to -60 metres is probably insufficient to allow genetic differentiation to occur between the western and Arafuran populations. Perhaps the latter stock existed even at sea levels of -150 metres. And, if the glacial-interglacial cycle had continued far longer than is presumed, then what differences have been derived prior to, and since the last glaciation? The King Sound/Fitzroy River basin is not so apparent at these levels (-60 metres; Figure 6), and fish there might have represented a population which utilized seasonal estuarine habitat fronting the Indian Ocean, or the Ord River basin population may have served as the point from which colonization occurred as habitat became available.

Geographically disparate populations must either have preconceived adaptation to their environment as manifested in a flexible biology, or selective mechanisms promote adaptation to changing environmental conditions, ensuring their reproductive success. Implicit in the next

section is the tenet of selection of locally adaptive traits, against that of gene flow, as the primary determinants of population structure.

2.1.4 Some applications of population genetics theory to issues in fishery management

2.1.4.1 Adaptive fitness

The differential reproductive success of individuals in their environment prompted the Darwinian theory of natural selection as one of fitness (Hartl & Clark, 1989). Each habitat is assumed to have a suitability for that species, and suitability is equivalent to fitness in evolutionary time, so that selection produces adaptation by altering relative allelic frequencies and eliminating individuals that are less fit (Krebs, 1985).

How natural selection enables a species to adapt to its environment is difficult to measure, however the study of how species survive in alien environments provides some theoretical basis. Simberloff (1981) concluded that the success of a species in a "new" environment would be dependent on "vacant niches". In contrast, Moyle, Li & Barton (1986) contend that by compressing the "realized niche" of one or more of the present species, the newcomer then "fits" into the environment. Barrett & Richardson (1986) argue that the inheritance patterns of traits conferring increased fitness might enable successful invasion. For example, Morrissy (1973) found the exotic Western Australian rainbow trout (*Oncorhynchus mykiss*) exist with a higher summer temperature tolerance than eastern fish.

In a comprehensive review of local adaptation in salmonids, Taylor (1991 and references therein) finds evidence of locally adapted traits in morphology, behaviour, developmental biology, physiology, disease resistance, and life history traits. Futuyama (1986) and Taylor (1991) acknowledge that a unified concept which demonstrates a genetic basis to local adaptation is elusive, and the methodology for establishing such a concept is not universally accepted (Ender, 1986). If three conditions were to demonstrate the argument, they would be that: the feature has a genetic basis; differential expression of the trait would be associated with differential survival

and reproductive capability among individuals in the same environment; and there is an ability to demonstrate a mechanism for selection and maintenance of the trait in the population (Barker & Thomas, 1987). It is indeed a formidable task.

In contrast to natural selection is artificial selection, and more importantly directional selection, where phenotypically superior traits are chosen for agricultural improvement. A form of artificial selection by fishery harvest has been attributed to declines in growth rates in some salmonids (Ricker, 1972). In many agricultural species, successful artificial selection by outcrossing has been accomplished for many years, provided that population turn-around has been maintained (Hartl & Clark, 1989). In fisheries this has not been the case, possibly due to the high fecundity of most species, resulting in a very small effective population size and a homogeneous population (see Keenan, 1995). Selective pressures resulting from an artificial environment are thought to have caused reduced adaptive potential of many hatchery stocks (Meffe, 1986), and have been blamed for the loss of disease resistance in rainbow trout (Ferguson & Drahuschak, 1990), and reduced genetic variation in many salmonid species (Allendorf & Phelps, 1980; Vuorinen, 1982; Ståhl, 1987; Gyllensten & Wilson, 1987; Verspoor, 1988). Whether the apparent decrease in fitness is due to population homogeneity (e.g. inbreeding depression), or to the typically crowded hatchery conditions in an otherwise "nil" environment is unknown. Under hatchery conditions, theoretical Castle-Hardy-Weinberg equilibria of gene frequencies is not achieved, implying environmental adaptation is a determinant of population structure.

There are arguments that challenge the pre-eminence of local adaptation (Larkin, 1981). The apparent flexibility in species of successful naturalizations (e.g. chinook salmon in New Zealand, Withler, 1982) could itself result from highly variable local environments through selection for phenotypic plasticity (Via & Lande, 1985). Some species, such as coho salmon in coastal Vancouver (Larkin, 1981) use alternative "home" streams in drought years, and might differ in their propensity to form locally adapted populations (Taylor, 1991).

2.1.4.2 Mutation and genetic neutrality

Mutation creates variation in heritable genetic material. The problem of whether mutation rates are important in adaptive fitness has resulted in a number of theoretical approaches in population genetics. In classical population genetics theory, the typically low phenotypic variation seen in species was thought to be due to natural selection cleansing the genome of inevitable mutational variation; the proposition being that genetic variability produced a "load" on the gene pool such that organismal fitness was diminished (see Wallace, 1970). Contrasting this theory was that genetic variability was much higher, and natural selection favoured genetic polymorphisms through "balancing" mechanisms such as the fitness superiority of heterozygotes (Dobzhansky, 1955), variation in fitness among habitats, or frequency-dependent fitness advantage (Ayala & Campbell, 1974). Of the models, it is only the theory of frequency-dependent selection that assumes fitness is dependent upon biotic factors, including intraspecific competition for resources. By considering fitness as a property of gene frequency, and by allowing rare genotypes to have higher fitness, the opportunity for the existence of two or more segregating phenotypes (polymorphism) in the population might increase (Hartl & Clark, 1989).

Contrary to this, Kimura (1968) suggested that most polymorphisms produce such small effects that they are selectively neutral, and their fate is ultimately determined by random genetic drift. The cornerstone of that assumption is that genic regions that are less functionally constrained are most like to harbour neutral variation and to display the greatest allelic or nucleotide substitution (Avice, 1994). Neutral theory does not challenge the Darwinian mode of adaptive evolution, rather it appears to be a response to the intellectual challenge provided by the unexpectedly high levels of molecular variability observed in species.

The role of mutation and neutral theory in selective processes remains a matter of conjecture (e.g. Liberman & Feldman, 1986), and has not diminished with the recent technological advances which have better equipped investigators to probe molecular sequences. Whilst the controversy remains, it was perhaps best summarized by Darwin (cited in Hartl & Clark, 1989):

Variations neither useful nor injurious would not be affected by natural selection, and would be left either a fluctuating element, as perhaps we see in certain polymorphic species, or would ultimately become fixed....We may easily err in attributing importance to characters, and in believing that they have been developed through natural selection;...many structures are now of no direct use to their possessors, and may never have been of any use to their progenitors....we are much too ignorant in regard to the whole economy of any organic being to say what slight modifications would be of importance or not.
(p. 349)

2.1.4.3 Genetic drift, effective population size and population founding

The chance process of changes in allele frequencies is random genetic drift, with its principal effect being genetic divergence between subpopulations (Gall, 1987). The magnitude of allele frequency change in each generation depends on population size, and becomes less important in larger populations (Hartl & Clark, 1989). It is the effect of population size on processes that change gene frequencies which heralded the arguments between Fisher (1931) and Wright (1931), and remains within the discourse of population genetics to this day. In any event, it is the loss of unique allelic characteristics through directed selection, or the subsequent hybridization and concomitant "pollution" (Sattaur, 1989) of discrete gene pools by transgenics that is the concern of fisheries biologists (Allendorf & Leary, 1988; Ferguson, 1990). Alteration of selective forces including predation, competition, environmental modification and disease are considered the sorts of processes which ultimately lead to gene pool modification through differential reproductive success and allelic drift (Krueger & May, 1991).

Mayr (1963) postulates that reproductive isolation by geographic distance enables genetic divergence because of random genetic drift and natural selection. A critical measure of whether effective genetic transmission to subsequent generations will alleviate any detrimental effects of random genetic drift and loss of variability is the concept of the minimum viable population (Soulé, 1987), dependent on the effective population size (N_e). N_e is extremely difficult to measure. It is usually very much smaller than the actual population size, with plenty of fish masking the fact that the progeny may derive from very few adults (Nelson & Soulé, 1987).

Other combined factors of skewed sex ratio and variance in lifetime family size are thought to reduce N_e by at least an order of magnitude (Nelson & Soulé, 1987; Sherwin, 1992).

A population that undergoes a severe temporary reduction in population size is said to experience a "bottleneck" effect (Hartl & Clark, 1989). A population bottleneck is a natural phenomenon, typically occurring when a small group of migrants leave a population to found a new population; the accompanying random genetic drift is known as a "founder effect" (Nei, Marayuma & Chakraborty, 1975). Bottlenecking of founding populations in hatcheries is thought to be a major contributor to the problems associated with the propagation of populations in artificial environments, where gene pools suffer a depletion in the genetic representation of their natural counterparts (Allendorf & Ryman, 1987). The low levels of heterozygosity are attributed to a restricted, homogenizing gene pool, such that the loss of genetic variation, perhaps only 10%, can have deleterious effects upon stock viability (Falconer, 1981). Low heterozygosity in Australian barramundi stocks have been attributed to founder effects (Keenan, 1994). An application of successful founding in hatcheries has been demonstrated by Ferguson, Ihssen & Hynes (1991), who implemented a controlled breeding program among trout species that showed comparable enzyme heterozygosity among hatchery and wild fish. Their work showed that maintaining allelic variation and N_e was species-specific and warranted a fairly equitable contribution to the population by all founders, thus promoting genic representation and minimizing the effects of genetic drift.

2.1.4.4 Inbreeding depression and heterosis

Closely related to the concept of genetic variation is inbreeding. Inbreeding is mating between relatives; with small N_e , individuals are more likely to mate with a relative by chance (Sherwin, 1992).

The tendency for hatcheries to restrict gene flow and to maintain closed populations (usually for disease quarantine), prompted Gall (1987) to question the long-term success of the industries. The effect of genetic drift by inbreeding depression has an interpretation in the

percentage increase in homozygosity caused by a particular mating system (pedigree) over the population average (Hartl & Clark, 1989). For example, Tave (1993) demonstrated the change in the coefficient of inbreeding (F) typically used by fish farmers to be about 3-5% per generation, sufficient to counteract the benefits of mass selective breeding programs. A reduction in fitness that is proposed to be due to genetic drift is usually measured by a decrease in fertility or progeny viability (Sherwin, 1992), and in its overall effect is analogous to inbreeding depression (Mayer, Charlesworth & Meyers, 1996). The poor breeding performance of stocked fish in impoundments was attributed by Tave (1993) to small N_e and the associated lack of genetic variability restricting adaptive fitness. Keenan (1995) found that larger founding populations in stocked reservoirs had levels of genetic variation similar to wild populations, and that as a result the introduction was more likely to be successful.

The homogenizing effect of hybridization, resulting in a decline in some characteristic relative to parental lines, is outbreeding depression (Krueger & May, 1991; Sherwin, 1992). Whilst this is the norm, it is not necessarily the rule; Allendorf & Leary (1988) found that crosses of rainbow trout (*Salmo gairdneri*) and westslope trout (*Salmo clarki lewisii*) resulted in reduced growth rates relative to parents, whereas the latter species crossed with yellowstone cutthroat trout (*Salmo clarki bouvieri*) yielded hybrids with increased development rates.

The situations where coadapted gene complexes of hybridizing parents remain intact to yield an F_1 generation of apparently increased fitness is hybrid vigour or heterosis, and is a common feature of modern agriculture (Hartl & Clark, 1989). Dobzhansky (1955) thought the preservation of some of the parental genic arrangements was the result of local selective pressures, and this may explain the event of heterosis among two parapatric freshwater fish in South America, *Poeciliopsis occidentalis* and *Poeciliopsis monarcha*, where there is a hybrid all-female form in the contact zone (Moore, 1977). There appears to be no empirical studies that have demonstrated F_2 fitness as a function of divergence between parental stocks. The data that are available invariably show lower fitness in F_2 hybrids than either parental stock, even if the F_1 generation displayed vigour (Erdler, 1977). Emien (1991) developed a model to evaluate the

consequences of hybridization of salmon populations adapted to various environments. The study (Emlen, 1991) concluded that periodic mixing of only 5-10% of the population may result in reductions of fitness that could require a number of generations to recover.

Queensland commercial barramundi hatcheries typically use fewer than 20 broodfish (Keenan, 1995). The hatcheries are retaining the highest performing males and fastest growing fish from particular production runs (F_1) as eventual broodstock, providing the bulk of fingerling production (F_2), (R. Garrett, Department of Primary Industry and Fisheries, Queensland, personal communication, March 28, 1996). This implies that if homogeneous fish populations in hatcheries and fishery enhancement programs have limited genetic variability, then hybridization with wild populations might impact upon the fitness of the latter, and the sustainability of those ventures is questionable.

2.1.4.5 Hybridization

The general ability and propensity of fish to interbreed and produce viable hybrid offspring are firmly established, with natural hybridization of fish thought more common than in other groups of vertebrates (Campton, 1987). Australia's fish fauna has so far avoided these effects because no introduced fish thought to be closely related to indigenous Australian families have yet been introduced here, although there is evidence of hybridization occurring among some exotic species (i.e. carp, Arthington, 1991).

The negative impacts of hybridization are typically associated with the interbreeding of lineages from remote or artificial environments. Shaklee et al. (1993) describe "domestication selection" where hatchery spawning and propagation typically selects traits for tolerance to crowding, decreased aggression, increased growth rate, and disease resistance, and yet in the wild each of these factors might normally be expected to affect the differential survival of individuals, and thus the genetic fitness of the stock (e.g. Hynes, Brown, Helle, Ryman & Webster, 1981; Gharrett & Shirley, 1985; Allendorf & Ryman, 1987; Verspoor, 1988; Gile & Ferguson, 1990). Indeed none of these characteristics may be representative of the source

stock, "with the hatchery acting as a sink for wild broodstock, thus reducing by attrition the very population it was designed to enhance" (Waples, 1991).

Hybridization typically increases average heterozygosity within the hybridizing populations, but also results in a loss of diversity between populations, so that locally adapted and "unique" traits are replaced by a smaller number of relatively homogeneous ones (Allendorf & Leary, 1988), and the occasional expression of "hidden" deleterious recessives (Emlen, 1991). Examples of this phenomenon have been documented for populations that have experienced little stocking, and for those that have experienced a history of stocking (e.g. Simon, 1972; Utter, Milner, Ståhl & Teel, 1989). The ecological and evolutionary potential of the species as a whole is reduced, and genetic diversity, which would normally be "expected to buffer total productivity for the resource against periodic or unpredictable environmental change", might render the population vulnerable to perturbations (Riggs cited in Waples, 1991). In the salmonid fisheries of the northern hemisphere, hybridization between hatchery and wild fish is common, with fears held for the viability of wild stocks (e.g. Sattaur, 1989; Evans & Willox, 1991; Krueger & May, 1991). Similarly, the back-crossing of hybrid fish (introgression) has been demonstrated to result in poor growth and survivorship to reproduction (Williamson & Carmichael, 1990; Philipp & Whitt, 1991; Philipp, 1991).

To summarize, the genetic and ecological consequences of decreased organismal fitness through small effective population sizes, hybridization and propagation in artificial environments among others, are demonstrated. The problems associated with gene flow or the lack thereof are often seen as a major threat to stock viability, and appear to largely ignore the role of mutation; the presumption being that mutation is either selectively neutral or will disappear with recombination. Genomes characterized by [presumably] no recombination and high mutation offer alternative hypotheses for how genetic variability amongst geographically disjunct populations might implicate local selective pressures rather than gene migration, as a major determinant of barramundi population genetic structure.

2.1.5 The mitochondrial gene complex

2.1.5.1 Introduction

Mitochondria are small organelles present in high numbers within the cytoplasm of aerobic cells. They fulfil most of the energy requirements of aerobic cells by coupling electron transfer reactions with the production of adenosine triphosphate or ATP (Darley-Usmar, Ragan, Smith & Wilson, 1994). The mitochondria contain mitochondrial DNA which encodes 13 component subunits of much larger protein complexes that form part of the metabolic pathway responsible for oxidative phosphorylation (Darley-Usmar et al. 1994).

Mitochondria are characterized by a high degree of genetic and metabolic autonomy (Darley-Usmar et al. 1994). Most proteins in mitochondria are encoded by nuclear genes, however their specific transport across the mitochondrial membrane remains poorly understood (Clayton, 1991; Holt & Jacobs, 1994). Evidence for the intergenomic transfer of genetic material between the nucleus and mitochondria has been found in all well-studied regions of the human mitochondrial genome (Zhang & Hewitt, 1996). Holt & Jacobs (1994) postulate that because the actual number of mitochondria is tissue-dependent, the replication of mtDNA is cell-cycle regulated, at least within a defined tissue type. This suggests that mtDNA replication is influenced by a “copy control mechanism”, rather than by replication occurring at the DNA-synthesis phase or S phase, as with nuclear DNA (Watson, Hopkins, Roberts, Steitz & Weiner, 1987). Thus, the control of replication is more relaxed than for nuclear DNA (Clayton, 1991).

2.1.5.2 Characteristics of the mitochondrial genome

The mitochondrial genome is a small, double-stranded, circular molecule of DNA (Attardi, 1985), which constitutes around 1% of total cellular DNA (Alberts et al. 1989). The morphological similarity of mitochondrial to plasmid DNA prompted the endosymbiotic hypothesis (Margulis, 1970); which proposes that mtDNA are evolutionary relics, prompting further hypotheses concerning the relative importance of the mitochondrial genome (Alberts et al. 1989). Despite this, the essential function of energy synthesis by mitochondria necessitates the conservation of highly constrained genes, and yet genomic regions differ considerably in genetic variability.

Animal mtDNA is non-recombining and appears to be almost exclusively maternally inherited (Hayashi, Tagashira & Yoshida, 1985; Olivo, Van de Walle & Laipis, 1983; Hurst, 1991), and the combination of specific genes in the mtDNA genome has been described by Avise et al. (1987), and Avise (1992, 1994) as "haplotypes".

Maternally inherited mtDNA is especially attractive as a population marker to fisheries biologists since it is more likely to show differences among populations than is nuclear DNA, because the effective genomic population size is halved, meaning that it is more susceptible to population bottlenecks (Nei & Tajima, 1981; Billington & Hebert, 1990). This would especially apply to founding stocks, whereas "source" populations would be expected to have more "fixed" haplotypes. Koehler, Lindberg & Brown (1991) believe the maternal inheritance of animal mtDNA to be mediated by the differential amplification of small numbers of specific germ-line mtDNA molecules from the mtDNA genotype of the previous generation, allowing paternal leakage of mtDNA. The extent and rate of leakage is thought to be small, but the precise proportion remains unknown (Gyllensten, Wharton & Josefsson, 1991).

Usually an organism has only one type of mtDNA, however if a zygote receives a large number of organelles through the egg, a chance mutation may enable the replication of more than one mtDNA type (heteroplasmy) (Attardi, 1985; Klug & Cummings, 1993).

2.1.5.3 Structure and function of mtDNA

In higher vertebrates including fish, the mitochondrial genome is about 16,500 base pairs (bp) in length (Brown, 1983; Meyer, 1994). The piscine mitochondrial gene order (Figure 7) is thought to comply with the "consensus" vertebrate gene order, containing 13 genes coding for proteins, 2 genes coding for ribosomal RNAs (the small 12S and larger 16S rRNA), 22 genes coding for transfer RNAs (tRNAs), and a major noncoding region (control region) that contains the initiation sites for mtDNA replication and RNA transcription (Meyer, 1994). The 13 genes coding for proteins are cytochrome *b*, 3 units of cytochrome oxidase (CO1, CO11, CO111), 2 subunits of oxidative phosphorylation (ATPase6, ATPase8), and 7 subunits of the mitochondrial

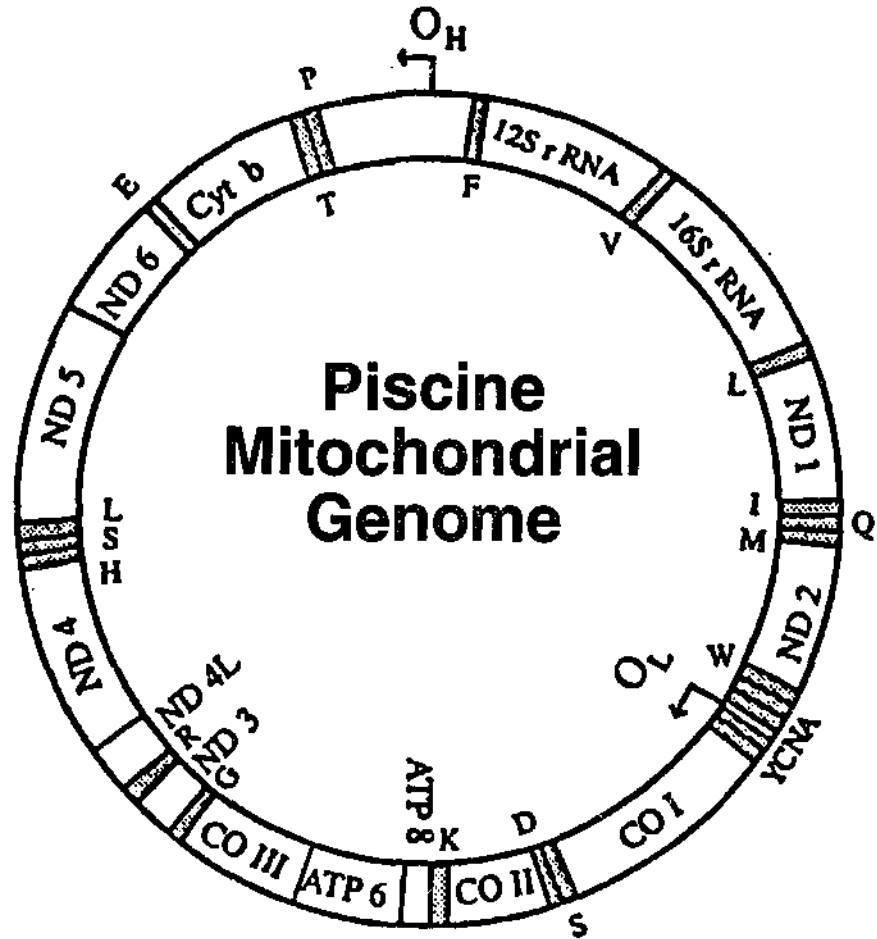


Figure 7. Piscine mitochondrial gene order. The origins of H- and L- strand replication are indicated in the figure. The origin of the H- strand is in the control region, and the origin of L- strand replication is in the YCNAW tRNA gene cluster. Transfer RNA genes are shown in shaded boxes. The coding sequences (templates) of all proteins (except ND 6) and the majority of the tRNA genes are on the H-strand. The tRNA genes encoded by the L- strand are labelled on the outside of the circle, and the tRNA genes encoded by the H- strand are labelled on the inside (from Meyer, 1994).

dehydrogenase complex (ND1,2,3,4,4L,5,6). Descriptions of subunit function are given in Attardi (1985) and Darley-Usmar & Schapira (1994).

The mtDNA genome consists of a light "L" and a heavy "H" strand, reflecting density differences in their respective guanine and thymine content (Clayton, 1991). All genes except ND6 and 8 tRNAs are encoded by the H-strand (Meyer, 1994). Within the control region, the synthesis of a short segment of H-strand DNA results in a three-stranded DNA structure known as the displacement loop (D-loop), and the short nascent H-strand is located at the origin of H-strand replication (O_H ; Clayton, 1991). The sequences adjacent to the D-loop contain both the O_H (Fig. 7), and the transcriptional promoters for the H- and L-strands (Holt & Jacobs, 1994). When the H-strand has replicated over two-thirds its length, the origin of L-strand replication (O_L) is exposed on the displaced H-strand, and initiation of L-strand synthesis begins in a tRNA cluster away from the control region (Fig. 7; Clayton, 1991). Other D-loop sequence elements of unknown function include three conserved sequence blocks which Holt & Jacobs (1994) suggested may have a role in promoting H-strand synthesis, although this remains poorly understood.

The transcription of mitochondrial DNA to RNA, and its translation to protein essentially follows the same pattern as in the "universal" biogenetic code (Alberts et al. 1989; Clayton, 1991). There are however several differences: nearly every nucleotide appears to be part of at least one coding sequence. The few regulatory and intervening sequences available throughout the genome means mutations are more likely to become "fixed" within a region, especially in the absence of recombination which typically erodes those features over generational time; only 22 tRNAs are required for mitochondrial protein synthesis, compared to at least 31 tRNAs specifying amino acids in the cytosol, which means that protein synthesis occurs with fewer tRNA molecules; and lastly, comparison of mitochondrial gene sequences and the amino acid sequences of the corresponding proteins indicate an altered genetic code, so that 4 of the 64 codons have "meanings" which differ from those present in other genomes (Alberts et al. 1989).

In summary, the mitochondrial genome appears to be highly efficient as it contains few duplicate or noncoding sequences (Gray, 1989). The mitochondrial genetic code is more degenerate and less constrained than its nuclear equivalent (Attardi, 1985; Alberts et al. 1989; Meyer, 1994), thus high mutation rates in isolated populations should reveal genetic differentiation by their characteristic genotypes. These features, along with non-recombination and the predominantly maternal mode of inheritance, make the molecule an attractive tool for studying the genealogy of populations. The following section evaluates the evolution of mtDNA from the viewpoint of intraspecific population divergence in Recent geologic time.

2.1.6 Evolution of the mitochondrial genome

2.1.6.1 Introduction

It has been observed that mtDNA variation is more pronounced between than within populations, and this allows mtDNA to be used to estimate phylogenies of populations and patterns of historical phylogeography (Joseph & Moritz, 1994; Dowling, Moritz, Palmer & Rieseberg, 1996). There has been wide application of mtDNA studies in piscine population genetics and fisheries management (see reviews in Ferris & Berg, 1987; Ryman & Utter, 1987; Hallermann & Beckmann, 1988; Ovenden, 1990; Dizon, Lockyer & Perrin, 1992; Avise, 1994). Investigations of mtDNA have shown sufficient intraspecific variation to permit the identification of fish stocks (e.g. Bartlett & Davidson, 1991, 1992; Carr & Marshall, 1991; McVeigh, Bartlett & Davidson, 1991; Avise, 1994), and to facilitate phylogenetic studies among closely related fish species (Kocher et al. 1989; Meyer, Kocher & Wilson, 1991; Sturmbauer & Meyer, 1992).

The mtDNA genome is thought to evolve at a rate about 10 times that of its nuclear equivalent (Brown, George & Wilson, 1979). The high rate of evolution is thought to be due to an unusually high rate of mutation (Brown et al. 1979; reviewed in Attardi, 1985; Thomas & Beckenbach, 1989; Holt & Jacobs, 1994). Alberts et al. (1989) consider the high degeneracy of the mtDNA code coupled with a reduced fidelity in replication and/or repair, to allow frequent base substitution (one purine for another, or one pyrimidine for another) without adversely affecting the organelle.

2.1.6.2 The molecular clock

Brown (1983) proposed that mitochondrial DNA evolved at a rate of approximately 2% per million years, or about 300 bp over this time, assuming a 15,000 bp molecule. This "molecular clock hypothesis" infers that populations which have colonized habitats since the end of the Pleistocene would show little divergence (Billington & Hebert, 1991), however this has not been the case (see review in Hillis, Mable & Moritz, 1996). There is no conclusive evidence that the forecast rate of evolution of the mtDNA genome (Brown et al. 1979) can be correlated with the time of predicted divergence, or universally applied across taxa. Martin, Naylor & Palumbi (1992) considered that unknown factors, apart from base or codon position bias, choice of sequence or selection, were responsible for an apparently slow rate of divergence in some groups of sharks. Avise (1992) suggested life history characteristics might confound the molecular clock, believing the wide, effective dispersal of the catadromous eel *Anguilla rostrata*, was responsible for little or no population sequence divergence, despite interglacial cycles dating to the Pliocene. Li & Graur (1991) considered the variability of divergence rates in nucleotide positions, different genes and different genomic regions to be undeniable. This same variability has been demonstrated among taxonomic groups (Li, 1993; Avise, 1994), casting further doubt upon the universality of a standard molecular clock.

The ability to discriminate between stocks of a fish species does not rely on divergence which postdates isolation, but instead may be based on pre-existing mtDNA polymorphisms (Bernatchez, Dodson & Boivin, 1989; Ward, Billington & Hebert, 1989; Bernatchez & Dodson, 1990; Avise, 1992), and any new variability within a stock might enhance discrimination. Recent coalescence of historically subdivided populations might be masked in nuclear genomic investigations (Hudson, 1990), however mtDNA polymorphisms in founding populations have been useful in stock discrimination (Avise et al. 1987; Meyer, 1994).

A final word on mitochondrial molecular clocks concerns generational time and senescence. The degeneration of mitochondria in old organisms has been described across species (Bittles, 1989). Hayashi et al. (1994) proposed that the primary reason for mitochondrial

dysfunction was associated with nuclear DNA rather than mitochondrial DNA mutation. Other hypotheses suggest the role of oxygen "free radicals" in disrupting respiratory gene organization (Bittles, 1992). Whichever theory is correct, analyses of human mtDNA have revealed large, age-dependent genomic deletions (Pang, Lee, Yang & Wei, 1994), and age-specific point mutations (Zhang, Linnane & Nagley, 1993). Whether mtDNA mutations are spontaneous or induced, the lack of replicative repair mechanisms are thought to be responsible for the rapid rate of evolution. The possibility that age-related mutations might be a significant factor in observed mtDNA differences within and between fish stocks has not yet been considered in fisheries population studies.

2.1.6.3 Tempo and mode of nucleotide sequence substitution

Of the three kinds of observed mtDNA sequence changes, nucleotide base substitutions are more common than additions or deletions (indels), and rearrangements are the least common form of mtDNA change (Meyer, 1994). The rate of silent substitutions (nucleotide base changes that do not result in amino acid changes) which are mainly transitions (changes of one purine for the other, or one pyrimidine for the other), is about 4-6 times that of replacement substitutions, which cause amino acid change (Brown et al. 1979).

Evolution of the mitochondrial genome is due principally to transitional differences (Wolstenholme & Clary, 1985). Transitions often outnumber transversions (purine-pyrimidine swapping) by a factor of 10-20 in within-species comparisons, and they are found in all positions of codons in all mitochondrial genes (Meyer, 1994), including the non-coding control region (Kocher & Wilson, 1991). The predominance of transitions over transversions is thought to have a mutational rather than a selective origin (Thomas & Beckenbach, 1989; Meyer, 1994) where the relative probabilities of substitutions between particular nucleotide base pairs can be asymmetric, resulting in biased base composition (Moritz & Hillis, 1996).

Substitutions at third positions of codons accumulate until they become saturated with transitions, however mutations at first and second positions may continue thereafter (Meyer,

1994). The transitional bias appears to decrease with increasing sequence divergence, and therefore time since common ancestry, at which stage transversions predominate (Wolstenholme & Clary, 1985). Transversional bias is believed to be symptomatic of evolutionary divergence, and has been demonstrated in specific fish phylogenies (e.g. Kocher et al. 1989; Meyer, Kocher & Basasibwaki, 1990; Fajen & Breden, 1992). The rate of transversion increase depends on factors governing base saturation. Base saturation depends on base compositional bias (DeSalle, Freedman & Prager, 1987), and the time taken for transversions to become fixed, which is probably determined by the differential functional constraints of coding regions (Holmquist, 1983).

2.1.6.4 Protein coding genes

Each mitochondrial protein coding gene varies in its evolutionary rate relative to functional constraints on the gene product and base compositional biases (DeSalle et al. 1987; Johansen, Guddal & Johansen, 1990). Substitutional patterns in these genes are relatively well understood, and are reviewed in Meyer (1994). Transitions in the third position of codons are the most frequent form of substitution (Edwards & Wilson, 1990). The second position of codons is thought to be the most conserved, and for this reason it is most similar among closely related taxa and increases in phylogenetic information among more distantly related species (Meyer, 1994).

The cytochrome *b* gene is the only fully functional mitochondrial-encoded protein that is not a subunit of a larger enzyme complex (Palumbi, 1996), and it is perhaps the most studied of mitochondrial genes. The evolution and structure of the cytochrome *b* gene is reviewed elsewhere (Irwin, Kocher & Wilson, 1991; Esposti Degli et al. 1993). Although functional constraints of the cytochrome *b* gene product tend toward generally high conservation of sequences, there is a heavy predominance of transitions, which occur at least 20 times more often than transversions (Edwards & Wilson, 1990). Meyer (1994) suggests that the transitional bias in itself is an unreliable indicator of descent in the absence of transversions, and this gene might be unsuitable for phylogenetic reconstruction in relatively recent evolutionary time. The

slow evolution of the gene (Martin et al. 1992) has been used to test deep evolutionary relationships in whales (Árnason & Gullberg, 1994) and sharks (Martin & Palumbi, 1993), with the latter noting various levels of amino acid conservation in different parts of the gene. Other workers (e.g. Bartlett & Davidson, 1991; Whitmore & Craft, 1996) have investigated sequence variation to establish genealogical relationships of fish species, however intraspecific differentiation is often more difficult to determine (Meyer, 1994), and is variable among taxa. For example, whilst McVeigh et al. (1991) found very low levels of intraspecific variation in Atlantic salmon, Carr & Marshall (1991) reported sufficient haplotype diversity to distinguish between populations of Atlantic cod.

The cytochrome oxidase complex evolves at similar evolutionary rates to the cytochrome *b* gene (Palumbi, 1996). Whilst deep evolutionary divergence has been demonstrated in Penaeid shrimps (Palumbi & Benzie, 1991) and other phyla (Palumbi, 1996), this gene has rarely produced meaningful intraspecific phylogenies (Meyer, 1994).

Meyer (1994) suspects the ATPase complex to be among the more variable of coding genes. It has been used for testing the relationships of Neopterygian sharks (Normark, McCune & Harrison, 1991), however it apparently awaits more widespread application, including comparisons of intraspecific phylogenies.

The mitochondrial dehydrogenase complex (ND genes) has similar characters to those ATPase subunits (Meyer, 1994). The specific variability of the mitochondrial-encoded genes is well exemplified in this gene complex; O'Connell, Skibinski & Beardmore (1995) found no significant haplotype variation in the ND5 and ND6 genes of Atlantic salmon, and concluded that meaningful variability in this gene depended on substantial evolutionary divergence within a species. These same genes were investigated by Hansen & Loeschcke (1996) to detect significant intraspecific variability in brown trout.

2.1.6.5 Transfer RNA genes

All vertebrate mitochondrial genomes contain 22 transfer RNAs (tRNAs), and have been found to display much base substitution variability, although they are still among the more slowly evolving of the mitochondrial genes (Meyer, 1994). This characteristic, and the small size of tRNAs (59-75 bp), have restricted their use in phylogenetic enquiry.

2.1.6.6 Ribosomal RNA genes

The two subunit ribosomal RNA (rRNA) genes (12S & 16S) have overall substitution rates about half those of protein coding genes (Mindell & Honeycutt, 1990; Hillis & Dixon, 1991), making them suitable for investigating groups within major phyla (see review in Hillis et al. 1996), and of more distantly related species within major fish groups (e.g. lutjanid snappers in Sarver, Freshwater & Walsh, 1996). Gene length mutation is more frequent in rRNA genes, by comparison with protein coding genes (Meyer, 1994), making alignment of sequences for phylogenetic inference can be difficult and potentially misleading (Swofford, Olsen, Waddell & Hillis, 1996).

2.1.6.7 The control region

The mitochondrial control region may evolve as much as five times faster than protein coding regions (Aquadro & Greenberg, 1983; Brown, 1985; Thomas & Beckenbach, 1989), although little is known of the mechanisms controlling this high mutation rate (Holt & Jacobs, 1994). Fumagalli, Taberlet, Favre & Hausser (1996) believed unidirectional replication slippage to be the dominant factor in the rapid evolution of the molecule. The structure of the control region is partially constrained to regulate replication and transcription (Clayton, 1991), whilst the sequences flanking those conserved sequence blocks are areas of high variability that contain many polymorphic sites within species (Meyer, 1994; Fumagalli et al. 1996; Palumbi, 1996).

For some time, authors have advocated investigating polymorphisms in the non-coding regions (Slatkin, 1987; Davidson et al. 1989). In the control region, nucleotide substitutions outnumber deletions and additions in closely related fish species (Meyer et al. 1990).

Stumbauer & Meyer (1992) compared the sequence divergence of cichlid fish (*Tropheus* spp.) and found only 2 substitutions in the cytochrome *b* gene, compared to 4% corrected sequence divergence in the control region. This region has been found to display the highest frequency of length mutations at the population level, and large numbers of duplicated tandem sequence repeats (e.g. Buroker, Brown & Gilbert, 1990).

Sequencing analyses of the control region have enabled specific differentiation over about a million years (Meyer et al. 1990); between generic fish taxa (Ong, Stabile, Wirgin & Waldman, 1996), and among geographically remote populations of the same species (Bernatchez, Guyomard & Bonhomme, 1992).

To conclude, the control region is often, but not always, the most informative region in the mitochondrial genome (Hansen & Loeschcke, 1996). For instance, those authors found the control region to be uninformative in differentiating Danish brown trout stocks, whereas the ND1, 5 and 6 genes contained the greatest polymorphic information. For many taxa the control region has proved to be useful as a fisheries population marker, but clearly there are exceptions.

Chapter 3

3.1 Materials and Methods

3.1.1 Introduction

The methods available for investigations of molecular systematics are many and varied, and reflect astounding technological advancement (see review and references in Hillis, Moritz & Mable, 1996). The analysis of DNA has several advantages over alternatives such as proteins for molecular systematics: the genotype rather than the phenotype is assayed; one or more sequences appropriate to a problem can be selected upon the basis of evolutionary rate or mode of inheritance; the methods of sequence analysis are typically general to all DNA types; and DNA can be prepared from small amounts of relatively stable tissue (Dowling et al. 1996). Investigations of recombinant DNA are not addressed herein, however it is noted that allozyme data (e.g. Pogson, Mesa & Boutilier, 1995) and tandemly repeated segments of "satellite" DNA (see Castelli, Philippart, Vassart & Georges, 1990; Bentzen & Wright, 1992; Wright & Bentzen, 1994; Heath, Bernier & Mousseau, 1995) remain as useful tools for depicting piscine population structure and the genetic "fingerprints" of individuals.

3.1.2 Systematic investigations of the mitochondrial genome: A review

Of the techniques available in DNA sequence investigations (see Dowling et al. 1996; Palumbi, 1996), this discussion will describe and compare those two that have received widest application: restriction fragment length polymorphisms (RFLPs) and direct sequence analysis.

RFLP analyses typically investigate base substitutions or insertion/deletion (indels) events. These are commonly detected using restriction endonucleases: enzymes isolated from

bacteria that cut DNA at a constant position within a specific recognition sequence (typically 4-6 bp in length), throughout the genome (Beckenbach, 1991). Indels, rearrangements and base substitutions, can create or eliminate cleavage sites for a particular enzyme(s), thereby altering the number and size of fragments detected by them. The cleavage of DNA at a characteristic, usually symmetrical recognition sequence, most often results in sequence overhang at either end (5' or 3'), but sometimes none (Dowling et al. 1996). The variable fragment patterns revealed following digestion of the DNA extract with restriction enzymes are the basis for polymorphic fragment length analyses, and have been successfully used in assessing population sub-structuring in fish (e.g. Billington & Hebert, 1988, 1990; Ward, Billington & Hebert, 1989; McVeigh et al. 1991; Billington, Barrette & Hebert, 1992; Danzmann, Ferguson & Arndt, 1993; Grewe et al. 1993; Crosetti, Nelson & Avise, 1994; Billington & Strange, 1995; Hall & Nawrocki, 1995; Pogson, Mesa & Boutilier, 1995; Tagliavini, Harrison & Gandolfi, 1995; Ward, Elliot & Grewe, 1995; Hansen & Loeschcke, 1996; Whitmore & Craft, 1996; but see Ovenden, Bywater & White, 1993; O'Connell et al. 1995).

In general, restriction enzymes that cleave at 4 bp sites will cleave more often than those that cleave at 6 bp sequences, thus producing more, albeit smaller fragments. The recognition that restriction enzymes vary in their efficiency for generating RFLPs (Dowling et al. 1996) has complicated the procedure. For example, whilst 4 bp-recognizing restriction enzymes are thought most suitable for closely related animal mtDNAs (Dowling & Brown, 1993), Dowling et al. (1996) later thought that enzymes which produced larger fragments tended to detect more fragment length polymorphism. McVeigh et al. (1991) expressed reservations for RFLP analyses of the mitochondrial genome, because random surveying of the entire genome included areas of differential functional constraints and variable mutational frequencies. For instance, Thomas,

Withler & Beckenbach (1986) thought that because of the frequency of changes in the mtDNA control region, restriction enzymes in this region would probably underestimate the level of sequence divergence due to an increased probability of multiple changes within a restriction site. Similar arguments have been offered by Thomas & Beckenbach (1989) and Billington & Hebert (1991), although Beckenbach (1991) appeared unsure whether RFLP analysis provided higher or lower estimates of sequence divergence, and concluded that direct sequencing of target areas within the mtDNA genome seemed a more rigorous approach.

Direct sequence comparisons between homologous DNA regions rather than restriction site similarities are possible through the selection of DNA primers [typically highly conserved oligonucleotide sequences which anneal to a complementary sequence of single-stranded DNA] specific to the molecule being studied (e.g. Kocher et al. 1989; Meyer et al. 1990), and the molecular cloning technique of the polymerase chain reaction [see below], (Chapman & Brown, 1991). For the mitochondrial genome, direct sequencing of variable coding and non-coding regions (Bartlett & Davidson, 1991; Carr & Marshall, 1991; McVeigh et al. 1991; Bernatchez et al. 1992; Sturmbauer & Meyer, 1992; Martin & Palumbi, 1993; Árnason & Gullberg, 1994; Ong et al. 1996; Sarver et al. 1996) have furthered understanding of the evolutionary relationships of fish species and population structure. Whilst direct sequencing of the mitochondrial genome seems closer to determining actual population divergence in specific genomic regions, the recent work of Santos, Ribeiro-Dos-Santos, Meyer & Zago (1996) demonstrated a 93% agreement between data obtained by RFLP and direct sequencing of American Indian D-loop mtDNA.

3.1.3 The Polymerase Chain Reaction

Although the exponential synthesis of DNA was first described some time ago (Kleppe, Ohtsuka, Kleppe, Molineux & Khorana, 1971), direct genomic sequencing required the laborious, technically demanding process of genomic cloning which typically precluded the analysis of large numbers of individuals in population studies (Carr & Marshall, 1991). The advent of enzymatic genome amplification (Saiki et al. 1985), followed by the use of heat stable polymerase (Mullis & Faloona, 1987) such as *Thermus aquaticus* (*Taq*), eventuated in the polymerase chain reaction, or PCR (Saiki et al. 1988).

For a detailed treatment of the PCR, see Erlich, Gelfand & Sninsky (1991), Mullis, Ferré & Gibbs (1994) and Palumbi (1996). Briefly, the PCR cycle consists of three major phases: denaturation, annealing and extension. In denaturation, heat is used to stop all enzymatic reactions and dissociate genomic DNA from double to single strands. In the annealing phase, the temperature is lowered so that oligonucleotide primers can bind to appropriate sites (target positions or flanking sequences) on the template DNA. The extension phase allows the enzyme to work, synthesizing the target DNA segment. As the temperature slowly rises from the annealing temperature, polymerization begins and is unidirectional. At this time, the polymerase recognizes the single-stranded template DNA and binds temporarily to this strand at a point adjacent to a double-stranded stretch of DNA. The polymerase also binds to deoxynucleotide triphosphates (dNTPs), and using the energy in the triphosphate bond, catalyzes a reaction that attaches an appropriate nucleotide to the second DNA strand. The polymerase enzyme then moves to the nascent end of lengthened, double-stranded DNA, and the process (cycle) is repeated. Once a few extra nucleotide bases have been added to the primer, the stability of the

primer-template complex is secure, and the polymerase will synthesize thousands of bases per minute.

The principal advantage of PCR is that it is very rapid. It can utilize tiny quantities of tissue, enabling non-lethal sampling (Doupé & Chandler, manuscript submitted see Appendix A), and is sufficiently robust to amplify even degraded tissue (Beckenbach, 1991). Notwithstanding this, the biggest assumption made about PCR is that the product produced is the product desired, and is typically indicated by comparing the fragment obtained with a fragment of known size (Palumbi, 1996). There is evidence that mitochondrial gene segments have been transferred into the nuclear genome, and are particularly susceptible to PCR incorporation (Zhang & Hewitt, 1996). The use of target-specific primers and capillary-feed, rather than temperature-gradient gel electrophoresis is thought to reduce the contamination by nuclear insertions in amplified mitochondrial DNA sequences (Zhang & Hewitt, 1996). Innis, Gelfand, Sninsky & White (1990 and references therein) provide a thorough description of methods and applications for PCR, including guidelines for minimizing product contamination and considerations for optimal target amplification.

3.2 Procedure

The many steps required to obtain the barramundi mtDNA sequences are potentially confusing in their complexity, and are summarized in Figure 8.

3.2.1 Sampling location, size and methodology

Baverstock & Moritz (1996) suggest samples should be taken from multiple populations representing a hierarchy from closely spaced to geographically distant sites, which allows the

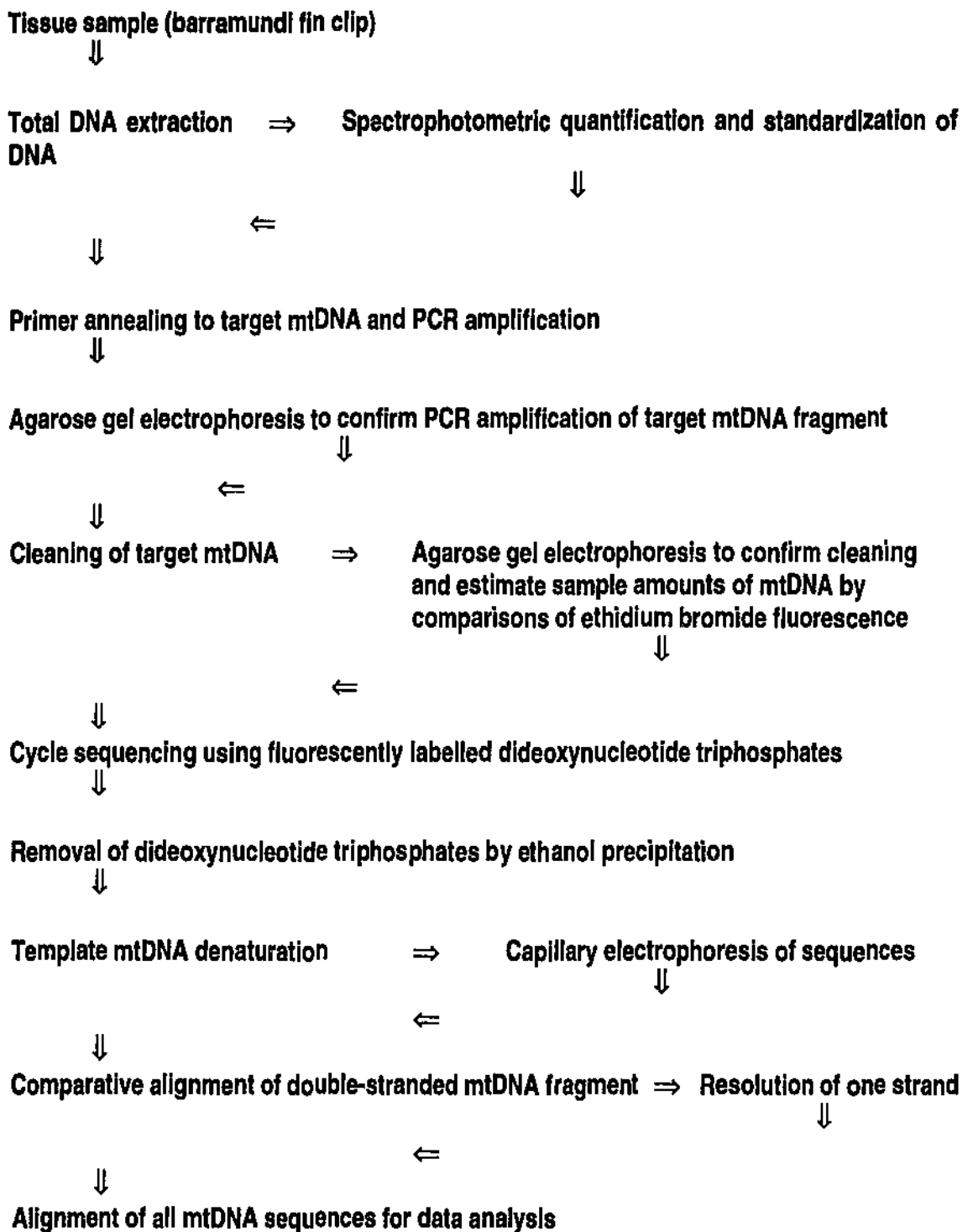


Figure 8. Synopsis of procedure taken to produce the final set of aligned barramundi mtDNA sequences.

identification of locally polymorphic haplotypes and those with widespread variation. This also allows assessments of the distribution of variation within versus among populations. A common problem in genetic surveys is the number of samples to be taken. Grewe et al. (1993) proposed a model which would allow sampling of at least one individual lake trout for every haplotype present 95% of the time. These workers set a conservative minimum sample size ($n = 80$) for their study involving RFLP analysis. Sequencing analysis might require considerably less samples due to target sampling of a specific region, rather than the entire genome. In sequencing studies of the mitochondrial control region, Bernatchez et al. (1992) sampled between 1-8 individuals in each of 24 populations to successfully discriminate geographically remote European brown trout populations. Ong et al. (1996) sequenced that genomic region to assess population divergence between approximately 20 individuals from 2 populations of Atlantic sturgeon. Sequencing investigations of other mtDNA regions like cytochrome *b* have identified genetically separate populations from small samples, often using only 1-10 individuals (e.g. McVeigh et al. 1991; Whitmore & Craft, 1996). Notwithstanding the potential to discriminate populations with comparatively few samples, the relatively high cost of sequencing analyses (approx. \$25 per fish) constrains the maximum number of samples for this study to approximately 50 individuals.

The spatial sampling regime of Australian barramundi is given in Figure 9. Sampling was biased in a westwards direction to sample polymorphism within the Kimberley populations, and to reveal polymorphic differences between those fish and adjacent but distant stocks that represent the genetic diversity present in hatchery stocks presumed to be representative of local wild fish. One barramundi sample each from Thailand and New Guinea, and one sample of the Nile perch, *Lates nilotica*, was obtained to provide comparative "out-groups" for phylogenetic

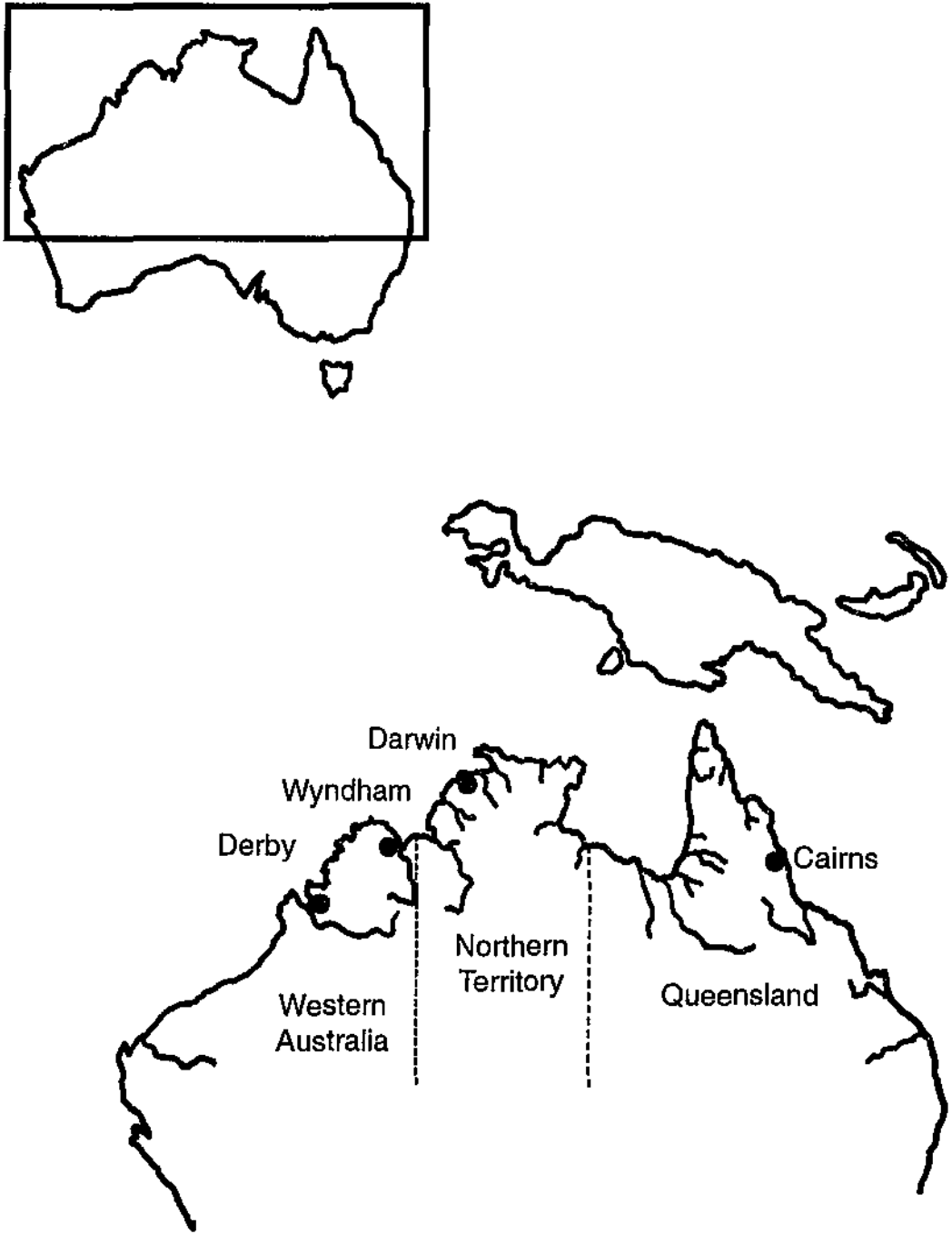


Figure 9. Location of sampling sites across northern Australia.

analyses, and to provide an indication of their evolutionary relationships to, and amounts of genetic divergence from Australian populations.

Kimberley fish were sampled from the professional net fishery. Derby (Fitzroy River) samples were taken from fish caught in nets set along tidal creeks flanking the Fitzroy River estuary. Fifteen fish (FIT 1-FIT 15) were sampled at random from the total catch over 5 days. Scissors and tweezers were used to take caudal fin samples as described by Doupé & Chandler (Appendix A). The tissue sample was placed in a marked 1.5mL cryogenic tube, and immediately stored in liquid nitrogen at -196°C. The sampling bench and implements were washed with seawater between each sampling. The procedure described was repeated for Wyndham fish, which were sampled from tidal creeks adjacent to the Ord River estuary. Fifteen fish (ORD 1-ORD 15) were sampled from the total catch over 3 days. On return to Perth, the Kimberley samples were stored at -20°C.

Darwin fish were obtained from the Australian Barramundi Culture P/L hatchery, south of Darwin. The 10 fish (DAR 1- DAR 10) were the F₁ generation of wild broodstock obtained from Shoal Bay, near Darwin (B. Richards, manager, personal communication, October 6, 1996).

Cairns fish were obtained from the Bluewater Barramundi P/L hatchery at Mourilyan, south of Cairns. The 5 fish (CAI 1-CAI 5) were the F₁ generation of wild fish taken from the Cairns area (G. Doyle, manager, personal communication, October, 8, 1996).

For Cairns and Darwin fish, the procedure was that juvenile fish (\approx 300g) were randomly selected from ponds and killed in a cold saltwater brine. The whole fish was

immediately packed in ice, packaged, and freighted to Perth by air. Fish were collected and transported to the University where they were stored at -20°C.

One partly processed (i.e. gilled, gutted & scaled) fish from a Thailand hatchery (SEA 1) was obtained from Sealanes Food Service P/L, Fremantle, Western Australia. Once processed in Thailand, this fish was stored at -20°C, and maintained at this temperature for transport and storage in Fremantle (P. Paino, manager, personal communication, October 25, 1996).

In the laboratory, frozen fish from Thailand, Cairns and Darwin were systematically taken from the freezer and fin clipped. Each sample was treated as for the Kimberley samples, except that the bench was thoroughly washed with tap water, and sampling instruments were washed in 0.2M HCL, and then distilled water between sampling. The tissue samples were first placed in marked 1.5mL cryogenic tubes, and then placed in liquid nitrogen overnight. Samples were then stored at -20°C.

The New Guinean sample (PNG) was a barramundi fillet purchased from a restaurant in northern New Guinea. This fish was reported to have been caught in the professional net fishery near Kerema, on the south coast of New Guinea (M. Vanderklift, tourist, personal communication, September 11, 1996). The muscle tissue sample was taken from the fillet and placed in a 200mL specimen vial containing 85% ethanol for 2 hours (Dessauer, Cole & Hafner, 1996). Once the alcohol had diffused through the tissue, the 85% ethanol was replaced with 70% ethanol for sample storage (Kocher et al. 1989).

One muscle tissue sample from a Nile perch (*Lates nilotica*, NP) was taken from a fillet supplied by Sealanes Food Service P/L, Fremantle, Western Australia. The fish was processed in Kenya, and the fillet was packed and maintained at -20°C for transport and storage in Fremantle (S. Paino, manager, personal communication, November 2, 1996). The sample was treated with alcohol as for the New Guinean sample.

3.2.2 Extraction and preparation of total DNA

For each sample, a small quantity of tissue (approx. 0.1g) was taken and placed in a marked 1.5mL eppendorf tube. Sample preparation and total DNA extraction occurred by those methods described in Doupé & Chandler (Appendix A), except that samples were not vortexed, but gently mixed. The New Guinean and Nile perch samples were first washed in distilled water, and then processed as for the others. Total DNA stock samples were then stored at 4°C.

3.2.3 Spectrophotometric quantification of total DNA

For each sample, a 1:10 dilution was performed by taking 10µL of DNA extract and adding 90µL of distilled water, to make a 100µL solution. That amount was placed in the Beckman™ DU640 spectrophotometer, following calibration using distilled water (500µL). A spectrophotometrical optical density (OD) reading was taken for each sample to determine the amount of total DNA and protein present in the sample at the respective 260nm and 280nm wavelengths. After each sample was analyzed, the cuvette was twice washed using purified water (approximately 100µL).

The OD_{260}/OD_{280} ratio estimates the purity of the nucleic acid, and the OD_{260} reading estimates the total nucleic acid concentration of the sample. An OD_{260} of 1 corresponds to

approximately 50µg/mL of double-stranded DNA (Sambrook, Fritsch & Maniatis, 1989).

Quantification of sample total DNA (µg/mL) was obtained using the formula:

$$\text{Total DNA } (\mu\text{g/mL}) = \text{OD}_{260} \times 50 \times \text{dilution}$$

3.2.4 Standardization of total DNA

The spectrophotometrical reading of total DNA (µg/mL) was used to create a standardized solution of 10µg/mL DNA (x) in 200µL distilled water for each sample using the equation:

$$x = 10\mu\text{g/mL} \times 200\mu\text{L}$$

$$x = \frac{2000\mu\text{L}}{\text{Total DNA } (\mu\text{g/mL})}$$

$$\text{distilled water to be added } (\mu\text{L}) = 200 - x$$

3.2.5 Amplification and visualization of target mtDNA

Light and Heavy strand primers were used to target variable sequences in the left flank of the mtDNA control region (see Figure 7). The light strand primer, designed specifically for the barramundi mtDNA control region (S. Chenoweth, Griffith University, personal communication, no date) was: BRC1L 5' TTT ATG CTA ACC AAT AAG T 3'. The heavy strand primer was: MT16498H 5' CCT GAA GTA GGA ACC AGA TG 3' (Meyer et al. 1990). Samples from all Australian fish, and the Thai and New Guinean sample were used in the control region investigation. Amplification of each strand was done concurrently using the PCR reactions and thermocycling conditions described in Doupé & Chandler (Appendix A), except that the total volume of each PCR reaction solution was increased from 10µL to 20µL (18µL of PCR reaction mix and 2µL of total DNA).

Light and Heavy strand primers known to target variable sequences in the mtDNA cytochrome *b* region (Kocher et al. 1989; see Figure 7) were used for one sample from each Australian locality (DAR 1 [DAR *b*], FIT 1 [FIT *b*], ORD 1 [ORD *b*], CAI 2 [CAI *b*]), and the New Guinean [PNG *b*], Thai [SEA *b*], and Nile Perch [NP *b*] samples. The light strand primer was: L14841 5' AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA 3' (Kocher et al. 1989). The heavy strand primer was: H15149 5' AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A 3' (Kocher et al. 1989). Amplification of each strand was done concurrently using the PCR reactions and thermocycling conditions described in Doupé & Chandler (Appendix A). The total volume of each PCR reaction mix was increased as described for the control region.

Gel electrophoresis of all PCR reactions was performed using the conditions and methods followed by Doupé & Chandler (Appendix A). Specifically, 2 μ L of 6 x Ficoll loading buffer was added to each of 5 μ L PCR reactions and to 4 μ L of 100ng/ μ L pUC19 plasmid DNA cut with a *Hpa*I restriction enzyme to produce standard fragments of known size (Biotech™). Electrophoresis time was extended to 40-50 minutes, and voltage was increased to 80 volts.

Once electrophoresis was complete, the electrophoretic gel was transferred to a tub and soaked in the 1 x TAE electrophoresis buffer containing 5 μ L of ethidium bromide for 5-10 minutes. Ethidium bromide molecules become intercalated into the DNA, and fluoresce in the presence of ultraviolet light (Sambrook et al. 1989). The gel was then irradiated with ultraviolet light, and the DNA visualized. The electrophoresed fragments for all reactions and for both genomic regions were compared to the pUC/*Hpa*I DNA standards for PCR fragment size confirmation (Doupé & Chandler, Appendix A). Figure 10 shows an example of an agarose gel

used routinely to determine the success of PCR reactions by visualization and comparison of the target fragment to the DNA size standards.

Where gels demonstrated the success of the PCR, sample PCR products were frozen and stored at -20°C. Where any PCR reaction failed (determined by no visual fragment on the agarose gel), the PCR was repeated by accessing sample total DNA stock. In that event, the total DNA concentration of the reaction was doubled from 2µL to 4µL, by subtracting 2µL of distilled water from the PCR reaction mix. Thermocycling and electrophoresis conditions were not altered. This procedure was repeated for the following mtDNA control region samples: DAR 6; ORD 2, 8, 15; FIT 4, 15; CAI 1, 3, 4, 5 & SEA 1.

Gel electrophoresis of fragments again indicated a PCR failure in 3 of the mtDNA control region samples: ORD 15, FIT 4 & SEA 1. Tissue sampling, total DNA extraction and PCR was repeated for these samples, using the procedure as described above. Electrophoresis of the 3 samples was performed as described. Ethidium bromide staining and ultraviolet fluorescence revealed that ORD 15 and FIT 4 had again failed to reveal any reaction product, whereas SEA 1 displayed a signal, albeit weak. It is difficult to determine why 2 of 30 samples failed to anneal to primers and promote polymerization, especially when all other samples for the Kimberley were treated with an identical procedure both in the field and the laboratory. Those 2 samples were omitted from the experiment, however the tissue samples and sample PCR products were retained.

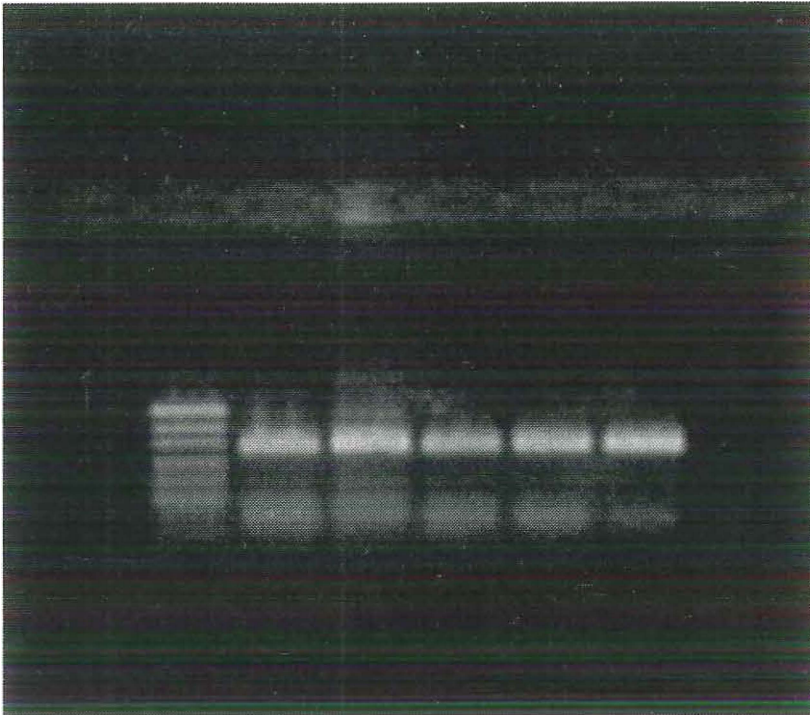


Figure 10. Agarose gel stained with ethidium bromide to show mtDNA control region PCR amplified sequences of *Lates calcarifer*. From left, the lanes are the pUC/*HpaII* DNA standard, DAR 5, ORD 3, FIT 6, CAI 2 and PNG.

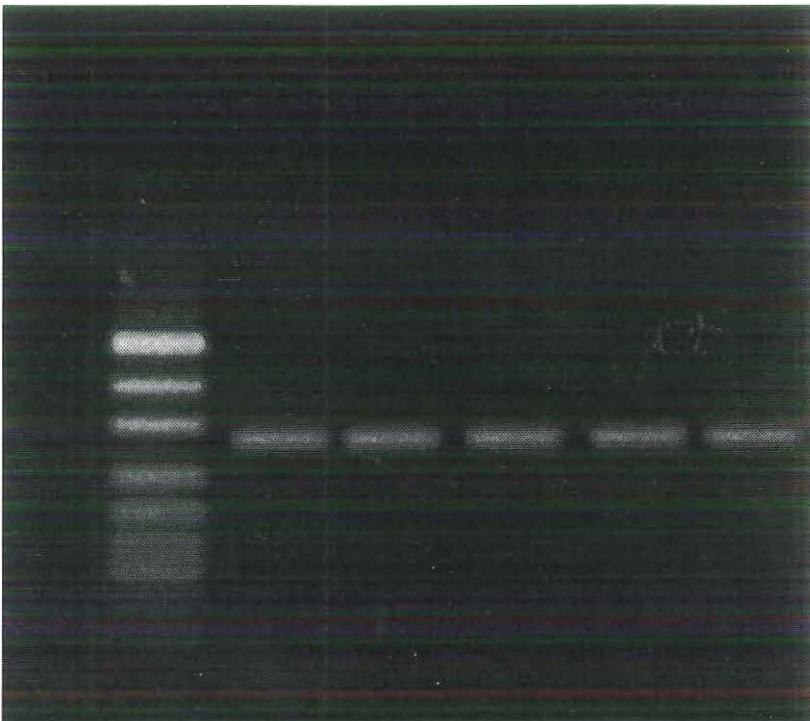


Figure 11. Agarose gel stained with ethidium bromide for fluorescent quantification of double-stranded DNA, and confirmation of cleaned DNA templates. Note the absence of primer-dimers beneath the template DNAs that can be seen in Fig. 10 before cleaning.

3.2.6 Preparation for sequencing templates

The PCR reaction products were then cleaned to remove all non-target DNA including primers, polymerases, and salts from the template DNA before thermal cycle sequencing. This was done using the protocol provided in the QIAquick™ PCR purification kit:

Allotments of 6 PCR reaction products were systematically removed from the freezer and briefly spun in a table-top centrifuge at 13,000 rpm. First, the volume of each template PCR reaction product was estimated by drawing the sample into a pipette set for a known volume. Distilled water was added to the estimated volume of PCR reaction product to make a 30µL dilute solution. For each sample, 5 volumes of PB buffer (150µL) was added to 1 volume of PCR reaction (30µL), and the 0.5mL eppendorf tube was hand mixed. A QIAquick™ spin column was placed in a marked 2mL collection tube, and the sample placed inside the column. The sample was then centrifuged at 13,000 rpm for 45 seconds. The flow-through was discarded, and the spin column was returned to the collection tube. The sample was washed by adding 0.75mL PE buffer (diluted with 100% ethanol) to the spin column, and centrifuged at 13,000 rpm for 45 seconds. The flow-through was discarded, and the spin column was returned to the collection tube. The sample was centrifuged for one minute at 13,000 rpm¹. The spin column was then placed in a clean, labelled 1.5mL eppendorf tube, and the collection tube was discarded. Template DNA elution was done by adding 30µL of distilled water directly onto the spin column membrane, and allowing the sample to stand for 1 minute. The template DNA was then centrifuged for a further minute at 13,000 rpm, and stored at -20°C.

¹ Product leakage from gel loading wells (see 3.2.7) was thought to be due to ethanol elution of DNA in some samples. Ethanol was removed by drying samples at room temperature in a Speed Vac™ SC110 vacuum centrifuge for 20 minutes at low speed and resuspended in 30µL of distilled water.

3.2.7 Ethidium bromide fluorescent quantification of double-stranded DNA: The minigel method

Sambrook et al. (1989) provided a variety of methods for quantifying the amount of nucleic acid present in a sample, even when the total is very small. Apart from spectrophotometric measurement, the sample amount of DNA can be estimated from the intensity of fluorescence emitted by ethidium bromide following electrophoresis on a minigel.

Two microlitres of 6 x Ficoll loading buffer was added to 5 μ L of template DNA and run on a 2% agarose gel in 1 x TAE electrophoresis buffer (Sambrook et al. 1989) at 80 volts for 1 hour. The last well was loaded with 2 μ L of 6 x Ficoll diluted with 3 μ L of distilled water and mixed with 3 μ L of 100ng/ μ L pUC19/*Hpa*II DNA standard. Gels were stained with ethidium bromide to observe DNA fluorescence, and to confirm that templates were cleaned (see Figure 11).

The concentration of template DNA on the minigel was estimated by first summing the individual fragment lengths (in base pairs) of the pUC/*Hpa*II DNA standard (2652 bp, Biotech manual). Then, the number of base pairs for each fragment was transformed to a percentage of the total pUC/*Hpa*II DNA molecule. Following this, the percentage DNA shared by each fragment was transformed to a DNA concentration (ng/ μ L), by knowing the DNA concentration of the pUC/*Hpa*II standard (100ng/ μ L). For each sample, the fluorescent intensity of the template DNA was compared to that DNA standard fragment which displayed comparable fluorescence, giving an estimation of the quantity of DNA in each sample. Those samples estimated to contain < 5ng/ μ L DNA (DAR 5, 6, 7; ORD 2, 11, 14; FIT 1, 7, 15; CAI 1; SEA [control region]; CAI *b*; SEA *b*; Nile Perch *b*) were dried at room temperature by vacuum centrifuge at low speed for approximately 60

minutes, and resuspended in 10 μ L of distilled water (approximately half volume), to increase the DNA concentration of the sample (see 3.32 below).

3.3 Direct DNA Sequencing

3.3.1 A review of direct DNA sequencing techniques and strategies

Of the available methods for DNA sequencing, the two techniques most widely used are the enzymatic method of Sanger, Nicklen & Coulson (1977) and the chemical degradation method of Maxam & Gilbert (1977). Both methods generate separate populations of radiolabelled or fluorescently labelled oligonucleotides that begin from a fixed point and terminate randomly at a fixed residue, or combination of residues. Because every nucleotide base in the DNA has an equal chance of being the variable terminus, each population consists of a mixture of oligonucleotides whose lengths are determined by the location of a particular base along the length of the original DNA (Sambrook et al. 1989). The populations of oligonucleotides are then resolved by electrophoresis under conditions that discriminate between individual DNAs that differ in length by as little as one nucleotide.

Maxam-Gilbert, or chemical DNA sequencing relies on the use of base-specific modification and cleavage reactions (Hillis et al. 1996). This method involves dividing the target DNA into four subsamples, which are treated with a series of base-specific chemical reagents that partially cleave the DNA. For example, a sample treated with dimethyl sulfate will methylate a few percent of the guanines in the sequence, and piperidine displaces the methylated guanines, thereby cleaving the DNA at those sites (Hillis et al. 1996). In all subsamples, random chemical cleavage occurs to only a few fragments among a large population of DNA fragments.

The radiolabelled fragments from the four subsamples are electrophoretically separated by size on a denaturing polyacrylamide gel, and the sequences are then read from an autoradiograph.

Sanger sequencing uses dideoxynucleotide analogues in primer-directed enzymatic DNA extension to produce discrete DNA fragments (Hillis et al. 1996). Oligonucleotide primers are annealed to the target DNA, and the sample is divided into four subsamples. For each sample, the four deoxynucleotide triphosphates (dNTP; i.e. dATP, dCTP, dGTP, dTTP), one of which is radioactively labelled or labelled with a fluorescent dye, are added. DNA polymerase and one of four dideoxynucleotide triphosphates (ddNTP; i.e. ddATP, ddCTP, ddGTP, ddTTP) is added to each subsample. The competition between chain elongation and termination is determined by the ratio of dNTP to ddNTP in each of the four sequencing reactions (Sambrook et al. 1989). The primer has a free 3' OH group to which additional nucleotides can be attached, and polymerization occurs by using the target DNA as a template (Hillis et al. 1996). On some strands in the sequencing reaction, a given ddNTP will be incorporated into the growing strand, at which point the polymerization is terminated, because the ddNTP lacks a 3' OH group (Sambrook et al. 1989). The labelled fragments are electrophoretically separated and interpreted, as for Maxam-Gilbert sequencing.

Both sequencing methods have been used extensively, however if there was to be a single argument for one method over the other, it would be a preference for Sanger sequencing because this method does not require prior knowledge of the restriction map of the target sequence. That information is required for Maxam-Gilbert sequencing, because it is necessary to cleave the DNA into manageable size pieces for sequencing (Hillis et al. 1996).

3.3.2 Cycle sequencing: The dideoxy-mediated chain termination method

Cycle sequencing is based on the dideoxynucleotide chain-termination method of Sanger et al. (1977). The reaction involves heat denaturation of double-stranded template DNA, allowing oligonucleotide primers access to a single strand, and subsequent extension by a thermostable DNA polymerase (*Taq*). Successive cycles of denaturation, annealing and synthesis result in the amplification of a fluorescently labelled product.

A single cycle sequencing reaction consisted of 4.5 μ L of terminator premix (fluorescently labelled ddNTPs: 1.58 μ M A-DyeDeoxy, 94.74 μ M T-DyeDeoxy, 0.42 μ M G-DyeDeoxy and 47.37 μ M C-DyeDeoxy, 78.95 μ M dITP, 15.79 μ M dATP, 15.79 μ M dCTP, 15.79 μ M dTTP, 168.42mM Tris-HCL (pH 9.0), 4.21mM (NH₄)₂SO₄, 42.10mM MgCl₂, 0.42 units/ μ L Amplitaq DNA polymerase), 20ng template DNA, 2.0 μ L of 1.6 μ M primer (L-strand or H-strand), and double distilled water to make a total volume of 10 μ L (Applied Biosystems Inc.). Double distilled water was added only to those reactions where samples were estimated to contain \geq 9ng/ μ L DNA. Where samples were estimated to contain less than that amount, no water was added, but 3.5 μ L (estimated to contain 15-20ng DNA) of template DNA was added to the reaction. The reagents were hand mixed in a 0.5mL eppendorf tube and briefly spun in a table-top centrifuge at 10,000 rpm. Each sample was transferred to a labelled 25 μ L PCR capillary tube for sequencing. This procedure was duplicated for each sample (light and heavy strands), and for both genomic regions.

The sequencing reactions were done on a MJ™ research minicycler. The minicycler was preheated to 96°C. Samples were placed in the minicycler for a denaturation step at 96°C for 30 seconds, followed by an annealing step of 50°C for 15 seconds, and a synthesis step of

60°C for 4 minutes. This cycle was repeated 25 times. When cycling was completed, the samples were held at 4°C before being transferred to labelled 0.5mL eppendorf tubes. Samples were stored at -20°C.

3.3.3 Purification of extension products by ethanol precipitation

Excess DyeDeoxy terminators may be removed from the completed sequencing reactions by a variety of centrifugation and precipitation protocols. The method used here was a modified version of the ethanol precipitation of spin column eluant (Applied Biosystems protocol 401388).

To each sample, 20µL of room temperature 95% ethanol and 1µL 3M Na acetate (pH 4.6) was added, and briefly vortexed. The sample was chilled at -20°C for 15 minutes, and then spun in a refrigerated centrifuge to 13,000 rpm for 30 minutes at 4°C. The supernatant was removed and discarded. The DNA pellet was washed with 200µL of ice-cold 70% ethanol and spun in a refrigerated centrifuge to 13,000 rpm for 10 minutes at 4°C. The supernatant was removed and discarded. The sample was dried at room temperature by vacuum centrifuge for approximately 15 minutes at low speed. Samples were then stored at -20°C.

3.3.4 Sequence analysis by capillary electrophoresis

Frozen samples were transported in crushed ice to the Lions Eye Research Institute at Nedlands, Perth. For each sample, 25µL of template suppression reagent (Applied Biosystems Inc.) was added. The sample was hand mixed and then briefly spun in a table-top centrifuge to 10,000 rpm. The template sequence was denatured by placing the sample in a Perkin Elmer™ 9600 thermocycler pre-heated to 95°C for 2 minutes, followed by immersion in crushed ice for

approximately 5 minutes. The sample volume (approximately 25 μ L) was transferred to a labelled 0.5mL electrophoresis tube (Applied Biosystems Inc.) and capped. Samples were then loaded into a ABI Prism™ 310 Genetic Analyzer.

Each sample was injected into a 47 cm long sequencing capillary containing 6% sequencing polymer with 6.6M urea (Applied Biosystems Inc.) for 30 seconds at 2.4kV. Samples were then electrophoresed at 7.5kV at 42°C. The sample migrated along the capillary for a length of 36 cm at which time the fluorescently labelled ddNTPs incorporated in each of the four sequencing reactions (see 3.31 & 3.32) were detected at the 590nm wavelength by tunable laser beam, which is stationary with respect to the electrophoresis apparatus. The detected fragments were then computationally interpreted as a nucleotide sequence by a series of pair-wise comparisons and base-spacing algorithms designed by the manufacturer (ABI Prism™) that compare light absorption wavelengths of the fluorescently labelled ddNTPs (Applied Biosystems Inc.). Electrophoresis continued for a total of 80 minutes for mtDNA control region samples, and for 100 minutes for the mtDNA cytochrome *b* gene, which was expected to yield longer sequences.

3.3.5 Comparative sequence analysis of double-stranded mtDNA

Double-stranded mtDNA sequences were analyzed using the software package Sequence Navigator version 1.0.1 (ABI Prism™). First, the L-strand and H-strand sequence for a given sample was selected, and then the reverse complement sequence of the H-strand sequence was obtained, allowing the complementary opposite sequences to be read in a parallel, 5'-3' direction.

The two sequences were then comparatively aligned. The L-strand sequence [which does not display the unlabelled forward primer, but begins with the first labelled ddNTP], was identified by locating the reverse complement sequence of the H-strand primer, found at the end of the target sequence (Figure 12). All nucleotide bases preceding the H-strand primer of that sequence were deleted from the data. The H-strand sequence was identified by locating the L-strand primer, which precedes the H-strand sequence (Figure 12). All nucleotide bases preceding the L-strand primer were deleted from the data.

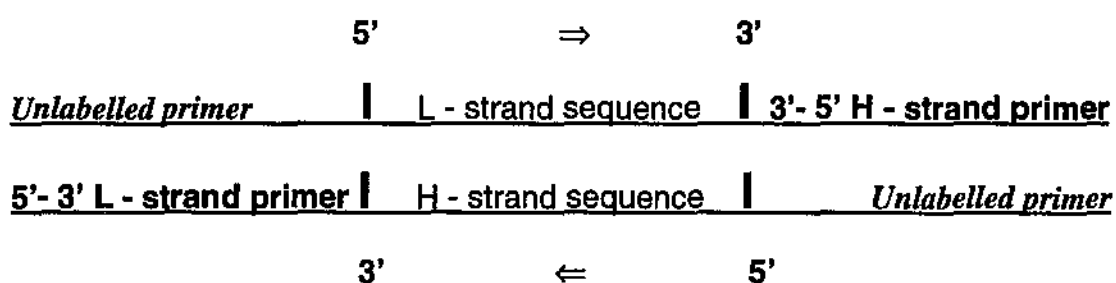
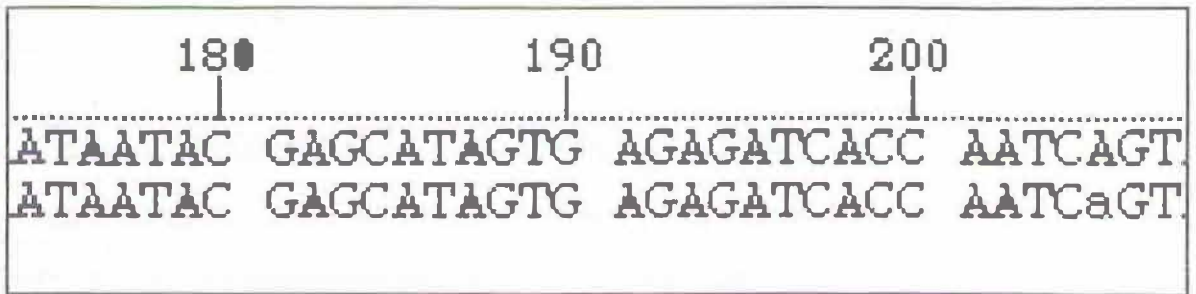
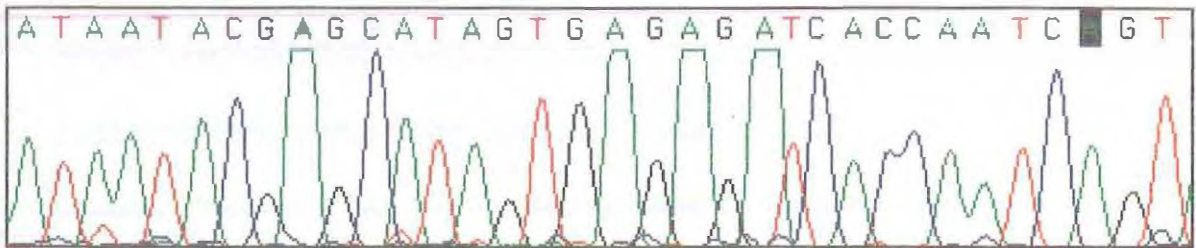


Figure 12. Schematic diagram showing positions of primers and nucleotide sequences in comparative alignment with the H-strand sequence in reverse complement.

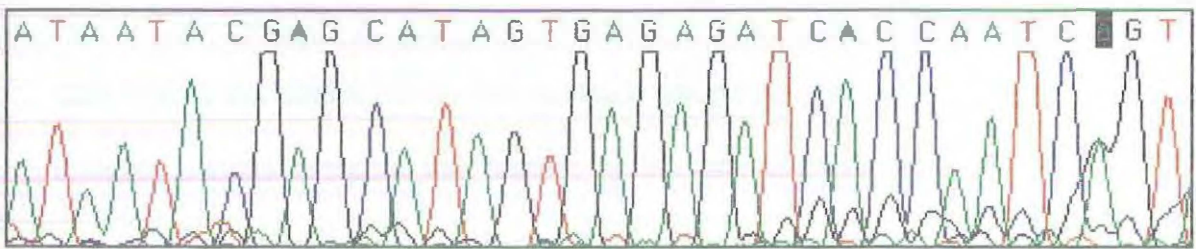
The target sequences were again comparatively aligned to allow cross-checking of the double-stranded nucleotide sequence (Figure 13a), and for comparisons of the electropherograms for each strand (Figure 13b,c). The relative intensity of fluorescence for each of the four labelled dideoxynucleotides corresponds to the peak height seen on the chromatograph (Figure 13b & c), with reliable reads depending on many factors, including template quality, current variation, and polymer injection variation (Hillis et al. 1996). Where fluorescent peaks stood independently, or when signal variation and other anomalies were minimal relative to minor background "noise", the nucleotide base resolved by the base spacing algorithm was accepted as being correct (see examples to the left of base 205 [highlighted] in



(a)



(b)



(c)

Figure 13. Example of partial mtDNA control region sequences and chromatographs for DAR 2 from the automated DNA sequencer. The height of the coloured lines indicates the relative intensity of fluorescence that corresponds to each of the four labelled dideoxynucleotides so the peaks are read directly as nucleotide sequences. Nucleotide bases are adenine (A), cytosine (C), guanine (G) and thymine (T). 13(a) shows nucleotide base pairs 174-207 in comparative alignment with the (lower) H-strand sequence in reverse complement arrangement. The lower two windows show the L-strand (13b) and H-strand (13c) in comparative alignment. 13 (c) is an example of base resolution at base pair no. 205 (highlighted), where two distinct peaks (A&G) in the L-strand chromatograph (13b) are displayed as a wide G peak in the H-strand chromatograph, creating background noise which obscured a smaller, yet defined A peak. In this instance an "a" base was nominated.

13b & c). Where nucleotide bases were not resolved by the sequencing analysis algorithms, the base was recorded as "N" or "-". In all sequences, this was encountered mostly at the 5' ends of each sequence, where the unlabelled primers create "noise" with respect to the pairwise comparisons of the initial, labelled sequences. This often resulted in the first 20-40 nucleotide bases of a given sequence remaining poorly or only partially resolved. Where ambiguities occurred, electropherograms were compared in consultation to the manufacturer's catalogue (Applied Biosystems Inc.), which describes a series of errors commonly encountered in sequencing analyses. Bases were only resolved where the comparative amplitude of fluorescent peaks was considered sufficient [relative to the background noise typically seen near the base of most peaks] for a nucleotide base to be confidently nominated. In those circumstances, lower case lettering was used to indicate that nucleotide manipulation had occurred (Figure 13c). The analyzed L-strand sequence was retained as the data sequence for each sample. This procedure was repeated for all samples, and for both genomic regions.

A generally poor base signal was a common characteristic of most cytochrome *b* electropherograms, resulting in ambiguous sequence resolution. The raw sequence data were re-analyzed using the variety of base-spacing algorithms available without significant improvement. Sequences were tentatively resolved for the Australian and New Guinean samples, however only the Kimberley samples were considered to be reasonably reliable. The Darwin, Cairns and PNG (H-strand) samples required extensive editing. The Thai and Nile perch samples contained large segments of no base signal, indicating total failure. The cytochrome *b* samples were removed from any further analysis.

It is commonplace to search nucleotide databases (e.g. Genbank) for comparable sequences, although this becomes difficult or ambiguous if the sequences are distantly related or come from non-protein-coding regions (Hillis et al. 1996). This was the case with the barramundi control region mtDNAs, where comparable sequences were not located in database searches. Representative sequences from the 44 sequences obtained for the barramundi mtDNA control region were compared with sample sequences of that region provided by S. Chenoweth (personal communication, no date) to confirm sequence homology.

3.3.6 Sequence comparison and alignment

Many authors (e.g. Sankoff, Morel & Cedergren, 1973; Felsenstein, 1988; Weir, 1990) argue that sequence alignment and phylogenetic analysis are not separate issues; assumptions based upon evolutionary models of base substitution and insertion/deletion events will influence how these incidents are weighted in a comparative sequence alignment matrix. For example, the mutational bias for transitions in the mtDNA control region is thought to be about 10:1 (Tamura & Nei, 1993), and popular sequence alignment analyses that arbitrarily weight mutation events (Needleman & Wunsch, 1970), or assume mutations have an equal chance of occurrence (Jukes & Cantor, 1969) might incorrectly influence subsequent analyses (Hillis et al. 1996). Considering this, Weir (1990) and Hillis et al. (1996) recommend that investigators should attempt aligning sequences by eye, especially where intraspecific phylogenies are concerned.

The sequences were organized within a square data matrix using the software package MacClade version 3.03 (Maddison & Maddison, 1992). Each sequence occupied a single line on the matrix, and "dummy" bases (e.g. ZZZZ) were added to the 5' end of each sequence so all samples were equal in length (287bp). The 3'-5' H-strand primer was removed from the 3' end of

the sequence, which gave a common starting point for alignment. Alignment was done by eye in a 3'-5' direction. Every attempt was made to align common nucleotide bases between sequences, and where a final alignment resulted in a gap, a base deletion was accepted to have occurred. Sequences that were not resolved in the comparative alignment analysis of double-stranded DNA were left unresolved. Alignment continued in a 5' direction until a common starting base was found, beyond which at least one base was ambiguous (i.e. a Z , N or a,t,c,g began the sequence). No attempt was made to weight any bases.

3.4 Data analysis

3.4.1 Introduction

The following methods describe how inferences for the evolution of geographically disparate barramundi stocks are obtained by estimates of nucleotide diversity within and between barramundi mtDNA populations, and to test correlations of genetic diversity over geographic distance. Genetic diversity estimates are also used to measure the extent of genetic population subdivision and their transformation to evaluations of gene flow between populations. Genetic distances are calculated upon models of mtDNA evolution to construct phylogenies for the barramundi populations, and to infer the historic relationships between stocks using a coalescent model of gene flow. In addition to this, "intuitive" analyses which might include visual comparisons of aligned sequences, the tallying of unique and shared genotypes, or the ratios of mutational events provide support for those inferences.

A summary of the methods of data analysis is provided in Figure 14, to demonstrate how inferences of population structure might explain the mtDNA phylogenies of selected populations of Australian barramundi.

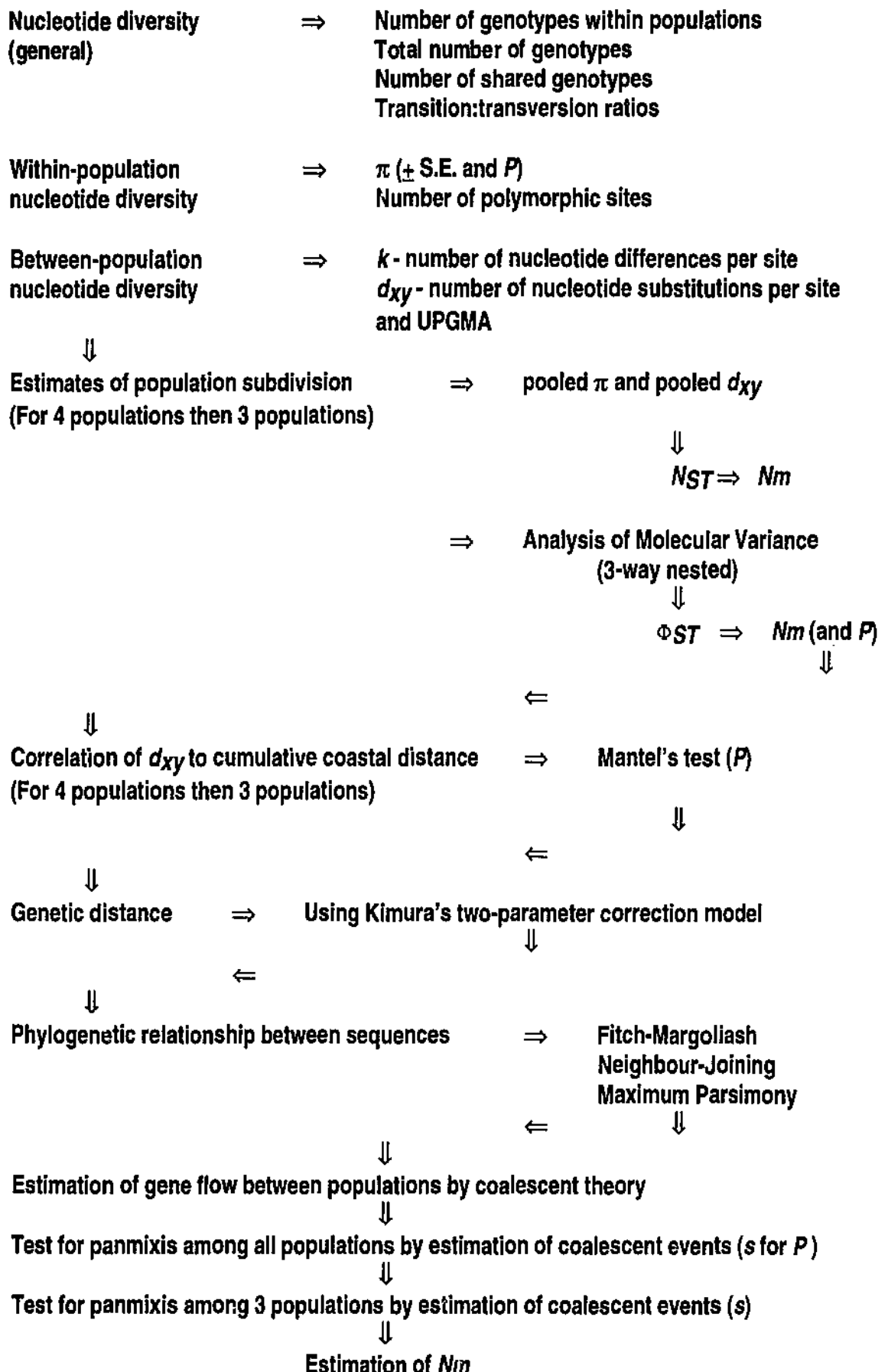


Figure 14. Synopsis of data analysis methods undertaken to infer the genetic population structure and phylogenies of Australian barramundi from mtDNA control region sequences.

3.4.2 Nucleotide diversity within and between barramundi populations

Since no homologous sequence for the barramundi mtDNA control region was obtained from a genetic database, a reference sequence was required from within the samples, however the criteria for a reference sequence would ideally include a sequence that was unique to a given geographic location (Hillis et al. 1996). The mtDNA sequences obtained for the Cairns fish fulfilled this criteria.

Nucleotide diversity was first calculated by counting the number of genotypes within each geographic location, and which genotype(s) were shared between localities. The number of insertion/deletion events for each sequence was counted and transition/transversion ratios were calculated.

The within-population nucleotide diversity estimate (π ; see equations 10.5 and 10.6 of Nei, 1987), and the standard error of the estimate and number of polymorphic nucleotide sites were calculated for each population using the DNA Sequence Polymorphism (DnaSP) software package version 1.0 (Rozas & Rozas, 1995). Confidence limits for the standard errors of each population (see equation 8.5 in Zar, 1984) were estimated using Bonferroni's correction (Chew cited in Trexler, 1988) for multiple tests [where $P < 0.02$] and with no correction [where $P < 0.05$]. Between-population nucleotide diversity was measured by the average number of nucleotide differences per site between populations (k ; see equation A3 in Tajima, 1983), and the average number of nucleotide substitutions per site between populations (d_{xy} ; see equation 10.20 in Nei, 1987). The d_{xy} values were clustered by the unweighted pair group method using arithmetic averages (UPGMA) clustering algorithm (Sneath & Sokal, 1973), using the software package Phentree (Constantine, Hobbs & Lymbery, 1994).

3.4.3 Nucleotide diversity estimates as a measure of population subdivision

The values obtained for π and d_{xy} were used to calculate an F_{ST} analogue (see Lynch & Crease, 1990) which estimates population subdivision at the nucleotide level (N_{ST}), by giving “the ratio of the average genetic distance between genes from different populations relative to that among genes in the population at large” (Lynch & Crease, 1990). N_{ST} estimates were obtained for all four Australian barramundi populations, and then for three populations by removing Cairns data.

First, the π values for each population were pooled, following Lynch & Crease (1990), equation 3:

$$\pi_p = \sum \hat{\pi}_p/n_p$$

where π_p = within population diversity for the i^{th} population
 n_p = number of populations in the sample

Then, d_{xy} values between populations were pooled, following Lynch & Crease (1990), equation 15:

$$d_p = 2 \sum d_{xy}/n_p(n_p - 1)$$

where d_p = pooled between population diversity
 d_{xy} = nucleotide diversity between the x^{th} and y^{th} populations

N_{ST} was obtained using equation 36 (Lynch & Crease, 1990):

$$N_{ST} = d_p/d_p + \pi_p$$

To estimate gene flow, the formula of Wright (1931) was followed:

$$Nm = (1/F_{ST} - 1)/4$$

where N = the effective population size
 m = is the effective proportion of migrants between populations

Excoffier, Smouse & Quattro (1992) present an analysis of molecular variance (AMOVA) for mtDNA restriction sequences which is derived from a matrix of squared-distances among all

pairs of genotypes. The analysis produces variance estimates and F_{ST} analogues that reflect the correlation of genetic diversity at different levels of hierarchical subdivision (Excoffier et al. 1992). The Analysis of Molecular Variance version 1.05 software package (Excoffier et al. 1992) was used to test for significant statistical subdivision of barramundi populations, by providing a PHI statistic (Φ_{ST}) that is analogous to traditional F_{ST} estimates (Reynolds, Weir & Cockerham, 1983).

Because barramundi may show variable genetic divergence between populations, input files were prepared as per the manufacturer's instructions, with the exception being that I used Kimura's (1980) two-parameter model (see 3.4.5) rather than the usual euclidean distance algorithms described in Excoffier et al. (1992).

To test for population subdivision between all four Australian barramundi populations, I compared all sequences represented only once (CAI 1, DAR 1, 3, 4 & 6, FIT 1, 3, 5, 6, 7, 8, 9 & 13, and ORD 4, 6, 7, 8, 9, 10, 11, 12 & 14). To test for significant population subdivision between Darwin and Kimberley populations, and then between only Kimberley populations, the Cairns and then Darwin distance estimates were systematically removed from the analysis. One thousand permutations for each analysis was performed to give statistical significance levels.

Estimates of Nm for each hierarchical test of population subdivision were made using the equation of Wright (1931).

3.4.4 The correlation of d_{xy} to cumulative coastal distance

The interrelationship between the genetic characteristics of each barramundi population and coastal distance [and thus the isolation by distance model of barramundi population genetic structure] was tested using estimates of the average number of nucleotide substitutions per site between populations (d_{xy}). This measurement was preferred to other distance measures, as d_{xy} estimates the differences in genetic diversity between populations, rather than differences in the mtDNA sequences themselves.

Cumulative coastal distances between Derby, Wyndham, Darwin and Cairns were estimated using the software package ARC-INFO version 7.0.4, (Environmental Systems Research Institute). Mantel's correlation (Rohlf, 1992) was chosen in favour of traditional tests for correlation because the test variables here cannot be considered independent (Zar, 1984). The statistical significance (P_g) of Mantel's correlation (Z) was tested by one hundred random resamples of the data (Numerical Taxonomy and Multivariate Analysis System [NTSYS] software package version 1.70; Rohlf, 1992). All populations were tested first, and then genetic and coastal distance data for Cairns were removed from the analysis.

3.4.5 Genetic distance between barramundi mtDNA sequences

Methods for measuring the extent of genomic difference between mtDNA sequences (i.e. genetic distance) are characterized by inherent assumptions concerning evolutionary models of mutation (see Nei, 1987; Weir, 1990; Swofford et al. 1996). For example, the unequal rates of base substitutional patterns in the mtDNA genome are well-established (Tamura & Nei, 1993; Meyer, 1994; Swofford et al. 1996), so that standard population classification measures (e.g. Nei,

1972; Rogers, 1972) that make a priori assumptions concerning rates of divergence (see Nei, 1987) as a function of allele frequencies are thought to miss the evolutionary information obtained from sequence data (Swofford et al. 1996). It is for this reason that Kimura's (1980) two-parameter corrective model was chosen to estimate the genetic distance between individual barramundi sequences. Kimura's model accounts for the observation that base transitions and transversions occur at different rates, but assumes equal frequencies of base change over the length of the sequence (Nei, 1987; Weir, 1990). The PHYLIP version 3.5 software package (Felsenstein, 1993) was used to estimate pairwise genetic distance between barramundi sequences.

3.4.6 Phylogenetic relationship between barramundi sequences

The construction of phylogenetic trees to graphically represent genetic distance measures also assume models of evolutionary change (Avice, 1994). For example, additive distance algorithms assume that empirical distances in the matrix are either underestimates or overestimates of their true values, with the net effect being that branch lengths connecting the operational taxonomic units (OTUs) vary in respect to their empirical distances (Avice, 1994). Average linkage clustering algorithms (e.g. UPGMA) assume the pairwise distance values reflect a constant rate of evolution along the dendrogram branches (i.e. ultrametric), thus any rate heterogeneity among taxa is not detected (Nei, 1987; Weir, 1990; Avice, 1994). Fitch & Margoliash (1967) and Saitou & Nei (1987) provide additive distance algorithms that account for this discrepancy so that branch lengths are analogous to genetic distance (Weir, 1990; Swofford et al. 1996). Cladistic parsimony methods use neither additivity nor ultrametricity, but reconstruct phylogenies on the basis of the minimum number of evolutionary steps required (see Felsenstein, 1983; Williams, 1992).

I used PHYLIP to construct Fitch-Margoliash and Neighbor-Joining trees from the matrix of genetic distance between sequences, and the Phylogenetic Analysis Using Parsimony version 3.1.1 software package (PAUP; Swofford, 1993) to construct maximum parsimony trees from the original sequence data. The PNG sequence was used as an outgroup taxon to root the trees. No single minimum-length tree was found using either branch and bound or heuristic searches in PAUP, but very similar trees were produced by the Fitch-Margoliash and Neighbor-Joining analyses in PHYLIP. The data for each classification were pooled and a consensus tree which summarizes the congruence of the rival trees (Swofford, 1991), was drawn. The consensus tree was compared to the Fitch-Margoliash and Neighbor-Joining trees. All trees were very similar, and the Fitch tree was retained as best representing the data.

3.4.7 A coalescent approach to estimations of gene flow and migration between barramundi populations

An alternative estimation of gene flow by methods other than F_{ST} analogues is based upon the coalescent model of Tavaré (1984; see also Hudson, 1990), and estimates by calculation of coalescent events, how closely a given phylogeny constructed for mtDNA samples approximates the random-mating island model of population genetic structure (see Slatkin & Maddison, 1989). The model (Slatkin & Maddison, 1989) assumes that mtDNA samples have been taken from distinct geographic areas, so each sampling location is regarded as a state character (see Forey et al. 1992) associated with each gene sampled, and is treated as an unordered multistate character (see Fitch, 1971). The algorithm (Slatkin & Maddison, 1989) assigns the character state of each location to the corresponding external node of the tree. Sets of states are then assigned down the tree toward the root. At each step, the rule for joining two sets is a simple majority-rules procedure, so the ancestral state is made of states that occur in both state sets that have been joined; if no states are present in both, then a migration event

must have occurred and the ancestor's state set is present in both sets. Summing the joinings gives the minimum number of migration events consistent with the data (Slatkin & Maddison, 1989).

I used the Fitch-Margoliash phylogeny, with the PNG sequence deleted, in the analysis. Hudson, Slatkin & Maddison (1992) and Edwards (1993) have demonstrated that estimates of gene flow using the method of Slatkin & Maddison (1989) are comparable across tree-making methods. The algorithm described in Slatkin & Maddison and implemented in MacClade version 3.03 (Maddison & Maddison, 1992) was used to estimate the number of migration steps (s) or coalescent events that have occurred to explain the Australian barramundi phylogeny. The phylogeny was resampled one thousand times to compare the observed s value with its null distribution given the number of populations and individuals sampled with random mating. This gives an s value that predicts where the barramundi phylogeny would demonstrate panmixis and how closely the observed phylogeny approximates an island model of population structure, thus inferring the likelihood of the observed s value supporting genetic population structuring among the barramundi populations.

To estimate Nm from s , taxa from the Darwin and Kimberley samples were randomly removed to gain equal sample sizes ($n=8$), and the smallest sample (Cairns) was discarded (following Slatkin & Maddison, 1989; see also Edwards, 1993). The modified tree was reconstructed and a smaller estimation for s was obtained. This value was multiplied by $2/r$, where r is each sampling location (Slatkin & Maddison, 1989), and the resulting value was used to estimate Nm by interpolation of the values from Table 1 in Slatkin & Maddison (1989).

Chapter 4

4.1 Results

4.1.1 Nucleotide polymorphism of barramundi mtDNA populations

This study produced a total of 44 barramundi mtDNA control region sequences, with each being 231 nucleotide bases in length. These sequences are shown in comparative alignment and match-first format in Table 1 where there are clear patterns of nucleotide base change between populations.

Mitochondrial DNA nucleotide polymorphism of barramundi is summarized in Table 2. There were 26 mtDNA genotypes among the samples, of which 4 were present in the Darwin sample and 10 in each of the two Kimberley rivers. The average number of genotypes per individual in the Darwin sample is 0.4, compared to a larger average among Kimberley fish of 0.7.

Table 2
Nucleotide base characteristics in barramundi mtDNAs

Population	Samples (n)	Genotypes (n)	Transitions a	Transversions b	Ratio a:b	Insertion/Deletions
Cairns	5	1	-	-		
Darwin	10	4	19	2	9.5:1	
Fitzroy R.	14	10	20	5	4:1	5
Ord R.	14	10	28	1	28:1	4
PNG	1	1	5	1	5:1	
Total	44	26	72	9	8:1	9

The Kimberley rivers shared the only mtDNA genotype that was present in two geographic locations. This genotype was shared by FIT 5, 10, 11, 14 & 15 and ORD 1, 5, 12 & 13. The Cairns fish were represented by a single genotype and although this lack of variation within the sample might simply be a function of the smaller sample size, one might expect more polymorphism from a randomly sampled population reputed to be the F₁ of wild broodstock (see Discussion). Darwin samples had multiple genotypes and were taken using the same criteria as Cairns fish.

The barramundi control region sequences were adenine and thymine rich, which is similar to other fish species (e.g. Bernatchez et al. 1992), but differs to some other vertebrates (e.g. birds, Edwards, 1993), and like other studies of mtDNA, transitions outnumbered transversions. Replacement substitutions (transversions) appeared in all samples except Cairns, with most being recorded from Darwin and especially Fitzroy River fish. Meyer et al. (1990) and Fajen & Breden (1992) have linked the comparatively higher number of transversions in some fish populations to evolutionary divergence. The large transitional bias in Ord River fish (28:1) indicates there is a relatively high rate of silent mutation occurring within that population, whilst the transition/transversion ratios within other populations including the total among samples, reflects the widely accepted model of piscine mtDNA evolution (i.e. 5-10:1, Meyer, 1994). Nine single insertion/deletion events were detected, but only in Kimberley fish.

The number of polymorphic sites for each of the barramundi mtDNA sequences is shown in Table 3. There were 46 polymorphic sites among all sequences with Cairns fish having nil polymorphic sites. Darwin sequences contained only 4 polymorphic sites which resulted in 4

mtDNA genotypes, whereas the Fitzroy River sample (10 genotypes) contained 4 times as many polymorphic sites as Darwin. The Ord River sequences (10 genotypes) contained 6 times more polymorphic sequences than Darwin and half as much again as the Fitzroy River.

Table 3
Within-population estimates of mtDNA nucleotide diversity

Population	Sequences (n)	Polymorphic sites (n)	π	S.E.
Cairns	5	0	0.00	± 0
Darwin	10	4	0.005	$\pm .003$
Fitzroy R.	14	17	0.016	$\pm .011$
Ord R.	14	25	0.04	$\pm .014$
Total	44	46		

The within-population estimates of nucleotide diversity (π) for these Australian barramundi mtDNA sequences from each geographic location are given in Table 3. The single genotype representing Cairns fish resulted in a diversity index of nil. The larger amounts of within-population nucleotide diversity (π) of Kimberley fish is well demonstrated here. For example, nucleotide diversity for Darwin mtDNAs was 0.005, which is three times less than the Fitzroy River samples (0.016), and more than 10 times less than samples from the Ord River (0.04). The nucleotide diversity in the Ord River sample was 2.5 times more than the Fitzroy River. Despite the large differences in within-population estimates of nucleotide diversity, they were not significant at the 5% confidence level (applied with a Bonferroni correction), which indicates more samples are required to reduce the standard errors associated with each within-population estimate.

Measurements of between-population diversity are presented in Table 4. The average number of nucleotide differences per site between populations (k) was distinctly higher where Cairns was compared to Darwin and Ord River fish, and where Darwin was compared to Kimberley sequences. The high k values demonstrate the substantial differences in polymorphic sites between these populations. Estimated k values were approximately halved where comparisons were made between Kimberley samples, and between Cairns and Fitzroy River populations, because these populations shared many similar nucleotide sites. For example, 85% of Fitzroy River samples differ from the Cairns sequence by between 4 and 6 (1.7-2.6%) nucleotide bases (see Table 1).

Table 4
Between-population barramundi mtDNA nucleotide diversity

Population	Sequences (n)	k	d_{xy}
Cairns v. Darwin	15	9.4	0.08
Cairns v. Fitzroy R.	19	4.12	0.02
Cairns v. Ord R.	19	8.31	0.03
Darwin v. Fitzroy R.	24	7.62	0.05
Darwin v. Ord R.	24	9.00	0.05
Fitzroy R. v. Ord R.	28	5.45	0.02

Similar patterns of the differences in nucleotide diversity between barramundi populations are shown in the average number of nucleotide substitutions per site (d_{xy}). Darwin and Kimberley fish (0.05), and Darwin and Cairns fish (0.08) have much higher d_{xy} values than those obtained from comparisons between Cairns and Kimberley fish (0.02 & 0.03), and for comparisons of only Kimberley fish (0.02). The data indicate that Darwin mtDNAs are quite different to all other populations, and that Cairns and Kimberley mtDNAs are more alike. This is shown in the UPGMA cluster analysis of d_{xy} values (Figure 15). The d_{xy} values involving Cairns

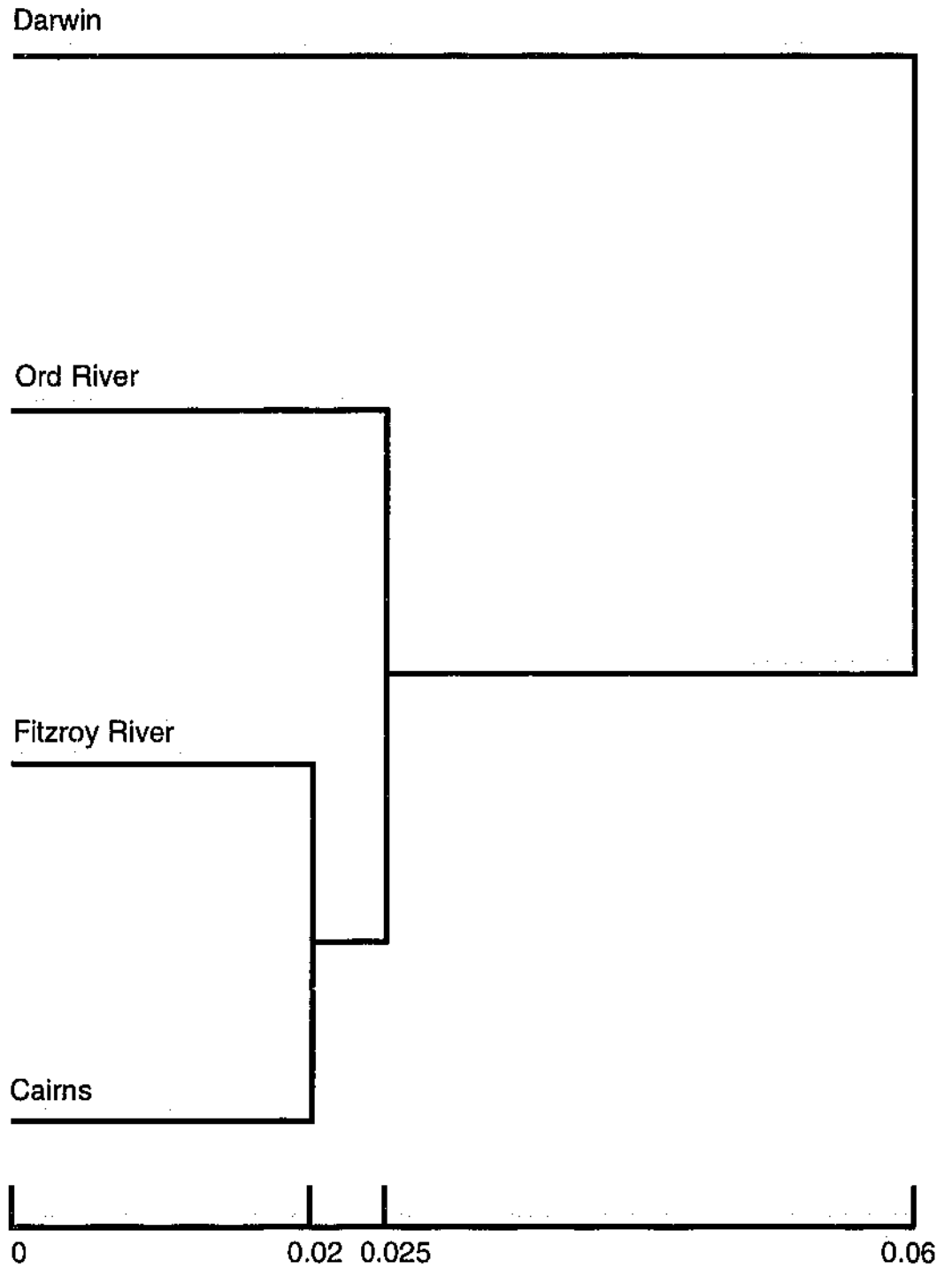


Figure 15. UPGMA dendrogram giving the relationship of the average number of nucleotide substitutions per site between populations (d_{xy}) of Australian barramundi.

fish may have been distorted by the small sample size and monomorphic character of those samples.

The pooled within-population estimates of nucleotide diversity π_p , and the pooled between-population estimates of the average number of nucleotide substitutions per site d_p , are given in Table 5.

Sample	π_p	d_p	N_{ST}	Nm
4 populations	.01525	0.041	0.72	0.1
3 populations	.02033	0.02	0.49	0.4

The average between-population nucleotide diversity for all 4 Australian barramundi populations yielded the high N_{ST} (read F_{ST} after Lynch & Crease, 1990) value of 0.72 (Table 5), indicating that 72% of the observed nucleotide diversity is due to diversity between populations (i.e. substantial genetic structuring). The range of allozyme-based F_{ST} estimates for barramundi populations given by Shaklee & Salini (1985), Shaklee et al. (1993) and Keenan (1994) are very much smaller than those indicated here (0.004-0.046 and 0.005-0.064). These results indicate that barramundi population subdivision is more clearly defined when estimated by the mitochondrial genome than for estimates made from the measurement of gene frequencies at variable nuclear loci (see Hallerman & Beckmann, 1988; Billington & Hebert, 1991). Further, when Cairns mtDNAs are removed from the analysis (Table 5), an N_{ST} value of 0.49 upholds the inference of well-defined genetic structuring within the Kimberley and Darwin populations, and

indicates that approximately half the average nucleotide diversity observed in Darwin and Kimberley fish is due to genetic differences between those populations.

The N_{ST} estimates for all four of these Australian populations (0.72) and for the Darwin and Kimberley populations (0.49) inferred an Nm value of 0.1 and 0.2 respectively. The higher N_{ST} value (and lower Nm estimate) gained where the Cairns population was included in the analysis is probably due to the nil genetic diversity in that population since N_{ST} is a function of between-population diversity compared to within-population diversity. These results indicate that the migration of genes between populations is insufficient to influence within-population genetic structure under an island model (Slatkin, 1987), and that other forces such as genetic drift and/or localized selective pressures might be the primary determinants of the observed genetic differentiation among barramundi populations.

4.1.2 Analysis of Molecular Variance

Results for the AMOVA analysis are given in Table 6, together with an AMOVA-based estimate of Nm (Excoffier et al. 1992).

Table 6
Analysis of Molecular Variance among selected Australian barramundi populations

Variance component	d.f.	Sums of Squares	Mean Squares	Φ_{ST}	P	Nm
Cairns v. Darwin v. Ord v. Fitzroy	3	0.4897	0.163	0.563	$P < 0.001$	0.2
Darwin v. Ord v. Fitzroy	2	0.3798	0.190	0.526	$P < 0.001$	0.2
Ord v. Fitzroy	1	0.0367	0.37	0.084	$0.1 > P > 0.05$	2.7

The among-populations variance estimate for all 4 Australian barramundi populations gave the highly significant Φ_{ST} value of 0.563 ($P < 0.001$) under the island model, which is equivalent to a low predicted gene flow between populations of 0.2 individuals per generation. Very similar results were obtained for among-populations variance estimates for Darwin and Kimberley populations ($\Phi_{ST} = 0.526$, $P < 0.001$), $Nm = 0.2$. The AMOVA for only Kimberley fish indicated there is substantial migration of fish between the Ord and Fitzroy Rivers ($Nm = 2.7$), and that within-population genetic structuring was not significant ($\Phi_{ST} = 0.084$). This Φ_{ST} estimate is still higher than Keenan's (1994) average F_{ST} estimate of 0.064 for 14 barramundi populations east of the Kimberley, for which he concluded there was moderate population differentiation.

4.1.3 The correlation of d_{xy} to cumulative geographic distance

Mantel's correlations of the average number of mtDNA nucleotide substitutions per site between populations (d_{xy}), and cumulative geographic distance matrices were not significant where all four Australian barramundi populations ($Z = 3490$, $P_g > 0.05$), or only Darwin and Kimberley populations ($Z = 674$, $P_g > 0.05$) were compared. The results suggest a non-linear relationship between d_{xy} and cumulative coastal distance, and provide conditional support for an "isolation by distance" type of population structure (Richardson, Baverstock & Adams, 1986; see Discussion).

4.1.4 Genetic distance between barramundi mtDNA genotypes

The genetic distance matrix calculated from Kimura's (1980) two-parameter corrective model is given in Appendix B. The Fitch-Margoliash phylogram generated from this matrix is shown in Figure 16 and shows the interrelationships of sequences as a function of genetic distance, and the common ancestry of sequences in an evolutionary context.

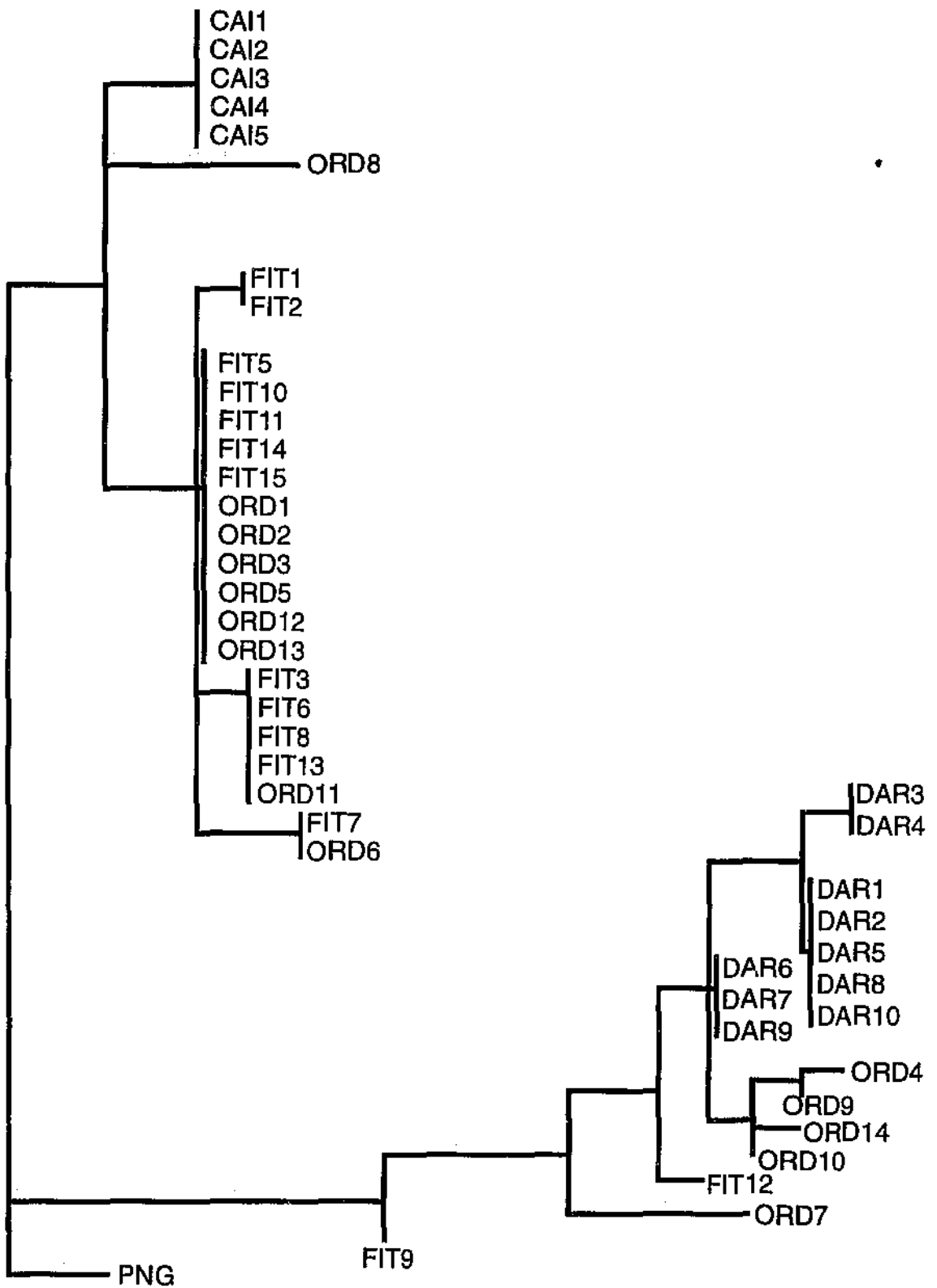


Figure 16. Fitch-Margoliash phylogram summarizing genetic distance relationships between Australian barramundi populations. Branch lengths reflect actual genetic distance as corrected after Kimura (1980). PNG is the outgroup taxon.

The Cairns fish form a separate group, but as with the estimates for d_{xy} (Figure 15), Cairns fish are associated with a major cluster that incorporates 75% of Kimberley fish. The Darwin population groups with the other significant cluster containing the remaining 25% of Kimberley mtDNAs. Within the Cairns cluster, that group, and a single divergent fish (ORD 8) are separate from a second cluster that contains 85% of the Fitzroy River mtDNAs and 57% of the Ord River mtDNAs. In this cluster, FIT 5, 10, 11 14 & 15 share genetic identity with ORD 1, 2, 3, 5, 12 & 13, suggesting there is some gene flow between those populations as indicated by their Φ statistic (Table 6). Other genotypes representing both Kimberley localities display varying amounts of evolutionary divergence by their variable branch lengths and their genetic dissimilarity from the major Fitzroy River-Ord River phylogeny.

The Fitch-Margoliash phylogram also shows that although only the Ord and Fitzroy River populations share identical sequences, the sharing of ancestral sequences is widespread. For example, the Fitzroy and Ord sequences in the first cluster are most closely related to the Cairns sequences than to the other sequences from the Kimberley. In the second cluster, ORD 4, 9, 10 & 14 are more closely related to the Darwin sequences than to FIT 12, ORD 7 & FIT 9.

4.1.5 Estimates of migration and gene flow between barramundi populations using a coalescent approach

The transformed Fitch-Margoliash phylogram is shown in Figure 17. Slatkin & Maddison's (1989) algorithm estimated a minimum number of 10 between-population migration steps ($s=10$ coalescent events) in the barramundi phylogeny (Figure 17).

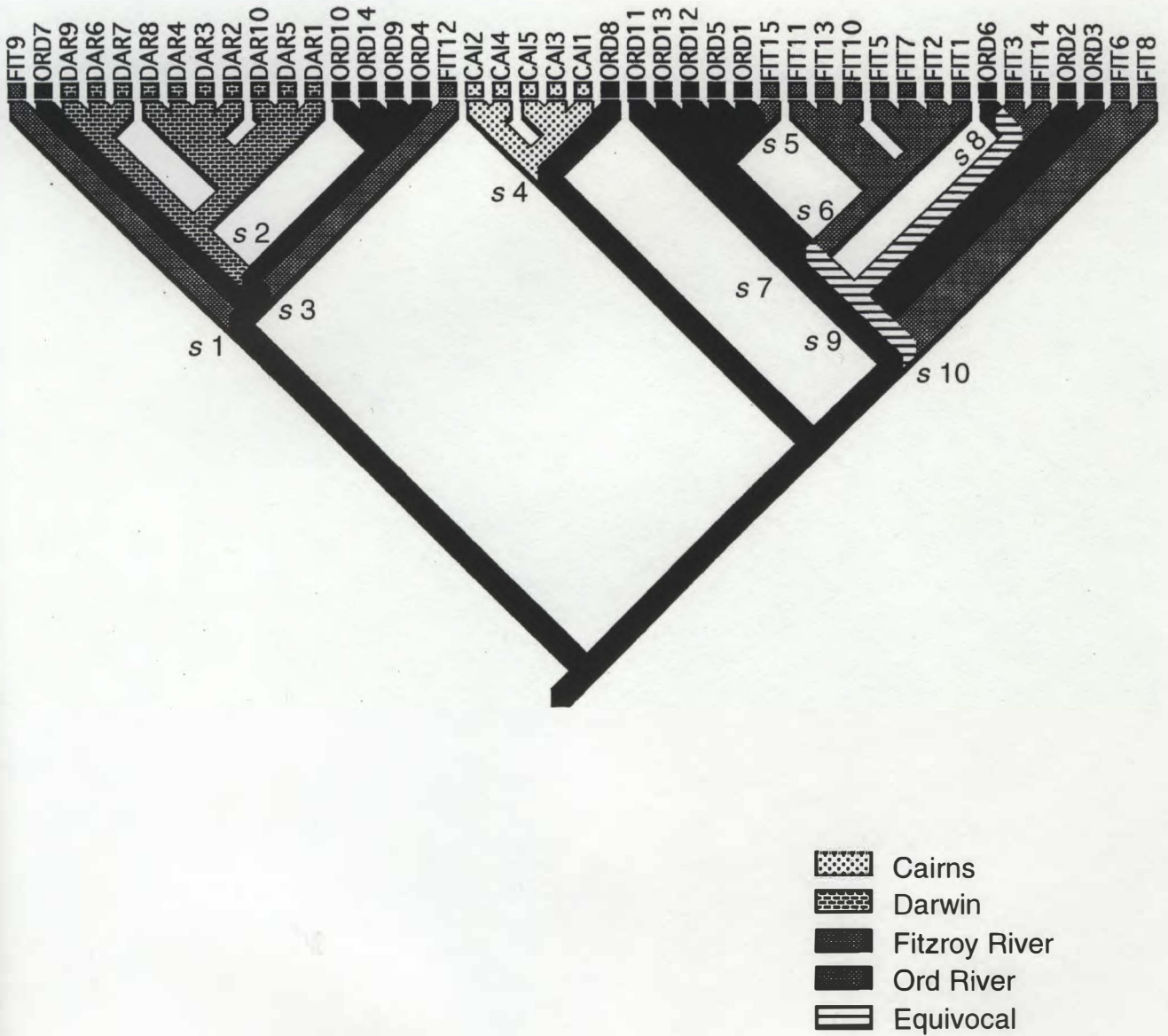


Figure 17. Transformed Fitch-Margoliash phylogram of Australian barramundi populations showing the number of inferred between-population coalescent events (s) as indicated on the tree. The equivocal area indicates the convergence of unresolved ancestral states.

The null distribution produced by random tree sampling ($n=1000$) of the phylogeny [given the number of populations and individuals sampled in an island model of genetic population structure], predicted an s value of 23 for the barramundi phylogeny (Figure 15) to characterize panmixis. Gene flow sufficient to promote random mating between these widespread Australian barramundi populations is therefore highly unlikely ($P<0.001$).

Migration rates between Darwin and Kimberley barramundi populations [where $s=6$] gave an Nm estimate of approximately 4.0 individuals migrating between populations per generation. This Nm estimate approaches Slatkin's (1987) prediction that under conditions of an island model, inter-population exchange of more than 5.0 individuals per generation is sufficient to counteract population divergence due to genetic drift.

The prediction by Slatkin & Maddison (1989) that coalescent analysis provides an insight into the historical associations of mtDNA phylogenies is well demonstrated here, and furthers the hypothesis given for the genetic distance phylogram presented in Figure 16. For example, the sharing of ancestral sequences between Kimberley and Darwin barramundi in Figure 16 is represented here as a number of coalescent events which predict at some time in the past, there was substantial migration ($Nm=4.0$) between these populations.

Chapter 5

5.1 Discussion

5.1.1 Evaluation of molecular techniques for phylogenetic enquiry

The method of caudal fin clipping as a minimally invasive sampling technique for the acquisition of genetic material (Doupé & Chandler, Appendix A) is well demonstrated by this study. In conjunction with alcohol preservation as used for the New Guinean sample, it presents a cost-effective, simple and straight-forward method of field tissue collection and storage. This is especially so given the clear advantages of target amplification by PCR followed by direct DNA sequencing. Notwithstanding this, the failure to amplify two samples from each Kimberley river indicates no method is absolute. The reasons for non-amplification might include the inexactness of the minigel method of DNA quantification (Sambrook et al. 1989). This might also account for the failure of some of the cytochrome *b* amplifications, however it appears that cycles of partial thawing and freezing of the Thai and African samples had resulted in the degradation of DNA. Further, the “universality” of these primers (sensu Kocher et al. 1989) does not necessarily hold for all taxa, and might not be the most appropriate probe for these species.

5.1.2 Sequence variation in the barramundi mtDNA control region

The large amounts of polymorphism thought to characterize the mtDNA control region of fish (Bernatchez et al. 1992; Meyer, 1994) is also a feature of barramundi populations. If numbers of polymorphic sites are equivalent to genotypic diversity as seems to be the case here (see Tables 2 & 3), then there are implications for Kimberley aquaculture and the wild fishery (see later): Darwin fish were represented by four fewer samples than those from the Kimberley,

yet the Kimberley had 5-7 times more polymorphic sites representing 2.5 times the number of genotypes when compared to the Darwin collection.

Estimates of mtDNA variability, π , for barramundi are generally far higher than those reported for a range of coastal and marine species including the catadromous eel, *Anguilla rostrata* (see Table 2 in Avise, 1992), however this is not a general characteristic of all barramundi populations in the present study. For example, Cairns fish show no genetic diversity from an albeit small sample, yet Kimberley fish [particularly Ord River samples] show 3-10 times more genetic diversity than Darwin samples, which is high relative to the difference in the number of samples.

The overall transition/transversion ratio complies with the general model (i.e. 8:1; see Meyer, 1994), but with two dominant features; first, mutation rates in this section of the mtDNA control region are very high, and second, the differences between populations suggests each is displaced from equilibrium and evolving at separate evolutionary rates. For instance, Fitzroy River fish showed a low ratio of 4:1 compared to Darwin (9.5:1) and the Ord River (28:1); the latter shows very high rates of silent mutation, whilst the numbers of transversions in the Darwin (2) and [especially] Fitzroy River (5) populations infer evolutionary divergence. This is providing that higher transversional bias in piscine mtDNAs is symptomatic of population differentiation (Kocher et al. 1989; Meyer et al. 1990; Fajen & Breden, 1992), as is the case with mammals (see Bernatchez et al. 1992).

5.1.3 Genetic diversity and population subdivision of barramundi

A goal of this study was to present a description of the genetic structure of barramundi across a broad geographic section of a still wider Australian range. The relatively low number of individuals and populations sampled risks underestimating existing genetic diversity thus limiting the usefulness of such an approach (but see Slatkin & Barton, 1989), however the N_{ST} estimate of 0.72 for all Australian populations indicates barramundi have a very high genetic structure (see Table 4 in Lynch & Crease, 1990; Bernatchez et al. 1992). This inference still holds when Cairns fish are removed from the analysis ($N_{ST}=0.49$). The equivalence of gene diversity estimates to traditional F_{ST} have been demonstrated by Chakraborty & Danker-Hopfe (1991), and statistical power for the presence of a well-defined barramundi population genetic structure is provided by AMOVA, with Φ_{ST} values approximating 0.5. This demonstrates highly significant ($P<0.001$) population subdivision between all populations, and again between Kimberley and Darwin populations.

Partial, but not significant ($0.1>P>0.05$) population subdivision was observed between the Ord and Fitzroy Rivers, and is often noted in larger populations covering a broad range (Lande & Barrowclough, 1987). Trends of population structuring are evident in the UPGMA dendrogram of population relationships (Figure 15), where Fitzroy and Ord River populations are separated by 0.025 nucleotide substitutions per site, but Darwin fish show substantial nucleotide divergence (0.06 substitutions/site). The clustering of Cairns fish with the Kimberley populations is more likely due to the small sample size and monomorphic nature of the Cairns sample, although the possibility of a deeper historical relationship between these lineages cannot be discounted.

5.1.4 Gene flow estimations and barramundi population genetic structure

Conditional support for a stepping-stone form of isolation by distance is provided by tests of Mantel's correlations of the average number of nucleotide substitutions per site between populations (d_{xy}), and cumulative geographic distance. Under the stepping-stone model in one dimension, the correlation of the variables decreases exponentially with geographic distance (Kimura & Weiss, 1964). This explains the asymptote in Figure 18a - a levelling off with increasing distance, but not the convex asymptotic relationship found here (schematically shown in Figure 18b). This deviation is partly a function of the statistic (see Peterson, 1996). For example, more samples would presumably yield more polymorphism, but only until base saturation by silent mutations would result in more common bases between populations (homoplasies; Meyer, 1994), and a concurrent levelling of the correlation. The nil diversity of the Cairns samples by chance alone may have resulted in their similarity to the most distant samples [Fitzroy River, see also Figure 15], and have consequently dragged the exponent sharply downwards (Figure 18b).

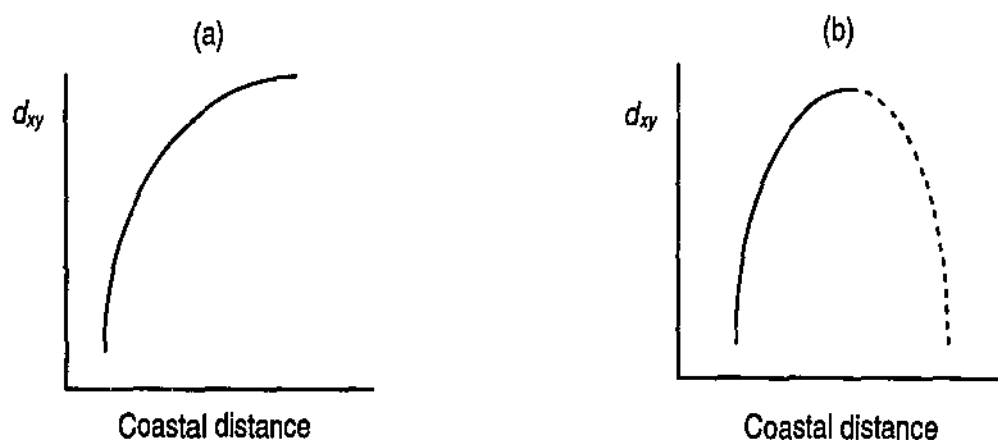


Figure 18. Schematic representation of an isolation by distance model of population structure predicting the relationship of d_{xy} and cumulative coastal distance (a), and the convex asymptotic relationship found for Australian barramundi in this study (b).

All frequency-based estimates of gene flow outside the Kimberley give $Nm < 0.5$, and predict that genetic drift could be causing the significantly high levels of population subdivision due to small effective population sizes (Nei et al. 1975). This might explain the low levels of genetic diversity beyond the Kimberley. The larger amounts of polymorphism and genotypes within the Kimberley might indicate these populations are sufficiently large to counteract the effects of genetic drift, however the sampling regime favoured this result to some extent.

Keenan (1994; table 4) compared estimates of Nm under the island and one-dimensional stepping-stone models, and demonstrated that Nm estimates are two orders of magnitude larger under the stepping-stone model, whilst maintaining significant subpopulation differentiation (see also Slatkin, 1985; Trexler, 1988; Slatkin & Barton, 1989). Isolation by distance will often produce statistically significant allelic divergence despite substantial exchange among adjacent populations, because exchange between genetically similar subpopulations results in each migrant being less effective in promoting divergence, as it has an increased probability of reproducing in a similar population from which it has emigrated (Allendorf & Phelps, 1981; Johnson, Clarke & Murray, 1988; Edwards, 1993). Goldstein & Holsinger (1992) demonstrated that one-dimensional population structure allowed far higher polygenic variation than did a two-dimensional model, despite gene flow. For instance, if gene flow is confined to only adjacent populations in a linear array, rather than to many more populations in two-dimensional space (as for an island of populations), population differentiation (i.e. F_{ST}) will be higher in one-dimensional space. Ward et al. (1994) showed that while total heterozygosity was similar in 49 freshwater (one dimension) and 57 marine species (two dimensions), subpopulation heterozygosity was significantly higher in freshwater species.

Recalculating Nm under a stepping-stone model requires estimations of mutation rate (μ) and effective population size (N_e), in addition to a quartering of N_e to account for non-recombination of mtDNA (Billington & Hebert, 1991). Accurately estimating μ in this rapidly evolving genomic region is important, as it may mislead frequency-based estimates of population subdivision, and therefore Nm (see Edwards, 1993; Milligan, Leebens-Mack & Strand, 1994). Further, estimating μ assumes selective neutrality (Porter, 1990; Edwards, 1993), although the debate concerning the selective role of mtDNA mutation remains unresolved (see Avise et al. 1987; Avise, 1992). Estimates of N_e are also problematic (Lande & Barrowclough, 1987) but necessary (Cockerham & Weir, 1993), and are confounded by the constraints of population genetic models.

For the purpose of demonstration, but notwithstanding all of the problems associated with estimating migration rates under a stepping-stone model, a comparison of migration estimates between the island and stepping-stone models can be made. In this example, we will apply the highly significant AMOVA estimate of population subdivision between Kimberley and Darwin barramundi ($\Phi_{ST} \approx 0.5$) as the analogue fixation index (F_{ST}). For the island model, the number of immigrants ($N_e m$) is solved using the equation:

$$N_e m = 0.25 [(1/F_{ST}) - 1]$$

For the one-dimensional stepping-stone model, the island model estimates of N_e and μ are used, and $N_e m$ is solved using equation 2 in Keenan (1994):

$$N_e m = 0.03125 \times N_e \mu^{-1} \times [(1/F_{ST}) - 1]^2$$

where N_e was estimated as 1000, 2000 and 3000, and calculated for mtDNA using the formula $N_e/4xm$, and a mutation rate, $\mu=1 \times 10^{-5}$ (C. Keenan, Department of Primary Industry & Fisheries, personal communication, March 24, 1997).

A range of values for effective population size adjusted for mtDNA was employed to estimate gene flow as measured by migration rates between populations (Table 7).

Table 7
Estimates of migration rate as a percentage ($m\%$) and number of immigrants ($N_e m$) as determined from the island model and one-dimensional stepping-stone model using an observed Φ_{ST} value (0.5) with a fixed mutation rate (μ) and a range of effective population sizes (N_e) for western Australian barramundi populations

Model	Φ_{ST}	μ	$m\%$ ($N_e m$)		
			$N_e = 250$	$N_e = 500$	$N_e = 750$
Island	0.5	1×10^{-5}	.09 (0.24)	.05 (0.24)	.03 (0.24)
Stepping-stone	0.5	1×10^{-5}	5.2 (13)	1.2 (6)	0.5 (4)

It is evident from Table 7 that variation in population size has no effect on the estimates of $N_e m$ derived from the island model, whereas the estimates derived from the one-dimensional model for the same population sizes range from 13-4, as N_e increases. Keenan (1994) attributed such differences to mutation rate μ , arguing that while mutation rate has little effect on the estimate of $N_e m$ in the island model and is usually ignored, the factor $N_e \mu^{-1}$ can have a large effect on $N_e m$ in the one-dimensional model because mutation becomes formally equivalent to long-range dispersal. Caution should be taken when estimating μ for at least two reasons; first, there is evidence of variable divergence and non-equilibrium conditions among these barramundi populations, and second, the mutation rate of 1×10^{-5} could be an underestimate for this rapidly evolving portion of the mtDNA genome. If that is the case, then higher mutation rates will reduce estimates of $N_e m$ (see Table 4 in Keenan, 1994). Estimating N_e can also differ even when the same model of population genetic structure is applied, because the population estimate appears dependent upon whether demographic or genetic data are used. For example, N_e for barramundi

under a stepping-stone model in one dimension was much lower when estimated from harvest rates (demographic data; Shaklee et al. 1993) than Keenan's (1994) estimate, where the proportional loss of heterozygosity between selected locations was compared [genetic data]. The recalculation of Nm for a stepping-stone model does increase migration estimates, however its accuracy depends upon genetic and demographic data, and both are only coarse estimates. For the interaction between these variables to explain the large genetic differentiation between barramundi populations, then more accurate measurements are required. This can only be accounted for through monitoring the change in gene frequencies in each population, and by the collection of ecological data that gives population life history information (Sherwin, 1992; Rannala & Hartigan, 1996).

The phylogenetic analysis of relationships between sequences showed a somewhat different picture of genetic structure than that provided by estimates of Nm from the variance of gene frequencies within and between populations. The new information provided by coalescent analysis showed that although extant sequences were largely confined to different populations, thereby giving high estimates of population subdivision and low estimates of gene flow, ancestral sequences were more widely spread, and indicated a substantial number of migration events. As shown by Edwards (1993) and Milligan et al. (1994), traditional F_{ST} methods make no distinction between the effects of genetic factors [such as mutation] and demographic factors [such as migration]. Phylogenetic methods using coalescent analysis separate these factors. The apparent discrepancy between substantial genetic structure and reconstructed migration events may be partly explained by the high mutation rates in mtDNA. Whilst coalescent analysis is thought to account for the historical nature of the relationship between sequences, and thus their genetic structure (Lymbery, 1995), estimates of migration remain dependent upon an island

model of population structure (Slatkin & Maddison, 1989) and the accuracy of the phylogram (Felsenstein, 1988; Templeton, Crandall & Sing, 1992; Hudson et al. 1992). The hypothesis that the historic barramundi phylogeny approximates a random-mating island model was shown to be highly unlikely ($P < 0.001$), despite a large number of migration events ($s=10$). When Cairns data are removed, estimated migration between Kimberley and Darwin populations ($Nm \sim 4.0$) becomes theoretically sufficient to prevent population differentiation by genetic drift under an island model (Slatkin, 1987; Slatkin & Maddison, 1989).

Determining how much gene flow still occurs between barramundi populations might well be confounded by estimations that are based upon equilibrium island models with no selection. Furthermore, isolating the effects of genetic data from demographic data (e.g. Edwards, 1993) might be theoretically expedient, but it is difficult to understand population dynamics under such simplifying constraints. Conflicting estimates of migration might be resolved in an historical context which indicates far higher levels of gene flow than is estimated by diversity-based indices, which may be better indicators of contemporary gene flow (Moritz, 1994; Barton & Wilson, 1995). For example, if populations have separated in Recent geologic time, migration estimates will be high, reflecting the retention of mitochondrial lineages; this is expected to decrease as the time since divergence increases relative to N_e (Edwards, 1993). High mutation in differentially isolated populations already experiencing the effects of diminished gene flow might explain the genetic subdivision inferred from diversity-based estimates, whilst the sharing of common sequences between only Kimberley populations reflects continuity of genetic exchange. Both situations might have occurred in the context of barramundi population extinction-recolonizations (see Lande & Barrowclough, 1987) across northern Australia (see below), where range expansion might cause a deviation from the genetic structure expected to

result from isolation by distance (Slatkin, 1993; Peterson, 1996). Such events are thought to confound estimates of high population subdivision and migration that are based on the island model (Lande & Barrowclough, 1987; Whitlock, 1992; Milligan et al. 1994).

The genealogical structure of these Australian barramundi possibly approximates an isolation by distance model in one dimension, however this classification is provisional for at least two reasons: first, larger samples from intermediate locations are required, and second, the isolation by distance model is applicable only when the Kimberley populations are pooled, and might not suit the Kimberley genealogy per se (see later). Contrasting Keenan's (1994) argument against gene flow estimates based on the island model is the overwhelming support throughout the literature for its simplicity (see also Slatkin & Barton, 1989; Porter, 1990). Further, Keenan (1994) summarizes the mechanism that produces barramundi population subdivision as "simply" mutation and genetic drift operating independently in different parts of the population. There are serious ramifications for simplifying genetic models from a management perspective; this change in philosophy has resulted in a paradigm shift from the role of selective adaptation in population processes (see especially Shaklee et al. 1993), to its dismissal as being inconsequential (Keenan, 1994). The corollary here is that the barramundi's physical environment, [and therefore local selection pressures], play no part in population dynamics, so habitat management is largely insignificant in the conservation of viable barramundi genetic variation.

5.1.5 Recent history and zoogeography of western Australian barramundi

The maternal inheritance, absence of recombination, and rapid evolution of the mtDNA control region provides further zoogeographic hypotheses for the Recent history of western

Australian barramundi populations; coalescent and genetic distance analyses provide historic information for adjacent Kimberley and Darwin populations (Figure 16), and a hypothesis for the shared ancestry of sequences and observed population subdivision is proposed in the context of alternate sea levels and population extinction-recolonization events:

If we consider sea levels 150m below present levels (Figure 6), sea level retreat and extinction of localized habitats and their populations results in a large population inhabiting the western coastline. The broad geographic coverage of the “western population” (sensu Keenan, 1994) has allowed peripheral population divergence [i.e. isolation by distance] into a northern (Darwin) and southern (Fitzroy River) population, with a hybrid contact zone (i.e. the Ord River population) separating the two. Partial coalescence of each population, as revealed by substantial gene flow at the contact point ($Nm \approx 4.0$), would provide larger amounts of genetic diversity and silent mutation in the hybrid population, with drift affecting variable sites away from the contact zone. Common ancestral sequences would be retained in all populations due to no recombination.

Coastal inundation to 60m below present levels provides a shifting land/sea interface, and the recolonization of habitats by dispersing barramundi. The Ord River basin is well developed at these levels, as is the “top end” fronting the Arafura Sea (Figure 6), resulting in decreased between-population contact and increased within-population differentiation due to mutation. Fitzroy and Ord River populations continue to exchange genes, however it is unclear whether the Ord River has served as the point from which other Kimberley populations have colonized.

Fluctuating sea levels during the period 70,000-10,000 years BP (Torgersen et al. 1983; Torgersen et al. 1985) would probably see cycles of at least partial extinction-recolonizations of barramundi populations along the western coastline. Gaggioti (cited in Taylor & Dizon, 1996) thought that variable dispersal over long periods results in increased population genetic differentiation.

An approximation of time since population divergence is possible, despite the problem of variable evolutionary rates between populations (Li, 1993; Avise, 1994). Assuming that the 16,500bp piscine mtDNA genome evolves at 2% or about 300bp per million years (Brown, 1983), then the control region (approx. 1100bp long; Shoffner & Wallace, 1995) which is thought to evolve five times faster than protein coding regions (Aquadro & Greenberg, 1983; Brown, 1985; Thomas & Beckenbach, 1989) might be expected to change by at least 100bp over this time. Clearly this has not occurred, however the magnitude of the differences between the Kimberley and other populations suggests there is evidence for longer-term isolation. For instance, if the populations have been separated for about 10,000 years, then the number of nucleotide base differences per site between populations (k) would be one or very few. If 100,000 years of isolation were allowed, then k values approximating 10 become more representative of the data where comparisons between Darwin and other locations are made, however these are coarse estimates that don't account for the periodic recombination of a western population, or the variable evolution of subpopulations. More accurate inferences are possible given more samples, and an investigation of a more slowly evolving region like the cytochrome *b* gene.

5.1.6 Gene flow, selection and population differentiation

Although genetic models are typically insensitive to selection and mutation, selective forces that vary geographically could affect population subdivision, and therefore estimates of Nm (Johnson et al. 1988). For example, Slatkin (cited in Johnson et al. 1988) argued that geographically variable selection should result in a large variance of private alleles; the greater numbers of genotypes, and the higher within-population estimates of nucleotide diversity and polymorphic sites found in the Kimberley fish might be explained by geographically variable selection. The differential effect of selection over gene flow and genetic drift reflects the relative importance of genetic adaptations to local environments (Slatkin, 1987), so permanent clines can result from the interaction between gene flow and selection (Haldane cited in Slatkin, 1985; Rockwell & Barrowclough, 1987). If we consider changes in selection intensities occurring along a geographical gradient, then environmental "pockets" may arise that favour certain alleles in certain areas, so that the genotypic frequencies are characteristic of localities (Slatkin, 1985).

For barramundi, geographic variation in localities for sex change (Moore, 1979; Davis, 1982), the presence of primary females, and absence of sex change (Moore, 1979; Maneewong, 1987), variable length/sex ratio relationships (Davis, 1982; Patnaik & Jena, 1976; Wongsomnuk & Manevonk, 1973; Moore, 1979), relative age/size classes (MacKinnon & Cooper, 1987), and the possibility of fully marine life histories (Pender & Griffin, 1996), are supposed to be due to a "flexible biology" (Keenan, 1994), and population differentiation is presumed to be due to genetic drift and a small N_e , if we assume genetic neutrality and no selection. It is difficult to comprehend that such wide variability in life-history traits is explained by a process that theoretically decreases genetic diversity and limits adaptation, particularly when considering habitat shifts and population instability in the Recent period. Like most studies of population genetic structure, this

study has investigated a single, presumably neutral genomic region. Given the polygenic basis of most life history traits (Holsinger, 1996), quantitative studies that separate the genetic from environmental effects on traits, and/or mapping genes which influence life history traits and then studying their population genetics, are required (e.g. Mitchell-Olds, 1995; Storfer, 1996).

In the context of periodic extinction-recolonization events, population differentiation due to genetic drift is negligible if the time of population persistence is less than the time required to fix neutral alleles, which is equivalent to population size (Slatkin, 1987). If migration (m) is opposed by selection (s), then population size is unimportant, because differentiation will occur where $s > m$ (Slatkin, 1987; Johnson et al. 1988; Trexler, 1988; Lymbery, 1993). Not knowing the effects of mutation or the role of selection pressures in determining population genetic structure remains a difficult problem (Allendorf & Phelps, 1981; Pogson et al. 1995), but cannot be ignored (see Altukhov, 1990; Taylor, 1991). Considering Wright's (1943) acknowledgement of the role environmental adaptation might contribute to population structure, and the wide concern for the effects of artificial selection in hatchery environments, surprisingly few investigators look to local conditions to explain their data, preferring to explain genetic differentiation by drift alone.

5.1.7 The role of local environments and selective processes in forecasting an alternative barramundi population structure in the Kimberley

Dunstan (1959) broadly classified the characteristics of Queensland rivers to describe five types of barramundi habitat relative to juvenile abundance. Morrissy (1985, pp.15-16) compared these to the Kimberley, and concluded there was a deficiency of habitat diversity in the region, with the most suitable juvenile habitat being confined to the areas of King Sound (Fitzroy River) and Cambridge Gulf (Ord River), with habitat on the remainder being "...sparse on the largely rocky, steep Kimberley coast". Historic annual rainfall statistics obtained from the Bureau

of Meteorology for each sampling location demonstrate the inconsistent nature of the tropical monsoon (Figure 19). Such differences in rainfall would be expected to affect the availability of juvenile habitat in the Kimberley (Morrissy, 1985), and the dispersal of fish.

Keenan (1994) discussed the movement of tagged barramundi along the Queensland coast, and with few exceptions, thought adult fish typically remained about their native rivers. He speculated that variable population boundaries might reflect the regional effects of flood pluming, and concluded that stochastic but extensive flooding of northern Australian rivers was probably the major source of juvenile transfer between localities, and hence gene flow between populations. Flood pluming, or more specifically, the effects of flood pluming on dispersal and juvenile habitat are stochastic events, and might provide some explanation for the genetic population structure found in the Kimberley. For example, Morrissy (1985, Figure 8) showed an inverse relationship between mean monthly rainfall and mean monthly catches at Wyndham. Assuming a direct association between rainfall and riverflow, then the decrease in catches might indicate dispersal of fish following the flood plume, or fish leaving the flooded areas to find more suitable salinities and spawning habitats, as noted elsewhere (Moore, 1982; Moore & Reynolds, 1982). Either situation arising from variable, local rainfall at or between these few major Kimberley rivers results in the infrequent, but probably large migration of fish across a coastline separating suitable habitats. The interaction of rainfall and juvenile habitat availability then provides a hypothesis for Kimberley barramundi population genetic structure and migration estimates found in the present study.

The relationship between gene flow and geographic distance differs for Kimberley barramundi, as it appears that Recent genetic exchange remains a characteristic of

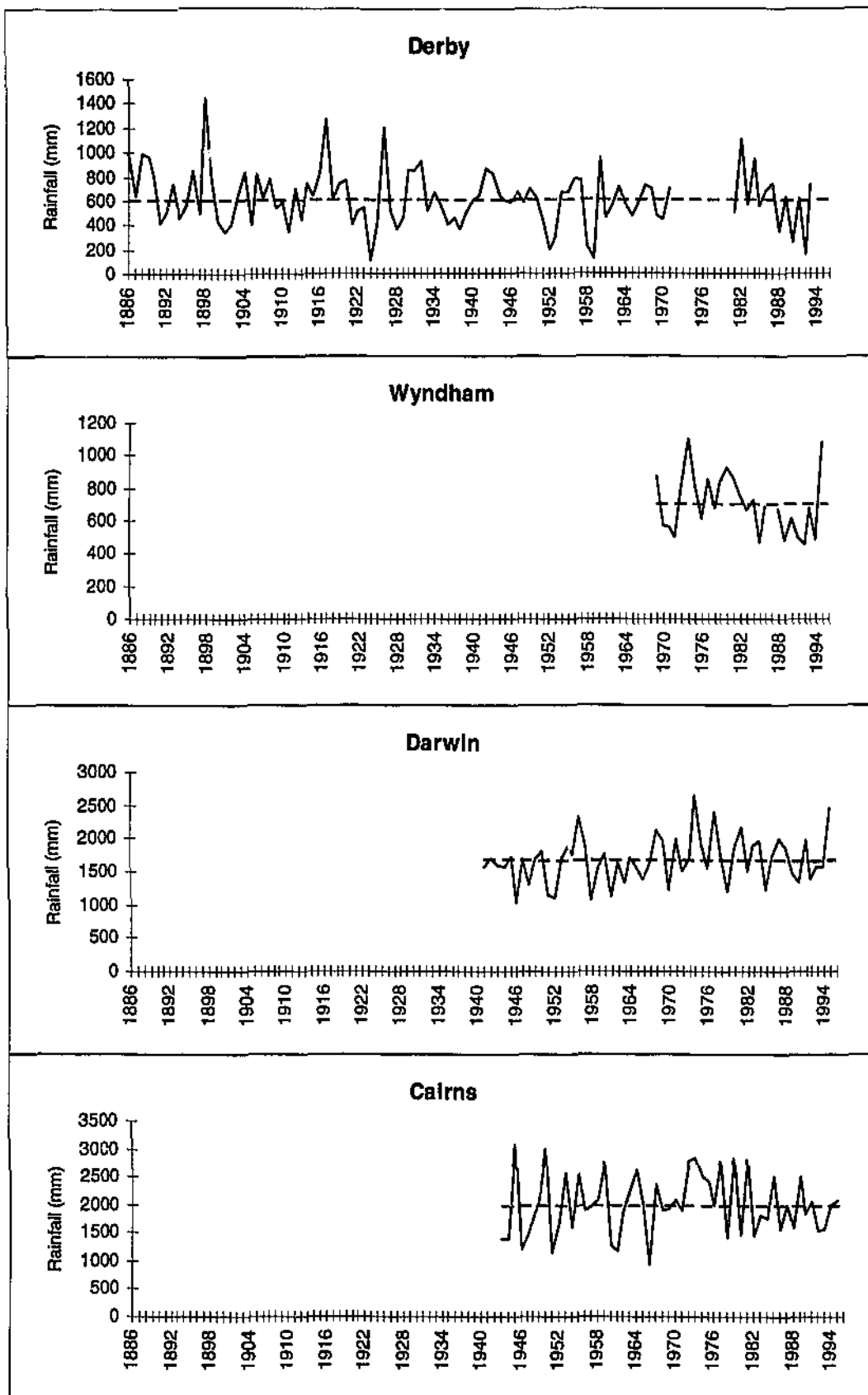


Figure 19. Annual rainfall data showing mean (- - -) at each sampling location. Breaks in annual rainfall indicate no data recorded at that station.

contemporary populations. This is inferred by insignificant population subdivision and estimated gene flow between the Ord and Fitzroy Rivers ($Nm=2.7$), which is sufficient to prevent population divergence by genetic drift under an island model of population structure. This change in population structure indicates a population genetic boundary exists between the Kimberley and the wider barramundi population dynamics occurring east of this region, and follows Keenan's (1994) finding of at least two population boundaries between the Ord River and Darwin ($\sim 850\text{km}$); its exact location is presently unknown. An east-trending increase in rainfall and numbers of larger rivers results in a distinct environmental cline across northern Australia, with mean annual rainfall in the Kimberley (Derby $\sim 600\text{mm}$ & Wyndham $\sim 700\text{mm}$) being far less than Darwin ($\sim 1700\text{mm}$) or Cairns ($\sim 2000\text{mm}$) (Figure 19), and corresponds to the genetic boundary. Keenan also recognized that Queensland barramundi population boundaries (500-600km) extend for much greater distances than in the Northern Territory, and this study indicates that the Kimberley population boundary is wider still at approximately 3,500km. The interplay of shifting seasonal habitat, selective pressures and long-distance gene flow in the Kimberley can only be better interpreted by knowing the geography of absolute survival values (Rockwell & Barrowclough, 1987) and more genetic information, however we cannot ignore the genetic separation of this population from adjacent populations occupying habitats that are at least seasonally, more widespread.

The geographic classification of the Kimberley in the Timor Sea Drainage Division for biological and management practicalities (Thorn, 1995) has direct applications for barramundi management in the sense that the Kimberley drainage basin coincides with a discrete population genetic province. Given that intraspecific population genetic differentiation is a criterion for the assessment of translocation proposals in Western Australia (see Thorn, 1995; Environmental

Protection Authority & Fisheries Department, in review), then appropriate management policy would embrace the biogeographical affinities (*sensu* Horwitz, 1997) of this barramundi population from a regional Kimberley perspective, rather than a continental approach. A similar policy has recently been endorsed in Queensland, where the Fisheries Management Authority has ceased the translocation of barramundi across population genetic boundaries (C. Keenan, personal communication, March 10, 1997).

5.1.8 Directions for Kimberley aquaculture and its implications for recreational fishery enhancement programs and conservation

Because Kimberley aquaculture and recreational fishery enhancement programs will occur in the same riverine environment that is inhabited by wild barramundi, albeit separated by dam walls, then their genetic management implications cannot be discussed independently. Furthermore, these implications should be discussed in the context of the management objective for the translocation of live aquatic species in Western Australia: "...to minimize the risk [of a proposed translocation] to...local aquatic environment with particular reference to maintenance of genetic and biodiversity" (Environmental Protection Authority & Fisheries Department, in review, p. 3).

The aquaculturalist is typically interested in the selection and mass production of traits for fast or optimal growth (Davidson et al. 1989; James, 1992). In the absence of a link between genetic markers and quantitative traits (see Storfer, 1996), then it is desirable that a large population of fish that yields the greatest potential for biological and genetic diversity is surveyed (Purdom, 1992). Although low levels of genetic diversity do not necessarily equate to reduced population fitness and viability (e.g. Caro & Laurenson, 1994; but see Altukhov &

Selmenkova, 1987; Ryman & Utter, 1987), the comparative differences between barramundi populations would indicate that Kimberley aquaculturalists would be better served by harvesting the existing genetic diversity found in the local river systems upon which these ventures are planned. Furthermore, no free polymorphic sites in Cairns or Darwin barramundi indicates a low potential for the genetic improvement of desirable production traits when compared to Kimberley fish. Against this opinion, is the argument that gene flow from exogenous individuals will increase genetic diversity (Moav et al. 1978; Kapuscinski & Lannan, 1984, 1986). Considering Cairns and Darwin fish are represented by one and four genotypes respectively, then it is difficult to detect a productive benefit arising from their hybridization with Kimberley fish. Of course, all of this is providing that the transplanted fish do in fact survive, or that hybridization has not caused the breakup of coadapted gene complexes that might affect the viability of the venture (Sherwin, 1992) and the wild fishery (Nelson & Soulé, 1987).

The implication to hatchery proprietors that larger broodstock populations should yield greater genetic diversity is possibly ill-founded, even when Kimberley fish are used to alleviate the potential problems arising from low genetic diversity. If broodstock are not changed, irrespective of their performance, then the effect of inbreeding depression has been shown by Tave (1993) to be sufficiently large to counteract the effects of mass selective breeding. Similar problems occur when only small founding population sizes are used (Ferguson et al. 1991). The number of samples taken from Darwin and the Kimberley are similar to broodstock sizes in a typical barramundi hatchery (Keenan, 1995), and while not statistically significant, the differences in genetic diversity between those samples demonstrate the need for producers to understand the relationship between genetic diversity and both the size and origin of the founding population.

The genetic diversity available to aquaculture by harvesting wild populations requires effective management of the wild fishery. The continued decline in wild barramundi populations (Davis, 1982; Shaklee & Salini, 1983; Russell & Rimmer, in press) possibly reflects both the demand by the various user groups for a finite resource and current management practices; the annual harvest of Kimberley barramundi is about 10% of the annual quota in Queensland and the Northern Territory. The low levels of genetic diversity in those samples might represent the low diversity available in wild stocks due to over-harvest (e.g. Ricker, 1972), or the effects of hatchery practices due to small effective population sizes and broodstock retention. Either scenario has clear implications for fishery enhancement programs in the Kimberley where hatchery stocks are used, and for state fishery policy to achieve management objectives for the maintenance of genetic diversity in the wild fishery.

The perception that aquaculture will offset the depletion of wild stocks (Nel, 1996) is flawed if the populations that are used to maximize genetic diversity are genetically impoverished in the first instance. For example, the release of many thousands of hatchery barramundi in the Cairns region over the past few years is aimed at enhancing the local recreational fishery (Russell & Rimmer, in press), and yet a sample of Cairns fish presumed to be the F₁ of wild stocks, show no genetic diversity in the mitochondrial region surveyed. In their study of Australian bluefish (*Pomatomus saltatrix*), Graves, Beardley, McDowell & Scoles (1992) sampled 18 hatchery fry that were believed to be the F₁ of wild fish, and found a single mtDNA genotype which they explained as a "result of a smaller effective population size", or "reflecting a longer period of population isolation", inferring population bottlenecks. The hybridization of these fish with other local fish is probably inevitable, and the effects of introgression would be expected to result in a loss of adaptive fitness in wild populations (e.g. Sattaur, 1989; Hynes et al.

1981; Allendorf & Ryman, 1987; Williamson & Carmichael, 1990; Philipp, 1991; Waples, 1991; Frankham, 1994 and others). If genetic variation within discrete populations is to provide a theme for the sustainability of aquacultural industries and serve as the basis for stock enhancement programs, then the goals of aquaculture and the release of populations into the wild must be clearly defined. If not, these objectives [and the management objective of maintaining genetic diversity] cannot be met by the one program (Dixon, 1990), and has little conservation value.

Escaped barramundi presently constitute a part of the non-target catch for the Lake Argyle catfish (*Arius* sp.) industry (N. Stewart, proprietor, personal communication, October 24, 1996), and reports of barramundi [presumably escapees] being caught in rivers to the south of Lake Argyle and in Spillway Creek (Figure 3) (S. Goodson, EKRFAC, personal communication, March 7, 1997) demonstrates that barramundi will leave Lake Argyle, and it appears that Lake Kununurra is being stocked with fish, albeit by default. A study where the mtDNA genotypes of barramundi found in Spillway Creek were compared to the Darwin and Ord River mtDNAs herein (Table 1) should confirm the origin of those fish. The movement of barramundi into the greater Ord River catchment is probably an acceptable, minimum impact method of recreational fishery enhancement of a translocated species (see Prokop, 1995), however the escapement of fish into Lake Kununurra and probably the lower Ord River (Bird, 1992) suggests the hybridization of genetically differentiated barramundi is imminent. This contrasts the requirement for mechanical barriers to prevent escapement (Thorn, 1995), and indicates the inadequacy of dam walls as such. But furthermore, the potential for introgression and genetic pollution (*sensu* Sattaur, 1989) of the Ord River barramundi population is imminent, and contravenes the management objective.

For the translocation of genetically differentiated barramundi to have no impact upon local stocks, then land-based farm production is desirable, but might be perceived as an unacceptable alternative. The desire to produce hatchery fish that are genetically and ecologically similar to the local population is a task confronting scientists and managers of hatchery and wild populations. Doyle et al. (1991) proposed that breeding programs designed to increase genetic diversity by producing breeds specially adapted to local environments would be an attractive conservation strategy, however such programs require natural-type hatchery environments for selective processes to be evaluated (e.g. Frankham, 1994). This situation is unlikely in fledgling aquaculture industries where short-term production success is vital to ensure economic viability. Clearly, assistance by government and industry is required if the goals of sustainable aquaculture and the conservation and maintenance of genetic diversity are to achieve a similar endpoint.

5.1.9 Conclusion

The results of this study indicate that barramundi in each of the Kimberley, Darwin and Cairns regions are readily identifiable as genetically differentiated stocks, and should be managed as such. A decision to ban the translocation of barramundi requires more than the identification of genetic markers that characterize gene frequencies and patterns of diversity, because the markers themselves are a result of the demographic properties that influence them (Milligan et al. 1994; Holsinger, 1996). Whilst sustainable barramundi aquaculture in the Kimberley should look toward the propagation of native stocks, demographic and ecological information for Kimberley barramundi is required, as this will enable the clarification of local selection pressures and genetic differentiation (Holsinger, 1996; Storfer, 1996).

If managers wish to adopt either the island or stepping-stone models to draw the analogy between the translocation of barramundi between genetically subdivided populations, and their migration [i.e. gene flow] among those populations without human agency, then Table 7 will serve as an example: If we consider $N_e=250$, then in an island model, 0.24 individuals or .09% of the population can be translocated per year [i.e. a generation]. The same estimate of N_e under the stepping-stone model allows 13 individuals or 5.2% of the population to be moved per year, whilst maintaining highly significant population subdivision. Clearly, the island model would be unsatisfactory to the proponent of the translocation, as it effectively stops the movement of barramundi, however the stepping-stone model would theoretically allow sufficient barramundi to found a hatchery. If that were the case and these fish were translocated from hatcheries in Darwin or Cairns, then we might expect comparable levels of genetic diversity as found here, and the potential problems associated with this have been previously outlined. These problems then contravene the management objective to maintain genetic and biodiversity, but that is the context that the translocation management of barramundi must be framed.

Managers should accept that genetic results concerning population differentiation and estimates of population size and gene flow between populations must be interpreted by models. For example, the island model is the simplest, yet it makes the most restrictive assumptions that constrain equal effective population sizes and results in the smallest level of genetic differentiation (i.e. N_e) for a specific level of dispersal, whereas stepping-stone models handle more complicated assumptions including variation in N_e , to yield higher dispersal (Taylor & Dizon, 1996). Neither model considers variable dispersal over time, but effective management would ideally allow for temporal variation in population demographics. The phylogenetic analysis provided herein provides some indication of how population dynamics might change over time.

Given the inherent problems associated with population genetic models, and an absence of ecological data, managers would do better to acknowledge frequency-based estimates of population differentiation which clearly show that the population genetic structure of Kimberley barramundi is vastly different to Darwin or Cairns fish. This restriction then shifts the burden of proof from the investigator, for which statistically significant levels of population subdivision have been inferred, to the proponent of a translocation proposal. That is, the proponent must demonstrate that there will be no significant change to the genetic structure as a result of the proposal, and that the management objectives will be maintained (see Horwitz, 1995 and Taylor & Dizon, 1996). If that cannot be shown, then a precautionary approach to the translocation of barramundi genetic material which dictates that disturbance might lead to loss or damage should be followed (*sensu* Horwitz, 1995). In the absence of such critical information, the translocation should not proceed.

If the genetic differentiation and diversity of the Kimberley barramundi is to be preserved, then a reactive government should reflect the desires of a community that understands the cost of management under increasing pressure for a finite resource (*sensu* Wardell-Johnson & Horwitz, 1996). This requires consideration for the capacity of future generations to experience environmental values which go beyond a perception of the Kimberley barramundi as quarry to which anglers have a "right" of access (Stagles, 1995), or as economic chattels to be moved around the landscape.

Chapter 6

6.1 References

- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., & Watson, J.D. (1989). *Molecular biology of the cell* (2nd Ed.). U.S.A.: Garland
- Allendorf, F.W., & Phelps, S.R. (1980). Loss of genetic variation in a hatchery stock of cutthroat trout. *Transactions of the American Fisheries Society*, 109, 537-543.
- Allendorf, F.W., & Phelps, S.R. (1981). Use of Allelic Frequencies to Describe Population Structure. *Canadian Journal of Fisheries and Aquatic Sciences*, 38, 1507-1514.
- Allendorf, F.W., & Ryman, N. (1987). Genetic management of hatchery stocks. In N. Ryman & F.M. Utter (Eds.), *Population Genetics & Fishery Management* (pp. 141-159). U.S.A.: University of Washington Press.
- Allendorf, F.W., & Leary, R.F. (1988). Conservation and distribution of genetic variation in a polytypic species, the cutthroat trout. *Conservation Biology*, 2, 170-184.
- Altukhov, Y.P. (1981). The Stock Concept from the Viewpoint of Population Genetics. *Canadian Journal of Fisheries and Aquatic Sciences*, 38, 1523-1538.
- Altukhov, Y.P., (1990). *Population Genetics: Diversity and Stability*. United Kingdom: Harwood Academic Publishers.
- Altukhov, Y.P., & Salmenkova, E.A. (1987). Stock Transfer Relative to Natural Organization, Management, and Conservation of Fish Populations. In N. Ryman & F.M. Utter (Eds.), *Population Genetics & Fishery Management* (pp. 333-343). U.S.A.: University of Washington Press.
- Anderson, I., Barlow, C., Fielder, S., Hallam, D., Heasman, M., & Rimmer, M. (1993). Occurrence of the picorna-like virus infecting barramundi. *Austasia Aquaculture*, 7, (2), 42-44.
- Aplin, K.P., Baverstock, P.R., & Donnellan, S.C. (1993). Albumin Immunological Evidence for the Time and Mode of Origin of the New Guinean Terrestrial Mammal Fauna. *Science in New Guinea*, 19, (3), 131-145.
- Aquaculture WA. (1995). *Barramundi Lates calcarifer*. No 9 in a series on aquaculture species. Fisheries Department of Western Australia.
- Aquadro, C.F., & Greenberg, B.D. (1983). Human mitochondrial DNA variation and evolution: analysis of nucleotide sequences from seven individuals. *Genetics*, 103, 287-312.

- Árnason, Ú., & Gullberg, A. (1994). Relationship of baleen whales established by cytochrome *b* gene sequence comparisons. *Nature*, 367, 726-728.
- Arthington, A.H. (1991). Ecological and genetic impacts of introduced and translocated freshwater fishes in Australia. In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. Canadian Journal of Fisheries and Aquatic Sciences, 48 (Suppl. 1), 33-43.
- Attardi, G. (1985). Animal Mitochondrial DNA: An Extreme Example of Genetic Economy. *International Review of Cytology*, 93, 93-145.
- Australian Fisheries Statistics. (1995). Australian Bureau of Agricultural and Resource Economics.
- Australian Water Resources Council. (1976). *Review of Australia's Water Resources*. Canberra: Australian Government Publishing Service.
- Avise, J.C. (1992). Molecular population structure and the biogeographic history of a regional fauna: a case history with lessons for conservation biology. *Oikos*, 63, 62-76.
- Avise, J.C. (1994). *Molecular Markers, Natural History and Evolution*. New York: Chapman & Hall.
- Avise, J.C., Arnold, J., Ball, R.M., Bermingham, E., Lamb, T., Neigel, J.E., Reeb, C.A., & Saunders, N.C. (1987). Intraspecific Phylogeography: The Mitochondrial DNA Bridge Between Population Genetics and Systematics. *Annual Review of Ecology and Systematics*, 18, 489-522.
- Awang, A.B. (1987). Sea bass (*Lates calcarifer*) larvae and fry production in Malaysia. In J.W. Copland & D.L. Grey (Eds.), *Management of Wild and Cultured Sea Bass/Barramundi (Lates calcarifer). Proceedings of an international workshop held in Darwin, Australia, 24-30 September 1986. ACIAR Proceedings No. 20* (pp. 144-147). Canberra: Australian Centre for International Agricultural Research.
- Ayala, F.J., & Campbell, C.A. (1974). Frequency-dependent selection. *Annual Review of Ecology and Systematics*, 5, 115-138.
- Baby Barra Boost. (1995). *Western Fisheries*, September, 22.
- Barker, J.S.F. (1992). The analysis of genetic structure of populations. In P.I. Dixon (Ed.), *Population Genetics and its Applications to Fisheries Management and Aquaculture. Proceedings of a workshop held at the University of New South Wales, Kensington, on 25-27 June, 1990.* (pp.77-97). New South Wales: Centre for Marine Science, University of New south Wales.
- Barker, J.S.F., & Thomas, R.H. (1987). A quantitative genetic perspective on adaptive evolution. In V. Loeschcke (Ed.), *Genetic Constraints on Adaptive Evolution* (pp. 3-23). Berlin: Springer.

- Barlow, C.G., & Rodgers, L.J. (1990). The Proposal To Introduce Nile Perch To Australia. In D.A. Pollard (Ed.), *Introduced and Translocated Fishes and their Ecological Effects. Proceedings of the Australian Society for Fish Biology workshop held at Magnetic Island, Townsville, Queensland, 24-25 August 1989. Proceedings No. 8* (pp.108-109). Canberra: Department of Primary Industries and Energy & Bureau of Rural Resources.
- Barrett, S.C.H., & Richardson, B.J. (1986). Genetic attributes of invading species. In R.H. Groves & J.J. Burdon (Eds.), *Ecology of biological invasions: an Australian perspective* (pp. 21-33). Canberra: Australian Academy of Science.
- Bartlett, S.E., & Davidson, W.S. (1991). Identification of *Thunnus* tuna species by the polymerase chain reaction and direct sequence analysis of their mitochondrial cytochrome *b* genes. *Canadian Journal of Fisheries and Aquatic Sciences*, 48, 309-317.
- Bartlett, S.E., & Davidson, W.S. (1992). FINS (forensically informative nucleotide sequencing): a procedure for identifying the animal origin of biological specimens. *Biotechniques*, 12, 408-411.
- Barton, N.H., & Wilson, I. (1995). Genealogies and geography. *Philosophical Transactions of the Royal Society of London. Biological Sciences*, 349, 49-59.
- Baverstock, P.R., & Moritz, C. (1996). Project Design. In D.M. Hillis, C. Moritz & B.K. Mable (Eds.), *Molecular Systematics* (2nd ed. pp. 17-28). U.S.A.: Sinauer Associates Inc.
- Beckenbach, A.T. (1991). Rapid mtDNA Sequence Analysis of Fish Populations Using the Polymerase Chain Reaction (PCR). In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. *Canadian Journal of Fisheries and Aquatic Sciences*, 48 (Suppl. 1), 95-98.
- Bentzen, P., & Wright, J.M. (1992). Nucleotide sequence and evolutionary conservation of a minisatellite variable number tandem repeat cloned from Atlantic salmon, *Salmo salar*. *Genome*, 36, 271-277.
- Benzie, J.A.H., Frusher, S., & Ballment, E. (1992). Geographical variation in allozyme frequencies of populations of *Penaeus monodon* (Crustacea: Decapoda) in Australia. *Australian Journal of Marine and Freshwater Research*, 43, 715-725.
- Berggren, W.A., Burckle, L.H., Cita, M.B., Cooke, M.B.S., Funnell, B.M., Gartner, S., Hays, J.D., Kennett, J.P., Opdyke, N.D., Pastouret, L., Shackleton, N.J., & Takayanagi, Y. (1980). Towards a Quaternary time scale. *Quaternary Research*, 13, 277-302.
- Bernatchez, L., & Dodson, J.J. (1990). Mitochondrial DNA variation among anadromous populations of cisco (*Coregonus artedii*) as revealed by restriction analysis. *Canadian Journal of Fisheries and Aquatic Sciences*, 47, 533-543.
- Bernatchez, L., Dodson, J.J., & Boivin, S. (1989). Population bottlenecks: influence on mitochondrial DNA diversity and its effect in coregonine stock discrimination. *Journal of Fish Biology*, 35 (Suppl. A), 233-244.

- Bernatchez, L., Guyomard, R., & Bonhomme, F. (1992). DNA sequence variation of the mitochondrial control region among geographically and morphologically remote European brown trout *Salmo trutta* populations. *Molecular Ecology*, 1, 161-173.
- Billington, N., & Hebert, P.D.N. (1988). Mitochondrial DNA Variation in Great Lakes Walleye (*Stizostedion vitreum*) Populations. *Canadian Journal of Fisheries and Aquatic Sciences*, 45, 643-654.
- Billington, N., & Hebert, P.D.N. (1990). Technique for Determining Mitochondrial DNA Markers in Blood Samples from Walleyes. *American Fisheries Society Symposium*, 7, 492-498.
- Billington, N., & Hebert, P.D.N. (1991). Mitochondrial DNA Diversity in Fishes and its Implications for Introductions. In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. *Canadian Journal of Fisheries and Aquatic Sciences*, 48 (Suppl. 1), 80-94.
- Billington, N., Barrette, R.J., & Hebert, P.D.N. (1992). Management Implications of Mitochondrial DNA Variation in Walleye Stocks. *North American Journal of Fisheries Management*, 12, 276-284.
- Billington, N. & Strange, R.M. (1995). Mitochondrial DNA Analysis Confirms the Existence of a Genetically Divergent Walleye Population in Northeastern Mississippi. *Transactions of the American Fisheries Society*, 124, 770-776.
- Bird, C. (1992). *Trial barramundi trapping and tagging program on the Ord River*. Unpublished report to the Bernard Bowen Fisheries Research Institute, Western Australia. Western Australia: Marine Research Laboratories.
- Bittles, A.H. (1989). The role of mitochondria in cellular ageing. In A.M. Warnes (Ed.), *Human ageing and later life* (pp. 29-37). London: Edward Arnold.
- Bittles, A.H. (1992). Evidence for and against the causal involvement of mitochondrial DNA mutation in mammalian ageing. *Mutation Research*, 275, 217-225.
- Bloom, A.L., Broeker, W.S., Chappell, J., Matthews, R.K., & Mesolella, K.J. (1974). Quaternary sea level fluctuations on a tectonic coast: new Th²³⁰ / U²³⁴ dates from New Guinea. *Quaternary Research*, 4, 185-205.
- Brown, W.M. (1983). Evolution of genes and proteins. In M. Nei & R.K. Koehn (Eds), *Evolution of genes and proteins* (pp. 62-88). U.S.A.: Sinauer Associates Inc.
- Brown, W.M. (1985). The mitochondrial genome of animals. In R.H. MacIntyre (Ed.), *Molecular evolutionary genetics* (pp. 95-130). New York: Plenum.
- Brown, W.M., George, M.Jr., & Wilson, A.C. (1979). Rapid evolution of animal mitochondrial DNA. *Proceedings of the National Academy of Sciences of the USA*, 76, (4), 1967-1971.
- Buroker, N.E., Brown, J.R., & Gilbert, T.A. (1990). Length heteroplasmy of sturgeon mitochondrial DNA: an illegitimate elongation model. *Genetics*, 124, 157-163.

- Cadwallader, P. & Kerby, B. (Eds.). (1995). Fish Stocking in Queensland: Getting it Right! *Proceedings of the Symposium held in Townsville, Queensland, 11 November 1995* Fortitude Valley: Queensland Fisheries Management Authority.
- Campton, D.E. (1987). Natural Hybridization And Introgression in Fishes: Methods of Detection and Genetic Interpretations. In N. Ryman & F.M. Utter (Eds.), *Population Genetics & Fishery Management* (pp.161-192). U.S.A.: University of Washington Press.
- Caro, T.M., & Laurenson, M.K. (1994). Ecological and Genetic Factors in Conservation: A Cautionary Tale. *Science*, 263, 485-486.
- Carr, S.M., & Marshall, D. (1991). DNA sequence variation in the mitochondrial cytochrome b gene of Atlantic cod (*Gadus morhua*) detected by the polymerase chain reaction. *Canadian Journal of Fisheries and Aquatic Sciences*, 48, 48-52.
- Castelli, M., Philippart, J-C., Vassart, G., & Georges, M. (1990). DNA Fingerprinting in Fish: A New Generation of Genetic Markers. *American Fisheries Society Symposium*, 7, 514-520.
- Chakraborty, R., & Leimar, O. (1987). Genetic variation within a subdivided population. In N. Ryman & F.M. Utter (Eds.), *Population Genetics & Fishery Management* (pp. 89-120). U.S.A.: University of Washington Press.
- Chakraborty, R., & Danker-Hopfe, H. (1991). Analysis of Population Structure: A Comparative Study of Different Estimators of Wright's Fixation Indices. In C.R. Rao & R. Chakraborty (Eds.), *Handbook of Statistics* (Vol. 8. pp.203-254). Netherlands: Elsevier Science Publishers.
- Chapman, R.W., & Brown, B.L. (1991). Mitochondrial DNA Isolation Methods. In D.H. Whitmore (Ed.), *Electrophoretic and Isoelectric Focusing Techniques in Fisheries Management* (pp. 107-129). U.S.A.: CRC Press.
- Chappell, J. (1974). Geology of coral terrains, Huon Peninsula, New Guinea: a study of Quaternary tectonic movements and sea level changes. *Geological Society of America Bulletin*, 85, 553-570.
- Chappell, J. (1983). A revised sea-level record for the last 300,000 years from Papua New Guinea. *Search*, 14, (3-4), 99-101.
- Chappell, J., & Shackleton, N.J. (1986). Oxygen isotopes and sea level. *Nature*, 27, 137-140.
- Clayton, D.A. (1991). Replication and transcription of vertebrate mitochondrial DNA. *Annual Reviews in Cell Biology*, 7, 453-478.
- Cockerham, C.C., & Weir, B.S. (1993). Estimation of gene flow by *F*-statistics. *Evolution*, 47, (3), 855-863.

- Collins, L.B., Wyrwoli, K.H., & France, R.E. (1991). The Abrolhos carbonate platforms: geological evolution and Leeuwin Current activity. In A.F. Pearce & D.I. Walker (Eds.), *The Leeuwin Current: an influence on the coastal climate and marine life of Western Australia. Proceedings of a Symposium of the Royal Society of Western Australia and the Western Australian Branch of the Australian Marine Sciences Association, held at CSIRO Laboratories, Perth, Western Australia, 16 March 1991* (pp. 47-57).
Journal of the Royal Society of Western Australia. Volume 74, 1991.
- Constantine, C.C., Hobbs, R.C., & Lymbery, A.J. (1994). Fortran program for analyzing population structure from multilocus genotypic data. *Journal of Heredity*, 85, (4), 336-337.
- Crosetti, D., Nelson, W.S., & Avise, J.C. (1994). Pronounced genetic structure of mitochondrial DNA among populations of the circumglobally distributed grey mullet (*Mugil cephalus*). *Journal of Fish Biology*, 44, 47-58.
- Danzmann, R.G., Ferguson, M.M., & Arndt, S.K.A. (1993). Mitochondrial DNA variability in Ontario and New York rainbow trout (*Oncorhynchus mykiss*).
Canadian Journal of Zoology, 71, 1923-1933.
- Darley-Usmar, V., & Schapira, A.H.V. (Eds.).(1994). *Mitochondria: DNA, Proteins and Disease*. U.S.A.: Portland Press Research Monograph V.
- Darley-Usmar, V., Ragan, I., Smith, P., & Wilson, M. (1994). The proteins of the mitochondrial inner membrane and their role in oxidative phosphorylation. In V. Darley-Usmar & A.H.V. Schapira (Eds.), *Mitochondria: DNA, Proteins and Disease* (pp. 1-25).
U.S.A.: Portland Press Research Monograph V.
- Davidson, W.S., Birt, T.P., & Green, J.M. (1989). A review of genetic variation in Atlantic salmon, *Salmo salar* L., and its importance for stock identification, enhancement programmes and aquaculture. *Journal of Fish Biology*, 34, 547-560.
- Davis, T.L.O. (1982). Maturity and sexuality in barramundi, *Lates calcarifer* (Bloch), in the Northern Territory and South-eastern Gulf of Carpentaria.
Australian Journal of Marine and Freshwater Research, 33, 529-545.
- Davis, T.L.O. (1984a). Estimation of fecundity in barramundi, *Lates calcarifer* (Bloch), using an automatic particle counter.
Australian Journal of Marine and Freshwater Research, 35, 111-118.
- Davis, T.L.O. (1984b). A population of sexually precocious barramundi, *Lates calcarifer*, in the Gulf of Carpentaria, Australia. *Copeia*, 1984, 144-149.
- Davis, T.L.O. (1985). Sexual Changes and Gonad Maturity, and Abundance of Larvae and Early Juveniles of Barramundi, *Lates calcarifer* (Bloch), in Van Diemen Gulf and the Gulf of Carpentaria. *Australian Journal of Marine and Freshwater Research*, 36, 177-190.
- Davis, T.L.O. (1986). Migration Patterns in Barramundi, *Lates calcarifer* (Bloch), in Van Diemen Gulf, Australia, with Estimates of Fishing Mortality in Specific Areas.
Fisheries Research, 4, 243-258.

- Davis, T.L.O. (1987). Biology of wildstock *Lates calcarifer* in northern Australia. In J.W. Copland & D.L. Grey (Eds.), *Management of Wild and Cultured Sea Bass/Barramundi (Lates calcarifer). Proceedings of an international workshop held in Darwin, Australia, 24-30 September 1986. ACIAR Proceedings No. 20* (pp. 22-29). Canberra: Australian Centre for International Agricultural Research.
- Dawkins, R. (1978). *The Selfish Gene*. Sydney: Granada Publishing Limited.
- Dawkins, R. (1983). *The Extended Phenotype*. Great Britain: Oxford University Press.
- Department of Commerce and Trade & Department of Resources Development. (1993). *Report Of The Kimberley Water Resources Development Advisory Board*. Perth: Department of Commerce and Trade & Department of Resources Development.
- DeSalle, R., Freedman, T., & Prager, E.M. (1987). Tempo and mode of sequence evolution in mitochondrial DNA of Hawaiian *Drosophila*. *Journal of Molecular Evolution*, 26, 157-164.
- Dessauer, H.C., Cole, C.J., & Hafner, M.S. (1996). Collection and Storage of Tissues. In D.M. Hillis, C. Moritz & B.K. Mable (Eds.), *Molecular Systematics* (2nd ed. pp. 29-50). U.S.A.: Sinauer Associates Inc.
- Dixon, P. (1990). Hatchery releases-Take care. In D.A. Pollard (Ed.), *Introduced and Translocated Fishes and their Ecological Effects. Proceedings of the Australian Society for Fish Biology workshop held at Magnetic Island, Townsville, Queensland, 24-25 August 1989. Proceedings No. 8* (p.151). Canberra: Department of Primary Industries and Energy & Bureau of Rural Resources.
- Dixon, P.I. (Ed.).(1992). *Population Genetics and its Applications to Fisheries Management and Aquaculture. Proceedings of a workshop held at the University of New South Wales, Kensington, on 25-27 June, 1990*. New South Wales: Centre for Marine Science, University of New south Wales.
- Dizon, A.E., Lockyer, C., & Perrin, W.F. (1992). Rethinking the stock concept: a phylogenetic approach. *Conservation Biology*, 6, 24-36.
- Dobzhansky, T. (1955). A review of some fundamental concepts and problems of population genetics. *Cold Spring Harbour Symposium on Quantitative Biology No. 20* (pp. 1-15).
- Doupé, R.G., & Chandler, D. (Manuscript submitted). Minimally invasive tissue sampling of fishes revisited. *Marine and Freshwater Research*.
- Dowling, T.E., & Brown, W.M. (1993). Population structure of the bottlenose dolphin (*Tursiops truncatus*) as determined by restriction endonuclease analysis of mitochondrial DNA. *Marine Mammal Science*, 9, 138-155.
- Dowling, T.E., Moritz, C., Palmer, J.D., & Rieseberg, L.H. (1996). Nucleic Acids 111: Analysis of Fragments and Restriction Sites. In D.M. Hillis, C. Moritz & B.K. Mable (Eds.), *Molecular Systematics* (2nd ed. pp. 249-320). U.S.A.: Sinauer Associates Inc.

- Doyle, R.W., Shackel, N.L., Basiao, Z., Matricia, T., & Talbot, A.J. (1991). Selective Diversification of Aquaculture Stocks: A Proposal for Economically Sustainable Genetic Conservation. In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. Canadian Journal of Fisheries and Aquatic Sciences, 48 (Suppl. 1), 148-154.
- Dunstan, D.J. (1959). The Barramundi *Lates calcarifer* (Bloch) in Queensland waters. *CSIRO Division of Fisheries & Oceanography, Technical Paper No.5*, 1-22.
- Dunstan, D.J. (1962). The barramundi in New Guinea waters. *Papua New Guinea Agriculture Journal*, 15, 23-31.
- Edwards, S.V. (1993). Mitochondrial Gene Genealogy and Gene Flow Among Island and Mainland Populations of a Sedentary Songbird, the Grey-Crowned Babbler (*Pomatostomus temporalis*). *Evolution*, 47, (4), 118-1137.
- Edwards, S.V., & Wilson, A.C. (1990). Phylogenetically Informative Length Polymorphism and Sequence Variability in Mitochondrial DNA of Australian Songbirds (*Pomatostomus*). *Genetics*, 126, 695-711.
- Ellstrand, N.C. (1992). Gene flow by pollen: implications for plant conservation genetics. *Oikos*, 63, 77-86.
- Elton, C.S. (1958). *The ecology of invasions by animals and plants*. London: Chapman & Hall.
- Emlen, J.M. (1991). Heterosis and outbreeding depression: a multi-locus model and an application to salmon production. *Fisheries Research*, 12, 187-212.
- Endler, J.A. (1977). *Geographic variation, speciation and clines*. New Jersey: Princeton University Press.
- Endler, J.A. (1986). *Natural Selection in the Wild. Monographs in Population Biology*. New Jersey: Princeton University Press.
- Environmental Protection Authority & Fisheries Department. (in review). *Translocation of Live Aquatic Non-Endemic Species into or within Western Australia*. Unpublished Memorandum Of Understanding between the Environmental Protection Authority and Fisheries Department.
- Erich, H.A., Gelfand, D., & Sninsky, J.J. (1991). Recent advances in the polymerase chain reaction. *Science*, 252, 1643-1651.
- Esposti Degli, M., De Vries, S., Crimi, M., Ghelli, A., Patarnello, T., & Meyer, A. (1993). Mitochondrial cytochrome *b*; evolution and structure of the protein. *Biochimica et Biophysica Acta*, 1143, 243-271.

- Evans, D.O., & Willox, C.C. (1991). Loss of exploited, indigenous populations of lake trout, *Salvelinus namaycush*, by stocking of non-native stocks. In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. Canadian Journal of Fisheries and Aquatic Sciences, 48 (Suppl. 1), 134-147.
- Excoffier, L., Smouse, P.E., & Quattro, J.M. (1992). Analysis of Molecular Variance Inferred From Metric Distances Among DNA Haplotypes: Application to Human Mitochondrial DNA Restriction Data. *Genetics*, 131, 479-491.
- Fajen, A., & Breden, F. (1992). Mitochondrial DNA sequence variation among natural populations of the Trinidad guppy, *Poecilia reticulata*. *Evolution*, 46, 1457-1465.
- Falconer, D.S. (1981). *Introduction to Quantitative Genetics* (2nd Ed.). London: Longman.
- Felsenstein, J. (1983). Parsimony in systematics: biological and statistical issues. *Annual Reviews in Ecology and Systematics*, 14, 313-333.
- Felsenstein, J. (1988). Phylogenies from molecular sequences: Inference and reliability. *Annual Review of Genetics*, 22, 521-565.
- Felsenstein, J. (1993). *PHYLIP (Phylogeny Inference Package)*, version 3.5c. Seattle: Department of Genetics, University of Washington.
- Ferguson, M.M. (1990). The genetic impact of introduced fishes on native species. *Canadian Journal of Zoology*, 68, 1053-1057.
- Ferguson, M.M., & Drahuschak, L.R. (1990). Enzyme heterozygosity and disease resistance in rainbow trout. *Heredity*, 64, 413-417.
- Ferguson, M.M., Ihssen, P.E., & Hynes, J.D. (1991). Are cultured stocks of brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) genetically similar to their source populations? In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. Canadian Journal of Fisheries and Aquatic Sciences, 48 (Suppl. 1), 118-123.
- Fernando, C.H. (1991). Impacts of Fish Introductions in Tropical Asia and America. In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. Canadian Journal of Fisheries and Aquatic Sciences, 48 (Suppl. 1), 24-32.
- Ferris, S.D., & Berg, W.J. (1987). The utility of mitochondrial DNA in fish genetics and fishery management. In N. Ryman & F.M. Utter (Eds.), *Population Genetics & Fishery Management* (pp.277-299). U.S.A.: University of Washington Press.
- Fisher, R.A. (1931). *The Genetical Theory of Natural Selection* (1st Ed.). Oxford; Clarendon.
- Fitch, W.M., & Margoliash, E. (1967). Construction of phylogenetic trees. *Science*, 155, 279-284.

- Fitch, W.M. (1971). Toward defining the course of evolution: minimum change for a specified tree topology. *Systematic Zoology*, 20, 406-416.
- Forey, P.L., Humphries, C.J., Kitching, I.J., Scotland, R.W., Siebert, D.J., & Williams, D.M. (Eds.). (1992). Cladistics: A Practical Course in Systematics. *Proceedings of a workshop of the Systematics Association in 1991*. (Publication No. 10). Great Britain: Oxford University Press.
- Frankham, R. (1994). Genetic management of captive populations for reintroduction. In M. Serena (Ed.), *Reintroduction Biology of Australian and New Zealand Fauna* (pp. 31-34). New South Wales: Surrey Beatty & Sons.
- Fumagalli, L., Taberlet, P., Favre, L., & Hausser, J. (1996). Origin and Evolution of Homologous Repeated Sequences in the Mitochondrial DNA Control Region of Shrews. *Molecular Biology and Evolution*, 13, (1), 31-46.
- Futuyama, D.J. (1986). *Evolutionary Biology* (2nd Ed.). U.S.A.: Sinauer Associates Inc.
- Gall, G.A.E. (1987). Inbreeding. In N. Ryman & F.M. Utter (Eds.), *Population Genetics & Fishery Management* (pp. 47-88). U.S.A.: University of Washington Press.
- Galloway, R.W., & Löffler, E. (1974). Aspects of geomorphology and soils in the Torres Strait Region. In D. Walker (Ed.), *Bridge and Barrier: The Natural and Cultural History of Torres Strait* (2nd ed., pp. 11-28). Canberra: Australian National University.
- Gharrett, A.G., & Shirley, S.M. (1985). A genetic examination of spawning methodology in a salmon hatchery. *Aquaculture*, 47, 245-256.
- Ghosh, A. (1973). Observations on the larvae and juveniles of the "bhekti", *Lates calcarifer* (Bloch), from the Hooghly-Matlah estuarine system. *Indian Journal of Fisheries*, 20, 372-379.
- Gile, S.R., & Ferguson, M.M. (1990). Crossing methodology and genotypic diversity in a hatchery strain of rainbow trout (*Oncorhynchus mykiss*). *Canadian Journal of Fisheries and Aquatic Sciences*, 47, 719-724.
- Glazebrook, J.S. & Campbell, R.S.F. (1987). Diseases of barramundi (*Lates calcarifer*) in Australia: a review. In J.W. Copland & D.L. Grey (Eds.), *Management of Wild and Cultured Sea Bass/ Barramundi (Lates calcarifer)*. *Proceedings of an international workshop held in Darwin, Australia, 24-30 September 1986*. *ACIAR Proceedings No. 20* (pp. 204-206). Canberra: Australian Centre for International Agricultural Research.
- Glazebrook, J.S., Heasman, M.P., & De Beer, S.W. (1990). Picorna-like viral particles associated with mass mortalities in larval barramundi, *Lates calcarifer* Bloch. *Journal of Fish Diseases*, 13, 245-249.
- Goldstein, D.B., & Holsinger, K.E. (1992). Maintenance of polygenic variation in spatially structured populations: roles for local mating and genetic redundancy. *Evolution*, 46, 412-429.

- Graves, J.E., Beardley, A.M., McDowell, J.R., & Scoles, D.R. (1992). Stock structure in the bluefish, *Pomatomus saltatrix*, along the mid-Atlantic coast. *Fishery Bulletin*, 90, 703-710.
- Gray, M.W. (1989). Origin and evolution of mitochondrial DNA. *Annual Review of Cell Biology*, 5, 25-50.
- Greenwood, P.H. (1976). A review of the family Centropomidae (Pisces, Perciformes). *Bulletin of the British Museum (Natural History) Zoology*, 29, 1-81.
- Grewe, P.M., Krueger, C.C., Aquadro, C.F., Bermingham, E., Kincaid, H.L., & May, B. (1993). Mitochondrial DNA Variation among Lake Trout (*Salvelinus namaycush*) Strains Stocked into Lake Ontario. *Canadian Journal of Fisheries and Aquatic Sciences*, 50, 2397-2403.
- Griffin, R.K. (1979). Recreational barramundi fishing-how important is it to the Northern Territory? *Australian Fisheries*, 38, (4), 25-26.
- Gyllensten, U., & Wilson, A.C. (1987). Mitochondrial DNA of salmonids. In N. Ryman & F. Utter (Eds.), *Population Genetics and Fishery Management* (pp. 301-317). U.S.A.: University of Washington Press.
- Gyllensten, U., Wharton, D., & Josefsson, A. (1991). Paternal inheritance of mitochondrial DNA in mice. *Nature*, 352, 255-257.
- Hall, H.J., & Nawrocki, L.W. (1995). A rapid method for detecting mitochondrial DNA variation in brown trout, *Salmo trutta*. *Journal of Fish Biology*, 46, 360-364.
- Hallerman, E.M., & Beckmann, J.S. (1988). DNA-Level Polymorphism as a Tool in Fisheries Science. *Canadian Journal of Fisheries and Aquatic Sciences*, 45, 1075-1087.
- Hansen, M.M., & Loeschcke, V. (1996). Genetic differentiation among Danish brown trout populations, as detected by RFLP analysis of PCR amplified mitochondrial DNA segments. *Journal of Fish Biology*, 48, 422-436.
- Hartl, D.L., & Clark, A.G. (1989). *Principles of Population Genetics*. (2nd ed.). U.S.A.: Sinauer Associates Inc.
- Hayashi, J-I., Tagashira, Y., & Yoshida, M.C. (1985). Absence of extensive recombination between inter- and intraspecies mitochondrial DNA in mammalian cells. *Experimental Cell Research*, 160, 387-395.
- Hayashi, J-I., Ohta, S., Kagawa, Y., Kondo, H., Kaneda, H., & Yonekawa, H. (1994). Nuclear but not mitochondrial genome involvement in human age-related mitochondrial dysfunction. *Journal of Biological Chemistry*, 269, 6878-6883.
- Heath, D.D., Bernier, N.J., & Mousseau, T.A. (1995). A single-locus minisatellite discriminates chinook salmon (*Oncorhynchus tshawytscha*) populations. *Molecular Ecology*, 4, 389-393.

- Hellberg, M.E. (1994). Relationships between inferred levels of gene flow and geographic distance in a philopatric coral, *Balanophylla elegans*. *Evolution*, 48, (6), 1829-1854.
- Hillis, D.M., & Dixon, M.T. (1991). Ribosomal DNA: molecular evolution and phylogenetic inference. *Quarterly Review of Biology*, 66, 411-453.
- Hillis, D.M., Mable, B.K., & Moritz, C. (1996). Applications of Molecular Systematics: The State of the Field and a Look to the Future. In D.M. Hillis, C. Moritz & B.K. Mable (Eds.), *Molecular Systematics* (2nd ed. pp. 515-543). U.S.A.: Sinauer Associates Inc.
- Hillis, D.M., Moritz, C., & Mable, B. (Eds.).(1996). *Molecular Systematics* (2nd ed.). U.S.A.: Sinauer Associates Inc.
- Hillis, D.M., Mable, B.K., Larson, A., Davis, S.K., & Zimmer, E.A. (1996). Nucleic Acids IV: Sequencing and Cloning. In D.M. Hillis, C. Moritz & B.K. Mable (Eds.), *Molecular Systematics* (2nd ed. pp. 321-381). U.S.A.: Sinauer Associates Inc.
- Holmquist, R. (1983). Transitions and transversion in evolutionary descent: an approach to understanding. *Journal of Molecular Evolution*, 19, 134-144.
- Holsinger, K.E. (1996). The scope and limits of conservation genetics. *Evolution*, 50, (6), 2558-2561.
- Holt, I.J., & Jacobs, H.T. (1994). The structure and expression of normal and mutant mitochondrial genomes. In V. Darley-Usmar & A.H.V. Schapira (Eds.), *Mitochondria: DNA, Proteins and Disease* (pp. 27-54). U.S.A.: Portland Press Research Monograph V.
- Horwitz, P. (1995). Recreational Fishing and Translocated Aquatic Species: Conservation Imperatives and Long-Lasting Solutions. In F.B. Prokop (Ed.), *Translocation Issues in Western Australia. Proceedings of a Seminar and Workshop held on 26th and 27th September 1994. Fisheries management paper No. 83* (pp. 96-103). Perth: Fisheries Department of Western Australia.
- Horwitz, P. (1997). Biogeographical Affinities of Macrocrustacean Groups in Northeast Tasmania. *Records of the Queen Victoria Museum and Art Gallery*, 103, 171-177.
- Hudson, R.R. (1990). Gene genealogies and the coalescent process. *Oxford Surveys in Evolutionary Biology*, 7, 1-44.
- Hudson, R.R., Slatkin, M., & Maddison, W.P. (1992). Examination of levels of gene flow from DNA sequence data. *Genetics*, 132, 583-589.
- Hurst, L.D. (1991). Sex, slime and selfish genes. *Nature*, 354, 23-24.
- Hynes, J.D., Brown, Jr., E.H., Helle, J.H., Ryman, N., & Webster, D.A. (1981). Guidelines for the culture of fish stocks for resource management. *Canadian Journal of Fisheries and Aquatic Sciences*, 38, 1867-1876.

- Innis, M.A., Gelfand, D.H., Sninsky, J.J., & White, T.J. (Eds.).(1990). *PCR Protocols: A Guide to Methods and Applications*. California: Academic Press Inc.
- Irwin, D.M., Kocher, T.D., & Wilson, A.C. (1991). Evolution of the Cytochrome *b* Gene of Mammals. *Journal of Molecular Evolution*, *32*, 128-144.
- James, J.W. (1992). Quantitative Genetics of Semi-Managed Populations. In P.I. Dixon (Ed.), *Population Genetics and its Applications to Fisheries Management and Aquaculture* (pp. 137-148). *Proceedings of a workshop held at the University of New South Wales, Kensington, on 25-27 June, 1990*. New South Wales: Centre for Marine Science, University of New South Wales.
- Johansen, S., Guddal, P.H., & Johansen, T. (1990). Organization of the mitochondrial genome of Atlantic cod, *Gadus morhua*. *Nucleic Acids Research*, *18*, 411-419.
- Johnson, M.S., Clarke, B., & Murray, J. (1988). Discrepancies in the Estimation of Gene Flow in *Partula*. *Genetics*, *120*, 233-238.
- Jones, M.R., & Torgersen, T. (1988). Late Quaternary evolution of Lake Carpentaria on the Australia-New Guinea continental shelf. *Australian Journal of Earth Sciences*, *35*, 313-324.
- Jones, S., & Sujarsingani, K.H. (1954). Fish and fisheries of the Chilka lakes with statistics of fish catches for the years 1948-50. *Indian Journal of Fisheries*, *1*, 256-344.
- Joseph, L., & Moritz, C. (1994). Mitochondrial DNA phylogeography of birds in eastern Australian rainforests: First fragments. *Australian Journal of Zoology*, *42*, 385-403.
- Jukes, T.H., & Cantor, C.R. (1969). Evolution in protein molecules. In H.N. Munro (Ed.), *Mammalian Protein Metabolism* (pp. 21-123). New York: Academic Press.
- Kapuscinski, A.R.D. & Lannan, J.E. (1984). Application of a conceptual fitness model for managing Pacific salmon fisheries. *Aquaculture*, *43*, 135-146.
- Kapuscinski, A.R.D. & Lannan, J.E. (1986). A conceptual genetic fitness model for fisheries management. *Canadian Journal of Fisheries and Aquatic Sciences*, *43*, 1606-1616.
- Karakousis, Y., & Triantaphyllidis, C. (1990). Genetic structure and differentiation among Greek brown trout (*Salmo trutta* L.) populations. *Heredity*, *64*, 297-304.
- Keenan, C.P. (1994). Recent Evolution of Population Structure in Australian Barramundi, *Lates calcarifer* (Bloch): An Example of Isolation by Distance in One Dimension. *Australian Journal of Marine and Freshwater Research*, *45*, 1123-48.
- Keenan, C. (1995). Genetic Implications of Fish Stocking Programs. In P. Cadwallader & B. Kerby (Eds.), *Fish Stocking in Queensland: Getting it Right! Proceedings of the Symposium held in Townsville, Queensland, 11 November 1995* (pp. 64-71). Fortitude Valley: Queensland Fisheries Management Authority.

- Keenan, C., & Salini, J. (1990). The Genetic Implications of Mixing Barramundi Stocks in Australia. In D.A. Pollard (Ed.), *Introduced and Translocated Fishes and their Ecological Effects. Proceedings of the Australian Society for Fish Biology workshop held at Magnetic Island, Townsville, Queensland, 24-25 August 1989. Proceedings No. 8* (pp.145-150). Canberra: Department of Primary Industries and Energy & Bureau of Rural Resources.
- Kendrick, G.W., Wyrwoll, K.H., & Szabo, B.J. (1991). Pliocene-Pleistocene coastal events and history along the western margin of Australia. *Quaternary Science Reviews, 10*, (5), 419-439.
- Kimberley Development Commission. (1994). *Annual Report of the Kimberley Development Commission*. Kununurra: Kimberley Development Commission.
- Kimberley Development Commission. (1995). Economic And Social Development Of The Kimberley Region. *Annual Report of the Kimberley Development Commission*. Kununurra: Kimberley Development Commission.
- Kimura, M. (1968). Evolutionary rate at the molecular level. *Nature, 217*, 624-626.
- Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution, 16*, 111-120.
- Kimura, M., & Weiss, G.H. (1964). The stepping stone model of population structure and the decrease of genetic correlation with distance. *Genetics, 49*, 561-574.
- Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., & Khorana, H.G. (1971). Studies on polynucleotides XCVI. Repair replication of short synthetic DNA's as catalyzed by DNA polymerases. *Journal of Molecular Biology, 56*, 341-361.
- Klug, W.S., & Cummings, M.R. (1993). *Essentials of genetics*. U.S.A.: Macmillan.
- Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo, S., Villablanca, F.X., & Wilson, A.C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA, 86*, 6196-6200.
- Kocher, T.D., & Wilson, A.C. (1991). Sequence evolution of mitochondrial DNA in humans and chimpanzees: Control region and a protein-coding region. In S. Osawa & T. Honjo (Eds.), *Evolution of Life* (pp. 391-413). Tokyo: Springer-Verlag.
- Koehler, G.M., Lindberg, G.L., & Brown, D.R. (1991). Replacement of bovine mitochondrial DNA by sequence variant within one generation. *Genetics, 129*, 247-255.
- Krebs, C.J. (1985). *Ecology. The Experimental Analysis of Distribution and Abundance*. (3rd Ed.). New York: Harper & Row.

- Krueger, C.C., & May, B. (1991). Ecological and genetic effects of salmonid introductions in North America. In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. Canadian Journal of Fisheries and Aquatic Sciences, 48 (Suppl. 1), 66-67.
- Lambeck, K., & Nakada, M. (1990). Late Pleistocene and Holocene sea-level change along the Australian coast. *Palaeogeography, Palaeoclimatology, Palaeoecology*, 89, 143-176.
- Lande, R., & Barrowclough, G.F. (1987). Effective population size, genetic variation, and their use in population management. In M. Soulé (Ed.), *viable Populations For Conservation* (pp.87-123). Cambridge: Cambridge University Press.
- Larkin, P.A. (1981). A perspective on population genetics and salmon management. *Canadian Journal of Fisheries and Aquatic Sciences*, 38, 1469-1475.
- Li, W.-H. (1993). So, what about the molecular clock hypothesis? *Current Opinions in Developmental Genetics*, 3, 896-901.
- Li, W.-H., & Graur, D. (1991). *Fundamentals of Molecular Evolution*. U.S.A.: Sinauer Associates Inc.
- Liberman, U., & Feldman, M.W. (1986). Modifiers of mutation rate: A general reduction principle. *Theoretical Population Biology*, 30, 125-142.
- Long, J.A. (1995). *The rise of fishes*. Sydney: University of New South Wales Press.
- Lymbery, A.J. (1993). Migration, Selection and Population Size in *Ostertagia ostertagi*. *Parasitology Today*, 9, (2), 37-38.
- Lymbery, A.J. (1995). Genetic Diversity, Genetic Differentiation and Speciation in the Genus *Echinococcus* Rudolphi 1801. In R.C.A. Thompson & A.J. Lymbery (Eds.), *Echinococcus and Hydatid Disease* (pp. 51-88). United Kingdom: CAB International.
- Lynch, M., & Crease, T.J. (1990). The Analysis of Population Survey Data on DNA Sequence Variation. *Molecular Biology and Evolution*, 7, (4), 377-394.
- MacKinnon, M.R. (1987). Rearing and growth of larval and juvenile barramundi (*Lates calcarifer*) in Queensland. In J.W. Copland & D.L. Grey (Eds.), *Management of Wild and Cultured Sea Bass/ Barramundi (Lates calcarifer)*. *Proceedings of an international workshop held in Darwin, Australia, 24-30 September 1986*. ACIAR Proceedings No. 20 (pp. 148-153). Canberra: Australian Centre for International Agricultural Research.
- MacKinnon, M.R., & Cooper, P.R. (1987). Reservoir stocking of barramundi for enhancement of the recreational fishery. *Australian Fisheries*, July, 1987.
- Maddison, W.P., & Maddison, D.R. (1992). *MacClade: Analysis of Phylogeny and Character Evolution*. U.S.A.: Sinauer Associates Inc.

- Mallen-Cooper, M. (1992). Swimming Ability of Juvenile Australian Bass, *Macquaria novemaculeata* (Steindachner), and Juvenile Barramundi, *Lates calcarifer* (Bloch), in an Experimental Vertical-slot Fishway. *Australian Journal of Marine and Freshwater Research*, 43, 823-834.
- Maneewong, S. (1987). Induction of spawning of sea bass (*Lates calcarifer*) in Thailand. In J.W. Copland & D.L. Grey (Eds.), *Management of Wild and Cultured Sea Bass/ Barramundi (Lates calcarifer). Proceedings of an international workshop held in Darwin, Australia, 24-30 September 1986. ACIAR Proceedings No. 20* (pp. 119-119). Canberra: Australian Centre for International Agricultural Research.
- Margulis, L. (1970). *Origin of Eukaryotic Cells*. U.S.A.: Yale University Press.
- Martin, A.P., & Palumbi, S.R. (1993). Protein Evolution in Different Cellular Environments: Cytochrome *b* in Sharks and Mammals. *Molecular Biology and Evolution*, 10, (4), 873-891.
- Martin, A.P., Naylor, G.J.P., & Palumbi, S.R. (1992). Rates of mitochondrial DNA evolution in sharks are slow compared with animals. *Nature*, 357, 153-155.
- Martinez, P., Arias, J., Castro, J., & Sanchez, L. (1993). Differential stocking incidence in brown trout (*Salmo trutta*) populations from Northwestern Spain. *Aquaculture*, 114, 203-216.
- Maxam, A.M., & Gilbert, W. (1977). A new method for sequencing DNA. *Proceedings of the National Academy of Sciences of the USA*, 74, 560-564.
- Mayer, S.S., Charlesworth, D., & Meyers, B. (1996). Inbreeding depression in four populations of *Collinsia heterophylla* Nutt (Scrophulariaceae). *Evolution*, 50, (2), 879-891.
- Mayr, E. (1963). *Animal Species and Evolution*. Cambridge: Harvard University Press.
- McGregor, A., & Chester, Q. (1992). *The Kimberley: Horizons Of Stone*. Australia: Hodder and Stoughton.
- McVeigh, H.P., Bartlett, S.E., & Davidson, W.S. (1991). Polymerase chain reaction/direct sequence analysis of the cytochrome *b* gene in *Salmo salar*. *Aquaculture*, 95, 225-231.
- Meffe, G.K. (1986). Conservation genetics and the management of endangered fishes. *Fisheries*, 11, (1), 14-23.
- Meyer, A. (1994). DNA technology and phylogeny of fish. In A.R. Beaumont (Ed.), *Genetics and Evolution of Aquatic Organisms* (pp. 219-249). London: Chapman & Hall.
- Meyer, A., Kocher, T.D., & Basasibwaki, P. (1990). Monophyletic origin of Lake Victoria cichlid fish suggested by mitochondrial DNA sequences. *Nature*, 347, 550-553.
- Meyer, A., Kocher, T.D., & Wilson, A.C. (1991). African fish-a reply. *Nature*, 351, 467-468.

- Milligan, B.G., Leebens-Mack, J., & Strand, A.E. (1994). Conservation genetics: beyond the maintenance of marker diversity. *Molecular Ecology*, 3, 423-435.
- Mindell, D.P., & Honeycutt, R.L. (1990). Ribosomal RNA in vertebrates: evolution and phylogenetic applications. *Annual Review of Ecology and Systematics*, 21, 541-566.
- Mitchell-Olds, T. (1995). The molecular basis of quantitative genetic variation in natural populations. *Trends in Ecology and Evolution*, 10, 324-328.
- Moav, R., Brody, T., & Hulata, G. (1978). Genetic improvement of wild fish populations. *Science*, 201, 1090-1094.
- Moore, R. (1979). Natural sex inversion in the giant perch (*Lates calcarifer*). *Australian Journal of Marine and Freshwater Research*, 30, 803-813.
- Moore, R. (1982). Spawning and early life history of barramundi, *Lates calcarifer* (Bloch), in Papua New Guinea. *Australian Journal of Marine and Freshwater Research*, 33, 647-661.
- Moore, R., & Reynolds, L.F. (1982). Migration Patterns of Barramundi, *Lates calcarifer* (Bloch), in Papua New Guinea. *Australian Journal of Marine and Freshwater Research*, 33, 671-682.
- Moore, W.S. (1977). An evaluation of Narrow Hybrid Zones in Vertebrates. *Quarterly Review of Biology*, 52, 263-278.
- Moritz, C. (1994). Applications of mitochondrial DNA analysis in conservation: a critical review. *Molecular Ecology*, 3, 401-411.
- Moritz, C., & Hillis, D.M. (1996). Molecular Systematics: Context and Controversies. In D.M. Hillis, C. Moritz & B.K. Mable (Eds.), *Molecular Systematics* (2nd ed. pp. 1-13). U.S.A.: Sinauer Associates Inc.
- Morrissy, N.M. (1973). Comparisons of strains of *Salmo gairdneri* Richardson from New South Wales, Victoria and Western Australia. *Australian Society for Limnology Bulletin*, 5, 11-20.
- Morrissy, N.M. (1980). Aquaculture. In W.D. Williams (Ed.), *An Ecological Basis for Water Resource Management* (pp. 215-226). Canberra: A.N.U. Press.
- Morrissy, N.M. (1983). *Potential Fisheries Capability of the Ord in the Short, Medium and Long Terms*. Report No. 59, Department of Fisheries and Wildlife, Western Australia.
- Morrissy, N.M. (1985). *The Commercial Fishery for Barramundi (Lates calcarifer) in Western Australia*. Report No. 68, Department of Fisheries and Wildlife, Western Australia.

- Morrissy, N.M. (1987). Status of the Barramundi (*Lates calcarifer*) Fishery in Western Australia. In J.W. Copland & D.L. Grey (Eds.), *Management of Wild and Cultured Sea Bass/ Barramundi (Lates calcarifer). Proceedings of an international workshop held in Darwin, Australia, 24-30 September 1986. ACIAR Proceedings No. 20* (pp. 99-102). Canberra: Australian Centre for International Agricultural Research.
- Moyle, P.B., Li, H.W., & Barton, B.A. (1986). The Frankenstein Effect: Impact of introduced fishes on native fishes in North America. In R.H. Stroud (Ed.), *Fish culture in fisheries management* (pp. 415-426). U.S.A.: American Fisheries Society.
- Mullis, K.B., & Faloona, F.A. (1987). Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods in Enzymology*, 155, 335-350.
- Mullis, K.B., Ferré, F., & Gibbs, R.A. (Eds.).(1994). *The Polymerase Chain Reaction*. Boston: Birkhauser
- Munday, B.L., Langdon, J.S., Hyatt, A., & Humphrey, J.D. (1992). Mass mortality associated with a viral-induced vacuolating encephalopathy and retinopathy of larval and juvenile barramundi, *Lates calcarifer* Bloch. *Aquaculture*, 103, 197-211.
- Murayama, T. (1970). Effective numbers of alleles in a subdivided population. *Theoretical Population Biology*, 1, 273-306.
- Needleman, S.B., & Wunsch, C.D. (1970). A general method applicable to the search for similarities in the amino acid sequence of two proteins. *Journal of Molecular Biology*, 48, 443-453.
- Nei, M. (1972). Genetic distance between populations. *American Naturalist*, 106, 283-292.
- Nei, M. (1973). Analysis of Gene Diversity in Subdivided Populations. *Proceedings of the National Academy of Sciences of the USA*, 76, 5269-5273.
- Nei, M. (1987). *Evolutionary Molecular Genetics*. New York: University Of Columbia Press.
- Nei, M., & Li, W. -H. (1979). Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences of the USA*, 76, 5269-5273.
- Nei, M., & Tajima, F. (1981). DNA polymorphism detectable by restriction endonucleases. *Genetics*, 97, 145-163.
- Nei, M., & Jin, L. (1989). Variances of the average numbers of nucleotide substitutions within and between populations. *Molecular Biology and Evolution*, 6, 290-300.
- Nei, M., & Miller, J.C. (1990). A Simple Method for Estimating Average Number of Nucleotide Substitutions Within and Between Populations From Restriction Data. *Genetics*, 125, 873-879.

- Nei, M., & Takahata, N. (1993). Effective Population Size, Genetic Diversity, and Coalescence Time in Subdivided Populations. *Journal of Molecular Evolution*, 37, 240-244.
- Nei, M., Maruyama, T., & Chakraborty, R. (1975). The bottleneck effect and genetic variability in populations. *Evolution*, 29, 1-10.
- Nei, S. (1996). *Kimberley Aquaculture Development Plan*. Fisheries Department of Western Australia and Kimberley Development Commission.
- Nelson, K., & Soulé, M. (1987). Genetical Conservation of Exploited Fishes. In N. Ryman & F.M. Utter (Eds.), *Population Genetics & Fishery Management* (pp. 345-368). U.S.A.: University of Washington Press.
- Normark, B.B., McCune, A.R., & Harrison, R.G. (1991). Phylogenetic relationships of Neopterygian fish, inferred from mitochondrial DNA sequences. *Molecular Biology and Evolution*, 8, 819-834.
- O'Connell, M., Skibinski, D.O.F., & Beardmore, J.A. (1995). Absence of restriction site variation in the mitochondrial ND5 and ND6 genes of Atlantic salmon amplified by the polymerase chain reaction. *Journal of Fish Biology*, 47, 910-913.
- Olivo, P.D., Van de Walle, J.J., & Laipis, J.P. (1983). Nucleotide sequence evidence for rapid genotype shifts in bovine mitochondrial DNA D-loop. *Nature*, 306, 400-402.
- Ong, T-L., Stabile, J., Wirgin, I., & Waldman, J.R. (1996). Genetic Divergence between *Acipenser oxyrinchus* and *A. o. desotoi* as Assessed by Mitochondrial DNA Sequencing Analysis. *Copeia*, 1996, (2), 464-469.
- 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., Bywater, R., & White, R.W.G. (1993). Mitochondrial DNA nucleotide sequence variation in Atlantic salmon (*Salmo salar*), brown trout (*S. trutta*), rainbow trout (*Oncorhynchus mykiss*) and brook trout (*Salvelinus fontinalis*) from Tasmania, Australia. *Aquaculture*, 114, 217-227.
- Palumbi, S.R. (1996). Nucleic Acids II: The Polymerase Chain Reaction. In D.M. Hillis, C. Moritz & B.K. Mable (Eds.), *Molecular Systematics* (2nd ed. pp. 205-247). U.S.A.: Sinauer Associates Inc.
- Palumbi, S.R., & Benzie, J. (1991). Large mitochondrial DNA differences among morphologically similar penaeid shrimp. *Molecular Marine Biology and Biotechnology*, 1, 27-34.
- Pang, C-Y., Lee, H-C., Yang, J-H., and Wei, Y-H. (1994). Human skin mitochondrial DNA deletions associated with light exposure. *Archives of Biochemistry and Biophysics*, 312, 534-538.
- Patnaik, S., & Jena, S. (1976). Some aspects of biology of *Lates calcarifer* (Bloch) from Chilka Lake. *Indian Journal of Fisheries*, 23, 65-72.

- Pender, P.J., & Griffin, R.K. (1996). Habitat History of Barramundi *Lates calcarifer* in a North Australian River System Based on Barium and Strontium Levels in Scales. *Transactions of the American Fisheries Society*, 125, 679-689.
- Peterson, M.A. (1996). Long-distance gene flow in the sedentary butterfly, *Euphilotes enoptes* (Lepidoptera: Lycaenidae). *Evolution*, 50, (5), 1990-1999.
- Philipp, D.P. (1991). Genetic Implications of Introducing Florida Largemouth Bass, *Micropterus salmoides floridanus*. In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. Canadian Journal of Fisheries and Aquatic Sciences, 48 (Suppl. 1), 58-65.
- Philipp, D.P., & Whitt, G.S. (1991). Survival and growth of northern, Florida, and reciprocal F₁ hybrid largemouth bass in central Illinois. *Transactions of the American Fisheries Society*, 120, 574-582.
- Pogson, G.H., Mesa, K.A., & Boutilier, R.G. (1995). Genetic Population Structure and Gene Flow in the Atlantic Cod *Gadus morhua*: A Comparison of Allozyme and Nuclear RFLP Loci. *Genetics*, 139, 375-385
- Pollard, D.A. (1990). Recommendations of the workshop. In D.A. Pollard (Ed.), *Introduced and Translocated Fishes and their Ecological Effects. Proceedings of the Australian Society for Fish Biology workshop held at Magnetic Island, Townsville, Queensland, 24-25 August 1989. Proceedings No. 8* (pp.180-181). Canberra: Department of Primary Industries and Energy & Bureau of Rural Resources.
- Pollard, D.A., & Burchmore, J.J. (1986). A Possible Scenario for the Future of Australia's Freshwater Fish Fauna. In P. De Deckker & W.D. Williams (Eds.), *Limnology in Australia* (pp. 615-636). Netherlands: CSIRO & Dr W. Junk.
- Porter, A.H. (1990). Testing nominal species boundaries using gene flow statistics: The taxonomy of two hybridizing admiral butterflies (*Limenitis*: Nymphalidae). *Systematic Zoology*, 39, (2), 131-147.
- Pownall, P.C. (1969). Increasing interest in aquaculture. *Australian Fisheries*, 28, (8), 3.4.8.
- Pressey, R.I., & Middleton, M.J. (1982). Impacts of flood mitigation works on coastal wetlands in New South Wales. *Wetlands (Australia)*, 2, 27-44.
- Prokop, F. (1995). What Translocation can do for Recreational Fishing Enhancement: Benefits v Costs. In F.B. Prokop (Ed.), *Translocation Issues in Western Australia. Proceedings of a Seminar and Workshop held on 26th and 27th September 1994. Fisheries management paper No. 83* (pp. 30-36). Perth: Fisheries Department of Western Australia.
- Purdom, C.E. (1992). Genetics in fish culture. In P.I. Dixon (Ed.), *Population Genetics and its Applications to Fisheries Management and Aquaculture* (pp. 155-170). *Proceedings of a workshop held at the University of New South Wales, Kensington, on 25-27 June, 1990*. New South Wales: Centre for Marine Science, University of New South Wales.

- Rannala, B., & Hartigan, J.A. (1996). Estimating gene flow in island populations. *Genetics Research, Cambridge*, 67, 147-158.
- Regional Development Council & Department of Commerce and Trade. (1996). *Challenges and opportunities for Western Australia's regions*. A discussion paper.
- Renault, T., Haffner, Ph., Baudin Laurencin, F., Brevil, G., & Bonami, J.R. (1991). Mass mortality in hatchery-reared sea bass (*Lates calcarifer*) larvae associated with the presence in the brain and retina of virus-like particles. *Bulletin of the European Association for Fish Pathology*, 11, 68-73.
- Reynolds, J., Weir, B.S., & Cockerham, C.C. (1983). Estimation of the coancestry coefficient: Basis for a short term genetic distance. *Genetics*, 105, 767-779.
- Reynolds, L.F., & Moore, R. (1973). Barramundi (*Lates calcarifer*) research in Papuan waters. *Fisheries Research Annual Report 1971-1972*, 21-28. New Guinea Department of Agriculture, Stock and Fisheries.
- Reynolds, L.F., & Moore, R. (1982). Growth rates of barramundi, *Lates calcarifer* (Bloch), in Papua New Guinea. *Australian Journal of Marine and Freshwater Research* 33, 663-670.
- Richardson, B.J., Baverstock, P.R., & Adams, M. (1986). *Allozyme Electrophoresis: a Handbook for Animal Systematics and Population Studies*. Sydney: Academic Press.
- Ricker, W.E. (1972). Hereditary and environmental factors affecting certain salmonid populations. In R.C. Simon & P.A. Larkin (Eds.), "The Stock Concept in Pacific Salmon", *H.R. MacMillan Lectures in Fisheries* (pp. 19-160). Vancouver: University of British Columbia.
- Rockwell, R.F., & Barrowclough, G.F. (1987). Gene Flow and the Genetic Structure of Populations. In F. Cooke & P.A. Buckley (Eds.), *Avian Genetics: A Population and Ecological Approach* (pp. 223-255). London: Academic Press.
- Rogers, J.S. (1972). Measures of genetic similarity and genetic distance. *Studies in Genetics VII. University of Texas Publication*, 7213, 145-153.
- Rohlf, E.J. (1992). *NTSYS Numerical Taxonomy and Multivariate Analysis System*. Version 1.7. New York: Exeter Software.
- Rozas, J., & Rozas, R. (1995). *DNA Sequence Polymorphism*. Spain: University of Barcelona.
- Russell, D.J., & Garrett, R.N. (1985). Early Life History of Barramundi, *Lates calcarifer* (Bloch), in North-eastern Queensland. *Australian Journal of Marine and Freshwater Research*, 36, 191-201.
- Russell, D.J., & Garrett, R.N. (1988). Movements of juvenile barramundi, *Lates calcarifer* (Bloch), in north-eastern Queensland. *Australian Journal of Marine and Freshwater Research*, 39, 117-123.

- Russell, D.J., & Rimmer, M.A. (in press). Assessment of Stock Enhancement of Barramundi *Lates calcarifer* (Bloch) in a Coastal River System in far Northern Queensland, Australia. *Proceedings of the 2nd World Fisheries Congress at Brisbane, Queensland, Australia, 28 July-2 August 1996*. (Editors & publishers unknown at time of writing).
- Rutledge, W.P. (1990). *Barramundi (Lates calcarifer, Bloch) Aquaculture and Recreational Stocking in Queensland*. Information Series Q190035. Brisbane: Queensland Department of Primary Industries.
- Rutledge, W., Rimmer, M., Russell, J., Garret, R., & Barlow, C. (1990). Cost benefit of hatchery-reared barramundi, *Lates calcarifer* (Bloch), in Queensland. *Aquaculture and Fisheries Management*, 21, 443-448.
- Ryman, N., & Utter, F. (Eds.).(1987). *Population Genetics & Fishery Management*. U.S.A.: University of Washington Press.
- Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., & Arnheim, N. (1985). Enzymatic Amplification of β -Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia. *Science*, 230, 1350-1354.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., & Erlich, H.A. (1988). Primer-directed enzymatic amplification of DNA with a thermostable polymerase. *Science*, 239, 487-491.
- Saitou, N., & Nei, M. (1987). The neighbor-joining method: a new method for constructing phylogenetic trees. *Molecular Biology and Evolution*, 4, 406-425.
- Salini, J., & Shaklee, J.B. (1987a). Stock structure of Australian and Papua New Guinean barramundi (*Lates calcarifer*). In J.W. Copland & D.L. Grey (Eds.), *Management of Wild and Cultured Sea Bass/Barramundi (Lates calcarifer)*. *Proceedings of an international workshop held in Darwin, Australia, 24-30 September 1986*. ACIAR Proceedings No. 20 (pp. 30-34). Canberra: Australian Centre for International Agricultural Research.
- Salini, J., & Shaklee, J.B. (1987b). Barramundi discoveries may change management. *Australian Fisheries*, 1987, (1), 2-6.
- Salini, J., & Shaklee, J.B. (1988). Genetic structure of barramundi (*Lates calcarifer*) stocks from Northern Australia. *Australian Journal of Marine and Freshwater Research*, 39, 317-329.
- Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*. (2nd ed.). U.S.A.: Cold Spring Harbour Laboratory Press.
- Sanger, F., Nicklen, S., & Coulson, A.R. (1977). DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences of the USA*, 74, 5463-5467.
- Sankoff, D., Morel, C., & Cedergren, R.J. (1973). Evolution of 5S RNA and the non-randomness of base replacement. *Nature*, 245, 232-234.

- Santos, S.E.B., Ribeiro-Dos-Santos, A.K.C., Meyer, D., & Zago, M.A. (1996). Multiple founder haplotypes of mitochondrial DNA in Amerindians revealed by RFLP and sequencing. *Annals of Human Genetics*, 60, 305-319.
- Sarver, S.K., Freshwater, D.W., & Walsh, P.J. (1996). Phylogenetic Relationships of Western Atlantic Snappers (Family Lutjanidae) Based on Mitochondrial DNA Sequences. *Copeia*, 1996, (3), 715-721.
- Sattaur, O. (1989). The threat of the well-bred salmon. *New Scientist*, 122, (1662), 36-40.
- Shackleton, N.J. (1987). Oxygen isotopes, ice volume and sea level. *Quaternary Science Reviews*, 6, 783-790.
- Shaklee, J.B., & Salini, J. (1983). Studies suggest multiple stocks of Australian barramundi. *Australian Fisheries*, 42 (2), 36-8.
- Shaklee, J.B., & Salini, J. (1985). Genetic variation and population subdivision in Australian barramundi, *Lates calcarifer* (Bloch). *Australian Journal of Marine and Freshwater Research*, 36, 203-218.
- Shaklee, J.B., Phelps, S.R., & Salini, J. (1991). Analysis of Fish Stock Structure and Mixed-Stock Fisheries by the Electrophoretic Characterization of Allelic Isozymes. In D.H. Whitmore (Ed.), *Electrophoretic and Isoelectric Focusing Techniques in Fisheries Management* (pp. 173-196). U.S.A.: CRC Press.
- Shaklee, J.B., Salini, J., & Garrett, R.N. (1993). Electrophoretic characterization of multiple genetic stocks of barramundi (*Lates calcarifer*) in Queensland, Australia. *Transactions of the American Fisheries Society*, 122, 685-701.
- Sherwin, W.B. (1992). Conservation of Genetic Resources. In P.I. Dixon (Ed.), *Population Genetics and its Applications to Fisheries Management and Aquaculture* (pp. 171-190). *Proceedings of a workshop held at the University of New South Wales, Kensington, on 25-27 June, 1990*. New South Wales: Centre for Marine Science, University of New South Wales.
- Shoffner, J.M., & Wallace, D.C. (1995). Oxidative Phosphorylation Diseases. In C.R. Scriver, A.L. Beaudet, W.S. Sly & D. Valle (Eds.), *The Metabolic and Molecular Bases of Inherited Disease* (Vol. 1. 7th Ed. pp. 1535-1609). U.S.A.: McGraw-Hill.
- Simberloff, D.S. (1981). Community effects of introduced species. In M.H. Nitecki (Ed.), *Biotic Crises in Ecological and Evolutionary Time* (pp. 53-81). New York: Academic Press.
- Simon, R.C. (1972). Gene frequency and the stock problem. In R.C. Simon & P.A. Larkin (Eds.), *The stock concept in Pacific salmo, H.R. Macmillan Lectures in Fisheries* (pp. 161-172). Vancouver: University of British Columbia.
- Slatkin, M. (1985). Gene flow in natural populations. *Annual Review of Ecology and Systematics*, 16, 393-430.

- Slatkin, M. (1987). Gene Flow and the Geographic Structure of Natural Populations. *Science*, 236, 787-792.
- Slatkin, M. (1993). Isolation by distance in equilibrium and non-equilibrium populations. *Evolution*, 47, 264-279.
- Slatkin, M., & Barton, N.H. (1989). A comparison of three indirect methods for estimating average levels of gene flow. *Evolution*, 43, (7), 1349-1368.
- Slatkin, M., & Maddison, W.P. (1989). A Cladistic Measure of Gene Flow Inferred from the Phylogenies of Alleles. *Genetics*, 123, 603-613.
- Smith, H.M. (1945). *Fresh-water Fishes of Siam or Thailand*. [Reprint, 1965]. Washinton: Smithsonian Institute.
- Sneath, P.H.A., & Sokal, R.R. (1973). *Numerical Taxonomy*. San Francisco: W.H. Freeman & Co.
- Soulé, M. (1987). Introduction. In M. Soulé (Ed.), *Viable Populations for Conservation* (pp. 1-10). Cambridge: Cambridge University Press.
- Stagles, I. (1995). Why Fishermen Want Species Translocated. In F.B. Prokop (Ed.), *Translocation Issues in Western Australia. Proceedings of a Seminar and Workshop held on 26th and 27th September 1994. Fisheries management paper No. 83* (pp. 91-93). Perth: Fisheries Department of Western Australia.
- Ståhl, G. (1987). Genetic population structure of Atlantic salmon. In N. Ryman & F. Utter (Eds.), *Population Genetics and Fishery Management* (pp. 121-140). U.S.A.: University of Washinton Press.
- Stoddart, J.A., & Trendall, J.T. (1990). Currents In The Barramundi Gene Pool. In D.A. Pollard (Ed.), *Introduced and Translocated Fishes and their Ecological Effects. Proceedings of the Australian Society for Fish Biology workshop held at Magnetic Island, Townsville, Queensland, 24-25 August 1989. Proceedings No. 8* (p.144). Canberra: Department of Primary Industries and Energy & Bureau of Rural Resources.
- Storfer, A. (1996). Quantitative genetics: a promising approach for the assessment of genetic variation in endangered species. *Trends in Ecology and Evolution*, 11, (8), 343-348.
- Stumbauer, C., & Meyer, A. (1992). Genetic divergence, speciation and morphological stasis in a lineage of African cichlid fish. *Nature*, 358, 578-581.
- Swofford, D.L. (1991). When are Phylogeny Estimates from Molecular and Morphological Data Incongruent? In M.M. Miyamoto & J. Cracraft (Eds.), *Phylogenetic Analysis of DNA Sequences* (pp. 295-333). New York: Oxford University Press.
- Swofford, D.L. (1993). *Phylogenetic Analysis Using Parsimony*. Version 3.1. U.S.A.: Laboratory of Molecular Systematics, Smithsonian Institute.

- Swofford, D.L., & Olsen, G.J. (1990). Phylogeny reconstruction. In D.M. Hillis & C. Moritz (Eds.), *Molecular Systematics* (pp. 411-501). U.S.A.: Sinauer Associates Inc.
- Swofford, D.L., Olsen, G.J., Waddell, P.J., & Hillis, D.M. (1996). Phylogenetic Inference. In D.M. Hillis, C. Moritz & B.K. Mable (Eds.), *Molecular Systematics* (2nd ed. pp. 407-514). U.S.A.: Sinauer Associates Inc.
- Tagliavini, J., Harrison, I.J., & Gandolfi, G. (1995). Discrimination between *Anguilla anguilla* and *A. rostrata* by polymerase chain reaction-restriction fragment length polymorphism analysis. *Journal of Fish Biology*, *47*, 741-743.
- Tajima, F. (1983). Evolutionary relationships of DNA sequences in finite populations. *Genetics*, *105*, 437-460.
- Takahata, N., & Nei, M. (1984). F_{ST} and G_{ST} statistics in the finite island model. *Genetics*, *107*, 501-504.
- Tamura, K., & Nei, M. (1993). Estimation of the Number of Nucleotide Substitutions in the Control Region of Mitochondrial DNA in Humans and Chimpanzees. *Molecular Biology and Evolution*, *10*, (3), 512-526.
- Tateno, Y., Nei, M., & Tajima, F. (1982). Accuracy of estimated phylogenetic trees from molecular data. 1. Distantly related trees. *Journal of Molecular Evolution*, *18*, 387-404.
- Tavaré, S. (1984). Line-of-descent and genealogical processes, and their applications in population genetic models. *Theoretical Population Biology*, *26*, 119-164.
- Tave, D. (1993). *Genetics for Fish Hatchery Managers* (2nd Ed.). New York: AVI.
- Taylor, E.B. (1991). A review of local adaptation in Salmonidae, with particular reference to Pacific and Atlantic salmon. *Aquaculture*, *98*, 185-207.
- Taylor, B.L., & Dizon, A.E. (1996). The Need to Estimate Power to Link Genetics and Demography for Conservation. *Conservation Biology*, *10*, (2), 661-664.
- Templeton, A.R., Crandall, K.A., & Sing, C.F. (1992). A Cladistic Analysis of Phenotypic Associations With Haplotypes Inferred From Restriction Endonuclease Mapping and DNA Sequence Data. III. Cladogram Estimation. *Genetics*, *132*, 619-633.
- Thomas, W.K., & Beckenbach, A.T. (1989). Variation in Salmonid Mitochondrial DNA: Evolutionary Constraints and Mechanisms of Substitution. *Journal of Molecular Evolution*, *29*, 233-245.
- Thomas, W.K., Withler, R.E., & Beckenbach, A.T. (1986). Mitochondrial DNA analysis of Pacific salmonid evolution. *Canadian Journal of Zoology*, *64*, 1058-1064.

- Thorn, C. (1995). Translocation Policy and Controls. In F.B. Prokop (Ed.), *Translocation Issues in Western Australia. Proceedings of a Seminar and Workshop held on 26th and 27th September 1994. Fisheries management paper No. 83* (pp. 12-29). Perth: Fisheries Department of Western Australia.
- Torgersen, T., Hutchinson, M.F., Searle, D.E., & Nix, H.A. (1983). General bathymetry of the Gulf of Carpentaria and the Quaternary physiography of Lake Carpentaria. *Palaeogeography, Palaeoclimatology, Palaeoecology*, *41*, 207-225.
- Torgersen, T., Jones, M.R., Stephens, A.W., Searle, D.E. & Ullman, W.J. (1985). Late Quaternary hydrological changes in the Gulf of Carpentaria. *Nature (London)*, *313*, 785-787.
- Treadwell, R., McKelvie, L., & Maguire, G.B. (1991). *Profitability of selected aquaculture species*. ABARE Discussion paper 91.11.
- Trexler, J.C. (1988). Hierarchical organization of genetic variation in the sailfin molly, *Poecilia latipinna* (Pisces: Poeciliidae). *Evolution*, *42*, 1006-1017.
- Utter, F., Milner, G., Ståhl, G., & Teel, D. (1989). Genetic population structure of chinook salmon, *Oncorhynchus tshawyatscha*, in the Pacific northwest. *Fisheries Bulletin*, *87*, 239-264.
- Verspoor, E. (1988). Reduced genetic variability in first-generation hatchery populations of Atlantic salmon (*Salmo salar*). *Canadian Journal of Fisheries and Aquatic Sciences*, *45*, 1686-1690.
- Via, S., & Lande, R. (1985). Genotype-environment interactions and the evolution of phenotypic plasticity. *Evolution*, *39*, 505-522.
- Vuorinen, J. (1982). Little genetic variation in the Finnish Lake Salmon, *Salmo salar sebago* (Girard). *Hereditas*, *97*, 189-192.
- Wallace, B. (1970). *Genetic Load*. New Jersey: Prentice-Hall.
- Waples, R.S. (1991). Genetic interactions between hatchery and wild salmonids: Lessons from the Pacific Northwest. In N. Billington & P.D.N. Hebert (Eds.), *International Symposium on "The Ecological and Genetic Implications of Fish Introductions (FIN)"*. *Canadian Journal of Fisheries and Aquatic Sciences*, *48* (Suppl. 1), 124-133.
- Ward, R.D., Billington, N., & Hebert, P.D.N. (1989). Comparison of Allozyme and Mitochondrial DNA Variation in Populations of Walleye, *Stizostedion vitreum*. *Canadian Journal of Fisheries and Aquatic Sciences*, *46*, 2074-2084.
- Ward, R.D., Woodwark, M., & Skibinski, D.O.F. (1994). A comparison of genetic diversity levels in marine, freshwater, and anadromous fishes. *Journal of Fish Biology*, *44*, 213-232.
- Ward, R.D., Elliot, N.G., & Grewe, P.M. (1995). Allozyme and Mitochondrial DNA Separation of Pacific Northern Bluefin Tuna, *Thunnus thynnus orientalis* (Temminck and Schlegel), from Southern Bluefin Tuna, *Thunnus maccoyii* (Castelnau). *Marine and Freshwater Research*, *46*, 921-930.

- Wardell-Johnson, G., & Horwitz, P. (1996). Conserving biodiversity and the recognition of heterogeneity in ancient landscapes: a case study from south-western Australia. *Forest Ecology and Management*, 85, (1996), 219-238.
- Watson, J.D., Hopkins, N.H., Roberts, J.W., Steitz, J.A., & Weiner, A.M. (1987). *Molecular biology of the gene* (4th Ed.). U.S.A.: Benjamin/Cummings.
- Weir, B.S. (1990). *Genetic Data Analysis*. U.S.A.: Sinauer Associates Inc.
- Wells, V. (1969). Aquaculture in Japan. *Australian Fisheries*, 28, (8), 14-17.
- Western Australian Tourism Commission. (1995). *Western Australian Travel Survey: Kimberley Region Summary of Key Findings*. Perth: Jet Visual.
- Whitlock, M.C. (1992). Temporal fluctuations in demographic parameters and the genetic variance among populations. *Evolution*, 46, 608-615.
- Whitmore, D.H. (Ed.).(1991). *Electrophoretic and Isoelectric Focusing Techniques in Fisheries Management*. U.S.A.: CRC Press.
- Whitmore, D.H., & Craft, C.M. (1996). Mitochondrial DNA cytochrome *b* variation reveals different largemouth bass genealogies. *Journal of Fish Biology*, 48, 695-705.
- Wild Stock Fisheries Summary. (1995). Department of Primary Industry and Fisheries (Northern Territory). Fisheries Division. January to December 1995.
- Williams, D.M. (1992). DNA analysis: theory and methods. In P.L. Forey, C.J. Humphries, I.L. Kitching, R.W. Scotland, D.J. Siebert & D.M. Williams (Eds.), *Cladistics: A Practical Course in Systematics. Proceedings of a workshop of the Systematics Association in 1991*. (Publication No. 10, pp. 89-123). Great Britain: Oxford University Press.
- Williams, W.D. (1970). On the proposed introduction of *Lates niloictus* (L.) to Australia. *Australian Society for Limnology Bulletin*, 3, 33-35.
- Williams, W.D. (1982). The Argument Against the Introduction of the Nile Perch to Northern Australian Fresh Waters. *Search*, 13, (3-4), 67-70.
- Williamson, J.H., & Carmichael, G.J. (1990). An aquacultural evaluation of Florida, northern, and hybrid largemouth bass, *Micropterus salmoides*. *Aquaculture*, 85, 247-258.
- Withler, R.E. (1982). Genetic consequences of fertilizing chinook salmon (*Oncorhynchus tshawytscha*) eggs with pooled milt. *Aquaculture*, 68, 15-25.
- Wolstenholme, D.R., & Clary, D.O. (1985). Sequence evolution of *Drosophila* mitochondrial DNA. *Genetics*, 109, 725-744.

- Wongsomnuk, S. & Manevonk, S. (1973). *Results of experiments on artificial breeding and larval rearing of the sea bass (*Lates calcarifer* Bloch)*. Thailand Division of Research and Investigations, Department of Fisheries, Sonkla Marine Fisheries Station Contribution No.5.
- Wright, J.M., & Bentzen, P. (1994). Microsatellites: genetic markers for the future. *Reviews in Fish Biology and Fisheries*, 4, 384-388.
- Wright, S. (1931). Evolution in Mendelian populations. *Genetics*, 16, 97-159.
- Wright, S. (1943). Isolation by distance. *Genetics*, 28, 114-138.
- Wright, S. (1951). The genetical structure of populations. *Annals of Eugenics*, 15, 323-354.
- Wright, S. (1965). The interpretation of Population structure by *F*-statistics with special regard to systems of mating. *Evolution*, 19, 395-420.
- Yingthavorn, P. (1951). Notes on pla-kapong *Lates calcarifer* (Bloch) culturing in Thailand. *Fish Biology Technical Paper No.20*.
- Zar, J.H. (1984). *Biostatistical Analysis*. (2nd ed.). U.S.A.: Prentice-Hall International, Inc.
- Zhang, C., Linnane, A.W., & Nagley, P. (1993). Occurrence of a particular base substitution (3243 A to G) in mitochondrial DNA of tissues of ageing humans. *Biochemical and Biophysical Research Communications*, 195, 1104-1110.
- Zhang, D., & Hewitt, G.M. (1996). Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology and Evolution*, 11, (6), 247-251.
- Zhivotovsky, L.A., Gharret, A.J., McGregor, A.J., Glubokovsky, M.K., & Feldman, M.W. (1994). Gene Differentiation in Pacific Salmon (*Oncorhynchus* sp.): Facts and Models with Reference to Pink Salmon (*O. gorbuscha*). *Canadian Journal of Fisheries and Aquatic Sciences*, 51 (Suppl. 1), 223-232.

Appendix A

Minimally invasive tissue sampling of fishes revisited.

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Abstract. Despite the desirability of gathering genetic data without killing subjects, the overwhelming majority of studies in fishery-oriented population genetics have utilized tissues from individuals that were sacrificed for the research. We investigate the amounts of total DNA available from a range of tissue in the barramundi, *Lates calcarifer* (Bloch), a commercially and recreationally important species. Quantitative comparisons of total DNA extracted from caudal fin clips against necropsied tissue (liver), demonstrate no significant difference, however caudal fin tissue provides a significantly cleaner product. Amplification and electrophoresis of total DNA, followed by direct sequencing of target mitochondrial genomic regions obviates the need for lethal tissue sampling and promotes the merits of fin clipping for the acquisition of comparative genetic material that is practical, ethical and attuned to the aims of conservation and better management.

Extra Keywords: total DNA; nonlethal sampling; fin clips.

Introduction

Whilst the acquisition of tissue samples for fisheries-based genetic research would ideally enable the release of sampled individuals, protein analyses and the subsequent discovery of apparently tissue-specific forms of virtually identical enzymes (isozymes), resulted in a shift toward necropsied tissues (e.g. liver, eye, heart, kidney and brain) (Morizot *et al.* 1990). A greater understanding of both organelle and nuclear DNA (i.e. total DNA) has been achieved with recent technological advances, such as the probing and/or amplification of DNA sequences (e.g. Kocher *et al.* 1989), enabling the discrimination of species, subspecies, and local populations and stocks.

Despite the desirability of gathering genetic data without killing subjects, the overwhelming majority of studies in fishery-oriented population genetics have utilized tissues from sacrificed individuals (Billington and Hebert, 1990; Morizot *et al.* 1990).

Morizot *et al.* (1990) stated a number of criteria for minimally invasive sampling procedures, rather than noninvasive, or low-risk sampling. The most salient of these include:

1. Lethality caused by the sampling technique should be extremely infrequent, and the effects on fish health and fitness should be minimal.
2. The collected tissue should allow extensive genetic analyses.
3. Sampling should be relatively easy to perform, [perhaps by relatively untrained persons], and post-sampling treatment of tissues should be minimal, allowing easy and convenient field storage.

In their comprehensive review of fishery-based nonlethal sampling procedures, Morizot *et al.* (1990) concluded that although liver tissue was the most potentially informative allozyme yielding material, the favoured minimally invasive approach of fin clipping was only slightly behind liver in information yield, and ahead of other tissues when compared to the above selection criteria. Few other workers have employed minimally invasive sampling techniques. Vuorinen and Piironen (1984) examined the enzyme products scored from the adipose fins of juvenile salmonids without significant detriment, as did Morizot *et al.* (1990) in their studies of catfish, with

the latter suggesting the high fat content of this tissue source causing difficulties in interpretation. More recently, Hall and Nawrocki (1995) sampled the adipose fin of brown trout *Salmo trutta* (L.), to successfully discriminate a number of populations. Wingo and Muncy (1984) established a procedure for sampling the blood of the walleye *Stizostedion vitreum* (L.). This technique was used by Billington and Hebert (1990) to vindicate blood sampling as a nonlethal sampling methodology, as did Pogson *et al.* (1995). Work by Mork and Heggberget (1984) and Danzmann *et al.* (1993) has confirmed the utility of fish gametes as a potentially minimally invasive sampling technique for the collection of genetic data, although this is typically a seasonal opportunity.

Rapid advances in genetic technologies have substantially increased the tools available to researchers in fisheries population studies, resulting in comparisons between various genetic techniques (e.g. Ward *et al.* 1989; Pogson *et al.* 1995; Ward *et al.* 1995), and a greater understanding of the mitochondrial genome of fish populations (Billington and Hebert, 1988; Kocher *et al.* 1989; Billington *et al.* 1992; Grewe *et al.* 1993; Billington and Strange, 1995), yet investigators mostly rely on tissue taken from sacrificed animals. This practice continues despite the demonstration of Whitmore *et al.* (1992) that mitochondrial DNA (mtDNA) restriction fragment length polymorphisms are available by sampling the scale epithelium of live fishes.

This study uses the barramundi, *Lates calcarifer* (L.), as a model for investigating the amounts of total DNA available from various tissues in a representative fish. We then seek to reiterate that the technologies of gene amplification and direct sequencing enables the development of molecular character sets from tissue sources obtained by our preferred minimally invasive sampling technique of caudal fin clipping.

Methods

Tissue selection

Eight areas of a freshly killed 400g barramundi were identified as potential sites for the extraction of sufficient amounts of total DNA for use in genetic analyses. We selected typically necropsied tissues (liver, gill, heart); “moderately” invasive tissue (eye); potentially minimally invasive tissue sampling of lateral scales and muscle biopsy (pectoral fin muscle and basal tail muscle); and the preferred minimally invasive tissue source, caudal fin clips. Blood samples were not taken, however liver, gill and heart tissues are areas of rich blood supply, and might serve as a blood analogue.

DNA preparation and quantification

Tissue samples were vortexed in 250 μ L 0.1% Triton X-100 (Sigma) for 1 min. 50 μ L of 100mg/mL proteinase K (Sigma) was added to the tube and the composite heated for 1 hour at 50°C with occasional mixing. Twenty five microlitres of SET buffer (5% SDS, 50mM EDTA, 500mM Tris pH 8) (Sigma) was added to the reaction and a single extraction performed with 500 μ L 1:1 Phenol:chloroform/isoamyl alcohol (Sigma). The aqueous layer was removed to a new tube and 25 μ L of 3M sodium acetate (Sigma) and 250 μ L isopropanol (BDH) added. DNA was precipitated from the solution at -20°C for 30 min and then pelleted at 13 000rpm for 15 minutes at room temp. The DNA pellet was washed once with 70% ethanol (BDH), dried and resuspended in 50 μ L of sterile water.

Each of 50 μ L samples were analyzed spectrophotometrically to provide both DNA and protein optical densities (OD) at their respective 260nm and 280nm wavelengths. The OD_{260nm}/OD_{280nm} ratio provides an estimate of the purity of the nucleic acid, with pure preparations having OD_{260nm}/OD_{280nm} values between 1.8-2.0 (Sambrook *et al.* 1989).

To compare total DNA yield and purity of caudal fin and liver tissue, we obtained a second fish (320g). The procedure followed was identical to that stated above except only caudal fin and liver were sampled. Caudal fin clips were taken at the distal margin of the fin, with

the angle of cutting being a rounded, "bite-like" cut. Five pieces of tissue were taken from each source.

DNA amplification and electrophoresis

We selected primers designed to amplify variable sequences within the control and cytochrome *b* regions of the barramundi mitochondrial genome using the polymerase chain reaction (PCR, Saiki *et al.* 1988). For the control region, we used a light-strand primer (S. Chenoweth, personal communication) and a heavy-strand primer (Meyer *et al.* 1990). The light and heavy strand primers used for the cytochrome *b* amplification were those described in Kocher *et al.* (1989). Amplification of each region was done separately for DNA samples extracted from the liver and caudal fin. PCR reactions consisted of polymerase reaction buffer (67mM Tris-HCL pH 8.8, 16.6mM (NH₄)₂SO₄, 0.45% Triton X-100, 2mg/mL gelatin) (Biotech), 0.05μM of each primer (Research Genetics), 0.5units of *Taq* polymerase (Biotech) 250μM dNTPs (Biotech), 2mM MgCl₂ (Sigma) and 20ng of target DNA in a 10μL reaction. Amplifications were done in capillary tubes on a MJ research minicycler. Conditions for the control region were an initial denaturing step of 94°C for 5 min followed by 35 cycles of 94/30 sec, 40/30 sec, 72/60 sec and a final step of 72/5 min. Thermocycling conditions for the cytochrome *b* region were 54° for 5 min followed by 30 cycles of 94/20 sec, 50/40 sec, 72/40 sec and a final step of 72/5 minutes. Five microlitres of each reaction was run on a 2% agarose gel in 1 x TAE buffer (Sambrook *et al.* 1989) at 70 volts for 30 min using pUC19 *Hpa*II (Biotech) standards of known fragment size. DNA was visualized by staining with ethidium bromide.

DNA template preparation and sequencing

Direct sequencing of the double-stranded PCR products requires the prior removal of DNA primers, salts and polymerases. One caudal fin PCR product each from the mtDNA control region and cytochrome *b* gene was cleaned following the manufacturer's protocol (QIAquick). Double stranded DNA was fluorescently quantified using the minigel method (Sambrook *et al.* 1989).

Thermal cycle sequencing involved the mixing of fluorescently labelled dideoxynucleotide triphosphates, template DNA and PCR primers to make a 10 μ L reaction (Applied Biosystems). The sequencing reactions were done in capillary tubes on a MJ research minicycler. Sequencing conditions involved 96°C for 30 sec, followed by 50/15 sec and 60/4 min. This cycle was repeated 25 times for both genomic regions.

Excess dideoxynucleotide triphosphates were removed from samples by ethanol precipitation (Applied Biosystems). For each sample, 25 μ L of template suppression reagent (Applied Biosystems) was added, briefly centrifuged, and denatured in a Perkin Elmer 9600 thermocycler preheated to 95°C for 2 min. Each sample was transferred to a ABI 310 Genetic Analyzer and electrophoresed for 80 min for the control region, and 100 min for the cytochrome *b* gene. The double-stranded sequences were aligned, and the light strand sequence was resolved using the Sequence Navigator version 1.0.1 software package (ABI Prism).

Data analysis

The data were tabulated and compared without statistical analyses. A simple factorial ANOVA was applied to replicate samples of caudal fin and liver tissues for differences in total DNA yields (μ g/mL) and purity of total DNA extracted (OD_{260nm}/OD_{280nm}).

Results

Total DNA extraction from source tissue

The results of the total DNA extraction, together with the conversion of optical density units to DNA concentrations (μ g/mL) are given in Table 1. The amounts of total DNA extracted from all tissues ranged from 6.0-107.5 μ g/mL.

Table 1. Spectrophotometrical results of DNA extraction analysis

Tissue source	DNA OD _{260nm}	Total DNA ($\mu\text{g}/\text{mL}$)	Protein OD _{280nm}	$\frac{\text{OD}_{260\text{nm}}}{\text{OD}_{280\text{nm}}}$
Liver	1.41	70.5	1.71	1.72
Eye	0.37	18.5	0.20	1.89
Lateral scales	2.15	107.5	1.13	1.90
Pectoral fin muscle	0.85	42.5	0.45	1.89
Basal tail muscle	0.30	15.0	0.17	1.82
Caudal fin	0.52	26.0	0.27	1.96
Gill	0.70	35.0	0.45	1.50
Heart	0.12	6.0	0.10	1.15

Quantity and quality of total DNA in caudal fin and liver tissue

Total DNA extracted from the caudal fin ranged from 34.0-78.5 $\mu\text{g}/\text{mL}$ (± 14.5 S.E.), compared to liver samples 25.5-55.0 $\mu\text{g}/\text{mL}$ (± 11.7 S.E.). There was no significant statistical difference ($F = 3.79$, d.f. 9, $P > 0.05$) in extracted total DNA ($\mu\text{g}/\text{mL}$) between caudal fin and liver samples.

The purity of DNA extracted ($\text{OD}_{260\text{nm}}/\text{OD}_{280\text{nm}}$) had a relatively small range in the caudal fin (1.65-1.79; ± 0.05 S.E.), compared to the higher variability found in the liver samples (1.02-1.62; ± 0.20 S.E.), and there was a statistically significant difference ($F = 11.58$, d.f. 9, $P < 0.05$) in the purity of total DNA extracted from the caudal fin and the liver, with DNA extracted from the caudal fin being significantly less contaminated by protein.

Amplification of the mitochondrial control region and cytochrome *b* gene was expected to yield DNA fragment sizes of \approx 300 base pairs (S. Chenoweth, personal communication; Meyer *et al.* 1990; Kocher *et al.* 1989). Products obtained from both liver and caudal fin DNA confirm this (Fig. 1).

Direct sequencing of caudal fin PCR products obtained from barramundi mtDNA demonstrate the resolution of a 279 base pair sequence for the mtDNA control region, and a 334 base pair sequence for the mtDNA cytochrome *b* gene (Fig. 2).

Discussion

The results of the DNA extraction analysis clearly demonstrate the utility of a range of tissue for further molecular examination. The lack of statistical variability between the amounts of total DNA extracted from the liver and fin clips indicates that the present accepted requirement for necropsied tissue has no clear scientific advantage, and negates the need for destructive sampling methodologies. We note the important contribution by commercial fisheries for tissue samples, however these are not always available. The method described herein provides a practical and ethical alternative.

Fin clipping has been an accepted identification method in fisheries research for many years, although some researchers (e.g. Billington and Hebert, 1990; Cadwallader, 1995) have criticized the technique as being inefficient and expensive. Nevertheless, whilst arguments might continue regarding fin clipping for capture and release studies (Bergstedt, 1985), this investigation promotes the merits of fin clipping for the acquisition of genetic material, perhaps in conjunction with physical identification. The described methodology has wide ranging applications in the establishment of genetic markers. For example, markers could be developed for the identification of individuals in both wild and captive environments; for identifying colonies of endangered species; for assessments of hybridization; and using genetic data in mark-release studies (Morizot *et al.* 1990).

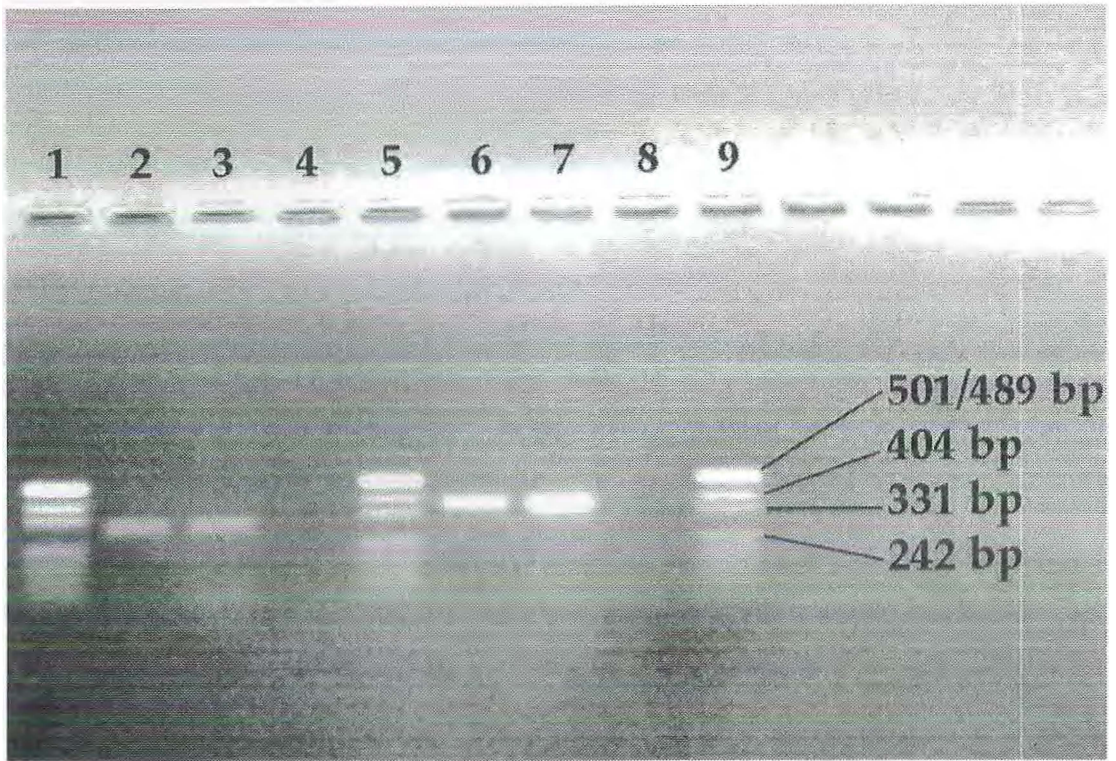


Fig. 1. Agarose gel stained with ethidium bromide to show PCR amplified sequences of *Lates calcarifer*. The fragment sizes of the pUC/*Hpa*II standard (lanes 1, 5 & 9) are given at right. Lane 2 is liver v. control region and lane 3 is caudal fin v. control region. Lane 6 is liver v. cytochrome *b* and lane 7 is caudal fin v. cytochrome *b*. Lanes 4 & 8 are water blanks.

a) GTGTAATGCATATATATATGCATTAATTAACATTTGCTTGTAAATCAAAGGACATACGTG 60
 CATTCAATGGTACTCGTAAATACAATGTACGGTAACCTATAATTAATGTACTTTAAGCAA 120
 TAATATTACATACTAATCATCAGCAATAATATTGAGCGTAGTGAGAGATCACCAATCAGT 180
 AGGTATTCAGAGTGTTGACGGTTCCTTGATAGTCAAGGACAGACACGGTGTGGGGGTTA 238
 CACAAATTGAACTATTACTGGCATCTGGTTCCTACTTCAGG 279

b) GCTCATATTAGCCTATGNCCTTATCTCCCAAATCCTAACAGGCCTATTCCTAGCCATACA 60
 CTACACTTCAGATATCGCAACAGCNTTACATCCGTGACACATATTTGTCGCGACGTCAA 120
 CTATGGATGACTTATTCGGAATATACATGCTAATGGCGCATCTTTCTTCTTCATCTGCAT 180
 CTACCTTCACATTGGTCGGGGTCTGTACTACGGCTCCTATCTCTACAAAGAACTTGAAA 240
 CATTGGAGTCATCCTCCTACTATTAGTTATAATAACTGCCTTCGTGGCTATGTTCTCCCC 300
 TGAGGACAAATATCATTCTGAGGGGCTGCAGTTT 334

Fig. 2. Barramundi mitochondrial DNA sequences from *a*, part of the control region, and *b*, part of the cytochrome *b* region. Bases are adenine (A), cytosine (C), guanine (G), and thymine (T). N indicates an unresolved nucleotide base.

Caudal fin clipping is possibly the only minimally invasive tissue sampling technique that reasonably addresses the criteria of Morizot *et al.* (1990). First, the method is non-lethal, and is likely to have minimal effect upon fish health or fitness, whereas “moderately” and potentially minimally invasive tissue extractions as described would be expected to impair fish health. For example, eye removal would have obvious implications, and one might anticipate an increased potential for fungal or other infections through muscle biopsy and scale removal. Whilst the scale removal technique of Whitmore *et al.* (1992) demonstrated similar results to ours, we argue that method has some significant shortcomings; the removal of scale epithelium leaves a large potential for infection (Morizot *et al.* 1990), and scale removal is possibly impractical for small fish. Stuart (1958) warns against the clipping of fins other than pelvic and caudal fins, due to possible interference in mobility and reproductive displays. The conclusions of Russell and Hales

(1992) and Russell (1995) were that the rapid regeneration of clipped fins in the barramundi rendered clipping for physical identification an ineffective practice. Such rapid recovery strongly suggests that, in species such as the barramundi, fin clipping is a preferred sampling technique in obtaining tissue for genetic analyses.

Second, the results of this study indicate that the amounts of total DNA extracted from the caudal fin are comparable to the liver. We amplified regions of the mitochondrial genome which constitute approximately 1% of total DNA (Alberts *et al.* 1989). This implies that total DNA available in caudal fins has direct application for other techniques involving the probing and amplification of specific nucleotide sequences.

Third, sampling is relatively simple; the tissue yielded plenty of DNA when only stored on ice, although warmer climates and prolonged periods in the field may increase molecular deterioration. Alternate means of sample storage, such as 70% ethanol preservation (Kocher *et al.* 1989), have been demonstrated to be effective (Whitmore *et al.* 1992; unpublished data), but rarely used.

Population genetics studies offer outstanding prospects for aquacultural improvement (Morizot *et al.* 1990). Additionally, if the aims of such studies in fisheries management are to identify the genetic similarities and differences between stocks of species, and these works are to be utilized in a manner that enables their conservation (especially where rare or restricted genomes have been identified), then it is timely that lethal sampling practices are reviewed. The evidence is that minimally invasive tissue sampling techniques are a potential source of genetic information at least for the barramundi, and probably many other bony fishes. As our natural resources continue to decline under the strain of exploitation and over-utilization, it is timely that we researchers continue to evaluate methodologies that are practical, ethical and reflect the broad objectives of conservation and better management.

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References

- Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson, J.D.** (1989). *Molecular biology of the cell* (2nd ed.). (Garland: New York).
- Bergstedt, R.A.** (1985). *Mortality of Fish Marked by Fin Clipping: An Annotated Bibliography*. National Fisheries Research Centre-Great Lakes Report 85-3.
- Billington, N., and Hebert, P.D.N.** (1988). Mitochondrial DNA Variation in Great Lakes Walleye *Stizostedion vitreum* L., Populations. *Canadian Journal of Fisheries and Aquatic Sciences* **45**, 643-654.
- Billington, N., and Hebert, P.D.N.** (1990), Technique for Determining Mitochondrial DNA markers in Blood Samples from Walleyes. *American Fisheries Society Symposium* **7**, 492-498.
- Billington, N., Barrette, R.J., and Hebert, P.D.N.** (1992). Management Implications of Mitochondrial DNA Variation in Walleye Stocks. *North American Journal of Fisheries Management* **12**, 276-284.

- Billington, N., and Strange, R.M.** (1995). Mitochondrial DNA Analysis Confirms the Existence of a Genetically Divergent Walleye Population in Northeastern Mississippi. *Transactions of the American Fisheries Society* **124**, 770-776.
- Cadwallader, P.** (1995). The Need for a Responsible Approach to Fish Stocking in Queensland. In "Fish Stocking in Queensland: Getting it Right!". (Eds P. Cadwallader & B. Kerby.) pp. 2-7. Proceedings of the Symposium held in Townsville, Queensland, 11 November 1995. (Queensland Fisheries Management Authority: Fortitude Valley).
- Danzmann, R.G., Ferguson, M.M., and Arndt, S.K.A.** (1993). Mitochondrial DNA variability in Ontario and New York rainbow trout *Oncorhynchus mykiss* L. *Canadian Journal of Zoology* **71**, 1923-1933.
- Grewe, P.M., Krueger, C.C., Aquadro, C.F., Bermingham, E., Kincaid, H.L., and May, B.** (1993). Mitochondrial DNA Variation among Lake Trout *Salvelinus namaycush* L., Strains Stocked into Lake Ontario. *Canadian Journal of Fisheries and Aquatic Sciences* **50**, 2397-2403.
- Hall, H.J., and Nawrocki, L.W.** (1995). A rapid method for detecting mitochondrial DNA variation in brown trout, *Salmo trutta*. *Journal of Fish Biology* **46**, 360-364.
- Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Pääbo S., Villablanca, F.X., and Wilson, A.C.** (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the USA* **86**, 6196-6200.
- Meyer, A., Kocher, T.D., Basasibwaki, P., and Wilson, A.C.** (1990). Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature* **347**, 550-553.

- Morizot, D.C., Schmidt, M.E., Carmichael, G.J., Stock, D.W., and Williamson, J.H. (1990).** Minimally invasive tissue sampling. In "Electrophoretic and Isoelectric Focusing Techniques in Fisheries Management". (Ed D.H. Whitmore.) pp. 143-156. (CRC Press: U.S.A.).
- Mork, J., and Heggberget, T.G. (1984).** Eggs of Atlantic salmon *Salmo salar* L., and trout *S. trutta*: identification by phosphoglucosomerase zymograms. *Fisheries Management* **15**, 59-67.
- Pogson, G.H., Mesa, K.A., and Boutilier, R.G. (1995).** Genetic Population Structure and Gene Flow in the Atlantic Cod *Gadus morhua* L.: A Comparison of Allozyme and Nuclear RFLP Loci. *Genetics* **139**, 375-385
- Russell, D.J. (1995).** Measuring the Success of Stock Enhancement Programs. In "Fish Stocking in Queensland: Getting it Right!". (Eds P. Cadwallader & B. Kerby.) pp. 72-78. Proceedings of the Symposium held in Townsville, Queensland, 11 November 1995. (Queensland Fisheries Management Authority: Fortitude Valley).
- Russell, D.J., and Hales, P.W. (1992).** Evaluation of techniques for marking juvenile barramundi *Lates calcarifer* (Bloch), for stocking. *Aquaculture and Fisheries Management* **23**, 691-699.
- Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988).** Primer-directed enzymatic amplification of DNA with a thermostable polymerase. *Science* **239**, 487-491.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989).** *Molecular cloning: a laboratory manual* (2nd ed.). (U.S.A.: Cold Spring Harbour Laboratory Press).

- Stuart, T.A.** (1958). Marking and Regeneration of Fins. *Freshwater and Salmon Fisheries Research Publication No.22.* (Scottish Home Department: Edinburgh).
- Vuorinen, J., and Piironen, J.** (1984). Electrophoretic identification of Atlantic salmon *Salmo salar* L., brown trout *S. trutta*, and their hybrids. *Canadian Journal of Fisheries and Aquatic Sciences* **41**, 1834-1841.
- Ward, R.D., Billington, N., and Hebert, P.D.N.** (1989). Comparison of Allozyme and Mitochondrial DNA Variation in Populations of Walleye, *Stizostedion vitreum* L. *Canadian Journal of Fisheries and Aquatic Sciences* **46**, 2074-2084.
- Ward, R.D., Elliot, N.G., and Grewe P.M.** (1995). Allozyme and Mitochondrial DNA Separation of Pacific Northern Bluefin Tuna, *Thunnus thynnus orientalis* (Temminck and Schlegel), from Southern Bluefin Tuna, *Thunnus maccoyii* (Castelnau). *Marine and Freshwater Research* **46**, 921-930.
- Whitmore, D.H., Thai, T.H., and Craft, C.M.** (1992). Gene Amplification Permits Minimally Invasive Analysis of Fish Mitochondrial DNA. *Transactions of the American Fisheries Society* **121**, 170-177.
- Wingo, W.M., and Muncy, R.J.** (1984). Sampling walleye blood. *Progressive Fish-Culturalist* **46**, 53-55.

