

Chapter 4

Progress in Carp Genetics Research

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4.1 Introduction

Ten years ago, very little was known about the genetics of most of the cyprinid species of interest to aquaculture in Asia, for example the Chinese carps, silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*) and grass carp (*Ctenopharyngodon idella*); Indian major carps, catla (*Catla catla*), rohu (*Labeo rohita*) and mrigal (*Cirrhinus cirrhosus*); and the silver barb (*Barbonymus gonionotus*). The exceptions to this would have been the common carp (*Cyprinus carpio*) and the goldfish (*Carassius auratus*). Still relatively little is known about the genetics of most of these species, but significant progress has been made in the last decade. Valuable information about the genetic structure of wild and captive populations and traits of interest to aquaculture are now becoming available and our knowledge of these areas should increase greatly over the next decade, as should the applications of genetics to the aquaculture of cyprinids.

This chapter reviews progress in genetics research that is relevant to aquaculture of cyprinid species in Asia. It begins with cytogenetic (4.2) and molecular (4.3) techniques that can be used to provide basic genetic descriptions of cyprinid species and populations, as well as having applications in aquaculture genetics. Hybridization studies, of which many have been carried out, are dealt with next (4.4). Quantitative genetics, the main focus of this publication (4.5), is divided into four subsections (inbreeding and negative selection; current status; stock/strain comparisons; and selective breeding). Chromosome set manipulations (4.6), sex determination and its manipulation (4.7), gene transfer (4.8) and finally applications of cryopreservation (4.9) and tissue culture (4.10) to aquaculture genetics research complete the review.

4.2 Cytogenetics

Basic cytogenetics studies, describing chromosome numbers and morphology, have

been carried out on many of the cyprinid species of interest to aquaculture in Asia. Much of this information, and equivalent information on other cyprinids (as well as other groups of fish), is summarized by Klinkhardt et al. (1995). It is evident from a frequency distribution of diploid chromosome numbers for cyprinid species taken from this database that $2n = 50$ is by far the most common diploid chromosome number in cyprinids, and also that polyploidisation (tetraploidy and hexaploidy) has occurred in several cyprinids (Fig. 4.1). Ancestrally tetraploid species (approximately 100 chromosomes) are found in the genera *Carassius*, *Cyprinus*, *Spinibarbus* and *Tor*, while *Schizothorax taliensis* and *S. yunnanensis* are ancestrally hexaploid (with 148 chromosomes). In addition to being ancestrally tetraploid, *C. auratus* and *C. gibelio* also have forms that are triploid (relative to the species' ancestrally tetraploid chromosome complement, i.e. they have approximately 150 chromosomes). Reddy (1999) described karyological studies on Indian cyprinid species in some detail. Griffith et al. (2003) demonstrated an increase in the lifespan of fish with larger genomes, from an analysis of a wide range of species. Within individual orders such as the Cypriniformes the relationship was positive but not significant, possibly because of the limited number of samples analyzed.

Perhaps curiously, the number of fundamental chromosome arms (NF) described by various authors in the database of Klinkhardt et al. (1995) showed much more intraspecific variation than the diploid chromosome counts. It is not clear if this reflects genuine polymorphism or differences in interpretation by different authors.

Nucleolus organizer regions (NORs) have been studied in several species of Asian cyprinids (e.g. Carman et al. 1993; John et al. 1993; George et al. 1994; Anjum and Jankun 1998). George et al. (1994) showed that the NORs varied between Indian major carp species. Lakra and Krishna (1996) reviewed chromosome banding

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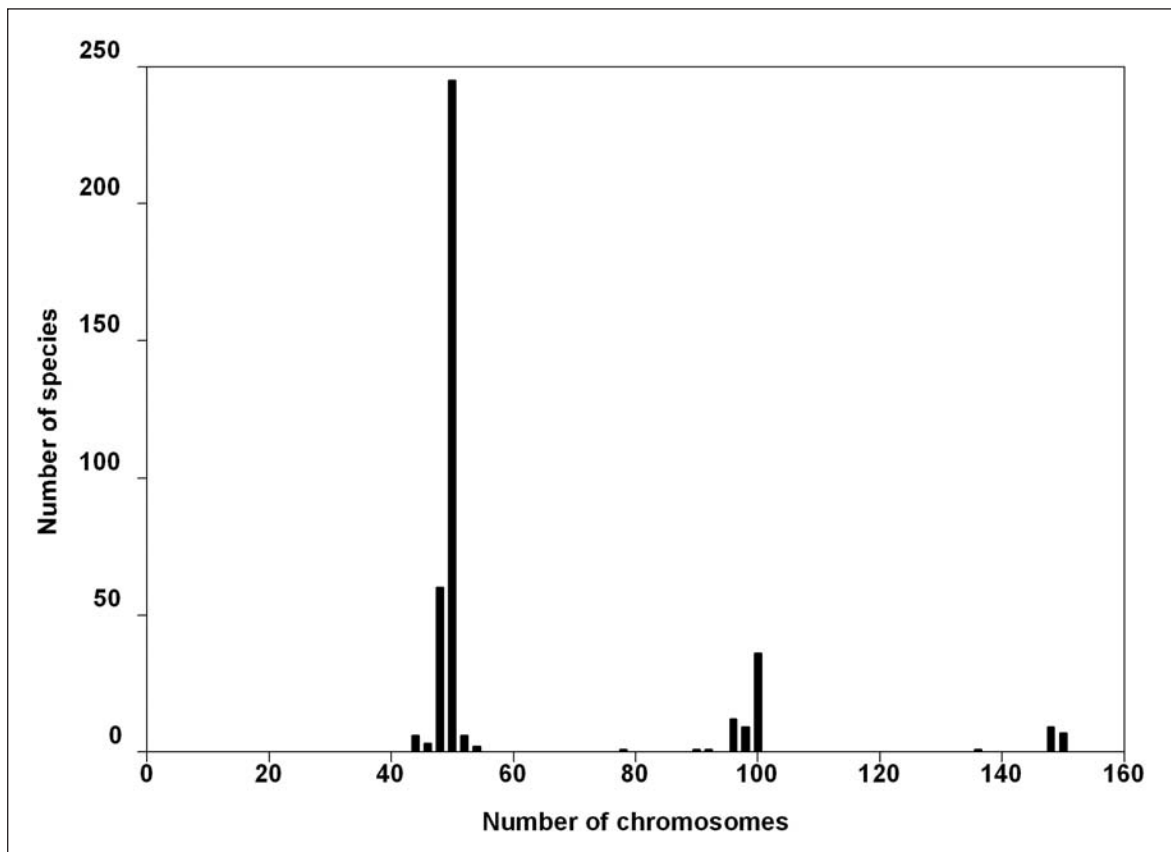


Fig. 4.1. Frequency distribution of the diploid number of chromosomes in cyprinid species (worldwide). Source: Klinkhardt et al. (1995). Where more than one value is given per species in the database, the most common value is shown (in the case of a tie, the lower value is shown). The only cyprinid species in the database that is not shown is *Ptychobarbus dipogon* ($2n = 446$)

techniques for fish and described G-banding for the first time in Indian major carps. Luo (1998) used trypsin digestion and Giemsa staining to produce chromosome-specific banding in the grass carp. This technique may go some way to overcoming some of the general difficulties in obtaining high resolution banding patterns in fish chromosomes (Goodier and Davidson 1993).

Chromosomes can be used as genetic markers in studies on hybridization, induced polyploidy, gynogenesis and androgenesis. While the techniques involved to prepare metaphase spreads from large numbers of fish or embryos are fairly laborious, they are also well standardized.

Distinguishing triploid and diploid fish using metaphase spreads prepared from hatched embryos is fairly routine and allows analysis at an early stage (Kligerman and Bloom 1977). However, assessment of polyploidy may be more easily carried out using other methods such as

semi-automated erythrocyte cell volume quantification (Harrell et al. 1998). Such methods are generally used in *C. carpio* due to the large number of chromosomes in this species (Cherfas et al. 1993; Klinkhardt et al. 1995).

Chromosome counts are a good way of verifying haploid controls in gynogenesis or androgenesis experiments, and are generally used in combination with morphological examination (haploid embryos show a typical "haploid syndrome") and scoring survival rates (nearly all haploid embryos die before diploid embryos reach the first feeding stage). Restoration of diploidy via temperature or pressure shocks given to the fertilized eggs following UV treatment of sperm or eggs can be verified by comparing the karyotypes of shocked batches (should be diploid) compared to the UV controls (should be haploid). However, to verify uniparental inheritance in diploid gynogenetic or androgenetic fish and biparental inheritance in diploid controls, molecular genetic techniques such as DNA fingerprinting are more informative.

Interspecific hybridization may not produce the expected diploid animals with one haploid set of chromosomes from each parental species (Chevassus 1983; see 4.4). Altered ploidy levels in such hybridizations should be easily detected, but for parental species with similar karyotypes, analyzing offspring metaphase spreads may not detect some of the more unusual outcomes of interspecific fertilizations, such as gynogenetic or androgenetic progeny. In many situations, allozyme or DNA markers, possibly in combination with karyotype analysis, may be more powerful.

Chromosome polymorphisms have been found in population genetics studies in some species of fish (e.g. variation in B chromosome frequency in the characid *Astyanax scabripinnis*: Néó et al. 2000) but are of limited general application.

Cyprinids are gonochoristic and several aquaculture species have been shown genetically to have apparently fairly simple sex determination systems (generally but not always XX/XY – see 4.7). However, in common with most other species of fish, heteromorphic sex chromosomes appear to be absent or very rare in cyprinids (e.g. Manna and Khuda-Bukhsh 1977; Buth et al. 1991).

Although studies of cyprinid karyotypes yielded some interesting information and applications – for example insights into the role of polyploidization in the evolution of this group, and chromosome counts can be used as markers in chromosome set manipulation experiments – otherwise limited progress has generally been made in such studies. To a large extent this is also true in other groups of fish, but rapid progress is now being made in cytogenetics in certain species of fish (e.g. physical mapping of genes and other DNA sequences). The species where such advances are being made tend to be either high-value aquaculture species or important model species for basic biological research. The latter group includes one cyprinid, the zebra danio (*Danio rerio*), a small cyprinid used as a model species in basic research.

4.3 Genetic markers: species, population and genome analysis

To characterize individuals, populations or species at the genetic level, polymorphic (variable) genetic markers are required. Historically, markers such as allozymes were used,

where the product of a gene rather than the DNA sequence itself is examined (polymorphism is detected in the form of bands in different positions in an electrophoresis gel after staining for enzyme activity in the case of allozymes). With the development of a variety of techniques for working directly with DNA sequences, it is now possible to detect DNA polymorphisms directly, both in the nuclear genome and the mitochondrial genome.

Standard techniques can be used for isozymes, multilocus DNA fingerprinting, RAPDs and mitochondrial DNA in different species with relatively little adaptation or prior knowledge of the species, while other markers such as microsatellite DNA loci may require considerable preliminary research to isolate relevant DNA sequences and develop appropriate PCR primers (some microsatellite loci will, however, cross-amplify in related species).

A variety of polymorphic genetic markers, primarily allozymes and different types of DNA markers, can be used for such purposes as phylogenetics, species and hybrid discrimination, population genetic analysis, comparison of levels of genetic variation in hatchery and wild stocks, pedigree analysis in hatchery and experimental breeding, and gene mapping.

Knowledge of the genetic structure of wild populations can be important in conserving genetic resources and managing capture fisheries and identifying potential source populations for the development of aquaculture brood stock. Allozyme analysis has been used to study populations of Chinese carps (*H. molitrix*, *A. nobilis* and *C. idella*) both between and within major river systems. Li et al. (1986) demonstrated differences between natural populations of *H. molitrix*, *A. nobilis* and *C. idella* from the Zhu Jiang (southern China), Chang Jiang (Yangtze: central China) and Heilong (northern China) rivers, using 16 enzyme loci. Allozyme analysis of these three species and black carp (*Mylopharyngodon piceus*) from different areas within the Yangtze River (Xia et al. 1996; Zhao and Li 1996) was carried out to help formulate conservation strategies. The results of Zhao and Li (1996) suggested that there was no significant genetic divergence and that one conservation area in the Yangtze River would be adequate (Swan Oxbow area in the middle reaches: described by Li et al. 1995). However, Lu et al. (1997) used PCR-RFLP analysis of mtDNA to demonstrate

that, for three of the four species studied (*H. molitrix*, *A. nobilis*, *M. piceus* but not *C. idella*, where mtDNA diversity was lower), juvenile fish from different nursery areas belonged to genetically distinct populations. This implied that these carps could not be managed as a single unit within the Yangtze River – genetically distinct stocks require individual management. The difference between the results from allozyme and mtDNA analyses emphasizes the value of using more than one technique in such studies.

Wang et al. (2003) studied the ND5/6 mtDNA region of *H. molitrix* from Poyang Lake, a major nursery area for this species, and two connected rivers, the Yangtze and the Ganjiang. From analysis of restriction fragment length polymorphisms, they were able to discern differences between the populations in the two rivers and to conclude that the fish in Poyang Lake consisted mainly of the Ganjiang River population.

Genetic markers that show fixed differences between species can be used to monitor hybridization and introgression where these are known or suspected to occur. A low frequency of hybridization occurs in the wild between many pairs of closely related species, but presumably natural selection prevents this from reaching significant proportions in most cases. In the hatchery environment, hybrids may be deliberately produced for aquaculture, or inadvertent hybridization may take place between similar species. In either case, if F_1 hybrids are used as brood stock, further hybrid or back-cross generations may be produced, which may lead to introgression between the species concerned. This may have deleterious effects on survival and may lead to altered feeding niches in farming systems.

Valenta et al. (1990, 1991) studied enzyme polymorphism in *A. nobilis* and *H. molitrix* in hatchery populations in what was then Czechoslovakia, and concluded that several loci could be used to distinguish between these species and to identify F_1 hybrids. Egelhof (1996) demonstrated that *H. molitrix* and *A. nobilis* used in aquaculture and enhanced fisheries in Bangladesh could be distinguished on the basis of allozyme variation. Zhang et al. (1999) concluded that RAPD analysis could also be used for the same purpose, as well as revealing intraspecific polymorphism. Song et al. (1994) constructed restriction maps of the mtDNA of

these two species. Differences between the species were observed: however the maternal inheritance of mtDNA does limit its usefulness in monitoring hybridization. Introgression between *H. molitrix* and *A. nobilis* has been suggested to be a major reason for the decline in performance of *H. molitrix* stocks in Bangladesh and a preliminary study using species-diagnostic microsatellite loci detected some hybrid genotypes in hatchery brood stock (Mia et al. 2002). Likewise, Thien and Tien (1988) suggested that one reason for a decline in the quality of Harmandi silver carp (*Hypophthalmichthys harmandi*) in Vietnam could be due to uncontrolled hybridization of this species and *H. molitrix* imported from China. Payusova and Tselikova (1993) described genetic (allozyme) differences between these two species, which could presumably be used to determine the extent of such hybridization.

Microsatellite DNA loci exhibit the highest levels of genetic variation that can currently be routinely detected and attributed to alleles of single loci (O'Connell and Wright 1997; Carvalho and Hauser 1998). They have tremendous potential to increase the power of genetic analysis of a number of situations. Kamonrat (1996), McConnell et al. (2001b) and Kamonrat et al. (2002) described microsatellite loci from *B. gonionotus*. Kamonrat (1996) applied some of these loci to study several population genetics- and aquaculture-related questions in this species in Thailand. A low but significant level of genetic discreteness was observed between natural populations from different watersheds (Chao Phraya and Mekong rivers), but mixed stock analysis indicated that 75 and 90 per cent of the fish captured from these two river systems, respectively, were of hatchery origin. This could be interpreted as a success in terms of the hatchery-based restocking program, but the lack of a specific policy for restocking and transferring brood stock between hatcheries located in the different watersheds carry the threat of eroding the genetic integrity of the natural populations.

The pedigrees of individual *B. gonionotus* in a large communally reared group could be successfully established by using one to five microsatellites (Kamonrat 1996). This allowed estimation of heritability values (which ranged from 0.193-0.523) without the requirement of separate rearing facilities and/or complex tagging systems, and removed several potentially large environmental sources of phenotypic variance from the experiment.

Polymorphic genetic markers have been applied to the analysis of *C. carpio* populations. This species is thought to have originated in the Caspian Sea area and later spread to Eastern Europe and China (Balon 1995). Both allozyme (Kohlmann and Kersten 1999) and mtDNA (Gross et al. 2002; Zhou et al. 2003) polymorphisms indicated a major division between the European and East Asian populations, with a further division between koi carp and the other East Asian populations studied (see also 4.5.2: given the long history of domestication of *C. carpio*, much of the analysis of further subdivision of Asian *C. carpio* populations is dealt with in this publication in the context of strain development). Microsatellites have also been developed for *C. carpio* (Crooijmans et al. 1997; Aliah et al. 1999). A study utilizing allozyme, microsatellite and mtDNA markers to look at *C. carpio* populations throughout its distribution range concluded that these populations clustered into two groups, Europe/Central Asia (*C. carpio*) and East/Southeast Asia (*Cc. haematopterus*) (Kohlmann et al. 2003), with no justification for a separate Central Asian subspecies (*C. aralensis*). David et al. (2003) estimated that about 60 per cent of 59 microsatellite primer pairs studied in *C. carpio* amplified duplicate loci, supporting other evidence for the tetraploid origin of this species. They furthermore suggested that the tetraploidy may have arisen by hybridization (allotetraploidy) and that this may have occurred relatively recently (approximately 12 million years ago).

Among the Indian major carps and other cyprinid species in South Asia, many species-specific markers have been detected, for example using esterases (Gopalakrishnan 1997), mtDNA RFLP (Padhi and Mandal 1995) and *Mbo*I satellites (Padhi et al. 1998). Reddy (1999) summarized much of this information. Padhi and Mandal (1997) detected inadvertent hybridization resulting from multispecies spawning in a major carp hatchery by probing *Eco* RI digested DNA with a ribosomal RNA gene probe. Barman et al. (2003) reported species-specific RAPD DNA markers for four major carp species.

In contrast, there have been few applications of such techniques to the study of intraspecific genetic variation in wild or captive cyprinid populations in South Asia. Polymorphism for several enzyme loci has been characterized in *L. rohita* (NBFG 1999) and an ongoing study is characterizing eight riverine populations of this

species. Naish et al. (1997) reported an ongoing study on wild and hatchery populations of *C. catla*, using tetranucleotide microsatellites to examine both the genetic structure of the wild populations and comparative levels of genetic variation in wild and hatchery populations. Basavaraju et al. (2000) and Mair et al. (2000) described preliminary results from this study and a parallel one using PCR-RFLP analysis of mtDNA on the same groups of fish (see 4.5.1). NBFG (1999) reported that the primers for one of the microsatellite loci developed for *C. catla* (G1: Naish and Skibinski 1998) also amplified a polymorphic locus in *L. rohita*, with four alleles being found in three wild populations. McConnell et al. (2001a) described five more polymorphic microsatellite loci from *C. catla*. A number of microsatellite sequences from *L. rohita* have been lodged in GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>).

Given the multiple threats to wild cyprinid populations in many parts of Asia including the observed declines in the populations of several species (e.g. Tsai et al. 1978; Dehadrai et al. 1994; Sreenivasan 1995; Lu et al. 1997), further genetic studies to help understand population structure, genetic diversity, etc., should have priority status.

Relatively few DNA sequences have been entered into the GenBank database for most of the cyprinids being considered in this publication (see list of species in Appendix 1). While *C. carpio* and *C. idella* had 11 140 and 591 nucleotide sequences respectively in the GenBank database in January 2004, none of the other species had more than 50 entries each, with most having very few. This can be contrasted to 584 674 entries for *D. rerio*, one of the major model species for genome analysis. While the zebra danio is the only cyprinid to have had the full capacity of modern genomics techniques applied to the analysis of its genome (e.g. Postlethwait et al. 1999; Kelly et al. 2000; Clark 2003), some of these resources have also been developed for *C. carpio*, including microsatellite markers (Crooijmans et al. 1997), a bacterial artificial chromosome library with two-fold coverage of the haploid genome (Katagiri et al. 2001) and an extensive range of ESTs and other DNA sequences (GenBank). It should be noted that the number of entries for *C. carpio* and *C. idella* has increased remarkably in approximately two years, reflecting the potential of genomics research to produce large numbers of microsatellite markers, expressed sequence tags (ESTs), etc., in a short time.

Microsatellites and other markers are already being applied to population genetics studies and for family identification in aquaculture genetics research, and in the longer term marker-assisted selection (using genetic markers closely linked to quantitative trait loci) is expected to improve the efficiency of selective breeding for at least some traits. Sun and Liang (2000) described a genetic linkage map that has been constructed for *C. carpio* and *Cyprinus pellegrini*, using microsatellites developed from *C. carpio*, crucian carp (*Carassius carassius*) and *D. rerio*, as well as RAPDs and other DNA markers. This map spanned 5 789 cM in 50 linkage groups.

4.4 Hybridization

Interspecific hybridization has been undertaken in a number of cyprinid species in many countries with the objective of manipulating sex ratio, producing sterile animals, improving growth and a few other traits. Although the desired or expected outcome of interspecific hybridization is generally an organism carrying one haploid set of chromosomes from each parental species, Chevassus (1983) described several different possible outcomes. These include diploid, triploid or tetraploid hybrids, haploid or diploid gynogenetics and haploid or diploid androgenetics. Several such “unexpected” progeny types have been observed from cyprinid hybridizations (Krasznai 1987; Wu 1990). For example, Stanley and Jones (1976) observed gynogenetic and androgenetic progeny as well as diploid hybrids in a *C. carpio* x *C. idella* cross. Thus it is accurate to describe the process as “heterospecific insemination” (Chevassus 1983), although there might be some uncertainty about the outcome of this process in terms of the genome of the progeny. Such possibilities emphasize the value of verifying the genetic nature of progeny from heterospecific inseminations, using for example karyotyping, allozymes or DNA markers in addition to morphological or morphometric analysis.

Wu (1990) reported that during 1960-90, over 100 combinations of “distant hybridizations” were carried out in China, including crosses between families, sub-families, genera and species (it was not explicitly stated that all of these hybridizations were between cyprinid species, but only one non-cyprinid species was referred to in the text and reference list of this review). In general, more distant combinations of parental species gave less viable hybrid offspring – no

hybrids between different families survived to the hatching stage; hybrids between sub-families gave some viable, sterile hybrids with very high rates of abnormalities, while five hybrids between different genera gave viable, fertile hybrid offspring. One of these intergeneric hybrids, *C. carpio* (Jian variety) x *C. carassius*, was evaluated for aquaculture in China (Sun et al. 1994) and considered to be promising. Liu et al. (2001) found that F₂ hybrids of red *C. carassius* x *C. carpio* produced diploid eggs and diploid sperm, resulting in tetraploidy in the F₃ and subsequent generations. These tetraploid hybrids were fertile, and gave rise to sterile triploid hybrids in backcrosses to either parent species. These have been produced on a large scale in China (Liu et al. 2001). This appears to be the first case of artificial synthesis of a bisexual hybrid tetraploid form in fish (Gomelsky 2003).

Research has also been carried out on hybridization between Chinese carp species elsewhere in the world, principally in India, Eastern Europe and the USA. Marian et al. (1986) described characteristic karyological, biochemical and morphological markers of *H. molitrix*, *A. nobilis* and their hybrid. Issa et al. (1986) described better growth and survival of the reciprocal hybrids over the parental species during the fingerling period, and Kammerad (1991) stated that the female *H. molitrix* x male *A. nobilis* showed faster growth and better survival than *H. molitrix* during three years of culture.

C. idella x *A. nobilis* hybrids were produced in the USA as a potential way to obtain sterile fish for aquatic weed control, in an attempt to mitigate the controversy over the use of *C. idella* in the country (Sutton et al. 1981). Induced triploidy and monosex female production were also investigated in *C. idella* for the same reason (Shelton 1987; Thomas 1994). The *C. idella* x *A. nobilis* hybrid progeny were a mixture of diploids and triploids (Allen and Stanley 1983; Beck and Biggers 1983a and b; Beck et al. 1984). The hybrids do not appear to be used at present, although triploid *C. idella* are (e.g. Thomas 1994).

Kowtal (1987) and Reddy (1999) reviewed the hybridizations of cyprinid species carried out in India. Research on cyprinid hybridization began in India shortly after the earliest successful induced breeding through hypophysation in 1957 (Kowtal 1987). More than 40 hybrids have been produced. These include hybrids between species within the genus *Labeo*, hybrids between

species in different genera of Indian carps (*Catla*, *Cirrhinus* and *Labeo*), hybrids between *C. carpio* and Indian major carps, hybrids between the Indian and Chinese carps, hybrids between *C. carpio* and Chinese carps, and hybrids between the Chinese carp species.

Almost all of the hybrids produced among Indian major carps exhibited intermediate traits of their parental species (Reddy 1999). The majority of these hybrids were also fertile. Langer et al. (1991) produced an F₂ generation of *C. catla* x *L. rohita* hybrids. Khan et al. (1990) and Reddy et al. (1990) described how three *C. carpio* x Indian major carps (*C. catla*, *L. rohita* or *C. cirrhosus*) hybrids were sterile, apparently due to aneuploidy (74–76 chromosomes) resulting from having one ancestrally tetraploid parent (*C. carpio*, 2n = 100 chromosomes) and one diploid parent (Indian major carp, 2n = 50 chromosomes). However, Khuda-Bukhsh et al. (1988) reported that *C. carpio* x *L. calbasu* progeny were gynogenetic in origin, following analysis of the karyotype and electrophoresis of haemoglobin and transferrin.

Some of these hybrids have been reported to have some potential for aquaculture and reservoir stocking, for example the *C. catla* x *L. rohita* hybrid for reservoir stocking (Natarajan et al. 1976; Somalingam et al. 1990); *C. carpio* x Indian major carp hybrids for aquaculture due to their high growth rate and flesh content, low seine net escapability and sterility (Khan et al. 1990); and *C. catla* x *L. fimbriatus* hybrids for higher dress out percentage due to smaller head and growth nearly equivalent to that of *C. catla* (Basavaraju et al. 1995). However, to date there has been little actual use of these hybrids.

A novel form of hybridization, “nucleocytoplasmic hybridization” was developed in fish species in China by microinjecting the nucleus of one species into the egg of another (Tung et al. 1973). Chen et al. (1990) took this a stage further by using electrofusion instead of microinjection to combine the nucleus and eggs of different cyprinid species. While the technique also has the potential to be used to produce clonal lines of animals (in a cytoplasmic environment which could be conspecific or heterospecific), to date it has mostly been used to study interactions between the nucleus and cytoplasm of different species, mostly cyprinids.

Despite the large body of research on hybridization among cyprinid species, there is

little evidence of significant production of any of these hybrids in Asia except for *C. carpio* hybrids in China (Bartley et al. 1997, 2000; FAO 1999), although Bartley et al. (1997) do point out that hybrid production in general may be underreported in FAO aquaculture statistics.

4.5 Quantitative genetics

Kirpichnikov (1981) stated that “selection should begin simultaneously with the domestication of new species of freshwater fishes; the delay with selective breeding may result in the decreased diversity of the genetic structure of the cultivated species or variety and may even lead to rapid degeneration.” In a newly domesticated species or population (i.e. when a captive breeding population is initiated from a wild one, and maintained over generations), some genetic changes will take place without any active intervention by the hatchery managers, as a result of the new environment and the changed selection pressures it places on the population. A lack of application of genetic principles, or misguided attempts to select for improved performance, may result in deterioration of the performance of the stock. Closely linked to this, and also frequently caused by poor management, is inbreeding.

Conversely, the application of genetic principles (in the context of the biology of the species and hatchery practices) should be able to prevent or minimize inbreeding and improve the performance of the population.

4.5.1 Hatchery management: Inbreeding and inadvertent selection

Cyprinids can be particularly prone to the negative effects of poor genetic management. High fecundity means that a new generation of brood stock can be produced from very few parents in most species. Long generation times in most cultured cyprinids can have both positive and negative effects. Long generation times should slow down the rate of inbreeding per year, but mixing fish from different classes in the same pond for future replacement brood stock and selecting later within such a group, may narrow the genetic base. It is also common practice in some hatcheries to retain fish for future brood stock from fry or fingerlings remaining after the bulk have been sold off. These are quite likely to have been subjected to some form of selection (e.g. size, net escapability) that may affect

performance traits. Similar practices may happen in combined hatchery/grow-out farms, where larger fish are sold for consumption and smaller fish retained for brood stock replacement (Hussain and Mazid 1999).

Negative effects of inbreeding on traits of importance to aquaculture have been demonstrated in several species of fish, including cyprinids. Kirpichnikov (1981), summarizing some of his earlier work, concluded that even moderate levels of inbreeding lead to inbreeding depression in *C. carpio*, with a reduced growth rate and survival and the appearance of “phenodeviants”. The same author also quoted work by Moav and Wohlfarth (1968) and Wohlfarth and Moav (1971), who concluded that one inbreeding generation after the crossing of sibs retarded the growth of *C. carpio* by 10-20 per cent, and was accompanied by decreasing viability and a marked increase in the number of malformations. Kincaid (1983) studied the effects of inbreeding on a variety of traits in rainbow trout (*Oncorhynchus mykiss*) by setting up a series of planned crosses that would give known levels of inbreeding (25 or 50 per cent) relative to an outbred control. For nearly every trait a significant reduction in performance was observed under both hatchery and field conditions.

While these negative effects can be demonstrated in planned inbreeding experiments, and practices that are likely to have negative genetic effects on performance can be identified, it is generally difficult to quantify the degree of inbreeding or negative selection and the effects of such practices in existing stocks of fish in hatcheries and farms. There are a number of possible reasons for this:

- Poor or non-existent records in the hatchery of the relevant data, e.g. number of spawners actually contributing to brood stock replacement (this may be lower than the total number of spawners used in breeding), age at first spawning, age at removal from brood stock, etc.

Ekmath and Doyle (1990) managed to estimate N_e (effective population size) and ΔF (rate of inbreeding) for major carp populations in hatcheries in Karnataka, India but few others have managed to obtain adequate data to estimate inbreeding levels in this way.

- Molecular genetics techniques (e.g. microsatellite DNA loci, mitochondrial DNA,

allozymes) have been used to estimate levels of genetic variation in hatchery populations (e.g. Allendorf and Ryman 1987; Billington and Hebert 1991; Ferguson 1995), but without access to the source population in its original state (or to samples from earlier generations in the same hatchery) these data may be of limited usefulness.

If samples were taken from the hatchery population at the time of its founding or if the hatchery population was taken from a single wild population that is still extant and presumably unaltered, then assessment of any genetic changes should be relatively straightforward. For example, Fiumera et al. (2000) used microsatellite loci to study a captive breeding program for *ex situ* conservation of a Lake Victoria cichlid, *Prognathochromis perrieri*, over several generations, and concluded that the effective population sizes in the subpopulations held in different institutes were 2.5-7.7, far below the actual number of adults maintained in each generation (mean 32-243). However, such closely-monitored situations are likely to be rare in the context of most Asian cyprinid hatcheries. More frequently, the source population will be unknown or unavailable. The hatchery may not operate as an entirely closed unit or the hatchery population may have been founded from more than one source.

Reed and Frankham (2001) surveyed studies on populations from a wide variety of species, and concluded that there was no consistent relationship between molecular genetic variation and quantitative genetic variation. This suggests that, in general, the results of molecular genetic studies have to be used with caution when making inferences about variation for quantitative genetics parameters, and that where possible quantitative genetic variation should be estimated directly, e.g. by estimating heritability for traits of interest.

- Perceived deteriorations in the performance of aquaculture stocks over time have led to suggestions of genetic deterioration (e.g. Hussain and Mazid 1999). However, accurate documentation of such changes is seldom available.

Even if reductions in performance can be verified, it is very difficult to separate genetic changes from environmental factors that may have contributed to declining performance (e.g. changes in culture practice or increasing water pollution). There is a

parallel between this and the measurement of genetic improvement through deliberate selection (see below), where genetic changes need to be separated from improvements in culture practices and other environmental factors. Genetic controls are used in selective breeding and could be used to estimate the degree of genetic change where inbreeding or negative selection has been suggested, e.g. fish taken from the source population and grown side-by-side with the tested stock, or cryopreserved sperm from the base hatchery population. Again, however, these are not often available.

Eknath and Doyle (1985 a, b) concluded that negative selection was taking place in *L. rohita* and *C. cirrhosus* hatchery populations in Karnataka, India, in that selection of larger brood stock fish for spawning actually selected slower growing, late maturing fish.

- In unmonitored hatchery populations, complex genetic changes may take place. For example, “green-fingered” selection may be having some positive effect but may be counteracted by inbreeding.

It is evident in the case of *C. carpio* that “unscientific” selection over many generations of domestication has led to genetic changes and to a wide variety of different body and fin shapes, growth rates, scale patterns and colors. Unintentional selection during domestication will also result in adaptation of wild populations to the hatchery environment. If these are operating at the same time as management practices that result in inbreeding, the net changes in the population are likely to be complex and hard to evaluate.

4.5.2 Current status of domestication and strain development

Distinct domesticated strains have only really been developed in *C. carpio*. From its natural origins in Central Asia, it spread first naturally into Asia and Europe and then more recently has been introduced to almost every country in the world with suitable habitat and climatic conditions (FAO 1998). During its long history of domestication (Balon 1995; Hulata 1995) in several areas, stocks and strains have been developed with a variety of characteristics. These include very obvious differences in scale pattern, coloration and body shape, as well as variation in growth rate and other quantitative traits. The most striking morphological variation is observed

among ornamental strains (koi). In many cases, the origins and development of such stocks and strains cannot be traced because records were not kept and the selection applied to develop such varieties was not scientifically applied or monitored over generations. Most countries in which aquaculture of *C. carpio* is important have stocks with genetic differences for traits of economic importance, good examples in Asia are in China, Indonesia and Vietnam (Li and Wang 2001; Sumantadinata 1995; Thien and Trong 1995). These have come from a mixture of importations and local differentiation.

Because of the long history of domestication and transfers, it has become very difficult to identify and study wild populations of *C. carpio*, or to be sure of the geographical origin of some captive populations. Brody et al. (1979) studied enzyme and transferrin loci in two domestic populations of European origin and two of Chinese origin. Differences in allele frequencies were found between the European and Chinese populations at most polymorphic loci. Kohlmann and Kersten (1999) studied enzyme polymorphism in sixteen populations of *C. carpio* in Germany. Four of these were of Asian origin (the Amur River in Russia; the Red River in Vietnam; Koi from Japan; Ropsha selected strain bred in Russia from wild Amur River carp and local domesticated carp). There was a clear differentiation between the populations of Asian and European origin. The authors concluded that these two clusters corresponded to the two still existing subspecies *C. carpio haematopterus* (East Asia) and *C. carpio carpio* (Europe). However, all of the countries that are the focus of this publication have imported *C. carpio* of European origin and used these in the development of commonly used aquaculture stocks, so even in the Asian countries such as China and Vietnam with indigenous *C. carpio* (Froese and Pauly 2001; Trong 1995), it is likely that some introgression has taken place between European and Asian genotypes. In the Asian countries with no indigenous *C. carpio*, it is likely that current stocks are predominantly or entirely of European origin, on the basis of the known history of introductions (see Chapter 6).

4.5.3 Stock/strain comparisons and cross-breeding

As *C. carpio* is the only cyprinid species in which distinct stocks/strains have been developed, it follows that with few exceptions stock/strain comparisons, including studies on heterosis and

genotype-environment interaction, have been conducted on this species. A few studies have been conducted on other cyprinid species to compare wild populations from different sources. Comparison between existing populations, stocks or strains can be an important initial part of developing a breeding program, so this is dealt with here, before selective breeding (see 4.5.4).

Israeli scientists conducted a series of experiments comparing different strains of *C. carpio* and their cross-breeds, much of which focussed on comparisons of Chinese and European carp. Moav et al. (1975) compared three European stocks (Našice, with a very high height:length ratio; Gold, an inbred line with gold body coloration; and Dor-70, the product of a selection experiment in Israel), one stock of Chinese origin (Big-Belly) and eight cross-breeds (mostly from crosses between these four stocks), in five different environmental conditions. The average growth rate of the Big-Belly carp across all five environments was much poorer than that of the European carp, while all of the cross-breeds showed heterosis. When the genotype-environment interaction of the four purebred groups was represented as a linear function of the environment (i.e. the mean weight of the group as a function of the overall mean weight of all of the fish), the European carp showed much higher responsiveness to changes in the environment. The cross-breeds showed an intermediate but complex responsiveness with a high degree of heterosis. Našice and Gold showed evidence of high levels of inbreeding, reflected in the superior performance of the Našice x Gold cross-bred over the parental strains, while Dor-70 did not show any evidence of inbreeding depression despite some inbreeding over the previous five generations.

Wohlfarth et al. (1983) conducted a similar study with less groups and a more refined experimental design to minimize the effects of inbreeding on the performance of the tested groups. All three groups of fish in the experiment were cross-breeds - Chinese origin (Taiwanese x Hong Kong), European origin (Dor-70 x Našice) and Chinese x European (Taiwanese x Dor-70). They were communally stocked into a variety of environments. In environments with a mean overall growth rate of $>5 \text{ g.day}^{-1}$, the European cross-bred were the fastest growing of the three groups, while in environments with overall mean growth rates of $2\text{-}5 \text{ g.day}^{-1}$, the Chinese x European cross was the fastest growing. The Chinese cross-bred grew faster than the European cross-bred in

the poorest environment (which had an overall mean growth rate of approximately 2 g.day^{-1}) and extrapolation of the results suggested that the Chinese cross-bred would also have grown faster than the Chinese x European group in environments with an overall mean growth rate of $<1 \text{ g day}^{-1}$. Wohlfarth et al. (1986) compared similar groups of fish in ponds with different inputs (manure and/or supplementary feeds), and observed that the Chinese strain showed the fastest relative growth in poor conditions, while the European strain showed the fastest growth under improved conditions. These results suggested that the European and Chinese *C. carpio* strains are each adapted to the typical culture environments in which they were developed, that genotype-environment interactions can be very important and that heterosis for growth can also be strong.

Wohlfarth et al. (1975) looked at a range of other traits in the same groups of carp studied by Moav et al. (1975). They concluded that sexual dimorphism for weight was greater in the Big-Belly than the European strain (cross-breeds were intermediate, suggesting additive inheritance for this trait), viability under poor conditions was higher in the Big-Belly strain (mortality rates were intermediate in the Big-Belly x European crosses but heterosis in the direction of lower mortality was observed in the European crosses, suggesting inbreeding in the latter) and the Big-Belly had much higher seine net escapability (Big-Belly x European crosses showed slight dominance in the direction of higher seine net escapability).

Hulata et al. (1980) found higher percentages of gonad abnormalities in F_1 Chinese x European *C. carpio* and in back-crosses between the F_1 and either parent. It was suggested that this was evidence of a partial reproductive barrier between the two geographic groups.

Evaluation of three strains of *C. carpio* in Thailand (local, Indonesian and fifth generation selected Vietnamese strain) and their cross-breeds indicated that the local strain grew faster than the Vietnamese strain, but survival was lower. The Indonesian strain grew faster than the local strain, but with lower survival. The Indonesian strain had significantly faster growth among the nine groups (six cross-breeds and three pure strains). No crosses performed better than the parent pure strains for growth. Hence it was planned to undertake combined family and mass selection for growth (Anon. 2001).

Wohlfarth (1993) reviewed heterosis for growth rate in *C. carpio*. He concluded that heterosis for growth in this species is a common but not universal phenomenon, limited by genetic factors, genotype-environment interactions, and age and weight of fish. It appeared that the cross-bred advantage was limited to relatively young and small fish and he suggested that this could be of particular importance in warm climates where *C. carpio* could reach market size during their first year. It is worth noting that Flajshans and Gall (1995) stated that 80 per cent of Hungarian and almost 100 per cent of Israeli *C. carpio* production was based on cross-breds and that they are also used commercially in the former Soviet Union (Hulata 1995). Linhart et al. (2002) stated that practically all *C. carpio* stocked in commercial ponds in the Bohemia area of the Czech Republic are F₁ cross-breds between two distant strains. Bakos and Gorda (1995) gave more details on the cross-breeding program for *C. carpio* in Hungary, including attempts to increase the heterosis effect by reproducing the parental lines by gynogenesis. One gynogenetic hybrid out of 21 tested resulted in a 10 per cent higher growth rate. No positive heterosis on the survival rate was observed in the gynogenetic hybrids. Wang and Xia (2002) suggested that heterosis should be higher in crosses involving more distantly related parent stocks: however, this study was based on very few crosses.

Sixteen crosses among four strains of *C. carpio* (Rajadanu, Wildan Cianjur, Majalaya and Sutisna Kuningan) were evaluated in Indonesia for developing a synthetic base population for selective breeding. Heterosis for growth ranged from 0.2 - 4.9 per cent and for survival from 1.6 - 4.7 per cent (Anon. 2001).

A number of intraspecific hybrids (cross-breds) of *C. carpio* have been produced and are being widely used in aquaculture in China. These include not only F₁ cross-breds between two parental strains, but also three-way crosses and populations derived from further generations of breeding from initial cross-breds (Anon. 2001, Li and Wang 2001; Zhu et al. Chapter 3.2, this vol.). After 587 days of monoculture, Jianhuang carp showed 12 and 32 per cent higher growth than female and male parents, respectively. Jianhe carp showed 21 per cent higher growth as compared to the male parent and 8 per cent lower than the female parent (Anon. 2001).

Gela et al. (2003) found evidence for significant genotype x environment interaction for several traits when four *C. carpio* crosses were compared under low (350 m above sea level) and high (750 m ASL) altitude conditions in Central Europe. A Hungarian strain (HSM) was used as a common maternal parent, crossed to four paternal strains (HSM, wild Amur carp, Ropsha carp and Tata carp) to produce the four evaluated crosses. The traits showing significant strain x altitude interaction included the total weight, fillet weight, index of head length and gonadosomatic index.

Li et al. (1987) studied the growth rates of wild and hatchery populations of *H. molitrix* and *A. nobilis* from the Changjiang (Yangtze) and Zhujiang rivers in China. Although the *H. molitrix* from the Zhujiang River grew faster than those from the Changjiang River at the fingerling stages, the Changjiang River fish grew faster in trials in the second year of life and onwards. The wild stocks outgrew the hatchery stocks in both cases. *A. nobilis* from the Changjiang River grew faster than those from the Zhujiang River. However, they suggested that poor nutrition and environmental conditions for captive brood stock could reduce the performance of their offspring compared to offspring from wild brood stock. This emphasizes that when comparing stocks of different origins, ideally the brood stock as well as the tested offspring should be held under identical environmental conditions to produce reliable estimates of genetic differences in the analyzed traits. Li et al. (1987) noted that fry from the Zhujiang River were distributed more widely due to the earlier spawning in the more southerly Zhujiang River region, but suggested that the Changjiang River fish were more suitable for culture. Yang and Li (1996) also noted faster growth rates of wild Yangtze River *H. molitrix* and *C. idella* compared to a hatchery population (three generations from the wild), although the differences were not significant. Li et al. (1987) opined that stocks of slow-growing *H. molitrix* introduced into Southeast Asian countries probably originated from the Zhu River system and suggested replacement with stocks from the Yangtze River.

Reddy et al. (2002) compared five riverine stocks and one farmed stock of *L. rohita* (Ganga, Yamuna, Gomati, Brahmaputra, Sutlej and CIFA farm stock) in earthen ponds in Orissa, India. These were grown communally either in monoculture or polyculture with *C. catla* and *C. cirrhosus*. The final body weight and survival rates of the wild

stocks were equal to or better than the farmed one, while large differences were found between half-sib and full-sib groups within the stocks. Ranking of groups between monoculture and polyculture was highly consistent, indicating a lack of genotype x environment interaction in this study. Gjerde et al. (2002) studied heterosis in two 3 x 3 diallel crosses involving the same stocks of *L. rohita*. They concluded that heterosis for harvest weight was low or negative and heterosis for survival was negligible, indicating little value in using cross-breeding for genetic improvement in these rohu stocks.

Comparison of three populations of *B. gonionotus* (introduced Thai and Indonesian strains and a "local" population of Thai origin) and six cross-breeds between these populations in Bangladesh did not show any significant differences in growth apart from one cross-bred that was significantly smaller at harvest than the other eight groups (Hussain et al. 2002). Evaluation of six indigenous populations of *B. gonionotus* in southern Vietnam indicated genotype x environment interaction (Anon. 2001).

Vandeputte et al. (2002) used a hemi-isogenic scaly *C. carpio* as an internal control in a comparison of the performance of three French mirror carp strains. The tested strains were grown separately from each other, in replicated ponds, and fish from the control strain were added to each pond. Although no significant differences were detected between the mirror carp strains (possibly because of a lack of genetic differentiation between these strains), the method did allow for an assessment of traits at the early stages (the first trait to be studied was at five weeks post-resorption) and had a greater power to detect any differences than a comparable experiment using replicated separate ponds for each stock without an internal control. In the latter design, environmental variation between "replicate" ponds reduces the ability of such a study to detect genetic differences between strains.

In summary, differences in performance between populations and strains of the same species have been clearly demonstrated in some cases, as have heterosis and genotype-environment interactions. However, it is hard to draw general conclusions about the prevalence of the latter two. Additionally, there may be significant amounts of additive genetic variance within populations as well as differences between populations. In developing a

breeding program where two or more different populations are initially available, it is probably best to start by comparing the populations (and if possible families within populations) and their F_1 cross-breeds in the relevant range of culture systems. F_1 cross-breeds have been extensively used for commercial production in *C. carpio* in China, Israel and some European countries, while crosses involving two or more different populations have been used as the basis for selective breeding programs in several species (see 4.5.4).

4.5.4 Selective breeding

Selective breeding has been the basis of nearly all of the genetic improvement that has taken place in terrestrial agricultural animals and plants since the earliest stages of domestication. This genetic improvement has been the result of centuries of on-farm breeding and the recent application of modern breeding and selection theory: it is estimated that selection has increased the productivity of modern breeds of farm animals by at least two to three times in the last 50 years (Bentsen and Olesen 2002). In contrast, Gjedrem (1997) estimated that less than 1 per cent of aquaculture production worldwide in 1993 came from genetically improved breeds.

Although aquaculture organisms differ from agricultural mammals and birds in several important ways (e.g. higher fecundity and smaller post-embryonic size), the principles of selective breeding can also be applied to their genetic improvement.

Some of the most important points in applying quantitative genetics theory to the selective breeding of fish are:

- The nature of the base population in which the selection is started: this should be made up from the best performing wild populations and/or captive stocks available and should contain good levels of genetic variation.
- The traits that are to be selected should be clearly defined, if necessary prioritized (e.g. on the basis of economic importance) and heritability values estimated in the base population. It is very hard to give "typical" heritability values for a trait or a species, since these will depend on the population and the environment in which the estimation is made. Fig. 4.2 shows a frequency distribution of heritability values for traits related to

aquaculture production from a range of fish species, including *C. carpio*.

- Conceptually, the simplest form of selection is what is known as “mass selection” (sometimes also called “individual selection”). This can often be very effective, but the lack of knowledge of family structure can lead to problems, e.g. inbreeding if individuals from only a few families are (inadvertently) being selected in each generation. Bentsen and Olesen (2002) describe ways of designing mass selection programs while avoiding high inbreeding rates. Within-family selection and between-family selection can be used, or combined selection, which should be the most efficient genetically. Falconer and Mackay (1996) described these in some detail, including some of the factors that may influence the choice of selection method.
- Cross-breeding (see 4.5.3) is sometimes a valuable alternative to selective breeding (e.g. if heritability values are low but heterosis is

high) or an additional technique (if selected lines show heterosis in crosses).

Five generations of mass selection for a faster growth rate (size at seven months in pond culture) in *C. carpio* in Israel did not yield any response, while selection for slower growth yielded a relatively strong response for the first three generations (Moav and Wohlfarth 1976). The authors suggested that this stock of *C. carpio* (“the local strain of the domesticated European carp”) had already reached a selection plateau for fast growth rate (and thus had diminished additive genetic variance for growth), due to a strong correlation between growth rate and reproductive fitness. They also suggested that the response to selection for slower growth demonstrated residual, mostly non-additive, genetic variance for growth rate, with a strong dominance component for fast growth. Inbreeding may have been a complicating factor in this study. Despite the lack of improvement through mass selection, subsequent between-group selection from within the “high” line

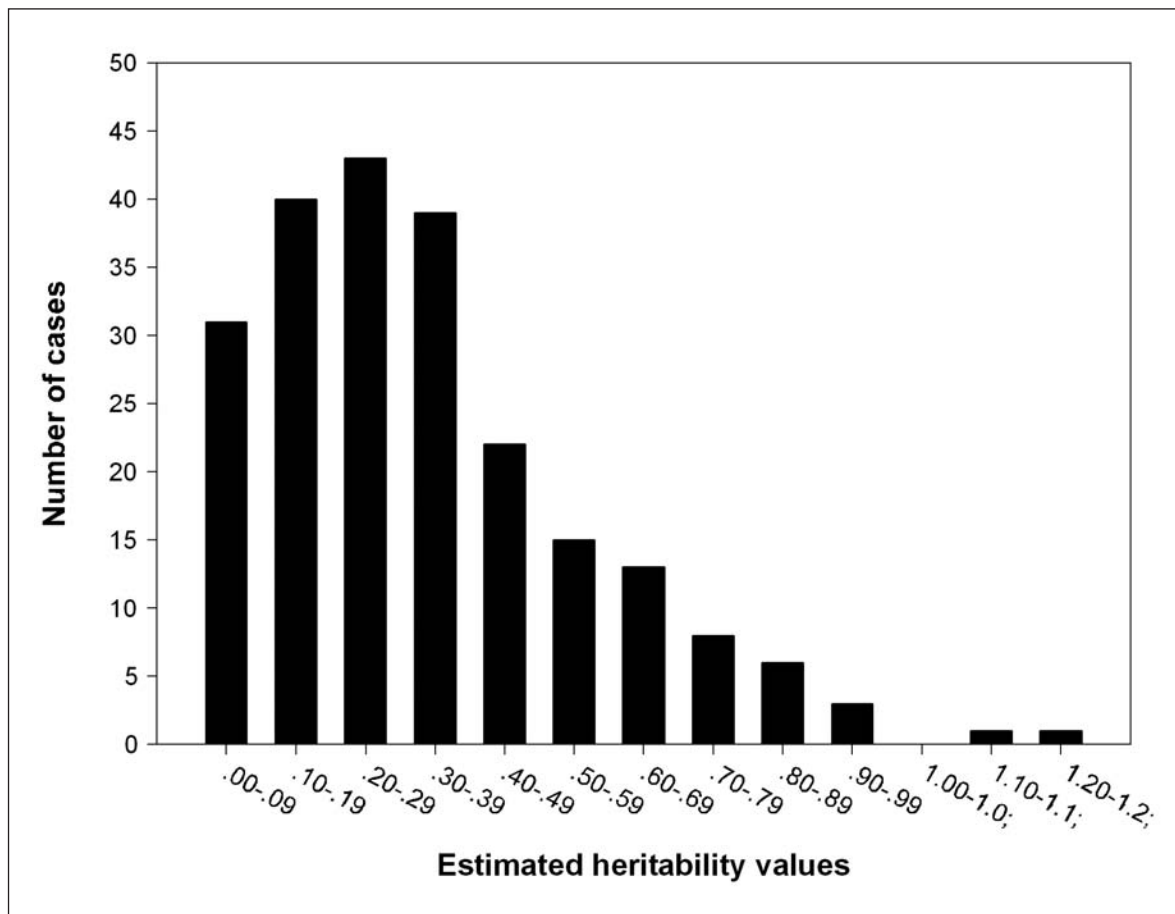


Fig. 4.2. Frequency distribution of heritability values for a range of production-related traits in *C. carpio*, *Oreochromis niloticus*, *Salmo salar*, *Oncorhynchus mykiss* and *Ictalurus punctatus* (data from Table 4.1 of Tave 1993)

resulted in the fast-growing Dor-70 line, which also produced high-yielding cross-breeds (Moav and Wohlfarth 1976) that became the basis of commercial carp production in Israel (Wohlfarth 1993). Although some inbreeding must have accompanied the selection that resulted in the Dor-70 line, cross-breeds involving Dor-70 as a parental line rarely exceeded the growth of Dor-70 by a meaningful amount (Wohlfarth 1993).

Eknath (1990) suggested that the lack of response to mass selection for growth rate in the experiments of Moav and Wohlfarth (1976) impeded the adoption of the selection approach to carp genetic improvement for a long time despite promising results obtained from other species.

Ankorion et al. (1992) estimated realized heritabilities for body shape (height/length ratio) in *C. carpio* through one generation of mass selection. Both up and down selection showed strong responses (heritability estimates were 0.47 ± 0.06 and 0.33 ± 0.10 , respectively). The authors considered that the heritability estimates might have been inflated due to the structure of the base population (which was composed of two cross-breeds, with a common female parent and two different male parents). There was no significant phenotypic correlation between body shape and growth rate.

Other selection programs on *C. carpio* have emphasized the importance of starting with a genetically diverse base population. In these examples, two or more different stocks have been used, sometimes with the aim of selecting more than one trait for improvement. Kirpichnikov (1972, 1981) and Babouchkine (1987) summarized the development of the Ropsha *C. carpio* by selecting from hybrids of the Galician European carp and the Amur wild carp. After producing groups of cross-breeds and back-crosses in the initial generations, mass selection was used within three different lines. The Ropsha carp possesses good resistance to low temperature, high general viability and rapid growth in the first and second years and is well adapted to cultivation in the northern and northwestern regions of the former USSR. Kirpichnikov et al. (1993) described the development of the Krasnodar *C. carpio*, which was selected for resistance to dropsy and also for high growth rate. The base populations for this program were a local mirror carp stock (L), the Ropsha carp (R) and a Ukrainian-Ropsha cross (UR). In initial tests, the Ropsha carp were

the most resistant to dropsy, while after five, six and seven generations of mass selection the UR fish were the most resistant. The effectiveness of selection in improving dropsy resistance in the R and L stocks was questionable, but UR x R and UR x L cross-breeds also showed higher resistance and UR x L cross-breeds showed some heterosis for embryonic survival and growth in two-year-old fish. Preliminary experiments on the L stocks after several generations of mass selection indicated that family selection could be successfully used to improve dropsy resistance.

Many strains/varieties of *C. carpio*, a principal aquaculture species in China with a production of 2.05 million tonnes in 1999 (20 per cent of total freshwater fish production: Li and Wang 2001) have been developed during its long history of culture. The Jian carp was developed in China through a six-generation combined breeding program involving family selection, inter-line crossing and gynogenesis (Zhang and Sun 1994). It has been described as the first "artificial" breed or variety of *C. carpio* in China (Doyle et al. 1994; Sun et al. 1995). Improvement of the growth rate in pond culture over several generations has been demonstrated (Zhang et al. 1995), although a highly significant genotype x environment interaction was observed when growth was compared in ponds and cages. The Jian carp were shown to grow faster than several other varieties of *C. carpio* in China (Sun et al. 1995), and is widely cultured in over 660 000 ha of ponds in China, with an estimated annual production of over 1 million tonnes (Anon. 2001). Further selection of Jian carp for one more generation during 1999-2000 resulted in increased growth by 6 per cent and 9 per cent in mono and polyculture (Anon. 2001).

Selective breeding studies are in progress in China to produce: (i) stable (color) fish with improved growth; and (ii) ornamental fish with different pigmentation patterns in Oujiang common carp. The results to date indicate the existence of strong genetic inheritance of pigmentation patterns (Li and Wang 2001).

From eight stocks of *C. carpio* in Vietnam, double hybrids between the Vietnamese white carp, Hungarian scaled carp and Indonesian yellow carp were used as the starting material for a selective breeding program (Thien et al. 1987; Thien and Trong 1995). Six generations of mass selection were carried out in three hybrid lines (Thien 1997). Thien (1997) reported that the

realized heritability in one of these lines in the early generations was 0.20-0.29. After five generations of mass selection, the growth rate had increased by 33 per cent compared to the base population. However, realized heritability decreased to nearly zero by the sixth generation. To overcome this, family selection has been initiated (Thien et al. 2001).

In cyprinid species other than *C. carpio*, selective breeding has generally been started from base populations entirely or partially composed of fish taken directly from the wild, which presumably ensures relatively high levels of genetic variation in most cases. Two generations of selective breeding of *L. rohita* for faster growth, from a base population composed of five wild riverine stocks and one farm stock (see 4.5.3), resulted in 35-40 per cent faster growth in field trials undertaken in different parts of India (Reddy, chapter 3.3, this vol.; Anon. 2002).

Additive gain in terms of growth from two generations of mass selection of *B. gonionotus* in Bangladesh from a synthetic base population formed from three strains (local, Thai and Indonesian) was estimated at a mean of 7.2 per cent per generation, while the synthetic base population grew 7.5 per cent faster than the nonselected local stock, giving a total gain of 21.9 per cent (Hussain, chapter 3.1, this vol.; Hussain et al. 2002). Mass selection of *B. gonionotus* undertaken in Thailand for two lines, the Chao Phraya and Mekong River, resulted in 30 per cent higher growth in the Chao Phraya line with a realized heritability of 0.4 for body weight at seven months (Anon. 2001).

Selective breeding of blunt snout bream (*Megalobrama amblycephala*) in China started in 1986 through the establishment of two selected lines, a control group and an inbred line (Li and Cai 2003). Five generations of mass selection resulted in an average improvement in growth of 4.0 and 5.8 per cent per generation in the two selected lines. Three generations of full-sib mating resulted in a decline in the growth rate of 17 per cent relative to the control line, i.e. an average of 5.7 per cent per generation. The reduction in growth rate through inbreeding was thus approximately equal to the gain from mass selection, which is a clear reminder of the potential damage that can be done by poor brood stock management.

In conclusion, it is apparent that there is much scope for the appliance of quantitative genetics to improve the performance of cultured carps. This may take the form of obtaining one or more stocks (from the wild or other domesticated stocks) on the basis of their performance and/or level of genetic variance, managing stocks to minimize inbreeding or avoid inadvertent selection, applying selective breeding to improve performance for important traits, or cross-breeding where heterosis is observed. Ideally, a breeding program should take all of these factors into consideration. In practice, limitations in financial, physical or human resources have often restricted the scale and complexity of breeding programs for carps in Asia. For example, selective breeding involving the identification of individual families is generally more efficient than mass selection, but requires additional resources for physical identification (separate rearing for each family followed by physical tagging or marking of each family before communal on-growing) or identification using highly polymorphic microsatellite markers.

4.6 Chromosome set manipulations and polyploidy

Gynogenesis occurs naturally in the genus *Carassius*, with many different clonal lines existing, some of them triploid (e.g. Nakanishi and Onozato 1987; Zhu 1990; Cherfas et al. 1994; Dong and Taniguchi 1996; Umino et al. 1997; Zhou et al. 2000). Induced gynogenesis involves inactivating the paternal (sperm) genome and then restoring diploidy either through suppression of the second meiotic division ("meiotic" gynogenesis) or suppression of the first mitosis ("mitotic" gynogenesis). Most of the more recent protocols for gynogenesis have used ultraviolet irradiation to inactivate the paternal genome, and temperature (cold or heat) shocks to restore diploidy.

Diploid gynogenesis has been induced experimentally in a wide variety of cyprinid species, including *M. piceus* (Rothbard and Shelton 1993), *C. idella* (Stanley 1976; Xia et al. 1990), Indian major carps (John et al. 1984; reviewed by Hussain 1996 and Reddy 1999), *B. gonionotus* (Siraj et al. 1993; Pongthana et al. 1995), *H. molitrix* (Mirza and Shelton 1988; Xia et al. 1990) and most notably in the *C. carpio* (Nagy et al. 1978; Linhart et al. 1986; Hollebecq et al. 1986; Komen et al. 1988; Komen 1990; Sumantadinata et al. 1990).

Androgenesis has been investigated less than gynogenesis. In general it appears to be more difficult to successfully produce diploid androgenetic fish than the equivalent mitotic gynogenetics, due to the difficulties of irradiating unfertilized eggs appropriately. However, one laboratory in the Netherlands has been highly successful in developing and using androgenesis in research on the *C. carpio* (Bongers et al. 1994, 1997a, b, c, 1999) and a few other laboratories have also carried out research on induced androgenesis in *C. carpio* (e.g. Grunina et al. 1995; Ponniah et al. 1995) and *C. carassius* (Fujikawa et al. 1993).

Gynogenesis and androgenesis have been used to investigate sex determination and to produce monosex female cyprinids (see 4.7), to study the effects of inbreeding (e.g. Komen et al. 1992c), to produce fully inbred clonal lines (Komen et al. 1991), to study gene-centromere recombination rates (reviewed by Komen 1990) and to study quantitative genetics parameters. Sumantadinata et al. (1990) studied variance in quantitative characters in gynogenetic *C. carpio* and were able to determine the genetic contribution to this. Bongers et al. (1997a and b) used androgenetic lines to investigate the genetic factors influencing gonad development in *C. carpio*. Bongers et al. (1998) reviewed the use of genetically uniform strains of *C. carpio* in experimental animal research.

While gynogenesis and androgenesis have made little direct impact on aquaculture apart from contributing to monosex female production and in some selective breeding programs, they provide interesting tools for basic research underpinning aquaculture genetics and are likely to make further contributions in the future.

Triploidy has been investigated in certain species of cyprinids, notably *C. carpio* (Gervai et al. 1980; Hollebecq et al. 1986; Cherfas et al. 1993, 1994; Reddy et al. 1998; Basavaraju et al. 2002) and *C. idella* (Cassani and Caton 1985; Thompson et al. 1987; Allen and Wattendorf 1987). In most of the studies on triploidy in *C. carpio* no clear advantages in terms of the growth rate have been demonstrated (Gervai et al. 1980; Cherfas et al. 1994, and papers cited therein). Triploidy could in theory be used to prevent ovary development and spawning, and increase growth rates, in early maturing strains, but the evidence for this is

ambivalent. Wu (1990) gave brief information on two trials in which the *C. carpio* triploids were more than twice the size of diploids at five months old. Reddy et al. (1998) described a trial conducted in a single pond in which a batch of *C. carpio* originating from heat-shocked eggs and containing approximately 80 per cent triploids/20 per cent diploids were grown (without any separate, unshocked, diploid controls). Nine months after stocking, the triploids were 53 per cent larger than their diploid siblings (mean weights 59.46 g and 38.83 g, respectively). Basavaraju et al. (2002) described several trials comparing the growth rates and other parameters in triploid and diploid *C. carpio* in Karnataka, India. Although these trials continued for several months beyond the typical time of first maturation in diploid common carp, the triploid fish did not show any superiority over diploids apart from higher dressout percentages.

In the grass carp, the induction of triploidy was investigated in connection with control of reproduction of this exotic species that is used for aquatic weed control in the USA (Allen and Wattendorf 1987). This is in fairly widespread use, e.g. in the state of Florida (Thomas 1994).

Triploidy could at least in theory be used anywhere to control reproduction of alien species, domesticated strains, transgenic fish, etc., and is already used in commercial aquaculture in a limited range of species. However, many Asian cyprinid hatcheries allow brood stock to spawn in tanks or ponds without stripping eggs and sperm. As *in vitro* fertilization is necessary for the induction of triploidy (to give access to newly fertilized eggs and to allow the precise timing of the shocks involved relative to fertilization), the incentive to produce triploids would have to be high to bring about such a change in the hatchery breeding technique. Crosses between diploid and tetraploid brood stock can produce triploid offspring without the need to induce triploidy (e.g. in rainbow trout: Myers and Hershberger 1991), but the production of tetraploid brood stock is very difficult in most species where this has been attempted (very low larval viability; poor fertility in females: Thorgaard et al. 1990; Pandian and Koteeswan 1998; Rothbard et al. 2000; Arai 2001) and fertilization rates in diploid female x tetraploid male crosses are also low, probably because of the large size of the sperm (Thorgaard et al. 1990).

4.7 Sex determination and its manipulation

Most of the cyprinid species that are cultured in Asia (e.g. Indian major carps, Chinese carps) are harvested long before sexual maturation and before any significant sexual dimorphism is observed in traits that are of relevance to aquaculture. However, there are exceptions. The two most noticeable are *B. gonionotus* and *C. carpio*. *B. gonionotus* generally matures at one year old, and even during growout significant sexual dimorphism in the growth rate may be observed, with females growing faster than males (Pongthana et al. 1999). Ripe ovaries are often found in harvested females, and are consumed in many countries where this species is grown, adding value to the females for culture. In some of the regions of Asia in which the *C. carpio* is cultivated, sexual maturation is observed in fish as young as six months old (Reddy et al. 1998; Basavaraju et al. 2002.). While this may or may not be a problem in terms of breeding and fry production before harvest, a significant proportion of the body weight at harvest can be gonad (the gonadosomatic index can exceed 20 per cent in females). Females also grow slightly faster than males. Sexual dimorphism for growth appears to be larger in Asian strains of *C. carpio* than in European strains (Wohlfarth et al. 1975).

The sex determination systems of several species of cyprinids of interest to Asian aquaculture have been investigated using gynogenesis and/or hormonal masculinization and progeny testing. All-female gynogenetics have been produced in *C. idella* (Stanley 1976), *C. carpio* (Nagy et al. 1978), *A. nobilis* (Shelton 1986a: cited by Castelli 1994), *H. molitrix* (Mirza and Shelton 1988) and *B. gonionotus* (Pongthana et al. 1995), while all-female progeny have been observed in at least some of the crosses between hormonally masculinized fish and normal females in *C. carpio* (Nagy et al. 1981; Wu et al. 1990), *C. idella* (Boney et al. 1984; Shelton 1986b) and *B. gonionotus* (Pongthana et al. 1999: there were also some batches containing a small proportion of males in this study). Bongers et al. (1999) produced viable YY *C. carpio* by androgenesis. These results suggest that female homogamety (i.e. an XX/XY type of sex determination mechanism) is found in these species. However, there are some species of cyprinids that have, or appear to have, other types of sex determination mechanisms. For example, fully inbred lines of the *D. rerio* contained both males and females (Streisinger et

al. 1981), and the European barbel (*Barbus barbus*) has a female heterogametic (ZW/ZZ) system (Castelli 1994); gynogenetic and control batches of the rosy barb (*Puntius conchoni*) showed very variable sex ratios (Powell 2000). Komen et al. (1992a and b) demonstrated that a recessive mutation (*mas-1*), first found in a homozygous inbred line of *C. carpio*, caused masculinization of genetic females (XX).

In Israel, all female *C. carpio* populations have been established by sex reversing XX-gynogenetic females to males and using these males for breeding. This resulted in a 10-15 per cent increase in yield (Gomelsky et al. 1994).

Mirza and Shelton (1988) developed a breeding plan for the production of monosex female *H. molitrix*, to control reproduction of an exotic species used for water quality improvement in the USA. Rothbard et al. (2000) produced triploid all-female *C. idella* by inducing triploidy (see 4.6) in eggs fertilized by XX neomales. In Asia, there has been some application of monosex female breeding plans for the species where sexual dimorphism is observed in aquaculture, namely *C. carpio* (Wu et al. 1990) and *B. gonionotus* (Pongthana et al. 1999). Fig. 4.3 shows the breeding plan initially developed for monosex female *B. gonionotus* by Pongthana et al. (1995, 1999), although this has subsequently been modified to combine mass selection for the growth rate with all-female production. In the modified plan, neomales are produced in each selected generation by gynogenesis and sex reversal.

Sex-reversal in carps using steroid hormones, required for purposes such as the breeding plans outlined above, appears to be more difficult to achieve routinely than in cichlids or salmonids. This appears to be because the onset of sexual differentiation (the "labile period" for sex-reversal) occurs later in cyprinids and may be dependant on size as well as age (Shelton et al. 1995), thus making the exact timing of treatments difficult. There may also be genetic variation in *C. carpio* for response to treatment with 17 α -methyltestosterone (Komen et al. 1993). Table 4.1 presents information from sex-reversal studies on several cyprinid species used in Asian aquaculture.

4.8 Gene transfer

The first published study on gene transfer in fish (Zhu et al. 1985) involved the introduction of

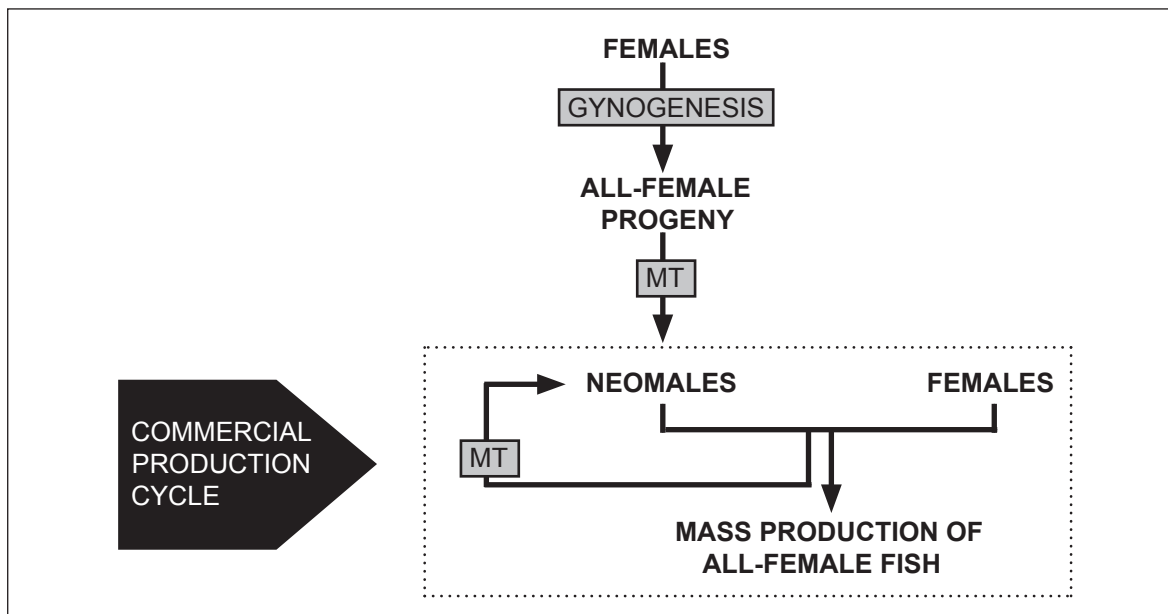


Fig. 4.3. Mass production of all-female *B. gonionotus*. MT = masculinization with 17α -methyltestosterone. After Pongthana et al. (1995, 1999)

Table 4.1. Examples of sex-reversal in cyprinid species. DPH = days post-hatch; MT = 17α -methyltestosterone; E2 = 17β -estradiol; n.d. = not determined

Species	Steroid treatment	Start time (DPH)	Duration of treatment (days)	Results and comments	Reference
<i>C. carpio</i>	MT (100 mg.kg ⁻¹ in feed); gynogenetic fry	26-62	36	71-86.7% males, rest undifferentiated when treated at 25°C; size effect; hermaphrodites at 20°C	Nagy et al. (1981)
	MT (100 mg.kg ⁻¹ in feed); normal fry	42	35	92.7% males (earlier or later treatments gave high % of sterile fish)	Komen et al. (1989)
	MT (100 mg.kg ⁻¹ in feed); gynogenetic fry	26-40	40	Highest % males (up to 96.6%) in control groups in recirculating systems	Gomelsky et al. (1994)
	E2 (25-125 mg.kg ⁻¹ in feed); normal fry	21-70	35	No effect on sex ratio	Komen et al. (1989)
<i>C. idella</i>	5 mg MT in silastic implant; normal fry	112	n.d.	98% male or intersex	Boney et al. (1984)
	5 mg MT in silastic implant; gynogenetic fry	70	n.d.	86% male or intersex	Boney et al. (1984)
<i>B. gonionotus</i>	MT (25 mg.kg ⁻¹ in feed); gynogenetic fry	14	28 or 35	33.9, 29.8% males	Pongthana et al. (1999)
<i>H. molitrix</i>	5 mg MT in silastic implant; normal fry	124 or 319	n.d.	78.9, 90.9% male or intersex	Mirza and Shelton (1988)

mammalian growth hormone coding sequences driven by mammalian metallothionein promoters (pMThGH) into *C. auratus* eggs. Zhu (1992) reviewed the early research on this subject and described how linearized cloned foreign genes were microinjected into fertilized, dechorionated

cyprinid eggs at the single-cell stage using the expelled second polar body as a guide to the target area for microinjection. This led to the generation of mosaic transgenic fish (with copies of the foreign gene integrated into the chromosomes of some, but not all, cells) and

germline inheritance of the foreign genes. Expression of the foreign genes could be detected through Northern blots, radioimmunoassay and enhanced growth in the transgenic fish. Transgenic *C. carpio* and *C. carassius* were respectively 9.4 per cent and 78.4 per cent larger than the controls. Changes in body proportions were also observed in transgenic carp and loach, with these fish being deeper in relation to length than the controls. Wei et al. (1993) and Fu et al. (1998) also described growth trials on *C. carpio* using the pMThGH fusion gene. Transgenic fish were 22.7 per cent to 52.1 per cent larger than the controls. Fu et al. (1998) concluded that the transgenic carp were more efficient in utilizing dietary protein than the controls across a range of 20-40 per cent dietary protein content.

Further developments in China described by Zhu (1992) included the gene transfer by electroporation and an "all-fish" growth hormone gene construct, pCAgGH, in which expression of a *C. idella* growth hormone gene was controlled by a *C. idella* β -actin promoter. This "all-fish" construct was developed with two aims: to try to increase the efficiency of growth enhancement and to produce transgenics that were more likely to be acceptable to consumers. It has been suggested that transgenics receiving only rearranged genes from the same species should be called "autotransgenics", as opposed to "allotransgenics", where all or part of the introduced DNA construct comes from another species (Beardmore 1997).

Other research groups attempted to enhance growth in *C. carpio* using DNA constructs that were at least partially of fish origin (see Table 4.2). A construct in which the *Oncorhynchus mykiss* growth hormone cDNA was expressed using the long terminal repeat promoter of the Rous sarcoma virus (RSVrtGHcDNA) were used by a group in the USA, while constructs using the carp β -actin promoter and *O. tshawytscha* (FV-1/csGH) or carp growth hormone (FV-2/cGH) were used by a group in Israel. These groups achieved similar levels of growth enhancement to that obtained with pMThGH. Transgenic carp were up to 58.5 per cent larger in the studies in the USA (Zhang et al. 1990; Chen et al. 1993). In the Israeli studies two groups of transgenic carp were 42.2 per cent and 70.6 per cent larger than the controls in a winter growth trial (although the controls were slightly larger than the transgenics at the start of the trial, the transgenics showed higher specific growth rates during the trial),

while three groups of transgenic *C. carpio* all showed a lower specific growth rate (SGR) than the controls in a summer growth trial (Hinits and Moav 1999).

Most research on growth enhancement via gene transfer in cyprinids has been carried out on *C. carpio*. While there is some data on growth in transgenic *C. carassius* (Zhu 1992; Xu et al. 1991) and *D. rerio* (Sheela et al. 1998), published information on other cyprinids is mostly related to methodology for gene transfer, e.g. on Indian major carps (Alok and Khillan 1989; Reddy et al. 1991; Tantia et al. 1991; Venugopal et al. 1998) and *M. amblycephala* (Wu et al. 1994).

While growth enhancement has been achieved in *C. carpio* using GH constructs, the levels of enhancement demonstrated in experiments to date do not compare with that achieved in salmonids (e.g. transgenic mean weight >10 times the size of control mean: Devlin et al. 1995) and tilapia (e.g. transgenic mean weight >3 times the size of control mean: Rahman and Maclean 1999). Dunham (1999), reviewing the literature on transgenic fish, suggested that the response (growth enhancement) appears to be greatest in unimproved stocks. This seems to be confirmed by experiments carried out by Devlin et al. (2001) in salmonids. Rainbow trout from a slow-growing wild stock showed dramatic growth enhancement following growth hormone gene transfer (OnMTGH1) while a fast-growing domesticated strain indicated relatively modest growth enhancement.

Given the extremely long history of domestication and selection for *C. carpio* (Balon 1995; Hulata 1995) and the relatively short history of domestication of most other cyprinids, it is attractive to speculate that transgenic growth enhancement in cyprinids such as Indian major carps and Chinese carps could be larger than that observed in *C. carpio*. However, the studies reported above on *C. carpio* utilized different DNA constructs from those used in salmonids and tilapia, which may also have been a factor. Promoter efficiency, other DNA sequences in the construct (introns, enhancers, vector, etc.), integration position affects, DNA methylation, etc., can all affect expression of transgenes (Iyengar et al. 1996).

Transgenic fish are now widely used for basic research on developmental biology, gene regulation and function, etc. (see Table 4.2 for

examples) *D. rerio* has become one of the most important model species for such studies. While proving valuable for basic research, some of the knowledge generated should also filter through into aquaculture-related developments. These can be divided into two broad areas: improved efficiency in generating transgenic organisms (transfer efficiency, targeted integration, control over location, timing and level of expression, etc.) and greater ability to modify a variety of commercially important traits. While attempts have been or are being made to modify traits of fish such as freeze and cold tolerance (Shears et al. 1991; Fletcher et al. 1992; Wang et al. 1995), disease resistance (e.g. Jiang 1993; Jia et al. 2000; Dunham et al. 2002; Sarmasik et al. 2002; Zhong et al. 2002), carbohydrate metabolism (Pitkänen et al. 1999) and sterility (Aléstrom et al. 1992; Uzbekova et al. 2000) in fish, to date the results from such research have generally been much less encouraging than those from transgenic growth enhancement, or the research is still at an early stage.

Major concerns exist over consumer issues and potential environmental impacts of transgenic fish. Wu et al. (2003) address some of these issues. "All-fish" DNA constructs were used to produce transgenic growth-enhanced *C. carpio*, to lessen consumer worries about eating fish expressing non-piscine genes. Trials were carried out on mice fed with transgenic or non-transgenic fish, and the digestibility of the growth hormone in the human digestive tract was considered. They concluded that the "all-fish" GH-transgenics met the principle of "substantial equivalence" to the non-transgenics. To address the potential environmental impact, Wu et al. (2003) also produced a sterile triploid transgenic, by crossing diploid transgenic *C. carpio* with tetraploid *C. carassius* x *C. carpio* hybrids (Liu et al. 2001). The benefits to fish farmers using the transgenics were estimated to be 52 per cent higher than from using normal common carp. Producing triploids in this way should avoid the possibility of a remnant small proportion of diploids (i.e. temperature or pressure shock "failures") that could breed. Wu et al. (2003) stated that the "all-

Table 4.2. Gene transfer in cyprinids: examples of DNA constructs including sequences from cyprinids (1-3) and DNA constructs that have been introduced into cyprinids (4-5). (*D. rerio* examples quoted represent only a sample of papers/subjects related to this species)

Role of cyprinid	DNA sequences	Species	References
1. Promoters/ regulatory sequences from cyprinids	Carp myosin, β -actin regulatory sequences	<i>C. carpio</i> , <i>C. idella</i>	Liu et al. (1989, 1990a and b); Cavari et al. (1993); Moav et al. (1993, 1995); Alam et al. (1996); Alam and Maclean (1997); Müller et al. (1997); Hinits and Moav (1999)
2. Coding sequences from cyprinids	α -globin gene	<i>C. carpio</i>	Yoshizaki et al. (1991a and b); Fu and Aoki (1992)
3. "All-cyprinid" DNA constructs	Actin promoter/ GH coding sequence	<i>C. carpio</i> / <i>C. idella</i> (respectively)	Zhu et al. (1993)
4. Cyprinid recipients for gene transfer (non-cyprinid DNA constructs)	Non-piscine GH construct	<i>C. idella</i> , <i>L. rohita</i> , <i>C. cirrhosus</i> , <i>M. ambycephala</i> , <i>C. carassius</i> , <i>C. auratus</i> , <i>D. rerio</i>	Zhu et al. (1985); Alok and Khillan (1989); Chen et al. (1989); Hayat et al. (1991); Hernandez et al. (1991); Pandian et al. (1991); Powers et al. (1991); Xu et al. (1991); Chen et al. (1993); Cui and Zhu (1993); Wei et al. (1993); Xie et al. (1993); Erdelyi et al. (1994); Wu et al. (1994); Chatakondi et al. (1995); Sun et al. (1995); Fu et al. (1998); Sheela et al. (1998)
	Piscine GH construct	<i>C. carpio</i> , <i>L. rohita</i> , <i>C. cirrhosus</i> , <i>C. catla</i> , <i>C. auratus</i> , <i>D. rerio</i>	Chen et al. (1990, 1993); Zhang et al. (1990); Hayat et al. (1991); Powers et al. (1992); Cavari et al. (1993); Zhang et al. (1993); Zhao et al. (1993); Venugopal et al. (1998)
	Rainbow trout vitellogenin gene	<i>C. carpio</i>	Hayat et al. (1991)
	Non-piscine reporter gene	<i>C. carpio</i> , <i>C. carassius</i> , <i>C. auratus</i> , <i>D. rerio</i> , <i>Puntius conchoniis</i>	Hallerman et al. (1990); Yoon et al. (1990); Zelenin et al. (1991); Khoo et al. (1992); Müller et al. (1993); Guillen et al. (1996); Kang et al. (1999)
	Rat Gap-43 gene construct	<i>C. carpio</i>	Shimbo et al. (1993)
	Bacterial plasmid	<i>D. rerio</i>	Stuart et al. (1988)
5. Cyprinid recipients for gene transfer (DNA construct partially or totally of cyprinid origin)	Mammalian <i>Hox</i> promoters	<i>D. rerio</i>	Westerfield et al. (1992)
	Reporter gene with carp myosin, β -actin regulatory sequences	<i>C. auratus</i> , <i>D. rerio</i>	Moav et al. (1993); Williams et al. (1996); Müller et al. (1997)
	Common carp β -actin promoter + salmon GH or carp GH	<i>C. carpio</i>	Moav et al. (1995); Hinits and Moav (1999)

fish" transgenic *C. carpio* were waiting for public acceptance and governmental authorization.

Cloned gene constructs have been shown to be expressed strongly when injected directly into the muscle of fish (Hansen et al. 1991; Rahman and Maclean 1992). From this and studies on mammals, "DNA vaccination", where proteins expressed from the injected DNA stimulate antibody production, is being developed as an alternative to more traditional vaccination techniques for fish (e.g. Heppell et al. 1998; Kanellos et al. 1999). A U.S. patent has been taken out on this process (Davis 2001). The injected DNA does not appear to integrate into the fish genome (Vaughan et al. 1999), so the vaccinated fish cannot be described as transgenics. Goldfish have been shown to produce anti-bodies to foreign proteins expressed from plasmids injected into muscle (Russell et al. 2000), while the carp β -actin promoter has been shown to be suitable for driving expression of foreign proteins in this situation (Gomez-Chiarri and Chiaverini 1999).

4.9 Cryopreservation

Successful cryopreservation of sperm of many cyprinids, like many other groups of fish, has become fairly routine at least under laboratory conditions (Withler 1982; Kumar, 1988, 1989; Gupta and Rath 1991; Chen et al. 1992a and b; McAndrew et al. 1993; Maise 1996; Ponniah et al. 1999). In contrast, cryopreservation of eggs and embryos has not been successful, and appears likely to remain elusive (Chen et al. 1988; Calvi et al. 1996; Lubzens et al. 1996; Zhang et al. 1997). While cryopreservation of sperm alone still has applications in relation to genetics research and practice in aquaculture, the inability to store eggs or embryos in the same way places major limitations on most of these. For example, while cryopreserved sperm can form a gene bank of sorts for a wild or domesticated stock of fish, without cryopreserved eggs from the same stock live fish can only be "reconstituted" from the gene bank using androgenesis (Penman et al. 1996), and even then the mtDNA will be of maternal origin. Otherwise eggs from a later generation of the same stock (if available) or from a similar stock will have to be used.

Cryopreserved sperm can be used to monitor the progress of selective breeding without maintaining a separate (live) control line in addition to the selected line(s). The difference

between the means of the current generation x current generation and current generation x earlier generation crosses should represent half of the genetic progress over this period, and the progress per generation can be calculated from this.

One factor that has limited the successful application of sperm cryopreservation at the field level has been poor reproducibility of freezing protocols (Rana and Gilmour 1996). While programmable coolers can give highly reproducible cooling rates in different samples, simpler types of apparatus (e.g. freezing straws in liquid nitrogen vapor in a polystyrene box or in the neck of a Dewar vessel) can give highly variable cooling rates and thus lower success. Improved portable sperm freezing systems have been designed (e.g. Magyary et al. 1996) and other attempts have been made to efficiently scale up sperm cryopreservation methods (Lubzens et al. 1997).

4.10 Tissue culture

Cultured cells can be used for a variety of purposes associated with research on genetics of cultured cyprinids. Examples of such applications are:

- (i) Short-term leucocyte culture to provide an abundant source of high-quality metaphase spreads for studies on the cytogenetics of the source species. In general, such cultures are a much better source of mitotic chromosome spreads than solid tissues. Chromosome preparations can also be made from longer term tissue cultures, but chromosome rearrangements and losses in such cultures may limit their usefulness for this purpose (e.g. Li et al. 1994).
- (ii) Tissue cultures can be used to test expression of gene constructs ultimately intended for gene transfer (Moav et al. 1991; Bearzotti et al. 1992; Fu et al. 1993; also see 4.8). Transformation and expression assays are often more easily carried out in tissue culture than in whole organisms.
- (iii) Fish embryonic stem (ES) cell lines could be genetically transformed and then introduced into developing embryos, giving rise to chimeric fish. Transgenic offspring would be generated from the gametes produced from the stem cells that had become part of the developing gonadal tissue in the host fish. ES lines could thus form a "bridge" between *in vitro* and *in vivo* manipulations of animal

genomes. The elements of such a system have been demonstrated in the mouse and the medaka, *Oryzias latipes* (Hyodo and Matsubashi 1994; Sun et al. 1995; Hong et al. 2000) but not to date in any cyprinids.

- (iv) Nucleus transplantation (see also 4.4). While nuclei of cells from dissociated early embryos have given rise to the most successful rates of nucleus transplantation (e.g. Tung et al. 1973), tissue culture could provide large numbers of cells for this process, particularly if scaling up using electrofusion instead of microinjection (Chen et al. 1990). Although nucleus transplantation has been achieved using donor cells from tissue culture, chromosome rearrangements and losses occurring in long-term cultures could limit the usefulness of such an approach.

Primary cultures and cell lines have been set up from several species of cyprinids, including *C. carpio* (e.g. Horiuchi et al. 1979; Fijian et al. 1983; Chen et al. 1987; Moritomo et al. 1996; Weyts et al. 1997; Degani et al. 1999), major carps (e.g. Rao et al. 1997; Sathe et al. 1997; Joseph et al. 1998), *C. idella* (e.g. Deng et al. 1985; Zuo et al. 1986; Li et al. 1988; Yang et al. 1992; Zeng et al. 1993) and gimbuna crucian carp (e.g. Hasegawa et al. 1997). Very few cyprinid cell lines are held in, and thus available from, centralized cell culture collections – the ECACC (European Collection of Cell Cultures: <http://www.ecacc.org/>) listed four cyprinid cell lines (CLC and EPC from *C. carpio*; CAR from *C. auratus*; and FHM from fathead minnow, *Pimephales promelas*) among 25 fish lines from a total of 985 lines, while the ATCC (American Type Culture Collection: <http://www.atcc.org/>) did not list any cyprinid cell lines among nine fish cell lines.

4.11 Summary

In the last ten years or so, progress has been made in several areas of basic and applied research on the genetics of the carp genetic resources of relevance to Asia. For example, China has continued its progress on selective breeding of *C. carpio* and all of the other countries where cyprinids are of importance to aquaculture now have breeding programs for one or more important cyprinid species. Most of these selective breeding programs have begun by comparing different stocks of the target species, including studies of genotype x environment interaction and heterosis, and have then used mass selection to improve growth-related traits. Some have used

more complex forms of selection or selected for more than one trait. In other directions, there have been some studies on population genetics of important cyprinid species, development of monosex female production in appropriate species, and further advances in research on transgenic fish.

With some notable exceptions, there has been limited impact of genetic improvement on aquaculture production to date in most of the countries in Asia where carps are important in aquaculture. Dissemination of improved breeds is now receiving a greater degree of attention, including the targetting of poorer farmers.

Priority areas for future research and development on the genetic resources of cyprinids in Asia should include:

- A greater understanding of the population genetic structure and biodiversity of indigenous cyprinids, to support both conservation efforts and exploitation for aquaculture.
- The continued application of genetics research to develop improved breeds for aquaculture.
- Greater interaction with fish farmers, hatcheries, etc., and dissemination of improved breeds and the technologies involved.

Molecular genetics is a rapidly advancing field that has already given valuable tools such as polymorphic molecular markers and gene transfer. It is likely that further developments in this area will have applications in research on the genetics of cyprinids in Asia, hopefully to the benefit of both conservation and exploitation of these resources.

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