Summary of Thesis submitted for the Degree of Doctor of Philosophy

by

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on

Inter-specific hybridization in the fish family Cyprinidae.

The breakdown of reproductive isolation leading to inter-specific hybridization is a widespread phenomenon amongst cyprinid fishes. There are seventeen cyprinid species occcurring in the British Isles, within five sub-families, giving rise to some ten different types of hybrid. Most of these belong to the sub-family Leuciscinae. The most commonly occurring hybrids are probably those between roach, *Rutilus rutilus* (L.), and common bream, *Abramis brama* (L.).

In this study the phenomenon of hybridization amongst species of the Cyprinidae was investigated through:

an experimental breeding programme to investigate the nature of inter-species and hybrid gamete compatibilities;

the identification of species and hybrids from the experimental breeding programme through the analysis of morphometric, meristic and genetic characters (genetic characters were analysed using enzyme electrophoresis);

the comparison of morphmetric, meristic and genetic information of natural fish with similar features of fish from the experimental breeding programme to identify the occurrence of post F1 hybridization in natural populations;

the use of restriction enzyme analysis of mitochondrial DNA to elucidate the importance of maternal ancestry in a natural hybrid population .

The breeding programme found, for the species in this study, that there was no success in cross-fertilization of taxa between different sub-families. Interspecific gamete compatibility was only found within the leuciscine sub-family. In cases where a hybrid cross produced progeny it was also noted that the reciprocal cross was successful. This suggests that there is not a genetic barrier to gamete compatibility resulting from the sexual directionality of a hybrid cross. Female roach/common bream hybrids also produced progeny when crossed with males of leuciscine species.

Identification of the progeny of the experimental breeding programme showed that the genetic techniques of enzyme electrophoresis was more reliable than the statistical analysis of meristic and morphometric traits in the identification of species and their F1 hybrids. However, genetic information alone cannot establish precisely the nature of post F1 hybrids and in the identification of backcrossed roach/common bream hybrids it was noted that meristic information was needed to support genetic data.

In the two natural hybrid populations of roach/common bream and rudd/common bream, from the Forty Foot Drain and Essex University Lake respectively, the analysis of morphometric, meristic and genetic characters found no evidence of post F1 hybridization in these waters. It is suggested that absence is due to either the limitations of the sampling methods or biological processes. Possible biological processes include factors such as the inappropriate mating behaviour of F1 hybrids or the inferior fitness of post F1 hybrids.

The analysis of mitochondrial DNA did not yield sufficient results to elucidate the importance of maternal ancestry in hybridization. It is suggested that this aspect of hybridization is of such critical importance that it must become the subject of a future research programme.

The importance of the causes and consequences of inter-specfic hybridization in fishes are discussed. It is suggested that, because they are rarely investigated in hybrid studies, these become incorporated into research programmes in the future. These areas of investigation will have implications for fisheries management, freshwater ecology, genetic conservation and species integrity.

TO MY PARENTS

THE UNIVERSITY OF HULL

INTER-SPECIFIC HYBRIDIZATION IN THE FISH FAMILY CYPRINIDAE

a Thesis submitted for the
Degree of Doctor of Philosophy
at the University of Hull

by

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ABSTRACT

The breakdown of reproductive isolation leading to inter-specific hybridization is a widespread phenomenon amongst cyprinid fishes. There are seventeen cyprinid species occcurring in the British Isles, within five sub-families, giving rise to some ten different types of hybrid. Most of these belong to the sub-family Leuciscinae. The most commonly occurring hybrids are probably those between roach, *Rutilus rutilus* (L.), and common bream, *Abramis brama* (L.).

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In the two natural hybrid populations of roach/common bream and rudd/common bream, from the Forty Foot Drain and Essex University Lake respectively, the analysis of morphometric, meristic and genetic characters found no evidence of post F1 hybridization in these waters. It is suggested that absence is due to either the limitations of the sampling methods or biological processes. Possible biological processes include factors such as the inappropriate mating behaviour of F1 hybrids or the inferior fitness of post F1 hybrids.

The analysis of mitochondrial DNA did not yield sufficient results to elucidate the importance of maternal ancestry in hybridization. It is suggested that this aspect of hybridization is of such critical importance that it must become the subject of a future research programme.

The importance of the causes and consequences of inter-specfic hybridization in fishes are discussed. It is suggested that, because they are rarely investigated in hybrid studies, these become incorporated into research programmes in the future. These areas of investigation will have implications for fisheries management, freshwater ecology, genetic conservation and species integrity.

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CHAPTER ONE
INTRODUCTION TO HYBRIDIZATION AMONGST FISHES

1.1 INTRODUCTION

1.1.1 The species concept

The species concept, and the characteristics and criteria for definition, has been the subject of much debate (e.g. Ray, 1686; Darwin, 1859; Dobzhansky, 1940; Sylvester-Bradley, 1956; Mayr, 1957a; 1957b; Beaudry, 1960; Simpson, 1961). The term species is the foundation upon which all biological studies are based. However, although the concept is a central theme in biology, it is not always defined clearly. Mayr (1963) in his description of species included the attributes which feature amongst most species definitions i.e. that they are units composed of populations of individuals which are morphologically similar, have inter-connected gene-pools and are isolated, in terms of their reproductive capabilities, from other such populations. Such a description implies there is a degree genetic incompatibility between some species.

1.1.2 Isolating mechanisms

For many species such a description is adequate. However, because some gametes are compatible the aspect of reproductive isolation requires precise definition. For example, it is well established that the cross of a horse with a donkey produces offspring called a mule. Indeed, there are many more circumstances where the boundaries of species and reproductive isolation are poorly defined. For example, Phillips (1915) was able to cross successfully the mallard (*Anas platyrhyncos*) and pintail (*Anas cuta*) duck species in captivity. However, these two species are not known to hybridize in the wild. In this case the mechanisms which maintain reproductive isolation between the species are not those of genetic incompatibility but instead are ones which involve reproductive behaviour.

Clearly, in natural conditions where there is the possibility for individuals of different species to cross-fertilize, the criteria for the description of reproductive isolation requires further qualification. It can be described strictly as 'the absence of any form of inter-specific reproductive activity,' or it may be more flexible as 'the absence of genetic exchange between the genomes of species.' What is clear is that if the species concept, as defined by the idea of reproductive isolation, is to remain at the heart of biological science, then it must maintain flexibility in its interpretation.

Mayr (1963) summarized the mechanisms maintaining reproductive isolation between different species into the following catagories:

Pre-mating mechanisms

- habitat isolation;
- different reproductive periods;
- incompatible courtship behaviour;
- anatomical/physiological incompatibilities;

Post-mating mechanisms

- gamete incompatibility
- zygote mortality
- hybrid inferiority
- hybrid sterility

The implication of the last two aspects of the post-mating isolating mechanisms is that hybridization, and the presence of hybrids in a population, does not imply that the criteria of reproductive isolation has been violated.

1.1.3 Hybridization and introgression

When these reproductive isolation mechanisms break down, or are incomplete, inter-breeding between species may occur. Such an event is termed hybridization. However, the term is somewhat difficult to define because the phenomenon is dependent upon a reliable definition of the term species (Section 1.1.1). Nevertheless, the phenomenon of hybridization calls into question one of the most important aspects in the definition of a species, that of reproductive isolation.

The definition of the term hybridization is dependent upon the context in which it is used. In this study the term hybridization refers to the cross-breeding of individuals which are members of two different populations which are at present assigned to two different taxa ie. inter-specific hybridization (Mayr, 1963; Woodruff, 1973). The resulting offspring of such an event are termed hybrids.

Where hybrid progeny may be reproductively active there is the possibility of back-crossing between the hybrid and one of the parent taxa which may lead to gene introgression. Introgression is the incorporation of the genes of one species into the

gene pool of another which has occurred as a result of hybridization (Anderson & Hubricht, 1938). This is a critical factor of genetic exchange between species, even where hybridization is rare, and hence an important source of genetic variatiability among species (Anderson, 1949). However, there is no clear definition as to the point where hybridization and backcrossing becomes introgression (Verspoor & Hammer, 1991).

1.1.4 Hybridization in fishes

The phenomenon of hybridization is more common among fishes than other groups of vertebrates (Lagler *et al.*, 1962). This ability to interbreed and produce viable offspring among fishes is illustrated by the 3,759 references relating to hybridization compiled by Schwartz (1972; 1981).

The relative ease with which fishes are able to cross-fertilise is accounted for by a number of attributes which appear to undermine pre-mating mechanisms of reproductive isolation (Hubbs, 1955):

- external mechanisms of fertilization;
- weak isolating mechanisms;
- parental species occurring in unequal abundances;
- competition for limited spawning habitat;
- susceptibility to secondary contacts between recently evolved species.

These factors are often enhanced by either natural or man-made environmental perturbations and alterations of local habitat. This is highlighted by the relatively low incidence of hybridization amongst fishes that occur in the more stable marine and tropical aquatic environments, in comparison to temperate and freshwater habitats (Hubbs, 1955).

1.2 THE FAMILY CYPRINIDAE (TELEOSTEI-CYPRINIFORMES)

1.2.1 Features of cyprinid fishes

The general features of the cyprinid fishes of North-West Europe are described by Wheeler (1969). Cyprinids posess a single dorsal fin which has either one or two spiny rays for support. The pelvic fins are situated mid-body and well behind the pectoral fins. All the body scales are cycloid, but the head is scaleless. The jaws are toothless

but the fifth gill-arch is modified into pharyngeal bones which are found situated ventrally in the throat, behind the gill cover.

1.2.2 Taxonomy and systematics of Cyprinidae

Cyprinidae is one of the largest families of vertebrates in the world. The family has a wide geographic distribution including mainland Eurasia, Japan, the East Indian Islands, Africa and North America. There are around 1700 species within approximately 220 genera (Howes, 1991). They have considerable morphological and physiological diversity which has allowed them to exploit a wide variety of habitats (Howes, 1991).

Cuvier (1817) established the Cyprinidae as a family and since this time many authors have sought to divide the group into sub-families (e.g. Fowler, 1924; Gosline, 1978; Jayaram, 1981; Arai, 1982; Chen *et al.*, 1984). The most recent summary recognises seven sub-families of Cyprinidae, these are Cyprininae, Gobionae, Acheilognathinae, Leuciscinae, Cultrinae, Alburninae and Rasborinae (Howes, 1991). Most of these groupings are dependent upon the structure of the barbels, if present, and the morphology of the pharyngeal bones. Using these features it is possible to identify the two major lineages of Leuciscini and Barbini (Bonaparte, 1846; Nikolsky, 1954). However, these classifications must be interpreted with caution because these sub-groupings may not represent phylogenetic lineages (Howes, 1991).

1.2.3 Cyprinids of the British Isles

Seventeen species of cyprinids occur in the British Isles. These are dominated by species in the Cyprininae and Leucisinae sub-families (Table 1.1). They predominate in the middle and lower sections of rivers as well as in the still waters of many lakes and reservoirs. Species which occupy the faster flowing waters of the middle reaches of rivers include barbel (*Barbus barbus* (L.)), chub (*Leuciscus cephalus* (L.)), dace (*Leuciscus leuciscus* (L.)) and gudgeon (*Gobio gobio* (L.)) (Wheeler, 1969; Maitland, 1972). Those species which dominate the lower reaches of rivers and still waters include bleak (*Alburnus alburus* (L.)), carp (*Cyprinus carpio* L.), common bream (*Abramis brama* (L.)), roach (*Rutilus rutilus* (L.)), rudd (*Scardinius erythrophthalmus* (L)), silver bream (*Blicca bjoerkna* (L.)) and tench (*Tinca tinca* (L.)) (Wheeler, 1969; Maitland, 1972).

Ten different hybrid types have been recorded in the freshwaters of the British Isles (Table 1.2). Most of these occur between members of the Leuciscinae sub-family and

Table 1.1 Common and scientific names of cyprinid fishes occurring in the British Isles, sub-families from Howes (1991), and scientific names from Wheeler (1992)

Sub-family	Scientific name	Common name
Cyprininae	*Barbus barbus* (Linnaeus, 1758)	barbel
Cyprininae	*Carassius auratus* (Linnaeus, 1758)	goldfish
Cyprininae	*Carassius carassius* (Linnaeus, 1758)	crucian carp
Cyprininae	*Ctenopharyngodon idella* (Valenciennnes, 1844)	grass carp
Cyprininae	*Cyprinus carpio* Linnaeus, 1758	carp
Cyprininae	*Tinca tinca* (Linnaeus, 1758)	tench
Leuciscinae	*Abramis brama* (Linnaeus, 1758)	bream
Leuciscinae	*Blicca bjoerkna* (Linnaeus, 1758)	silver bream
Leuciscinae	*Leuciscus cephalus* (Linnaeus, 1758)	chub
Leuciscinae	*Leuciscus idus* (Linnaeus, 1758)	ide (orfe)
Leuciscinae	*Leuciscus leuciscus* (Linnaeus, 1758)	dace
Leuciscinae	*Phoxinus phoxinus* (Linnaeus, 1758)	minnow
Leuciscinae	*Rutilus rutilus* (Linnaeus, 1758)	roach
Leuciscinae	*Scardinius erythrophthalmus* (Linnaeus, 1758)	rudd
Gobioninae	*Gobio gobio* (Linnaeus, 1758)	gudgeon
Acheilognathinae	*Rhodeus sericeus* (Pallas, 1776)	bitterling
Alburninae	*Alburnus alburnus* (Linnaeus, 1758)	bleak

Table 1.2 Typical cyprinid hybrids known to occur in British fresh-waters

Hybrid	Relevant authors.
common bream x roach	Brassington & Ferguson (1976); Child & Soloman (1977); Cross (1978); Cowx (1983); Mulrooney & Fahy (1985); Wood & Jordan (1987); Adams & Maitland (1991).
rudd x roach	Wheeler (1969); Brassington & Ferguson (1976); Wheeler (1976); Cross & O'Rouke (1978); Burrough (1981); Thompson & Iliadou (1990).
common bream x silver bream	Wheeler (1969); Swinney & Coles (1982).
common bream x rudd	Wheeler (1969); Child & Soloman (1977).
roach x silver bream	Swinney & Coles (1982).
roach x bleak	Wheeler (1969).
roach x chub	Wheeler & Easton (1978).
bleak x chub	Wheeler (1978).
silver bream x rudd	Wheeler (1969).
bleak x rudd	Wheeler (1969).

the most common appear to be those between roach/rudd, roach/common bream and rudd/common bream. Hybrids probably occur more frequently between these species than others because they are among the most common and widespread of the cyprinids. Factors which also contribute to hybridization among these species include their similarity of preferred spawning habitat, the temporal overlap of their spawning activities and the large scale modification of their habitats by human activities (Weisel, 1954; Hubbs, 1955).

1.3 IDENTIFICATION OF CYPRINID SPECIES AND THEIR HYBRIDS

The ecology of many of the British cyprinid species has received much attention (e.g. Cowx *et al.*, 1993 and references therein). However, the majority of work upon cyprinid hybrids has concentrated merely on their identification. Indeed, an important part of any study of hybridization is to establish reliable methods of identification of both parental species and their hybrids. However, it has not been possible to verify the identity of putative hybrids in every case because of the reliability of the traditional methods used. Hence, there is a need to confirm the taxonomy of pure-species and hybrids from controlled breeding experiments and more reliable methods of identification, using genetic techniques, of fish from natural waters.

1.3.1 Identification by morphology

Traditionally cyprinid species and their hybrids have been identified on the basis of their anatomical features (Figure 1.1). Features used in identification include meristics, morphometrics and pharyngeal bone morphology (Wheeler, 1969; Maitland, 1972; Bagenal, 1973). Such features are robust for species identification, but may not always be suitable for distinguishing hybrids.

Morphometric characters include:

- body, fin and eye coloration;
- body depth (a);
- head width;
- mouth shape and size (b);
- size and position of the eye (c);
- position of dorsal (d) and pelvic fins (e);
- shape of dorsal (d) and anal fins (f).

(Letters in parenthesis refer to Figure 1.1).

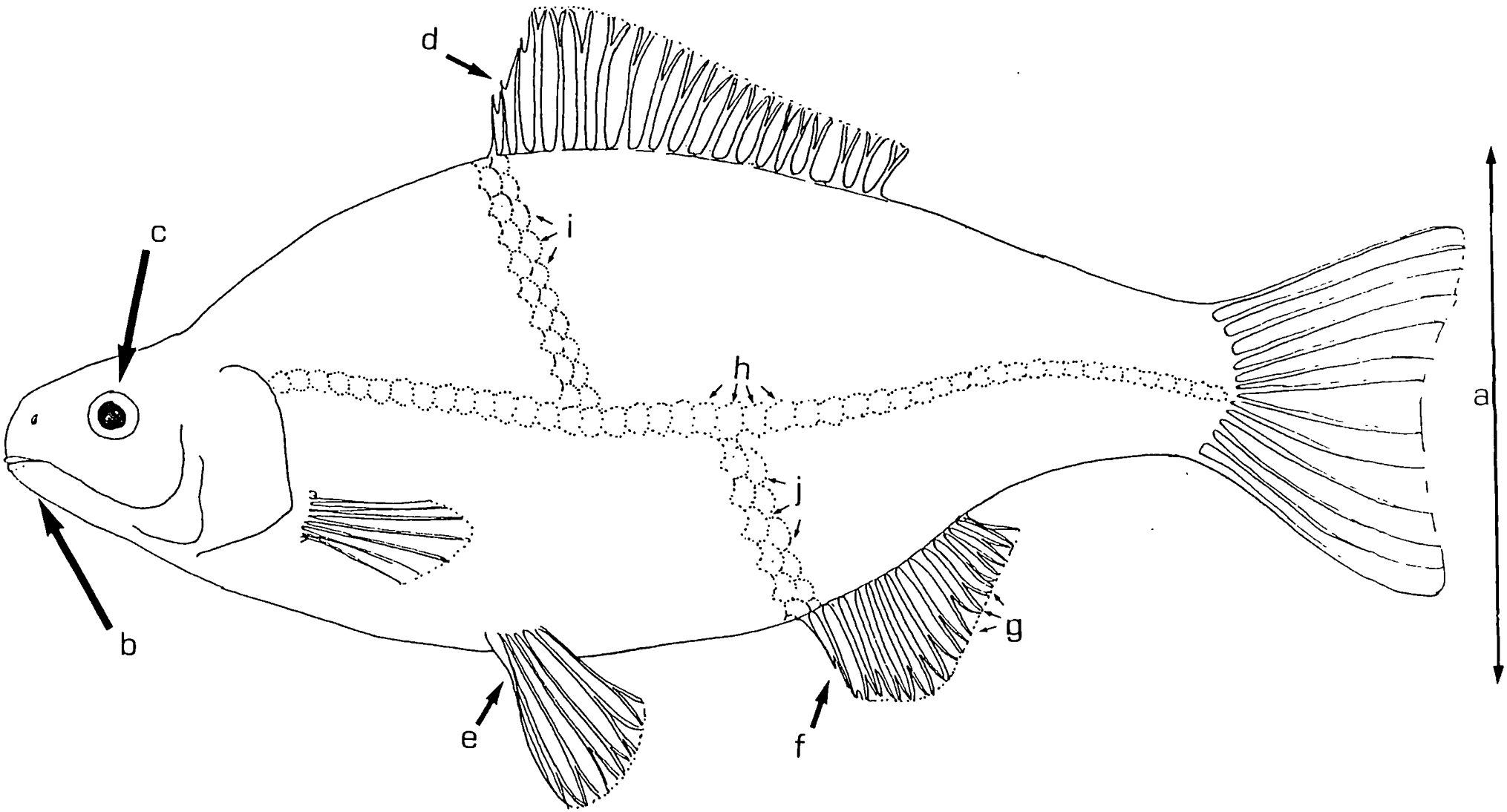


Figure 1.1 Meristic and morphometric features used in the identification of cyprinid species and their hybrids

Meristics characters include:

- the number of anal fin rays (g);
- the number of lateral line scales (h);
- the number of scales between the lateral line and the dorsal fin (i);
- the number of scales between the lateral line and the anal fin (j).

(Letters in parenthesis refer to Figure 1.1).

Pharyngeal bone characters include:

- structure and dentition;
- crenulation;
- pectination.

Some of the published meristic data which have been used in the identification of cyprinid species and their hybrids are given in Appendix A. The similarity amongst the data, for each species and hybrid, suggests that some degree of reliability can be attached to them. However, there are many assumptions implicit within such data which may limit their application to hybrid studies:

- the parent species of the hybrid cross are already known;
- data for the hybrids will be intermediate between the apparent parent species;
- the hybrids are of the F1 generation.

It is apparent from these assumptions that there is a need in the taxonomy of hybrids to characterise the features of pure-bred species and their hybrids from controlled breeding experiments. To date, the only reliable study of this kind in the British Isles was conducted by Wood & Jordan (1987), upon roach and common bream hybridization. Once these assumptions have been analysed reliable conclusions can be established which will enhance all future hybrid studies.

1.3.2 Identification by genetics

Enzyme electrophoresis is a genetic technique which examines enzyme molecule polymorphisms, i.e. differences in the size, shape and structure. This is assessed by measuring the degree of mobility of an enzyme molecule as it migrates, from a fixed point, across a buffered gel which has an electrical field applied across it. The buffered gel acts as a molecular sieve and variation in the mobility of the enzyme molecules between species, which arise from the differences in size, shape and electrical charge can be detected. These features of the enzyme are determined by the amino-acid composition of the enzyme, which, in turn, are determined by the DNA sequence at the

point on the chromosomes which codes for the enzyme. This point is referred to as a gene locus. Each gene locus in an individual's genome is composed of two alleles and each is inherited independently: one from each parent.

At each gene locus there are two possible alternatives. Either the two alleles may be identical, in which case the individual is termed homozygous at the gene locus, or the alleles may be different and the individual is heterozygous. Where individuals of two parent species are both homozygous at a gene locus, but are different between the species then their F1 hybrids will express a heterozygous condition for the gene. If the differences for an allele at a locus is fixed between the species, then the gene locus is termed diagnostic and may be used to detect hybridization using enzyme electrophoresis.

When the electric field has been applied to the gel for a sufficient period of time differences in mobility of the enzymes can be detected. The location of an enzyme on the buffered gel can be depicted by coloured bands using enzyme specific stains. If these procedures are repeated for a number of enzymes, the patterns which appear on the gel can be used to interpret parental ancestry.

If the genetic differences between the alleles can be detected by variations in the molecular mobility on an electrophoresis gel the following enzyme banding patterns will be observed. For a monomeric enzyme (enzyme molecule has only one sub-unit), the pattern observed for the parental species and their hybrid similar to that shown in Figure 1.2a. For dimeric and tetrameric enzymes (two and four sub-unit enzyme molecules respectively), heteromeric bands form (Figures 1.2b and 1.2c). Enzyme electrophoresis can also be used to ascertain whether hybridization in a population has progressed past the F1 generation. This is observed when a mixture of heterozygosity and homozygosity is expressed for an individual hybrid at gene loci which are diagnostic.

Detailed descriptions of various techniques and methods of electrophoresis are outlined in Shaw & Prasad (1970), Harris & Hopkinson (1976), Sambrook *et al.* (1983), Richardson *et al.* (1986) and Hebert & Beaton (1989).

Hence, enzyme electrophoresis can be used to detect genetic differences between species and hybrids by dissimilarities in the enzyme structure (Ferguson, 1977; Ferguson, 1980; Campton, 1987). Despite the clear advantages over traditional methods, there have been few studies which have applied these techniques to cyprinid hybrid studies (Brassington & Ferguson, 1976; Child & Solomon, 1977; Cross, 1978; Valenta, 1978; Berrebi *et al.*, 1989; Thompson & Iliadou, 1990).

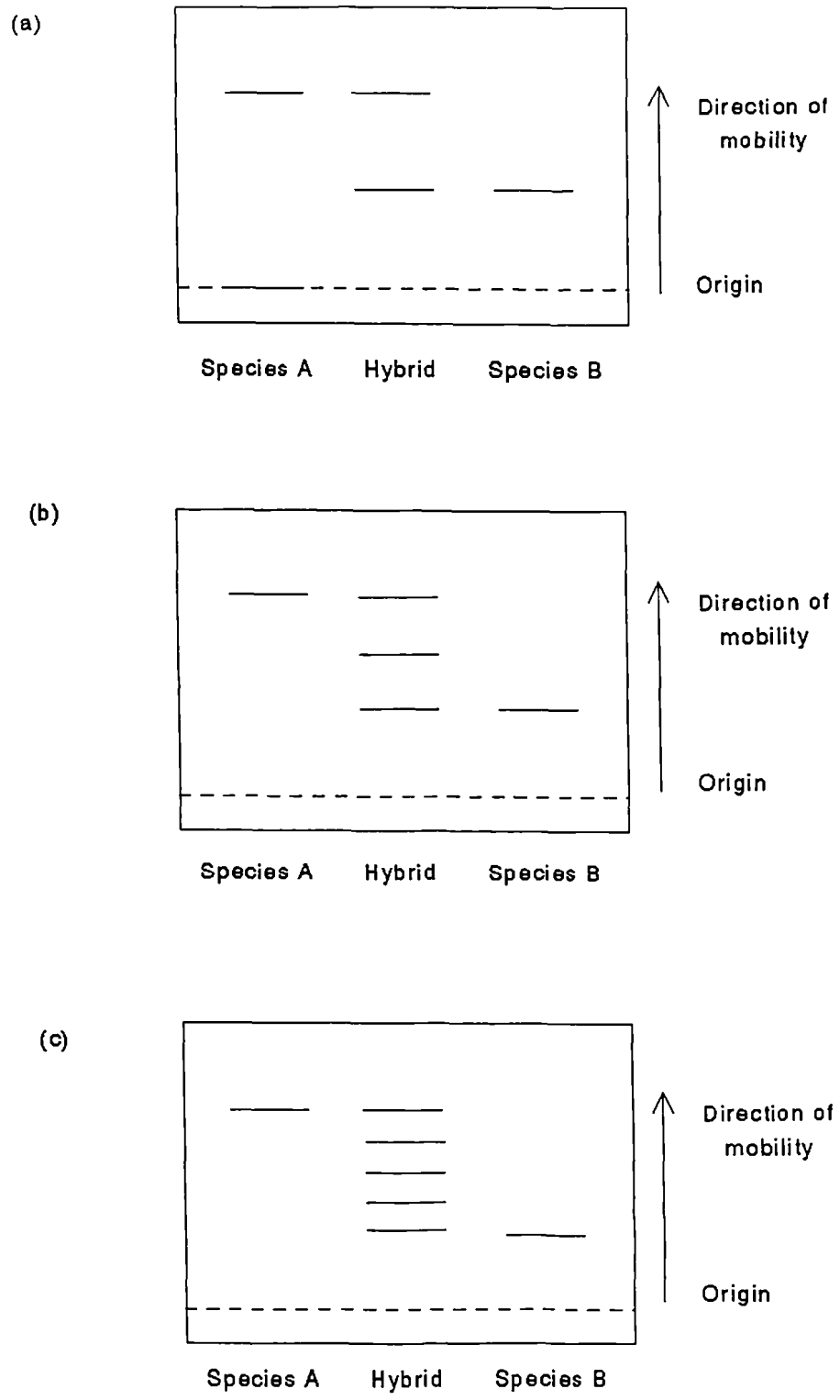


Figure 1.2 Diagrams of electrophoresis gels showing hybrid banding patterns for monomeric (a), dimer (b), and tetrameric (c) enzymes

1.3.3 Species-specific sexual ancestry

Where there is a breakdown of mechanisms of reproductive isolation it is possible to discover which species is the maternal parent and, hence, which species is the paternal parent. This can be ascertained by the genetic analysis of the mitochondrial DNA (mtDNA). Mitochondrial DNA is a closed circular molecule, found only in the mitochondria, of approximately 16-18 kilobase pairs and is inherited maternally (Hutchinson *et al.*, 1974) and therefore can be used to identify the maternal parent species in an inter-specific hybrid.

Genetic assessment of mtDNA involves the use of restriction enzymes which cut the mtDNA at specific gene sequences on the molecule. This creates smaller fragment molecules which are linear. These smaller mtDNA fragments can be separated according to their size, using agarose gel electrophoresis. The patterns can then be visualized under ultra-violet light. Different banding patterns will be observed for different species because of changes in the DNA sequence. Once the banding patterns of the parent species have been properly characterised, the patterns of the hybrids can be compared to discover the maternal parent species. An example of the type of result expected for two species and their F1 hybrid is illustrated (Figure 1.3). In this example the maternal parent is species A.

This technique has been used widely to assess the genetic relationships both between and within different fish species (Billington & Hebert, 1988; Hynes *et al.*, 1989; Baby *et al.*, 1991; Seyoum & Kornfield, 1991). The technique has also been used in combination with allozyme studies to determine maternal ancestry of sunfish hybrids in North America (Avise & Saunders, 1984). Indeed, using the techniques of enzyme electrophoresis and mtDNA analysis together can yield a great deal of information upon the nature of hybridization. However, this combination of techniques has yet to be applied to cyprinid hybridization in either the British Isles or the rest of Europe.

1.4 AIMS AND OBJECTIVES

Little is known regarding inter-specific hybridization among cyprinids in comparison to aspects of this phenomenon among salmonids (e.g. Campton, 1987; Gyllensten & Wilson, 1987). Firstly, there is a need to establish the potential for inter-specific hybridization between cyprinid species and the possibility of post-F1 generation hybridization through a controlled cross-breeding programme (e.g. Burroughs, 1981; Cowx, 1983; Wood & Jordan, 1987), i.e. Chapter 2. The progeny of these experiments can be used to examine the assumptions of cyprinid hybrid identification with both

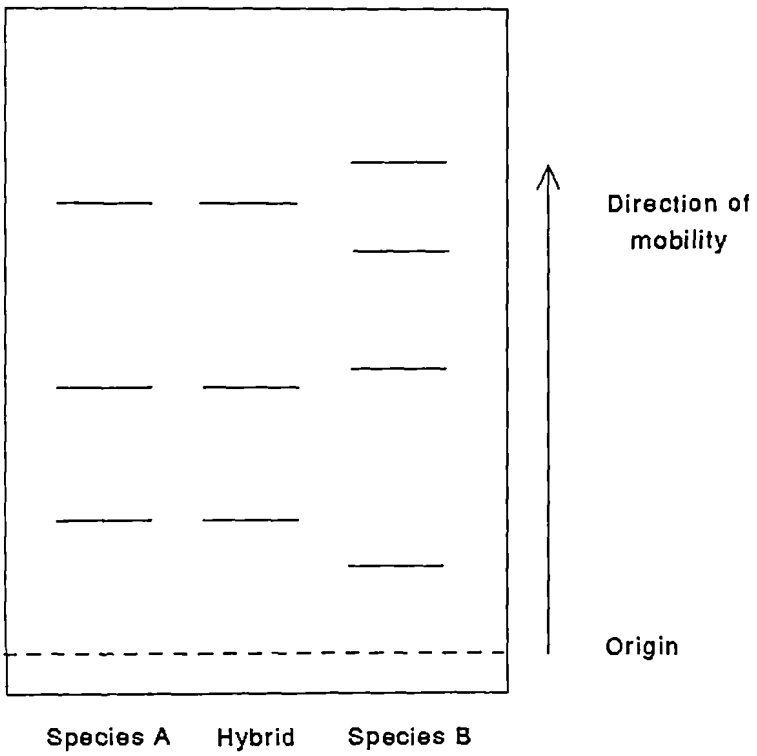


Figure 1.3 Diagram of an electrophoresis gel showing mtDNA fragments for hybrid and parent species

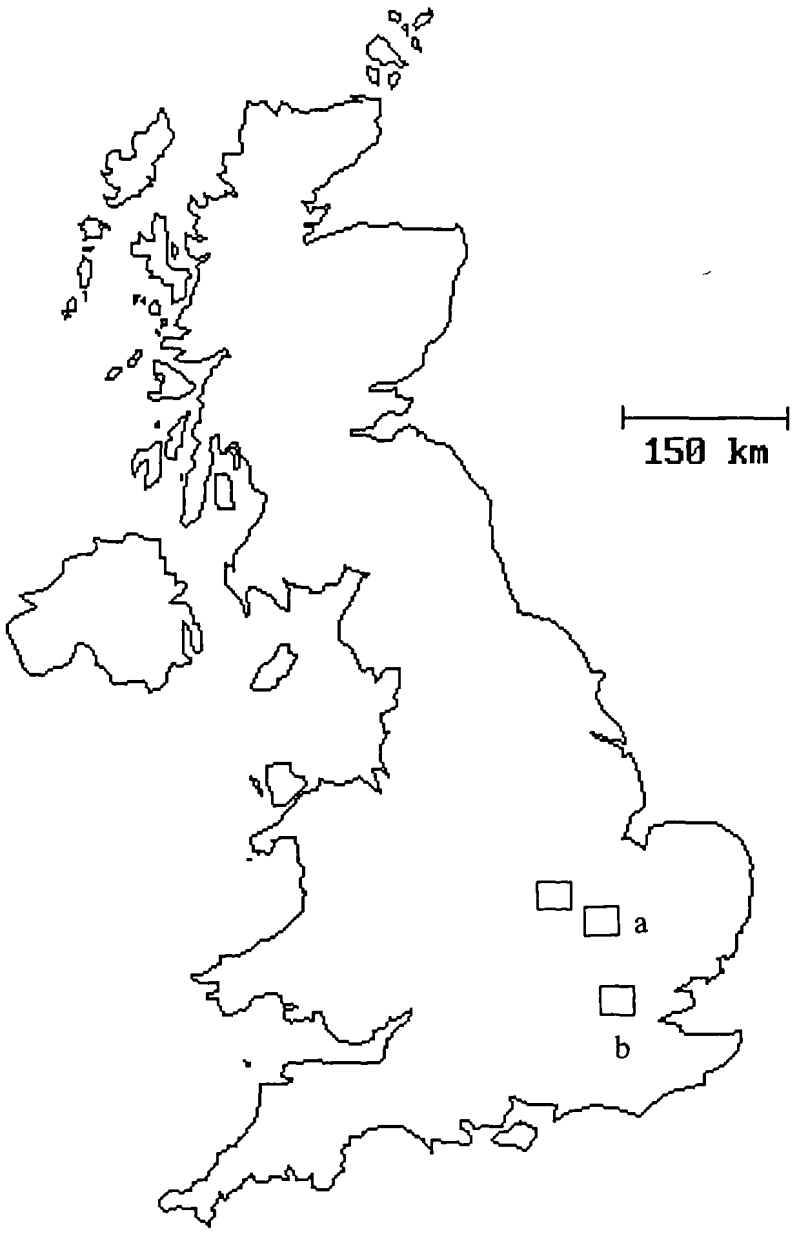


Figure 1.4 Geographic location of the Forty Foot Drain (a), Essex University Lake (b) and Peterborough Rowing Lake (c)

traditional and genetic techniques. Data from these experimental crosses can then be compared with hybrids from wild populations to examine the extent to which hybridization is occurring (Chapters 5 & 6). Roach/common bream hybrids and rudd/common bream hybrids were examined from the Forty Foot Drain (NGR TL 357882) and Essex University Lake (TM 031242) respectively (Figure 1.4).

At present there are no studies on cyprinid hybrids of the British Isles to establish the causes and consequences of the phenomenon particularly with respect to species-specific sexual ancestry. Such information would prove to be invaluable to strategies of fisheries management, particularly since many authors have suggested that human activities may enhance hybridization (e.g. Weisel, 1954; Hubbs, 1955; Criveli & Dupont, 1987). Hence, it is important to establish maternal ancestry among cyprinid hybrids in natural populations to ascertain information on aspects of both the hybridization event and hybrid appearance (Chapter 7). An attempt was made to examine roach/common bream hybrids from Peterborough Rowing Lake (NGR TL 172980) using mtDNA analysis. However, this was not successful.

The results and conclusions of the present study are discussed with respect to species integrity, fisheries management and future investigations (Chapter 8).

CHAPTER TWO.
EXPERIMENTAL CROSS-FERTILIZATION OF CYPRINID FISHES.

2.1 INTRODUCTION

2.1.1 Genetic compatibility

There is considerable potential for the gametes of one species of fish to come into contact with those of another (Section 1.1.3). However, little is known about the genetic compatibility between the gametes of different cyprinid species. Ryabov (1981a,b) reviewed a several studies which examined cyprinid hybridization to establish the level of taxa divergence which prevents genetic compatibility of the gametes. However, these reviews were not based upon direct observations and, because of inconsistencies between the methods of each author, was therefore not able to examine accurately the potential for natural hybridization among cyprinid species.

2.1.2 Species-specific sexual ancestry

There is a paucity of information on species-specific sexual ancestry among hybrids, i.e. which is the paternal and which is the maternal species. This is true for cyprinids. It is often suggested that hybrids in natural waters are more similar in appearance to one of the parent species than the other. In these circumstances, it is often cited that the species which the hybrid is most similar to in appearance is the maternal parent (Ryabov, 1981a; Collares-Pereira & Coehlo, 1983; Economidis & Sinis, 1988). This suggests that the hybrid population is composed of individuals which are the product of the same maternal and paternal parent species crosses. However, in circumstances where this may occur, it is not known whether this is because of factors related to species-specific sexual incompatibility of the gametes, species-specific sexual behaviour when spawning or inferior fitness of the hybrids of the reciprocal cross during early development.

2.1.3 Complex hybridization

It is reported by some authors that mature hybrids found in the wild may be able to produce viable gametes (Cowx, 1983; Fahy *et al.*, 1988). Also, the viability of roach/common bream hybrid gametes has been demonstrated under controlled conditions by Wood & Jordan (1987). Thus, despite the occurrence of post-F1 hybrids having not been verified in natural cyprinid populations in British Isles, circumstantial

evidence indicates that there may be the potential for hybrid populations with complex inter-bred ancestry. Indeed, this has already been suspected in some hybrid populations (e.g Wheeler, 1976).

2.1.4 Aims and objectives

To assess the potential of these aspects of gamete compatibility an experimental breeding programme was devised using as many cyprinid species as possible (Burrough, 1981; Cowx, 1983). The principal aims of the breeding programme were, in terms of the genetic compatibility of the gametes:

i) to identify the potential for hybridization among cyprinids species in the UK;
ii) to identify whether species-specfic sexual ancestry is of significance;
iii) to attempt to determine the viability of the gametes of F1 cyprinid hybrids.

2.2 MATERIALS AND METHODS

2.2.1. Cross breeding programme

To achieve these objectives an experimental breeding programme to produce hybrid fish of known ancestry was set up at the Regional Fisheries Laboratory (RFL), National rivers Authority (NRA) Anglian Region, Brampton, Cambridgeshire, UK. The experimental breeding programme took place between the 20th April and 16th of June in 1990, 1991 and 1992. The programme was restricted to crosses involving the following fish types because of the availability of suitable broodstock:

- barbel *Barbus barbus* (L.);
- common bream *Abramis brama* (L.);
- chub *Leuciscus cephalus* (L.);
- goldfish *Carassius auratus* (L.) (female only);
- roach *Rutilus rutilus* (L.);
- rudd *Scardinius erythrophthalmus* (L.);
- RBHF1 Presumed F1 roach x common bream hybrid (female only).

2.2.2 Sources of broodstock

The broodstock fish for the programme were obtained from known spawning grounds and site facilities provided by the NRA (Table 2.1), just before spawning activity was expected to commence. Each year the timing of broodstock collection varied slightly, but for the species concerned and their locations this usually occurred in early-mid May. Where necessary, broodstock fish were caught with the assistance of staff from the RFL and fisheries survey teams of the NRA Anglian Region. Two techniques were used to catch the broodstock fish: seine netting and pulsed DC electricfishing. The method employed depended upon the topography of the site and the species concerned. Where appropriate, the broodstock fish were transported to the RFL in aerated tanks. On arrival they were segregated into holding tanks (*ca* 3000-l volume), according to species and sex, where they were held before ovulation and sperm release were induced.

2.2.3. Broodstock identification

Broodstock fish species were identified with the aid of published keys (Wheeler, 1969; Maitland, 1972; Bagenal, 1973). The external anatomical features used for identification, both morphometric and meristic, are described in Section 1.3.1 and illustrated in Figure 1.1.

The roach/common bream hybrids were identified as first filial generation (F1), because their morphometric and meristic characteristics were intermediate of their presumed parent species (Wheeler, 1969; Child & Solomon, 1977; Cowx, 1983).

2.2.4 Artificial production of gametes

Ovulation and sperm release were induced, using methods similar to those described by Easton & Dolben (1980) and Wood & Jordan (1987), i.e. with injections of gonadotrophic hormone prepared from carp pituitary extract (CPE). To reduce stress during handling procedures, broodstock fish were anaesthetised in benzocaine (1:10 000 dissolved in acetone) prior to injection.

CPE doses (10 $mg.ml^{-1}$) were prepared in 0.7% w/v saline. The preparation was centrifuged at 3 000 x *g* for 15 minutes to leave a clear supernatant which was suitable for injection. Fish were weighed to calculate the required CPE doses. A 1-ml sterile syringe and a 23G hypodermic needle were used to inject the CPE into either the musculature of the caudal peduncle (IMCP) or the peritoneal cavity (IP). The injection

Table 2.1 Sources of broodstock fish for the experimental breeding programme.

Site	National Grid Reference (NGR)	Fish types.
Costessey Fish Farm, Norwich.	TG 180120	Roach and rudd
The Cut-Off Channel, Cambridgeshire.	TL 620995	Common bream and roach/bream hybrids
River Cam, Cambridgeshire.	TL 492458	Chub
Great Ouse, Linford, Buckinghamshire.	SP 850435	Barbel and chub
Eastmoor Farm Pond, Doddington, Cambridgeshire.	TL 407901	Roach and rudd
Brampton RFL	TL 205705	Goldfish

protocols used for each species, in terms of dosage and timing are summarized in Table 2.2. After injection fish were returned to their original holding tanks. Mature female fish required two CPE injections, a low concentration priming dose which was adminstered immediately after arrival at the RFL and a high concentration resolving dose which was given 12 hours later. Mature males only required a single CPE injection which was administered 12 hours after arrival at the RFL i.e. at the same time as the resolving dose for females.

Each fish was examined, at intervals of 4-6 hours, to check for ovulation and sperm production and then returned to their holding tanks. When ovulation appeared to be imminent the equipment for gamete stripping and fertilization was prepared.

2.2.5 Fertilization

Broodstock fish were again anaesthetised in benzocaine (1:10 000 dissolved in acetone), prior to gamete stripping. Eggs were then stripped from the anal vent of an ovulating female into a number of plastic spawning bowls. The number of spawning bowls used for each female corresponded to the number of species for which males were available for fertilisation. Approximately 2 ml of eggs from a female were deposited into each spawning bowl. An additional batch of eggs was taken from each female and left unfertilized. These eggs acted as a control to compare the development and mortality of fertilised eggs. During egg stripping it was imperative that all equipment and anaesthetised female broodstock fish were dry. If there was moisture the eggs would become sticky and the fertilization procedure would become difficult to perform successfully. Sperm were transferred from the anal vent of anaesthetised males to each egg batch with a pasteur pipette. Before sperm were transferred they were first examined under a high-powered microscope to ensure motility. If the sperm were motile it was assumed that, providing gametes were compatible, they would be capable of fertilising eggs. Approximately 0.5 ml of sperm were transferred from each male to each batch of eggs. Woynaravich solution (0.15% w/v urea, 0.2% w/v NaCl in distilled water) was added to each batch of eggs and sperm. The gametes were mixed in this solution for 5 minutes, with a clean, dry feather, to ensure that fertilization was possible. Woynarovich solution was used as the mixing medium because it prolongs sperm motility but at the same time prevents the eggs becoming sticky. After mixing the excess Woynarovich solution was drained off and the egg batches were rinsed twice in Woynarovich solution to displace excess sperm.

Table 2.2 CPE injection protocols and time to ovulation after each resolving dose.

Broodstock	Sex	Priming dose mg ml^{-1}	Resolving dose mg ml^{-1}	Ovulation time (h)
Common bream	F	1.0	5.0	15-30
	M		2.5	
Chub	F	1.0	5.0	18-24
	M		2.5	
Roach	F	1.0	5.0	8-24
	M		2.5	
Rudd	F	1.0	5.0	15-30
	M		2.5	
RBH F1	F	1.0	5.0	8-30
Barbel	F	1.0	5.0	8-24
	M		2.5	
Goldfish	F	1.0	5.0	8-16

2.2.6 Egg husbandry

When the fertilization procedure was complete each egg batch was sub-divided into two portions. The first portion of eggs from each cross (*ca* 100 eggs) was placed into 250-ml Pyrex crystallizing dishes containing approximately 200 ml of water which was dosed to 0.1 ppm malachite green. Malachite green is an anti-fungal agent. Dishes were then incubated at 17°C and the water was changed every 24 hours. Observations were made at 12-hour intervals to record the number of viable eggs and the development of the embryos. These observations were considered important because they may have indicated if there were critical stages at which the development of a hybrid embryo terminated. Eggs which were not viable were recognised as those which had turned opaque as a result of globulins precipitating in the perivitalliene fluid. Fertilization rates where calculated when all eggs in a control batch were dead. This occurred approximately 36 hours after gamete stripping. At this point it was assumed that all eggs that were still viable in the experimental batches, from each respective female, had been fertilised. Hatching rates for a particular cross were calculated from the number of eggs that were still viable when the first hatches were observed.

The second portion of eggs, from each cross, was placed onto Rokalene mesh which was suspended in 15-litre plastic tanks. The progeny produced within these tanks were reared for use in other studies (Chapters 3 & 4). The tanks were aerated and had under-gravel filtration to maintain water quality. Temperature was maintained between 15°C and 18°C in a temperature-controlled room. Tanks were dosed daily to 0.1 ppm malachite green until the first embryos hatched.

2.2.7 Nomenclature

Where a hybrid cross is referred to in the form of roach/common bream, this classes the both reciprocal hybrid crosses of roach and common bream together. Where the parental ancestry of a cross is referred to in the form roach x common bream, the famale parent is the first named species i.e. hybrid has the parental ancestry of female roach and male common bream. However, this is not the case when referring to the F1 roach/common bream hybrid since the parental ancestry was not known.

2.3 RESULTS

2.3.1 Crosses performed

During the experimental programme 34 crosses were performed using the artificial spawning techniques. All possible reciprocal crosses, including pure species self-crosses, were performed for broodstock fish for which both male and female broodstock had been available. These were barbel, common bream, chub, roach and rudd. Four inter-species crosses were performed using a female goldfish and five crosses, 2 back-crosses and 3 triple-crosses, were performed with female F1 roach/common bream hybrids (Table 2.3).

The experimental breeding programme was bound by two constraints. First, variability in the timing at which each species achieves maturity of their gametes precluded the use of some species (e.g. dace). This restricted the number of different crosses that were possible. Second, injections of gonadotrophic hormone did not always induce ovulation in females which appeared to have reached maturity of their gametes. This made the planning and the co-ordination of the desired experimental breeding programme difficult.

2.3.2 Fertilization rates

The absence of replications of the crosses prevented the use of techniques which could assess the statistical significance between the results of the crosses. Fertilisation rates were high for all pure bred and all reciprocal inter-species crosses between common bream, chub, roach and rudd (Table 2.4). Indeed, some inter-species hybrid crosses were more successful than pure-bred crosses. This suggests that, under controlled conditions, the gametes of these species are genetically compatible and that species-specific sexual ancestry is not important. However, fertilization rates involving female roach with male common bream (46.6%) and female roach with male chub (65.2%), appeared to be much lower than their reciprocal crosses involving male roach with female common bream (68.9%) and male roach with female chub (88.2%).

The fertilisation rate observed in the pure-bred barbel cross was also low (34.7%) in comparison to other pure-bred crosses (Table 2.4). Observations of the barbel eggs in the Pyrex dishes suggests that the environment was not suitable for their development. The eggs of all other fish types used in the programme adhered to the glass surface of the dishes. Thus when observations were made there was minimal disturbance and no movement of the eggs. However, the barbel eggs did not adhere to the glass surface of

Table 2.3 Year in which experimental cross performed.

Broodstock sex			Male		
Female	Common bream	Chub	Roach	Rudd	Barbel
Common bream	1990	1990	1990	1990	1991
Chub	1991	1991	1991	1992	1991
Roach	1991	1991	1991	1992	1991
Rudd	1992	1992	1992	1992	1992
Barbel	1991	1991	1991	1992	1991
RBH F1	1990	1990	1990	1990	1991
Goldfish	1991	1991	1991	--	1991

Table 2.4 Percentage of eggs fertilised for each experimental cross.

	Male				
Female	Bream	Chub	Roach	Rudd	Barbel
Common Bream	78.4	84.2	68.9	87.8	0.9
Chub	80.0	97.2	88.2	88.8	1.6
Roach	46.4	65.2	87.5	91.4	0.0
Rudd	77.0	91.1	97.7	90.0	0.0
Barbel	0.0	0.0	0.0	0.0	34.7
RBH F1	83.5	70.0	81.6	64.9	0.0
Goldfish	11.9	10.4	7.4	--	0.0

the dishes. As a consequence, slight disturbance of the dishes, which occurred during observations, resulted in extensive egg movement and probably high mortality. In comparison to the fertilisation rates recorded for the inter-species crosses involving either male or female barbel (0.0-1.6%), the pure-bred barbel fertilization rate does appear to be relatively high. This suggests that the gametes of barbel are not genetically compatible with the other fish categories used in the programme (common bream, chub, goldfish, roach, roach/common bream hybrids and rudd).

Fertilization rates involving inter-specific crosses with the female goldfish were also low. From these data it appears that the gametes of the female goldfish are not genetically compatible with the male of the other species used in the programme. However, interpretation of these data are difficult because there are no results from a pure-bred goldfish cross with which a comparisons can be made.

Fertilization rates were high for the female F1 roach/common bream hybrid back-crosses with both male common bream and roach. Similarly, fertilisation rates were high for the female F1 roach/common bream hybrid triple-crosses with either chub or rudd. However, no developing embryos were observed when the F1 female hybrid was triple-crossed with male barbel. These results suggest that hybrid gametes are not only capable of back-crossing with their original parent species, but also are genetically compatible with the gametes of some of the other species.

2.3.3 Hatching rates

First hatching was observed between 120 and 140 hours (85.0-99.2 degree days) after incubation in crosses in which fertilisation was successful. With four exceptions, hatching rates were comparatively high for all pure-bred and for all reciprocal inter-specific crosses between common bream, chub, roach and rudd (Table 2.5). However, hatching rates were low for the barbel x barbel, common bream x chub, roach x common bream and roach x chub crosses. In the barbel x barbel and common bream x chub crosses there were large differences between the fertilization and hatching rates. These results may be attributed to poor water quality which was observed in the Pyrex crystallizing dishes of these crosses. This was caused by growths of fungi on decaying eggs. This problem persisted despite all attempts to maintain good water quality. The low hatching rates observed for the roach x bream and roach x chub crosses (Table 2.5), are because of low fertilization rates for these crosses (Table 2.4). There was no hatching of eggs in any of the crosses involving male or female barbel and none in the crosses in which eggs from the female goldfish were used. In cases where barbel eggs

Table 2.5 Percentage of eggs hatching for each experimental cross.

	Male				
Female	Bream	Chub	Roach	Rudd	Barbel
Common Bream	70.0	35.5	68.9	84.1	0.0
Chub	78.3	97.2	88.2	75.6	0.0
Roach	28.9	53.1	79.2	88.5	0.0
Rudd	68.7	76.7	94.2	85.5	0.0
Barbel	0.0	0.0	0.0	0.0	0.0
RBH F1	82.5	69.1	72.1	62.2	0.0
Goldfish	0.0	0.0	0.0	--	0.0

were fertilised, including the barbel x barbel cross, all embryos died in the early stages of development (*ca* 60 hours after fertilization).

2.3.4 Embryonic development

The general pattern of development obseved for pure-bred common bream, chub, roach and rudd embryos are given (Table 2.6). Inter-species hybrid crosses also showed similar patterns of development. However, abnormalities in development were observed in crosses involving female goldfish. In these crosses most of the eggs which were fertilised only survived as viable embryos until they were approximately 100 hours old. At this point all viable embryos of this cross died. Furthermore, embryo development had ceased in the late neurogenesis to early organogenesis phase (Table 2.6), which was achieved after approximately 50 hours. This suggests that this stage in development is critical in the genetic compatibility of goldfish eggs with sperm from common bream, chub and roach. In all crosses which produced progeny the embryo development was in agreement with patterns observed by previous authors (Gulidov & Popova, 1981; Penaz & Gajdusek, 1979; Penaz & Sterba 1969; Herzig & Winkler, 1986).

2.4 DISCUSSION

2.4.1 Broodstock identification

Genetic studies, such as enzyme electrophoresis, are the only method by which broodstock identification can be verified (Ferguson, 1977). These methods were not performed because of logistic and ethical reasons (electrophoretic analysis requires the fish to be sacrificed), and therefore the identity of the broodstock fish cannot be confirmed with absolute certainty. However, since all broodstock fish appeared to fit the descriptions of Wheeler (1969), Maitland (1972) and Bagenal (1973), in terms of their meristics morphometrics and general appearance, it was deemed that these fish were probably pure-bred species.

It was not possible to identify the presumed F1 roach/common bream hybrid with certainty because it has never been characterised from controlled breeding experiments. In terms of the features of its morphology, meristics and pharyngeal bone formation, it was clearly intermediate between the presumed parent species. In addition, the features of the presumed F1 roach/common bream hybrid conformed to those observed in a previous study, in which genetic information was used to support meristic data to

Table 2.6 General development of embryos surviving to hatching of crosses between common bream, chub, roach, rudd and presumed F1 roach/bream hybrids.

Time (hours)	Development phase
0-20	Morulation & blastulation
20-50	Neuralation & organogenesis
50-70	Trunk segmentation & tail elongation
70-90	Tail segmentation & rudimentary gills
90-100	Movement, circulation & eye pigmentation
100-120	Yolk sac much reduced & circulation 2 beats sec^{-1}
120-140	Extensive musculature & first hatches

identify them as being F1 roach/common bream hybrids (Child & Solomon, 1977). The meristic, morphometric and pharyngeal bone characteristics of the presumed F1 roach/common bream hybrid were also similar to those observed in previous studies where such hybrids were tentatively identified (Pethon, 1978; Cowx, 1983; Mulrooney & Fahy, 1985; Wood, 1985). Subsequent morphometric and genetic investigations into the offspring of this fish also indicated that it was probably an F1 roach/common bream hybrid (Chapter 3; Chapter 4).

2.4.2 Inter-specific gamete compatibility

In cases where the gametes of different species were compatible with each other, statistical significance could not be attached to the differences in either the fertilization or hatching rate data between the experimental crosses because there were no replications or psuedo-replications of each cross (C. McGowan, *pers. comm.*). Also, despite there being information for salmonid reciprocal hybrid crosses (Alm, 1955; Dumas *et al.*, 1992; McGowan & Davidson, 1992), there are few inter-specific cyprinid hybrid studies in the published literature with which these data can be compared, with the exception of the study by Wood & Jordan (1987). In contrast to the results presented here, Wood & Jordan (1987) were not able to produce successfully F1 hybrid progeny from the common bream x roach cross. This may be attributed to differences in the cross-fertilization methods employed. The results presented here have been produced by inducing gamete production with gonadotrophic hormone injections. Eggs and sperm were then stripped and mixed by hand from anaesthetised fish to ensure complete mixing of gametes. Although Wood & Jordan (1987) induced gamete production using the same method, the broodstock fish were left to spawn in the holding tanks and under these circumstances it is not possible to control all factors which may affect the mixing of gametes. Clearly, unlike the procedure employed in this study, the method of Wood & Jordan (1987) is not able to answer with certainty the question of inter-species gamete compatibility because their method introduced aspects of behaviour.

In terms of relating these results to hybridization studies in the wild, it would be beneficial to perform investigations which combine both these methods of cross-fertilization. The results could be enhanced further if they are supported by field observations of spawning behaviour and mitochondrial DNA investigations, determine the maternal ancestry of hybrid fish (Dowling *et al.*, 1989).

Although there is no statistical analysis, the most important output of these artificial crosses is the extent of the potential for hybridization among cyprinid species which

occur sympatrically in fresh waters. These results show unequivocably that the isolating mechanisms between these taxa are not ones of genetic incompatibility of the gametes. Therefore, there must be other mechanisms maintaining reproductive isolation and genetic integrity of the species involved (Mayr, 1963; Solomon, 1977; Bloom & Perlmutter, 1978; Kwak & Skelly, 1992), e.g. either pre-spawning isolating mechanisms such as differences in spawning behaviour, habitat and timing, or post-spawning isolating mechanisms such as inferior fitness of the hybrid progeny (Section 1.1.2).

2.4.3 Reciprocal crosses

Some studies examining hybridization amongst different cyprinid taxa by experimental cross-fertilization have noted differences in the success of fertilization and hatching between reciprocated crosses (Makeyeva, 1972; Ryabov, 1981; Burkhead & Williams 1991). For example, Makeyeva (1972) was successful in crossing male *Aristichthys nobilis* (Rich.) with female *Cyprinus carpio* L. and achieved up to 98% hatching success, but the reciprocal cross proved to be incompatible. This suggests that, in some cases at least, there may be a genetic barrier determined by the sexual directionality of the hybrid cross and that this may restrict the potential of species to cross-fertilise. In circumstances where the hybridizing species spawn at similar times and on similar substrate, the behaviour in mate choice may be critical in maintaining mechanisms of genetic isolation and integrity. However, the results of the fertilization and hatching rates of each of the successful reciprocal crosses in the present study appeared not to be affected by such a genetic barrier. Hence, genetic compatibility between the gametes of cyprinid species appears not to be reflected by the direction of the hybrid cross.

Some discussion is necessary with respect to some of the differences in the success of the roach/common bream, common bream/chub and roach/chub reciprocal crosses. In comparison to the common bream x roach and chub x roach crosses, the reciprocal roach x common bream and roach x chub crosses show much lower fertilization and hatching rates. Although there may be a limited genetic barrier between the species which affects their potential to hybridize in the environment, it is more likely that the differences are the result of inefficiencies in the artificial spawning procedure during gamete mixing. In the case of the chub x common bream cross, where the hatching rate was much lower than the fertilization rate, embryo mortalities occurred gradually throughout the incubation period. It was noted that the developing hybrid embryos of this cross appeared to be healthy at each observation and that the occurrence of the mortalities did not occur in an instantaneous manner as would be expected if there had been genetic incompatibility. Therefore, it was concluded that the low hatching rates were the result of the apparent water quality problems in this particular incubating dish

and not because of genetic incompatibility. Reasons for the apparent poor water quality problems could not be identified. These problems persisted throughout the incubation period despite all attempts to maintain water quality.

2.4.4 Fertility of F1 hybrids

Some authors have demonstrated that hybrid fish are probably infertile through, for example, the abnormal development of gonads (Hulata *et al.*, 1980; Down & Leatherland, 1989). However, the present study has shown that the female F1 roach/common bream hybrid is in fact fertile. Indeed, the results of Wood & Jordan (1987) also indicated that F1 hybrids are fertile, but that the degree of fertility was limited by the male hybrid. Furthermore, although Kennedy & Fitzmaurice (1968) have observed spent F1 hybrids in the wild, there is no conclusive evidence that hybrids are reproductively active. Many authors have tentitively identified post F1 cyprinid hybrids in the wild (Cross; 1978; Fitzmuarice 1981; Fitzmuarice 1984; Mulrooney & Fahy, 1985), and such occurrences which may lead to populations with complex inter-bred ancestry (Wheeler, 1976; Burrough, 1981). By contrast other studies have concluded that hybrids are probably sterile in terms of the absence of spawning activity (Pepin *et al.*, 1970; Brassington and Ferguson, 1976; Pethon, 1978; Cowx, 1983). The results presented here suggest that while the F1 female roach/common bream hybrids are fertile and able to produce viable gametes, this does not necessarily imply that post-F1 hybridization will occur in wild populations (Section 5.4.2).

The results contradict the findings of Nikolukin (1946) regarding the success of the crosses of the female F1 roach/common bream hybrid back-crossed with bream and triple-crossed with rudd. While Nikolukin (1946) found that the embryos of these crosses were in poor condition with very few surviving to become normal larvae, the experimental crosses performed here produced high proportions of healthy embryos and larvae. The reasons for the differences between the success of these crosses may lie in the method of cross-fertilization and/or embryo maintenance. Unfortunately, these methods are not detailed by Nikolukin (1946) and therefore it is difficult to explain the apparent differences in the results. The success of the triple-crosses highlights the need to understand the spawning behaviour of hybrids in the wild because multi-species hybrid complexes will present problems to fishery managers that are both difficult to detect and resolve. Indeed, at present nothing is known about the spawning behaviour of hybrids in the wild.

2.4.5 Phylogenetic studies

Most studies of cyprinid taxonomy and phylogeny are based on morphological, physiological and geographical considerations (e.g. Bonaparte, 1846; Nikolsky, 1954; Howes, 1981; Chen *et al.*, 1984). Nevertheless, cross-fertilization data may be of great value in phylogenetic studies (Hubbs, 1970; Hester, 1970). As the taxa which are crossed become more distantly related, genetic similarity of the gametes decreases leading to their incompatibility (Soule, 1967). Hence, information on gamete compatibility may be of importance to phylogenetic studies in particular circumstances, because taxa which are more closely related genetically are those which are most likely to hybridize (McAllister & Coad, 1978).

According to Chen *et al.* (1984) and Howes (1991), the species of Cyprinidae involved in the present study belong to three sub-families;

- Leuciscinae: chub, common bream, roach, rudd and F1 roach/common bream hybrid;
- Cyprininae: goldfish;
- Barbinae: barbel.

These results, show that while cross-fertilization was successful between species within Leuciscinae, there was no success in outbreeding of taxa beyond the sub-family level. Some authors have suggested that if two species are able to produce viable offspring they should be included in the same genus (Dubois, 1981; Plateaux, 1981). However, there are many examples of hybidization between species belonging to different genera among Cyprinidae in both British and European waters (Kanno, 1968; Wheeler, 1978; Bianco 1982; Blatchuta & Witkowski, 1984; Crivelli & Dupont 1987). Furthermore, the reviews of cyprinid hybridization by Ryabov (1981a; 1981b), and the more recent study by Burkhead & Williams (1991), suggest that cyprinid species are able to outbreed beyond the sub-family level. If such data are incorporated into phylogenetic studies a reassessment of the systematics may be necessary.

There appears to be no definitive rules regarding cyprinid taxonomy and the potential for cross-fertilization. If this problem is to be resolved a rigorous scientific investigation of gamete compatibilities is required. This would include examinations using cryogenic techniques to determine the species, which are separated by spawning time and geographical location, which have compatible gametes. This needs to be supported by work on morphology, physiology, genetics, cytology and geographic distribution patterns because gamete compatibility is clearly only one aspect to be considered in phylogenetic studied.

2.4.6 Potential impacts

The genetic compatibility of the gametes of these species illustrates the potential threat to the integrity of many cyprinid species because of the high frequency of hybridization of sympatric taxa (Schwartz, 1972; 1981). Many of these species are young in terms of evolutionary history and exhibit great plasticity in their spawning behaviour and habitat (Holcik & Hruska, 1966). In comparison to gamete compatibility through breeding experiments, Pepin *et al.* (1970) concluded that those species which had similar spawning habits (i.e. the weakest pre-spawning isolating mechanisms), would have the highest probability of hybridization in wild populations. The vulnerability of species integrity must be considered further since many cyprinid species occupy niches in the still waters of lakes and slow flowing waters of lowland rivers. Both these habitats are greatly influenced by human activities such as pollution and habitat degradation (Cowx *et al.,* 1993), which are factors that have been recognised as enhancing hybridization in fishes (Hubbs, 1955). Consequenty, to elucidate the factors enhancing hybridization, further work is required which identifies the causes of this phenomenon (Weisel, 1954; Hubbs 1955; Campton, 1987).

CHAPTER THREE
IDENTIFICATION OF PURE-BRED AND HYBRID CROSSES USING MERISTIC CHARACTERISTICS AND PHARYNGEAL BONE FEATURES

3.1. INTRODUCTION

3.1.1 The assumptions of identifying hybrids

The need to characterise cyprinid hybrids with known ancestry has been highlighted by a number of authors (Burrough, 1981; Cowx, 1983; Wood & Jordan, 1987). At present there is a great deal of literature which tentatively proposes the putative parent species of a small number of hybrid individuals (Child & Solomon, 1977; Wheeler, 1978; Wheeler & Easton, 1978; Swinney & Coles, 1982). In all these examples it is assumed that the hybrids in question are of the F1 status. Furthermore, it is also assumed that the characteristics of the F1 hybrid are intermediate between those of their parent species. These assumptions must be verified, since F1 hybrids are known to be able to produce gametes which are compatible with their parent species (Wood & Jordan, 1987; Chapter 2) and, although there is no conclusive evidence, some authors have proposed that complex hybridization maybe occurring in natural waters (Wheeler, 1976; Burrough, 1981). Compounded upon these uncertainties, it is suggested that the expression of the genes relating to appearance of a hybrid are dominated by the maternal parent species (Witkowski & Blachuta, 1980; Collares-Pereira & Coelho, 1983; Blachuta & Witkowski, 1984). However, these claims have still to be verified from either controlled breeding experiments or appropriate genetic analysis of field specimens.

3.1.2 Aims and objectives

Despite the considerable number of hybrids that have been recognised from fresh waters in the British Isles, there does not appear to have been any attempt to verify their identity from controlled breeding experiments. To examine these aspects of hybrid appearance the progeny of some of the crosses of the experimental breeding programme (Chapter 2), in which parental ancestry is known, were examined and analysed to distinguish between:

- F1 hybrids and their parent species;
- reciprocal F1 hybrid crosses;
- F1 hybrids, backcrossed F2 hybrids and their parental species.

The results were used to evaluate the influence of parental genotype on the phenotypic expression of meristic and pharyngeal bone characters of hybrid fish.

3.2. MATERIALS AND METHODS

3.2.1 Maintenance of progeny

The progeny of the experimental breeding programme were reared in 15-l plastic tanks. The progeny produced from each cross were placed into separate tanks. The tanks were aerated and maintained at a temperature between 15°C and 18°C. Undergravel filters maintained the water quality in each tank. After approximately 20 days the progeny were transferred from the RFL at Brampton to the aquarium facilities of the Department of Applied Biology at the University of Hull. Here they were maintained in 60-l glass tanks at a temperature of 18°C to 22°C. Water quality within these tanks was again maintained by undergravel filtration.

3.2.2 Diet of progeny

The progeny were fed on a boiled chicken egg yolk suspension until they were able to feed on dried trout and carp feeds. This period lasted between 3 to 5 days. In the first 8 weeks this diet was supplemented with live *Artemia* sp. Thereafter, the diet was supplemented occasionally with *Artemia* sp. and live *Daphnia* sp.

3.2.3 Data collection

When the progeny were approximately 18 months old, and fully scaled, approximately 25 fish from each cross were killed in benzocaine (1:10 000 dissolved in acetone). The fork-length of the fish at this time varied between 5cm and 8cm. Five meristic characters were recorded from the progeny of each of the crosses of the experimental breeding programme (Table 3.1). These characters were counted using a blunt seeker under a low power microscope (x4). The fish had stunted body forms because they were reared in confined conditions, and therefore morphometric measurements were not considered suitable for identification purposes.

Meristic data were entered into a LOTUS 123 spreadsheet for collation and manipulation. For the purposes of statistical analyses the progeny of the cross-fertilization programme were classed into four groups according to the species crossed

Table 3.1 Meristic characters recorded from the progeny of the experimental breeding programme.

Meristic feature	Abbreviation
Lateral line scale counts	LLS
Dorsal fin ray counts	DFR
Anal fin ray counts	AFR
Dorsal fin to lateral line scale counts	DLS
Anal fin to lateral line scale counts	ALS

and their respective hybrids (Table 3.2). Statistical analyses were performed separately on each of these four groupings using the statistical software package SPSS/PC+.

3.2.4 Analysis of variance

The meristic characteristics recorded from each of the crosses were compared with each of the other crosses within the same progeny group (Table 3.2), using the SPSS/PC+ sub-program ONEWAY. Statistically significant differences between the means of each of the meristic characteristics, for each progeny cross, were compared using SCHEFFE (0.05) *a posteriori* contrasts. A Bartlett's Box probability was calculated in each case to check that variances were homogenous.

Progeny groups 1, 2 and 3, involved comparisons between the progeny of four crosses. These consisted of two pure-bred species crosses and both their reciprocal inter-species hybrid crosses (Table 3.2). For the analysis of crosses in group 4, comparisons were made between the two pure-bred species, the two back-crossed F2 hybrid crosses and a single F1 hybrid group which was made up of the combined meristic data from each of the reciprocal F1 crosses.

3.2.5 Discriminant analysis

Discriminant analysis is a multivariate statistical technique which optimises the separation of pre-determined classes. In the cases analysed here, these classes are the different progeny crosses within a progeny grouping (Table 3.2). This is done on the basis of the differences in the measured variables between each of the crosses (i.e. the meristic characteristics). The technique uses linear combinations of the values of these meristic characteristics to maximise the differences between the progeny classes, but minimise the differences within the same progeny class. The linear combinations form equations called 'Discriminant Functions.' These Discriminant Functions may then be used to predict the identity of fish, on the basis of its meristic characteristics, in cases when the ancestry is not certain.

The SPSS/PC+ sub-program DISCRIMINANT was performed on the meristic characteristics of each of the four progeny groups (Table 3.2). In each of the four cases the option to select only a single Discriminant Function equation was taken. In all four progeny groups both of the reciprocal hybrid crosses were treated as a single F1 hybrid cross. Therefore, in progeny groups 1,2 & 3 the Discriminant Function equations had to distinguish between three crosses and group 4 had to discriminate between five crosses.

Table 3.2 Progeny groups used for statistical analysis of meristic data.

Progeny group	Female parent	Male parent	Cross type	Cross code
Group 1	common bream	common bream	pure-bred	A
	common bream	chub	F1 hybrid	B
	chub	common bream	F1 hybrid	C
	chub	chub	pure-bred	D
Group 2	common bream	common bream	pure-bred	A
	common bream	roach	F1 hybrid	B
	roach	common bream	F1 hybrid	C
	roach	roach	pure-bred	D
Group 3	chub	chub	pure-bred	A
	chub	roach	F1 hybrid	B
	roach	chub	F1 hybrid	C
	roach	roach	pure-bred	D
Group 4	common bream	common bream	pure-bred	A
	RBH F1	common bream	F2 backcross	B
	common bream	roach	F1 hybrid	C
	roach	common bream	F1 hybrid	C
	RBH F1	roach	F2 backcross	D
	roach	roach	pure-bred	E

(RBH F1 = Presumed F1 roach/common bream hybrid).

All data were $\log_{10}$ transformed, to ensure homogeneity of variances, before they were interrogated with DISCRIMINANT. The success of the function equations were then assessed by discriminating between the crosses in each group using frequency histograms.

3.2.6 Pharyngeal bones

The pharyngeal bones were removed from progeny by dissection, placed into 15-ml labelled vials and were steamed for 15 minutes. Excess tissue was removed from the bones with an artist's brush. They were then cleaned, dried and stored until required for examination at a later date. The bones were examined under low power microscopy (x4) to record tooth formulation, bone structure (the length of the descending limb or *pars ventralis*), pectination and crenulation.

3.3 RESULTS

3.3.1 Analysis of group 1 data (common bream/chub)

Descriptive statistics

The summary of the meristic data from the progeny of the crosses in Group 1 showed that there were differences in the mean values of the meristic features recorded between pure-bred chub, pure-bred common bream and their F1 hybrids (Table 3.3). However, considerable overlaps were apparent between the ranges of some of the meristic features between the F1 hybrid and pure-bred progeny crosses (eg. DFR).

The means of the meristic features for the progeny of both the F1 hybrid crosses were found to be intermediate between those of the pure-bred common bream and chub. In addition, the observed mean values of each of the meristic features were found to be similar for both of the reciprocal F1 hybrid crosses. There were also considerable overlaps in the ranges of each of the meristic characters between the reciprocal F1 hybrid crosses (Table 3.3).

Analysis of variance

Statistical analyses of the differences using ONEWAY (Table 3.4) and Scheffe's test showed that there were statistically significant differences ($p < 0.05$), for the meristic characteristics AFR, LLS, DLS and ALS, between the following crosses within this progeny group (Table 3.5):

Table 3.3 Summary of meristic data for Group 1 progeny crosses (common bream, chub and their hybrids).

Meristic	Cross	Mean	95% C.L.	Range	N
AFR	Common bream	27.3	0.55	24-29	24
	Common bream/chub	14.6	0.31	13-16	24
	Chub/common bream	14.4	0.27	14-16	19
	Chub/	8.1	0.28	7-9	22
DFR	Common bream	8.5	0.20	8-9	24
	Common bream/chub	8.5	0.20	8-9	24
	Chub/common bream	8.5	0.23	8-9	19
	Chub	8.7	0.19	8-9	22
LLS	Common bream	57.1	0.56	55-60	24
	Common bream/chub	49.8	0.57	47-52	24
	Chub/common bream	49.6	0.67	48-52	19
	Chub	44.6	0.33	43-46	22
DLS	Common bream	13.3	0.30	12-14	24
	Common bream/chub	10.2	0.17	10-11	24
	Chub/common bream	10.3	0.26	9-11	19
	Chub	7.5	0.21	7-8	22
ALS	Common bream	7.6	0.28	6-9	24
	Common bream/chub	5.5	0.20	5-6	24
	Chub/common bream	5.6	0.23	5-6	19
	Chub	3.6	0.20	3-4	22

Table 3.4 ONEWAY output for Group 1 progeny comparisons (common bream, chub and their hybrids).

Meristic	Source	DF	Sum sq.	MSS	F	P
AFR	Between	3	4519.9	1506.7	1758.6	<0.0001
	Within	85	72.8	0.9		
	Total	88	4592.7			
DFR	Between	3	0.96	0.32	1.3	=0.28
	Within	85	21.1	0.25		
	Total	88	22.0			
LLS	Between	3	1846.4	615.5	359.1	<0.0001
	Within	85	145.7	1.7		
	Total	88	1992.1			
DLS	Between	3	380.9	127.0	384.6	<0.0001
	Within	85	28.1	33		
	Total	88	409.0			
ALS	Between	3	183.7	61.2	190.6	<0.0001
	Within	85	27.3	0.3		
	Total	88	211.0			

Table 3.5 Comparisons of mean values of group 1 progeny: Underlined means are not significantly different.

Meristic	Cross codes			
	A	B	C	D
AFR	27.3	14.6	14.4	8.1
DFR	8.5	8.5	8.5	8.7
LLS	57.1	49.8	49.6	44.6
DLS	13.3	10.2	10.3	7.5
ALS	7.6	5.5	5.6	3.6

(see Table 3.2 for cross codes)

- common bream and chub;
- common bream and common bream/chub hybrid;
- common bream and chub/common bream hybrid;
- chub and chub/common bream hybrid;
- chub and common bream/chub hybrid.

However, statistically significant differences were not observed for the DFR meristic character among these crosses ($p = 0.28$). In addition, statistically significant differences were not observed between the reciprocal chub/common bream hybrid and common bream/chub hybrid crosses for any of the meristic features recorded.

Discriminant analysis

Further analysis with the single discriminant function equation differentiated between common bream, chub and the F1 hybrids (Equation 3.1; Figure 3.1). The features of greatest importance in discriminating between chub and common bream and their hybrids were the number of lateral line scales (LLS) and the number of rays in the anal fin (AFR).

Equation 3.1

$$D.F.\ 1 = 30.96\ AFR + 4.09\ DFR + 34.02\ LLS + 8.74\ DLS + 6.22\ ALS - 111.33$$

In this analysis, the reciprocal F1 hybrid crosses were grouped together because the analysis of variance had not revealed any significant differences between the meristic characters. For the purposes of Discriminant Analysis it was therefore assumed that the progeny of these crosses could be considered as a single type. The equation explained 99.63% of the variance amongst the groupings ($p. < 0.0001$) and was able to predict the correct group classification for each individual fish. Group means for each of the three groupings were -12.21 for chub, 11.95 for common bream and -0.42 for their reciprocal F1 hybrid group (Equation 3.1; Figure 3.1).

Pharyngeal bones

The pharyngeal bones from the chub had a shorter *pars ventralis* and a second row of teeth, were very different from those of common bream (Table 3.6; Plate 3.1). The observations made on the pharyngeal bones of both the F1 hybrid crosses showed their features to be intermediate between those of the pure-bred species. In both the reciprocal hybrid crosses the pharyngeal bones had a long *pars ventralis* and possessed a second, inner row of teeth. From these differences it was possible to distinguish between common bream, chub and their hybrids. However, the characteristics of the bones could not be used to distinguish between the two reciprocal F1 hybrid crosses.

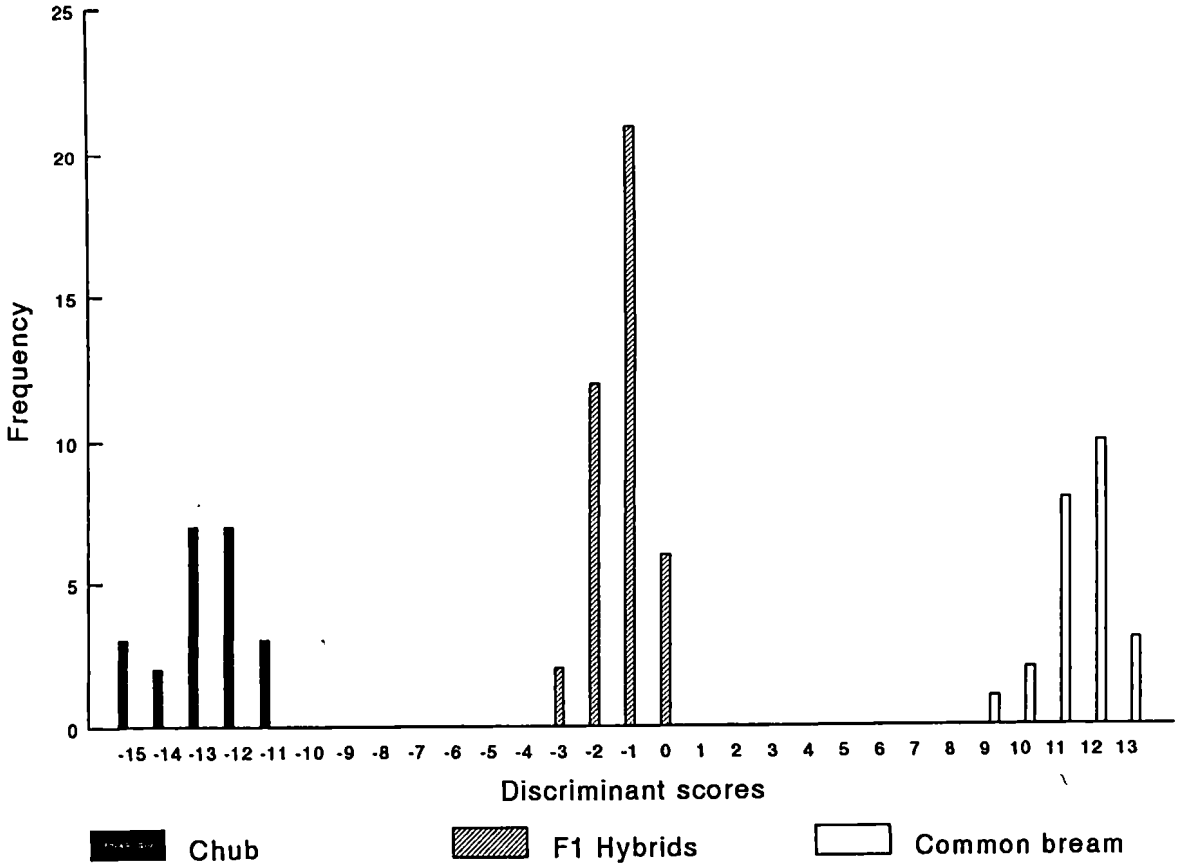


Figure 3.1 Discriminant analysis frequency distribution for chub, common bream and their F1 hybrids from the experimental breeding programme

Plate 3.1 **Pharyngeal bones of chub (top), common bream (bottom) and their hybrid (middle) from the experimental breeding programme.**

Table 3.6 Description of pharyngeal bone and tooth formulation of common bream, chub and their reciprocal F1 hybrids.

Fish	Formulation	Description
Common bream	5:5 6:5	Long ventral extension (*pars ventralis*) Teeth set high on bone and hooked All teeth have smooth masticatory surface Last tooth stocky and conical shape No teeth crenulate
Common bream/chub	2.5:5.0 1.5:5.1 1.5:5.2	Long *pars ventralis* Outer row teeth 1 & 2 hooked strongly Slight crenulation on exposed tooth surfaces Last tooth conical and stocky Inner row teeth hooked
Chub/common bream	1.5:5.1	Long *pars ventralis* All teeth strongly hooked First two in outer row especially hooked Fifth tooth conical shape Crenulations on exposed surface
Chub	1.5:5.1 2.5:5.2 1.5:5.2	Shorter *pars ventralis* Outer row teeth hooked First two hooked strongly Fifth tooth conical and stocky Crenulations on exposed surfaces

3.3.2 Analysis of group 2 data (roach/common bream)

Descriptive statistics

There were differences in the mean values of the meristic features recorded between pure-bred common bream, pure-bred roach and their F1 hybrids (Table 3.7). The means of the meristic characteristics of both of the reciprocal F1 hybrid crosses were intermediate between those observed for pure-bred common bream and roach progeny. Distinct ranges were noted between the pure-bred species and the F1 hybrids for the AFR and LLS meristic features. However, there were overlaps between the ranges of the recorded features, between hybrids and the pure-bred crosses for DLS and LLS (Table 3.7).

The mean values were similar for the progeny of both the reciprocal F1 hybrid crosses and considerable overlaps were observed between the ranges of the meristic characters of the hybrid crosses (Table 3.7). The meristic character DFR was not recorded for these crosses.

Analysis of variance

The analysis of variance and Scheffe's test showed statistically significant differences for the meristic characteristics AFR, LLS, DLS and ALS ($p < 0.05$), between the following crosses within this group (Table 3.8; Table 3.9):

- common bream and roach;
- common bream and common bream/roach hybrid;
- common bream and roach/common bream hybrid;
- roach and roach/common bream hybrid;
- roach and common bream/roach hybrid.

However, Scheffe's test was not able to show statistically significant differences ($p < 0.05$), between the roach/common bream hybrid and common bream/roach hybrid crosses for any of the four meristic features recorded.

Discriminant analysis

The single discriminant function, which was derived from the analysis, used to differentiate between common bream, roach and the F1 hybrids is given in Equation 3.2; Figure 3.2). The most important features which discriminated between the groups were the number of lateral line scales (LLS) and the number of fins in the anal fin (AFR). The reciprocal F1 hybrid crosses were grouped together because the analysis of variance did not identify significant differences in the means of any of the recorded

Table 3.7 Summary of meristic data for Group 2 progeny crosses (common bream, roach and their F1 hybrids).

Meristic	Cross	Mean	95% C.L.	Range	N
AFR	Common bream	27.3	0.55	25-29	24
	Common bream/roach	17.1	0.36	16-19	25
	Roach/common bream	16.7	0.29	16-18	23
	Roach	10.5	0.27	9-12	24
LLS	Common bream	57.1	0.56	56-60	24
	Common bream/roach	49.6	0.52	48-52	25
	Roach/common bream	49.4	0.52	48-52	23
	Roach	42.6	0.48	40-44	24
DLS	Common bream	13.3	0.30	12-14	24
	Common bream/roach	10.8	0.21	10-12	25
	Roach/common bream	10.5	0.24	10-12	23
	Roach	7.8	0.24	7-9	24
ALS	Common bream	7.6	0.28	6-9	24
	Common bream/roach	5.8	0.20	5-7	25
	Roach/common bream	5.7	0.18	5-6	23
	Roach	4.1	0.14	4-5	24

Table 3.8 ONEWAY output for Group 2 progeny comparisons (common bream, roach and their hybrids).

Meristic	Source	DF	Sum sq.	MSS	F	P
AFR	Between	3	3494.5	1164.8	1269.3	<0.0001
	Within	92	84.4	0.92		
	Total	95	3578.9			
LLS	Between	3	2526.2	842.0	497.7	<0.0001
	Within	92	155.7	1.7		
	Total	95	2681.9			
DLS	Between	3	343.3	114.4	207.6	<0.0001
	Within	92	50.7	0.6		
	Total	95	393.9			
ALS	Between	3	147.4	49.1	179.0	<0.0001
	Within	92	25.3	0.3		
	Total	95	172.7			

Table 3.9 Comparisons of mean values of group 2 progeny: Underlined means are not significantly different.

Meristic	Cross codes			
	A	B	C	D
AFR	27.3	17.1	16.7	10.5
LLS	57.1	49.6	49.4	42.6
DLS	13.3	10.8	10.5	7.8
ALS	7.6	5.8	5.7	4.1

(see Table 3.2 for cross codes)

meristic features between these two groups. The equation explained 99.73% of the variance (p. <0.0001) and was able to predict correct group classification in every case. The group means were 11.75 for common bream, -11.34 for roach and -0.20 for their reciprocal F1 hybrid group (Figure 3.2).

Equation 3.2

D.F. 1 = 31.76 AFR + 42.02 LLS + 9.92 DLS + 7.04 ALS - 129.99

Pharyngeal bones

Roach, common bream and the F1 could be distinguished from each other using the pharyngeal bones (Table 3.10; Plate 3.2). The principal differences were identified as the length of the *pars ventralis*, which was longer in common bream, and the degree of "stockiness" of the bone, which was a feature of roach. The bones of both of the F1 hybrid crosses were intermediate between the pure-bred crosses for both of these characteristics. The bones from the reciprocal F1 hybrid roach/common bream crosses were identical in appearance. Hence, it was not possible to identify features which could be used to distinguish between them.

3.3.3 Analysis of group 3 data (roach/chub)

Descriptive statistics

The mean values for the meristic features recorded for pure-bred chub, pure-bred roach and their hybrids were all similar (Table 3.11). Furthermore, although the mean values of the hybrids were found to be intermediate in most cases the roach/chub hybrid exhibited higher mean values for the DFR and DLS meristic counts than for the pure-bred crosses. There were also considerable overlaps, for all crosses within this progeny group, of ranges for all the meristic features that were recorded (Table 3.11).

Analysis of variance

Further analyses of these data using ONEWAY and Scheffe's test found that statistically significant differences (p <0.05), were only found for the meristic characteristic AFR, between the following crosses within this group (Table 3.12; Table 3.13):

- roach and chub;
- roach and roach/chub hybrid;
- roach and chub/roach hybrid;
- chub and chub/roach hybrid;
- chub and roach/chub hybrid.

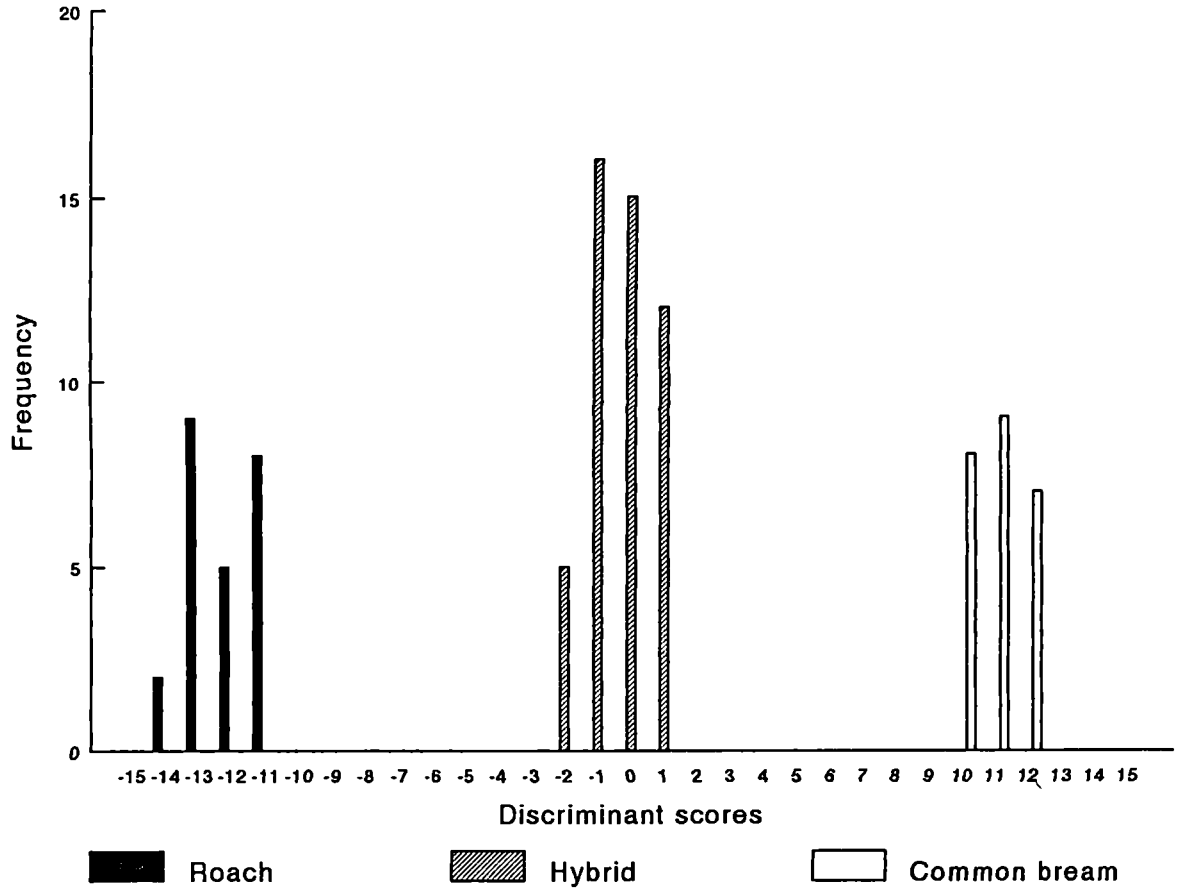


Figure 3.2 Discriminant analysis frequency distribution for roach, common bream and their F1 hybrids from the experimental breeding programme

Plate 3.2 **Pharyngeal bones of roach (top), common bream (bottom) and their F1 hybrid (middle) from the experimental breeding programme.**

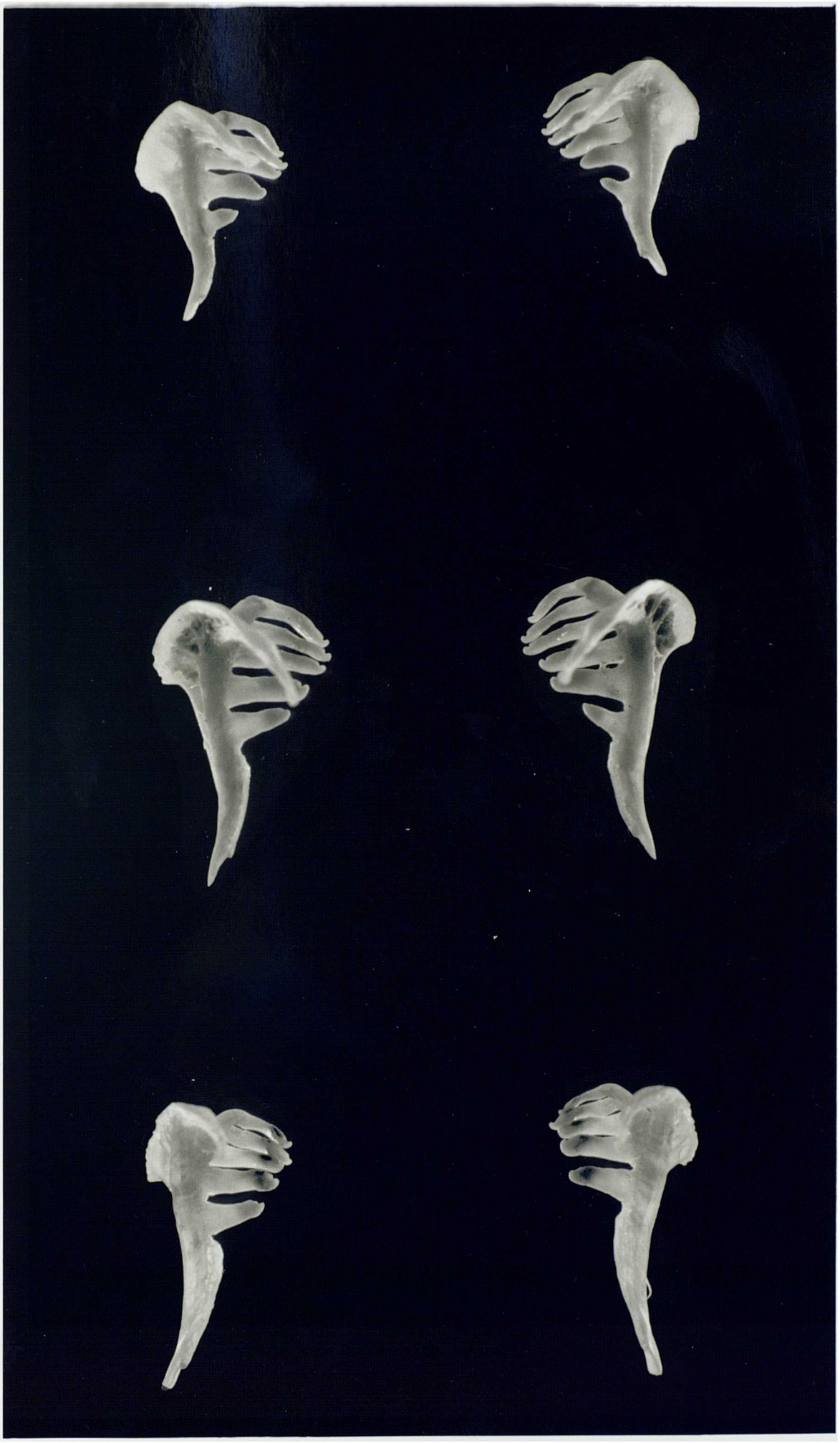


Table 3.10 Description of pharyngeal bone and tooth formulation of common bream, roach and their reciprocal F1 hybrids.

Fish	Formulation	Description
Common bream	5:5	Long ventral extension (*pars ventralis*) Teeth set high on bone and hooked All teeth have smooth masticatory surface Last tooth stocky and conical shape No teeth crenulate
Common bream/roach	6:5 5:5	Bones similar to roach (stocky) Intermediate *pars ventralis* Teeth 1 & 2 smooth masticatory surface Teeth 3,4 & 5 slightly hooked Fifth tooth conical
Roach/common bream	6:5 5:5	Bones similar to roach (stocky) Intermediate *pars ventralis* Teeth 1 & 2 smooth masticatory surface Teeth 3,4 & 5 slightly hooked Fifth tooth conical
Roach	6:5 5:5	Bones stocky Short *pars ventralis* First four bones hooked Smooth masticatory surface Last tooth conical

Table 3.11 Summary of meristic data for Group 3 progeny crosses (roach, chub and their hybrids).

Meristic	Cross	Mean	95% C.L.	Range	N
AFR	Chub	8.1	0.28	7-9	22
	Chub/roach	9.7	0.25	8-10	20
	Roach/chub	9.3	0.21	9-10	20
	Roach	10.5	0.26	9-12	24
DFR	Chub	8.7	0.19	8-9	22
	Chub/roach	8.7	0.29	8-10	20
	Roach/chub	9.1	0.17	8-10	20
	Roach	8.9	0.22	8-10	24
LLS	Chub	44.6	0.33	43-46	22
	Chub/roach	43.7	0.35	42-45	20
	Roach/chub	43.2	0.41	42-45	20
	Roach	42.6	0.48	40-44	24
DLS	Chub	7.5	0.21	7-8	22
	Chub/roach	7.7	0.21	7-8	20
	Roach/chub	7.9	0.21	7-9	20
	Roach	7.8	0.24	7-9	24
ALS	Chub	3.6	0.20	3-4	22
	Chub/roach	3.7	0.10	3-4	20
	Roach/chub	3.9	0.21	3-4	20
	Roach	4.1	0.14	4-5	24

Table 3.12 ONEWAY output for Group 3 progeny comparisons (roach, chub and their hybrids).

Meristic	Source	DF	Sum sq.	MSS	F	P
AFR	Between	3	66.6	22.2	60.3	<0.0001
	Within	82	30.2	0.4		
	Total	85	96.8			
DFR	Between	3	1.9	0.6	2.0	=0.0900
	Within	82	22.5	0.3		
	Total	85	24.3			
LLS	Between	3	47.4	15.8	17.1	<0.0001
	Within	82	76.0	9.3		
	Total	85	123.4			
DLS	Between	3	1.5	0.5	1.8	=0.1400
	Within	82	22.2	0.3		
	Total	85	23.7			
ALS	Between	3	3.5	1.2	7.4	=0.0002
	Within	82	12.9	0.2		
	Total	85	16.3			

Table 3.13 Comparisons of mean values of group 3 progeny: Underlined means are not significantly different.

Meristic	Cross codes			
	A	B	C	D
AFR	8.1	9.7	9.3	10.5
DFR	8.7	8.7	9.1	8.9
LLS	44.6	43.7	43.2	42.6
DLS	7.5	7.7	7.9	7.8
ALS	3.6	3.7	3.9	4.1

(see Table 3.2 for cross codes)

Similar significant differences were observed for the LLS meristic characteristic, with exception of the roach and roach/chub hybrid comparison which was not found to be significant ($p < 0.05$). For the meristic characteristic ALS, significant differences were only found between the pure-bred roach and roach/chub hybrid and pure-bred roach and pure-bred chub ($p < 0.05$).

Discriminant analysis

The discriminant equation used to differentiate between common bream, chub and both their reciprocal F1 hybrids, is given below (Equation 3.3; Figure 3.3). The features which were of greatest importance in the Discriminant analysis were the lateral line scales (LLS) and the anal fin ray counts (AFR). This equation explained 99.13% of the variance (p. <0.0001). However, the equation was only able to predict correct group classification 76.7% of cases. Group means were 1.96 for chub, -2.48 for common common bream and 0.18 for their reciprocal F1 hybrid group (Fig. 3.3).

Equation 3.3

$$D.F.\ 1 = 29.50\ AFR + 1.55\ DFR + 38.04\ LLS + 3.13\ DLS + 7.24\ ALS + 30.77.$$

Pharyngeal bones

The principal differences between the bones of roach and chub are that chub bones exhibit a second, inner row of teeth and that they are crenulate (Table 3.14; Plate 3.3). Examination of the pharyngeal bones of both the F1 hybrid crosses showed that they were intermediate in character between the pure-bred crosses. Although the bones of the hybrids were similar in appearance to roach they had some crenulations and most had an inner row of teeth. It was possible to distinguish between roach, chub and hybrid by the pharyngeal bones, but the bones could not be used to identify differences between the reciprocal hybrid crosses.

3.3.4 Analysis of group 4 data (common bream/roach/hybrids)

As it was not possible to distinguish differences between the reciprocal F1 roach/common bream and common bream/roach hybrid crosses (Section 3.2.2), these progeny were treated as a single F1 hybrid cross for further analyses with F2 backcrossed hybrids and pure-bred progeny.

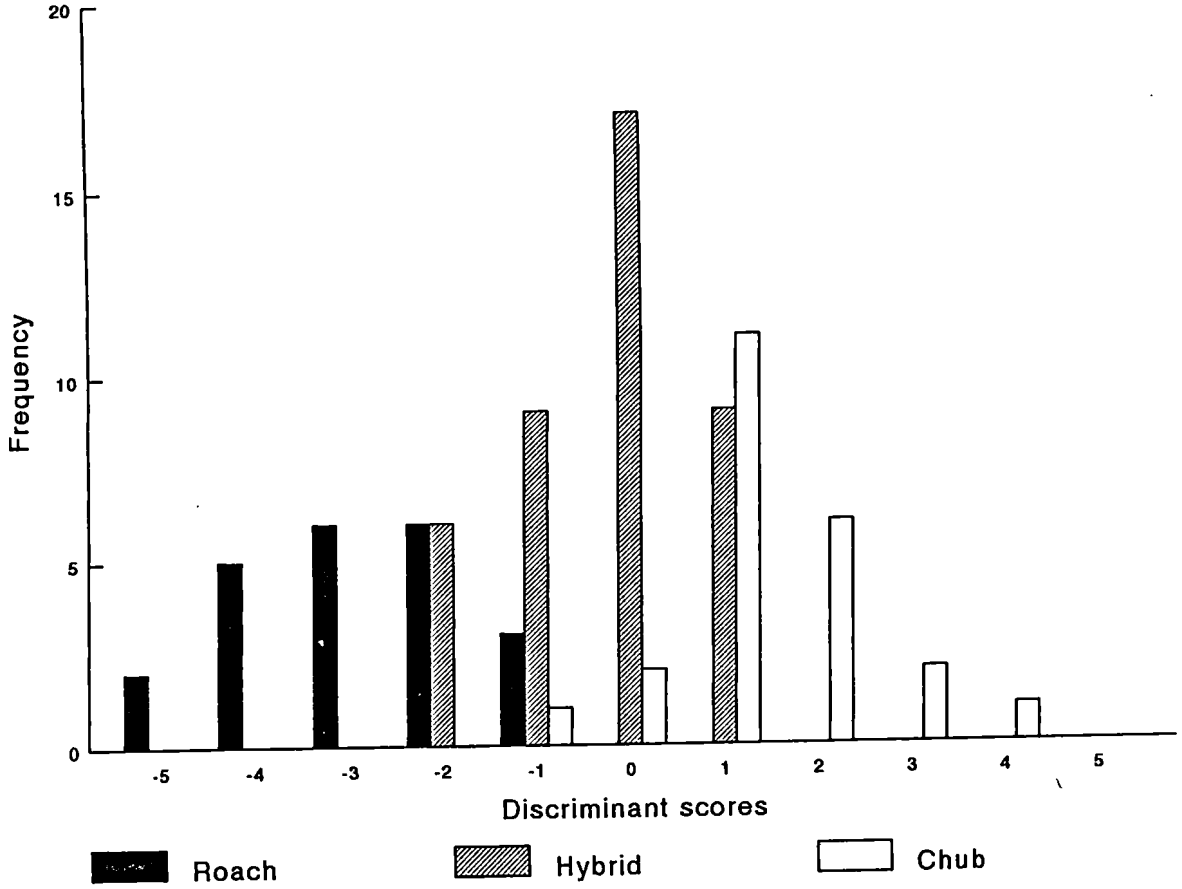


Figure 3.3 Discriminant analysis frequency distribution for roach, chub and their F1 hybrids from the experimental breeding programme

Plate 3.3 **Pharyngeal bones of roach (top), chub (bottom) and their hybrid (middle) from the experimental breeding programme.**

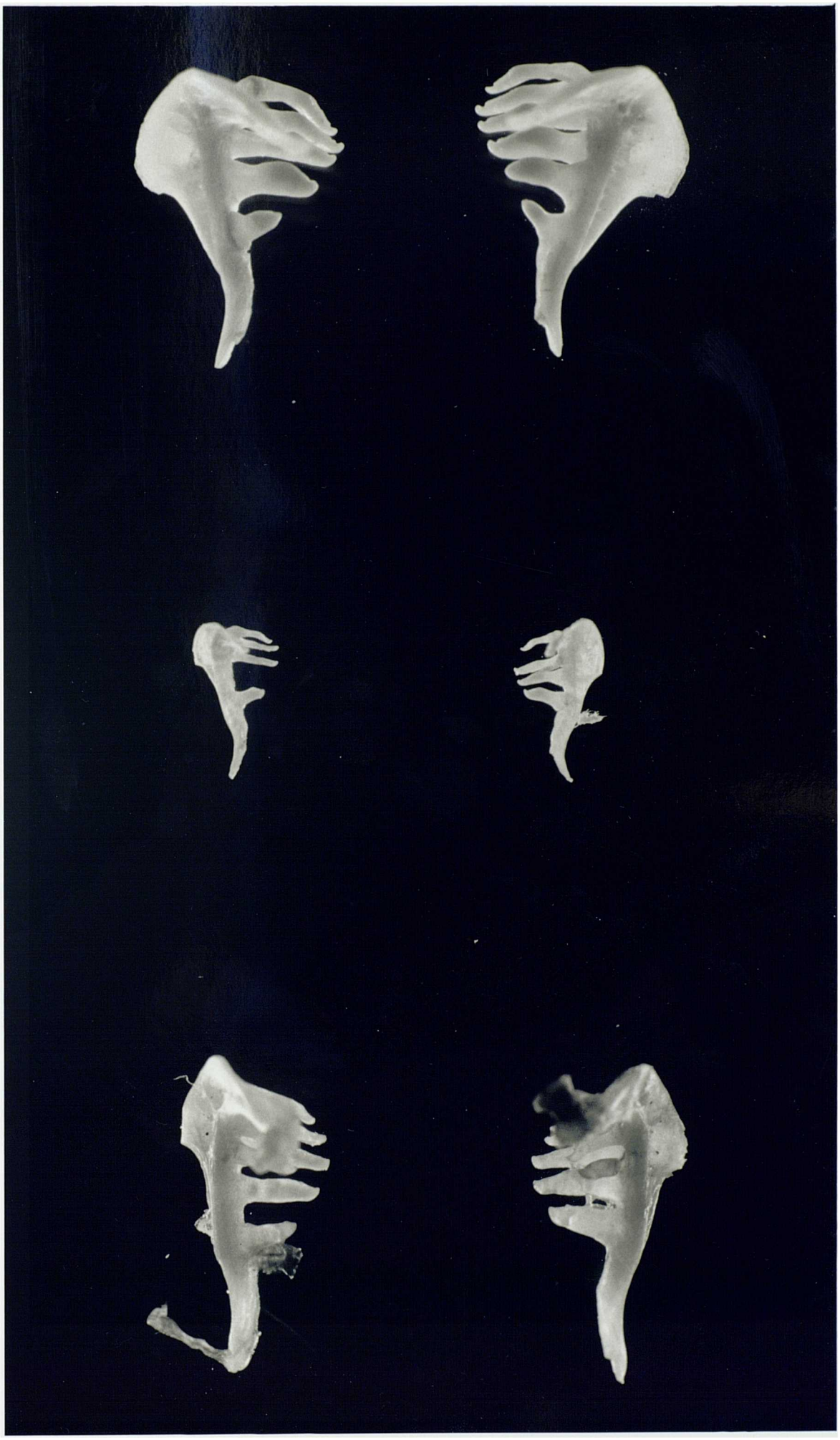


Table 3.14 Description of pharyngeal bone and tooth formulation of common bream, chub and their reciprocal F1 hybrids.

Fish	Formulation	Description
Roach	6:5 5:5	Bones stocky Short *pars ventralis* First four bones hooked Smooth masticatory surface Last tooth conical
Roach/chub	0.5:5.0 1.5:5.1	Similar to roach bones First two teeth are strongly hooked Fifth tooth stocky and conical Short *pars ventralis* Pectinate and crenulate
Chub/roach	0.5:5.0 0.5:5.1 1.5:5.1	Similar to roach bones First two teeth are strongly hooked Fifth tooth stocky and conical Short *pars ventralis* Pectinate and crenulate
Chub	1.5:5.1 2.5:5.2 1.5:5.2	Short *pars ventralis* Outer row teeth hooked First two hooked strongly Fifth tooth conical and stocky Crenulations on exposed surfaces

Descriptive statistics

The summary of the meristic data for the progeny in Group 4 indicated that there were different mean values for each cross for all the features observed (Table 3.15). In all cases the means of the F1 hybrids were intermediate of the pure-bred progeny. The means of the meristic counts for the F2 hybrid crosses, where the female F1 hybrid was backcrossed with common bream and roach, were intermediate between the F1 hybrid and pure-bred common bream, and the F1 hybrid and roach respectively. Overlaps were observed, between the crosses, in the ranges of all the the meristic features that were recorded (Table 3.15).

Analysis of variance

ONEWAY analysis of variance and Scheffe's test showed that there were statistically significant differences ($p < 0.05$), between all the meristic characters recorded (AFR, LLS, DLS and ALS)(Table 3.16). These were found between the following crosses within this progeny group (Table 3.17):

- Common bream and roach;
- Common bream and F1 hybrids;
- Common bream and F2 hybrids (F1 hybrid/common bream);
- Common bream and F2 hybrids (F1 hybrid/roach);
- Roach and F1 hybrids;
- Roach and F2 hybrids (F1 hybrid/common bream);
- Roach and F2 hybrids (F1 hybrid/roach);
- F2 hybrids (F1 hybrid/common bream) and F2 hybrids (F1 hybrid/roach);
- F2 hybrids (F1 hybrid/common bream) and F1 hybrids;
- F2 hybrids (F1 hybrid/roach) and F1 hybrids;

The meristic character DFR was not recorded among the crosses of this progeny group.

Discriminant Analysis

The single discriminant equation was able to differentiate between common bream, roach, the F1 hybrids and both the backcrossed F2 hybrid crosses within this progeny group (Equation 3.4; Figure 3.4). The most important features were the lateral line scales (LLS) and the anal fin ray counts (AFR). The equation explained 99.09% of the variance between the crosses ($p. < 0.0001$) and was able to predict correct group classification in every case. The group means for the crosses were 12.51 for common bream, -13.36 for roach, 0.18 for the F1 hybrids, 6.94 for the the F1 hybrid/common bream cross and -5.52 for the F1 hybrid/roach cross (Figure 3.4).

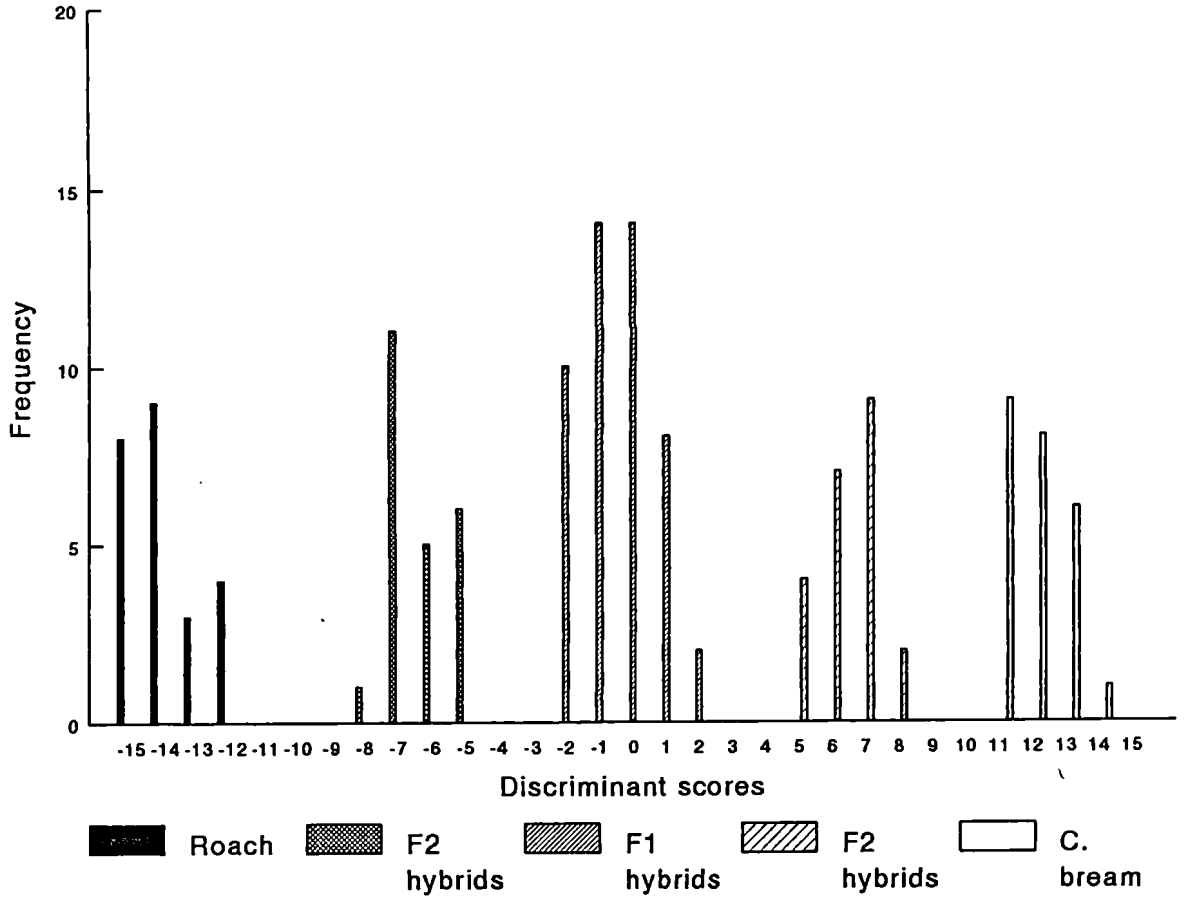


Figure 3.4 Discriminant analysis frequency distribution for roach, common bream, their F1 hybrids and their back-crossed F2 hybrids from the experimental breeding programme

Table 3.15 Summary of meristic data for Group 4 progeny crosses (common bream, roach, F1 hybrids and F2 hybrids).

Meristic	Cross	Mean	95% C.L.	Range	N
AFR	Common bream	27.3	0.55	25-29	24
	F1 hybrid/common bream	22.2	0.45	19-25	22
	F1 hybrids	16.9	0.24	16-19	48
	F1 hybrid/roach	13.7	0.23	12-16	23
	Roach	10.5	0.26	9-12	24
LLS	Common bream	57.1	0.56	55-60	24
	F1 hybrid/common bream	54.3	0.40	52-56	22
	F1 hybrids	49.5	0.36	48-52	48
	F1 hybrid/roach	46.0	0.23	44-48	23
	Roach	42.6	0.48	40-44	24
DLS	Common bream	13.3	0.30	12-14	24
	F1 hybrid/common bream	11.5	0.28	10-13	22
	F1 hybrids	10.7	0.16	10-12	48
	F1 hybrid/roach	8.8	0.27	8-10	23
	Roach	7.8	0.24	7-9	24
ALS	Common bream	7.6	0.28	6-9	24
	F1 hybrid/common bream	6.5	0.28	6-8	22
	F1 hybrids	5.8	0.14	5-7	48
	F1 hybrid/roach	5.7	0.22	5-7	23
	Roach	4.1	0.14	4-5	24

Table 3.16 ONEWAY output for Group 4 progeny comparisons (common bream, roach, F1 hybrids and F2 hybrids).

Meristic	Source	DF	Sum sq.	MSS	F	P
AFR	Between	4	4310.9	1077.7	1249.3	<0.0001
	Within	136	117.3	0.9		
	Total	140	4428.2			
LLS	Between	4	3307.6	826.9	565.3	<0.0001
	Within	136	198.9	1.5		
	Total	140	3506.6			
DLS	Between	4	445.9	111.5	274.7	<0.0001
	Within	136	55.2	0.4		
	Total	140				
ALS	Between	4	157.8	39.5	130.5	<0.0001
	Within	136	41.1	0.3		
	Total	140	198.9			

Table 3.17 Comparisons of mean values of group 4 progeny : Underlined means are not significantly different.

Meristic	Cross codes				
	A	B	C	D	E
AFR	27.3	22.2	16.9	13.7	10.5
LLS	57.1	54.3	49.5	46.0	42.6
DLS	13.3	11.7	10.7	8.8	7.8
ALS	7.6	6.5	5.8	5.7	4.1

(see Table 3.2 for cross codes)

Equation 3.4

D.F. 1 = 31.76 AFR + 42.02 LLS + 9.92 DLS + 7.04 ALS - 129.99

Pharyngeal bones

Differences between the pharyngeal bones of common bream, roach and the F1 hybrids have been commented upon previously (Section 3.2.2). The characteristics of the pharyngeal bones of both the F2 hybrid crosses appeared to be intermediate between those of the F1 hybrids and their respective pure-bred species (Table 3.18). However, apart from their apparent intermediacy it was not possible to identify features of these bones, in either of the backcrosses, which could distinguish them from the F1 hybrids.

3.4. DISCUSSION

3.4.1 Pure-bred progeny

The mean values and ranges observed for the meristic characteristics of the progeny of the pure-bred crosses (i.e. common bream, chub and roach), appeared to be similar to those which have been published previously for these species (Table 1.2). Similarly, the observations of the configurations and formulations made on the pharyngeal bones also conform to descriptions which have been noted by other authors (Table 1.2). Hence these data suggest that the parental broodstock used for the experimental cross-breeeding programme can be assumed to be pure-bred species (Chapter 2). Furthermore, the probable identity of the F1 hybrid progeny of the experimental breeding programme may also be confirmed.

3.4.2 F1 hybrid progeny

The meristic characteristics of the progeny of the F1 hybrid crosses were strictly intermediate between those of their parental species for the chub/common bream and roach/common bream crosses. Furthermore, this intermediacy facilitated the identification of the hybrids from their parent species using Discriminant Analysis. This was not the case for the roach/chub hybrid cross where the values of the meristic characters were similar for both the pure-bred species and their hybrids and therefore intermediacy could therefore not be identified using Discriminant Analysis. However, in the two former cases, these data provide evidence to suggest that hybrid appearance is intermediate between those of their parent species and that the phenotypic expression

Table 3.18 Description of pharyngeal bone and tooth formulation of common bream, roach, F1 hybrids and F2 hybrids.

Fish	Formulation	Description
Common bream	5:5	Long ventral extension (*pars ventralis*) Teeth set high on bone and hooked All teeth have smooth masticatory surface Last tooth stocky and conical shape No teeth crenulate
F2 hybrids (hybrid/common bream)	5:5	Intermediate between c. bream and F1 hybrid Similar to common bream except that the *Pars ventralis* is slightly shorter
F1 hybrids	6:5 5:5	Bones similar to roach (stocky) Intermediate *pars ventralis* Teeth 1 & 2 smooth masticatory surface Teeth 3,4 & 5 slightly hooked Fifth tooth conical
F1 hybrids (hybrid/roach)		Intermediate between roach and F1 hybrid Similar to roach except that the *Pars ventralis* is slightly longer
Roach	6:5 5:5	Bones stocky Short *pars ventralis* First four bones hooked Smooth masticatory surface Last tooth conical

of these characters are the result of the product of half of the genotypes of the parent species involved.

Meristic data for the roach/common bream hybrids are similar to values observed for this hybrid in natural populations (e.g, Cowx, 1983; Mulrooney & Fahy, 1985; Adams & Maitland, 1991), as are also those for roach/chub hybrids (Wheeler & Easton, 1978). However, Discriminant Analysis was not able to distinguish the hybrids from their parent species. Indeed, Wheeler & Easton (1978) concluded that external morphometric and meristic features could not enable separation of roach/chub hybrids from their parent species and that reliable identification had to be based upon pharyngeal bone morphology.

The pharyngeal bones of the common bream/roach and chub/roach F1 hybrid progeny were similar to the features recorded from the bones of these hybrid crosses from natural waters (e.g. Cowx, 1983 and Wheeler & Easton, 1978 respectively). However, the progeny of the common bream/chub cross cannot be compared with published accounts of their occurrence, because they have not been identified from natural waters. Nevertheless, the observations made on the pharyngeal bones also support the theory that their penotypic expression is the result of the combination of both parental genotypes. Furthermore, it was possible to distinguish between parental species and F1 hybrids, using the characteristics of the pharyngeal bones, in all three cases. This highlights the great value of such pharyngeal bone features and this has also been noted by a number of authors where they have been used successfully to identify hybrids in natural populations (Wheeler, 1969; Wheeler,1976; Wheeler & Easton, 1978; Wheeler, 1978; Burrough, 1981; Cowx 1983).

3.4.3 The influence of species-specific sexual ancestry

As mentioned above (Section 2.1.2) some authors have indicated that there may be evidence to suggest that the genome of the maternal parent may exert a greater influence in the overall appearance of hybrid offspring (Witkowski & Blachuta, 1980; Collares-Pereira & Coelho, 1983; Blachuta & Witkowski, 1984). However, these results could not distinguish between the reciprocal F1 hybrid crosses on the basis of the meristic data and pharyngeal bone configuration in any of the three examples. It must be emphasised that in the published examples where this aspect of the inheritance of hybrid appearance has been suggested (Witkowski & Blachuta, 1980; Collares-Pereira & Coelho, 1983; Blachuta & Witkowski, 1984), the conclusions regarding the role of maternal influence have been somewhat subjective. In all these studies the species-specific sexual ancestry of the hybrids in these natural waters was inferred and was not

assessed using suitable genetic techniques (i.e. restriction enzyme analysis of mitochondrial DNA). Hence, the conclusions of these studies were based upon information which was not reliable.

3.4.4 Identification of back-crossed hybrids of roach and common bream

Unfortunately, the features of the back-crossed F2 roach/common bream hybrids cannot be compared with similar data. This is because the characteristics of the meristic features and pharyngeal bones have not been verified fully either from controlled breeding experiments or natural populations. However, AFR counts for the F1 hybrid x roach backcrosses were comparable with those observed by Wood & Jordan (1987) for female roach/common bream hybrid x male roach (AFR = 12-16) and female roach x male roach/common bream hybrid (AFR = 12-15) crosses. Furthermore, the strict intermediacy of the meristic characters of the F2 hybrids, between those of the F1 hybrid and their respective parental backcross, appears to support the idea that the appearance of these fish is the product of half of the genotypes of both parental genomes.

The Discriminant Analysis was able to distinguish sucessfully between the parental species, their F1 hybrids and their F2 backcrosses in every case for the fish in progeny group 4. Although the analysis did not include data from progeny of F1 male roach/common bream backcrosses or F1 x F1 hybrids, the model provides an invaluable tool in the detection of post F1 hybridization in roach/common bream hybrid populations in the wild.

However, the applicability of the model is dependent upon the following two assumptions:

- F2 hybrids are more probably the result of a hybrid-parent backcross than a hybrid -hybrid cross (Stebbins, 1971);

- that the phenotype of a F2 backcross hybrid is independent of the parental ancestry of the fish, i.e. the progeny of a female F1 hybridx male parent backcross should have identical meristic features of the progeny of its reciprocal cross using a female parent x male F1 hybrid (Wood & Jordan, 1987).

The use of the pharyngeal bones requires further analytical work before they can be used as a reliable tool in the identification of post F1 cyprinid hybrids. For example, Cowx (1983) analysed quantitatively the features of pharyngeal bones using a $\log_{10}$

regression relationship of the length of the *pars ventralis* and the section housing the teeth of common bream, roach and their suspected hybrids from the River Exe. These analyses could be extended further to include F2 and backcrosses. However, the small size of the pharyngeal bones from the progeny of the experimental breeding programme limited their use for such analysis. Nevertheless, this does not preclude the development of this analysis in natural populations providing that they are supported by reliable genetic data.

3.4.5 Applicability of the multivariate approach to hybrid identification

Multivariate approaches have distinct advantages over univariate methods in the identification of hybrids. Among these data there are not only overlaps of the ranges of the features observed, but in some cases it was also difficult to find statistically significant differences between species and hybrids. However, by combining the differences of a number of features into overall scores for each fish, it was possible to eliminate some of the overlaps between hybrids and their parent species.

The earliest of such techniques was the hybrid index developed by Hubbs & Kuroma (1942). This model combines data to give overall values which range between 0 and 100. When the overall value for a fish lies between 30 and 70 this indicates that it is a probable hybrid (Hubbs *et al.,* 1943; Gilbert, 1978; Menzel, 1978; Mayhew, 1983). However, this method is not sufficiently robust to deal with hybrid identification in all circumstances. This is because it is dependent on the intermediacy of the measured characteristics of the hybrid between its parent species (Hubbs & Hubbs, 1947).

Many authors have used multivariate statistical techniques, such as Principal Componant Analysis (PCA), on morphometric and meristic data as tools for identifying hybridization in fishes (Smith, 1973; Neff & Smith, 1979; Butcher, 1980; Dowling & Moore, 1985). This method has advantages over the Hybrid Index because it is based on different mathematical principles and does not depend upon the intermediacy of the hybrid characters between those of their parent species. These methods have been applied to hybridization in natural populations (e.g. Crivelli & Dupont, 1987). However, this method alone can only provide circumstantial evidence of hybridization in natural populations and to achieve more reliable results these data need to be supported by evidence from breeding experiments (Burrough, 1981; Cowx, 1983; Wood & Jordan 1987) and/or genetic techniques (Ferguson, 1977; Ferguson 1980).

The method applied here, Discriminant Function Analysis (DFA), is a more powerful multivariate technique than PCA because the results can be used to predict hybrid

identification using the meristic data (Crivelli & Dupont, 1987). A principal requirement of such a model is that ancestry is known *a priori*, and therefore they are only suitable in a limited number of situations, i.e. either where data were taken from the progeny of breeding experiments or if morphological/meristic data are supported by genetic information for fish from natural populations (Chapter 4; Chapter 5; Chapter 6).

3.4.6 Limitations of these data

The applicability of these morphometric and meristic data to multivariate analysis of cyprinid hybrid studies is limited because;

- the analyses need to be extended to include data from other crosses;
- they are not suitable for discrimination in every case (i.e. roach/chub);
- it is possible that hybrids maybe the product of multiple crosses (Chapter 2).

This latter situation has not been found in natural populations. However, the gametes of F1 roach/common bream hybrids are known to be compatible with rudd and chub (Chapter 2). Under these circumstances identification using traditional methods may be impossible and hence the use of genetic techniques will be essential to discriminate between multiple-crossed hybrids.

The progeny analysed in this study, in all crosses, are the product of the gametes of just two individual fish and there is only a limited scope for genetic variation. As a result intra-specific and intra-hybrid variation in meristic and morphometric characteristics is limited. This aspect is critical because the use of morphometric and meristic characters in identification of species and hybrids has been questioned in a number of circumstances since it is known that environmental effects can influences morphology (Ali & Lindsey, 1974; MacGregor & MacCrimmon, 1977; Leary *et al.*, 1983a; Lyagina, 1985; Ferguson & Danzmann, 1987). Hence, it is essential that when these multivariate methods are applied to natural hybridization they must be supported by genetic information (Ferguson, 1977) and, where possible, environmental data.

CHAPTER FOUR
IDENTIFICATION OF HYBRIDS USING ENZYME ELECTROPHORESIS

4.1. INTRODUCTION

4.1.1 Limitations to meristic and morphometric data

There have been many reports of inter-specific hybrids amongst cyprinids in the British Isles based upon evidence from morphometric and meristic characteristics (Table 1.3). However, there are some fundamental limitations to the application of these methods of identification for these purposes (Ferguson, 1977; Chapter 3). For example, it is assumed that such data, for hybrids, will be intermediate between those of their putative parent species. However, the traits of the morphometric and meristic characteristics are polygenic i.e. the cumulative effect of a large number of genes. As such they may be influenced by environmental effects which may result in large amounts of intra-species and intra-hybrid variation amongst these types of characteristics (Ali & Lindsey, 1974; MacGregor & MacCrimmon, 1977; Lyagina, 1981; Angus & Schultz 1983; Libsovarsky & Ruban, 1985; Leary *et al.*, 1983a; Ferguson & Danzmann, 1987). These problems may become further complicated if the two parental species are similar in appearance e.g. roach and chub; roach and rudd. These factors may further limit the use of such characteristics in situations where complex and introgressive hybridization may be occuring (Wheeler, 1976; Burrough, 1981).

Therefore methods of identification are required which are independent of environmental influences, morphological similarities of the parental species and are able to ascertain whether either complex or introgressive hybridization is occurring. Enzyme electrophoresis examines the molecular structure of enzymes, as determined by genetics (Section 1.3.3; Section 3.4.6). Hence, such a method is independent of the limiting factors outlined above and is, therefore, probably a more suitable technique in the assessment of cyprinid hybridization.

4.1.2 Aims and objectives

Although the problem of cyprinid hybridization is a well known phenomenon in the British Isles, there have been no published studies which have ascertained genetic differences between pure-bred species and their hybrids from a controlled experimental breeding programme. Hence, enzyme electrophoresis was carried out upon the progeny of the crosses of the experimental breeding programme. The information gained from these investigations was used to examine the genetic differences between the following:

- parental species and their F1 hybrids;
- reciprocal F1 hybrid crosses (i.e. the influence of species-specific sexual ancestry);
- F1 hybrids, back-crossed F2 hybrids and their parental species.

4.2. MATERIALS AND METHODS

4.2.1 Tissue preparation

Six individuals of each cross were retained for enzyme electrophoresis after the analysis of meristic and pharyngeal bone features were complete (Chapter 3). Each individual was prepared by removing the head, tail, gut and scales. The remaining tissues were homogenised in 0.5ml 100mM Tris-HCl buffer, pH 7.4. When the sample and buffer were completely homogenised, the samples were centrifuged at 1000 x *g* for 5 minutes and stored at -80°C, in 1.5 ml Eppendorf vials, until they were required for enzyme electrophoresis. Enzyme electrophoresis was carried out according to two methods, these were either horizontal cellulose acetate gel (Section 4.2.3), or vertical polyacrylamide gels (Section 4.2.4).

4.2.2 Enzyme electrophoresis

Twenty enzyme systems were studied (Table 4.1). Most enzymes were examined using cellulose acetate procedures (Section 4.2.3). However, this method failed to resolve esterase and glutamate dehydrogenase which were examined using vertical polyacrylamide gel procedures (4.2.4).

4.2.3 Cellulose acetate

Enzyme electrophoresis using cellulose acetate gels was carried out according to the methods of Hebert & Beaton (1989). Cellulose acetate gels, supplied by Helena U.K. Ltd. (Cat. No. 3024), were prepared by soaking in Tris-Glycine tank buffer (25 mM tris, 200 mM glycine, pH 8.5) for 12 hours. Excess buffer was removed by blotting and samples were applied to the gels using an application kit supplied by Helena U.K. Ltd (Cat. Nos. 4090, 4094 & 4096). The application kit allowed 12 samples to be loaded onto one gel. In cases where enzyme activity was low sample loading was repeated 2-4 times. This ensured that sufficient enzyme was present to react with the substrate stains

Table 4.1 Enzyme systems examined using electrophoresis.

Enzyme	Code	E.C. No.	Structure
Adenylate kinase	AK	2.7.4.3	Monomer
Alcohol dehydrogenase	ADH	1.1.1.1	Dimer
Aspartate amino-transferase	AAT	2.6.1.1	Dimer
Creatine kinase	CK	2.7.3.2	Dimer
Esterase	EST	3.1.1.1	Monomer/dimer
Fumerate hydratase	FUM	4.2.1.2	Tetramer
Glutamate dehydrogenase	GDH	1.4.1.3	Complex
Glucose-6-phosphate dehydrogenase	G6PDH	1.1.1.49	Tetramer
Glycerol-3-phosphate dehydrogenase	GPDH	1.1.1.8	Dimer
Hexokinase	HEX	2.7.1.1	Monomer
Isocitrate dehydrogenase	IDH	1.1.1.42	Dimer/monomer
Lactate dehydrogenase	LDH	1.1.1.27	Tetramer
Malate dehydrogenase	MDH	1.1.1.37	Dimer
Malic enzyme	ME	1.1.1.40	Tetramer
Mannose phoshate isomerase	MPI	5.3.1.8	Monomer
Peptidase	PEP	3.4.11/13	Dimer/monomer
Phosphglucomutase	PGM	2.7.5.1	Monomer
6-Phosphogluconate dehydrogenase	6PGDH	1.1.1.44	Dimer
Phosphoglucose isomerase	PGI	5.3.1.9	Dimer
Sorbitol dehydrogenase	SDH	1.1.1.14	Tetramer

and so indicate the molecular mobility of the enzyme. Gels were then placed onto wicks which were soaked in Tris-Glycine tank buffer in a purpose built electrophoresis chamber. Enzyme electrophoresis was carried out at 200 volts and 2 mA at room temperature for 30 minutes.

4.2.4 Polyacrylamide procedures

Non-denaturing polyacrylamide gels and a discontiuous buffer system was used with the 'SE 250' gel unit supplied by Hoefer Scientific Instruments. Gel and buffer recipes were prepared according to Sambrook *et al.,* (1983) with the omission of Sodium Dodecyl Sulphate (SDS) using a polyacrylamide solution mix (Table 4.2). A 5% stacking gel (Table 4.3) and an 8% resolving gel (Table 4.4) were employed with a tris-glycine tank buffer system (Table 4.5). Sample aliquots of 10 µl were added to an equal volume of loading buffer (Table 4.6) and a 6 µl aliquot was dispensed into gel loading slots. A current of 10 mA was applied until the loading buffer reached the resolving gel (*ca* 60 minutes). The current was then increased to 20 mA until the loading buffer reach the end of the resolving gel (*ca* 2.5 hours).

4.2.5 Gel staining

When electrophoresis was complete the gels were dismantled from the apparatus and placed into shallow perspex trays (10.0 cm x 10.0 cm x 0.5 cm). The respective stain ingredients, for each enzyme, were added to 4 ml of the appropriate stain buffer (Appendix C) and 2 ml of 2% w/v molten agar. The stain was poured onto the gel and allowed to set. Gels were incubated at 37°C in the dark until the enzyme bands had stained on the gel sufficiently to permit interpretation. The gel was then scored to describe electrical mobility of the enzyme and hence allelic variation.

4.2.6 Gel scoring

For the purposes of gel scoring, enzymes were referred to in the abbreviated format XYZ. The encoding loci for each enzyme were referred to in the format Xyz. If there were multiple loci present these were indicated by the use of hyphenated numerals after the abbreviation (eg. Xyz-2). These were numbered in increasing order of electrophoretic mobility from the cathode end of the gel. Allelic variation at a particular locus is referred to in parenthesis in relation to the mobility of the homomeric band of a standard allele. A homomeric enzyme band is one where the base sequence of

Table 4.2 Components of polyacrylamide solution mix.

Amount	Ingredient
29g	Acrylamide
1g	N,N'-bisacryamide
100ml	Deionised water

Table 4.3 Components of 5% stacking gel solution mix.

Amount	Ingredient
6.80ml	Distilled water
1.70ml	Acrylamide mix (Table 4.2)
1.25ml	1.0M Tris, pH 6.8
0.10ml	Ammonium persulphate (10% w/v)
0.01ml	TEMED

Table 4.4 Components of 8% resolving gel solution mix.

Amount	Ingredient
6.90ml	Distilled water
4.00ml	Acrylamide mix (Table 4.2)
3.80ml	1.5M Tris, pH 6.8
0.15ml	Ammonium persulphate (10% w/v)
0.01ml	TEMED

Table 4.5 Components of tank buffer solution.

Amount	Ingredient	
25mM	Tris)	pH 8.3
250mM	Glycine)	

Table 4.6 Components of gel loading buffer solution.

Amount	Ingredient
50mM	Tris, pH 6.8
100mM	Dithiothreitol
0.1%	Bromophenol blue
10.0%	Glycerol

the nucleic acid, on the portion of the DNA which codes for the enzyme in question, is identical on both strands of the chromosome. The standard allele, which is arbitrarily designated 100, is the most frequent one exhibited in the roach (*Rutilus rutilus* (L.)). An allele which produces bands which migrate in a cathodic direction from the origin is preceded by a hyphen (eg. Xyz-2(-100)).

4.2.7 Genetic analyses

Allele frequencies were recorded at each loci, for each progeny group, using;

$$2Ho + Hc / 2N \qquad \text{(Equation 4.1);}$$

where Ho is the number of homozygotes for that allele, Hc was the number of heterozygotes for that allele and N was the number of individuals examined.

Analyses performed made comparisons between groups of progeny. Three comparisons were made between the genetic data of progeny groups using the procedures outlined above. These were:

(i) comparisons between chub, roach and their reciprocal F1 hybrids;
(ii) comparisons between chub, common bream and their reciprocal F1 hybrids;
(iii) comparisons between roach, common bream, their reciprocal F1 hybrids and their back-crossed F2 hybrids.

4.3 RESULTS

For interpretations of Tables refer to Sections 1.3.2, 4.2.6 and 4.2.7.

4.3.1 Chub and roach crosses

Of the twenty enzyme systems examined, seven of the loci were found to be diagnostic between the chub and roach progeny (Table 4.7). The progeny of pure-bred chub and pure-bred roach were homozygous at each of these loci. In addition, all the F1 hybrid fish from both of the reciprocal chub and roach crosses showed only heterozygosity at these diagnostic loci. These data from experimental progeny, indicate that F1 roach/chub hybrids display strict genetic intermediacy between their parent species; roach and chub.

4.3.2 Chub and common bream crosses

Amongst the twenty enzyme systems which were investigated, thirteen of the loci appeared to be diagnostic between the pure-bred chub and common bream progeny (Table 4.8). All individuals which were pure-bred chub or common bream were homozygous at these loci. However, all F1 hybrid progeny from both of the reciprocal chub and common bream hybrid crosses exhibited heterozygosity at each of these diagnostic loci. These data indicate that the enzyme patterns of F1 chub/common bream hybrids conformed to the expected and showed strict genetic intermediacy between chub and common bream.

4.3.3 Roach and common bream crosses

For the common bream and roach progeny twelve loci were found to be diagnostic (Table 4.9). The progeny of pure-bred roach and pure-bred common bream were homozygous at these diagnostic loci. All F1 hybrid progeny from both reciprocal common bream and roach crosses showed only heterozygosity at these diagnostic loci. The heterozygous patterns observed for F1 roach/common bream hybrids, from the experimental progeny, indicate that they are genetically intermediate between their parent species.

4.3.4 F1 hybrid roach/common bream back-crosses

Individual fish which were produced by backcrossing the female F1 roach/common bream hybrid with either male common bream or male roach were found to be heterozygous at some of the diagnostic loci but homozygous at others. It was also noted that there was no common genetic pattern, among the individual fish, for either of the two back-crossed progeny groups. Hence, these data do not indicate strict intermediacy of these progeny groups between F1 hybrid roach/common bream and either pure-bred roach or pure-bred common bream progeny. Hence, these data show that the fish in these groups are probably not F1 roach/common bream hybrid or pure-bred parental species. Therefore, these data cannot be used to confirm that these fish are F2 back-crossed progeny.

Table 4.7 Allele frequencies at diagnostic loci for pure bred chub, pure bred roach and both their reciprocal F1 hybrid crosses (* = female parent named first).

Loci	Alleles	Chub	*Chub x Roach	*Roach x Chub	Roach
Aat	85	1.00	0.50	0.50	0.00
	100	0.00	0.50	0.50	1.00
Ck	75	1.00	0.50	0.50	0.00
	100	0.00	0.50	0.50	1.00
Est-2	100	0.00	0.50	0.50	1.00
	110	1.00	0.50	0.50	0.00
Est-3	90	1.00	0.50	0.50	0.00
	100	0.00	0.50	0.50	1.00
Gpdh	100	0.00	0.50	0.50	1.00
	120	1.00	0.50	0.50	0.00
Ldh-3	100	0.00	0.50	0.50	1.00
	115	1.00	0.50	0.50	0.00
Sdh	40	1.00	0.50	0.50	0.00
	100	0.00	0.50	0.50	1.00

Table 4.8 Allele frequencies at polymorphic loci for pure bred common bream, chub and both their reciprocal F1 hybrid crosses (* = female parent named first).

Loci	Alleles	Common bream	*C.bream x chub	*Chub x c.bream	Chub
Aat	85	0.00	0.50	0.50	1.00
	100	1.00	0.50	0.50	0.00
Adh	80	1.00	0.50	0.50	0.00
	100	0.00	0.50	0.50	1.00
Ck	75	0.00	0.50	0.50	1.00
	88	1.00	0.50	0.50	0.00
Est-2	85	1.00	0.50	0.50	0.00
	110	0.00	0.50	0.50	1.00
Est-3	90	0.00	0.50	0.50	1.00
	100	1.00	0.50	0.50	0.00
Gpdh	100	1.00	0.50	0.50	0.00
	120	0.00	0.50	0.50	1.00
Hex	60	0.00	0.50	0.50	1.00
	85	1.00	0.50	0.50	0.00
Idh-2	90	1.00	0.50	0.50	0.00
	100	0.00	0.50	0.50	1.00
Mpi	70	1.00	0.50	0.50	0.00
	100	0.00	0.50	0.50	1.00
Pgi-1	100	0.00	0.50	0.50	1.00
	200	1.00	0.50	0.50	0.00
Pgi-2	100	0.00	0.50	0.50	1.00
	120	1.00	0.50	0.50	0.00
6Pgdh	80	1.00	0.50	0.50	0.00
	100	0.00	0.50	0.50	1.00
Sdh	40	0.00	0.50	0.50	1.00
	150	1.00	0.50	0.50	0.00

Table 4.9 Allele frequencies at polymorphic loci for pure bred common bream, pure bred roach, their reciprocal F1 hybrid crosses and their backcrossed F2 hybrid crosses (* = female parent named first).

Loci	Allele	Roach	*F1 hybrid x roach	*Roach x c.bream	*C.bream x roach	*F1 hybrid x c.bream	Common bream
Aat	100	1.00	0.67	0.50	0.50	0.50	0.00
	120	0.00	0.33	0.50	0.50	0.50	1.00
Adh	80	0.00	0.17	0.50	0.50	0.67	1.00
	100	1.00	0.83	0.50	0.50	0.33	0.00
Est-1	75	0.00	0.08	0.50	0.50	0.67	1.00
	100	1.00	0.92	0.50	0.50	0.33	0.00
Est-2	85	0.00	0.25	0.50	0.50	0.62	1.00
	100	1.00	0.75	0.50	0.50	0.38	0.00
Idh-2	90	0.00	0.17	0.50	0.50	0.75	1.00
	100	1.00	0.83	0.50	0.50	0.25	0.00
Ldh-3	100	1.00	0.42	0.50	0.50	0.92	1.00
	115	0.00	0.58	0.50	0.50	0.08	0.00
Mpi	70	0.00	0.38	0.50	0.50	0.75	1.00
	100	1.00	0.62	0.50	0.50	0.25	0.00
6Pgdh	80	0.00	0.38	0.50	0.50	0.50	1.00
	100	1.00	0.62	0.50	0.50	0.50	0.00
Pep-A	80	0.00	0.17	0.50	0.50	0.68	1.00
	100	1.00	0.83	0.50	0.50	0.32	0.00
Pgi-1	100	1.00	0.82	0.50	0.50	0.42	0.00
	200	0.00	0.18	0.50	0.50	0.58	1.00
Pgi-2	100	1.00	0.96	0.50	0.50	0.28	0.00
	120	0.00	0.04	0.50	0.50	0.72	1.00
Sdh	100	1.00	0.88	0.05	0.05	0.45	0.00
	150	0.00	0.12	0.05	0.55	0.55	1.00

4.4 DISCUSSION

4.4.1 Comparisons with previous studies

Comparative genetic data are available from previous studies for roach, common bream and their presumed F1 hybrids (Brassington & Ferguson, 1976 [LDH; PGI; PGM; EST]; Child & Solomon, 1977 [PGM; LDH; EST]; Cross, 1978 [AAT; LDH; PGI; PGM]). Comparative data are also available for all cyprinid species found in the British Isles (Midgelow-Marsden, 1993). The data presented here are in general agreement with those observed by these authors. Although there were some differences in band mobilities for AAT, PGI and LDH, which may be attributed to differences in the methods used, the general banding patterns were identical. However, the enzymes PGM and EST could not be compared with data from previous studies because they were not detected in the samples of skeletal muscle tissue taken from the experimental progeny. These enzymes are clearly more active in liver and heart tissues than skeletal muscle tissue.

4.4.2 Suitability of enzyme electrophresis techniques

Enzyme electrophoresis data were more successful than meristic and pharyngeal bone information in distinguishing between pure-bred species, their F1 hybrids and their F2 back-crossed hybrids from the experimental breeding programme. Genetic data were able to identify correctly the parental species of each fish examined. However, this was not possible using meristic and pharyngeal bone information without supportive data genetic data (e.g. roach/chub):

- of the assumptions regarding hybrid appearance (Section 3.4.6);
- the results are independent of environmental influences;
- they are able to distinguish between pure-bred species, F1 hybrids and F2 backcrossed hybrids.

However, the technique cannot be used to detect the extent of post F1 hybridization, i.e. differences between F2, F3 or further introgressed generations. Therefore, the limitation of enzyme electrophoresis investigations in natural hybrid populations is that it can only provide evidence that F1 hybrids are reproductively active.

The interpretations of the genetic data, of each fish examined from the same parental cross showed consistency, i.e. all alleles for each species and hybrid were fixed at each of the loci examined. Intra-specific genetic variability was not observed among these

data because each fish in a progeny group was the product of the same maternal and same paternal parent fishes. In natural populations many fish are involved in reproductive activities and so there is more scope for genetic variability. Nevertheless, the results presented here provide an important bench-mark against which comparisons can be made with natural populations.

It is not possible to identify fixed, sex-linked, diagnostic loci among the species investigated, because the method of enzyme electrophoresis is not sufficiently sensitive to detect species-specific sexual ancestry in hybridization. The most suitable genetic method of determining species-specific sexual ancestry available is restriction enzyme analysis of mitochondrial DNA (Hutchinson *et al.*, 1974; Chapter 7).

4.4.3 Further applications of enzyme electrophresis

Enzyme electrophoretic data have proven invaluable in taxonomic, systematic and phylogenetic studies (Avise & Selander, 1972; Avise, 1975; Ferguson, 1977; Buth & Burr, 1978; Shaklee & Tamaru, 1981; Thorpe, 1983; Buth, 1984; Ryman & Utter, 1987). To gain greater understanding of cyprinid hybridization genetic distance data (Nei, 1972), should be examined alongside information on ecology, behaviour, morphology, physiology, and artificial breeding experiments to examine the relationships which exist between related species. These investigations will elucidate relationships between genetic distance and the probability of hybridization. Such studies are important not only to biologists, but also to fishery managers who are required to maintain and develop fish stocks because they permit assessments of management policies.

4.4.4 Limitations of enzyme electrophoretic data

While enzyme electrophoresis is a great advance on the traditional techniques of taxonomy and phylogeneic investigations, it does underestimate the amount of genetic variation in DNA. This arises from differences in the DNA sequence not always resulting in changes to the enzyme structure which can be detected by electrophoresis. It is estimated that between 25-32% of DNA sequences result in enzyme structure changes that are detectable using electrophoresis (Lewontin, 1974; Ferguson, 1980). Furthermore, caution should be used in the use of such data because electrophoresis examines only a small proportion of the total genome of any one individual which may lead to error in interpretation. Electrophoretic data are also limited in their application

in higher taxonomic classifications (Avise, 1975). The development of more sensitive techniques will therefore be useful in future taxonomic and phylogenic investigations.

CHAPTER FIVE
HYBRIDIZATION AND INTROGRESSION BETWEEN ROACH AND COMMON BREAM IN THE FORTY FOOT DRAIN

5.1 INTRODUCTION

5.1.1 Natural occurrence of common bream/roach hybrids

On the basis of the evidence from meristic, morphometric and pharyngeal bone characters (Spillman, 1961; Pethon, 1978; Wood & Jordan, 1987; Fahy *et al.*, 1988; Economidis & Wheeler, 1989; Kennedy & Fitzmaurice, 1968; Adams & Maitland, 1991) and genetic information from enzyme electrophoresis (Brassington & Ferguson, 1976; Child & Solomon, 1977; Cross, 1978), the occurrence of hybrids between common bream and roach have been found to be widespread throughout the British Isles and Europe.

Roach and common bream are species which are found in similar habitats, these being the slow flowing areas of lowland rivers and the still waters of canals, lakes and reservoirs (Wheeler, 1969; Maitland, 1972). In addition, these two species show both spatial and temporal overlaps in their spawning activities (Wheeler, 1969) and are consequently susceptible to hybridization.

F1 generation common bream/roach hybrids are known to produce viable gametes which are capable of back-crossing with either of their parent species (Wood & Jordan, 1987; Chapter 2). Furthermore, spent roach/common bream hybrids have been observed in the wild (Cowx, 1983; Fahy *et al.*, 1988). If such fish are reproductively active in natural stocks, then this has serious implications for fisheries management and the genetic integrity of the species concerned.

Despite these implications the occurrence of post-F1 generation hybrid fish, in natural populations has still to be verified. Indeed, the occurrence of many inter-specific F1-cyprinid hybrids are often treated as rare specimens with great novelty value both by anglers and fishery managers.

5.1.2 Aims and objectives

Relatively few studies have paid attention to the possible consequences of the presence of post F1 cyprinid hybrids in natural populations (e.g. Wheeler, 1976; Burrough, 1981; Berrebi *et al.*, 1989; Thompson & Iliadou, 1990). Hence, a study to examine the

possibility of back-crossing of roach/common bream hybrids was carried out in a natural population. To detect the occurrence of back-crossed F2 generation fish in a natural population meristic data (Chapter 3) and genetic data (Chapter 4) from common bream, roach and their F1 and back-crossed F2 hybrids were collected from fish produced in the experimental breeding programme (Chapter 2). These data were compared with identical information collected from putative common bream, roach and their associated hybrids from a natural population, the Forty Foot Drain, Cambridgeshire, UK. The results were used to determine if introgressive hybridization, through back-crossing, is a occurring in the fish population in the Forty Foot Drain.

5.2 MATERIALS AND METHODS

5.2.1 Site description

The Forty Foot Drain is a man-made agricultural drainage ditch situated near Chatteris, Cambridgeshire (Figure 1.4). The ditch is typical of many in this area of the U.K. The geology of the area is composed mainly of clays and the landscape is predominantly flat. Such geographical features give rise to poorly drained soils on which the dominant land use is arable agriculture.

The drain is 24 km long and runs in an east-west direction from a site north of Ramsey (NGR TL 299881) to a site east of Chatteris (NGR TL 421874). Throughout its course it is linked extensively to other land drains in the area. The drain is approximately 15m wide and the maximum depth varies between 2m and 3m. The drain is uniformly straight with minimal aquatic or marginal vegetation. Where present the vegetation consists of grasses and low herbage. The drain is managed intensively through dredging and weed-cutting (A. Taylor *pers. comm.*), but there is no evidence available to suggest that the Forty Foot Drain has been subjected to problems with water quality.

Fisheries surveys by the NRA and their predecessors, the Anglian Water Authority (Anon, 1987), indicate the presence of a small but substantial roach/common bream hybrid population (Forty Foot Drain Fish Population Survey, AWA Internal Report, September 1987).

5.2.2 Sampling procedure

Quantitative sampling was performed at six sites on the drain by the NRA, Anglian region, in August 1991 as part of their routine fisheries survey (Table 5.1). At each site

Table 5.1 Location of sampling sites on the Forty Foot Drain.

Site No.	National Grid Reference
1	TL 315881
2	TL 335880
3	TL 351880
4	TL 363883
5	TL 383886
6	TL 404880

the sampling area was approximately 2000 m^2. Quantitative sampling was carried out using a drag-down and wrap-round technique with seine nets (Coles *et al.*, 1985). The dimensions of the seine nets were 30 m x 4 m, with a knot to knot mesh size of 25 mm and twine type 'Z'. For the purposes of the NRA survey, fish were identified using gross morphological features outlined previously (Section 2.2.3).

5.2.3 The Forty Foot Drain fishery

The 1991 NRA fishery survey reported the occurrence of eleven species of fish, belonging to four families, in the Forty Foot Drain (Table 5.2). The report found that the overall biomass and density were 33.1 gm^{-2} and 0.84 fish m^{-2} respectively. The survey also showed that the fish community appeared to be dominated by roach, common bream and silver bream (Table 5.3).

5.2.4 Specimen collection and treatment

One hundred and five fish were returned to the laboratory for further investigation. On the basis of their gross morphology the sample of fish was found to contain 34 pure-bred roach, 32 pure-bred common bream, 27 roach/common bream hybrids, 6 pure-bred rudd and 6 pure-bred silver bream. Both of these latter species are also known to hybridize with roach and common bream. Some genetic analyses were performed on the specimens of rudd and silver bream to ensure the pure-bred and hybrid nature of the 93 roach/common bream specimens.

The specimens were killed in benzocaine (1:10 000 dissolved in acetone) and were stored at -80°C until required for further examinantion. They were thawed individually, weighed to the nearest gramme and the fork-length was measured to the nearest millimetre. Meristic counts were recorded on all fish for AFR, LLS, DLS and ALS (Table 3.2 explains abbreviations). The pharyngeal bones were removed, using methods described previously (Section 3.2.4), and their morphology was recorded. Samples of eye, heart, liver and muscle tissue (*ca* 0.25-0.50g) were removed from each fish and homogenised in 0.5ml 100mM Tris-HCl buffer, pH 7.4.

5.2.5 Genetic and statistical analysis of specimens

Genetic analysis was performed on each specimen using identical procedures to those described previously for enzyme electrophoresis (Sections 4.2.1 - 4.2.7). When genetic

Table 5.2 The common and scientific names of the fish species found in the Forty Foot Drain.

Family	Common name	Species name
Anguillidae	Eel	*Anguilla anguilla* (L.)
Cyprinidae	Bleak	*Alburnus alburnus* (L.)
	Common Bream	*Abramis brama* (L.)
	Silver Bream	*Blicca bjoerkna* (L.)
	Roach	*Rutilus rutilus* (L.)
	Rudd	*Scardinius erythrophthalmus* (L.)
	Tench	*Tinca tinca* (L.)
Esocidae	Pike	*Esox lucius* L.
Percidae	Ruffe	*Gymnocephalus cernua* (L.)
	Perch	*Perca fluviatilis* L.
	Zander	*Stizostedion lucioperca* (L.)

Table 5.3 Fish population survey of the Forty Foot Drain, August 1991.
Source: Anon (1991).

Species	Biomass g. m^{-2}	Biomass %	Density No. m^{-2}	Density %
Roach	16.1	49	0.70	83
Common Bream	7.0	21	0.04	5
Silver Bream	1.4	4	0.03	4
Others	8.6	24	0.05	6
Hybrids	0.5	2	0.02	2
Total	33.1	100	0.84	100

analysis was complete the identity of each fish was confirmed. Statistical analyses were carried out on the meristic data of common bream, roach and their hybrids. Group means were compared using the SPSS/PC+ program ONEWAY (Section 3.2.3). The meristic data from the fish of the Forty Foot Drain were transformed (Log_{10}) and entered into the predictive equation derived by discriminant analysis which was used to distinguish between common bream, roach their F1 hybrids and their backcrossed F2 hybrids (Equation 3.4; Figure 3.4).

5.3 RESULTS

For interpretations of Tables refer to Sections 1.3.2, 4.2.6 and 4.2.7.

5.3.1 Genetic analysis

Initial enzyme screening revealed that silver bream differed genetically from common bream and roach for the enzyme Mpi. It was concluded from the results that silver bream were not implicated in the hybridization occurring in the Forty Foot Drain. In addition, these data indicated that rudd was also not involved in hybridization as they differed from roach for the enzymes Ldh, Pgm and Pgi (Table 5.4).

Enzyme electrophoresis revealed that 17 of the 30 loci examined were diagnostic between roach and common bream. In addition to the 12 diagnostic loci found amongst the roach and common bream progeny produced artificially, a further 5 loci were found to be polymorphic between these species in the Forty Foot Drain (Table 5.4). Two of the screened enzymes also exhibited intra-specific polymorphisms these were Malic enzyme and 6-Phosphogluconate dehydrogenase.

5.3.2 Pharyngeal bone structure

The features of the pharyngeal bones were sufficiently different to distinguish between roach, common bream and their hybrids (Plate 5.1). The structure of the pharyngeal bones for each of the groups (Table 5.5), was similar to the bones described from pure-bred roach and common bream and F1 hybrids produced in the experimental breeding programme (Table 3.10).

Table 5.4 Allele frquencies at 30 loci for roach, common bream and their hybrids from the Forty Foot Drain, Cambridgeshire, UK.

Loci	Allele	Rudd n=6	Roach n=34	Roach/common bream hybrids n=27	Common bream n=32	Silver bream n=6
Aat	100	1.00	1.00	0.50	0.00	0.00
	120	0.00	0.00	0.50	1.00	1.00
Ak-1	100	1.00	1.00	1.00	1.00	1.00
Ak-2	100	1.00	1.00	0.50	0.00	0.00
	110	0.00	0.00	0.50	1.00	1.00
Adh	80	0.00	0.00	0.50	1.00	1.00
	100	1.00	1.00	0.50	0.00	0.00
Ck	88	0.00	0.00	0.50	1.00	1.00
	100	1.00	1.00	0.50	0.00	0.00
Est-1	75	0.00	0.00	0.50	1.00	1.00
	100	1.00	1.00	0.50	0.00	0.00
Est-2	85	0.00	0.00	0.50	1.00	1.00
	100	1.00	1.00	0.50	0.00	0.00
Est-3	100	1.00	1.00	1.00	1.00	1.00
Fum	100	1.00	1.00	1.00	1.00	1.00
G-6pdh	100	1.00	1.00	1.00	1.00	1.00
Gdh-1	100	1.00	1.00	1.00	1.00	1.00
Gdh-2	100	1.00	1.00	1.00	1.00	1.00
Gpdh	100	1.00	1.00	1.00	1.00	1.00
Hex	100	1.00	1.00	0.50	0.00	0.00
	120	0.00	0.00	0.50	1.00	1.00
Idh-1	100	0.00	1.00	1.00	1.00	1.00
Idh-2	90	1.00	1.00	1.00	1.00	1.00
	100	1.00	1.00	0.50	0.00	0.00

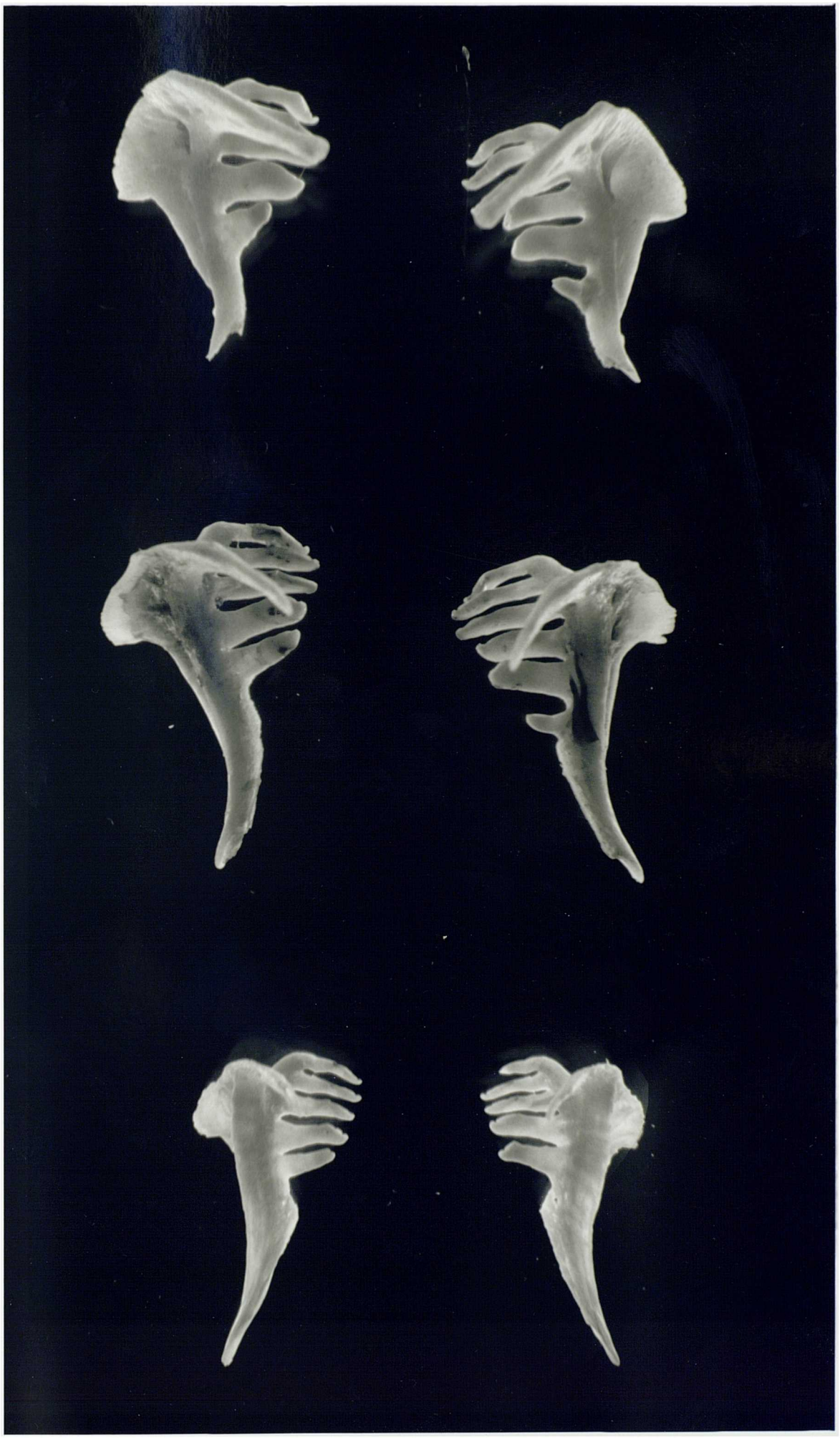
Table 5.4 contiued.

Loci	Allele	Rudd n=6	Roach n=34	Roach/common bream hybrids n=27	Common bream n=32	Silver bream n=6
Ldh-1	-100	1.00	1.00	1.00	1.00	1.00
Ldh-2	100	1.00	1.00	1.00	1.00	1.00
Ldh-3	100	0.00	1.00	0.50	0.00	0.00
	115	1.00	0.00	0.50	1.00	1.00
Mdh-1	100	1.00	1.00	1.00	1.00	1.00
Mdh-2	100	1.00	1.00	1.00	1.00	1.00
Me	95	0.00	0.00	0.00	0.09	0.00
	100	1.00	1.00	0.50	0.00	0.00
	110	0.00	0.00	0.50	0.91	1.00
Mpi	70	0.00	0.00	0.50	1.00	0.00
	100	1.00	1.00	0.50	0.00	1.00
Pep-A	80	0.00	0.00	0.50	1.00	1.00
	100	1.00	1.00	0.50	0.00	0.00
6-Pgdh	100	1.00	0.78	0.50	0.24	0.00
	120	0.00	0.10	0.21	0.34	0.00
	180	0.00	0.12	0.29	0.42	1.00
Pgi-1	100	0.00	1.00	0.50	0.00	0.00
	200	1.00	0.00	0.50	1.00	1.00
Pgi-2	100	0.00	1.00	0.50	0.00	0.00
	120	1.00	0.00	0.50	1.00	1.00
Pgm-1	92	1.00	0.00	0.50	1.00	1.00
	100	0.00	1.00	0.50	0.00	0.00
Pgm-2	100	1.00	1.00	1.00	1.00	1.00
Sdh	100	1.00	1.00	0.50	0.00	0.00
	150	0.00	0.00	0.50	1.00	1.00

Table 5.5 Description of pharyngeal bone and tooth formulation of common bream, roach and their F1 hybrids from the Forty Foot Drain.

Fish	Formulation	Description
Common bream	5:5	Long ventral extension (*pars ventralis*) Teeth set high on bone and hooked All teeth have smooth masticatory surface Last tooth stocky and conical shape No teeth crenulate
Common bream/roach	6:5 5:5	Bones similar to roach (stocky) Intermediate *pars ventralis* Teeth 1 & 2 smooth masticatory surface Teeth 3,4 & 5 slightly hooked Fifth tooth conical
Roach	6:5 5:5	Bones stocky Short *pars ventralis* First four bones hooked Smooth masticatory surface Last tooth conical

Plate 5.1 **Pharyngeal bones of roach (top), common bream (bottom) and their hybrid (middle) from the Forty Foot Drain, Cambridgeshire.**



5.3.3 Statistical analysis of meristic data

There were differences between the means of all meristic data for pure-bred common bream, roach and their F1 hybrids from the Forty Foot Drain (Appendix D; Table 5.6). The mean values for the hybrids were found to be intermediate between those of their parent species. There were no overlaps between the ranges of the meristic characters AFR and LLS, but there were considerable overlaps between the ranges for both ALS and the DLS meristic characters (Table 5.6). Comparisons of the means and ranges with published data appear to confirm the status of the pure-bred species and the F1 roach/common bream hybrids (Appendix A; Chapter 3).

Analysis of variance and Scheffe's *a posteriori* test ($P < 0.05$) found that there were statistically significant differences for all the meristic characters between roach, common bream and their hybrids (Table 5.7; Table 5.8).

When the $\log_{10}$ transformed data were entered into the predictive discriminant function equation (Equation 3.4; Figure 3.4), the frequency histogram obtained indicated that all fish appeared to be either pure-bred roach, common bream or their F1 hybrids (Figure 5.1). Hence, these meristic data support the findings of the genetic information, and pharyngeal bone morphology, that F2 back-crossed hybrids were not detected among the specimens of the Forty Foot Drain.

5.4 DISCUSSION

5.4.1 Interpretation of genetic data

There was more inter-specific polymorphism detected among the species and hybrids in the Forty Foot Drain than in the progeny of the experimental breeding programme because the genetic analyses were performed on a wider range of body tissues. It is known that a particular enzyme may exhibit more activity in one tissue (e.g. liver), than in comparison to another (e.g. muscle). Hence, where a wider range of tisues are screened, i.e. in the fish from the Forty Foot, enzyme electrophoresis will detect a greater amount of inter-specific genetic polymorphism. The activities of the enzymes AK, CK, HEX, ME & PGM were undetectable in the skeletal muscle tissue extracts from the fish of the experimental breeding programme (Chapter 4). However, the activity of these enzymes was sufficiently greater in the liver and heart tissues of the fish from the Forty Foot Drain to permit their detection.

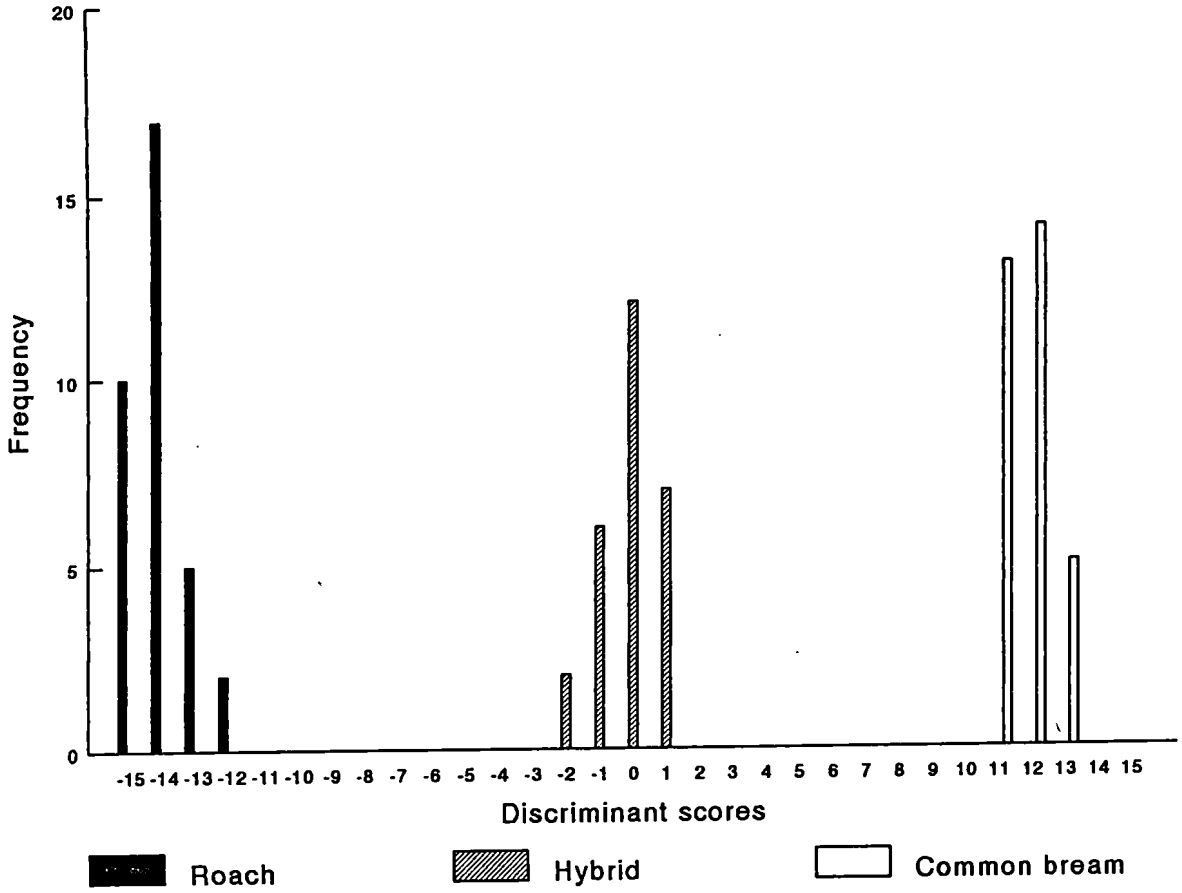


Figure 5.1 Discriminant analysis frequency distribution for roach, common bream and their F1 hybrids from the Forty Foot Drain, Cambridgeshire

Table 5.6 Summary of meristic data for common bream, roach and their F1 hybrids from the Forty Foot Drain, Cambridgeshire, UK.

Meristic	Cross	Mean	95% C.L.	Range	N
AFR	Common bream	27.1	0.41	25-29	32
	Common bream/roach	17.3	0.39	15-19	27
	Roach	10.4	0.25	9-12	34
LLS	Common bream	57.3	0.54	54-60	32
	Common bream/roach	50.4	0.46	48-52	27
	Roach	42.7	0.39	40-44	34
DLS	Common bream	13.1	0.26	12-14	32
	Common bream/roach	10.9	0.29	10-12	27
	Roach	8.0	0.08	7-9	34
ALS	Common bream	7.5	0.21	6-8	32
	Common bream/roach	5.7	0.18	5-6	27
	Roach	4.1	0.11	4-5	34

Table 5.7 Oneway analysis of variance of meristic data from common bream, roach and their hybrids from the Forty Foot drain, Cambridgeshire, UK.

Meristic	Source	DF	Sum sq.	MSS	F	P
AFR	Between	2	4628.4	2314.2	2333.2	<0.0001
	Within	92	89.3	0.99		
	Total	94	4717.7			
LLS	Between	2	3537.9	1769.0	1004.3	<0.0001
	Within	92	158.5	1.8		
	Total	94	3696.5			
DLS	Between	2	436.7	218.4	555.9	<0.0001
	Within	92	35.4	0.4		
	Total	94	472.1			
ALS	Between	2	188.6	94.3	40.2	<0.0001
	Within	92	21.2	0.3		
	Total	94	209.8			

Table 5.8 Comparisons of mean values of meristic characters of fish from the Forty Foot Drain: Underlined means are not significantly different.

Meristic	Common bream	Hybrids	Roach
AFR	27.1	17.3	14.4
LLS	57.3	50.4	42.7
DLS	13.1	10.9	8.0
ALS	7.5	5.7	4.1

Intra-specific polymorphisms, for the enzymes ME and 6-PGDH, were observed in the populations of the Forty Foot Drain. An explanation of the lack of intra-specific polymorphism amongst the progeny of the experimental breeding programme has been discussed previously (Section 4.4.3). Hence, there is an increased probability of intra-specific genetic variability in the Forty Foot Drain, in comparison to the experimental breeding programme, because more individual fish are involved in reproduction.

5.4.2 Post F1 hybridization in natural populations

The results suggest that F2 backcrossed hybrids were not detected in the Forty Foot Drain. However, sexually mature cyprinid hybrids have been observed in natural populations (Kennedy & Fitzmaurice, 1968; Cowx, 1983; Fahy *et al.*, 1985). Indeed, Kennedy & Fitzmaurice (1968) have found both fertile and spent hybrids of rudd and common bream in fresh waters in Ireland. Furthermore, F1 hybrids have been proven to be fertile (Wood & Jordan, 1987; Chapter 2) and some authors have proposed that complex hybridization may be occurring among cyprinids in natural waters (Wheeler, 1976; Burrough 1981). The occurrence of post F1 hybrids in natural waters has still to be confirmed using genetic techniques and some authors have concluded that, while F1 hybrids may become sexually mature, they may not be reproductively active (Cowx, 1983). However, there are a number of possible explanations why F2 hybrids were not detected in the Forty Foot and these include either biological reasons which maintain isolation between the species or limitations in the methodology.

Pre-mating isolation mechanisms

The mating behaviour of the mature roach/common bream hybrids may be inadequate or inappropriate to commence spawning activities with either other F1 hybrids or with either of the parent species. Barton & Hewitt (1983, 1985) have suggested that in each of the parent species there are co-adapted gene complexes controlling reproductive activities which have evolved independently. Indeed, this aspect of genetic evolution within a species, particularly with respect to reproduction, ensures that there is coincidence of reproductive behaviour between the individuals and is therefore a critical feature in maintaining species survival. However, when inter-specific hybridization occurs there is a fusion of genes from each of the parent species in the resulting F1 hybrids and that the genes controlling reproductive activities are no longer co-adapted. Hence, there is a breakdown of the gene complexes controlling mating behaviour and this may result in the absence of reproductive activities in the hybrids.

Post-mating isolation mechanisms

The absence of F2 backcrossed hybrids in the population maybe due to the inferior fitness of the F2 hybrids, in comparison to the pure-bred species and F1 hybrids, if the F1 roach/common bream hybrids are reproductively active. Avise & van den Avyle (1984) have advanced this theory to explain why there was a high frequency of F1 bass hybrids, but only a low frequency of F2 hybrids, in the Savannah river, USA. The suggested explanation for this is that while F1 hybrids may suffer from the breakdown of co-adapted gene complexes, as outlined above, their relative fitness may be enhanced by a phenomenon known as heterosis. This is where a hybrid with a high proportion of heterozygous gene loci is able to benefit from genetic advantages because it is able to exploit the genes of two species. However, in F2 hybrids the gene complexes are disrupted, but there is only a low proportion of heterozygous loci and so do not benefit from heterosis.

If either of the above situations is true for roach/common bream hybrids then it is possible that stable F1 hybrid populations may establish without threatening the genetic integrity of the parent species (Moore,1977). The result of this scenario is the continual loss of genes through hybridization from the gene pools of both species. Hence, as a consequence there is a threat of the loss of genetic variation and conservation. Ultimately this could result in the loss of unique populations of the species concerned.

Limitations of the sampling methods

The F1 hybrid population constituted a low proportion of the entire fish stocks of the Forty Foot Drain (<1%). The consequence of such a low F1 hybrid population is that an even lower proportion of F2 hybrids maybe expected. Therefore, they may also have a low probability of capture by the sampling technique. Furthermore, it is assumed that if F2 backcrossed hybrids were captured they can be distinguished from their parental species on the basis of their gross morphological features.

Limitations of the data

The analytical techniques used assume that the most probable F2 hybrids will be the result of a F1 hybrid with one of its parent species (Mayr, 1963; Stebbins, 1971). Furthermore, the results presented here for the F2 hybrids from the artificial breeding experiments, are the result of a female F1 hybrid backcrossed with males of the parental species and do not account for progeny which are the result of an F1 x F1 cross. There may be problems identifying the progeny of an F1 x F1 cross, based on either meristic or genetic data alone. Genetic data alone cannot distinguish F1 x parental backcrosses from F1 x F1 crosses. Meristic data alone cannot discriminate between F1 generation hybrids and F2 hybrids which are the product of an F1 x F1 cross because both types of hybrids show strict intermediacy between the parent species (Wood & Jordan, 1987).

However, when both sets of information are combined, the techniques are able to elucidate the differences between these types of hybrid.

The data are further limited in this study because it is assumed that F2 hybrid backcrosses which result from a male F1 hybrid and female parental species should in no way differ, in either meristic features or genetics, from the reciprocal crosses.

Limitations of the techniques

Interpretation of these genetic analyses is somewhat limited by the sensitivity of the techniques employed in detecting hybridization in natural populations (Section 3.4.6; Section 4.4.4). Although, enzyme electrophoresis is a reliable method, present day techniques are much more advanced in their capabilities. More recent genetic techniques are able to determine genetic differences through the direct examination of the DNA sequences (Hallerman & Beckman, 1988; Saiki *et al.*, 1988). These techniques will be of great benefit in future hybridization studies to determine precisely the extent of hybridization and introgression.

5.4.3 The importance of genetic studies in cyprinid hybridization

Many reports of cyprinid hybridization in the published literature are confined purely to the identification of a few F1 hybrid specimens on the basis of gross morphology (e.g. Swinney & Coles, 1982). Similarly, where genetic analysis is performed only a few specimens have been examined (Brassington & Ferguson, 1976; Child & Solomon, 1977). Although these studies are important in the examination of the hybridization phenomenon, genetic research to assess the consequences of F1 hybrid fertility in natural waters has only been previously adopted in two European studies (Berrebi *et al.*, 1989; Thompson & Iliadou, 1990). However, hybrid fertility and reproductive activity is of critical importance to both species integrity and fisheries managenment. It is therefore essential to develop techniques further to examine the phenomenon of cyprinid hybridization. Such studies are of particular importance where F1 hybrids are known to constitute a considerable proportion of the fish stock (e.g. Mulrooney & Fahy, 1985), since the probability of post F1 hybridization is much greater in these stocks.

CHAPTER SIX
HYBRIDIZATION AND INTROGRESSION BETWEEN THE RUDD AND THE COMMON BREAM IN ESSEX UNIVERSITY LAKE

6.1 INTRODUCTION

6.1.1 Natural occurrence of common bream/rudd hybrids

Hybrids between common bream and rudd have been reported throughout Europe (Berg, 1949; Kennedy & Fitzmaurice, 1968; Wheeler, 1969, Kennedy & Fitzmaurice, 1974; Child & Solomon 1977; Economidis & Wheeler, 1989). Both species occur sympatrically in the slow flowing reaches of rivers and the still waters of canals, lakes and reservoirs. These two species exhibit temporal and spatial overlaps in their spawning periods which occurs from mid-April to late-June. The spawning behaviour of these species occurs upon vegetation in shallow water (Svardson, 1949; Kennedy & Fitzmaurice, 1968; Wheeler, 1969, Kennedy & Fitzmaurice, 1974). The gametes of both species are known to be compatible in both of the reciprocal F1 hybrid crosses (Chapter 2). Furthermore, both fertile and spent common bream/rudd hybrids have been reported in natural populations (Kennedy & Fitzmaurice, 1974) and this may have important implications in fisheries where hybrids occur.

6.2.2 Aims and objectives

Presented here is the characterization of common bream (female) x rudd (male) hybrids from progeny of the experimental breeding programme on the basis of their meristic and pharyngeal bone characteristics. These results were compared with those from a natural common bream/rudd hybrid population from a lake in the grounds of Essex University. The results were also used to assess if back-crossing had occurred.

6.2 MATERIALS AND METHODS

6.2.1 Maintenance of progeny and data collection

Progeny from the pure-bred common bream, pure-bred rudd and their F1 female common bream/male rudd hybrids (Chapter 2), were maintained under identical conditions to those described previously (Sections 3.2.1 & 3.2.2). Meristic features and descriptions of the pharyngeal bones were assessed as described previously (Sections 3.2.3 & 3.2.6).

6.2.2 Data analysis of experimental progeny

Analyses were carried out upon the meristic characteristics to examine if there were statistically significant differences between common bream, rudd and their F1 hybrids using the SPSS/PC+ sub-program ONEWAY with Scheffe' (0.05) *a posteriori* contrasts. The data were then $\log_{10}$ transformed and the SPSS/PC+ sub-program DISCRIMINANT was then used to interrogate these data to ascertain if they are suitable for classifying the progeny.

6.2.3 Site description, sampling method and examination of specimens

Specimens of rudd, common bream and their presumed hybrids were obtained from a lake in the campus grounds of Essex University (NGR: TM 031242) in eastern England (Figure 1.4). The lake is approximately 4 hectares in area. However, water quality, biological and fish community data are not available.

Sampling of the lake occurred throughout 1991 and 1992. Sampling was carried out by NRA staff using seine nets which were identical to those described previously (Section 5.2.2). Specimens were obtained by systematically seine netting sections of the lake margins. Specimens were killed using benzocaine dissolved in acetone (1:10,0000). Each fish was bagged, labelled and stored at -20°C. At a later date they were transported, on ice, in cool boxes to the University of Hull. Upon arrival they were refrozen at -20°C until they were required for further examination.

Fish were defrosted individually and meristic characteristics and the formation of the pharygeal bones were recorded as described previously (Sections 3.2.3 & 3.2.6). The fish were not examined using genetic techniques because of financial considerations and time constraints.

6.2.4 Analysis of meristic data

The SPSS/PC+ sub-program ONEWAY and Scheffe's (0.05) *a posteriori* contrasts were performed on the meristic data to examine if there were statistically significant differences btween the pure-bred species and hybrids. The meristic data were $\log_{10}$ transformed and entered into the DISCRIMINANT equation derived from meristic data from common bream, rudd and their hybrids from the experimental breeding program.

6.3 RESULTS

6.3.1 Progeny of the experimental breeding programme

Meristic data

There were distinct mean values observed for the meristic data of the pure-bred rudd, common bream and their F1 hybrids. The values of the hybrids were strictly intermediate between those of the parental species (Table 6.1). Furthermore, distinct differences in the ranges of the meristic values were observed, although there was a slight overlap for ALS. These ranges were comparable with those observed for the pure-bred parental species and F1 common bream/rudd hybrids observed in previous studies (Table 1.3). Oneway analysis of variance and Scheffe's *a posteriori* test ($p < 0.05$), revealed that there were statistically significant differences among the mean values of the meristic features recorded (Table 6.2; Table 6.3).

Pharyngeal bone morphology

Using the features of the pharyngeal bones it was possible to identify differences between common bream, rudd and their hybrids. The most important features were the length of the *pars ventralis* and the amount of tooth pectination (Table 6.4; Plate 6.1).

Discriminant analysis

The predictive discriminant equation (Equation 6.1), was able to classify all progeny successfully. The equation was able to explain 99.18% of the variance ($p < 0.0001$). the group means were 8.99, -2.34 and -13.00 for common bream, F1 hybrids and rudd respectively (Figure 6.1).

Equation 6.1

$$D.F. = 38.76\,AFR + 35.50\,LLS + 6.36\,DLS + 3.29\,ALS - 119.13$$

6.3.2 Fish from Essex University Lake

Meristic data

Distinct differences were observed among the means and ranges of the meristic values recorded for common bream, rudd and their putative F1 hybrids (Appendix D; Table 6.5). The mean and range values observed for the meristic data are comparable with those recorded in previous studies (Table 1.3) and with those from the experimental breeding programme (Table 6.1). Furthermore, ONEWAY and Scheffe's test revealed that the differences between the means were all significant ($p < 0.05$)(Table 6.6 & 6.7).

Table 6.1 Summary of meristic data for common bream, rudd and female common bream x male rudd F1 hybrids.

Meristic	Cross	Mean	95% C.L.	Range	N
AFR	Common bream	27.3	0.55	25-29	24
	Common bream/rudd	17.7	0.32	16-19	24
	Rudd	11.2	0.33	10-12	12
LLS	Common bream	57.1	0.56	55-60	24
	Common bream/rudd	47.9	0.45	46-50	24
	Rudd	42.7	0.61	39-43	12
DLS	Common bream	13.3	0.30	12-16	24
	Common bream/rudd	9.9	0.29	9-11	24
	Rudd	7.8	0.25	7-8	12
ALS	Common bream	7.6	0.28	6-9	24
	Common bream/rudd	4.9	0.23	4-6	24
	Rudd	4.1	0.16	4-5	12

Table 6.2 ONEWAY output of meristic data for common bream, rudd and female common bream x male rudd F1 hybrids.

Meristic	Source	DF	Sum sq.	MSS	F	P
AFR	Between	2	2372.6	1186.3	1352.4	<0.0001
	Within	57	50.0	0.9		
	Total	59	2422.6			
LLS	Between	2	1955.0	977.5	648.5	<0.0001
	Within	57	85.9	1.5		
	Total	59	2040.9			
DLS	Between	2	274.4	137.2	384.6	<0.0001
	Within	57	26.6	.5		
	Total	59	301.0			
ALS	Between	2	133.9	67.0	187.4	<0.0001
	Within	57	20.4	0.4		
	Total	59	154.3			

Table 6.3 Comparisons of mean values of meristic characters of fish from the experimental breeding programme: Underlined means are not significantly different.

Meristic	Common bream	Hybrids	Rudd
AFR	27.3	17.7	11.2
LLS	57.1	47.9	42.7
DLS	13.3	9.9	7.8
ALS	7.6	4.9	4.1

Plate 6.1 **Pharyngeal bones of rudd (top), common bream (middle) and their hybrid (middle) from the experimental breeding programme.**

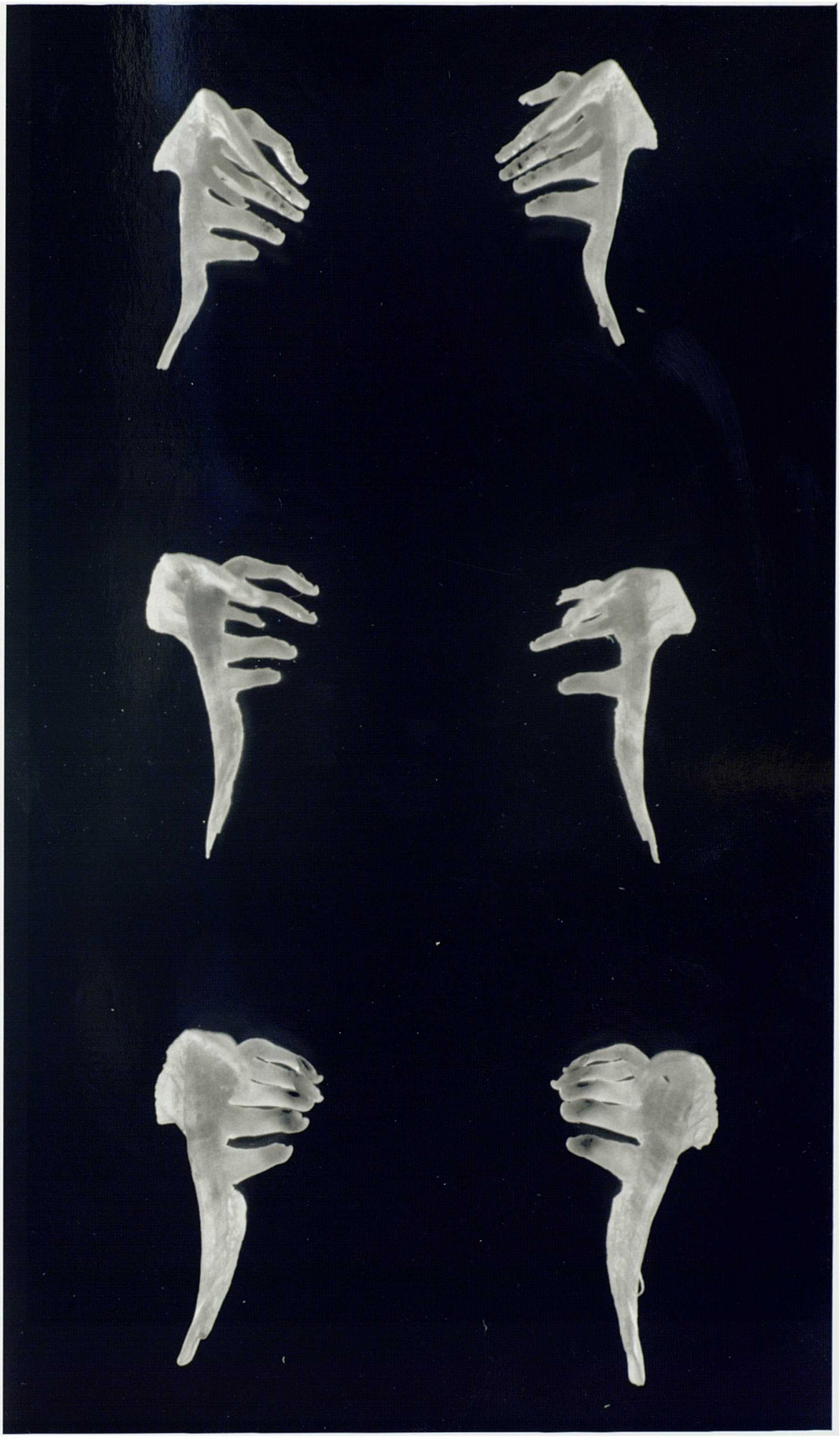


Table 6.4 Description of pharyngeal bone and tooth formulation of common bream, rudd and female common bream/male rudd F1 hybrids.

Fish	Formulation	Description
Common bream	5:5	Long ventral extension (*pars ventralis*) Teeth set high on bone and hooked All teeth have smooth masticatory surface Last tooth stocky and conical shape No teeth crenulate
Common bream/rudd	2.5:5.2	Intermediate length *pars ventralis* Outer row teeth 1 & 2 hooked strongly Teeth pectinate Slight crenulation on exposed tooth surfaces Inner row teeth hooked
Rudd	3.5:5.3	Short *pars ventralis* Outer & inner rows of teeth hooked Fifth tooth conical and stocky Crenulations on exposed surfaces Heavily pectinate.

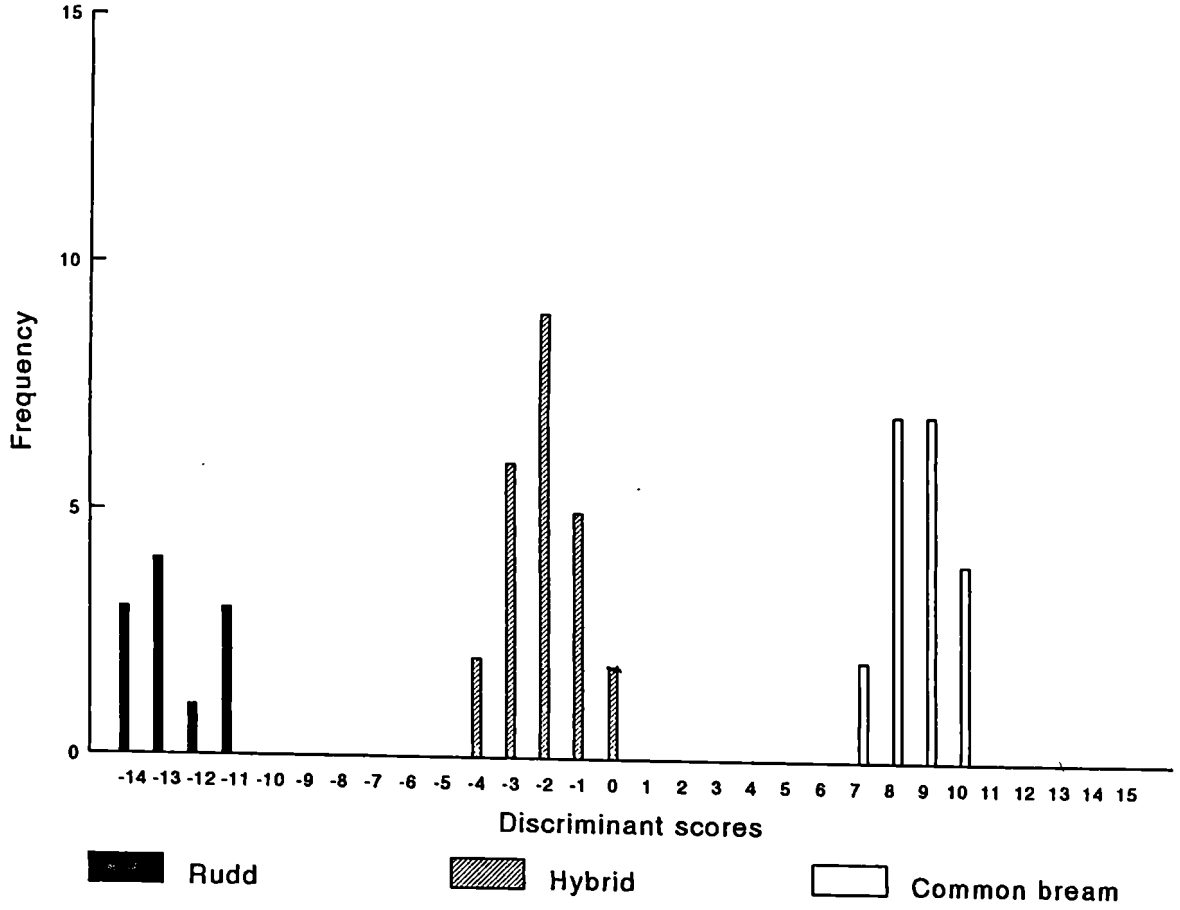


Figure 6.1 Discriminant analysis frequency distribution for rudd, common bream and their F1 hybrids from the experimental breeding programme

Table 6.5 Summary of meristic data of common bream, rudd and their hybrids from Essex University Lake.

Meristic	Cross	Mean	95% C.L.	Range	N
AFR	Common bream	27.5	0.55	25-30	23
	Common bream/rudd	17.8	0.49	16-20	18
	Rudd	11.7	0.33	11-13	19
LLS	Common bream	56.4	0.57	55-60	23
	Common bream/rudd	48.6	0.66	47-52	18
	Rudd	41.7	0.36	43-46	19
DLS	Common bream	13.6	0.44	12-14	23
	Common bream/rudd	9.4	0.29	10-11	18
	Rudd	7.3	0.20	7-8	19
ALS	Common bream	8.0	0.34	6-9	23
	Common bream/rudd	5.6	0.24	5-6	18
	Rudd	4.1	0.14	3-4	19

Table 6.6 ONEWAY output common bream, rudd and their hybrids from Essex University lake.

Meristic	Source	DF	Sum sq.	MSS	F	P
AFR	Between	2	2662.1	1331.0	1107.0	<0.0001
	Within	57	68.5	1.2		
	Total	59	2730.6			
LLS	Between	2	2266.5	1133.3	688.2	<0.0001
	Within	57	93.9	1.7		
	Total	59	2360.4			
DLS	Between	2	440.0	220.0	352.2	<0.0001
	Within	57	35.6	0.6		
	Total	59	475.6			
ALS	Between	2	167.7	83.8	225.5	<0.0001
	Within	57	21.2	0.4		
	Total	59	188.9			

Table 6.7 Comparisons of mean values of meristic characters of fish from Essex University Lake: Underlined means are not significantly different.

Meristic	Common bream	Hybrids	Rudd
AFR	27.3	17.8	11.7
LLS	56.4	48.6	41.7
DLS	13.6	9.4	7.3
ALS	8.0	5.6	4.1

Pharyngeal bone morphology

The pharyngeal bone features could be used to distinguish between common bream, rudd and their hybrids from the lake (Table 6.6). The bones were similar to those from the progeny of the experimental breeding programme (Table 6.3). However, some of the teeth of the inner row were missing in the rudd and the hybrids from the lake (Table 6.8; Plate 6.2).

Discriminant analysis

The meristic data which were $\log_{10}$ transformed and entered into the predictive discriminant function equation (Equation 6.1), produced a frequency histogram which indicated that all fish present were rudd, common bream or their F1 hybrids (Figure 6.2).

The meristic data and pharyngeal bone morphology indicated that the fish from the lakes at Essex University were all common bream, rudd or their F1 hybrids. F2 back-crossed hybrids which would show values intermediate between those of the F1 hybrids and the parental species appeared to be absent.

6.4 DISCUSSION

6.4.1 Features of F1 rudd/common bream hybrids

The features of both the pure-bred species and the F1 hybrid progeny are comparable to suspected F1 hybrids observed from natural populations (Appendix A). In addition, the meristic features of the hybrids and parent species from the Essex University Lake are similar to data from other natural populations. Unfortunately, the features of F1 hybrids between common bream and rudd could not be verified because their features have never been characterised from controlled breeding experiments. There have not been any genetic studies on rudd/common bream hybrids from the wild. Hence their identity also could not be verified with meristic data, supported by genetic information, from natural populations.

The meristic and pharyngeal bone features of the artificially produced F1 common bream/rudd hybrids were intermediate between those of their parental species (Table 6.1; Table 6.4). Similar observations were made on the F1 hybrids of the other inter-specific cyprinid crosses (Chapter 3).

Table 6.8 Description of pharyngeal bone and tooth formulation of common bream, rudd and their hybrids from Essex University Lake.

Fish	Formulation	Description
Common bream	5:5	Long ventral extension (*pars ventralis*) Teeth set high on bone and hooked All teeth have smooth masticatory surface Last tooth stocky and conical shape No teeth crenulate
Common bream/rudd	2.5:5.2 1.5:5.2 0.5:5.1	Intermediate length *pars ventralis* Outer row teeth 1 & 2 hooked strongly Teeth pectinate Slight crenulation on exposed tooth surfaces Inner row teeth hooked
Rudd	3.5:5.3	Short *pars ventralis* Outer & inner rows of teeth hooked Fifth tooth conical and stocky Crenulations on exposed surfaces Heavily pectinate.

Plate 6.2 **Pharyngeal bones of rudd (top), common bream (bottom) and their hybrid (middle) from Essex University Lake.**

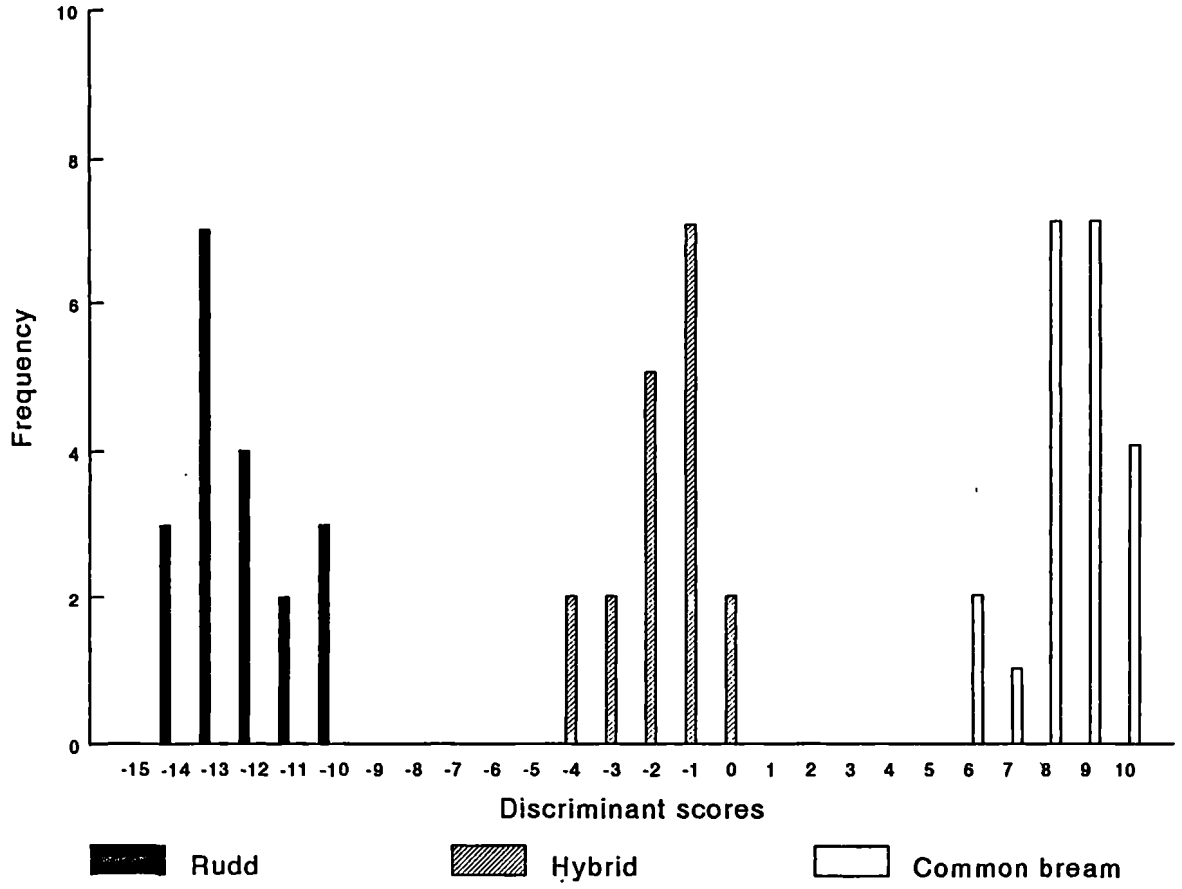


Figure 6.2 Discriminant analysis frequency distribution for rudd, common bream and their F1 hybrids from Essex University Lake

6.4.2 Post F1 hybridization

F1 hybrids between rudd and common bream have not been proven to be fertile under controlled experimental conditions and hence the genetic and meristic characteristics of F2 hybrids have not been verified. Furthermore, the presence of post F1 hybrids between rudd and common bream have not been confirmed in natural populations using genetic techniques. However, hybrids between rudd and common bream are thought to be fertile (Kennedy & Fitzmaurice, 1974) and their presence would have implications for the fishery in Essex University Lake. In consequence, it is difficult to make firm conclusions regarding either the presence, or absence of post F1 hybrid fish in Essex University Lake. However, if the assumptions regarding the use of meristic data and pharyngeal bone features for detecting post F1 generation cyprinid hybrid fish hold true:

- they are the product of half the genes of both parent species, i.e. intermediacy;
- the phenotypic expression of these characters is independent of species-specific
- sexual ancestry
- F2 hybrids are more probably the result of an F1 x parent species backcross than an F1 x F1 cross).

Then it would appear that post F1 hybrid specimens of rudd and common bream are not present in Essex University Lake. The reasons for the absence of post F1 cyrinid hybrids in natural populations have been discussed previously (Section 5.4.2). In addition, gamete compatibility must also be posed as a possible post-mating isolating mechanism because F1 hybrid gametes have not been proven to be fertile.

There were some problems in the use of the pharyngeal bones of the fish from Essex University Lake, because some of the teeth were missing from the inner row of the pharyngeal bones from some of the specimens of rudd and common bream/rudd hybrid. It is not thought that this is an indication of either backcrossing or introgressive hybridization, because these teeth may be lost through natural processes. Indeed all other features of the bones suggested that their descriptions conformed to those in previous studies for pressumed F1 rudd/common bream hybrids (Appendix A). Nevertheless, this highlights the need to use genetic techniques to investigate hybridization as there are a number of problems regarding the reliability of the meristic and pharygeal bone data alone.

CHAPTER SEVEN
MITOCHONDRIAL DNA ANALYSIS OF COMMON BREAM, ROACH AND THEIR NATURAL F1 HYBRIDS

7.1 INTRODUCTION

7.1.1 Background

The analysis of mitochondrial DNA (mtDNA) using restriction endonucleases is now applied widely to the taxonomy and population genetics of fish (e.g. Berg & Ferris, 1984; Ferris and Berg, 1987; Hynes *et al.*, 1989). This is primarily because differences in the DNA base sequence can be detected more efficiently than using enzyme electrophoresis (Brown *et al.*, 1979; Brown, 1983; Brown & Vinograd, 1984). However, it is the evaluation of the maternal inheritance of mtDNA (Hutchinson *et al.*, 1974; Giles *et al.*, 1980; Lansman *et al.*, 1981; Gyllensten *et al.*, 1985), that is of particular importance in hybrid studies (Section 1.1.3). In conjunction with other investigations (e.g. enzyme electrophoresis), it can be used to indicate the direction of a F1 hybrid cross, i.e. maternal inheritence and therefore the species-specific sexual ancestry (Section 1.3.3).

The determination of species-specific sexual ancestry is of importance in hybrid studies because it can reveal whether the maternal parent of a population of roach/common bream hybrids is roach, common bream or a mixture of both. If the mtDNA of the hybrids is characterised and is found to be similar to only one of the parent species this raises the need to perform further investigations to discover the reasons why this should be the case. For example, Avise & Saunders (1984) identified that among sunfish hybrids in the North America the maternal species was always the one which was the rarer in the fish stock. However, if the hybrid population has a mixture of the mtDNA genotypes then this implies that the factors controlling hybridization are independent of aspects related to species-specific sexual ancestry.

7.1.2 Aims and objectives

Although mtDNA can reveal important relationships regarding hybridization there has been no published research into the use of mtDNA studies on cyprinid hybrids in either the British Isles or Europe. To determine the maternal parent species of roach/common bream hybrids a sample of fish from a lake in eastern England, which included roach, common bream and their hybrids, were examined using mtDNA restriction enzyme

techniques. The results were discussed with respect to the possible scenarios leading to hybridization between these two species.

7.2 MATERIALS AND METHODS

7.2.1 Fish supply and treatment prior to analysis

A sample of 40 fish weas obtained from a commercial fish farmer comprising of 10 common bream, 10 roach and 20 common bream/roach hybrids. The fish originated from Peterborough rowing lake (NGR: TL 172 980) in November 1992 (Figure 1.4). The fish were transferred to aquarium facillities at the University of Hull where they were maintained, in a similar manner to those described previously (Section 3.2.1), until they were required for analysis.

7.2.2 Extraction of mitochondria

The method of mitochondrial extraction used was similar to that described by Lansman *et al.* (1981). The specimens of fish were killed in MS 222 and heart and liver tissues were extracted. Approximately 0.5-2.0 g of tissue was homogenised in 15 ml MSB-EDTA buffer, pH 7.5 (0.21M Mannitol; 0.07M Sucrose; 0.05M Tris; 0.01M EDTA). The homogenised tissue samples were then transferred to Nalgene tubes (50 ml volume) and placed into a Sorval SS-34 centrifuge head. The tissue samples were centrifuged twice at 1000 x *g*, for 10 minutes at 4°C, to pellet cellular debris. The supernatants were retained on each occasion and the cellular debris was discarded. To isolate mitochondria the remaining supernatant was centrifuged at 48,000 x *g* for 20 minutes at 4°C. The supernatant was discarded and the mitochondria rich pellet was retained and resuspended in 5 ml STE buffer, pH 8.0 (0.10M NaCl; 0.05M Tris; 0.01M EDTA).

7.2.3 Isolation of mitochondrial DNA

Mitochondria were lysed by the addition of 0.32 ml of 20% w/v SDS solution to each sample. The samples were left for 15 minutes at room temperature. Caesium chloride (1.6g) was added and the samples were left at 4°C for one hour. A further 3 ml of STE buffer was added and each sample was centrifuged at 5000 x *g* for 30 minutes at 4°C. 7.2 g of CsCl was added to 8 ml of this solution and allowed to dissolve. The samples were dispensed into 10 ml Beckman polycarbonate tubes (Cat. No. 355603) containing 0.5 ml ethidium bromide (10 $ml.mg^{-1}$). The tubes were balanced using paraffin oil.

Mitochondrial DNA was purified by differential gradient centrifugation in a Beckman Ultracentrifuge, with a 70 T*i* head at 90,000 x *g* for 40 hours. Two DNA bands were visualised by ultra-violet illumination, the lower, mitochondrial DNA, band was removed with a sterile syringe and hypodermic needle and stored in an Eppendorf tube.

7.2.4 Refinement of mitochondrial DNA

Ethidium bromide was removed from the samples by three butanol extractions. Caesium chloride was removed by dialysis in STE buffer for 4 hours followed by dialysis in TE buffer, pH 8.0 (0.05M Tris; 0.01M EDTA), twice for 4 hours. Residual proteins were removed by a single phenol/chloroform extraction followed by three ether extractions. Mitochondrial DNA was precipitated with a mixture of 0.5 ml isopropanol, 0.1 ml 3M sodium acetate and tRNA. The sample was left for one hour at -70°C before centrifugation at 12,000 x *g* for 10 minutes in a bench-top microfuge. The mitochondrial DNA pellet was washed with 70% ethanol, dried and resuspended in 50ml TE buffer.

7.2.5 Restriction enzyme digests

The restriction enzyme EcoRI was obtained through Northern Biological Ltd. Enzyme digests were carried out for one hour, using one unit of enzyme per mg of mtDNA, in 0.5 ml Eppendorf tubes at 37°C in 10 ml buffer (Table 7.1).

7.2.6 Agarose gel electrophoresis

The restriction enzyme digest mtDNA fragments were separated out using a 0.8% agarose gel using a continuous TBE buffer (0.045M Tris-Borate, 0.001M EDTA). Ethidium bromide (0.5 mgl^{-1}) was added to each gel to allow mtDNA fragment examination using short-wave ultra-violet light. Samples of 10 ml mtDNA digests, with 2 ml loading buffer (0.25% w/v bromophenol, 15% w/v ficoll), were loaded into the gels alongside a 10 ml, 100 ng, 1-kb DNA ladder (with 2 ml loading buffer), which was used to estimate the size of the DNA fragments.

Table 7.1 Reaction buffer for restriction enzyme digest.

Enzyme	Buffer components
EcoR I	100mM Tris-HCl, pH 7.5; 50mM NaCl; 6mM $MgCl_2$; 6mM b-Mercaptoethanol.

7.3 RESULTS

7.3.1 Roach

The method was successful for samples of mtDNA taken from three different roach. The results show that three mtDNA fragments were seen at 9kb, 5kb and 4kb for the enzyme EcoR I (Figure 7.1). This indicates that the restriction enzyme digest cut the mtDNA in three places to give three fragments.

7.3.2 Common bream and hybrids

It was not possible to obtain results for either common bream or roach/common bream hybrids.

7.4 DISCUSSION

The results of this study were not conclusive because of the difficulties encountered in adapting the mtDNA techniques for cyprinid fishes. Precise reasons explaining the apparent lack of success could not be identified. It has been suggested that there may have been insufficient mitochondrial rich tissue in the samples taken from the heart and livers of the cyprinids. A possible solution is to increase the amount of mtDNA using a technique called the Polymerase Chain Reaction (PCR). This technique amplifies the amount of DNA in a sample and should be employed after the mitochondrial DNA has been extracted and refined. The method has been used previously, to examine cytochrome b gene sequences, to establish the phylogenetic relationships between genera of the salmonid family (McVeigh & Davidson,1991). Similar techniques could be adapted to examination of mtDNA using restriction enzyme digests and agarose gel electrophoresis to establish the maternal ancestry of inter-specific hybrid fish. However, these techniques were not used here because considerable time, attention and finance would be required to refine such a method for cyprinids. Indeed, such a project should be the focus of future studies into both cyprinid systematics and hybridization.

Despite the lack of success of the methods used here it is still possible to discuss the possible scenarios among a hybrid population. These scenarios fall into two catagories and depend on whether only one or both parental mtDNA genotype was found in the hybrid population.

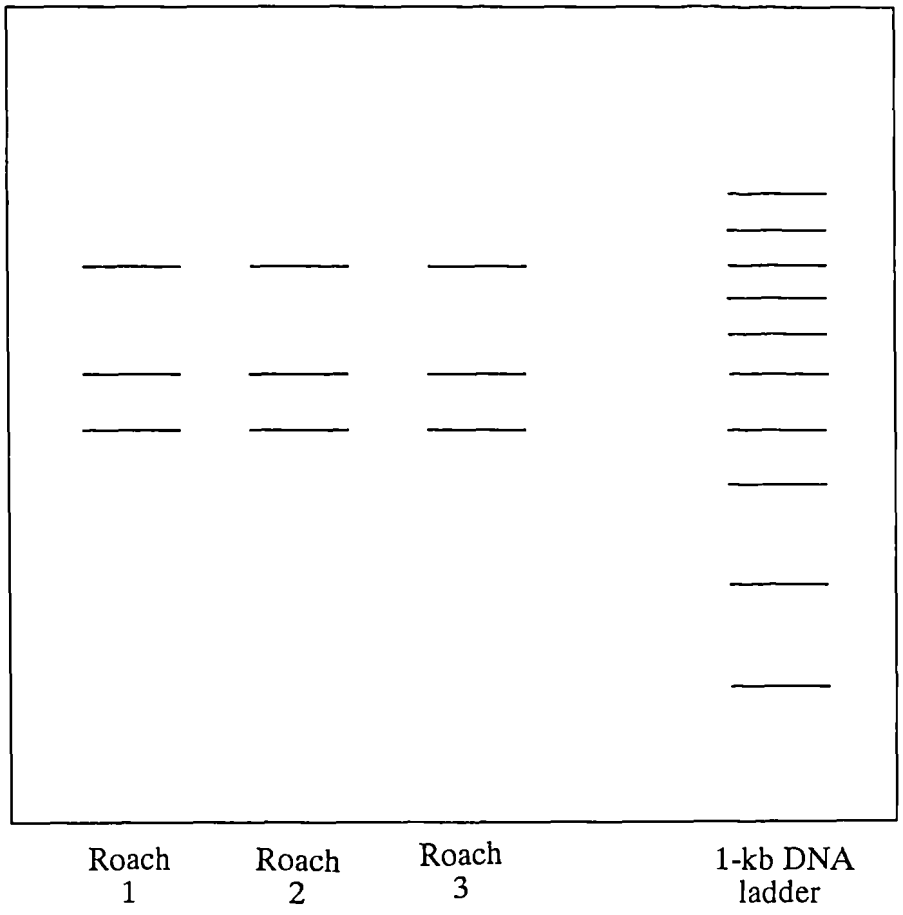


Figure 7.1 Diagram of mtDNA restriction enzyme digests of roach using EcoR1

7.4.1 Only one parental mtDNA genotype found

In hybrid populations where the mtDNA analysis indicates that the maternal parent is exclusively of only one of the two species, this would imply that one of the following factors is not independent of species-specific sexual ancestry in hybridization between roach and common bream.

Stock composition

Hubbs (1955) in his studies on sunfishes (*Lepomis*), suggested that hybridization is often enhanced where one species is very rare and the other is in great abundance. In later studies, using mtDNA, Avise & Saunders (1984) and Avise *et al.* (1984) identified that among hybrid sunfish populations the maternal ancestral species was the one which was least common in the fish stock. In the British Isles many fish communities are dominated numerically by roach with fewer dominated by common bream. It is not known whether the rarer species is the maternal parent in cases where hybrids are present in these fish stocks. Hence, an important aspect of hybridization would be to investigate the effect of stock composition upon maternal ancestry. This type of study could be extended to include hybrid other than roach/common bream, e.g. rudd/common bream and roach/rudd.

Spatial and temporal aspects of spawning

The spatial and/or temporal spawning requirements of one of the species and/or sexes may have greater plasticity. For example, the reproductive activities of the male of species A may occur for a much longer period than the female of that species, and that this longer period may overlap with the spawning activities of the female of species B. However, the same may not be true for the male of species B, i.e. there is not a temporal overlap in the spawning activities with the female of species A. Under these circumstances, if hybridization occurs then the resulting hybrids will probably be the result of a female species B x male species A cross. Similarly, the spawning requirements of one species and/or sex may be more flexible than another, e.g. although the preferred spawning habitat of roach is aquatic vegetation they have also been observed spawning upon gravels (Holcik & Hruska, 1966).

Behavioural and physiological differences in spawning

Fabricius (1950) suggested that spawning in fish is generally a response to non-specific factors such as sunlight, temperature and water velocity. These non-specific environmental cues are usually mediated by visual cues. For example, spawning behaviour of male grayling has been observed to be stimulated by the feeding activities of barbel on the spawning grounds (Poncin, *Unpubl.*). However, Bloom & Perlmutter (1978), while working on species of the cyprinid genus *Brachydanio*, concluded that

spawning in fishes may be controlled by pheromones which attract mates of the same species, but repel those of another. If these conclusions can be verified, such pheromones may be more influential in hybridization for one species and/or sex than the other.

Observations of inter-specific spawning events, between roach and common bream, have not been recorded in the scientific literature. However, there have been studies which have observed the spawning behaviour of these species individually (e.g. Svardson, 1949; Fabricius, 1951; Diamond, 1985), and these indicate that male common bream are more aggressive and territorial than male roach. This being the case, it is important to establish whether male common bream aggressiveness is influential on hybridization with female roach, or if male roach non-territoriality is an important factor which permits them to mix with female common bream during spawning.

Gamete compatibility and embyonic development

There are many examples in the literature which show that while species A male x species B female crosses produce an F1 hybrids, but the reciprocal cross is incompatible. Burkhead & Williams (1991), demonstrated this for European cyprinid rudd (*Scardinius erythrophthalmus* (L.)) with the North American minnow species the golden shiner (*Notemigonus crysoleucas*). In this example the cross of female rudd with male golden shiner was successful, but the reciprocal cross was not. However, in the case of many of the Leuciscinae of the British Isles, including the roach x common bream reciprocal crosses, this is not the case and cannot be used as an argument for the absence of the maternal genotypes in a hybrid population (Chapter 2).

Post hatch survival and fitness

Hybrids which are the product of one cross, e.g. a cross of a male of species A with a female of species B may be substantially less fit than hybrids of the reciprocal cross. These hybrids may therefore not survive to the stage where they can be detected in the adult fish stock and hence one of the parental mtDNA genotypes (species B), will be absent.

7.4.2 Both parental mtDNA genotypes found

Where both of the parental mtDNA genotypes were identified among the hybrid population, the presence of hybrids is probably the result of the chance meeting of gametes and is independent of:

- the proportions of the parent species in the fishery;
- habitat preferences and temporal aspects of spawning;
- aspects of mating behaviour of each species;
- physiological constraints on reproductive activities between the species;
- reciprocal cross gamete compatibility at fertilization;
- differences in embryo development and/or zygote mortalities in reciprocal hybrid crosses;
- that the competitive fitness of an individual hybrid being determined by species-specific sexual ancestry;

7.4.3 Further investigations

The simple cross-breeding programme (Chapter 2) concluded that gamete incompatibility is not an obstacle in reciprocal hybrid crosses. However, to yield sufficient information about species-specific sexual ancestry in hybrid populations in the wild more information is required. To gain further insight into the phenomenon the development of mtDNA analysis as a tool in hybrid studies is of paramount importance. The technique needs to be refined until reliable results can be obtained for cyprinid species (Section 7.4.). Once this has been established additional investigations will be required from hybrid populations in the wild which include:

- assessments of population compositions;
- observations of spawning requirements and behaviour in different hybrid populations;
- comparisons of performance/fitness of recirocal crosses in the wild and under laboratory conditions.

The presence/absence of a particular parental mtDNA genotype among hybrid populations may be the result of the environmental circumstances. More extensive investigations will be required to explain why in some circumstances one of the parental mtDNA genotypes is present in the population but is absent in another. For example, thermal effluents at a location may greatly influence the reproductive activities of two species which have the potential to hybridize. At this location both of the parental mtDNA genotypes might be observed in the hybrid population. However, at another location which is not affected by such an impact only one of the parental mtDNA genotypes might be observed among the hybrids. To assess more fully the reasons enhancing hybridization these locations must be compared with sites which do not contain hybrid populations.

CHAPTER EIGHT
GENERAL DISCUSSION

The results of the experimental breeding programme are important because they indicate clearly that there is not a genetic barrier to gamete compatibility between some of the cyprinid species occurring in the British Isles. Hence, there is great potential for cross-fertilization to occur in natural populations. Indeed, hybridization appears to be widespread among the cyprinid fish stocks of the British Isles. In a recent survey of angling clubs by Midgelow-Marsden (1993), it was propsed that hybrids may be present in 73.6% of fish populations which were fished by anglers. Perusal of records also show that surveys conducted in the central area of the Anglian region of the NRA show that in recent years hybrids are occurring at more sites and in greater numbers (A. Taylor, *pers. comm.*). It is probable that a similar situation exists in other regions. Irrespective of this, it also appears that the fisheries management agencies in the British Isles have conducted little research into either the causes or consequences of hybridization amongst cyprinids. It is essential that this problem is addressed in future work.

While much is known about the ecology of cyprinid species there has been little work which investigated the factors determining species distributions in the UK (Cowx *et al.*, 1993a; 1993b) or their inter-specific hybrids. Hence, it is important to establish the geograhical, macro-habitat features and micro-habitat characteristics which affect the distribution of both species of freshwater fishes and their hybrids. Such assessments are feasible. Indeed Crivelli & Dupont (1987) observed that strong year classes of hybrids in Lake Micri Prespa occurred in years when higher than usual water levels were observed between April and June.

8.1 CAUSES OF HYBRIDIZATION

The reproductive mechanism of external fertilization in water is the critical factor which facilitates inter-specific hybridization amongst fishes. Furthermore, many of the cyprinid species of the British Isles are members of the reproductive guild known as "broadcast spawners" (Balon, 1975) and this particular mechanism of reproduction may increase the possibility of gamete mixing and consequential hybridization. Many cyprinid species also exhibit similar preferences for spawning habitat and the time at which they spawn, e.g. roach and common bream are both phytophilus spawners with spawning activity occurring in late spring to early summer (Wheeler, 1969). The similarity of habitat preference by cyprinids was also noted by Diamond (1985) who observed both roach and common bream spawning on the roots of *Salix* sp. in spite of

the availability of other types of vegetation which were suitable for spawning by both species. Hence, with overlaps in reproductive activities in both space and time, the only mechanism of pre-mating reproductive isolation available to maintain species integrity are those of spawning behaviour (Kwak & Skelly, 1992).

The probability of hybridization among cyprinid species appears to be dependent upon the spawning requirements of each fish (Pepin *et al.*, 1970). These authors suggested that inter-specific hybrids occurred in two different groupings of cyprinids, these being species such as roach, rudd and common bream which belong to a phytophilus reproductive guild, and bleak, chub and gudgeon which are lithophilus spawners. On the basis of this information it may be concluded that inter-specfic hybridization should be confined in the main to species within the same reproductive group. However, this may not always be the case as Wheeler & Easton (1978) have recorded the occurrence of hybrids between chub and roach which belong to different ecological guilds according to Pepin *et al.* (1970) and Balon (1975). The occurrence of this hybrid may be explained by the observations of Holcik & Hruska (1966) who noted roach spawning upon gravels which are also the preferred spawning habitat of chub. They explain this through the high plasticity and adaptability not only of cyprinids in general, but of spawning activity roach in particular, which are contributory factors to hybridization between species which are phylogenetically related. Furthermore, the results presented here regarding gamete compatibility suggest that these two species are capable of hybridizing.

Compounded upon these, many papers suggest that the primary factor which leads to hybridization amongst fish species, including the cyprinids of the British Isles, is the interaction between species for available spawning habitat. Several authors have suggested that there are a number of environmental factors which increase these species interactions which may be either natural or man-made (Weisel, 1954; Hubbs, 1955; Gilbert, 1978; Cooper, 1980; Whitmore & Hellier, 1988). Midgelow-Marsden (1993) attempted to establish the environmental/management factors which enhanced hybridization in the UK. Unfortunately, this met with little success, probably because there is too little information regarding the natural distributions of species and their hybrids. Hence, there is a need for detailed examination of human interferences upon habitats with respect to species and hybrid distributions.

8.1.1 Natural factors

Hubbs (1955) described the natural factors which may lead to hybridization among fishes as those which result in habitat alterations and disturbances. Hubbs (1955)

concluded that hybridization is rare where habitats are more stable i.e. in marine tropical waters. However, in comparison temperate fresh waters have a much less stable environment and therefore have a more frequent occurrence of hybridization. In addition, the climatic changes observed since the Pleistocene have dramatically altered freshwater environments in the temperate regions of the Northern Hemisphere (Campton, 1987). Such alterations to habitat may bring together species which were previously geographically isolated, but are capable of cross-breedng, and so increasing the incidence of hybridization.

8.1.2 Man-made factors

The man-made factors which enhance hybridization may be divided into those which have a direct impact upon the interactions between species for spawning sites, e.g. species introductions, and those which have indirect influences, e.g. habitat alteration. Human impacts have been suggested as being particularly important in some North American studies in which hybridization was observed (e.g. Rakocinski, 1980; Busack & Gall, 1981; Graham & Felley, 1985). By increasing the competition/interaction for spawning sites the probability of hybridization is enhanced between species which are co-dominant, i.e. where both species are able to spawn in the presence of the other. However, where one species is dominant and the other species is not able to co-spawn, the submisssive species will be forced to spawn on secondary habitat reducing the probability of hybridization. It will be necessary to consider these effects in detail when examining the precise nature of these man-made influences because Thoma & Rankin (1988) showed that hybrids of *Notropis chrysocephalus* and *Notropis rubellus* were more common in locations which were relatively undisturbed. Thus, in the investigation of the causes of hybridization the examination of both pristine and impacted sites is required.

Species introductions

Introductions of species has been a practice to enhance poor fisheries. However, the consequences of species introductions have rarely been assessed. This is essential since many authors have cited this factor as one which encourages hybridzation among fishes (Weisel, 1954; Hubbs, 1955; Nelson, 1966; Nelson, 1973; Hambrick, 1976; Daget & Moreau, 1981; Verspoor, 1988; Whitmore & Hellier, 1988). A similar situation probably exists amongst the cyprinids of the British Isles. Indeed, hybridization has been reported to be an important impact of cyprinid introductions, particularly that of roach, into Ireland (Kennedy & Fitzmaurice 1968; Kennedy & Fitzmaurice 1974; Fitzmaurice 1984).

Stocking

Most lowland coarse fisheries have been subject to stocking activities at some time or another (Pearce, 1983; Hickley, 1994). In general, these are for the purposes of fishery enhancement. However, this may cause imbalances in species abundances and therefore enhance the possibility of competition for spawning habitat between the species (Hubbs, 1955; Whitmore & Hellier, 1988).

Habitat alteration

Canalization, river engineering works and aquatic management practices, e.g. weed cutting, have been referred to as contributing factors to the phenomenon of hybridization amongst species of fish. Such works result in the reduction of the available spawning habitat and so increases the inter-specific competition for what remains (Weisel, 1954; Hubbs, 1955; Edwards, 1979; Bianco, 1982; Miller & Behnke, 1985; Bianco, 1988; Elvira, 1990). In the British Isles these aquatic habitats are usually inhabited by many of the native cyprinid species (Wheeler, 1969). Furthermore, many of the cyprinids which are found in these habitats display similar preferences in terms of their spawning requirements. Such habitats must therefore be the focus of investigations to determine the mechanisms through which human impacts result in enhanced hybridization. Unfortunately, little is known, in quantitive terms, regarding either the habitat requirements or the impact of man on the cyprinid populations of the British Isles (Cowx *et al.* 1993a; Cowx *et al.*, 1993b).

Obstructions to migration

Many species of cyprinid are known to have spawning migrations and these include both roach (L'Abee-Lund & Vollested, 1985; Vollested & L'Abee-Lund, 1987) and common bream (Whelan, 1983). If there are obstructions to these migration runs, in the form of dams, locks, weirs or poor water quality fish may spawn among habitats which are far from their preferred area and are occupied by other species. This raises the potential for cross-fertilization and such obstructions have been cited on two occasions as the cause of inter-specific hybridization among European cyprinids (Economidis & Sinis, 1988; Balon, 1992).

Navigation.

Boat traffic in aquatic environments is also known to have impacts upon the marginal vegetation habitat (Murphy & Eaton, 1983), which is the preferred spawning habitat of phytophilous spawners. This is because of damage caused by boat hulls, propellors and their backwashes which increases bankside erosion and disturbs marginal vegetation. As a result the amount of available spawning habitat may be reduced which will increase competitive pressures on remaining spawning grounds and hence the probability of cross-fertilizations giving rise to inter-specific hybrids.

8.2 DETECTION OF HYBRIDIZATION

A primary objective of fisheries management is to understand how fish stocks interact with each other and their environment. To meet these objectives it is important to make accurate assessments of the fish stocks and this requires reliable methods of fish identification. Hence, it is important to have techniques which are capable of detecting hybridization and are able to distinguish between pure-bred species, F1 generation hybrids and post-F1 generation hybrids. The study has shown that the traditional techniques of taxonomy, such as meristics characters, may not be suitable in all cases where hybrid identity needs to be established. This is because of the assumptions associated with hybrid identification:

- the identity of the parent species are assumed;
- the hybrids are of the F1 generation;
- and that hybrid appearance is intermediate between their parent species.

These assumptions have implications when assessing hybridization using traditional techniques in the following circumstances:

- one or both parent species exhibit great morphological variation;
- the appearance of the hybrid shows morphological variability;
- post-F1 hybridization occurrs.

Clearly as this study has suggested in Chapters 5 & 6, the adoption of genetic techniques, such as enzyme electrophoresis, alongside the analysis of meristic and morphometric information, will provide great advantages in understanding what is happening in fish stocks because these methods determine with reliability whether:

- fish are pure species or hybrids;
- the hybrids are F1 or post F1 generation.

The advantages of such techniques are enhanced further when, as was attempted in this study in Chapters 3 & 4, pure-bred species and hybrids of known ancestry can be used as references.

In terms of stock assessment these methods detect the occurrence and the extent of F1 hybridization. However, what is more important for fishery management, and its role in maintaining the genetic integrity of species, is that they are also able to establish whether post F1 hybrids are present and therefore provide evidence that F1 hybrids may be reproductively active. It is known that F1 hybrids are fertile (Wood & Jordan, 1987;

Chapter 2), but few studies have been able to establish whether F1 hybrids are reproductively active in wild populations because the traditional methods of identification have precluded this evaluation.

8.3 HYBRIDIZATION IN NATURAL POPULATIONS

On the basis of the meristic and genetic information available it was concluded that there were no post-F1 hybrid fish in the two wild populations studied here. The reasons for their absence were outlined in Section 5.4.2. If the reasons for not detecting these fish are biological then it may be suggested that species integrity is indeed being maintained because genetic exchange will not be occurring (Mayr, 1963). Other authors working on cyprinid hybrids in Europe have also observed that appearance was strictly intermediate between their parents and therefore concluded that post F1 hybrids were not present, e.g. Cowx, (1983); Crivelli & Dupont, (1987). However, these and other studies also observed fertile and spent hybrids, e.g. Cowx, (1983); Crivelli & Dupont, (1987); Fahy *et al.*, (1988). Hence, providing that the identification of F1 hybrids in these studies are reliable, it would appear that in these cases the absence of post F1 hybrids is the result of either the lack of reproductive activities (Cowx, 1983) or post-hatch mortality (Greenfield & Deckert, 1973; Avise & van den Avyle, 1984). This aspect is a possibility as Bigelow (1965) described the theoretical existence and maintenance of stable hybrid popualtions, in which species integrity is maintained through selection against the F1 hybrids in spite of the extent of F1 inter-specific hybridization. This corroborates to some extent the explanations of Barton & Hewitt (1981; 1983; 1985), that co-adapted gene complexes may play a role in the maintenance of stable F1 hybrid populations (Moore, 1977; Section 5.4.2).

Although genetic isolation is maintained under these circumstances it does not agree with the theory that pre-mating mechanisms of genetic isolation are more efficient than post-mating mechanisms in terms of ecological energetics (Greenfield & Greenfield, 1972). In this scenario it is assumed that, if some of the energy of reproduction of the species concerned is invested in cross-fertilization, the mechanisms of reproduction are inefficient in terms of energy conservation. Hence, the question of the evolutionary importance of post-mating isolating mechanisms must be assessed since recently evolved taxa appear to be more likely to hybridize. The presence of F1 hybrids between the species is evidence that they are closely related in terms of genetics. Indeed, they may have evolved from a common ancester. However, in circumstances where genetic integrity is maintained by post-mating isolation mechanisms, there will be gene loss from the two species. This may have evolutionary significance because this may be a mechanism of genetic divergence between the species.

8.4 HYBRIDIZATION AND EVOLUTION

8.4.1 Hybridization and introgression

The inter-specific cross-fertilization observed in the natural cyprinid fish stocks of the British Isles is that which is termed sympatric hybridization (Woodruff, 1973), the extent of which is determined by local circumstances. Where the degree of hybridization is low the potential for post F1 hybridization is also low. This is a major contributory factor to the absence of post F1 hybrids in many studies including this one. In some cyprinid fisheries however, suspected F1 hybrids may account for up to 40% of the stock as recorded in Ireland by Fahy *et al.* (1988). Hence, in these circumstances there is a much greater probability for hybrids to become involved in reproductive activities. Where post F1 hybridization is frequent there may be the potential for the complete breakdown of reproductive isolation which results in a single merged hybrid population which exhibits a range of characteristics between the two species. In cases where post F1 hybridization is less common gene introgression may occur (Anderson & Hubricht 1938). Introgression is the incorporation of the genes from one species into the gene pool of another and could be an important source of genetic variation within fish taxa (Verspoor & Hammer, 1991), particularly if these genes are advantageous (Barton & Hewitt, 1985). This is an issue which is of critical importance to biodiversity and its management.

Introgression has not been identified in cyprinid stocks in the British Isles or continental Europe. However, in North America Menzel (1976; 1977) and Dowling *et al.* (1989) have suggested that gene introgression, as a result of hybridization, has played a critical role in the evolution of the cyprinid genus *Notropis*. This highlights the need for more genetic assessments of fish populations in the UK to identify the importance of gene introgression in the cyprinid species which are known to hybridize.

There are two problems in assessing the importance of introgression in hybridization studies. The first is the point at which hybridization results in introgression is difficult to define since there is no pre-determined basis for deciding which generation of back-crossing that the genes are considered to be introgressed (Verspoor & Hammer, 1991). Furthermore, identifying introgressed genes is problematic because genes may also be inherited through common ancestral origins of the two species and it is also possible that identical genes may be acquired independently (Avise & Saunders, 1984). In considering these points Verspoor & Hammer (1991) concluded that the extent and evolutionary importance of introgression would remain uncertain until genetic techniques are capable of dealing with the problem.

8.4.2 Unisexual Hybridization

The classical form of hybridization discussed in this study is that of heterospecific cross-fertilization which is the fusion of two haploid gametes from two species to form a diploid hybrid. Chevassus (1983), however, identified that other forms of hybridization were possible and that the form of these was dependent upon the ploidy of the genetic input of the parental gemetes:

- triploid hybrid (female haploid x male diploid);
- triploid hybrid (female diploid x male haploid);
- tetraploid hybrid (female diploid x male diploid).

Indeed, there are many accounts of these types of hybridization in the literature (e.g. Climino, 1972; Joswiak *et al.*, 1982; Dawley *et al.*, 1987; Collares Pereira, 1989). In many of these examples the polyploid F1 hybrids are all females and this maintains the F1 status and precludes gene exchange between the species. Nevertheless, the high DNA levels of these polyploids plays an important role in their evolution through adaptive radiation and hence, ultimately speciation (Schultz, 1977). The importance of these processes in the cyprinids of the British Isles is unknown and it is clear that rigorous karyological and biochemical analysis of hybrids, from wild populations and those of experimental breeding, are necessary.

8.5 PERFORMANCE OF HYBRIDS

Whether they are fertile or not, the presence of F1 hybrid fish in natural populations poses important questions regarding their impact upon aquatic ecosystems, e.g:

- do they occupy a particular niche and what is the impact on the parent species?
- is their performance similar, superior or inferior?

8.5.1 Heterosis and hybrid vigour

The impact of hybridization may have direct and detrimental impacts upon species. For example, Amarasinge & De Silva (in press) have provided evidence of how hybridization may have a damaging effect on the reproductive capabilities of tilapian species in reservoir fisheries. However, in many animal populations specimens which are the product of inter-specific hybridization may exhibit superior characteristics of performance in comparison to their parent species. This feature is attributed to

heterosis, where hybrid benefit from the acquisition of dominant favourable genes from both species to obtain genetically improved stocks, and hybrid vigour, which is the diversion of energy from reproduction into growth.

Where this occurs in natural fish populations there may be serious implications for the ecology of the system. For example, Svardson (1949) observed a faster growth rate of trout (*Salmo trutta*) x charr (*Salvelinus alpinus*) hybrids in Norway. Where performance of cyprinid hybrids have been examined however, the conclusions have been somewhat contradictory. Kanno (1968) and Crivelli & Dupont (1987) observed the enhanced growth performance of *Leuciscus cephalus* x *Alburnus alburnus* and *Rutilus ribilio* x *Alburnus alburnus* hybrids respectively when compared to their parent species. In contast Bianco (1982) working on *Alburus albidus* x *Leuciscus cephalus cabeda* hybrids and Pethon (1978), Cowx (1983) and Fahy *et al.* (1988), working on hybrids of roach and common bream, all observed that hybrid growth was intermediate to the parent species and so concluded that there was an absence of heterosis. Cowx (1983) noted that up to year class IV the growth of roach x common bream hybrids in the River Exe matched that of common bream, the faster growing species, but thereafter the growth of common bream was much greater than the hybrids.

In an associated study scales were taken from hybrids and parent species from the Forty Foot Drain and Essex University Lake natural populations (Bracewell, 1994). A rigorous analysis of the growth patterns and performance of these fish was precluded because of the restrictions imposed on removal of fish for the study, i.e. only those smaller than 15 cm. However, from the scales available average lengths at each age were back-calculated for fish up to age class IV. This was carried out for hybrids and parent species from both the Forty Foot Drain and Essex University Lake (Tables 8.1 & 8.2). The expected lengths for the hybrid groups were calculated by taking the mean of the back-calculated length at age for the parent species, i.e. the expected length of two year old roach x common bream hybrids in the Forty Foot Drain was calculated thus ((89.7+114.2)/2) = 102.0. The expected mean lengths of the hybrids were then compared with the observed back-calculated lengths. In all age classes and at both locations the expected hybrid lengths were greater than the observed lengths suggesting that the growth rates of the hybrids were inferior to the parent species (Table 8.1; 8.2). Analysis of the results indicated that statistically significant differences occurred between the observed and expected values for age groups II and III for the roach/common bream hybrids and age groups II and IV of the rudd/common bream hybrids (Bracewell, 1994).

Table 8.1 Back-calculated length at age for roach, common bream and their hybrids in the Forty Foot Drain, Cambridgeshire.

	Age class			
Fish type	I	II	II	IV
Roach (n=72)	69.9	89.8	111.5	135.7
Common bream (n=41)	79.9	114.2	158.1	196.2
Hybrids - expected	74.9	102.0	134.8	165.9
Hybrids - observed (n=46)	57.6	84.5	112.7	143.1

Table 8.2 Back-calculated length at age for rudd, common bream and their hybrids in the Forty Foot Drain, Cambridgeshire.

	Age class			
Fish type	I	II	II	IV
Rudd (n=72)	89.4	103.1	113.1	121.8
Common bream (n=41)	94.8	125.3	150.7	178.7
Hybrids (expected)	92.1	114.3	131.7	150.2
Hybrids (observed) (n=46)	75.4	91.6	113.8	136.8

8.5.2 Mortality rates

Bracewell (1994) also examined mortality rates of the fish in the Forty Foot Drain by linear regression analysis of $\log_e$ numbers against age. This was done using information from the NRA on the age class structure of roach and common bream and from the limited amount of data that could be used from the hybrid fish which were removed from the fishery. The mortality rates for roach and common bream were calculated to be 0.36 and 0.42 respectively. However, the mortality rates of the hybrids was found to be much greater at 0.51, suggesting that hybrid survival was inferior to both of its parent species.

The reasons for the apparent poor survival of the hybrids is not known, but similar survival was also observed in bass hybrids in North America (Dowling & Moore, 1985). Dupont & Crivelli (1988) noted that hybrids of *Rutilus rubilio* x *Alburnus alburnus* in Lake Micri Prespa, Greece, had higher than expected parasite loadings when compared to their parent species, and that this may account for the absence of post F1 hybrids in the population. The authors considered this increased parasite loading on the hybrids as being the result of their spatial distribution which overlapped both of those of their parent species, their more varied feeding habits and a reduction in their parasite defence mechanisms.

At both locations examined in this study the meristic and genetic evidence suggested that all hybrids were of the F1 variety. If this is the case it may be suggested that the inferior growth performance and survival rates may be a major factor contributing to the absence of post F1 hybrids in these populations since they may be not able to achieve sexual maturity.

8.5.3 Feeding and diet analysis

The potential competitive interactions of feeding may also be used to explain the performance of hybrids in comparison to their parent species. Dietary studies on hybrids will demonstrate the extent of niche overlap with the parent species and so will suggest whether competition exists in their trophic interactions. Dupont & Crivelli (1988) suggested from diet analysis, that the trophic niche of *Rutilus rubilio* x *Alburnus alburnus* hybrids was distinct from both of their parent species. However, Cowx (1983) working on the River Exe and Fahy *et al.*, (1988) working on Leixlip Reservoir found that the diet of roach x common bream hybrids was similar to roach. This may explain why *Rutilus rubilio x alburnus alburnus* hybrids had an enhanced growth rate but roach x common bream hybrids had an intermediate growth rate in these studies.

Such observations in natural populations emphasise the need for further field studies to investigate the ecological aspects of hybrid performance. the field studies should be supported by habitat information to ascertain whether hybrids are able to perform better under particular environmental circumstances. In addition, if this information could be supported with data from mtDNA studies it may be possible to discover if there is an influence of the maternal ancestry of the hybrids in determining performance. These types of field studies could be greatly enhanced if supported by laboratory investigations into competition using hybrids of known ancestral origin.

8.6 IMPLICATIONS FOR FISHERIES MANAGEMENT

8.6.1 Aquaculture

Aquaculture can utilize hybridization to exploit heterosis and hybrid vigour to obtain improved production. Developments of this kind are more important in countries where fish produce is the major source of protein in the diet. However, most of the hybrid fish produced for such purposes have had little practical value (Purdom, 1993). For example, Foerster (1968) experimented unsuccessfully with inter-species crosses of *Oncorhycus nerka*, a species which produced good quality flesh, and *Oncorhyncus keta*, a fish which grows rapidly. Bass hybrids between *Morone saxatilus* and *Morone chrysops* have however, been shown to be superior to their parent species, e.g. Bishop (1968), Yeager (1985) & Jahn *et al.* (1987). Detailed accounts of the use of hybrids in aquaculture are reviewed by Lovshin (1982) for cichlids and Hickling (1968) for cyprinids.

8.6.2 Rehabilitation

The decline of the native lake trout (*Salvelinus namaycush*) was observed in the North American Great Lakes. It was concluded that its decline was the result of the effects of human activities and predation by lampreys (Christie, 1960). To combat this Tait (1970) experimented with crosses of lake trout and brook trout (*Salvelinus fontinalis*), to produce hybrids known as splake trout. The hybrids had increased swim-bladder function, which allowed them to forage in deeper water away from lampreys, and matured earlier than the native lake trout. However, the project met with only limited success.

8.6.3 Aquatic management

Hybrids may come to play a vital role in the management of aquatic systems because, where applicable, their sterility will ensure that there is no long-term impact on the gene pools of the system.

Hybrids of male grass carp (*Ctenopharyngodon idella* (Val)) and female bighead carp (*Hypophthalmichthys nobilis* (Rich.)) have been used in North America for the biological control of weeds (Sutton *et al.*, 1981; Cassini & Caton, 1983). They have been used as a substitute for grass carp, which is a non-native species to North America, because the hybrids are triploids and so are theoretically sterile (Marian & Kraznai, 1978; Beck *et al.*, 1980). Grass carp had been introduced for weed control in a number of cases in North America. However, this is now prohibited because of the apparent reproductive success of grass carp which may pose a threat to the native species. Hence, the hybrids can be used as part of a controlled stocking programme with little long-term impact on the environment. There are obvious benefits of using this type of weed management, in comparison to pesticides or weed-cutting, since their is minimal long-term impacts on the environment.

8.6.4 Genetic conservation

The cyprinids of the British Isles are a valuable biological resource and they are important not just for their wildlife value, but also in social and economic terms to the two million anglers in the UK.

Both species diversity and genetic variability are valuable resources for sustained evolution and adaptation. This is further emphasised by the increased influence of human activities on the environment. Hence, it is important to assess the levels of genetic variability that exists in the present day cyprinid species on a nationwide scale and to identify the populations which show genetic importance in terms of their genetic diversity.

Hybridization poses a threat to this genetic variability among species and under some circumstances could result in the loss of unique genes or even genetically distinct populations. Therefore, in addition to genetic studies, assessments of the environmental influences which enhance hybridization on popualtions are also required. Once these have been evaluated measures may be taken to mitigate the impact of these influences particularly where populations are thought to be important for biodiversity. At present

there does not appear to be a practical strategy for the genetic conservation of fish stocks (Ryman, 1991). However, it is probable that the methods adopted will vary with circumstance and will depend on the extent of the pressures of human activities. While it may be more aesthetically favourable to conserve these stocks through pollution and habitat management, the reality of many situations may preclude this. Indeed, in some situations the formation of gene-banks or hatchery reared stocks may be the only viable solution (Taggart, 1981).

8.7 SUGGESTIONS FOR FUTURE WORK

The suggested outline for future hybridization studies follows two strategies:

- An extensive experimental cross-fertilization programme;
- A more focussed assessment of one hybrid type, probably roach/common bream.

8.7.1 Experimental breeding programme

It is essential to repeat and broaden the number of species in an experimental breeding programme. Where necessary cryogeneic preservation of gametes will be used to allow the inclusion of cyprinids such as tench and dace, whose spawning periods lie outside those of the majority of cyprinids in the British Isles. These techniques will also permit the inclusion of European and North American cyprinids and so elucidate further phylogenetic relationships on the basis of their gamete compatibilities. Where and when possible hybrids should be included in the programme to investigate their fertility and potential to produce post F1 hybrids.

These studies should be supported by studies which investigate the biochemical and karyological aspects of hybridization amongst cyprinid species. This will ascertain which crosses produce diploid, triploid, tetraploid and unisexual hybrids.

8.7.2 Research focussed on a particular hybrid type (e.g. roach/common bream)

To assess a greater understanding of the problem of hybridization in wild populations it is suggested that research should be concentrated into the following five areas.

Cross-breeding

Cross-breeding experiments to investigate reciprocal cross success and the fertility of both male and female F1 hybrids. This should be repeated for a wide number of populations to ensure that there is consistency in the results of gamete compatibilities.

Spawning activities

Observations of the species spawning in the wild should be carried out alongside environmental surveys to compare the conditions under which interbreeding does or does not occur. These observations may also be used in conjunction with other methods, e.g. mtDNA, to assess aspects of mating behaviour where hybridization is occurring.

Laboratory studies

An array of laboratory studies to compare the performance of hybrids to their parent species. These could include the following:

- long-term competition studies;
- investigations into prey capture efficiencies of species and their hybrids using techniques developed by Winfield (1983);
- physiological experiments to investigate the effects of differing environmental circumstances, e.g. temperature and oxygen;
- swimming abilities of the fry of hybrids and parent species.

Natural population studies

Natural population studies of hybrids are necessary to investigate the following:

- identify parent species and hybrids using genetic techniques;
- determine the conditions which are favourable to hybridization;
- compare preformance using growth analysis, condition factors, mortality and fecundity ;
- assess niche overlaps with extensive feeding and diet studies.

Mitochondrial DNA studies

The development and use of mitochondrial DNA techniques will be essential for all aspects of future hybrid work. In the first instance the techniques may be used to determine maternal ancestry. However, it will also be possible to use the techniques in conjunction of other aspects of hybrid investigation, e.g. the influence of maternal ancestry on hybrid ecology and *vice versa*. For example, Dowling *et al.*, (1989) found

that there were differences between the mtDNA genotype of hybrids between *Notropis chrysocephalus* and *Notropis cornutus* depending upon whether the drainage systems were flowing eastwards or westwards.

Management actions

The environmental data which are collected maybe used to identify the conditions which enhance hybridization. When these have been identified it will be possible to identify possible ameliorative management options. These will be variable and will depend upon individual circumstances. The ease with which the problem can be solved, the extent of other pressures and the importance of the stock itself, will all be issues taken into consideration regarding the type of action taken.

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Appendix A1. Meristic features of *Rutilius rutilus* (L.) according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Spillman (1961)	42-45	7-8	4-5	9-11	5:5,6:5
Berg (1949)	41-48	7-8.5	3-4.5	10-11	5:5,6:5
Wheeler (1969)	42-45	7-8	4-5	9-11	5:5,6:5
Barr, Evans & Jones (1972)	41-48	7-8	3-4	9-12	5:5,6:5
Maitland (1972)	42-45	-	-	-	5;5,6:5
Wheeler (1978)	42-45	-	-	9-11	5:6
Witkowski & Blachuta (1980)	40-46	7-9	3-4	10-12	-
Cowx (1983)	40-48	7-9	4-5	8-14	5:5,6:5
Blachuta & Witowski (1984)	42-44	8-10	3-4	10-12	5:5
Wood (1985)	42-46	9-10	4-6	10-13	5:6,5:5
Economidis & Wheeler (1989)	42-44	-	-	10-11	5:5,6:5
Adams & Maitland (1991)	41-44	8-10	-	10-12	-

Appendix A2. Meristic features of *Abramis brama* (L.) according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Spillman (1961)	50-58	10-15	6-8	23-28	5:5
Wheeler (1969)	51-60	-	-	24-30	5:5
Maitland (1972)	49	11-15	-	25	-
Bagenal (1973)	51-60	-	-	24-30	5:5
Witkowski & Blachuta (1980)	49-56	10-14	6-8	23-29	-
Cowx (1983)	53-64	10-15	7-10	23-30	5:5
Blachuta & Witkowski (1984)	51-57	13-15	5-7	23-37	5:5
Mulrooney & Fahy (1985)	49-57	-	-	24	-
Wood (1985)	53-59	12-15	7-9	25-31	5:5
Economidis & Wheeler (1989)	55-57	-	-	23-25	5:5
Adams & Maitland (1991)	53-57	13-14	-	25-28	-

Appendix A3. Meristic features of roach/common bream hybrid according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Spillman (1961)	47-51	8-11	5-6	15-17	5:5,5:6
Wheeler (1969)	42-54	-	-	15-20	5:5 ??
Child & Solomon (1977)	-	-	-	18-19	-
Pethon (1978)	43-57	-	-	14-19	-
Witkowski & Blachuta (1980)	48	9	4-5	15-17	5:5
Cowx (1983)	48-55	8-11	5-7	16-21	5:5,6:5 or 6:5.1
Blachuta & Witkowski (1984)	44	9	4	14	5:5
Mulrooney & Fahy (1985)	47-52	-	-	15-19	-
Wood (1985)	47-52	9-12	6-7	15-19	5:5,6:5 or 5:6.1
Economidis & Wheeler (1989)	48	-	-	15	5:5
Adams & Maitland (1991)	48-52	11-12	-	16-18	-

Appendix A4. Meristic features of *Scardinius erythrophthalmus* (L.) according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Spillman (1961)	40-45	7-8	3-6	12-14	3.5:5.3
Berg (1964)	38-42	-	-	13-14	3.5:5.3
Wheeler (1969)	40-45	7-8	3-5	10-11	3.5:5.3
Barr, Evans & Jones (1972)	40-44	7-8	3-4	14-17	3.5:5.3
Maitland (1972)	40-43	-	-	-	3.5:5.3
Bagenal (1973)	40-45	-	-	10-11	3.5:5.3
Wheeler (1976)	40-45	-	-	-	3.5:5.3
Burrough (1981)	-	-	-	-	3.5:5.3
Economidis & Wheeler (1989)	38-42	-	-	11	3.5:5.3 2.5:5.2

Appendix A5. Meristic features of *Blicca bjoerkna* (L.) according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Spillman (1961)	45-49	8-10	5-6	19-22	2.5:5.2
Wheeler (1969)	44-48	-	-	21-23	2.5:5.2
Maitland (1972)	50	8-11	-	27	-
Bagenal (1973)	44-48	-	-	21-23	2.5:5.2
Wheeler (1978)	44-48	-	-	21-23	2.5:5.2

Appendix A6. Meristic features of roach/rudd hybrid according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Spillman (1961)	39-42	-	-	10	5.1 or 5.2
Wheeler (1969)	39-42	-	-	10	5.1 or 5.2
Wheeler (1976)	-	-	-	-	5:6 or 5:5 or 1.5:5,5:5.1 or 1.5:5.1
Mulrooney & Fahy (1985)	41-43	-	-	11	-

Appendix A7. Meristic features of rudd/common bream hybrid according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Wheeler (1969)	46-50	-	-	15-18	5:5 to 2.5:5.2
Mulrooney (1985)	46-50	-	-	15-18	-
Economidis & Wheeler (1989)	48	-	-	16-17	6.1:2.5 5.2:2.5

Appendix A8. Meristic features of common bream/silver bream hybrid according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Spillman (1961)	-	-	-	21-25	5.1:2.5
Wheeler (1969)	48-55	-	-	23-26	5.1:2.5
Swinney & Coles (1982)	51-56	9-14	7-8	22-26	-

Appendix A9. Meristic features of roach/silver bream hybrid according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Spillman (1961)	42-45	-	4	14-18	5:5,5:6 5.1:5.2
Wheeler (1969)	43-46	-	-	14-16	5:5,5:6 5.1:5.2
Penczak (1978)	47-48	8-9	5	-	5,5
Swinney & Coles (1982)	42-46	9	5-6	15-17	5:5,5:6 5.1:5.2

Appendix A10. Meristic features of *Leuciscus cephalus* (L.) according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Wheeler & Easton (1978)	44-46	-	-	7-9	5.2:2.5

Appendix A11. Meristic features of roach/chub hybrid according to recent authors

Author	LLS	DLS	ALS	AFR	PBF
Wheeler & Easton (1978)	43-44	-	-	8-9	5.1:1.6

APPENDIX B Enzyme stains used for gel electrophoresis

AK	E.C. 2.7.4.3
4.0ml	Tris, pH 7.0
20.0mg	D-glucose
10.0mg	ADP
15.0ul	Hexokinase
5.0ul	G-6PDH
0.5ml	$MgCl_2$
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

ADH	E.C. 1.1.1.1
4.0ml	Tris, pH 7.0
0.5ml	ethanol
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

AAT	E.C. 2.6.1.1
4.0ml	Tris, pH 8.0
0.5ml	1.0M NaOH
20.0mg	L-aspartic acid
10.0mg	a-ketoglutaric acid
	Adjust pH 8
25mg	Fast Blue BB
2.0ml	Agar

CK	E.C. 2.7.3.2
4.0ml	Tris, pH 8.0
20.0mg	D-glucose
10.0mg	Creatine phosphate
10.0mg	ATP
5.0ul	G-6PDH
15.0ul	Hexokinase
0.5ml	$MgCl_2$
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

EST E.C. 3.1.1.1

4.0ml	Tris, pH 8.0
0.2ml	Naphthyl acetate solution
	20mg 1-naphthyl acetate
	20mg 2-naphthyl acetate
	2ml Acetone
2.0ml	Agar

FUM E.C. 4.2.1.2

4.0ml	Tris, pH 8.0
50.0mg	Sodium fumarate
30.0ul	MDH
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

GDH E.C. 1.4.1.3

4.0ml	Tris, pH 8.0
50.0mg	Glutamic acid
	Adjust pH 8
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

G-6PDH E.C. 1.1.1.49

4.0ml	Tris, pH 8.0
20.0mg	D-glucose
1.5ml	NADP
0.5ml	$MgCl_2$
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

GPDH E.C. 1.1.1.8

4.0ml	Tris, pH 8.0
40.0mg	DL a glycerophsphate
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

HEX	E.C. 2.7.1.1
4.0ml	Tris, pH 7.0
20.0mg	D-glucose
10.0mg	ATP
0.5ml	$MgCl_2$
10.0ul	G-6PDH
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

IDH	E.C. 1.1.1.42
4.0ml	Tris, pH 7.0
10.0mg	Isocitric acid
0.5ml	$MgCl_2$
1.5ml	NADP
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

LDH	E.C. 1.1.1.27
4.0ml	Tris, pH 7.0
1.0ml	Lactic acid
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

MDH	E.C. 1.1.1.37
4.0ml	Tris, pH 8.0
10.0mg	Malic acid
	Adjust pH 8
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

ME	1.1.1.40
4.0ml	Tris, pH 8.0
10.0mg	Malic acid
	Adjust pH 8
0.5ml	$MgCl_2$
1.5ml	NADP
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

MPI E.C. 5.3.1.8

4.0ml	Tris, pH 8.0
10.0mg	D-mannose-6-phosphate
5.0ul	PGI
20.0ul	G-6PDH
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

PEP E.C. 3.4.11

4.0ml	Tris, pH 7.0
5.0mg	Val-Leu
0.5ml	Dimethyl sulphoxide
5.0mg	Amino acid oxidase
5.0mg	Peroxidase
0.5ml	$MgCl_2$
4.0mg	3-amino-9-ethyl carbozole
2.0ml	Agar

PGM E.C. 2.7.5.1

4.0ml	Tris, pH 8.0
10.0mg	Glucose-1-phosphate
20.0ul	G-6PDH
0.5ml	$MgCl_2$
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

6PGDH E.C. 1.1.1.44

4.0ml	Tris, pH 8.0
10.0mg	6-phosphogluconic acid
0.5ml	$MgCl_2$
1.5ml	NADP
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

PGI E.C. 5.3.1.19

4.0ml	Tris, pH 8.0
10.0mg	Fructose-6-phosphate
10.0ul	G-6PDH
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar

SDH E.C. 1.1.1.14

4.0ml	Tris, pH 8.0
50.0mg	Sorbitol
1.5ml	NAD
0.5ml	MTT
0.5ml	PMS
2.0ml	Agar