Stock delineation of the pink ling (*Genypterus blacodes*) in Australian waters using genetic and morphometric techniques

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OBJECTIVES:

1. Delineate stock structure of pink ling in Australian waters so that separate management plans for each stock can be developed if required.

2. Examine the nature and relationship of orange and pink forms of ling so that management plans for the deepwater and inshore components of the fishery can be properly integrated.

3. To collect biological information, particularly in regards to life history and reproduction, that can be used together with stock delineation results and other information to develop yield estimates so that TACs can be reviewed.

1. NON-TECHNICAL SUMMARY

Lings (genus *Genypterus*) are now amongst the most commercially important fishes of the South East Fishery (SEF) with an annual catch of 2200 tonnes worth A$5.6 million. Increased consumer acceptance, demand and price, have been accompanied by pressures to expand the fishery, particularly in the western sector. The fishery managers, the Australian Fisheries Management Authority, have highlighted concerns that need to be addressed before the total allowable catch is increased. Two basic research needs are to resolve species composition and stock structure of ling in the fishery.

Two species of ling are currently taken in the fishery. A minor commercial species, the estuarine rock ling (*G. tigerinus*), is rarely caught in quantity by trawlers. The pink ling (*G. blacodes*), which occurs more widely on the continental shelf and upper slope, forms the bulk of the SEF catch. Pink ling is known to occur in two colour forms: a shallow-water orange morph and a deep-water pinkish morph. It had been suggested that these might represent separate species. Pink ling are currently managed as a single unit stock implying that increased fishing pressure in one area would affect biomass across the fishery. However, industry noted

differences in catchability and size composition between different fishing grounds that could be due to multiple stocks.

A multi-disciplinary study using several genetic and morphological approaches was undertaken by CSIRO Marine Research (CMR) to examine both species and stock structure of pink ling. The genetic techniques included allozyme electrophoresis, DNA microsatellites, and mitochondrial DNA sequencing. The morphological analyses used a combination of meristic data (fin ray, vertebrae, and pyloric caecae counts), head and body morphometry, and otolith shape. The study was linked to other CMR projects focusing on ling stock assessment and life history.

Genetic and some of the non-genetic techniques were able to distinguish between pink ling and rock ling. Pink ling, rock ling and the closely related South African kingklip (*G. capensis*) were distinguished by the only genetic technique, mitochondrial DNA sequencing, that was applied to all three species. However, no evidence was found from any of the techniques used to indicate that the pink and orange morphs are different species. Hence we suggest that they should be regarded as different forms of the same species. Pink ling from Australia and New Zealand are also considered to be the same species.

Pink ling populations from eastern Tasmania, western Tasmania, eastern Victoria, western Victoria, and New South Wales were examined to determine their stock structure. Genetic data were gathered for three intraspecifically variable allozyme loci and nine variable microsatellite loci. Morphometric and meristic data were also collected from each population. An initial comparison of morphological data from the extremities of the SEF region provided some evidence for more than one stock. However, later in the project, analyses including genetic and morphological data from across the region, were unable to refute the working hypothesis of a single stock.

Specimens obtained from western subregions tended to have wider and thicker otoliths than those from eastern subregions. However, otolith dimension also varied with fish size - an effect that could not be entirely eliminated by statistical methods. Fish from the western subregions were mostly larger than those from the eastern subregions so it was not possible to confidently distinguish between the effects of size and possible regional differences. To further complicate matters, otolith shape varies significantly aomng ling of similar size and from the same population.

The average pectoral-fin ray count for NSW specimens exceeded the average for eastern Tasmania. However, other subregions had intermediate averages and when all subregions were compared, differences between subregions were not statistically significant. None of the other meristic characters (i.e. dorsal-fin ray, anal-fin ray, pectoral-fin ray, precaudal vertebrae, caudal vertebrae, and pyloric caecae) differed significantly between subregions. Pink ling appear to be exceptionally variable in dorsal and anal-fin ray counts, both within the species and within populations, compared to other bony fishes making them poor candidates to perform this type of analysis.

The average relative head and jaw lengths of material from NSW, and western Tasmania appeared to be shorter than those of specimens from other subregions. However, this

difference is more likely to be attributable to errors associated with shrinkage and distortion during freezer storage than stock differences.

In conclusion, the evidence does not enable us to reject the hypothesis of a single stock. There is some weak morphological evidence that direct mixing between some sectors of the SEF may be partly restricted. However, all the genetic evidence indicates sufficient mixing to eliminate regional differences in the characters examined.

KEYWORDS: pink ling, *Genypterus blacodes*, stock structure.

2. BACKGROUND

The ling catch of the South East Fishery (SEF) has grown rapidly in size and significance in recent years. The pink ling is now the third most valuable species with a landed catch value of A$5.6 million in 1997. The total ling catch for that year was 2200 tonnes, making it the fifth largest in the fishery (Caton *et al*., 1998; Tilzey, 1999). However, a lack of information on stock structure together with unresolved problems surrounding species identification has hindered effective management of the fishery.

Lings (genus *Genypterus*) are elongate, cod-like fishes that are confined to the continental shelf and slope of the Southern Hemisphere (Cohen and Nielsen, 1978). Two species occur in Australian waters: the rock ling (*G. tigerinus*) which lives on inshore reefs and estuaries; and a SEF quota species, the pink ling (*G. blacodes*) which occurs more widely on the continental shelf and slope. The pink ling is much more important commercially than the rock ling. In Australia, the pink ling has two colour forms: a shallow water orange form and a deeper water pink form. Until recently, it was thought that these forms may be separate species of ling but a recent study (FRDC project 94/152) suggested that they may represent different growth stages of the same species. This issue has never been fully resolved.

The biology of lings in Australian waters is not well understood. Pink ling live in a variety of habitats but appear to be most abundant on soft muddy substrates into which they burrow. In Australia, spawning probably takes place during winter and spring (Kailola *et al*., 1993; Tilzey, 1994). Pre spawning pink ling are known to aggregate during autumn in northern New Zealand (Roberts, 1987). Pelagic ling larvae of both ling have been caught in surface waters around the southeastern Australian continental shelf and upper slope in all months of the year except June (Bruce and Furlani, pers. com.). Pink ling larvae reach at least 24 mm before settlement, suggesting that they probably remain in the water column for several months (Bruce, pers. com.). Maturity is attained at sizes of 50 cm or over. Adults are caught on the upper slope in 300–800 m (Last, 1983) whereas juveniles occur in shallower water on the continental shelf and upper slope. Pink ling attain a maximum size of about 160 cm and 20 kg (Yearsley *et al.*, 1999) and reach 26 years (Tilzey, 1999). Typical trawl caught ling are 50–90 cm, 0.6–4.5 kg (Yearsley *et al.*, 1999) and 3–6 years old (Kailola *et al*., 1993). New Zealand studies found that small ling eat mainly crustaceans whilst larger animals eat mainly fish. (Mitchel, 1984; Clark, 1985). There have been fewer studies of the food habits of pink ling in Australia. Industry representatives have suggested that Australian ling may move onto commercial fishing grounds to scavenge discarded bycatch. The stomachs of large South African ling, or kingclip (*G. capensis*), caught on commercial grounds often contain the heads of discarded fish bodies **(**Macpherson, 1983**)**.

Pink ling have traditionally been caught by demersal trawling throughout the SEF region, as well as within the Great Australian Bight (GAB) and Western Australian trawl fisheries. Major commercial catches are taken in NSW between Cape Howe and Uladulla and off the west coast of Tasmania. In past decades, the Australian ling catch has been taken primarily as by-catch after targeting grenadier, gemfish and royal red prawns (Tilzey, 1994). Whilst the catch values of some SEF quota species, such as orange roughy, have declined over recent years, the value of the ling component of the fishery has increased in response to market demand.

The total catch has increased from 790 tonnes in 1984 to around 2200 tonnes (for all methods) in 1997 (Tilzey, 1999).

Prior to 1998 there were no restrictions on non-trawl targeting of ling. More inshore vessels are now targeting ling and other vessels are beginning to use more efficient fishing techniques to increase catches. New Zealand landings of ling have doubled in the last decade through the introduction of longliners equipped with autoline gear (Colman, 1995). This equipment has now been installed on one Australian vessel and its wider adoption could bring the fishery under increased pressure. Other catches of ling in Australian waters using non-trawl methods such as droplining and set-netting increased rapidly until 1998 when the ling total allowable catch (TAC) became global (including both trawl and non-trawl methods) and was set at 2191 t. Presently, there are no yield estimates for ling (Tilzey, 1994) and a conservative approach to managing the fishery has been taken. They are being managed as a single stock and the size of this stock is being assessed in a separate CMR study. In recent samples from Eden and Ulladulla there was a marked decline in the proportion of older fish and an increased estimate of total mortality compared to that of the mid to late 1980's (Tilzey, 1999).

Knowledge of the stock structure of ling essential for their management has been examined in some detail by other countries. In New Zealand, morphometric studies have found evidence of at least three separate stocks in the New Zealand EEZ. Allozyme and morphometric studies indicated that populations in the sub Antarctic Zone and to the south of the South Island are isolated from those to the west of the South Island and from the Chatham Rise (Smith and Francis, 1982; Tilzey, 1994; Colman, 1995). Studies of a closely related species, the kingklip, on the south-east coast of South Africa have identified a total of three stocks based on otolith morphology and growth rate (Payne, 1977; 1985).

Despite the vulnerable nature of pink ling, almost nothing is known of its stock structure in Australian waters. Differences in catchability to the east and west of Bass Strait raise the possibility of separate stocks occurring in the eastern and western sectors of the SEF. Industry has also reported differing size compositions of catches between these sectors. The recent study by Colman (1995), which has developed an understanding of stock differences in New Zealand ling, highlights the potential for similar differences to exist in Australian waters.

Various genetic and non-genetic methods have been used to determine the structure of fishery stocks. Traditionally simple genetic methods, such as allozyme electrophoresis have tended to be more conservative (i.e. less likely to find stock differences) than other methods. However, more powerful, more expensive, contemporary techniques, such as DNA microsatellites, have found differences between populations that were not evident when other genetic techniques were used. Mitochondrial DNA sequencing is particularly useful for identifying differences between species.

Morphological methods have been used more often than genetic techniques to examine the stock structure of species. They are often more successful in finding population differences (e.g. Elliot *et al.*, 1995). However, morphometric and meristic characters are known to be affected by inherited factors as well as non-inherited factors such as water temperature and specimen size. These variables can make observed differences between populations difficult to

interpret. With genetic studies the results are easier to interpret because the results only reflect inherited factors.

The relative merits of genetic and non-genetic techniques have been debated widely in the literature with little consensus on the best individual method. A multi-disciplined study combining both general methodologies is considered to be the most powerful overall approach giving an estimate of the level of population mixing as well as the likely number of stocks.

3. NEED

There are two basic needs for the ling fishery that are addressed by this study:

1) It is important to determine whether pink ling from WA, the GAB, and the eastern and western sectors of the SEF represent separate stocks. If so, there may be a need for a separate management plan for each. Otherwise, a single large stock is probably best managed as a single entity. Industry has noted the potential for development of the fishery in the western sector. Hence, it is essential to establish whether or not the developing western fishery is distinct from the more fully exploited eastern fishery. Stock delineation work needs to be carried out before reliable biomass and yield estimates of stocks can be derived, and to assist with the development of existing management plans.

2) It is necessary to establish whether the shallower-water orange colour morph is the juvenile form of the pink ling, or whether it is a distinct species. This information is necessary to integrate non-trawl and trawl components of the fishery. Gear types, such as set netting, that can affect the smaller orange ling biomass may need to be managed if the morph is found to be a juvenile. The lack of such protection may otherwise impact on recruitment to the deepwater part of the fishery, which represents almost all the economic value of ling. Whilst preliminary allozyme analysis indicates the two colour forms of ling are the same species, there is a need to confirm this using more powerful genetic techniques.

4. OBJECTIVES

1. Delineate stock structure of pink ling in Australian waters so that separate management plans for each stock can be developed if required.

2. Examine the nature and relationship of orange and pink forms of ling so that management plans for the deepwater and inshore components of the fishery can be properly integrated.

3. To collect biological information, particularly in regards to life history and reproduction, that can be used together with stock delineation results and other information to develop yield estimates so that TACs can be reviewed.

5. METHODS

5.1. Acquisition and processing of specimens

Samples were obtained from across the SEF - New South Wales, eastern and western Victoria, and eastern and western Tasmania (Table 5.1). Difficulties were experienced in acquiring material from other regions. Only one specimen was obtained from Western Australia and only four from South Australia. Specimens were collected by several means—from industry sources, state fisheries agencies, CMR field surveys, and by industry liaison including the circulation of specimen request posters (Appendix C). Where possible, samples from each of the following depth categories were obtained from each region: shallow (<150 m), medium (150–350 m) and deep (>350 m). Collectors were asked to obtain ling between 45 and 65 cm total length (TL). This target range was selected to minimise size effects on shape, ensure that specimens were large enough to sex, and minimise the purchase costs of samples.

Table 5.1: Collection details for ling samples.

Subregion	n	Locality	Depth (m)	Collector	Date
NSW	22	Disaster Bay	78	R. Daley, CSIRO	Feb-94
NSW	10	Brush Island	117	K. Graham, NSW Fisheries	Mar-94
NSW	35	Bermagui	455	A. Jubb	Feb-98
NSW	33	Ulladulla	no record	D. Makin, NSW Fisheries	Jun-98
VIC(E)	42	Lakes Entrance	150	K. Smith, ISMP	Dec-97
VIC(E)	29	Lakes Entrance	150	K. Smith, ISMP	Feb-98
VIC(E)	6	miscellaneous	various	various	various
VIC(W)	26	Portland	756	Ken Smith, ISMP	Jul-97
VIC(W)	34	Portland	200	Ken Smith, ISMP	Feb-98
TAS(E)	5	D'Entrecasteau Channel	54	A. Faulkner, AMC	Jan-98
TAS(E)	5	Derwent River	no record	CSIRO	no record
TAS(E)	3	Fortescue Bay	117	A. Faulkner, AMC	May-94
TAS(E)	2	Hippolyte Rock	79	A. Faulkner, AMC	May-94
TAS(E)	9	Maria Island	50	A. Faulkner, AMC	Nov-97
TAS(E)	3	miscellaneous	various	various	various
TAS(E)	54	Hippolyte Rock	216	G. Carney	Jan-98
TAS(E)	2	Tasman Island	270	A. Faulkner, AMC	Mar-94
TAS(E)	8	Maria Island	510	S. Davenport, CSIRO	Jul-93
TAS(E)	8	Maria island	54	C. Massey	Mar-94
TAS(E)	12	Tasman Island	423	W. Baker	Apr-98
TAS(E)	11	Hippolyte Rock	400	A. Faulkner, AMC	Apr-94
TAS(W)	190	off Strahan	430	M. Wilson, AMC	Oct-96
TAS(W)	3	west coast TAS	530	Petuna Seafoods	Oct-95
TAS(W)	20	west coast TAS	400	Petuna Seafoods	Jan-97

Samples were stored frozen at -20ºC at the CMR laboratories in Hobart. Specimens were later thawed in batches, weighed and measured, and their body colour recorded. They were then partly dissected to remove tissues for genetic analysis: the right eye, part of the liver, and

approximately 2 g of muscle from the right side. After removing the tissue samples, the body cavity was cut open and pyloric caecae were counted before the gut was removed. Specimens longer than 400 mm were sexed. The pectoral fin and first gill arch were then dissected from the right side of each specimen and both saggital otoliths were dissected from the cranium. Specimens were then x-rayed, re-frozen and then stored until the end of the project. Gill rakers and dissected pectoral fins were preserved in 10% formalin for at least 1 week and then transferred to 70% ethanol. All morphological data were collected by the same person (i.e. Ross Daley) to keep the methodology as consistent as possible.

5.2. Research strategy

The combination of methods was needed to meet the objectives of the study and a two phase structure was proposed over two years. An initial investigation using morphological and allozyme techniques commenced in the first year of the study. The results were then reviewed based on this initial investigation. As part of the data provided some evidence for more than one stock the project continued into a second year using more sophisticated microsatellite genetics.

5.3. Morphological study

5.3.1. Meristics

Lings have long dorsal and anal fins comprised of many fin rays that are often obscure and difficult to count. To minimise the likelihood of errors, dorsal and anal-fin rays, as well as vertebral centra, were counted from radiographs using a hand lens where necessary. Vertebral counts include the last tail vertebrae or urostyle. Pectoral-fin counts were taken by dissecting the fin from the body, skinning the fin and then counting the rays under a stereo microscope with transmitted light. Counts made without removing the thick fleshy covering over the fin were often inaccurate. Pyloric caecae and gill rakers were counted using a stereo dissecting microscope. Atypically short caecae were still counted as one.

5.3.2. Body morphometry

The following standard measurements were taken using vernier callipers:

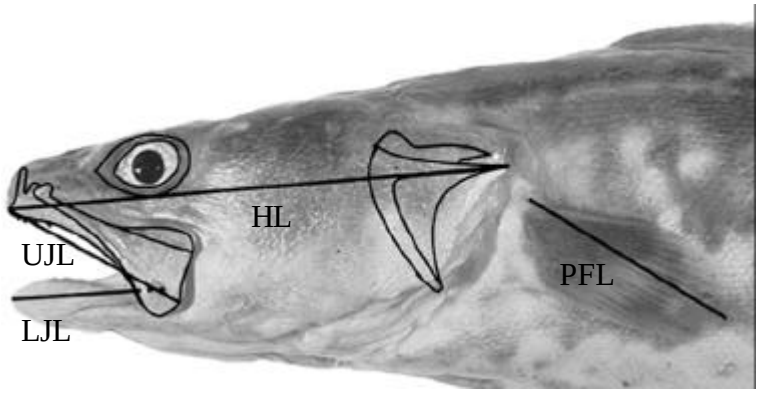
standard length (SL);
total length (TL);
head length (HL);
interorbital width (IOW);
upper jaw length (UJL);
lower jaw length (LJL);
pectoral-fin length (PFL); and

snout–anus distance (S–A).

These measurements follow Hubbs and Lagler (1958) and Colman (1995), with the following modifications and clarifications:

Standard length, total length and snout–anus were taken as minimum horizontal distances, the remaining measurements were taken directly from point to point on the left side of the body. Measurements taken from the snout tip were taken from the anterior tip of the upper jaw by pressing lightly on the soft tissue. Head length was measured from the snout to the tip of the opercular spine. Head length, upper and lower jaw, and pectoral-fin lengths follow Figure 5.1.

Figure 5.1: Head and pectoral-fin measurements



5.3.3. Otolith morphometry

The following otolith measurements were taken from point to point using Vernier callipers:

maximum length;
maximum width; and
maximum thickness, excluding any abnormal protuberances.

5.3.4. Morphological analyses

Data sets were analysed using several techniques on pooled and un-pooled data. Data pooling was required within some subregions in this study to obtain sufficient sample sizes. Pooling of sub-samples within geographic subregions is often considered to be undesirable. It can lead to within sample temporal or spatial variation which may affect the resolving ability of analyses as well as minimising between sample variation. In addition, certain factors are able to obscure genuine morphometric differences between regions, or highlight differences that do not really exist. These include differences in average specimen length, proportional measurements that change with size (allometry), non-normally distributed data, and sexual dimorphism. Various steps were taken to eliminate these undesirable effects.

Body and otolith measurements were initially expressed as proportions of total length or head length. Ratios and counts were then regressed against TL to determine if size was likely to bias the analyses. Average total lengths and sex ratios were compared between subregions using t-tests.

Measurements were normalised using the following equation in an attempt to eliminate the effects of allometry (Elliot *et al.*, 1995):

$$M_1 = M_0(TL_x/TL_1)^r$$

Where:

M_1=the normalised measurement;
M_0=the original measurement;
TL_x =the mean total length for all specimens examined for a particular character;
TL_1=the total length of the specimen from which M_1 was taken for a particular character; and
r=the slope of the log log regression of the measurement being examined and TL.

Normalised characters were again regressed against total length. Where normalised characters had slopes not significantly different from zero it was considered that the effect of size had been eliminated. These normalised measurements were then log transformed. This is a commonly used transformation for allometric characters (Haddon and Willis, 1995) and ensures the data are normally distributed.

One-way ANOVA of normally distributed data was used to identify characters that varied significantly between subregions. Two-way ANOVA was used to examine whether depth or the interaction between depth and subregion contributed to within and between group variance. For otolith measurements, where significant differences were found, Bonferroni pre-adjusted post-hoc tests were used to examine which sample sites were most dissimilar (SYSTAT 9 software) for these tests the significance level is 0.05. For other multiple tests undertaken the significance level α of 0.05, was adjusted using Bonferroni procedures. Generally, the α level was divided by the number of tests to derive a new α level, and *P* values had to be less than this corrected α value to be deemed significant.

All counts except dorsal and anal-fin ray counts were non-normally distributed. Therefore between subregion and between depth comparisons were made using chi-square analysis with CHRXC software **(**Zaykin and Pudovkin, 1993).

Discriminant function analyses using SYSTAT 9 software **(**SPSS Software, 1999**)** were used to examine possible structure in populations of the SEF and to determine how well members of a particular geographic group define that group. When populations within the subregion defined by groups can be plotted as distinct entities, and/or a high proportion of specimens are reassigned to their group of origin, structural differences in the populations are inferred. The jackknifed classification matrix was used to validate the classification and prevent the analysis from producing over optimistic classifications (SYSTAT 9 software).

Ling have been found to display significant within sample variation in otolith and head shape due to sexual dimorphism (Colman, 1995) so morphometric data for the sexes were grouped separately. However, ling are not known to display meristic dimorphism so these data were pooled within subregions.

5.4. Genetic study

5.4.1. Allozyme electrophoresis

Allozyme variation was examined using Helena Titan III cellulose acetate plates with a Tris-glycine (pH 8.5) (0.02 M tris, 0.192 M glycine; see Hebert and Beaton (1989) for further details) or a 75 mM Tris-citrate (pH 7.0) buffer system (see Richardson *et al.*, 1986 for further details). Small pieces of liver or muscle were placed in 1.5 ml micro centrifuge tubes, homogenised manually with a few drops of distilled water, and spun in a micro centrifuge at 10 000 g for 5 min. The supernatant was used for electrophoresis. Table 5.2 lists the enzymes and buffers used. Tris-glycine gels were run at 200V at room temperature, typically for 30 min. Tris-citrate gels were run at 100v at 4°C, typically for 60 min. Staining procedures follow those of Hebert and Beaton (1989) and Richardson *et al.*, (1986). Three different dipeptides were used in the aminopeptidase stains: *APlgg* used leucyl-glycyl-glycine, *APlt-1* and *-2* used leucyl-tyrosine, and *APpp* used phenylalanyl-proline. Coomassie blue was used for the general protein stain. Where two loci are suffixed -1, -2 or -3, the -1 suffix denotes the fastest migrating enzyme.

Notes on some of the enzymes

AAT.
AAT run using Tris-glycine (TG) reveals more alleles for *AAT-1* than AAT run on Tris-citrate (TC). The two fastest alleles on TG resolve as a single allele on TC, as do the next two mobility classes. In total, TG resolves 5 alleles and TC 3 alleles. However, TC reliability and ease of scoring is greater than using TG, and the results given here are for TC runs.

CK.
CK-2 gives two banded heterozygotes. This is typical for teleosts (Ferris and Whitt, 1978; Elliott and Ward, 1992), although the enzyme is known to be dimeric. The *CK-1* polymorphism in rock ling could be scored on coomassie blue protein stained gels and is none of the general proteins *PROT-1* to *PROT-3*.

IDH.
The liver-specific *IDH-1* and muscle-specific *IDH-2* have very similar mobilities.

GPI.
The products of the two GPI loci migrate close together, with *GPI-1* being a little faster and less active.

PROT.

The pink ling had a clear fast migrating protein band encoded by the *PROT-1* locus which was very weak or absent in rock ling. Hence pink ling was monomorphic for an active allele at this locus, termed 'm' in Table 5.3, while the rock ling was monomorphic for a null allele, termed 'o' in Table 5.3. The protein fingerprints are shown in Daley *et al*., (1997) and Yearsley *et al*., (1999).

Table 5.2: Allozyme loci assayed with buffer type.

Enzyme or protein name	Locus	EC No.	Buffer	Tissue	Structure
Acid phosphatase	*ACP*	3.1.3.2	TG	l	monomer
Adenosine deaminase	*ADA-1*	3.5.4.4	TG	l, m	monomer
Alcohol dehydrogenase	*ADH*	1.1.1.1	TG	l	dimer
Aspartate aminotransferase	*AAT-1*	2.6.1.1	TC/TG$^+$	m, l	dimer
	AAT-2	2.6.1.1	TC/TG	m, l	
Aminopeptidase	*APlgg*	3.4.11/13	TG	m	dimer
	Aplt-1	3.4.11/13	TG	m	dimer
	Aplt-2	3.4.11/13	TG	m	
	APpp	3.4.11/13	TG	m	dimer
Creatine kinase	*CK-1*	2.7.3.2	TG	e	
	CK-2	2.7.3.2	TG	m	monomer$^+$
	CK-3	2.7.3.2	TG	e	dimer
Esterase-D	*ESTD-1*	3.1.1.1	TG	l	
	ESTD-2	3.1.1.1	TG	l	
Fumarase	*FH*	4.2.1.2	TG	m	
Glyceraldehyde-phosphate	*GAPDH-1*	1.2.1.12	TG	e, m	
dehydrogenase	*GAPDH-2*	1.2.1.12	TG	m	
Glycerol-phosphate	*GPDH-1*	1.1.1.8	TG	m	
dehydrogenase	*GPDH-2*	1.1.1.8	TG	l	
Glucose phosphate	*GPI-1*$^+$	5.3.1.9	TG	m	dimer
isomerase	*GPI-2*$^+$	5.3.1.9	TG	m	dimer
Iditol dehydrogenase	*IDDH*	1.1.1.14	TG	l	tetramer
Isocitrate dehydrogenase	*IDH-1*$^+$	1.1.1.42	TC	l	dimer
	IDH-2$^+$	1.1.1.42	TC	m	
Lactate dehydrogenase	*LDH-1*	1.1.1.27	TG	e	
	LDH-2	1.1.1.27	TG	m	
Malate dehydrogenase	*MDH-1*	1.1.1.37	TG	m	
	MDH-2	1.1.1.37	TG	m	
Malic enzyme	*ME*	1.1.1.40	TG	m	
Mannose-6-phosphate isomerase	*MPI*	5.3.1.8	TG	l	monomer
Octanol dehydrogenase	*ODH*	1.1.1.73	TG	l	dimer
Phosphoglucomutase	*PGM-1*	5.4.2.2	TG	l, m	monomer
	PGM-2	5.4.2.2	TG	m	monomer
Phosphogluconate dehydrogenase	*PGD*	1.1.1.44	TC	l	dimer
General protein stain	*PROT-1*	-	TG	m	
	PROT-2	-	TG	m	
	PROT-3	-	TG	m	
Superoxide dismutase	*SOD*	1.15.1.1	TG	l	

Tissue used: m = white muscle, l = liver, e = eye, preferred tissue first. Assumed quaternary structure (from heterozygote banding patterns) given for polymorphic enzymes.$^+$ see text for details.

Table 5.3: Allozyme allele frequencies in *G. tigerinus* (rock ling) and *G. blacodes* (pink ling), and in the pink and orange morphs of *G. blacodes*.

Locus	Allele	*G. tigerinus*	*G. blacodes*	*G. blacodes*	
				pink	orange
Species-diagnostic loci					
IDDH	*m*	1.000	-	-	-
	s	-	0.857	1.000	0.818
	vs	-	0.143	-	0.182
	n	6	14	3	11
SOD	*m*	1.000	-	-	-
	s	-	1.000	1.000	1.000
	n	6	16	4	12
ME	*m*	1.000	-	-	-
	s	-	1.000	1.000	1.000
	n	6	10	3	7
APlt-1	*f*	-	0.031	-	0.042
	m	-	0.969	1.000	0.958
	s	1.000	-	-	-
	n	6	16	4	12
APpp	*m*	1.000	0.031	-	0.042
	s	-	0.969	1.000	0.958
	n	6	16	4	12
PROT-1	*o*	1.000	-	-	-
	m	-	1.000	1.000	1.000
	n	6	16	4	12
Variable but non-diagnostic loci					
ADH	*f*	-	0.094	0.125	0.083
	m	0.583	0.906	0.875	0.917
	s	0.417	-	-	-
	n	6	16	4	12
ODH	*f*	-	0.031	-	0.042
	m	1.000	0.906	0.875	0.917
	s	-	0.063	0.125	0.042
	n	6	16	4	12
AAT-1	*f*	-	0.250	0.500	0.182
	m	1.000	0.750	0.500	0.818
	n	6	14	3	11
MPI	*f*	0.167	0.071	-	0.091
	m	0.833	0.929	1.000	0.909
	n	6	14	3	11
PGM-1	*f*	0.167	-	-	-
	m	0.833	0.962	1.000	0.950
	s	-	0.038	-	0.050
	n	6	13	3	10
PGM-2	*f*	0.083	0.133	-	0.167
	m	0.917	0.867	1.000	0.833
	n	6	15	3	12
ADA	*f*	0.083	-	-	-
	m	0.750	0.667	0.667	0.667
	s	0.083	0.300	0.333	0.250
	vs	0.083	0.033	-	0.083
	n	6	15	3	12
IDH-1	*f*	0.083	0.094	0.125	0.083

	m	0.917	0.906	0.875	0.917
	n	6	16	4	12
PGD	*f*	-	0.033	-	0.042
	m	1.000	0.967	1.000	0.958
	n	6	15	3	12

Table 5.3 Continued

Locus	Allele	*G. tigerinus*	*G. blacodes*	*G. blacodes*	
				pink	orange
CK-2	*m*	0.750	1.000	1.000	1.000
	s	0.250	-	-	-
	n	6	16	4	12
CK-3	*f*	0.083	-	-	-
	m	0.917	1.000	1.000	1.000
	n	6	16	4	12
ACP	*f*	0.083	-	-	-
	m	0.917	1.000	1.000	1.000
	n	6	10	3	7
APlgg	*f*	-	0.219	0.375	0.167
	m	1.000	0.781	0.625	0.833
	n	6	16	4	12
APlt-2	*m*	1.000	0.969	1.000	0.958
	s	-	0.031	-	0.042
	n	6	16	4	12
GPI-1	*f*	-	0.031	0.125	-
	m	1.000	0.969	0.875	1.000
	n	6	16	4	12
GPI-2	*m*	1.000	0.969	1.000	0.958
	s	-	0.031	-	0.042
	n	6	16	4	12
Invariant loci					
AAT-2	*m*	1.000	1.000	1.000	1.000
	n	6	15	3	12
GPDH-1	*m*	1.000	1.000	1.000	1.000
	n	6	16	3	12
GPDH-2	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
LDH-1	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
LDH-2	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
CK-1	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
GAPDH-1	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
GAPDH-2	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
IDH-2	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
MDH-1	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
MDH-2	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
FH	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12
ESTD-1	*m*	1.000	1.000	1.000	1.000
	n	6	10	3	7
ESTD-2	*m*	1.000	1.000	1.000	1.000
	n	6	10	3	7
PROT-2	*m*	1.000	1.000	1.000	1.000

	n	6	16	4	12
PROT-3	*m*	1.000	1.000	1.000	1.000
	n	6	16	4	12

5.4.2. Microsatellite DNA markers

Construction of microsatellite library

A pink ling genomic DNA library was constructed from the DNA of a single individual. DNA was extracted from 10 samples of 50 mg of muscle tissue (Sambrook *et al*., 1989). Approximately 12 *m*g of genomic DNA was digested to completion with 100 units of *Sau3*AI restriction enzyme to generate small fragments. These fragments were size-fractionated on a 1% TBE (Tris-Borate-EDTA) agarose gel. The sample was loaded with size standards (100 base pair (bp) marker, Promega) on either side to assist with size selection. Exposure time of the gel to UV light was minimised to prevent DNA degradation and to allow good recovery of DNA from the gel. DNA was shielded from UV light during DNA detection by placement of foil strips over the sample area. The gel was photographed with size standards visible and with a ruler alongside to assist in sizing.

The 500–750 bp fraction was excised from the gel and the DNA extracted and purified by a GenecleanII gel extraction kit (Bio101). The fragments were then ligated into the dephosphorylated *Bam*HI site of the plasmid pGEM3Zf(+) (Promega) by T4 DNA ligase. The plasmid vector, pGEM® 3Zf(+) (Promega), was prepared by digesting approximately 19 *m*g of plasmid with 80 units of *Bam*HI, followed by treatment with 1 unit of calf intestinal phosphatase (Pharmacia) to remove the 5' phosphate groups. The enzyme was heat inactivated at 75°C and the vector purified by phenol/chloroform extraction, followed by ethanol precipitation of the DNA.

Ligations were set up as 10 *m*l reactions to which was added 1 mM ATP and 1 Weiss unit of T4 DNA ligase (Pharmacia). Reactions were incubated for 4 hours at 16°C. The optimum molar ratio of vector to insert cohesive ends, providing the highest proportion of insert containing clones, was determined as 1:2. This ratio was achieved using 400 ng vector and 78 ng insert. Half of the ligation reaction was transformed into Stratagene XL-1 Blue supercompetent cells by heat shock treatment, according to the recommended protocol. Aliquots of 80 *m*l of transformation mix were spread onto LB (Luria-Bertani) agar plates, containing ampicillin, as well as X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) and IPTG (isopropylthio-β-D-galactoside) for blue/white color colony selection. Following overnight incubation at 37°C, recombinant clones were detected as white colonies.

The library, consisting of about 11 000 recombinant clones, was screened for the presence of dinucleotide (CA) and trinucleotide (AAT, AAC) microsatellites, by the method outlined below.

Non-radioactive screening of microsatellite library

The library was screened using the DIG non-radioactive screening kit (Boehringer Mannheim) using conditions recommended by the manufacturer but with notable exceptions as described in Elliott and Reilly (1998).

End-labeling of probes

$(CA)_9$, $(AAT)_9$ and $(AAC)_8$ oligonucleotides, to be used for screening for microsatellite repeats, were 3' end-labeled with DIG molecules. Briefly, 100 pmol of each oligonucleotide was separately labeled with Digoxigenin-11-ddUTP (2',3'-dideoxyuridine-5'-triphosphate, coupled to digoxigenin) using terminal transferase, in a total volume of 20 *m*l. The mixture was incubated at 37°C for 15 minutes. The reaction was stopped in the presence of 0.4M EDTA (disodium ethylenediaminetetracetic acid) and ethanol precipitated in the presence of glycogen and lithium chloride. Each probe was then dissolved in 20 *m*l sterile water.

Colony hybridisation

The plating of 80 *m*l of transformed library mix provided a density of between 800 and 1300 colonies on each 135mm LB agar plate. Plates were refrigerated for about 1 hour before replica plating the colonies to nylon uncharged membrane filters (Boehringer Mannheim). Colonies on membranes were grown on fresh LB ampicillin plates to approximately 1-2 mm in size, and master plates were incubated for 2 to 3 hours to regenerate colonies.

Membranes were soaked for 15 minutes in a solution containing 0.5M NaOH, 1.5 M NaCl, 0.1% SDS to denature the double-stranded DNA to single-stranded. Membranes were then neutralised by soaking in 1M TrisCl, pH 7.5, 1.5 M NaCl for 15 minutes, and then finally washed in 2 x SSC (sodium chloride, sodium citrate solution). DNA was UV fixed to the membrane by exposing each side of the membrane to UV light for 10 seconds. Bacterial proteins were then removed by proteinase K treatment. Cellular debris were removed by blotting between damp filter papers. Membranes were prehybridised at the calculated melt temperature (T_m) in standard hybridisation buffer containing 5 x SSC, 1% block solution, 0.1% N-lauroyl, 0.02% SDS (sodium dodecyl sulfate). The probe was added at about 60 pmol in 20 mL of hybridisation solution and allowed to hybridise at the calculated T_m for between 2 hours to overnight. For the dinucleotide screening, hybridisation was performed at 52°C, whereas for the trinucleotide screening, the $(AAT)_9$ and $(AAC)_8$ probes were combined and hybridised at 54°C. The membranes were then washed in 2 x SSC, 0.1% SDS at room temperature with gentle rocking. This was followed by stringency washes in 0.5 x SSC, 0.1% SDS at the hybridisation temperature.

Detection

The membrane was equilibrated in washing buffer (150 mM NaCl, 100 mM maleic acid; pH 7.5), with 0.3% Tween 20, and then blocked in a 2% block solution. Anti-DIG alkaline phosphatase conjugate was added at a 10 000-fold dilution in block solution and incubated for 30 minutes. Membranes were then thoroughly washed in washing buffer, before equilibrating in 100 mM TrisCl, pH9.5, 100 mM NaCl. CPD-Star™ substrate, diluted 100-fold in washing buffer, was added at 500 *m*l per membrane for chemiluminescent detection. After the addition of the substrate, the membranes were sealed in plastic and incubated at room temperature for 5 minutes. Filters were exposed to x-ray film for about 10 minutes before developing.

Approximately 120 clones hybridised with the CA dinucleotide probe out of approximately 7000 of the clones screened i.e. ~ 1.6%; and a dozen putative trinucleotide positives were

found amongst the remaining ~ 4000 i.e. ~ 0.3%. Glycerol stocks were prepared for each of the positive clones. Overnight LB/Ampicillin cultures were resuspended in fresh LB media and an equal volume of sterile glycerol added. Cultures were snap frozen in liquid nitrogen and stored at -80°C.

Sequencing and microsatellite primer design

Positive clones were cultured overnight in LB medium – containing ampicillin. Double-stranded plasmid DNA was then prepared as a template for sequencing using the alkaline lysis method (Sambrook *et al*., 1989). DNA was suspended in 20 *m*l of double-distilled water.

The nucleotide sequence of the positive clones was determined with ABI PRISM™ BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer), according to kit instructions, and using 500 ng DNA template per reaction. Extension products were ethanol precipitated (Perkin Elmer Protocol). Sequencing reactions were run on 4% denaturing acrylamide gels on an ABI PRISM 377 DNA sequencer (PE Applied Biosystems).

In some cases sequence was unsuitable for primer design either due to lack of overlap with forward and reverse sequences, poor sequence quality or due to very long microsatellites, complex microsatellites or due to many other repeats in the flanking sequence. Ling appeared very microsatellite-rich considering the frequent occurrence of additional unrelated microsatellites found in flanking regions. Out of 28 clones sequenced, 15 appeared suitable for design of primers.

PCR primer pairs were designed for conserved flanking regions of the microsatellite repeat, using the Oligo primer design package. It was important to avoid regions of repetitive nucleotide sequence and primers were designed so products were smaller than about 200 bp. Other design considerations included avoidance of sequences that may cause primer-dimer formation and internal looping, and assuring to match annealing temperatures of the primer pair. Oligonucleotides were synthesised by Pacific Oligos and the forward primer was labeled with a fluorescent tag.

PCR amplification of microsatellite loci

The 15 loci, for which primers were designed, were evaluated for suitability for population assessment. The evaluation involved PCR amplification of several randomly selected individuals. Some degree of PCR optimisation was required for most loci. PCR products were checked on 2% TBE agarose gels and subsequently profiles were analysed on microsatellite gels and assessed for polymorphism and ease of scoring. Of the 15 loci, nine were selected for the study. The microsatellite motifs and PCR primer sequences for each locus are presented in Table 5.4. Total genomic DNA was isolated as a template from white muscle by either of 2 methods- from 50 mg of tissue by a modified CTAB protocol (Grewe *et al*., 1993) or from 25 mg of tissue using QIAamp tissue kit (Qiagen).

PCR amplifications were performed in a Perkin-Elmer 9600 thermocycler. Individual amplifications were made as 25 µl PCR reactions containing 67 mM Tris-HCl, pH 8.8; 16.6 mM $(NH_4)_2SO_4$; 0.45% Triton X-100; 0.2 mg/ml gelatin; 1.5 or 2.5 mM $MgCl_2$, 0.4 µM forward primer (fluorescent labeled), 0.2 µM reverse primer, 200 µM dNTPs, 0.5 units Taq F1 DNA polymerase (Fisher Biotech); and ~ 20 ng genomic DNA template. Denaturation for 3 minutes at 95°C was followed by 35 cycles made up of 30 seconds at 96°C, 30 seconds at the annealing temperature (Table 5.4) and 1 minute at 72°C. The final step was a prolonged extension of 20 min at 72°C.

The amplified products were diluted, mixed with formamide loading dye containing ABI Prism Gene Scan 350 Tamra size standards (PE Applied Biosystems), denatured by heating to 95°C for 2 minutes and loaded on a 4.8% 6 M Urea denaturing poly gel. The samples were run on an ABI PRISM 377 DNA sequencer and analysed with accompanying software (GENOTYPER®1.1.1.).

Loci were named following the convention used in the Hobart lab. For example, cmrGb5.5 denotes CMR, Gb denotes *Genypterus blacodes* (the species donating the DNA used in clone preparation and subsequent primer sequence identification), and 5.5 identifies the clone from which the primer pair was designed. A locus labeled as "B", describes a different microsatellite locus within the same clone. This means, for instance, that cmrGb5.8 and cmrGb5.8B are unlikely to be truly independent loci.

Of the 15 microsatellite loci evaluated, 2 loci (cmrGb3.8.1 and cmrGb4.11) were not optimised mainly due to time constraints. Other loci were omitted from the study due to poor performance on microsatellite gels. CmrGb4.12 gave only a weak signal, perhaps due to poor efficiency of fluorescent labeling of the oligo; cmrGb5.10 produced many stutter peaks causing scoring difficulties; locus cmrGb4.6 was monomorphic. The remaining loci were all demonstrated to be very polymorphic. Although cmrGb5.2 appeared useful it was not selected for this study since it could not be conveniently co-loaded with various combinations of other loci.

Nine loci were thus selected for the population study. Five of these loci were examined on one gel (cmrGb4.2, blue; cmrGb4.11B, green; cmrGb5.2B, green; cmrGb5.5, yellow; cmrGb5.9, blue) and four loci on a second gel (cmrGb2.6.1, yellow; cmrGb4.2B, blue; cmrGb5.8, green; cmrGb5.8B, yellow). These loci are subsequently referred to as 4.2, 4.11B etc. PCR reactions were co-loaded rather than multiplexed.

Table 5.4: Microsatellite motif, PCR primer sequences (5'– 3'), number of alleles observed, expected size of alleles and locus-specific annealing temperatures (°C) for 15 pink ling microsatellite loci.

Locus	Motif	Primer sequences	[Mg] (mM)	Allele Size[1]	^{o}C
cmrGb4.11B	$(gaca)_{11}$.. $(gaca)_5$..$(ga)_9$	CCT GAG TGC TTA AAG AGG A GAG GAG GAG ACG ATG AAA	2.5	234	54
cmrGb5.5	$(gt)_8tt(gt)_{28}$	ACT CCT GGA CTG GAT CTA A TGC AAA TTT CAT GTA AAT G	1.5	135	50
cmrGb5.9	$(ca)_{11}$	AGG GTC ACT TTC AGT TTT A TGC AGA ACA CAC TCC AC	1.5	137	56
cmrGb4.2	$(taaa)_8$	ATC GGG CAG TTC CTT GCT AT GGG AAG CTT TTG TGA GCA TC	1.5	191	56
cmrGb5.2B	$(cttt)_{19}$	CGG TCT GAG CAA TGA TAC GA TAC AGA GGG GAG GTA AAT CAA GTC	1.5	155	50
cmrGb2.6.1	$(gtt)_9$	AGA ACT AAA CCA GCA GAA TC CAC AAC AAG AGG GAA CTC	1.5	121	53
cmrGb5.8B	$(gt)_{29}$	CAC TTT GGG GCT TCT CCT C CCC GAT TCA TTC ATC CAT C	1.5	151	60
cmrGb4.2B	$(ct)_{16}t(ct)_7$.. $(gt)_{27}$	GAG TTG GTG TTT GCC CTG A GTC TGG AGT GTT TTG GAT CAT T	1.5	170	54
cmrGb5-8	$(gt)_{20}ga(gt)_5$	AAC CTC TGG CAT CCA TTT C CCC AAA GTG CTG CTA CTG	1.5	145	54
cmrGb4.6	$(gga)_2aga(gga)_5($ $aac)_3$	ACA GAT CAG AGC CCT CAG TGG TGG AGC AGA CAG AGT	2.5	119	65
cmrGb4.11	$(ca)_{20}$	AGA CTT GGC TGA GGT ATT CA GAT GGT TTG GGG AAG G	?	184	~52
cmrGb4.12	$(gt)_{32}$	ATT TTA TTT CCC TTG GAC A ACT TGC AGG CAT ACA CAT	1.5	101	50
cmrGb5.2	$(gt)_{30}gc$ $(gt)_2gc$ $(gt)_2gc$ $(gt)_5$	AAA CAG TGT TCG CGT TAC T CCT GAC ATG TGT CGT TGA	1.5	194	54
cmrGb3.8.1	(caa)9	ACG AAC ACG CAG AAG GAC GGT CGT TTC AGG ACA TTA CA	?	111	~56

cmrGb5.10	$(ca)_{55}$	AGA AAT GGG CCA CGG TCA C GGG GGA TAC GGC AAG CAC T	1.5	155	60

The (..) among motif descriptions respesents a gap of several non-repetitive nucleotides. [1] *Expected PCR product size (bp) based on cloned pink ling allele of specified repeat number.* [o] *C = annealing temperature. ?=not optimised*

5.4.3. Mitochondrial DNA sequence analysis

mtDNA extraction

For investigation into mtDNA sequence differences between deep and shallow individuals of *G. blacodes* (pink ling) and interspecific comparisons among several ling species, total genomic DNA was extracted from approximately 150mg of frozen tissue per individual using a modified CTAB (hexadecyltrimethylammonium bromide) extraction protocol described in Grewe *et al*., (1993). Three *G. blacodes* (two shallow; < 200 m and one deep; > 200 m), three *G. tigerinus* (rock ling, estuarine <30 m) and two *G. capensis* (kingklip) were examined (Table 5.5). After precipitation with isopropanol and ethanol, genomic DNA pellets were resuspended in 150 µl of deionized H_2O and stored at 4°C. Stock DNA was diluted to a 1:5 ratio for DNA sequencing.

Table 5.5: Source of ling individuals used in DNA sequencing study.

Species	Depth	Location	Sampling Year
G. blacodes (Pink1)	<200m	eastern Victoria	1998
G. blacodes (Pink2)	120m	eastern Tasmania	1998
G. blacodes (Pink3)	279m	eastern Tasmania	1998
G. tigerinus (Rock1)	<30m	Tasmania	1993
G. tigerinus (Rock2)	<30m	Tasmania	1993
G. tigerinus (Rock3)	<30m	Tasmania	1993
G. capensis (Kingklip1)	?	South Africa	1993
G. capensis (Kingklip2)	?	South Africa	1993

mtDNA cytochrome B amplification

Sequence variation in the three ling species was examined in the cytochrome B (cyt B) region of the mtDNA genome. The cytochrome B gene is well characterised in vertebrate species including several fish species (Kocher *et al*., 1989; Bartlett and Davidson, 1991; Carr and Marshall 1991; Fournier Lockwood *et al*., 1993 and Bennetts *et al*., 1999). Amplification of this region employed the universal cytochrome B primers CB1-L (L14817) (5'-CCATCCAACATCTCAGCATGATGAAA-3') and CB2-H (H15175) (5'-CCCTCAGAATGATATTTGTCCTCA-3') (where L and H designate the light and heavy strands respectively) described by Kocher *et al*. (1989). Double stranded PCR amplifications were performed in a PE-Applied Biosystems 9600 thermocycler in a total volume of 50 µl per individual. Individual amplifications consisted of 200 µM dNTP's, 10 mM TrisHCL pH 8.3, 50 mM KCL, 1.5 mM $MgCl_2$, 0.2 µM CB1-L and CB2-H, 0.025 µ/µl Amplitaq Gold (Perkin Elmer) and 10 µl of the 1/5 dilution of genomic DNA. Negative controls (containing no DNA) were included to screen for possible cross contamination.

After an initial cycle of 93°C × 10 minutes, 50°C × 45 seconds and 72°C × 2 minutes, samples were subjected to 93°C × 30 seconds, 50°C × 1 minute and 72°C × 2 minutes for 40 cycles with a final extension step of 72°C × 10 minutes. Five µl of the resulting PCR

fragments were run on a 2.5% 1X TBE agarose gel (containing ethidium bromide) at 120 volts for 60 minutes to confirm successful amplification. A 100 base pair ladder (Promega) was loaded on the gel to enable sizing of the fragment. Resulting fragments were visualised under UV light and photographed using a digital camera.

mtDNA cytochrome B gene sequencing

Cyt B double stranded PCR fragments were then purified using Wizard™ PCR Preps DNA Purification System (Promega) according to the manufacturer's instructions. Five *m*l of the column purified PCR fragments were run out on a 2.5% 1X TBE agarose gel (containing ethidium bromide) at 120 volts for 60 minutes against a PGEM 100 base pair ladder (Promega) to estimate concentration of the purified products.

The sequence of each individual was then determined with an ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) according to the manufacturer's instructions and using 8–14 ng of each purified PCR product. Cycle sequencing was performed with 25 cycles of 96°C × 10 seconds, 50°C × 5 seconds and 60°C × 4 minutes in a PE-Applied Biosystems 9600 thermocycler in a total volume of 20 μl. Unincorporated dye terminators were removed using an ethanol/sodium acetate precipitation method as described in Perkin Elmer's Automated DNA Sequencing Chemistry Guide. Sequencing reactions were run on a 5% denaturing polyacrylamide Long Ranger Singel (FMC) on an ABI Prism 377 DNA sequencer (PE Applied Biosystems). Products were analysed in both the forward and reverse directions.

5.4.4. Statistical analysis of allozyme and microsatellite data

Genetic distances between species and colour morphs were estimated from allozyme data using Nei's methods, as implemented in BIOSYS (Swofford and Selander, 1989). Standard values of distance and identity were from Nei (1972), and unbiased values (which take sample size into account) were from Nei (1978). Nei's genetic distance takes a range of 0 (total similarity) to infinity (total dissimilarity), and identity ranges from 1 (total similarity) to 0 (total dissimilarity). Tests of homogeneity of allelic and genotypic frequencies were assessed using standard Monte-Carlo chi-square methods in the programs CHIRXC and CHIHW (Zaykin and Pudovkin, 1993) with 5000 randomisations of the data to estimate p values.

Statistical analysis of microsatellite data used predominantly two software packages: Arlequin v.1.1 (Schneider *et al*., 1997) and GENEPOP v.3 (Raymond and Rousset, 1995).

ARLEQUIN was used for an analysis of variance of allele frequencies within and between populations, a process termed AMOVA (analysis of molecular variance) and based on Excoffier *et al*. (1992). ARLEQUIN also permitted multi-locus estimates of F_{ST}, the proportion of the total genetic variation attributable to population differentiation. F_{ST} statistics were originally developed by Wright (1951, 1965) to assess population structure in terms of inbreeding coefficients. The fixation index F_{ST} is the same as the weighted average of F_{ST} over loci defined by Weir and Cockerham (1984). Pairwise population estimates of F_{ST} can be used to estimate migrant numbers. If it is assumed that mutation rate is negligible with respect to migration rate, and that populations are at equilibrium between migration and drift, then the absolute number of migrants exchanged between two populations, M, is related to F_{ST} by $M = (1- F_{ST})/2 F_{ST}$, where M = 2Nm (N = population size, m = migration rate). ARLEQUIN was also used for microsatellite locus tests of Hardy-Weinberg equilibrium, i.e.

to test whether the observed genotype distributions within populations were in agreement with binomial expectations given the observed allele frequencies.

GENEPOP was used for various tests: (1) tests of linkage disequilibrium. This package establishes pairwise locus tables of genotypes, then performs a probability test using a Markov chain for each table. (2) to estimate the extent of deviation of microsatellite data from Hardy-Weinberg equilibrium using the fixation index F_{IS}. (3) to analyse contingency tables testing for spatial differentiation of allozyme and microsatellite alleles and establishing F_{ST} values for each locus

Other programs used were:

BOOT-IT, a program written by R. Ward and modified by P. Grewe. This quantifies the extent of genetic differentiation among collections using Nei's (1973) gene diversity (G_{ST}) statistics. G_{ST} represents the proportion of genetic diversity that can be attributed to differences between collections. It is operationally virtually identical to F_{ST}. A bootstrapping procedure was used to estimate the magnitude of G_{ST} that could be attributed to sampling error alone. This quantity is termed $G_{ST\text{-}null}$ and a mean value of $G_{ST\text{-}null}$ was estimated for each locus from 1000 replications. The number of times each of the 1000 estimates of $G_{ST\text{-}null}$ was equal to or greater than the observed G_{ST}, divided by 1000, gave the probability of obtaining the observed G_{ST} by sampling error. This procedure was used for the allozyme data alone to facilitate comparisons with past data collected and analysed in our laboratory (e.g. Elliott and Ward, 1992).

NULLTEST, a program written Bill Amos, Department of Zoology, Cambridge, CB2 3EJ, UK. This estimates best fit null allele frequencies from microsatellite or minisatellite data sets. Ninety five percent confidence intervals are estimated for any null allele as 1.96 standard deviations based on 100 randomisations of the data set.

In all analyses involving multiple tests, such as testing for the same effect in each of several loci, or in each of several populations, the predetermined experimentwise significance level, α, of 0.05 was adjusted using Bonferroni procedures. Generally, the α level was divided by the number of tests to derive a new α level, and *P* values had to be less than this corrected α value to be deemed significant.

5.4.5. Statistical analysis of mitochondrial DNA sequences

DNA sequences were aligned using Sequence Navigator Version 1.01 (Perkin Elmer). Neighbour joining (NJ) (Saitou and Nei, 1987) and unweighted pair-group method with arithmetic means (UPGMA) (Sneath and Sokal, 1973) trees were constructed from proportion of nucleotide differences (p) and Jukes and Cantor (1969) (JC) distance estimates using MEGA Version 1.01 (Kumar *et al*., 1993) where:

proportion (p) of nucleotide sites at which compared sequences are different is $p=n_d/n$ (no. of nucleotide differences /by the total number of sites)

variance $V(p)=[p(1-p)]/n$

JC assumes the rate of nucleotide substitution is same for all pairs of the four nucleotides and gives a maximum likelihood estimate of the number of nucleotide substitution (d) between compared sequences
$d=-3/4\log_e(1-4/3p)$ where $p=n_d/n$
variance $V(d)=p(1-p)/[(1-4/3p)^2 n]$

NJ produces an unrooted tree and in the absence of outgroup Operational Taxonomic Units (OTU's), the root is given at the midpoint of the longest route connecting two OTU's in the tree under the assumption of a constant rate of evolution (Kumar *et al*., 1993).

UPGMA assumes the rate of nucleotide substitution is the same for all lineages and produces a rooted tree (Kumar *et al*., 1993).

Bootstrapping examined the reliability of each interior branch, i.e., whether or not it was significantly different from 0. Bootstrap confidence levels (BCL) for branches of the trees were assessed with 2000 replicates of the bootstrap test (Felsenstein, 1985) in MEGA. High percentage values mean the branch was well substantiated.

6. RESULTS/DISCUSSION

6.1. Taxonomy and nomenclatural issues

Australian lings belong to the family Ophidiidae, a large group of eel-like marine fishes known as cusk-eels (Nelson, 1994). Lings are abundant on the continental slope and shelves in temperate parts of the Southern Hemisphere. In a review of ophidiiform fishes, Cohen and Nielsen (1978) recognise the existence of at least 5 valid species worldwide: *G. blacodes* (Forster, 1801) from Australasia and South America; *G. capensis* (Smith, 1847) from southern Africa; *G. chilensis* (Guichenot, 1848) and *G. maculatus* (Tschudi, 1846) from south America; and *G. microstomus* Regan, 1903 from Australia. However, they stressed that the literature is confused with misidentifications and the genus is in need of revision. In the late 1970's, independent studies were initiated by C.R. Robins and one of the authors (P. R. Last) but a full revision of the group has never been completed.

Four nominal species have been recorded from Australian seas (Paxton *et al*., 1989): *G. blacodes*, *G. microstomus*, *G. tigerinus* Klunzinger, 1872, and *G. australis* Castlenau, 1872. Only two species, *G. blacodes* (pink ling) and *G. microstomus* (rock ling), have been generally considered valid (Scott *et al*., 1974). After examining types, *G. tigerinus* was resurrected for the rock ling (Last *et al*., 1983), and *G. microstomus* and *G. australis* are now considered junior synonyms of *G. blacodes* (Paxton *et al*., 1989).

The greatest ling diversity occurs off South America, along with those mentioned above, *G. brasiliensis* (Regan, 1903) which is a valid species (Nakamura *et al*., 1986) occurs off Brazil, totaling four species from this continent.

The taxonomic composition of *G. blacodes* has remained problematic. Some authors have considered that the most widely distributed member of the genus may be conspecific with the south African kingklip, *G. capensis* (Ayling and Cox, 1982). Additional material, residing in the British Museum in London, from the Falkland Islands and Tristan da Cunha in the central South Atlantic has been identified as *G. blacodes*. Also, two colour forms of *G. blacodes* exist off southern Australia - an orange form on the continental shelf and a pink form that occurs mainly on the slope. Clearly two specific issues needed to be resolved for Australian lings. Is *G. blacodes* the correct name for Australian pink ling? Is the pink ling variable in colour or does a shallow-water orange morph constitute a second species?

Unpublished data by Last collected in 1980 from museums in Hamburg, Paris and London have provided evidence that populations of *G. capensis* and populations of South American and central Atlantic *G. blacodes* are not conspecific with Australian populations of *G. blacodes*. However, based on morphological data, *G. blacodes* from Australia and New Zealand appear to be conspecific. The type specimen of *G. blacodes*, now missing, was taken from New Zealand. Therefore, use of this name for the Australian pink ling is endorsed. The synonyms, *G. microstomus* and *G. australis*, were coined more than half a century after *G. blacodes* so, according to the 'Principle of Priority' (Article 23, International Code of Zoological Nomenclature, 1999), the oldest available name is the valid name of the taxon.

The possible occurrence of the two closely related species for the pink and orange colour morphs presently both identified as *G. blacodes* is investigated in the following sections.

6.2. Morphology

6.2.1. Size and sex ratio

The overall mean TL of specimens sampled was 593 mm and there were slightly fewer females (46%) than males (54%) (Table 6.1). Some specimens could not be sexed because the gonads were not developed, or the gut had been removed or had become rotten due to poor storage before acquisition. Only the western Victorian samples had a statistically different sex ratio (36% females, 64% males) to the overall average (46% females, 54% males).

For all subregions except eastern Victoria, the mean TL was significantly different to the overall mean ($P<0.01$) (t-test). The NSW and eastern Tasmania specimens were smaller than the overall mean and the western Victoria and Tasmania material were larger than the mean. When the regional mean total lengths were compared pair wise, only the eastern Tasmania and eastern Victoria samples were statistically equal ($P=0.113$). All other pairs were statistically different ($P<.0001$). Differences in mean TL between regions was undesirable, particularly for morphometric and otolith shape analysis as differences in size, as well as stock differences, may have contributed to between area differences in shape.

Table 6.1: Sizes and sex ratios compared between SEF subregions.

Sub-region	Depth category	n total	n females	n males	n not sexed	Sex ratio % female	Mean TL females	Mean TL males	Mean TL overall
NSW	Shallow	33	1	5	27	NA	NA	NA	327
NSW	?	33	19	13	1	59%	525	541	548
NSW	Deep	35	12	13	10	48%	558	557	557
NSW	TOTAL	101	32	31	38	51%	549	540	480
VIC(E)	Shallow	77	26	27	24	49%	553	535	560
TAS(E)	Shallow	27	9	7	11	56%	490	476	483
TAS(E)	Medium	56	19	34	3	36%	523	513	514
TAS(E)	Deep	41	16	18	7	47%	666	576	616
TAS(E)	TOTAL	124	44	59	21	43%	566	527	542
VIC(W)	Medium	34	8	18	8	31%	582	547	561
VIC(W)	Deep	26	10	14	2	42%	844	721	775
VIC(W)	TOTAL	60	18	32	10	36%	728	620	650
TAS(W)	Deep/whole	23	3	20	0	13%	609	686	676
TAS(W)	Deep/otoliths	197	104	91	2	53%	906	747	832
TAS(W)	TOTAL	220	107	111	2	49%	897	737	815

6.2.2. Meristics

Meristics are important in the identification of ophidiiform fishes in general (Cohen and Nielsen, 1978). Overall ranges for each of the three fin counts examined differed significantly from published ranges (Last *et al*., 1983; Gomon *et al*., 1994) for both *G. blacodes* and *G. tigerinus*: dorsal-fin ray counts for *G. blacodes* ranged from 131–159 rays (142–160 in the literature) and for *G. tigerinus* from 144–166 rays (versus 144-178); anal-fin ray count ranges were 98–119 (versus 104–126) and 107–124 (versus 107–124) for *G. blacodes* and *G. tigerinus* respectively; and pectoral-fin ray counts ranged from 22–28 (versus 19–24) for *G. blacodes*. *G. blacodes* had 3–6 dorsal pyloric caecae whereas *G. tigerinus* had 4–6. *G. tigerinus* tended to have more ventral caecae (3–4) than *G. blacodes* (1–3).

Vertebral counts for *G. blacodes* showed comparatively low levels of variation within and between groups. Total vertebrae for *G. blacodes* ranged from 67–71 centra and did not differ significantly from *G. tigerinus*: which ranged from 68–71 centra (n=12). The mean number of vertebrae for both *G. blacodes* and *G. tigerinus* was 69.4 centra. These results are similar to published values for *G. capensis*: 66–75 centra, mode=70 (Payne, 1985). All specimens of *G. blacodes* and *G. tigerinus* (n=12) had 4 complete gill rakers with additional partial rakers that were difficult to count.

Meristic data did not indicate population differences across the SEF region in *G. blacodes*. Dorsal-fin ray counts (ranging from 130–158 rays) varied greatly compared to most bony fishes (Figure 6.1). There was no clear mode, even for the eastern Tasmanian sample, which included over 100 specimens, and it is doubtful whether a mode could be established even if sample sizes were doubled. These factors suggest that dorsal-fin rays would not be effective for distinguishing between stocks. Similarly, anal-fin counts varied greatly with a broad overall range (98–119 rays) and showed no clear mode (Figure 6.2). Even the western Tasmanian sample, which only included 13 specimens, had a large range: 100–118 rays.

Pectoral-fin ray counts showed moderate levels of intraspecific variation. The modal pectoral fin count for all subregions was 25 (modal range 24–26) and the overall range was 22–28 (Figure 6.3). Eastern Tasmanian and eastern Victorian specimens more commonly had 24 than 26 rays, whereas individuals with 26 rays were more common than those with 24 rays in the sample from NSW. For western Victoria, about equal numbers of individuals had 24 and 26 rays whereas for western Tasmania there were too few specimens to form a trend.

Other meristics exhibited very low levels of variation. All specimens had 4 complete gill rakers so this character was eliminated from data collection early in the study. Precaudal and caudal vertebral counts showed very little intraspecific variation and the modes were the same for each subregion (Figures 6.4–6.5). Similarly, dorsal and ventral caecae showed little variation. The modal ventral caecae count was two for all subregions (Figure 6.7) but the modal dorsal caecae count varied between subregions (Figure 6.6).

Plots of the residuals indicated that dorsal and anal-fin ray counts were normally distributed. Means for these counts were compared using ANOVA. The remaining counts were not normally distributed and were therefore compared using chi-squared analyses (Zaykin and Pudovkin, 1993). For all tests the significance level (α) of 0.05 was adjusted using

Bonferroni procedures by dividing by the number of characters examined (7 in this case) so the new level of significance became 0.007.

The NSW and eastern Tasmania subregions were initially selected to examine the effect of depth on meristics as only samples from these subregions contained both deep and shallow water specimens (Table 6.2A–C). Neither depth nor subregion showed any significant effect for the meristics examined. This suggests that the deep and shallow populations of pink ling do not represent different species based on meristic data.

Depths were then pooled so that counts could be compared between all subregions (Table 6.3A–C). This analysis did not provide any reliable evidence that the data represents more than one stock. Only pyloric caecae counts displayed statistically significant overall regional variation. The statistical significance of this variation was largely due to the higher number of specimens with low counts in western Victoria compared to most other subregions (Figures 6.6–6.7). Given that the outcome has been largely influenced by only a few specimens, combined with difficulties in counting caecae (see methods), the probability value associated with this statistical measure is dubious.

Table 6.2A: Meristics, NSW and TAS(E), deep and shallow compared.

Subregion	Stat.	Dorsal rays	Anal rays	Pectoral rays	Precaudal vertebrae	Caudal vertebrae	Dorsal caecae	Ventral caecae
NSW	Mean	147.66	107.94	25.15	19.65	49.42	4.58	2.23
shallow	SE	0.92	0.72	0.17	0.09	0.45	0.11	0.07
	n	32	31	32	31	31	26	26
NSW	Mean	146.76	107.88	25.18	19.96	48.28	4.48	2.20
deep	SE	1.04	0.82	0.17	0.10	0.50	0.10	0.07
	n	25	24	33	25	25	27	30
TAS(E)	Mean	144.38	107.42	24.55	19.86	49.46	4.48	2.13
shallow	SE	1.02	0.78	0.18	0.10	0.49	0.10	0.08
	n	26	26	29	26	26	31	24
TAS(E)	Mean	145.17	107.78	24.70	19.72	49.50	4.36	2.07
deep	SE	1.23	0.94	0.18	0.00	0.58	0.11	0.07
	n	18	18	29	18	18	28	27

Table 6.2B: Probabilities associated with mean comparisons for NSW and TAS(E) deep and shallow normally distributed meristic characters. Derived from ANOVA analysis. a =0.007

Probability test	Dorsal rays	Anal rays
P: region	0.059	0.265
P: depth	0.640	0.583
P: region x depth	0.717	0.603

Table 6.2C: Probabilities associated with mean comparisons for NSW and TAS(E) deep and shallow non-normally distributed meristic characters. Derived from Chi-square analysis. a =0.007

Probability test	Pectoral rays	Precaudal vertebrae	Caudal vertebrae	Dorsal caecae	Ventral caecae
P: region	0.040	0.112	0.144	0.371	0.089
P: deep vs. shallow NSW	0.383	0.051	0.852	0.689	1.000
P: deep vs. shallow TAS(E)	0.300	0.435	0.730	0.428	0.647

Table 6.3A: Meristics, SEF subregions compared, depths pooled.

Region	Statistic	Dorsal rays	Anal rays	Pectoral rays	Precaudal vertebrae	Caudal vertebrae	Dorsal caecae	Ventral caecae
NSW	Mean	146.69	108.00	25.13	19.80	48.80	4.56	2.22
	SE	0.56	0.44	0.10	0.05	0.27	0.06	0.05
	n	91	87	82	89	90	89	88
VIC(E)	Mean	146.94	108.29	24.78	19.53	49.77	4.45	2.15
	SE	0.67	0.50	0.13	0.06	0.30	0.83	0.07
	n	64	65	56	65	64	49	46
TAS(E)	Mean	145.50	107.64	24.78	19.84	49.62	4.40	2.13
	SE	0.55	0.41	0.10	0.05	0.30	0.60	0.05
	n	98	97	110	100	98	109	105
VIC(W)	Mean	145.76	107.61	24.90	19.82	49.62	4.31	2.02
	SE	0.77	0.57	0.12	0.07	0.40	0.08	0.07
	n	49	51	59	51	50	49	58
TAS(W)	Mean	144.67	108.46	25.09	19.75	49.92	4.57	2.23
	SE	1.55	0.13	0.20	0.15	0.70	0.12	0.10
	n	12	13	22	12	12	23	22

Table 6.3B: Probability associated with mean comparisons between SEF sub- regions for normally distributed characters, depths pooled. Derived from ANOVA analysis. a =0.007

Dorsal rays	Anal rays
0.423	0.819

Table 6.3C: Probabilities associated with mean comparisons between SEF sub- regions for non-normally distributed characters, depths pooled. Derived from Chi-square analysis. a =0.007

Pectoral rays	Precaudal vertebrae	Caudal vertebrae	Dorsal caecae	Ventral caecae
0.734	0.010	0.076	0.003	0.003

Figure 6.1: Dorsal-fin ray counts, five subregions compared. Vertical axis: number of specimens, horizontal axis: number of dorsal-fin rays.

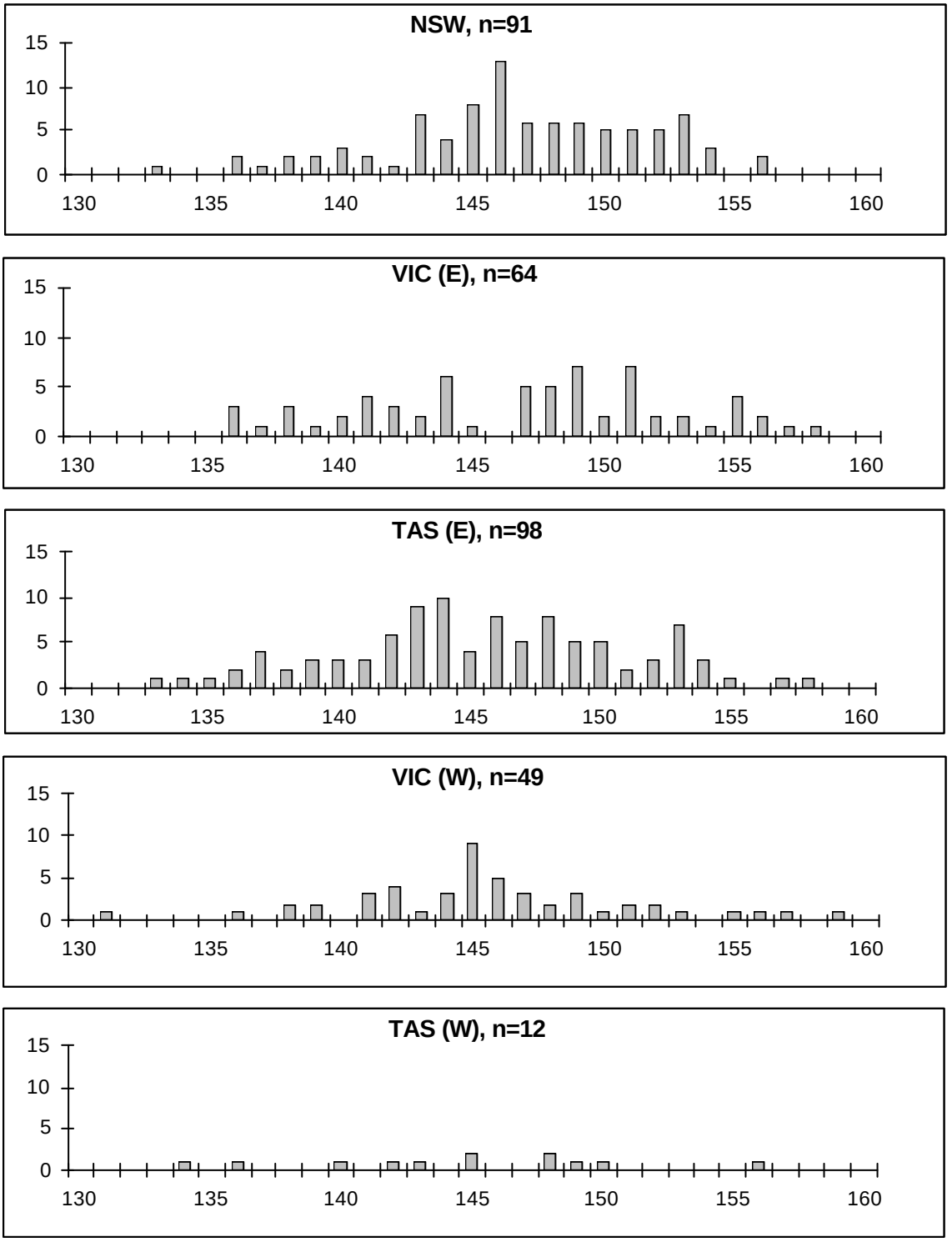


Figure 6.2: Anal-fin ray counts, five subregions compared. Vertical axis: number of specimens, horizontal axis: number of anal-fin rays.

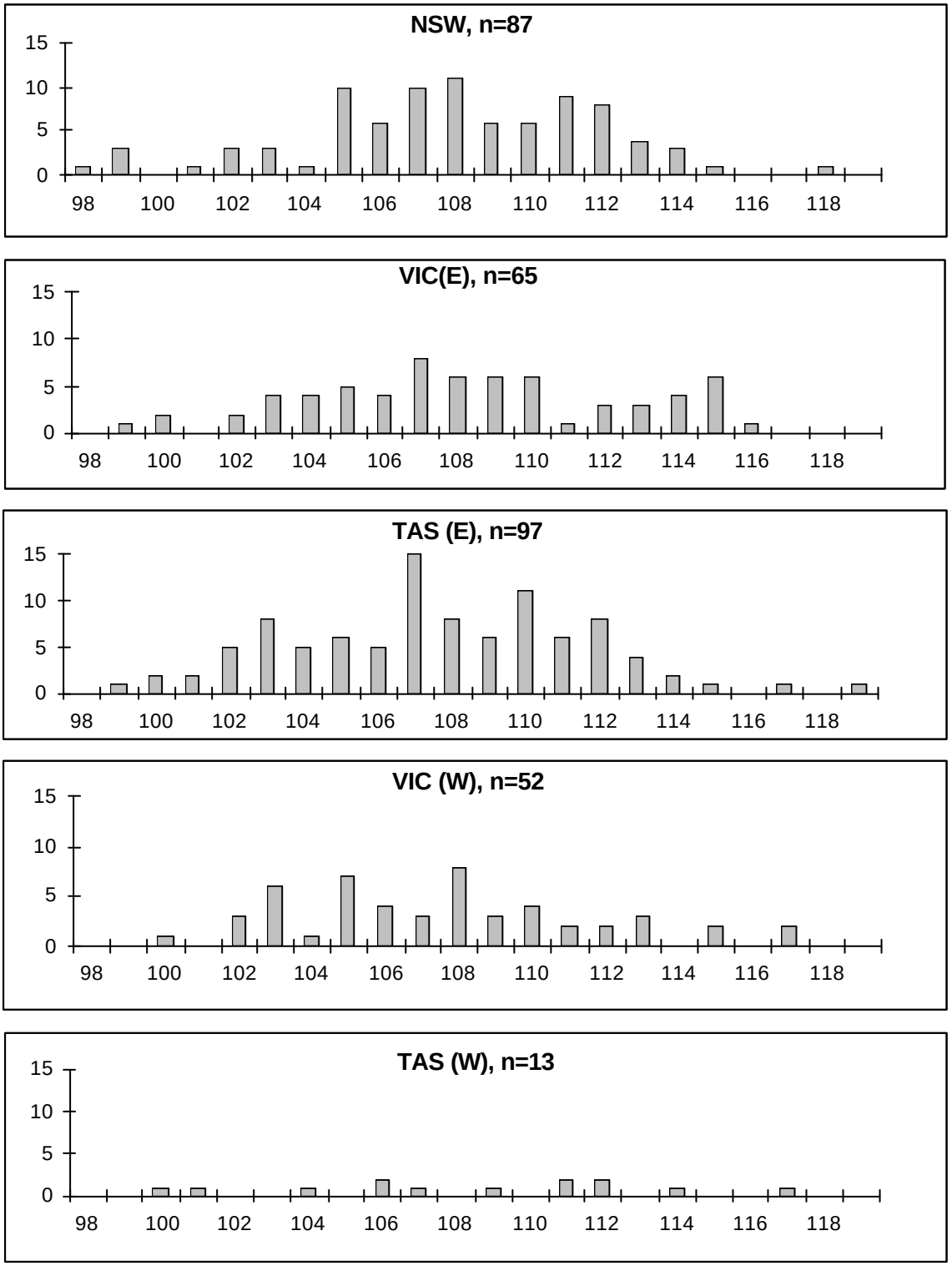


Figure 6.3: Pectoral-fin ray counts, five subregions compared. Vertical axis: number of specimens, horizontal axis: number of pectoral-fin rays.

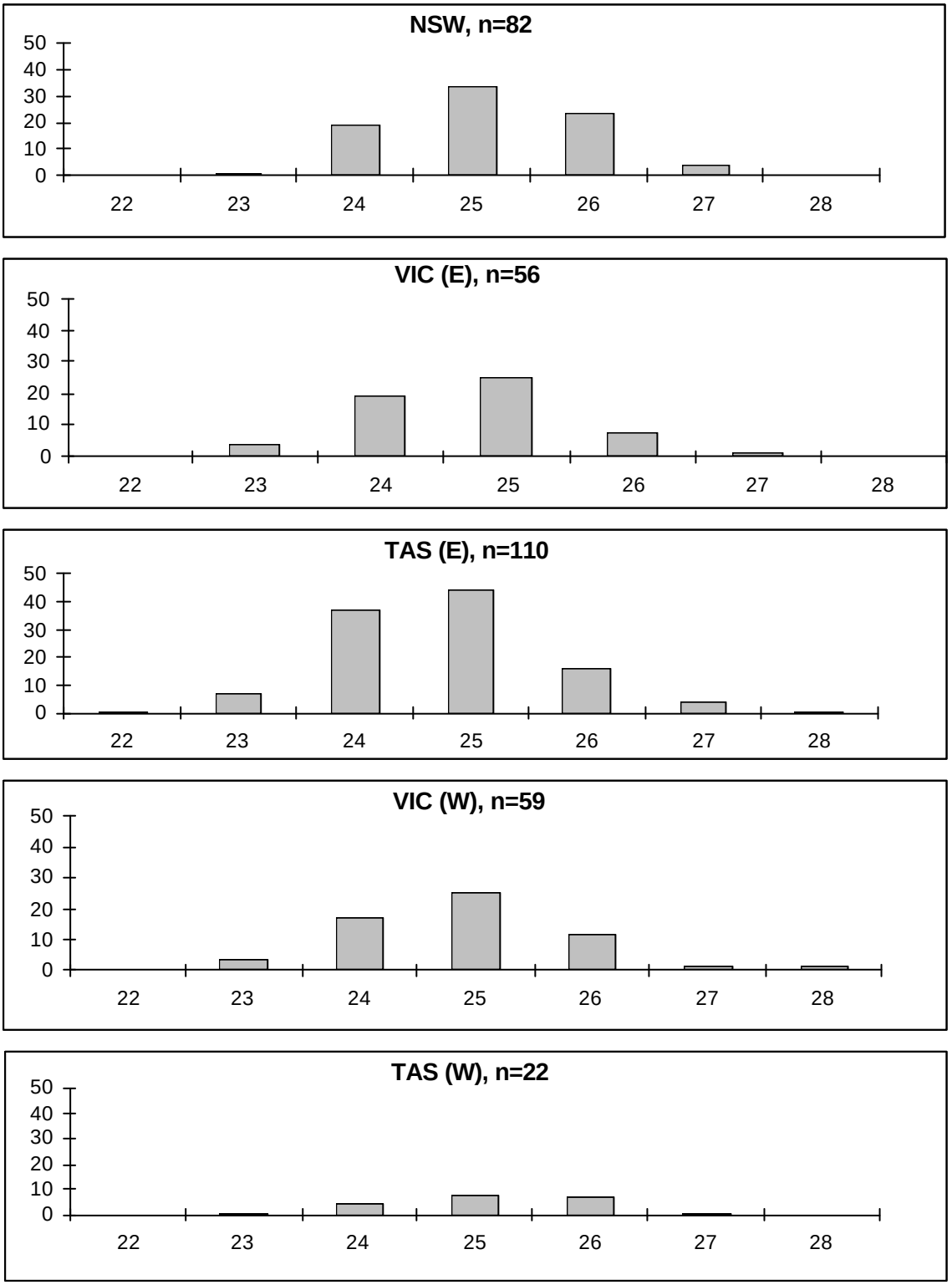


Figure 6.4: Precaudal vertebrae counts, five subregions compared. Vertical axis: number of specimens, horizontal axis: number of precaudal vertebrae.

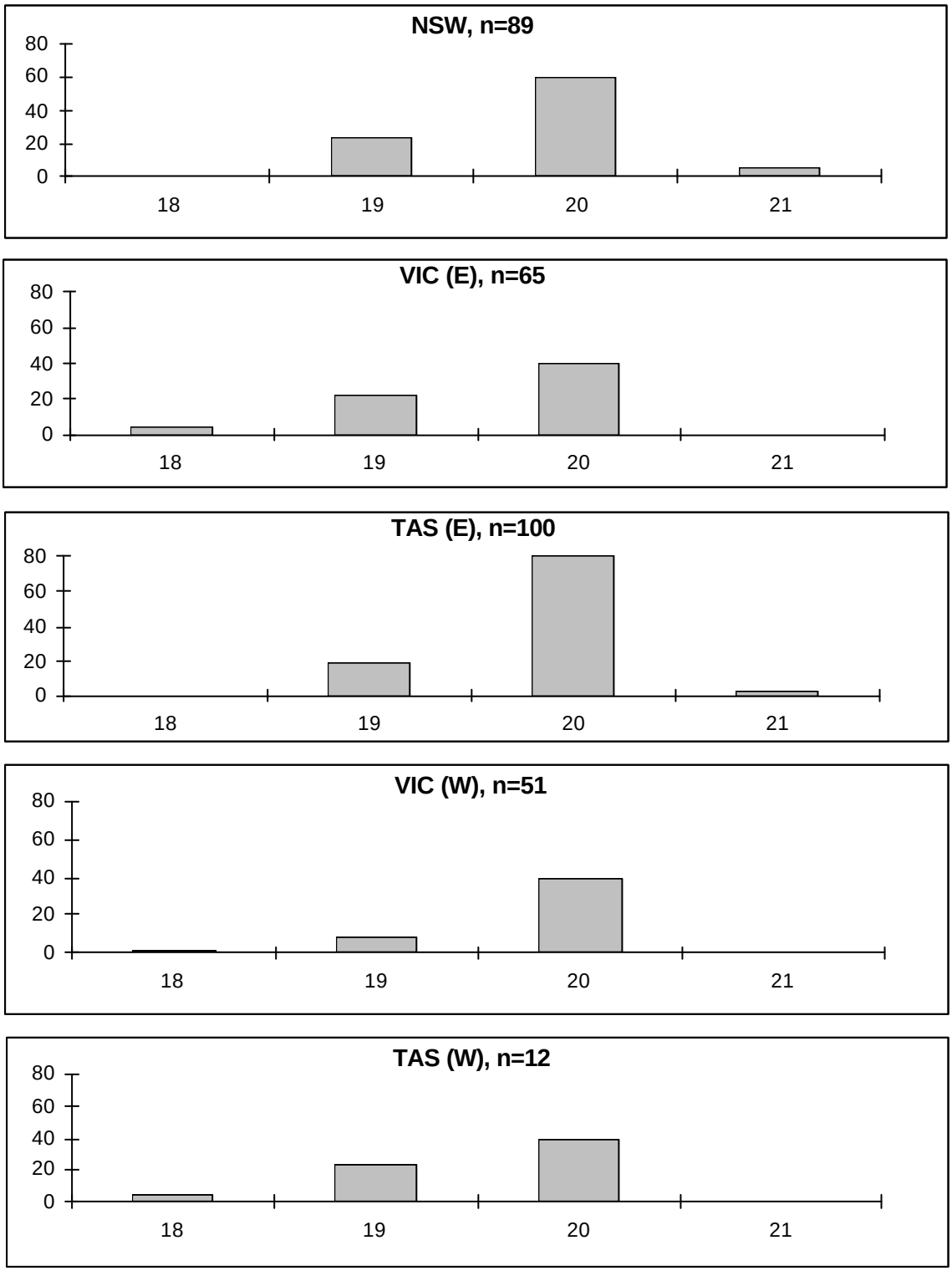


Figure 6.5: Caudal vertebrae counts, five subregions compared. Vertical axis: number of specimens, horizontal axis: number of caudal vertebrae.

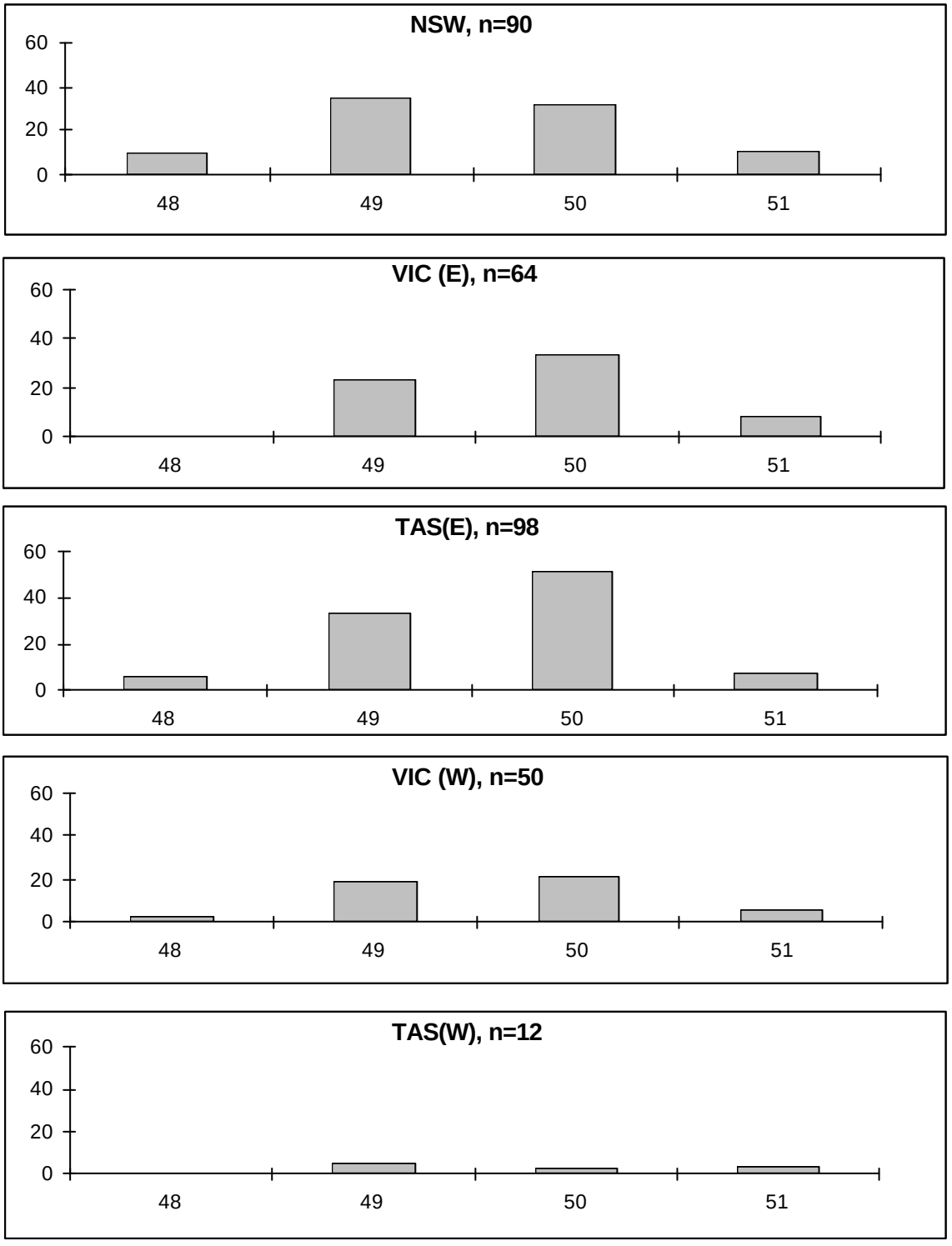


Figure 6.6: Dorsal pyloric caecae counts, five subregions compared. Vertical axis: number of specimens, horizontal axis: number of dorsal pyloric caecae.

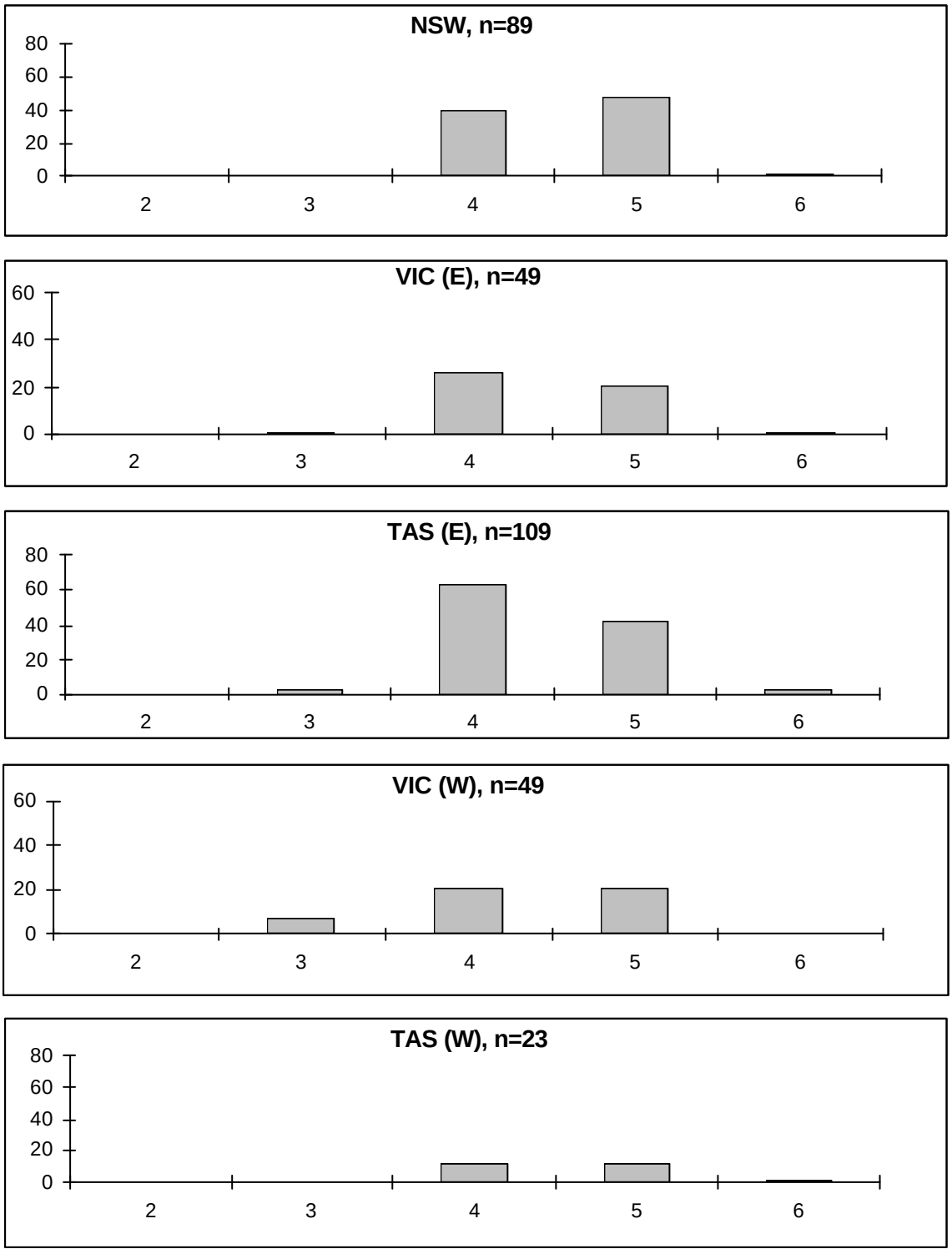
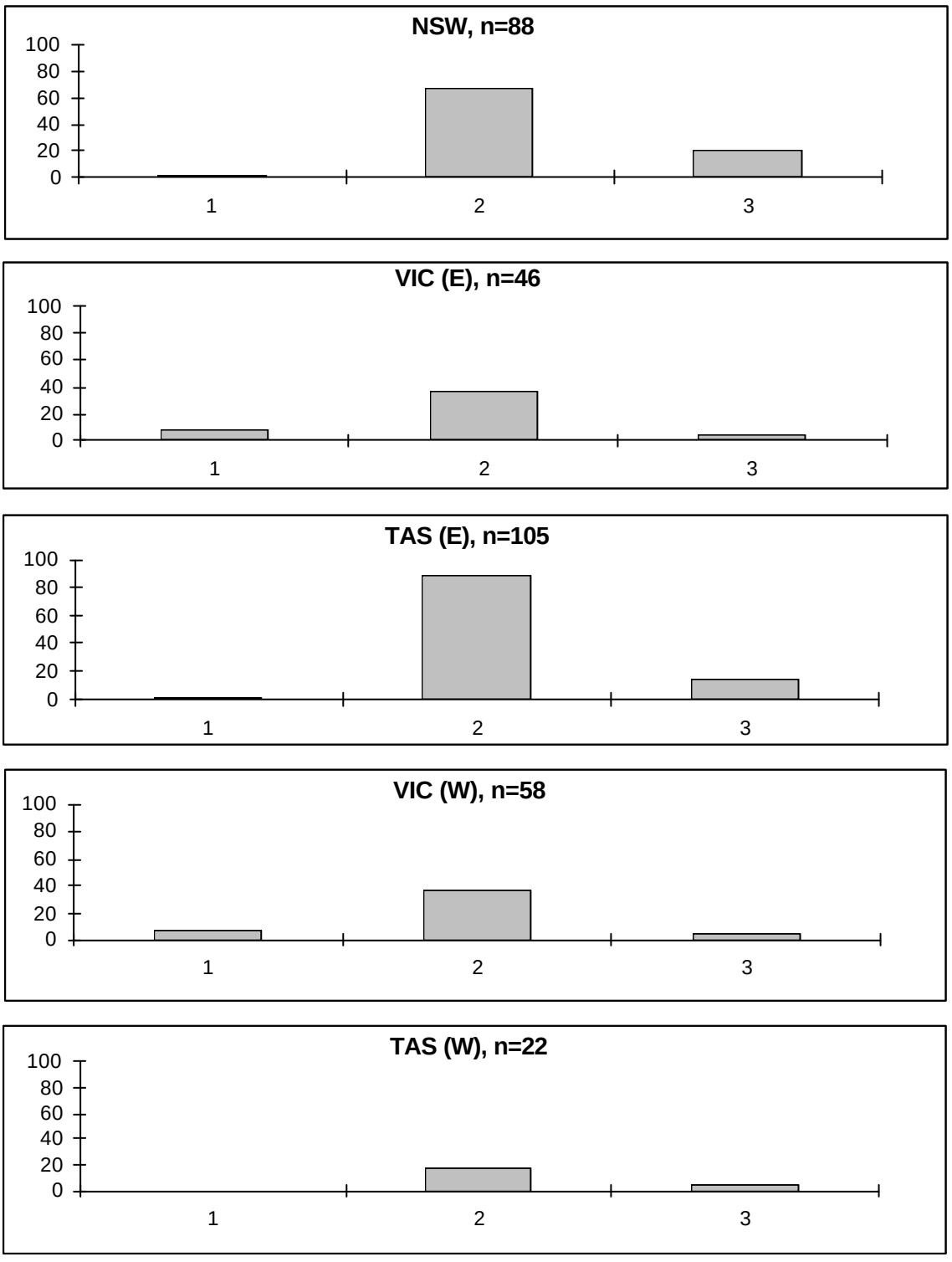


Figure 6.7: Ventral pyloric caecae counts, five subregions compared. Vertical axis: number of specimens, horizontal axis: number of dorsal and ventral caecae.



6.2.3. Body morphometry

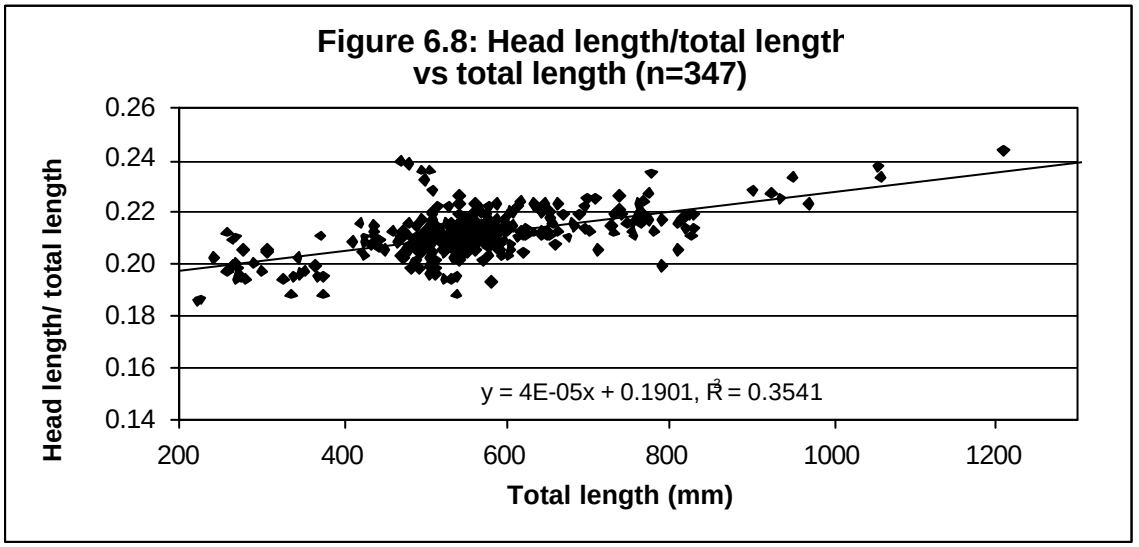
Preliminary assessment

Six head and body measurements were examined for outliers and then regressed against TL to test for allometry. Of the six, only pectoral-fin length did not have a slope significantly different from zero (Figure 6.13).

Head length (Figure 6.8) increased in proportion to TL with growth. Similarly, upper and lower jaw lengths increased proportionally to TL which was evident when jaw length measurements were regressed against head length (Figure 6.9–6.10).

Interorbital width and snout–anus distance had more complex relationships with TL. Interorbital width increased in proportion to TL more rapidly than head length, with a slight reduction in the rate of increase with size (Figure 6.11). Similarly, snout–anus distance increased in proportion to TL with a slight reduction in the rate of increase with size. (Figure 6.12).

All raw data were then normalised (see methods). Regressions of the normalised data against TL indicated that normalisation had successfully eliminated the effects of size from the data (Figures 6.14–6.19). The normalised data were then log transformed. Regional means are compared in Figures 6.20–6.25. NSW and western Tasmania specimens tended to have shorter heads, upper jaws and lower jaws than specimens from other subregions (Figures 6.20–6.22).



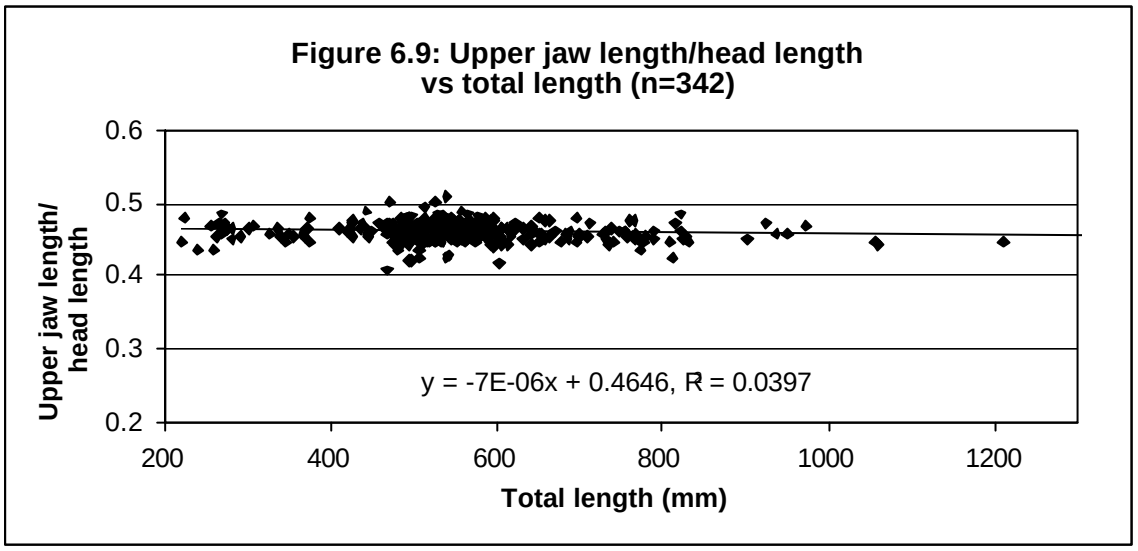


Figure 6.9: Upper jaw length/head length vs total length (n=342)
Upper jaw length/ head length
0.6
0.5
0.4
0.3
0.2
y = -7E-06x + 0.4646, R² = 0.0397
200
400
600
800
1000
1200
Total length (mm)

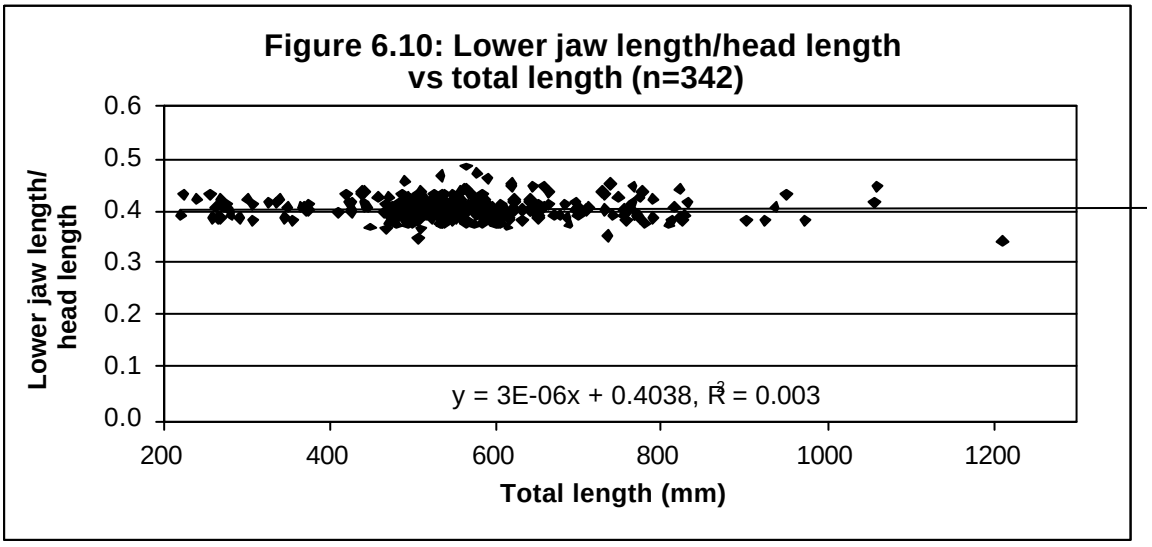


Figure 6.10: Lower jaw length/head length vs total length (n=342)
Lower jaw length/ head length
0.6
0.5
0.4
0.3
0.2
0.1
0.0
y = 3E-06x + 0.4038, R² = 0.003
200
400
600
800
1000
1200
Total length (mm)

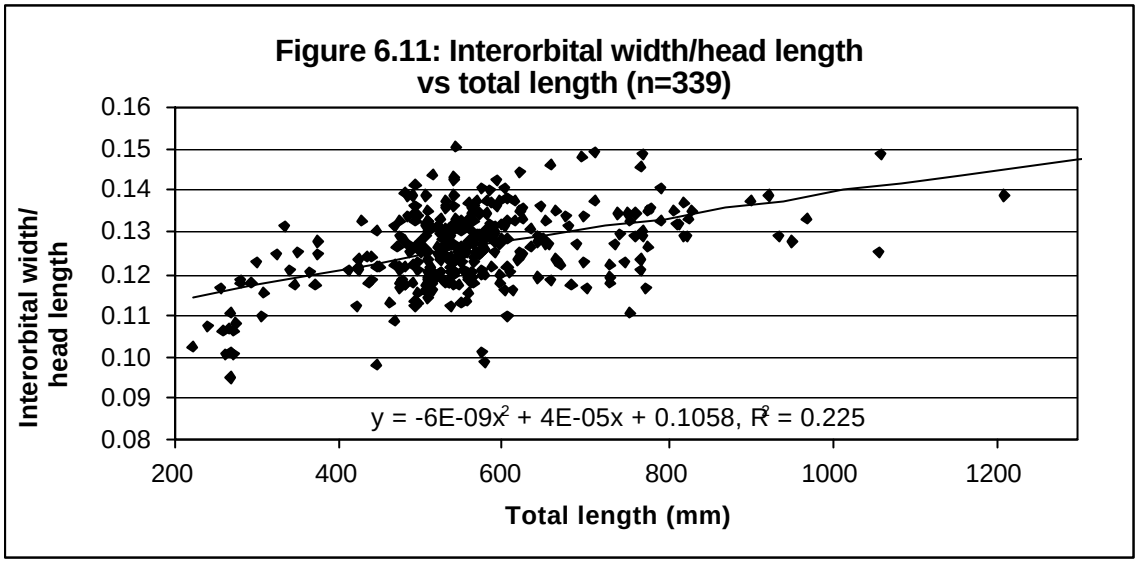


Figure 6.11: Interorbital width/head length vs total length (n=339)
Interorbital width/ head length
0.16
0.15
0.14
0.13
0.12
0.11
0.10
0.09
0.08
y = -6E-09x2 + 4E-05x + 0.1058, R2 = 0.225
200
400
600
800
1000
1200
Total length (mm)

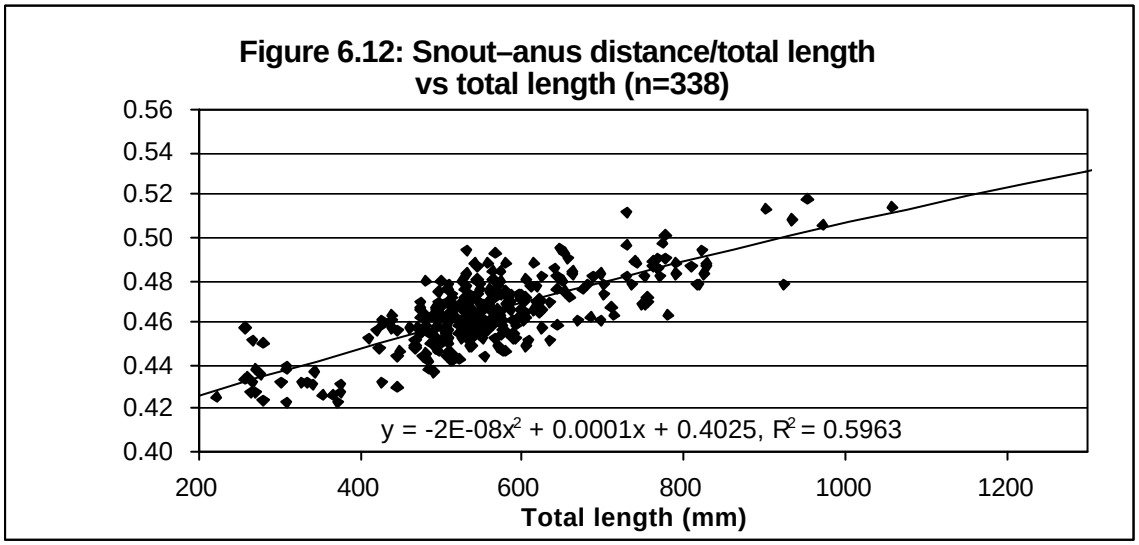


Figure 6.12: Snout–anus distance/total length vs total length (n=338)
0.56
0.54
0.52
0.50
0.48
0.46
0.44
0.42
0.40
y = -2E-08x2 + 0.0001x + 0.4025, R2 = 0.5963
200
400
600
800
1000
1200
Total length (mm)

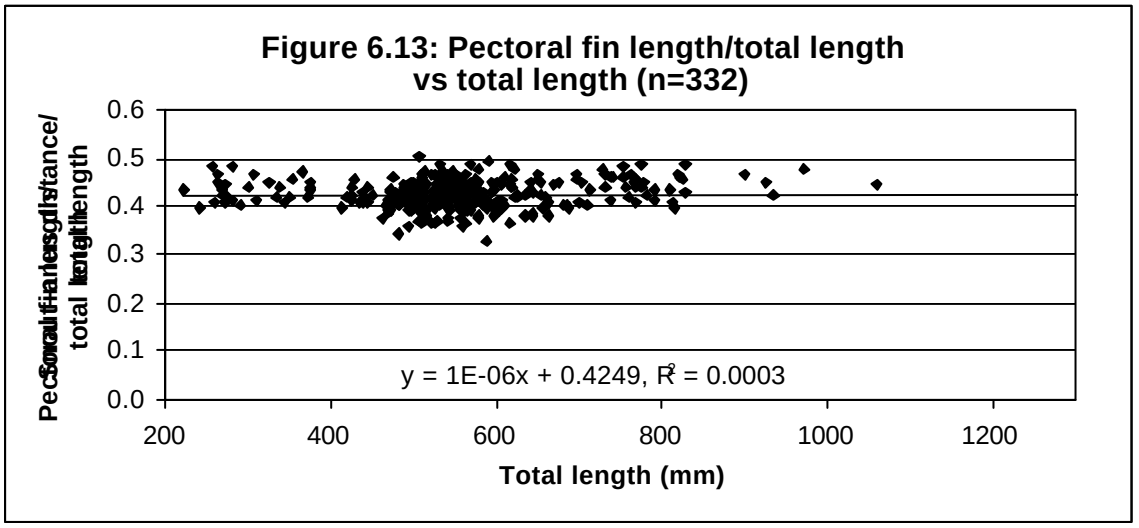


Figure 6.13: Pectoral fin length/total length vs total length (n=332)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
y = 1E-06x + 0.4249, R2 = 0.0003
200
400
600
800
1000
1200
Total length (mm)

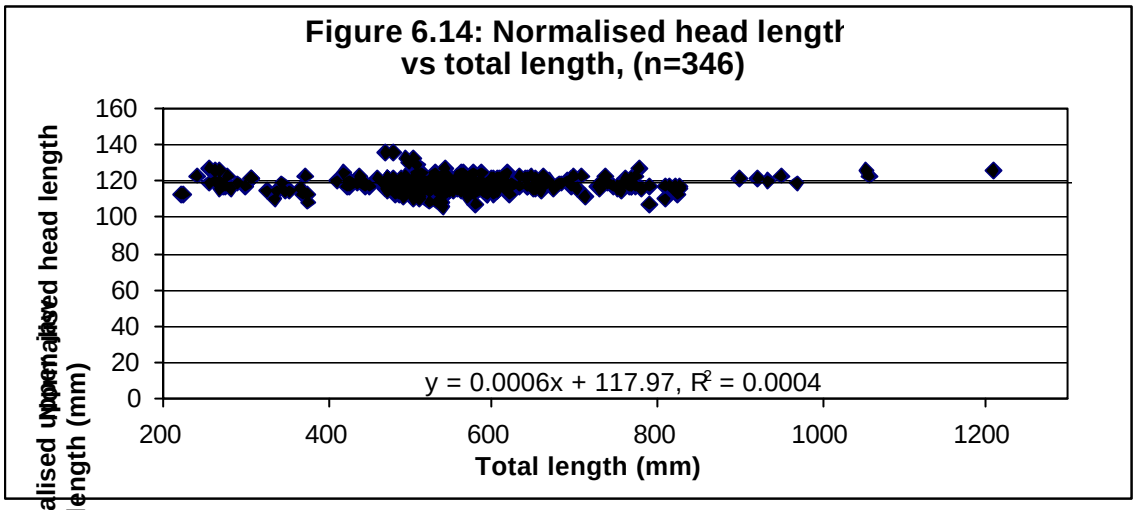


Figure 6.14: Normalised head length vs total length, (n=346)
Normalised upper jaw head length length (mm)
160
140
120
100
80
60
40
20
0
200
400
600
800
1000
1200
Total length (mm)
y = 0.0006x + 117.97, R2 = 0.0004

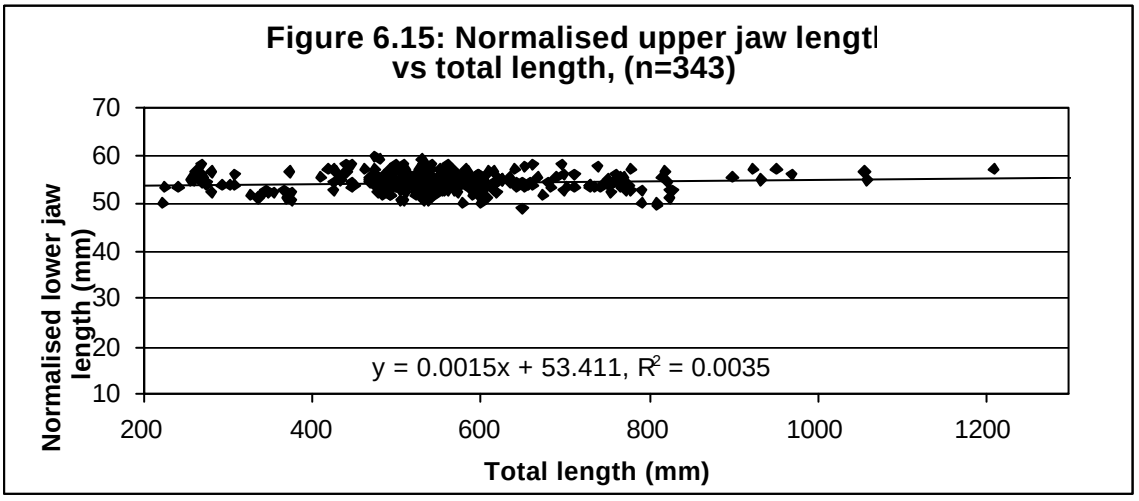


Figure 6.15: Normalised upper jaw length vs total length, (n=343)
Normalised lower jaw length (mm)
70
60
50
40
30
20
10
200
400
600
800
1000
1200
Total length (mm)
y = 0.0015x + 53.411, R2 = 0.0035

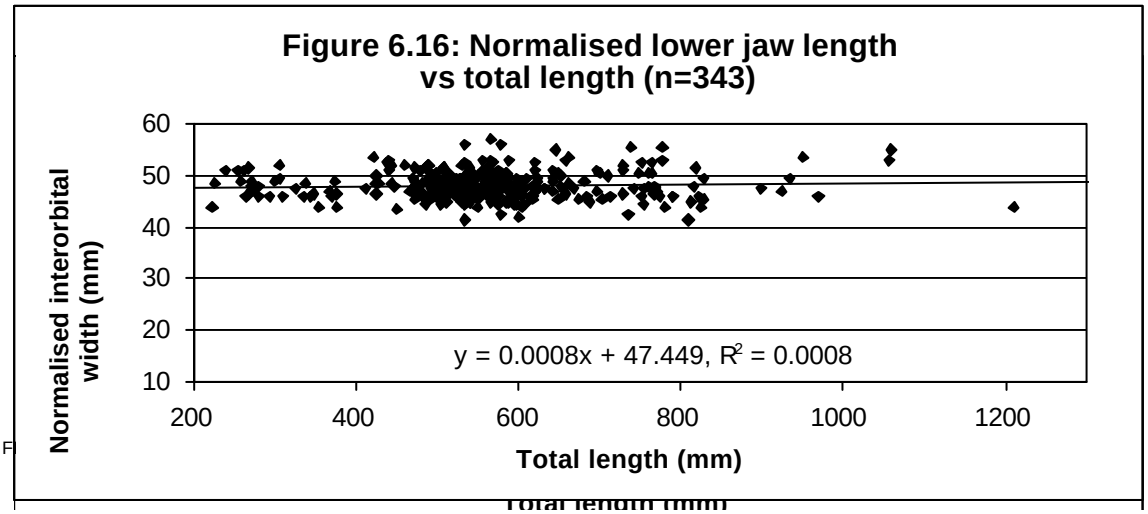


Figure 6.16: Normalised lower jaw length vs total length (n=343)
Normalised interorbital width (mm)
60
50
40
30
20
10
200
400
600
800
1000
1200
Total length (mm)
y = 0.0008x + 47.449, R2 = 0.0008

Figure 6.18: Normalised snout–anus distance vs total length (n=338)

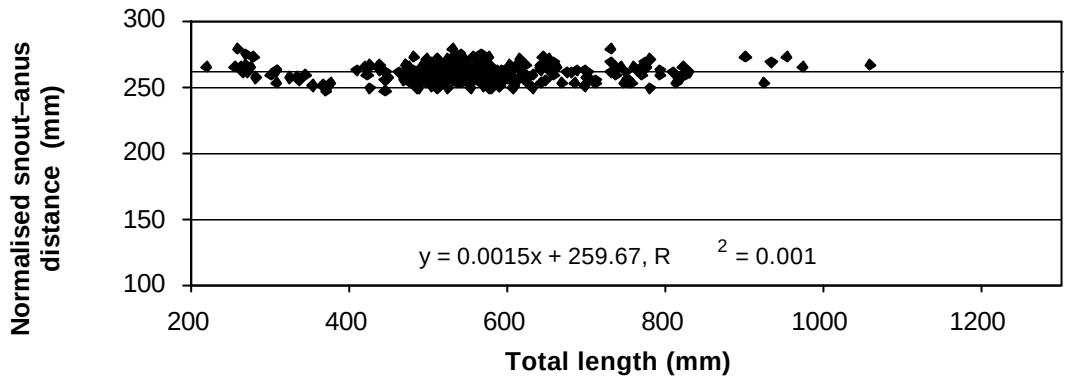


Figure 6.19: Normalised pectoral fin length vs total length (n=334)

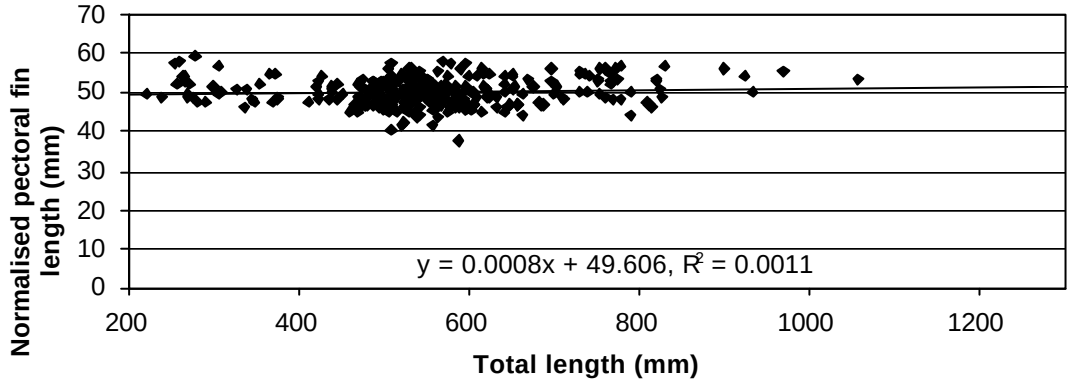


Figure 6.20: Regional variation in log transformed and normalised head length

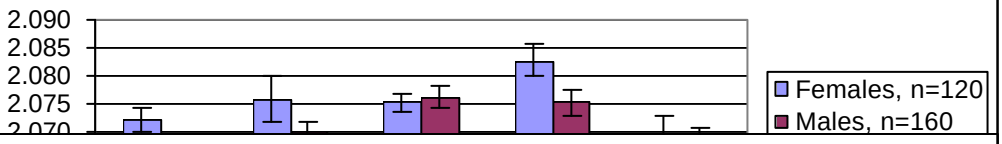


Figure 6.21: Regional variation in log transformed and normalised upper jaw length

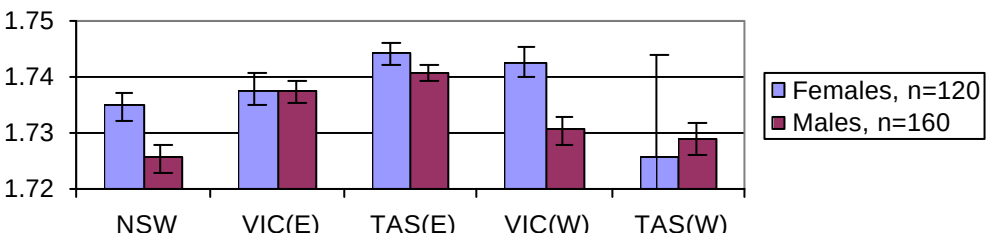


Figure 6.22: Regional variation in log transformed and normalised lower jaw length

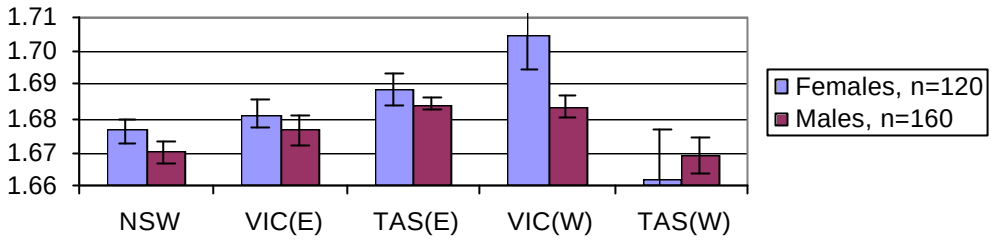


Figure 6.23: Regional variation in log transformed and normalised interorbital width

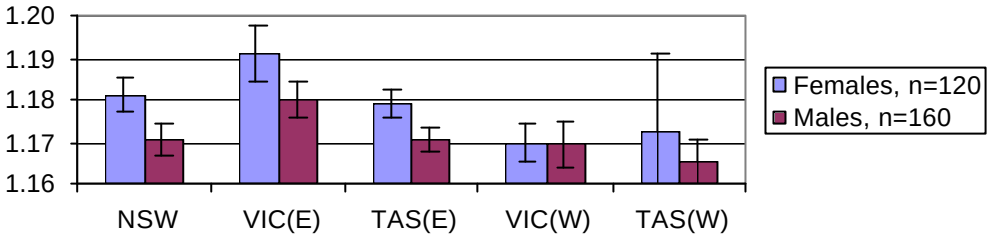


Figure 6.24: Regional variation in log transformed and normalised snout–anus distance

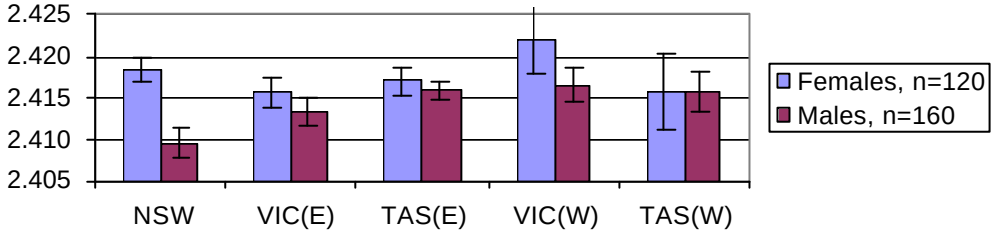
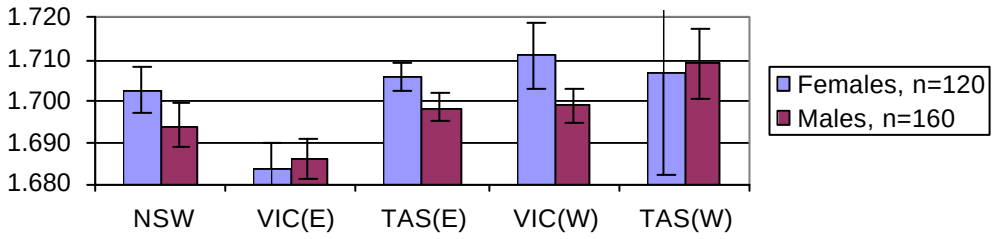


Figure 6.25: Regional variation in log transformed and normalised pectoral-fin length



Statistical Analysis, ANOVA

The sexes were treated separately to eliminate the effects of sexual dimorphism which has been reported for pink ling (Colman, 1995). Unfortunately this meant that sample sizes were reduced as many of the small sexually indistinct individuals had to be eliminated from the analyses. The data were then analysed using two-way ANOVA to test for the effect of depth and locality on head and body measurements. The 0.05 probability level was adjusted by dividing by the number of characters (6 in this case) hence the corrected probability level was 0.008. Once again, NSW and eastern Tasmanian males were selected for this comparison because only these subregions had both deep and shallow depth categories represented. Females could not be compared in this way because of an excess of incomplete data and unequal sample sizes. Depth had no significant effect on any of the characters examined (Table 6.4). The effect of the interaction between region and depth on pre-anus length was close to significant (*P*:0.013). Subregion had a significant effect on head length, upper jaw length, and snout–anus distance.

Table 6.4: Probabilities associated with mean proportional body measurement comparisons between NSW and TAS(E), deep and shallow males. Derived from ANOVA analyses. a **= 0.008**

Probability	Head length	Upper jaw length	Lower jaw length	Inter orbital	Snout–anus	Pectoral fin-length
P: sub- region	0.005	0.001	0.044	0.669	0.000	0.515
P: depth	0.893	0.771	0.489	0.085	0.978	0.834
P: subregion x depth	0.367	0.133	0.526	0.864	0.013	0.602

Depth categories were then pooled and the analysis was repeated with all subregions included. Males and females were treated separately. Three characters varied significantly between subregions for males (Table 6.5). Head length, upper jaw length and lower jaw length tended to be shorter for NSW and western Tasmanian samples than for most other subregions (Figure 6.20–6.22). Only lower jaw length differed significantly between sites among females (Table 6.5). Lower sample sizes for females may have reduced the ability of the analysis to highlight subregional differences in other characters.

Table 6.5: Probabilities associated with mean proportional body measurement comparisons between all five subregions after pooling depths. Derived from ANOVA analyses. a **= 0.008**

Probability	Head length	Upper jaw length	Lower jaw length	Inter orbital	Snout–anus	Pectoral fin-length
P: subregion, females	0.055	0.010	0.000	0.207	0.393	0.055
P: subregion, males	0.000	0.000	0.003	0.267	0.030	0.087

Statistical Analysis, discriminant function analysis

Subregions were then compared using discriminant function analysis with the sexes treated separately. Classification success was greatest when all variables were incorporated in the analysis. However, when more than two variables were used, the jack-knife validation indicated that an excess of predictors in the model had led to an over optimistic result. This restricted the number of variables that could be used to two. This was undesirable as performing this type of analysis with only two variables reduces its power significantly.

As upper and lower jaw lengths and head length were found to be highly correlated lower jaw length and head length were eliminated from the analysis. Pectoral-fin length was the next most important variable.

When only upper jaw length and pectoral-fin length data were used in the classification function the overall success rate for males was 34%. This is significantly (Wilks' Lambda P=0.0000) higher than the 20% that could be expected by chance which suggests that the shape of the specimens examined can be used to predict their geographic origin. However, closer examination of the classification matrix indicates that factors other than stock differences are the most likely causes of this significant result (Figure 6.21). Upper jaw length, which accounted for 80% of the total dispersion between groups, dominated the discriminant function.

Of the eastern Tasmanian males, 41 of 57 individuals were classified as originating in either eastern Tasmania or eastern Victoria (Table 6.6) suggesting that eastern populations mixed more with each other than with those of other subregions. This observation is credible as these subregions are adjacent and this scenario is consistent with our current understanding of the regional biogeography. Conversely NSW specimens were more likely to be misclassified as western Tasmanian than material from either eastern Tasmania or eastern Victoria. Primary populations off western Tasmania and NSW are well separated and based on biogeography it is most unlikely that ling from these subregions would form a unique common stock.

Table 6.6: Discriminant function analysis of body morphometry, classification matrix for males.

Origin	n	Classification results					% Correct
		NSW	VIC(E)	TAS(E)	VIC(W)	TAS(W)	
NSW	24	8	3	2	2	9	33%
VIC(E)	25	3	13	5	2	2	52%
TAS(E)	57	5	20	21	3	8	37%
VIC(W)	28	7	4	6	2	9	7%
TAS(W)	19	6	1	3	1	8	42%
Total	153	29	41	37	10	36	34%

A more likely explanation for the similarity of NSW and western Tasmania specimens is measurement error, which may be due to inconsistent freezer storage between subregions. Alternatively, unexpected environmental influences or unexpected mixing patterns may be occurring due to temporal variation or larval dispersion that is not understood.

Females were not compared using discriminant analysis as there were too few specimens from western Tasmania (n=4) to detect possible undesirable effects on the female data that were evident for males.

6.2.4. Otolith morphometry

Effect of specimen size and other factors on otolith shape

Otolith shape varied considerably with growth. The otoliths of larger individuals were generally smoother and more rounded (Figure 6.26). The length, width and thickness tended to decrease in proportion to the overall size of the animal during growth from 200 mm TL to 1200 mm TL (Figures 6.28–6.30). The relationship between otolith length and TL was best described by the curve linear relationship $y=-1E-05x^2+0.0279x$ (Figure 6.34) indicating that otolith growth declined steadily with age. This relationship does not change at age of maturity for pink ling as it does for kingklip (Payne, 1985).

Even among specimens of similar size (Figure 6.27) there was considerable variation in the shape of otoliths, this may have been due to differences in age. It would have been desirable to compare only specimens of a similar age but the cost of aging all specimens was prohibitive and much larger sample sizes would have been needed.

Data normalisation and transformation

Regressions of the normalised measurements (see methods) against TL suggested that normalisation had successfully eliminated the effect of size from the data (Figures 6.31–6.33). The normalised measurements were then log transformed before being analysed.

Statistical analysis, ANOVA

The adjusted significance level was 0.017 ($\alpha=0.05/3$). Females displayed no significant subregional differences in otolith shape. However, significant subregional differences in otolith width and thickness were evident in males (Table 6.7, Figures 6.36–6.37). Otolith length did not vary between subregions in males (Figure 6.35).

Bonferroni adjusted subregional pairwise comparisons of probabilities for male otolith width and thickness are given below (Tables 6.8–9). This method of test takes into account the number of tests involved hence $\alpha=0.05$. The most significant subregional differences in otolith width existed between ling from western Tasmania and New South Wales, between western Victoria and NSW, and between western Victoria and eastern Tasmania. New South Wales ling otoliths were on average the narrowest and western Victorian otoliths were the widest (Figure 6.36). Western Tasmanian male otoliths were on average the thickest (Figure 6.37) and these were significantly thicker than the subregions with the thinnest average male otoliths - the NSW and eastern Tasmanian subregions (Figure 6.37).

Table 6.7: Probabilities associated with subregional differences in mean otolith measurements. Derived from ANOVA analyses. a **=0.017**

Probability	Otolith length	Otolith width	Otolith thickness

P: Subregion, females	0.422	0.524	0.474
P: Subregion, males	0.419	0.000	0.000

Table 6.8: Bonferroni adjusted pairwise comparisons of subregional means for male otolith width. a **=0.05**

	NSW	VIC(E)	TAS(E)	VIC(W)	TAS(W)
NSW	1.000				
VIC(E)	0.025	1.000			
TAS(E)	0.580	0.964	1.000		
VIC(W)	**0.000**	1.000	**0.006**	1.000	
TAS(W)	**0.003**	1.000	0.375	0.322	1.000

Table 6.9: Bonferroni adjusted pairwise comparisons of subregional means for male otolith thickness. a **=0.05**

	NSW	VIC(E)	TAS(E)	VIC(W)	TAS(W)
NSW	1.000				
VIC(E)	0.497	1.000			
TAS(E)	1.000	0.964	1.000		
VIC(W)	0.160	1.000	0.334	1.000	
TAS(W)	**0.000**	1.000	**0.000**	1.000	1.000

Statistical analysis, discriminant function analysis

An initial discriminant function analysis compared male otolith proportions between all major sites (Table 6.10). The overall classification success using the jack-knifed matrix was 31% which was significantly higher than the 20% that could be expected by chance (Wilks' lambda *P*=0.0000). Western Victoria had the highest classification success suggesting that the mixing rate of ling from this subregion is lowest. The classification success for eastern Victoria individuals was zero, raising the possibility that the locality details for these specimens may have been misreported. However, when locality details were checked with the observer (ISMP) program, no anomalies were found.

Table 6.10: Discriminant function analysis of otolith morphometry, classification matrix for males.

		Classification results					
Origin	n	NSW	VIC(E)	TAS(E)	VIC(W)	TAS(W)	% correct
NSW	28	11	2	7	2	6	39%
VIC(E)	25	6	0	6	8	5	0%
TAS(E)	54	17	4	14	11	8	26%
VIC(W)	28	4	1	2	13	8	46%
TAS(W)	108	26	4	10	31	37	34%

Overall	243	64	11	39	65	64	31%

An additional discriminant function analysis was then performed to investigate possible biases created by subregional differences in mean size of fish. Male otoliths for all subregions were pooled and then divided into small (<500 mm TL), medium (500–749 mm TL) and large (750–1000 mm TL) size classes (Table 6.11). The overall classification success was 52% which is significantly higher than the 33% that could be expected by chance (Wilks' lambda = 0.0002). The two larger size classes in particular, which had the largest sample sizes, had very high classification successes. This suggests that the initial result had been biased by fish size variability, even though plots of the corrected measurements indicate that the effect of size of fish had been eliminated from the corrected data (Figures 6.31–6.33).

Table 6.11: Discriminant function analysis of otolith morphometry. Classification matrix for various size classes of male ling.

		Classification results			
Size class	**n**	**<500 mm**	**500–749 mm**	**750–10000 mm**	**% Correct**
<500 mm	33	5	18	10	15
500–749 mm	154	25	88	41	57
750–10000 mm	56	6	17	33	59
Overall	243	36	123	84	52

Figure 6.26: Change in otolith shape with growth. Males only.

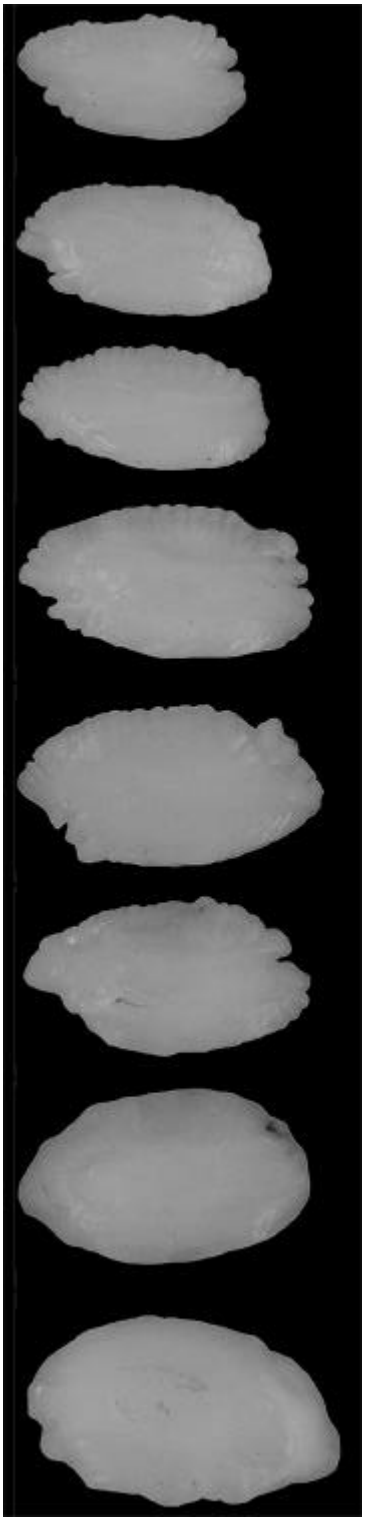


Figure 6.27: Variability in ling otolith shape among fish of similar size. Females only.

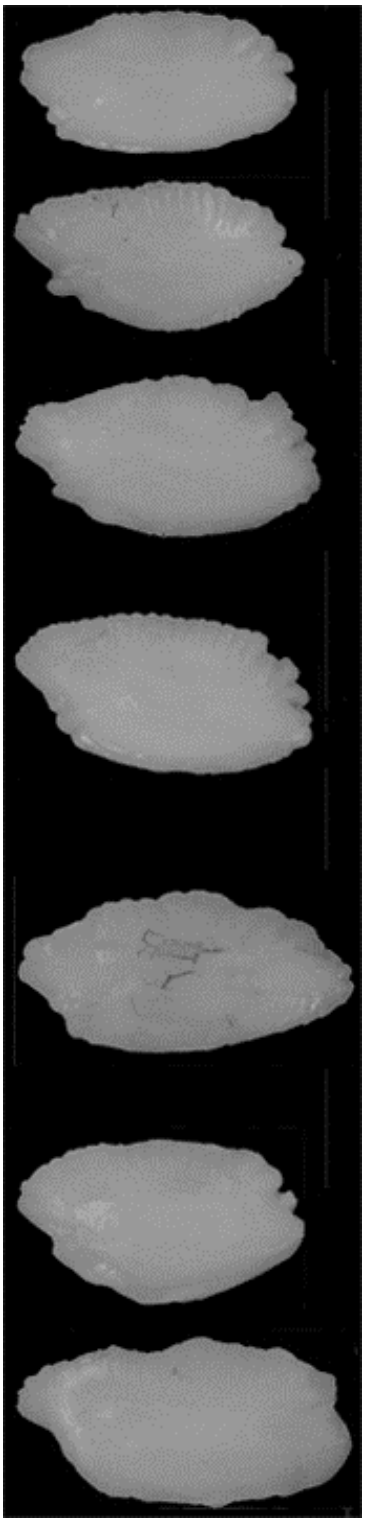


Figure 6.28: Otolith length/total length vs total length, sexes combined (n=529)

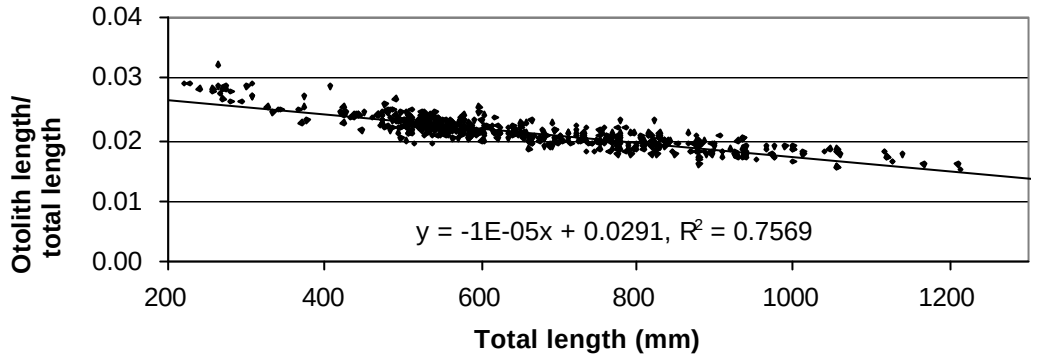


Figure 6.29: Otolith width/total length vs total length, sexes combined (n=527)

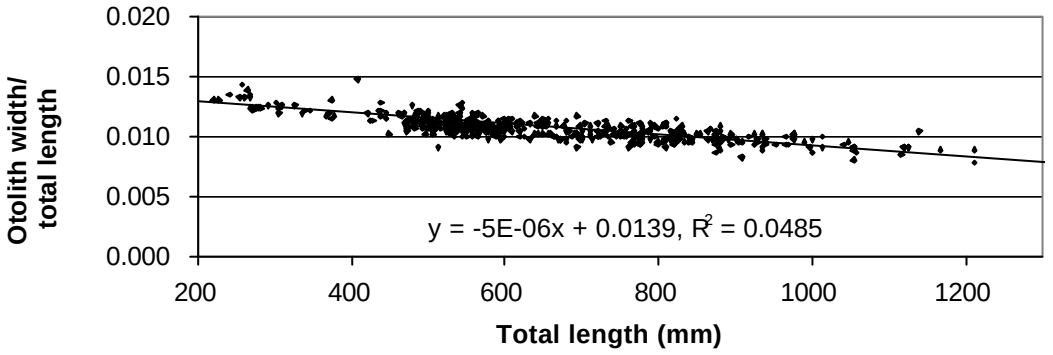


Figure 6.30: Otolith thickness/total length vs total length, sexes combined (n=527)

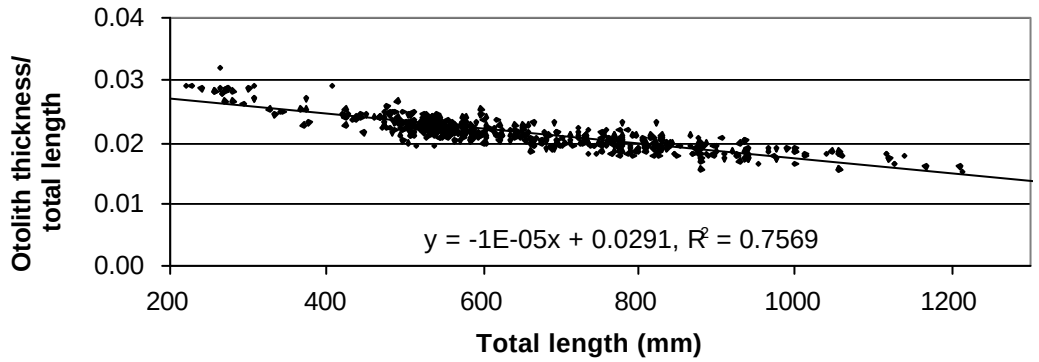


Figure 6.31: Normalised otolith length vs total length, sexes combined (n=520)

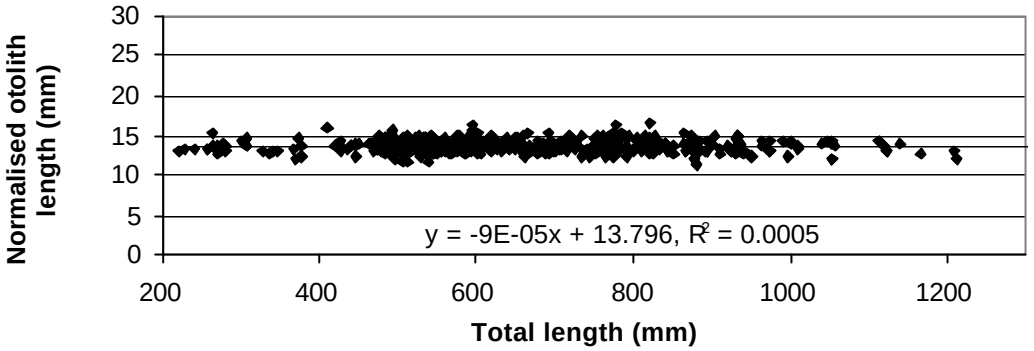


Figure 6.32: Normalised otolith width vs total length, sexes combined (n=520)

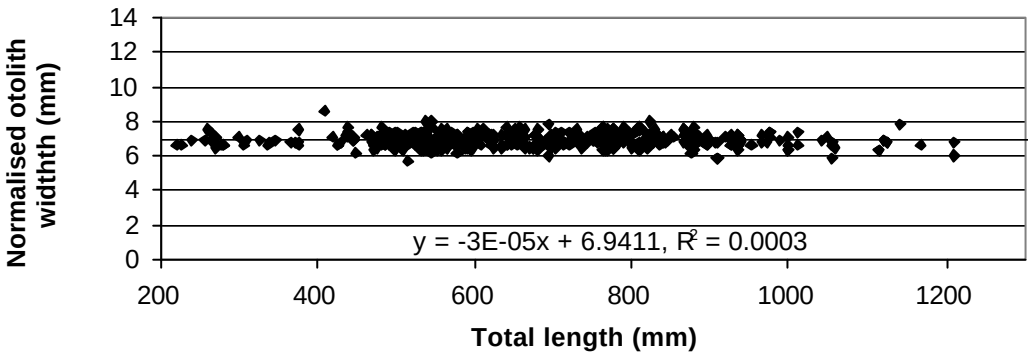


Figure 6.33: Normalised otolith thickness vs total length, sexes combined (n=520)

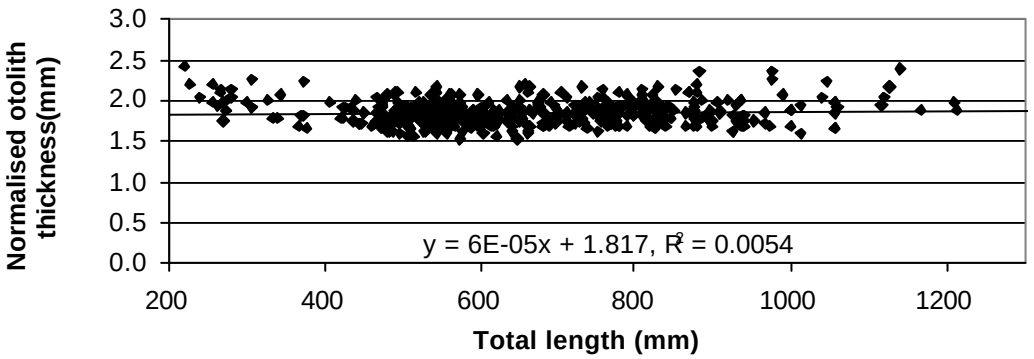


Figure 6.34: Otolith length vs total length

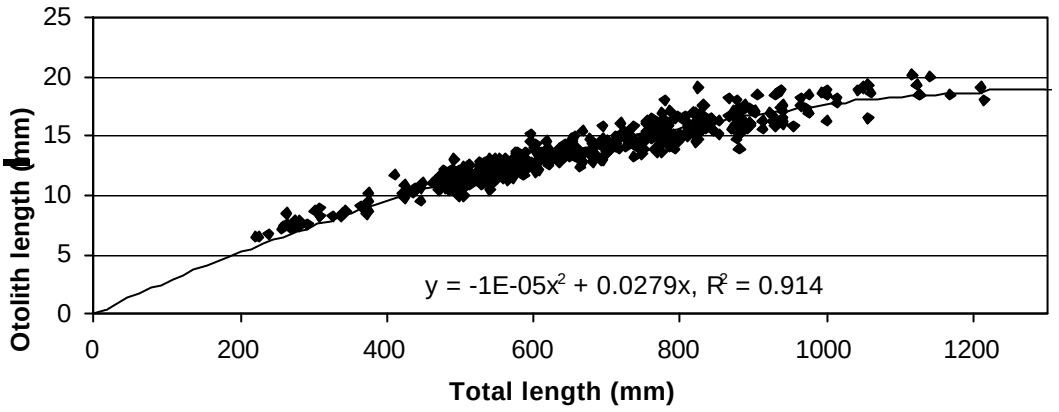


Figure 6.35: Regional variation in log transformed and normalised otolith length

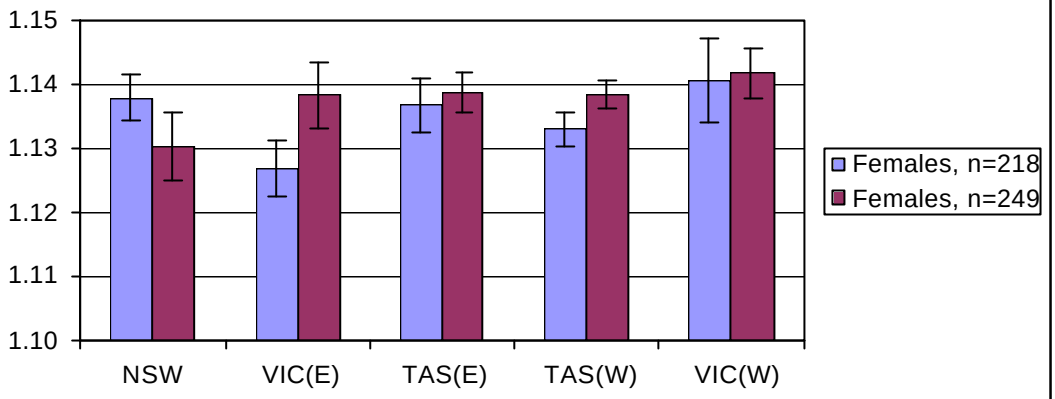


Figure 6.36: Regional variation in log transformed and normalised otolith width

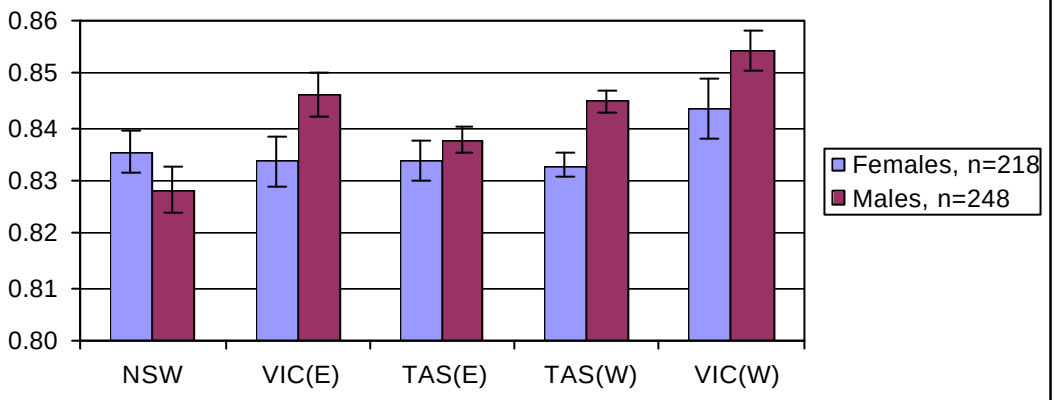
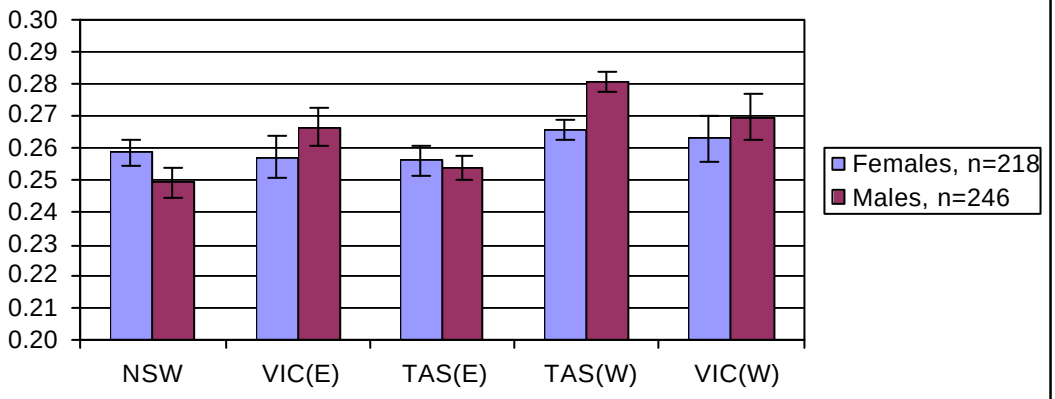


Figure 6.37: Regional variation in log transformed and normalised otolith thickness



6.3. Genetics

6.3.1. Allozyme electrophoresis and DNA microsatellites

Taxonomic issues

The major taxonomic issue to be resolved was whether the pink (deepwater) and orange (shallow water) forms of pink ling (*Genypterus blacodes*) were simply ecomorphs or two separate species. These two forms were compared by allozyme electrophoresis, both with one another and with the closely related, but distinct, rock ling (*G. tigerinus*). Allele frequencies are given in Table 5.3, and genetic variation (degree of polymorphic loci, heterozygosity) parameters are summarised in Table 6.12. Heterozygote banding patterns were consistent with known quaternary structures (Ward *et al*., 1992).

Table 6.12: Summary of allozyme genetic variation in two species

Species or morph	No. loci	Mean no. per locus	Mean no. alleles per locus	Percent loci polymorphic (0.95 crit.)*	HW expected heterozygosity	
					biased	unbiased
G. tigerinus	38	6.0±0.0	1.29±0.10	23.68	0.064±0.021	0.070±0.023
G. blacodes	38	15.3±0.2	1.47±0.10	23.68	0.072±0.019	0.075±0.020
pink	38	3.8±0.1	1.18±0.06	18.42	0.060±0.023	0.070±0.027
orange	38	11.6±0.2	1.45±0.10	26.32	0.073±0.019	0.076±0.020

*****Loci defined as polymorphic if the most common allele has a frequency of 0.95 or less.*

There were no differences in allele frequencies at any of the 38 allozyme loci examined between the pink and orange forms of *G. blacodes*. The genetic identity of these two morphs was extremely high (Nei standard I = 0.993; Nei unbiased I = 1.000) and conversely the genetic distance very low (Nei standard D = 0.007; Nei unbiased D = 0.000).While the numbers of individuals examined were quite low, the number of loci examined was high. In analyses of genetic distance, the numbers of loci examined are more important than numbers of individuals (Nei, 1978; Gorman and Renzi, 1979) and this particular study was therefore quite powerful. There was no evidence of any allozyme separation of the two forms, which may safely be regarded as forms of a single species (*G. blacodes*).

This conclusion was confirmed with microsatellite data. Eastern Tasmanian samples of pink ling (the area with largest sample sizes) were ascribed to shallow (50–120 m, n = 20), medium (200–325 m, n = 56) or deep (400–525 m, n = 25) collections. An AMOVA, across all nine loci across these three depth ranges (Table 6.13) was carried out—the percentage of variation attributable to collection (population) difference was very small, non-significant (P>0.99), and indeed negative (Table 6.13). The F_{ST} value was also very small and similarly negative and non-significant (F_{ST} = -0.0046, P>0.99). There was a random

distribution of genotypes across the three depth strata (P = 1.0, 200000 Markov chain steps). There is therefore no evidence for microsatellite separation of fish from these different depth strata.

Table 6.13: Results of AMOVA analysis at nine microsatellite loci in shallow, medium and deep samples of pink ling from eastern Tasmania.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among populations	2	5.322	-0.017	-0.46
Within populations	199	730.154	3.669	100.46
Total	201	735.475	3.652	

F_{ST} = -0.00462. The P that a randomly generated value would be greater than or equal to the among population variance component, or the F_{ST}, following 10100 permutations, was 0.997±0.001. Arlequin software.

On the other hand, six allozyme loci (*IDDH, SOD, ME, APlt-1, APpp* and *PROT-1*) enabled unequivocal separation of *G. tigerinus* from *G. blacodes*. The genetic identity of these two species was 0.825 (standard; unbiased I = 0.829) and the genetic distance 0.192 (standard; unbiased = 0.188). Clearly, the genetic data indicate that these two species are reproductively isolated from one another.

Genetic population structure of pink ling

A. Allozymes

Thirty-eight allozyme loci were examined in pink ling in the initial stage of the project (Table 5.3). Eight of these loci were polymorphic using the 0.95 criterion: *IDDH, ADH, AAT-1, MPI, PGM-2, ADA, IDH-1* and *APlgg*. Three of these loci could be scored easily and repeatedly from white muscle tissue – *AAT-1, PGM-2* and *APlgg* – and these were chosen for the population analysis.

Fish from five areas were examined, and allele frequencies determined (Table 6.14). Fifteen (five areas x three loci) Hardy-Weinberg tests were conducted using the CHIHW program (Table 6.15). Three of these tests gave *P* values <0.05, but after Bonferroni correction for 15 tests (α falling from 0.05 to 0.05/15 = 0.003), only one test was significant. This was *APlgg* in western Victoria, with *P* = 0.002. The other two loci from this area did not show significant deviations (*P* values of 0.478 and 0.762). We ascribe the *APlgg* discrepancy to errors in genotyping or stochastic noise rather than to any biologically important phenomenon.

Allele frequencies across the five areas (Table 6.14) did not show evidence of significant spatial heterogeneity. GENEPOP analysis of contingency tables (numbers at each allele x population, for each locus), using 400 batches and 4000 iterations per batch, gave non-significant values for each locus. Combining data across loci gave a non-significant result. F_{ST} values were correspondingly all very close to zero (Table 6.16). No pairwise population comparisons gave a significant *P* value for any locus (data not shown).

Approximately 1 per cent of the allele frequency variation could be attributed to differences among samples from a G_{ST} analysis (Table 6.16), but this was not significantly greater than that expected of stochastic noise in the given sample sizes (G_{STnull}, Table 6.16).

The conclusions from these analyses all indicate that there is no significant spatial partitioning of genetic diversity at the allozyme level in pink ling.

Table 6.14: Allozymes, allele frequencies in pink ling populations

Locus	Allele	Population NSW	VIC(E)	TAS(E)	VIC(W)	TAS(W)	Homogeneity *P*
AAT-1	*s*	0	0.008	0.018	0	0.050	0.117
	m	0.772	0.715	0.726	0.754	0.700	
	f	0.228	0.277	0.256	0.246	0.250	
	n	101	65	82	57	20	
APlgg	*s*	0.005	0.006	0.005	0	0	0.850
	m	0.776	0.808	0.779	0.814	0.750	
	f	0.219	0.186	0.216	0.178	0.250	
	vf	0	0	0	0.008	0	
	n	96	78	95	59	16	
PGM-1	*s*	0.034	0.065	0.080	0.052	0	0.341
	m	0.845	0.766	0.760	0.810	0.925	
	f	0.121	0.162	0.150	0.129	0.075	
	vf	0	0.006	0.010	0.009	0	
	n	103	77	100	58	20	

P = estimated probability of population homogeneity following 5000 randomisations of data.

Table 6.15: Results of Hardy-Weinberg tests of genotype distributions at each locus in each population of pink ling.

Locus	NSW		VIC (E)		TAS (E)		VIC (W)		TAS (W)	
	n	*P*	n	*P*	n	*P*	n	*P*	n	*P*
AAT-1	101	0.399	65	1.000	82	0.222	57	0.478	20	0.223
APlgg	96	0.647	78	0.034	95	0.511	59	0.002	16	0.502
PGM-1	103	0.283	77	0.092	100	0.035	58	0.762	20	1.000

P = probability of fit to Hardy-Weinberg equilibrium following 5000 randomisations of data.

Table 6.16: Contingency tests and FST and GST analyses of allozyme allele frequencies in pink ling populations

Locus	n	P±SE	F_{ST}	G_{ST}	G_{STnull}? SD	*P**

AAT-1	325	0.381±0.004	-0.0042	0.0047	0.0082±0.0068	0.652
APlgg	344	0.968±0.001	-0.0065	0.0110	0.0096±0.0082	0.318
PGM-1	358	0.193±0.004	0.0052	0.0180	0.0080±0.0055	0.056
overall		0.508	-0.0018	0.0107	0.0086±0.0042	0.230

P±SE and F_{ST} are results of contingency tests of spatial differentiation of the five populations at individual loci, and Weir and Cockerham's F_{ST} values, respectively. Results from GENEPOP (using 400 batches and 4000 iterations per batch). P estimates the probability that G_{ST} and G_{STnull} are not different following 1000 randomisations of the data.*

B. Microsatellite loci

Allele frequencies were determined (Appendix D). All loci were highly variable (Table 6.17). Total numbers of alleles per locus ranged from 11 to 52, with an average of 34.0. Had the sample sizes been larger than the average of about 60 per population, more alleles would have been detected. The average observed heterozygosity per locus was about 82%, with an average Hardy-Weinberg expected heterozygosity of about 90%.

Tests of genotypic disequilibrium (non-random assortment of genotypes) among loci were carried out (by the GENEPOP package) using fishers method to assess whether variation at each microsatellite loci was independent of variation at other microsatellite loci (Table 6.18). The Markov chain analysis used 400 batches and 4000 iterations per batch. These pair-wise locus tests were first carried out within each population. There were 36 pairwise locus comparisons within each population, so the α level of significance within populations was reduced from 0.05 to 0.05/36 = 0.00139. Two instances were significant at this *P* level: eastern Tasmania, 5.8A & 4.2B, *P* = 0.00011 and eastern Victoria, 2.6.1 and 5.5A, *P* = 0.00130. If the number of pairwise comparisons is considered to be 165 (5 populations by 36 comparisons, minus 15 pairwise comparisons within western Tasmania which GENEPOP stated carried 'no information' as all row or column totals equaled one), α falls to 0.00030, and only eastern Tasmania 5.8A and 4.2B remains significant).

Only one comparison was significant following corrections for multiple tests (α becoming 0.00139). This was for the pair 5.8A and 4.2B, where *P* = 0.00001. This locus pair was tested from eastern Victoria, western Victoria, NSW and eastern Tasmania (western Tasmania having 'no information'), with respective *P* values of 0.0029, 1.0000, 0.0227 and 0.0001. This inconsistency of *P* values across populations suggests that there is no true association between genotypes at these two loci. Furthermore, an earlier run of GENEPOP (admittedly with only 200 batches and 2000 iterations rather than the final 400 batches and 4000 iterations) yielded a *P* value of 0.0126, far from significant given the 36 tests. As a final statistical check, the software package ARLEQUIN was used to evaluate linkage disequilibrium. This analyses data in a different way, by determining expected frequencies of di-locus genotypes assuming Hardy-Weinberg equilibrium. The *P* value for 5.8A and 4.2B in eastern Tasmania was estimated at a non-significant 0.055±0.020 (c.f. 0.00011 from GENEPOP). It is also noteworthy that the two pairs of loci that we know to be linked as each member of the pair was isolated from a single clone (i.e. 5.8A and 5.8B, and 4.2B and 4.2A) did not show significant disequilibrium. It is therefore unlikely that two independently-derived loci such as 5.8A and 4.2B would be in true disequilibrium. We therefore consider all nine microsatellite loci to be independent loci, and all are used in ensuing analyses.

Tests of goodness-of-fit of genotype distributions to Hardy-Weinberg expectations were carried out for each locus in each population (Table 6.19). Within each population there were nine tests (equal to the number of loci), so α values for hypothesis testing were set at 0.05/9 = 0.0056 for each population. Significant deviations from equilibrium expectations were observed in seven instances: eastern Victoria, two loci, 5.8B (*P*<0.001), 5.8A (*P*<0.001); western Victoria, one locus, 5.8A (*P*<0.001); NSW, two loci, 5.8A (*P*<0.001), 4.2A (*P*<0.001); eastern Tasmania, one locus, 5.8B (*P*=0.003); western Tasmania, one locus, 4.11 (*P*=0.001). In each instance, there was an excess of homozygotes (a positive F_{IS}

value, averaging 0.198, Table 6.19). Even if the α value is set at 0.001 (from 0.05/45, where the 45 tests come from 9 loci x 5 populations), five of these instances remain significant.

An excess of homozygotes can be caused by several factors. One possibility is the presence of null (non-amplifying) alleles, which appear to be relatively common for microsatellites in a wide variety of species (e.g. Chinook salmon, Scribner *et al*., 1996; deer, Pemberton *et al*., 1995; Pacific oysters, McGoldrick and Ward, unpublished). Using the genotype data sets, best fit null allele frequencies can be estimated (using NULLTEST). These, along with associated 95% confidence intervals, are given in Table 6.19. Three of the five instances of $P<0.001$ in the Hardy-Weinberg tests are associated with estimated null allele frequencies of about 0.10–0.20 (eastern Victoria, 5.8B, 5.8A; western Victoria, 5.8A), and two with estimated null allele frequencies of about 0.05 (NSW, 5.8A, 4.2A). There is also evidence for (low frequency) null alleles in two further instances (western Victoria, 5.8B; eastern Tasmania, 5.8B), one of which (eastern Tasmania, 5.8B) showed evidence of Hardy-Weinberg deviations ($P=0.003$). Overall, this analysis suggested that null alleles existed at loci 5.8A and 5.8B, with an average frequency of about 0.10. These null alleles can account, at least partly, for the observed homozygote excess, as an expressed allele/null allele heterozygote will be mistakenly scored as the expressed allele homozygote. The null alleles appear to be found in all populations. Expressed allele frequencies can be adjusted to account for the estimated null allele frequencies, but since we cannot, in the absence of further data (such as breeding data), be certain that null alleles truly exist, we have chosen to use the allele frequency data from Appendix D in tests of population structure.

The first population structure analysis considered all five populations at each locus separately. Contingency tables (numbers at each allele x population) were constructed and analysed using GENEPOP (Table 6.20). The corrected α value was 0.05/9 (9 loci) = 0.0055. At this α value, only one locus showed evidence of differentiation. This was 5.8B ($P=0.002$). The P value over all nine loci was 0.025, just significant at the 0.05 level.

Populations were compared pairwise for locus 5.8B, to locate the source of the apparent differentiation at this locus (Table 6.21). There were ten pairwise comparisons, one of which (western Victoria and NSW) was just significant ($P=0.004$) at the adjusted α value of 0.05/10 = 0.005. Another population pair (western Victoria and eastern Tasmania) bordered on significance ($P=0.006$). It therefore seems that the western Victoria population was responsible for most of the heterogeneity. Inspection of the allele frequency data shows that much of the heterogeneity appeared to be associated with allele 155. This allele appears to be reasonably common in W VIC (frequency=0.100) and uncommon in the other populations (frequencies 0–0.06). On the other hands, flanking alleles 153 (f=0.009) and 157 (f=0.027) are less common in western Victoria than in other populations (f=0.024–0.090 and f=0.045–0.081, respectively, excluding western Tasmania which has a small sample size). This suggests that there might have been some inconsistencies in scoring the alleles in this subregion. Grouping alleles 153, 155 and 157 into a single composite allele and retesting increases the probability of homogeneity from 0.002 to 0.016 (this 'retested' value came from a bootstrapped Monte-Carlo contingency test, with 1000 randomisations), a value non-significant after Bonferroni correction for multiple tests. The overall nine locus P value becomes 0.069, i.e. non-significant.

The individual locus F_{ST} values were all very low (Table 6.20), ranging from 0.0030 to 0.0004. The value for locus 5.8B was 0.0012. Over all loci, the value was 0.0006. This is

extremely close to zero. Less than 0.1% of the variation was attributable to population differentiation.

The data set was also analysed using the package ARLEQUIN. This enabled a statistical analysis of variation across all loci, but did not permit a partitioning to individual loci. The AMOVA (Table 6.22) showed that the percentage of variation attributable to among population differences was -0.06. This is, in effect, zero. The F_{ST} value was -0.00058, again effectively zero. Both figures have a *P* value of 0.871

There were no statistically significant F_{ST} values among the ten possible pairwise population comparisons (Table 6.23). F_{ST} values are often used to estimate M (numbers of migrants exchanged per generation). The majority of pairwise comparisons, as expected from preceding analyses, give F_{ST} values of zero, corresponding to M values of infinity (Table 6.24). Two of the three pairwise comparisons with M values less than infinity involve the western Victoria population, which was responsible for most of the heterogeneity recorded earlier for the locus 5.8B. However, we do not believe that any reliance should be placed upon the few M values which are less than infinity - their (unestimated) standard errors will be extremely large. They are given here more for the sake of completeness than because they provide evidence of population structuring.

ARLEQUIN also performed a contingency test of differentiation between all populations. This gave a *P* value of 1.0 (for non-differentiation) and all pairwise population comparisons also gave *P* values of 1.0 (for non-differentiation).

The various GENEPOP and ARLEQUIN analyses fail to establish any significant evidence of population differentiation. The data are consistent with a hypothesis of panmixia.

Table 6.17: Microsatellites, summary of variability.

Loci	Ave. sample size per pop.	Ave. no. alleles per pop.	Total no. alleles	Average expected het.	Average observed het.
2.6.1	61.0±12.3	7.60±0.75	11	0.634±0.026	0.545±0.020
5.8B	61.2±12.2	34.6±2.79	52	0.966±0.001	0.789±0.038
5.8A	54.0±12.2	24.6±2.56	36	0.944±0.003	0.787±0.023
4.2B	59.8±12.3	20.6±1.54	26	0.924±0.003	0.924±0.008
4.2A	60.2±11.8	22.6±2.06	33	0.907±0.003	0.859±0.026
4.11	60.4±12.2	33.4±2.84	49	0.961±0.002	0.900±0.026
5.2B	61.2±12.5	20.2±0.97	25	0.938±0.001	0.949±0.015
5.9A	60.8±11.8	24.2±1.85	32	0.936±0.002	0.872±0.014
5.5A	58.4±12.2	22.4±3.34	42	0.845±0.027	0.780±0.023
Overall mean	59.7±0.76	23.4±2.63	34±4.24	0.895±0.034	0.823±0.040

Table 6.18: Microsatellites, genotypic disequilibrium for pairs of loci across all populations, *P* values above diagonal. Chi-square values and degrees of freedom below diagonal.

Loci	2.6.1	5.8B	5.8A	4.2B	4.2A	4.11	5.2B	5.9A	5.5A
2.6.1	----	0.9942	0.0018	0.2206	0.3618	0.9248	0.7378	0.3483	0.0331
5.8B	2.239 10	----	1	0.7388	0.8211	1	1	1	0.8855
5.8A	19.629 8	0 8	----	<0.0001	1	1	1	1	0.9361
4.2B	13.056 10	6.858 10	37.506 8	----	0.3372	1	0.8146	0.235	0.9112
4.2A	10.946 10	5.931 10	0 8	11.266 10	----	1	0.9524	1	0.813
4.11	4.449 10	0 10	0 8	0 10	0 10	----	0.4518	1	0.9999
5.2B	6.869 10	0 10	0 8	6.008 10	3.884 10	9.872 10	----	0.2703	0.8539
5.9A	8.930 8	0 8	0 8	10.447 8	0 8	0 8	9.925 8	----	0.1986
5.5A	19.612 10	5.085 10	2.973 8	4.686 10	6.027 10	0.924 10	5.519 10	11.056 8	----

df=10 where all populations contributed data, and 8 where only four populations contributed data.

Table 6.19: Microsatellites, results of Hardy-Weinberg tests of genotype distributions at each locus in each population of pink ling.

Population	Locus	Hardy-Weinberg tests		Null allele tests		
		P	F_{IS}	null present?	null frequency	95% CI
NSW	2.6.1	0.217	0.106	no	0.051	0.066
	5.8B	0.015	0.104	(yes)	0.051	0.022
	5.8A	<0.001	0.120	(yes)	0.059	0.030
	4.2B	0.756	0.007	no	<0.001	0.032
	4.2A	<0.001	0.130	(yes)	0.066	0.044
	4.11	0.124	0.045	no	0.020	0.027
	5.2B	0.649	0.015	no	0.005	0.027
	5.9A	0.807	0.050	no	0.021	0.029
	5.5A	0.014	0.138	(yes)	0.071	0.051
VIC(E)	2.6.1	0.166	0.137	no	0.088	0.095
	5.8B	<0.001	0.337	yes	0.198	0.022
	5.8A	<0.001	0.232	yes	0.126	0.027
	4.2B	0.017	-0.033	no	-0.020	0.037
	4.2A	0.961	-0.039	no	-0.026	0.045
	4.11	0.023	0.074	(no)	0.034	0.029
	5.2B	0.245	0.017	no	0.005	0.033
	5.9A	0.276	0.112	(yes)	0.058	0.028
	5.5A	0.769	0.053	no	0.023	0.054
TAS(E)	2.6.1	0.161	0.106	no	0.057	0.068
	5.8B	0.003	0.186	yes	0.100	0.020
	5.8A	0.037	0.115	(yes)	0.058	0.027
	4.2B	0.835	0.016	no	0.006	0.025
	4.2A	0.703	0.026	no	0.011	0.026
	4.11	0.362	-0.010	no	-0.007	0.022
	5.2B	0.835	-0.036	no	-0.026	0.027
	5.9A	0.063	0.093	(yes)	0.047	0.028
	5.5A	0.169	0.053	no	0.023	0.033
VIC(W)	2.6.1	0.319	0.055	no	0.023	0.094
	5.8B	0.069	0.151	yes	0.066	0.028
	5.8A	<0.001	0.219	yes	0.118	0.041
	4.2B	0.064	-0.005	no	-0.009	0.042
	4.2A	0.730	0.043	no	0.019	0.037
	4.11	0.415	0.049	no	0.020	0.030
	5.2B	0.317	0.010	no	0.001	0.035
	5.9A	0.140	0.054	no	0.022	0.041
	5.5A	0.444	0.114	no	0.058	0.058
TAS(W)	2.6.1	0.014	0.281	(no)	0.151	0.144
	5.8B	0.025	0.147	(yes)	0.066	0.047
	5.8A	0.243	0.156	(no)	0.072	0.066
	4.2B	0.602	0.016	no	-0.007	0.077
	4.2A	0.264	0.104	no	0.044	0.077
	4.11	0.001	0.162	(yes)	0.074	0.054
	5.2B	0.770	-0.067	no	-0.046	0.068
	5.9A	0.801	0.038	no	0.009	0.057
	5.5A	0.844	-0.112	no	-0.085	0.113

P = probability of fit to Hardy-Weinberg equilibrium following Markov chain analysis with a chain length of 200,000 (Arlequin program). F_{IS} estimated using Weir and Cockerhams's method, as

implemented in GENEPOP. Null allele statistics estimated using NULLTEST. Null present? summarises whether a null allele is likely to be present based on the estimated null allele frequency and its 95% confidence limits. Figures in brackets indicate weak evidence.

Table 6.20: Microsatellites, results of contingency tests of spatial differentiation of the five populations at individual loci, with Weir and Cockerham's F_{ST} values.

Locus	*P*±SE	F_{ST}
2.6.1	0.300±0.007	0.0030
5.8B	0.002±0.001	0.0012
5.8A	0.200±0.007	-0.0007
4.2B	0.877±0.005	-0.0004
4.2A	0.968±0.002	-0.0013
4.11	0.276±0.009	0.0005
5.2B	0.876±0.004	-0.0016
5.9A	0.024±0.002	0.0022
5.5A	0.219±0.009	0.0035
Overall	0.025	0.0006
(chisquare=31.47, d.f.=18)		

Results from GENEPOP (using 400 batches and 4000 iterations per batch).

Table 6.21: Microsatellites, results of pairwise population comparisons for locus 5.8B (see Table 6.20). *P* values (±SE) for null hypothesis of no significant differentiation.

	VIC(E)	NSW	TAS(W)	VIC(W)
TAS(E)	0.534±0.005	0.080±0.002	0.522±0.004	0.006±0.001
VIC(E)		0.049±0.002	0.162±0.003	0.079±0.002
NSW			0.032±0.001	0.004±0.001
TAS(W)				0.087±0.002

Table 6.22: Microsatellites, AMOVA analysis across all nine loci for the five populations. Arlequin package.

Source of variation	Degrees of freedom	Sum of squares	Variance components	Percentage of variation
Among populations	4	13.483	-0.00210	-0.06
Within populations	625	2265.776	3.62524	100.06
Total	629	2279.259	3.62314	

F_{ST} = -0.00058. The P that a randomly generated value would be greater than or equal to the among population variance component, or the F_{ST}, following 10100 permutations, was 0.871±0.003.

Table 6.23: Microsatellites, matrices of pairwise population comparisons over all loci. F_{ST} (above diagonal) and *P* estimates (below diagonal).

	TAS(E)	VIC(E)	NSW	TAS(W)	VIC(W)
TAS(E)	----	0.00025	-0.00121	-0.00085	-0.00048
VIC(E)	0.454	----	-0.00127	-0.00318	0.00207
NSW	0.923	0.913	----	-0.00661	0.00120
TAS(W)	0.655	0.924	0.993	----	-0.00189
VIC(W)	0.680	0.109	0.221	0.759	----

The SE associated with P were all less than 0.01 (4970 permutations)

Table 6.24: Migration numbers, M, estimated from F_{ST} values.

	VIC(E)	NSW	TAS(W)	VIC(W)
TAS(E)	2039.26	infinity	infinity	infinity
VIC(E)		infinity	infinity	240.58
NSW			infinity	415.77
TAS(W)				infinity
VIC(W)				

6.3.2 Mitochondrial DNA sequence analysis

Primers amplified a fragment of approximately 300 base pairs (excluding the size of primers) of the cyt *B* gene. Direct fluorescent sequencing was used to determine the amount of genetic variation present in a 259 base pair fragment of the cyt *B* gene that could be unambiguously aligned in all nine individuals. Sequences are provided as the coding strand of the fragment in Appendix E. Five mtDNA haplotypes were observed; two in pink and rock ling and a single haplotype in kingklip ling. Based on these haplotypes, both intraspecific and interspecific DNA variation were observed. Among interspecies sequence comparisons, a total of 61 variable nucleotide sites were identified and these were used in subsequent phylogenetic analysis (Table 6.25). All negative controls (those without DNA) were clean, indicating no aerial contamination.

Table 6.25: Matrix of total numbers of nucleotide site differences in pairwise comparisons among mtDNA cytochrome B for individuals from three ling species.

Sample*	Pink1	Pink2	Pink3	Rock1	Rock2	Rock3	Cap1
Pink1 A							
Pink2 B	3						
Pink3 A	0	3					
Rock1 C	42	41	42				
Rock2 D	38	39	38	4			
Rock3 C	42	41	42	0	4		

Cap1 E	23	26	23	61	57	61	
Cap2 E	23	26	23	61	57	61	0

Pink1=G. blacodes shallow morph; Pink2=G. blacodes shallow morph; Pink3=G. blacodes deep morph;
Rock1, 2 & 3= *G. tigerinus*; Cap1 & 2=*G. capensis.* ***MtDNA Haplotyypes A-E based on sequence comparisons.***

Intraspecific comparisons

Two haplotypes were observed in the three pink ling. Type A was found in one deep and one shallow individual and type B was found in the other shallow individual. Three nucleotide sites were different between the two haplotypes. The base differences consisted of two 3rd base changes (one transition & one transversion) and one 1st base transition change (Table 6.26). The mean number of transitions and transversions among the three pairwise comparisons was 1.3 and 0.6 respectively. Sequence divergence among the three individuals, ranged from 0–1.16%. The average sequence divergence between the deep and shallow individuals was 0.58%. This was smaller than the sequence divergence observed between the two shallow individuals (1.16%).

Two different haplotypes, C and D, were found in rock ling. Two fish were type C, one was type D. There were four nucleotide site differences between these two haplotypes, consisting of three 3 rd base transition changes and one 3 rd base transversion (Table 6.26). The mean number of transitions and transversions among the three pairwise haplotype comparisons was 2.0 and 0.6 respectively. Sequence divergence ranged from 0–1.5.

Only one mtDNA genotype, E, was observed in the two kingklip individuals (Table 6.26).

Overall, 14 base differences were observed in seven pairwise comparisons between individuals of the same species. Ten were transitions (8, T→C; 2, A→G), and four were transversions (all A→C). As previously indicated in the literature (Kocher *et al*., 1989; Bartlett and Davidson, 1991; Carr and Marshall, 1991; McVeigh *et al*., 1991; Bennetts *et al*., 1999), the majority of base changes observed between close relatives (of the same species) were transitions.

Interspecific comparisons

The interspecific variation was much greater than intraspecific variation. Between the 'A' pink ling haplotype and the 'E' kingklip ling haplotype, 23 variable nucleotide sites were observed. Between the same pink ling haplotype and the 'C' rock ling haplotype, 42 variable sites were observed. Sixty-one variable sites were observed between the 'C' rock ling haplotype and the 'E' kingklip ling haplotype. Numbers of transitions and transversions are given in Table 6.26 for pairwise comparisons between individuals of different species.

Table 6.26: Matrix of number of transitions (below diagonal) and transversions (above diagonal) in pairwise comparisons among mtDNA cytochrome *B* for individuals from three ling species.

Sample	Pink1	Pink2	Pink3	Rock1	Rock2	Rock3	Cap1	Cap2
Pink1 A	-----	1	0	21	20	21	13	13
Pink2 B	2	-----	1	20	19	20	14	14
Pink3 A	0	2	-----	21	20	21	13	13
Rock1 C	21	21	21	-----	1	0	32	32
Rock2 D	18	20	18	3	-----	1	31	31
Rock3 C	21	21	21	0	3	-----	32	32
Cap1 E	10	12	10	29	26	29	-----	0

Cap2 E	10	12	10	29	26	29	0	-----

Pink1=G. blacodes shallow morph; Pink2=G. blacodes shallow morph; Pink3=G. blacodes deep morph;
Rock1, 2 & 3= G. tigerinus; Cap1 & 2=G. capensis

Between the pink and rock ling individuals, depending on individual sequence, 38–42 bases were different, representing a minimum of 14% to a maximum of 16% sequence divergence. The number of transitional base changes (182) approximated the transversional differences (183) in the nine pairwise comparisons, giving a transition:transversion ratio of 1:1.

Between 23–26 bases were different between the pink and kingklip ling individuals, representing between 8.8–10% sequence divergence. In six pairwise comparisons, 64 transitional and 80 transversional base differences were observed giving a transition:transversion ratio of 1:1.25

The greatest numbers of nucleotide sites (56–60) were different between the rock and kingklip ling individuals with sequence divergence between 22–23%. In six pairwise comparisons, the number of transversional changes (190) out-numbered transitional base changes (168) giving a transition:transversion ratio 1:1.13.

The mean number of transitions across the 21 pairwise comparisons was 19.7 and 21.6 for transversions. The mean total number of transitions and transversions (mnd) across 21 pairwise comparisons was 41.2 and the overall ratio of transitions to transversions (ns/nv) was 1:1.10. Of the base pair differences among the 21 pairwise comparisons, the ratio of pyrimidine transitions (T→C; 260) to purine transitions (A→G; 154) is 1:0.59 between individuals of different species. Base change transversions of type A→T (168) and A→C (186) were the most common transversions observed in the pairwise comparisons between individuals of different species. Transversions were more evident as individuals of more distantly related species were compared (Table 6.26).

As previously noted in the literature (Kocher *et al*., 1989; Bartlett and Davidson, 1991; Carr and Marshall, 1991; McVeigh *et al*., 1991; Bennetts *et al*., 1999), 3 rd codon substitutions (36/61 variable sites) predominated over 18, 1 st base and seven, 2 nd base changes among species.

Pairwise genetic distance (p & JC) estimates are given in Table 6.27. Both distance estimates gave similar values. In all instances, *G. capensis* was most distantly related to *G. tigerinus*, and *G. blacodes* and *G. capensis* were most closely related.

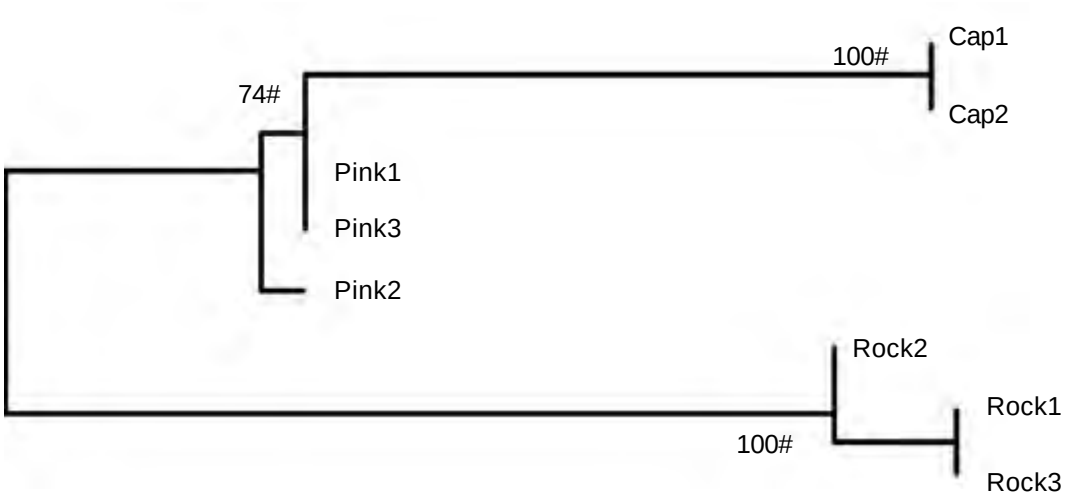
Table 6.27: Matrix of genetic distances among individuals (p distance estimates below the diagonal, JC distance estimates above).

Sample	Pink1	Pink2	Pink3	Rock1	Rock2	Rock3	Cap1	Cap2
Pink 1	-----	0.017	0.000	0.184	0.164	0.184	0.099	0.099
Pink 2	0.016	-----	0.012	0.179	0.169	0.179	0.102	0.102
Pink 3	0.000	0.012	-----	0.184	0.164	0.184	0.099	0.099
Rock 1	0.168	0.159	0.163	-----	0.016	0.000	0.280	0.280
Rock 2	0.143	0.151	0.147	0.016	-----	0.262	0.267	0.267
Rock 3	0.168	0.159	0.163	0.000	0.016	-----	0.286	0.286
Cap 1	0.081	0.101	0.089	0.236	0.221	0.236	-----	0.000
Cap 2	0.081	0.101	0.089	0.236	0.221	0.236	0.000	-----

Pink1=G. blacodes shallow morph; Pink2=G. blacodes shallow morph; Pink3=G. blacodes deep morph;
Rock1, 2 & 3= G. tigerinus; Cap1 & 2=G. capensis

Figure 6.38 shows a genealogical analysis of the relationships between the three species. Only the NJ tree employing the JC distance estimate is given here as caution needs to be exercised when using UPGMA trees based on short sequences (as in the current study) because of the assumption of a constant rate of evolution (Fournier Lockwood *et al*., 1993; Kumar *et al*., 1993). However, with our data the NJ and UPGMA tree topologies were very similar. The NJ tree shows BCL values obtained after 2000 bootstrap replicates. All external branches displayed BCL values of 100% and all other internal clusters were strongly supported. The tree strongly supported the division of the three species with *G. tigerinus* branching off separately from *G. blacodes* and *G. capensis.*

Figure 6.38: Genealogical NJ tree among ling species, constructed using JC distance estimates.



#Numbers on internal branches refer to the BCL (%) of each node. External BCL's though not given are each 100%

Discussion of mitochondrial DNA results

The main objective of the sequencing study was to determine if the shallow (orange) and deep (pink) morphs of pink ling (*Genypterus blacodes*) were genetically distinct. In addition, the study aimed to determine if three recognised species of ling could be identified using mtDNA cytochrome *B* gene sequences.

Based on the 259 base pair fragment, the three species could be unambiguously identified. Both intraspecific and interspecific site variations were evident. The two *G. capensis* individuals were of the same haplotype (E) while two haplotypes (A and B) were identified in the three *G. blacodes* and two haplotypes (C and D) in the three *G. tigerinus*.

The average sequence difference between the shallow and deep morphs of *G. blacodes* was very small and no greater than that observed between the two shallow individuals. This lack of differentiation suggests that the two morphs are just morphological variants of *G. blacodes*, rather than different species. This result concurs with previous findings of no significant differences between the two morph types based on either allozyme or microsatellite loci (see previous results in this report). The orange morph is believed to be the juvenile form and found on the shelf up to a depth of 200 m while the pink morph is most likely the adult form and found on the slope (at depths greater than 200 m).

The most divergent species in this study was *G. tigerinus*. The *G. blacodes* (from Australia) and *G. capensis* (from South Africa) individuals are more closely related to each other than either was to the *G. tigerinus* individuals (from Australia). The rock ling is a shallow-water estuarine and coastal fish (Gomon *et al*., 1994) while both pink and kingklip ling are deepwater marine species. The close phylogenetic relationship and similar ecologies between the pink and kingklip cytochrome *B* sequences suggests that these two species diverged from an ancestral marine rather than estuarine form. Intraspecific sequence divergence ranged from 0–1.5% whereas interspecific variation was, as expected, much larger and ranged from 8.8–23%.

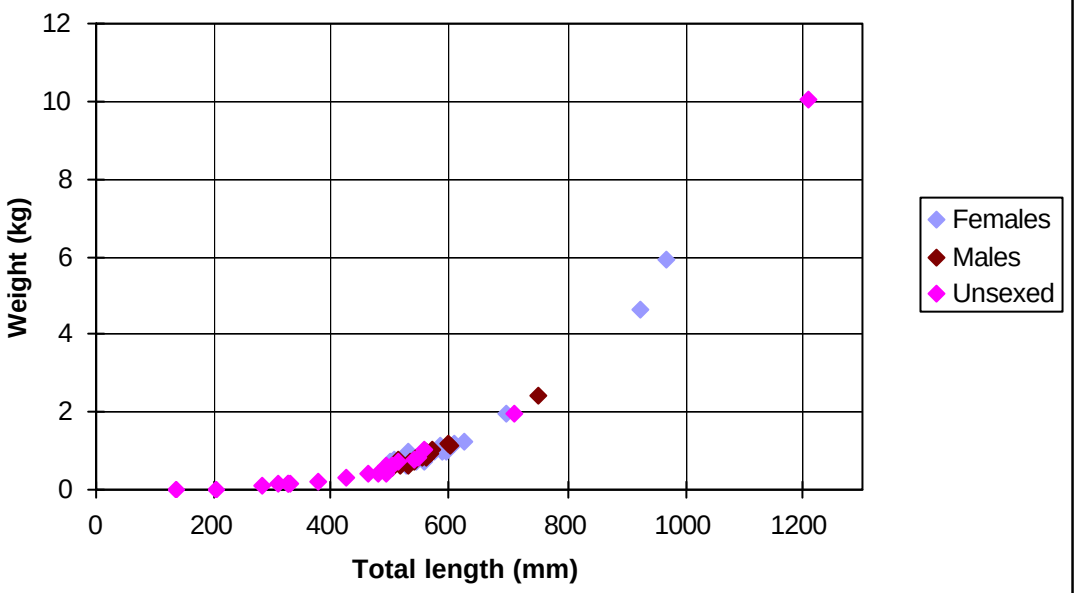
6.4. Biology

Some general biological observations on pink ling are included below.

6.4.1. Length/weight curve

Length/weight curves are useful for assigning weight estimates to fish of known length when conditions are not suitable for weighing, such as in remote areas or when working at sea. The length/weight curve derived from 66 specimens examined during the study is presented below (Figure 6.39).

Figure 6.39: Length weight curve



6.4.2. Size at maturity

The smallest specimens that could reliably be identified as males without magnification were in the size range 414–462 mm TL. The ovaries of seven females that ranged in size from 500–576 mm TL were poorly developed. The maximum ovum diameter was 20 *m*m and the maximum Gonosomatic Index (GSI - gonad weight expressed as a proportion of total weight) for the largest of these small females was 0.6%. However, one specimen of 566 mm TL had a well-developed ovary (GSI 1.0% with eggs ranging in size from 200–800 *m*m and the largest of these yolked) suggesting that the other females may have been taken outside the breeding season. This is smaller than published sizes of maturity for pink ling which range from 600–740 mm TL (Tilzey, 1994). Three other females with well-developed ovaries were much larger (809–932 mm TL).

6.4.3. Spawning time

Well developed ovaries from the following females were collected at various times during the year.

566 mm TL	TAS(E)	50 m	9/97
809 mm TL	TAS(W)	400 m?	11/98
889 mm TL	TAS(W)	423 m	4/98
932 mm TL	TAS(W)	423 m	4/98

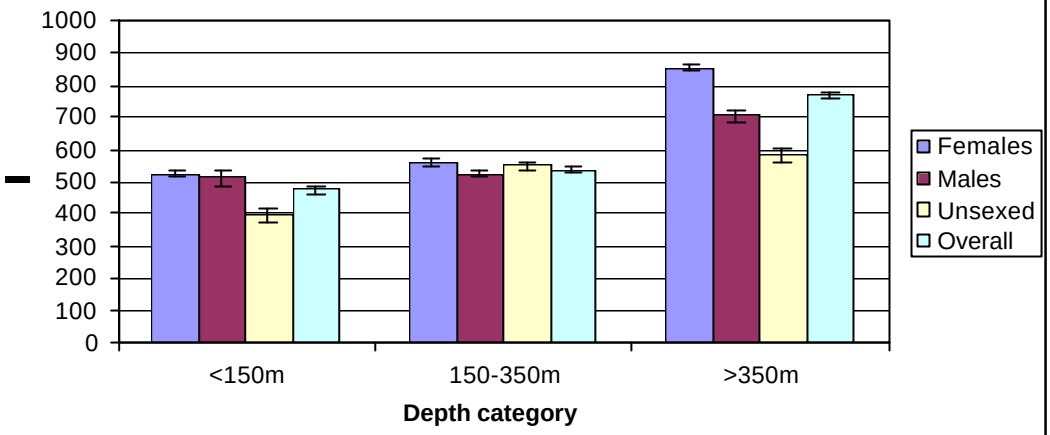
Ling larvae have been collected around Tasmania in all months except June (Furlani, 1998). One dissected ovary contained a mixture of yolked and unyolked eggs. These factors suggest that spawning may not be restricted to only certain parts of the year. It would be desirable to closely examine (by dissecting microscope) additional ovaries to determine whether or not spawning peaks occur in winter and spring as has been suggested (Tilzey, 1994).

6.4.4. Size variation with depth

The mean size of ling assigned to three depth categories were compared to investigate possible ontogenetic depth preferences (Figure 6.40). There was a slight overall increase in size between the <150 m and the 150–350 m depth categories which is consistent with previous findings (Furlani, unpublished data). There was also a significant difference in size between the >350 m category and the shallower categories. The average size of ling caught from the two shallower categories was below the published sizes of maturity whereas the average size of ling taken from deeper than 350 m exceeded published sizes of maturity (Tilzey, 1994). This suggests that young ling mainly develop to maturity in shallow waters before moving into deeper waters, and this has important implications for recruitment into the fishery.

Mean total length (mm)

Figure 6.40: Mean total length vs depth category



6.4.5. Diet

The diet of pink ling in New Zealand waters was reported to consist mainly of fish and crustaceans (Mitchell, 1984; Clark, 1985). Fish increase in importance with size as crustaceans become increasingly less important. There have been fewer studies of ling diet in Australia with the most detailed of these examining the diet of adults along the continental slope off eastern Tasmania (Blaber and Bulman, 1987). Major prey items were found to vary seasonally with blue grenadier consumed most in autumn, crustaceans in winter, and whiptails in summer .

The stomach contents of 18 specimens were examined during the study. Of these, 15 were adolescent ling collected from the continental slope off western Tasmania during October 1999. The remaining specimens were juveniles collected from the mid-shelf off NSW during February 1994. The few ling stomachs from NSW contained fish whereas those from Tasmania contained mainly invertebrates (Table 6.28).

Table 6.28: Ling stomach contents.

Specimen	Locality	Depth	Sex	TL	Prey items		
					Description	n	Size (mm)
RD007	Disaster Bay, NSW	78	?	275	bony fish	1	44
RD015	Disaster Bay, NSW	78	?	292	*Helicolenus* sp.	1	48
RD017	Disaster Bay, NSW	78	?	294	Bothidae	1	80
RD634	TAS(W)	700?	F	555	empty	0	
RD635	NW of Strahan, TAS(W)	730	F	500	nematode	20	10
					eye lense	1	2
					Decapoda	1	10
RD636	TAS (W)	700?	M	541	salps?	40	6
RD637	NW of Strahan, TAS(W)	730	F	532	eye lense	1	2
RD638	NW of Strahan, TAS(W)	730	M	514	salps?	15	6
RD639	TAS(W)	700?	M	565	salps?	10	5
					eye lense	1	2
RD640	TAS(W)	700?	F	545	Decapoda	3	20
					Macrouridae	1	20
RD641	TAS(W)	700?	M	561	empty	0	
RD642	off Low Rocky Point	600	M	532	empty	0	
RD643	TAS(W)	700?	F	576	salps?	10	6
					Decapoda	1	20
RD644	NW of Strahan, TAS(W)	730	F	505	bony fish	1	140
RD645	TAS(W)	700?	F	500	empty	0	
RD64R6	TAS(W)	700?	M	515	empty	0	
RD647	NW of Strahan, TAS(W)	730	M	560	empty	0	
RD648	TAS(W)	700?	M	505	Decapoda	1	100

6.5. General discussion

General

In the past, stock structure studies have tended to rely only on either genetic or non-genetic techniques. This simple type of approach has considerable inherent risk. Genetic techniques alone tend to be conservative in finding stock differences whereas it is possible for non-genetic techniques to find artificial differences rather than true stock diversity.

The combination of techniques used in the present study were used successfully to address a series of stock and species problems in the ling fishery. In summary, genetic and non-genetic techniques were used to validate each other and detect the effects of non-inherited regional differences that may have otherwise confounded the conclusions. These outcomes, other observations, and problems encountered during the project are discussed below.

Problems with specimen collection

Various problems arose with acquiring specimens from industry during the project that hampered progress to some extent. Industry input was less beneficial than in other similar projects for several important reasons. Firstly fishers reported that catches were slow in the year that the project commenced. This meant that fishers had difficulties in meeting commitments to buyers and were reluctant to provide specimens for research, even when full market value was offered. For the same reason, those specimens that were obtained early in the study tended to be too small to be of significant commercial value. This situation, combined with regional size differences among wild populations, made it impossible to obtain specimens of the same average size for each region. This led to heterogeneous data that created significant problems with the morphometric and otolith shape analyses.

In 1988, the ling quota became global (including both trawl and non-trawl) and some non-trawl operators were restricted to smaller catches than previous years. This resulted in fewer opportunities to obtain specimens and considerable ill feeling towards the administration of the fishery. We believe that this negativity may have contributed in some cases to a lack of willingness to assist the project. Hence, most specimens were obtained from State fishery agencies and the trawl sector of the fishery.

Although collectors were asked to provide details of location and depth, these were often sketchy for specimens obtained from fishers, e.g. Lakes Entrance < 150 m. For similar future studies, the risks associated with relying largely on industry for specimen collection need careful consideration in the project planning phase. The benefits of more travel (i.e. face to face contact with fishers and collecting directly from fishing vessels) to ensure that appropriate material is obtained need to be weighed up against the extra financial cost.

Species composition

Species problems were resolved successfully and the pink and orange forms were found to be the same species using both genetic and non-genetic methods. Among the genetic techniques, mitochondrial DNA sequencing was found to be very effective compared to other genetic

techniques that have been used for solving species problems. Pink ling *(G. blacodes*) populations from Australia and New Zealand are considered to be conspecific but the related African kingklip (*G. capensis*) is a distinct species of ling. Based on biogeographic grounds this raises questions about the conspecificity of South American and Australasian *G. blacodes* populations. This question was not investigated in this study.

Problems associated with stock structure techniques

A variety of techniques were applied to the ling fishery during the present study. This provides a good opportunity to highlight their relative strengths and weaknesses. Among the morphological techniques used here, meristic characters are the least plastic. Once laid down during larval development they are fixed and will not be affected by subsequent environmental changes. Meristic characters thus reflect genetic factors plus environmental conditions during the critical developmental period. Morphometric characters on the other hand are more plastic and can be affected by environmental states at any time, including pre-catch conditions, as well as by genetic factors. Interpretation of morphometric characters is therefore less certain than for meristic characters.

The levels of intraspecific variability differed between characters. Dorsal and anal-fin ray counts were extremely variable within a population and were even of limited use in distinguishing between different species of ling. Pectoral-fin counts showed moderate levels of variation and could be suitable for examining stock structure in ling species. Vertebrae and pyloric caecae counts showed low levels of variation and are likely to be most useful for addressing species problems but are not suitable for stock structure analyses.

A variety of complex statistical methods were used with limited success in an attempt to compensate for regional differences in mean fish size. This highlights the need to obtain fish of similar size in morphometric and otolith shape studies. Other factors that may confound measurement data are differences in size at age, and length, type and quality of storage of study material. Certain regional differences in non-inherited factors such as size, size at age, bottom type, and market forces, can make it impossible to collect specimens of similar average size for each region being considered.

Stock structure was also assessed using two different genetic techniques: allozyme and microsatellite analysis. The allozyme analysis used data from three polymorphic loci, the microsatellite analysis used nine polymorphic loci. Neither provided convincing evidence for any subpopulation structure. The three allozyme loci averaged around 3.3 alleles per locus with an average expected heterozygosity of 38%, whereas the nine microsatellites averaged 34.0 alleles per locus with an average observed heterozygosity of 90%. Clearly the microsatellite loci were much more variable, but hypervariability can sometimes be a weakness in population studies. For example, if genotypes in all fish were different from each other at each locus, then tests of genetic population structure would have no power. In our case, the most variable microsatellite loci (5.8B, 4.11, and 5.5A, with 52, 49 and 42 alleles per locus respectively) probably had rather low resolving power, given the sample sizes available. On the other hand, locus 2.6.1, with just 11 alleles, would have had high resolving power. There are also sometimes problems in ascribing alleles to a particular category when allele number is very high, and this appeared to be a problem with 5.8B here. Optimum numbers of alleles per

locus given sample sizes of around 100 would probably be around 8–15, but the time necessary to develop and screen enough microsatellite loci to reach this ideal degree of variability was not available in this project. However, be that as it may, there was no significant evidence of stock structure for the microsatellite or allozyme loci; both types of genetic data were in accord.

One general caveat with genetic stock structure analyses should be added. Where differences are found, then this is generally strong evidence for reproductive isolation of different stocks and is likely to carry a recommendation for management as discrete stocks. However, where differences are not detected, as here (and in about 50% of genetic case studies of species in the south-east fishery, Ward and Elliott, 2000), then the degree of genetic connectivity between the locations sampled is unclear. Gene flow levels of as low as 1% or as high as 50% are both consistent with a lack of genetic differentiation given the sampling efforts normally deployed. Clearly, then, our genetic data are consistent with the null hypothesis of a single stock, but do not (and cannot) prove that the null hypothesis is true. In our case, other (non-genetic) data also fails to reject the null hypothesis of a single stock, increasing our confidence that the null hypothesis is likely to be correct.

Comments on stock structure studies of ling in New Zealand waters.

Studies of pink ling in New Zealand waters have concluded that there is more than one stock present.

The genetic stock structure of ling in New Zealand waters was studied by Smith (1979) and Smith and Francis (1982). Smith (1979) examined two loci, *PGM* (phosphoglucomutase) and *GPI* (glucosephosphate isomerase) in 395 ling from different New Zealand waters including off the North and South Islands. The *PGM* locus is likely to be the same as the polymorphic one we studied (*PGM-1*), but the *GPI* locus may not be the same as either of the two nearly monomorphic *GPI* loci (*GPI-1* and *GPI-2*) we found but did not use in our routine screening. Smith found no allelic differences for *PGM* throughout the range sampled, but found some evidence of a *GPI* separation of Pukaki Rise (south east of the South Island) from other populations. In fact, evidence for this separation is extremely weak and far from convincing, with no probability levels of significant differentiation of less than 0.05 from any of the various tests he carried out, and there is no significant differentiation across all samples (P=0.49). A later and larger study of New Zealand fish (n=1743) used *GPI* alone (Smith and Francis, 1982). The 25 samples showed no significant allelic heterogeneity (P=0.26). After pooling regional samples, and after considering hydrological conditions, Smith and Francis suggested that there was evidence of two or three stocks, one around the mainland, one from the northern part of the Southern Plateau, and perhaps one from around Campbell Island. However, we believe that the genetic evidence for multiple stocks around New Zealand is weak.

A morphological study of the stock structure of pink ling in New Zealand was undertaken by Colman (1995). He found that ling from the west coast of the South Island and from the Chatham Rise and Canterbury waters generally had longer and narrower heads and thinner otoliths than ling from southern areas. There was evidence for at least three separate ling

stocks in the New Zealand EEZ. The same techniques were employed in the current study but no convincing evidence of more than one stock was found.

Regional and depth related variation in size and biology

Although the present study suggests that there are no regional differences in inherited factors within the fishery it is important to note that some non-inherited factors may vary between regions for ling. Industry has noted regional differences in size and catchability between different fishing grounds. This anecdotal information is consistent with size differences in catches sampled during the study between different regions and depths. These differences could be due to substrate/habitat variation, cohort differences, gear efficiency or even behavioural heterogeneity between regions.

Ling biology and future research needs

It is important to consider possible regional differences in the following aspects of ling biology which need further research:

* Spawning periodicity and locations
* Larval dispersal
* Diet
* Bottom type and habitat and their affect on catchability
* Depth preferences of juvenile fish
* Migration along shelf and slope of adults and movement into deeper water with age

Close involvement in the sampling process is an integral part of any such study because precise locality information must be recorded and stomach contents must be obtained in good condition. Industry sources often tend to approximate locality information and ling stomachs rot very quickly if not frozen immediately - they are also gutted at sea by most commercial vessels as fish are typically chilled on ice rather than frozen. Larval dispersion is being examined in a separate project by CMR.

7. BENEFITS

The key achievements of the study are:

1 The number of pink ling stocks was examined. There was no evidence of more than one stock.

2 The number of species in the fishery has been determined.

3 Possible subregional and depth related biological differences have been highlighted as important areas for future studies.

4 Some problems with the application of some well recognised stock structure techniques have been highlighted.

The understanding of species composition and stock structure of the ling fishery gained during the study can now be used to refine management plans. Both the fishing industry and the community should benefit from these refinements through increased sustainability of the resource.

Similar indirect benefits will be achieved as other researchers incorporate results of the present study into their work which will also be used to further refine management practices. For example, understanding of stock structure is critical to the current stock assessment of the ling fishery by CMR.

Longer term benefits for the industry may be achieved when additional biological research needs, highlighted in the present report, are addressed.

The use of a variety of genetic and non-genetic methods in the study has allowed direct comparison of the various methods. This has highlighted advantages, disadvantages and potential pitfalls of these methods when applied to species composition and/or stock structure problems. This will benefit researchers seeking to apply similar methods to other species.

8. FURTHER DEVELOPMENT

The findings of the study have particular relevance to the following bodies that should receive copies of the report:

South East Trawl Management Advisory Committee and
South East Fishery Non-trawl Quota Arrangements Working Group

The findings of the study were presented to industry at a workshop in Canberra on 29 February 2000.

Results from this study have been incorporated the current stock assessment of the ling fishery by CMR.

9. CONCLUSION

The first objective of the study was to delineate stock structure of pink ling in Australian waters so that separate management plans for each stock could be developed if required. None of the genetic or non-genetic techniques used were able to refute the working hypothesis of a single stock. This implies that fishing effort in any area of the fishery will ultimately affect the entire fishery and is consistent with the current management arrangements which treat the fishery as a single unit stock

The second objective of the research was to examine the nature and relationship of orange and pink forms of ling so that management plans for the deepwater and inshore components of the fishery can be properly integrated. Both genetic and non-genetic techniques indicate that the morphs of the pink ling are forms of the same species - the pink ling (*Genypterus blacodes*). The orange form primarily represents juvenile ling typically caught shallower than 200 m. This has important implications for recruitment into the deeper part of the fishery which contains the larger adults and represents most of the commercial value of the fishery.

The third objective of the study was to collect biological information, particularly in regards to life history and reproduction that can be used together with stock delineation results to develop yield estimates so that TACs can be reviewed. The collection of biological data focussed on understanding the nature of the pink and orange forms in order to meet the second objective. Early life history is the subject of a current project by CMR. Some of the biological information obtained during the study, together with the stock structure results, was discussed at an industry workshop on ling stock assessment in Canberra on 29 February 2000. The results of this stock assessment will be considered by AFMA when reviewing TACs. Additional biological information is still desirable to assist in the development of yield estimates. These future research needs are discussed in Section 6.5.

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APPENDIX A: INTELLECTUAL PROPERTY

Two components of intellectual property arose from this research.

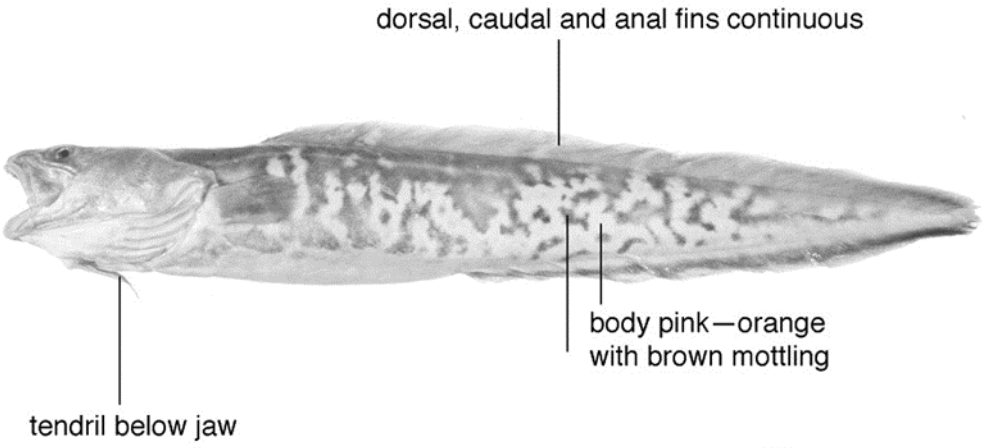
1. Copyright in this report
2. DNA library

APPENDIX B: STAFF

Ross Daley	CSOF3	Principal investigator, collection and analysis of allozyme and morphological data, report preparation.
Bob Ward	CSOF7	Genetics supervisor
Peter Last	CSOF7	Morphology
Anne Reilly	CSOF3	DNA library development; collection of genetic data
Sharon Appleyard	CSOF3	Mt DNA; genetics statistical analysis
Daniel Gledhill	CSOF3	Report preparation assistance
Helen Webb		Report preparation assistance

APPENDIX C: SPECIMEN REQUEST POSTER

WANTED, SA **PINK LING** *Genypterus blacodes*



similar species: tusk, not required

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APPENDIX D: MICROSATELLITES, ALLELE FREQUENCIES IN PINK LING POPULATIONS.

Locus	Allele	Population				
		VIC(E)	VIC(W)	NSW	TAS(E)	TAS(W)
2.6.1	104	0	0	0	0.005	0
	107	0	0.018	0.015	0.005	0
	110	0	0	0.022	0.01	0
	113	0.158	0.116	0.184	0.116	0.205
	116	0.017	0.036	0.029	0.03	0.023
	119	0.542	0.634	0.493	0.606	0.5
	122	0.217	0.143	0.184	0.182	0.114
	125	0.05	0.018	0.044	0.02	0.136
	128	0.017	0.027	0.029	0.02	0.023
	131	0	0.009	0	0	0
	161	0	0	0	0.005	0
		60	56	68	99	22
5.8B	117	0.008	0	0	0	0
	121	0	0.018	0	0	0
	125	0.024	0	0.015	0.01	0
	127	0.008	0.018	0.03	0	0.022
	129	0	0.045	0	0.01	0
	131	0	0	0.007	0	0
	135	0.008	0.018	0	0	0
	137	0.032	0.018	0.03	0.005	0
	139	0.024	0.045	0.037	0.025	0.022
	141	0	0	0	0.01	0.022
	143	0.024	0.036	0	0.005	0
	145	0.008	0	0.045	0.03	0
	147	0.008	0.018	0.015	0.01	0.022
	149	0.065	0.018	0.045	0.035	0.043
	151	0.032	0	0.03	0.045	0.022
	153	0.024	0.009	0.09	0.040	0
	155	0.04	0.1	0.06	0.025	0
	157	0.081	0.027	0.045	0.045	0.022
	159	0.04	0.073	0.045	0.056	0
	161	0.032	0.045	0.022	0.066	0.043
	163	0.024	0.027	0.03	0.025	0.022
	165	0.016	0.036	0.052	0.04	0.043
	167	0.032	0.009	0.007	0.056	0.022
	169	0.024	0.036	0.067	0.056	0.087
	171	0.056	0.027	0.007	0.035	0.065
	173	0.048	0.082	0.037	0.066	0.109
	175	0.032	0.045	0.022	0.035	0.087
	177	0.032	0.055	0.045	0.015	0.043
	179	0.032	0.018	0.052	0.025	0
	181	0.016	0	0.015	0.025	0
	183	0.024	0.009	0.015	0.035	0.065
	185	0.024	0.045	0.015	0.02	0.022
Locus	Allele	Population				

		VIC(E)	VIC(W)	NSW	TAS(E)	TAS(W)
	187	0.008	0.009	0.03	0.01	0.065
	189	0.04	0.036	0.007	0.03	0.022
	191	0.016	0.009	0	0.02	0.022
	193	0.008	0.009	0.015	0.015	0
	195	0	0	0.015	0.015	0.043
	197	0.04	0	0	0.005	0
	199	0.024	0.009	0.015	0.015	0
	201	0.008	0.009	0	0.005	0
	203	0.008	0	0	0	0
	205	0	0.018	0.015	0.01	0.022
	207	0	0.018	0.007	0.005	0.022
	209	0	0	0	0	0.022
	211	0.008	0	0	0.005	0
	213	0.008	0	0	0	0
	215	0	0	0	0.005	0
	221	0	0	0	0.005	0
	223	0.008	0	0	0	0
	235	0	0	0.007	0	0
	245	0	0	0.007	0	0
	N	62	55	67	99	23
5.8A	119	0	0	0.036	0.016	0
	129	0.045	0.043	0.018	0.026	0.026
	131	0.027	0.011	0.027	0.032	0.026
	133	0.018	0	0	0.005	0
	135	0.009	0	0.027	0.005	0
	137	0.045	0	0	0.026	0
	139	0.009	0.022	0	0.005	0.026
	141	0	0.011	0.018	0	0
	143	0.045	0.054	0.073	0.021	0.132
	145	0.009	0.033	0.027	0.047	0.053
	147	0.045	0.065	0.064	0.084	0.026
	149	0.064	0.043	0.055	0.058	0.053
	151	0.145	0.098	0.109	0.084	0.158
	153	0.073	0.109	0.073	0.111	0.053
	155	0.064	0.087	0.082	0.053	0.079
	157	0.027	0.022	0.018	0.047	0.026
	159	0.091	0.065	0.082	0.032	0.158
	161	0.036	0.054	0.073	0.053	0.053
	163	0.036	0.022	0.036	0.011	0
	165	0.064	0.098	0.018	0.047	0.053
	167	0.027	0.011	0.009	0.005	0
	169	0.036	0.011	0.045	0.058	0
	171	0.027	0	0.018	0.042	0
	173	0.018	0	0.018	0.032	0.026
	175	0.009	0.033	0.036	0.011	0.026
	177	0.018	0.065	0.018	0.011	0
	179	0	0.022	0	0.016	0
Locus	**Allele**	**Population**				
		VIC(E)	**VIC(W)**	**NSW**	**TAS(E)**	**TAS(W)**

	181	0	0	0.009	0.005	0
	183	0	0.011	0	0.005	0
	185	0	0	0	0.005	0
	187	0	0	0	0.005	0
	199	0	0	0	0.011	0
	203	0.009	0	0	0.021	0
	211	0	0	0	0.011	0
	213	0	0	0.009	0	0.026
	239	0	0.011	0	0	0
		55	46	55	95	19
4.2B	143	0	0.009	0	0.005	0
	147	0	0	0.007	0	0
	155	0.008	0	0	0.005	0
	157	0.008	0.028	0.022	0.016	0
	159	0	0.019	0.022	0.016	0.025
	161	0.04	0.057	0.052	0.031	0.05
	163	0.04	0.066	0.045	0.052	0.05
	165	0.063	0.028	0.045	0.063	0
	167	0.071	0.009	0.037	0.036	0.05
	169	0.167	0.113	0.142	0.083	0.15
	171	0.103	0.094	0.09	0.109	0.05
	173	0.063	0.085	0.045	0.073	0.15
	175	0.087	0.179	0.119	0.125	0.2
	177	0.04	0.028	0.015	0.063	0.025
	179	0.127	0.094	0.104	0.109	0.05
	181	0.048	0.075	0.037	0.042	0.075
	183	0.024	0.028	0.075	0.057	0.025
	185	0.016	0.028	0.03	0.021	0
	187	0.008	0.009	0.015	0.016	0
	189	0.024	0.009	0.037	0.042	0.05
	191	0.032	0.019	0.03	0.01	0.025
	193	0.008	0	0.007	0.005	0
	195	0.008	0	0	0.005	0
	197	0.008	0	0.015	0	0
	199	0	0.019	0.007	0.005	0.025
	201	0.008	0	0	0.01	0
		63	53	67	96	20
4.2A	179	0	0	0	0	0.024
	183	0.057	0.061	0.045	0.037	0.048
	187	0.008	0.026	0.015	0.021	0
	189	0.008	0	0	0.005	0
	191	0.23	0.149	0.231	0.168	0.19
	193	0	0.009	0	0.005	0
	195	0.107	0.123	0.127	0.168	0.214
	197	0.025	0.026	0.03	0.026	0
	199	0.033	0.053	0.037	0.032	0.024

Locus	**Allele**	**Population**				
		VIC(E)	**VIC(W)**	**NSW**	**TAS(E)**	**TAS(W)**

	201	0	0.009	0	0	0
	203	0.025	0.009	0.022	0.026	0
	205	0.016	0.009	0.015	0.011	0
	207	0.008	0	0.007	0.011	0
	209	0.057	0.061	0.037	0.021	0.024
	211	0.016	0.009	0.03	0.011	0.024
	215	0.049	0.035	0.022	0.053	0
	217	0.016	0	0.007	0	0
	219	0.156	0.175	0.082	0.137	0.119
	221	0	0	0	0.011	0.024
	223	0.016	0.035	0.045	0.037	0.071
	225	0.057	0.07	0.067	0.063	0.071
	229	0.049	0.044	0.075	0.089	0.048
	231	0	0	0	0.005	0
	233	0.008	0.018	0.022	0.021	0.071
	235	0.033	0.035	0.03	0.011	0
	237	0	0.009	0.015	0.011	0
	239	0	0.009	0.007	0.005	0
	241	0.008	0	0	0	0
	243	0	0	0.007	0.005	0.024
	245	0.016	0.009	0.007	0.005	0
	247	0	0.009	0.007	0	0.024
	249	0	0.009	0.007	0	0
	251	0	0	0	0.005	0
		61	57	67	95	21
4.11	188	0	0.009	0	0	0
	190	0	0	0.008	0	0
	192	0	0	0.015	0.01	0.024
	198	0	0	0.008	0	0
	200	0.008	0	0.031	0.01	0
	202	0.008	0	0	0	0
	204	0.008	0.027	0.008	0.01	0
	208	0	0	0.023	0	0.024
	210	0.008	0.009	0.008	0.021	0.024
	212	0.031	0.018	0.031	0.052	0.024
	214	0.016	0.027	0.023	0.005	0
	216	0.055	0.036	0.031	0.031	0
	218	0.023	0.009	0.038	0.01	0.024
	220	0.031	0.027	0.031	0.031	0.048
	222	0.055	0.118	0.077	0.082	0.095
	224	0.063	0.073	0.031	0.057	0.071
	226	0.055	0.036	0.023	0.072	0.095
	228	0.031	0.027	0.023	0.026	0.048
	230	0.039	0.082	0.054	0.036	0.024
	232	0.023	0.064	0.015	0.015	0
	234	0.07	0.018	0.069	0.088	0.071
	236	0.063	0.027	0.038	0.041	0.095
Locus	**Allele**	**Population**				
		VIC(E)	**VIC(W)**	**NSW**	**TAS(E)**	**TAS(W)**

	238	0.078	0.027	0.077	0.067	0
	240	0	0.027	0.031	0.015	0.024
	242	0.031	0.018	0.031	0.036	0
	244	0	0.018	0	0.015	0
	246	0.023	0.064	0.031	0.026	0
	248	0.008	0.018	0.015	0.026	0.024
	250	0.039	0.073	0.038	0.046	0.095
	252	0.023	0.036	0	0.021	0.024
	254	0.023	0.045	0.062	0.036	0.024
	256	0.008	0.009	0.023	0.015	0.024
	258	0.086	0.009	0.023	0.01	0.024
	260	0.008	0	0.023	0.01	0.024
	262	0.008	0.009	0	0.005	0.024
	264	0.008	0.009	0.008	0.005	0
	266	0.008	0	0.008	0.021	0
	268	0	0.009	0.008	0.005	0.048
	270	0.008	0.009	0	0.015	0
	272	0.008	0	0.008	0.005	0
	280	0	0	0	0.005	0
	274	0.016	0	0	0	0
	276	0	0	0.015	0	0
	284	0	0.009	0	0.005	0
	286	0.008	0	0	0.005	0
	300	0.008	0	0	0	0
	336	0.008	0	0.015	0	0
	344	0	0	0	0.005	0
	346	0.008	0	0	0	0
		64	55	65	97	21
5.2B	116	0	0	0	0.01	0
	124	0.015	0.009	0	0.005	0
	132	0.038	0.009	0.008	0.015	0.025
	136	0.008	0.009	0.015	0.02	0
	140	0.023	0.009	0.038	0.026	0.05
	144	0.023	0.061	0.03	0.041	0.05
	148	0.038	0.044	0.023	0.01	0.025
	152	0.031	0.053	0.068	0.056	0.025
	156	0.062	0.061	0.053	0.061	0.025
	160	0.069	0.114	0.076	0.102	0.1
	164	0.092	0.061	0.076	0.097	0.075
	168	0.1	0.105	0.076	0.056	0.075
	172	0.1	0.079	0.121	0.087	0.1
	176	0.054	0.061	0.076	0.112	0.075
	178	0	0.009	0	0	0
	180	0.077	0.044	0.091	0.077	0.125
	184	0.092	0.053	0.076	0.056	0.025
	188	0.031	0.07	0.053	0.056	0.15
	192	0.077	0.088	0.03	0.056	0.025
Locus	**Allele**	**Population**				
		VIC(E)	**VIC(W)**	**NSW**	**TAS(E)**	**TAS(W)**
	196	0.023	0.026	0.038	0.015	0.025

	200	0.031	0	0.038	0.015	0.025
	204	0.015	0.018	0.008	0.005	0
	208	0	0.018	0	0.015	0
	220	0	0	0	0.005	0
	224	0	0	0.008	0	0
		65	57	66	98	20
5.9A	131	0	0	0.008	0	0
	133	0.008	0	0.015	0.011	0.024
	137	0.133	0.043	0.053	0.063	0.071
	139	0.023	0.009	0.008	0.005	0
	141	0.008	0	0.008	0.016	0
	143	0.023	0.026	0.008	0.016	0
	145	0.039	0.017	0.091	0.116	0.048
	147	0.008	0.017	0.038	0.021	0.024
	149	0.016	0.069	0.091	0.068	0.024
	151	0.039	0.052	0.053	0.053	0.119
	153	0.117	0.086	0.068	0.053	0.071
	155	0.141	0.138	0.136	0.105	0.143
	157	0.086	0.112	0.083	0.079	0.143
	159	0.07	0.121	0.061	0.111	0.048
	161	0.039	0.017	0.008	0.042	0
	163	0	0.078	0.045	0.026	0.024
	165	0.023	0.034	0	0.021	0.048
	167	0.031	0.026	0.038	0.037	0.024
	169	0.039	0.017	0.038	0.016	0.048
	171	0.016	0.069	0.03	0.026	0.024
	173	0.039	0.009	0.03	0.016	0.048
	175	0.016	0.034	0.023	0.011	0
	177	0.008	0.009	0.038	0.021	0
	179	0.008	0	0.008	0.016	0.048
	181	0.008	0	0	0.005	0.024
	183	0.016	0.009	0.008	0.032	0
	185	0.023	0	0	0	0
	187	0.016	0	0	0	0
	189	0	0	0.008	0.005	0
	191	0.008	0	0.008	0.005	0
	201	0	0	0	0.005	0
	203	0	0.009	0	0	0
		64	58	66	95	21
5.5A	95	0	0	0.007	0	0
	99	0.033	0.102	0.103	0.06	0.059
	101	0	0	0.015	0.016	0
	103	0	0	0	0	0.029
	105	0	0	0	0.005	0
	107	0	0.019	0.022	0.027	0.029
Locus	**Allele**	**Population**				
		VIC(E)	**VIC(W)**	**NSW**	**TAS(E)**	**TAS(W)**
	109	0.131	0.148	0.162	0.13	0.029
	111	0.254	0.296	0.265	0.223	0.5

113	0	0	0.007	0.016	0
117	0.098	0.056	0.037	0.065	0.059
119	0.008	0	0	0.005	0.029
121	0.016	0	0.037	0.022	0
123	0.016	0.037	0.022	0.011	0
125	0	0.019	0	0.005	0
127	0.008	0	0	0	0
129	0	0.009	0.007	0.011	0
131	0.025	0	0.029	0.038	0.029
133	0.033	0.009	0.015	0	0
135	0.033	0	0.022	0.005	0
137	0.172	0.213	0.154	0.163	0.118
139	0.033	0.019	0.007	0.049	0.029
141	0.033	0.019	0.015	0.038	0
143	0.016	0.019	0	0.011	0.029
145	0.008	0	0.007	0.005	0
147	0	0	0.015	0.005	0
149	0	0.009	0	0.011	0
151	0.008	0	0.007	0	0
153	0	0	0	0.005	0
155	0	0	0	0.005	0
161	0.008	0	0.007	0	0
163	0.008	0	0	0.011	0
165	0	0.009	0.015	0	0
167	0.008	0.009	0.007	0.005	0
169	0	0	0	0.005	0
171	0	0	0	0	0.029
173	0.008	0	0	0.005	0
177	0.008	0	0	0	0
179	0.008	0	0	0.022	0
183	0	0	0.007	0.005	0
187	0.025	0.009	0.007	0.005	0.029
189	0	0	0	0.005	0
	61	55	68	92	17

APPENDIX E: SEQUENCE VARIATION IN A 259 BASE PAIR REGION OF THE MITOCHONDRIAL DNA CYTOCHROME B GENE FOR INDIVIDUALS FROM THREE DIFFERENT LING SPECIES.

P1#A##	CAA	ATC	CCC	ACA	GGA	CTA	TTC	CTA	GCC	ATG	CAC	TAC	TCA	CCA	GAC	ACC	48
P2#B##	***	***	***	***	***	***	**T	T**	***	***	***	***	***	***	***	***	48
P3#A##	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	48
R1#C##	***	**T	*T*	***	***	***	**T	**G	***	**A	***	***	**C	**T	***	*T*	48
R2#D##	***	**T	*T*	***	***	***	***	***	***	**A	***	***	**C	**T	***	*T*	48
R3#C##	***	**T	*T*	***	***	***	**T	**G	***	**A	***	***	**C	**T	***	*T*	48
C1#E##	***	***	A**	***	***	***	***	***	***	***	***	***	***	***	***	G**	48
C2#E##	***	***	A**	***	***	***	***	***	***	***	***	***	***	***	***	G**	48
P1#A##	TCC	TCC	GCC	TTT	TCA	TCA	ATC	GCC	CAC	ATC	AGT	CGA	GAC	GTA	AAC	TAC	96
P2#B##	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	96
P3#A##	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	96
R1#C##	A**	**A	***	**C	***	***	G**	***	***	***	T*C	***	***	***	***	***	96
R2#D##	A**	**A	***	**C	***	***	G**	***	***	***	T*C	***	***	***	***	***	96
R3#C##	A**	**A	***	**C	***	***	G**	***	***	***	T*C	***	***	***	***	***	96
C1#E##	**A	A**	***	***	***	***	***	***	***	***	*C*	***	***	***	**T	**T	96
C2#E##	**A	A**	***	***	***	***	***	***	***	***	*C*	***	***	***	**T	**T	96
P1#A##	GGC	TGA	CTC	ATT	CGC	TAC	CTT	CAC	GCC	AAC	GGC	GCC	TCA	TTA	TTC	TTT	144
P2#B##	***	***	**A	***	***	***	***	***	***	***	***	***	***	***	***	***	144
P3#A##	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	144
R1#C##	***	***	**A	***	**G	*A*	G**	***	***	***	***	**A	***	**C	**T	***	144
R2#D##	***	***	**A	***	**G	*A*	G**	***	***	***	***	**A	***	**C	**T	***	144
R3#C##	***	***	**A	***	**G	*A*	G**	***	***	***	***	**A	***	**C	**T	***	144

C1#E##	***	***	A**	**C	***	***	***	***	***	**T	***	***	***	A**	***	***	144
C2#E##	***	***	A**	**C	***	***	***	***	***	**T	***	***	***	A**	***	***	144
P1#A##	ATC	TGC	CTC	TAC	CTA	CAC	ATC	GGG	CGA	GGG	CTA	TAC	TAC	GGG	TCA	TAC	192
P2#B##	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	192
P3#A##	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	192
R1#C##	***	***	A*T	***	A*G	***	***	**C	***	**C	***	***	***	**A	**T	***	192
R2#D##	***	***	A*T	***	A*G	***	***	**C	***	**C	***	***	***	**A	**T	***	192
R3#C##	***	***	A*T	***	A*G	***	***	**C	***	**C	***	***	***	**A	**T	***	192
C1#E##	***	***	***	*T*	***	***	***	***	***	**C	***	**T	***	**A	***	*TT	192
C2#E##	***	***	***	*T*	***	***	***	***	***	**C	***	**T	***	**A	***	*TT	192
P1#A##	CTG	TAC	TAA	GAA	ACC	TGA	AAC	ATT	GGA	GTT	ATC	CTC	CTG	CTT	GCA	ACT	240
P2#B##	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	240
P3#A##	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	***	240
R1#C##	***	**T	A**	***	**A	***	***	G**	***	**A	***	***	***	***	CT*	G**	240
R2#D##	***	***	A**	***	***	***	***	G**	***	**A	***	***	***	***	CT*	G**	240
R3#C##	***	**T	A**	***	**A	***	***	G**	***	**A	***	***	***	***	CT*	G**	240
C1#E##	**C	***	*C*	***	***	***	***	**C	**C	A**	***	***	***	***	***	***	240
C2#E##	**C	***	*C*	***	***	***	***	**C	**C	A**	***	***	***	***	***	***	240
P1#A##	ATA	GCA	ACC	GCC	TTC	ATA	G										259
P2#B##	***	***	***	***	***	***	*										259
P3#A##	***	***	***	***	***	***	*										259
R1#C##	***	A**	***	**T	***	G*T	*										259
R2#D##	***	A**	***	**T	***	G*T	*										259
R3#C##	***	A**	***	**T	***	G*T	*										259
C1#E##	***	***	**A	***	***	***	*										259

C2[#]E[##] *** *** **A *** *** *** * 259

** represents bases same as Pink1 A haplotype*
[#]P1=G. blacodes shallow morph; P2=G. blacodes shallow morph;P3=G. blacodes deep morph; R1, 2 & 3= G. tigerinus; C1 & 2=G. capensis
[##]mtDNA haplotype