Population Genetics of Common Carp (*Cyprinus carpio* L.) in the Murray-Darling Basin



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A thesis submitted to the Faculty of Veterinary Science, The University of Sydney, in fulfilment of the requirements for the Degree of Doctor of Philosophy

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Declaration

This thesis is submitted to the University of Sydney in fulfilment of the requirement for the Degree of Doctor of Philosophy

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. Apart from the assistance mentioned in the acknowledgements and the contribution of the research paper co-authors listed below, the work described in this thesis was executed by the author, who also had substantial input into planning of the projects. I hereby declare that I have not submitted this material, either in full or in part, for a degree at this or any other institution.

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Contribution of Co-authors

Chapter 3: ***Population genetics of a globally invasive species, common carp (Cyprinus carpio L.), in the Murray-Darling Basin, Australia: evidence for multiple introductions and genetic structure, with suggested management units***

Dr. D.M. Gilligan selected the sample sites, organised and coordinated the collection of fish samples from the Murray-Darling Basin and Prospect Reservoir and assisted in manuscript preparation.

Dr. P. Grewe offered extensive technical advice, made genotyping equipment available and assisted with manuscript preparation.

Prof. F. Nicholas supervised the project, contributed to extensive discussions concerning data analysis and interpretation, and performed extensive manuscript editing.

Chapter 4: ***Invasive common carp (Cyprinus carpio L.) in Australia: origin of founding strains and population genetics of coastal waterways***

Dr. D.M. Gilligan selected the sample sites, organised and coordinated the collection of common carp samples from the Murray-Darling Basin, Prospect Reservoir, the Hunter and Hawkesbury-Nepean catchments and assisted in manuscript preparation.

Dr. P. Grewe offered extensive technical advice, made genotyping equipment available and assisted with manuscript preparation.

Prof. F. Nicholas supervised the project, contributed to extensive discussions concerning data analysis and interpretation, and performed extensive manuscript editing.

Prof. C. Moran assisted with manuscript preparation and provided technical and analytical advice.

Chapter 6: **Rapid identification of maternal lineages in common carp (*Cyprinus carpio* L.) using real-time PCR and high resolution melt-curve analysis**

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Prof. F. Nicholas supervised the project, contributed to extensive discussions concerning data analysis and interpretation, and performed extensive manuscript editing.

Dr. K.R. Zenger offered the original idea for the research paper and assisted with manuscript preparation.

I certify that the above statement about my contribution to the research papers in this Ph.D. thesis is true and accurate, and give Gwilym Haynes full permission to submit these journal articles as part of his Ph.D. thesis.

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Abstract

Common carp (*Cyprinus carpio* L.) are a highly invasive species of freshwater fish in Australia. Native to Eurasia, they can be separated into 3-4 different subspecies and innumerable aquaculture and ornamental strains. They have been introduced into Australia on a number of occasions and were established in the Murray-Darling Basin (MDB), Australia's largest and most important river system, by the 1920s. The release of a new aquaculture strain in the late 1960s, followed by extensive flooding in the mid 1970s, resulted in an explosion of common carp numbers. They are now the dominant species in this river system, and cause extensive ecological damage by competing with native freshwater species and by their feeding mode, in which they suck up mud, filter it through their gill rakers and expel water and fine particles through their gill opening. This feeding mode has been linked to increases in water turbidity, algal blooms, damage to river banks, loss of aquatic vegetation, alterations to the trophic cascade of ecosystems and declines in native fish. However, the effects of carp are difficult to discern from other factors degrading waterways and affecting native fish, such as flow regulation, irrigation and land clearing.

There is substantial public interest in the control of common carp. Australians find them unpalatable, considering them too bony and their flesh poor in taste. Subsequently, they are undesirable for recreational fishing and few commercial markets exist in Australia. In addition, as mentioned above, they are suspected of exerting a detrimental effect on the aquatic environment. In fact, carp are currently considered by fisheries biologists as the worst freshwater pest fish in many of the countries where they have been introduced. The cost of management in Australia has been estimated at a total of $15.8 million annually, with $2 million spent on research, $2 million on management, and $11.8 million on remediation of environmental impacts.

Previous population genetic studies on carp in Australia identified four strains: Prospect, Boolara, Yanco and Japanese koi. Interbreeding has been recorded between the Yanco and Boolara strain, and there is no reason to believe that it cannot occur between the other carp strains also. Hybridisation between carp and goldfish (*Carassius auratus*) has also been detected in the MDB, but the level of introgression between the two species has not been quantified. Some genetic structuring of carp within the MDB has been identified previously, although there was little clear pattern to this structuring.

The main aims of this Ph.D. study were:

1. to characterise the population genetic structure and level of genetic diversity of carp in the MDB;
2. to discern the history of introduction and spread of carp in the MDB;
3. to identify barriers to gene flow in the MDB, and from this data propose management units for control programs.

In addition, a number of side projects were also initiated with the following aims:

4. to discern the origin of the different strains of common carp that have been introduced into Australia;
5. to investigate the population genetics of three carp populations in separate waterways on the east coast of Australia;
6. to optimise PCR of microsatellite loci in both carp and goldfish;
7. to characterise the level of introgression between feral carp and goldfish in the MDB; and
8. to develop a protocol for the screening of sequence variants in the mitochondrial control region using real-time PCR and high-resolution melt-curve analysis technology.

Common carp were collected from every major river in the MDB. In rivers with major dams, carp were collected from both above and below these impoundments. Additionally, feral carp were collected from Prospect Reservoir (source of the Prospect strain) in the Sydney Basin; Japanese koi carp and domestic mirror-scaled carp were sourced from fish breeders; wild carp were sourced from the River Danube in Germany; and Russian Ropsha strain carp were obtained from a live gene bank in the Czech Republic. All carp were characterised for 14 microsatellite loci.

The core aims of this Ph.D. (the aims #1-3 above) are addressed in *Chapter 3*. Because of the expected lack of genetic equilibrium of the carp population under study, a range of analyses was utilized and consensus among results was interpreted as approaching biological reality. Genetic structuring between regions was detected, especially across the large impoundments at river headwaters. Evidence was found for three discernable strains of carp (Prospect, Yanco and Boolara) accounting for the majority of genetic variation

within the MDB, with a very minor contribution from ornamental Japanese koi carp. A history of introduction and colonisation is proposed from the genetic and non-genetic evidence. The basin was divided into 15 management units for future control programs. Most regions had high levels of genetic diversity, with multiple strains present and no evidence of recent population bottlenecks, implying that the invasiveness of carp is associated with high levels of genetic diversity. This project serves as a guide for other research groups looking to understand the population structure of invasive fish species as a step towards their control.

Chapter 4 builds on the research presented in *Chapter 3*. In this study, the origins of the strains are investigated (aim #4) by comparing representative of each strain with carp populations from Europe, using assignment tests and factorial component analysis (FCA). As isolated populations were not available for all strains, groups of individuals representative of the Boolara and Yanco strains were inferred from the assignment tests performed in *Chapter 3*. The population genetics of carp in the east coast of Australia was also investigated (aim #5). It was found that the Prospect, Boolara and Yanco strains are descended essentially from the European/central-Asian subspecies *C. carpio carpio*. Coastal populations exhibited levels of genetic variation comparable with domestic populations, were non-panmictic, and contained different proportions of each strain, consistent with independent histories of introduction. Recommendations are made for preventing the further spread of carp throughout the rest of Australia.

In *Chapter 5*, PCR was optimised for microsatellite loci in both carp and goldfish (aim #6), and introgression between the two species in the MDB was quantified (aim #7). Goldfish were collected opportunistically along with carp from the MDB, as were 23 putative carp-goldfish hybrids, identified as such by the presence of aberrant barbels around their mouths. Goldfish were also collected from local pet stores. Eight of the fourteen microsatellites that amplified in carp in *Chapter 3* also amplified in goldfish. A closed population of feral goldfish was genotyped for these eight markers, five of which proved to be suitable for analysis. All remaining goldfish and hybrids were genotyped for these five loci, and genotyping results were combined with results of genotyping of carp from *Chapter 3*. Assignment analyses were implemented in STRUCTURE and NEWHYBRIDS to determine whether the suspected hybrids had ancestry from both species, and to investigate undetected mixed-ancestry in individuals in the MDB and Prospect Reservoir.

The relationship between the individuals was visualised using two-dimensional FCA. In addition, UPGMA and Maximum Likelihood phylogenetic trees were constructed from the mitochondrial control region sequences of all the putative hybrids and from a number of carp, goldfish and related cyprinids. The assignment analyses and FCA confirmed the mixed nuclear-genome ancestry of all 23 putative hybrids, with 20 classes as F1 generation and 3 classified as F2 generation. Putative mixed ancestry was also detected in 15 individuals from the MDB phenotypically identified as carp, and one individual identified as goldfish. Overall, approximately 1.6% of the genetic diversity of carp in the MDB was found to be sourced from goldfish, and approximately 1% of feral goldfish genetic diversity is sourced from carp. There was some evidence that carp-goldfish hybridisation was biased in favour of male carp, namely that 21 of the 23 putative (phenotypic) hybrids had goldfish mitochondrial sequence,. However, too few individuals and loci were analysed to resolve this issue with any certainty. Although low, this level of introgression is still of concern, as it may introduce new adaptive alleles (e.g. for disease resistance) into invasive carp populations.

In *Chapter 6*, a protocol for using real-time PCR and high-resolution melt-curve (HRMC) analysis to score polymorphisms in the mitochondrial DNA control region of common carp is presented (aim #8). This is the first time HRMC analysis has been used in an aquacultural species. The technique is accurate, robust and rapid to apply. It has a number of advantages over other existing techniques for scoring DNA polymorphisms: it is rapid, taking less than three hours from start to finish; all procedures take place in closed PCR tubes, reducing the risk of contamination and human error; cycling conditions in the Rotor-gene 6000 PCR machine used in the methodology are more homogenous than in traditional block-based PCR machines; and the progress and success of each individual PCR is monitored in real-time. The primers were designed to score a greater number of polymorphic sites than in previous studies, and specifically target a section of the control region that is polymorphic amongst European carp races, which otherwise have very little mitochondrial DNA variation. The technique was used to accurately identify three common carp and one goldfish haplotype, with no haplotypes incorrectly identified. Although the method outlined here is optimised for scoring common carp mitochondrial haplotypes using the Rotor-gene 6000 machine, real-time PCR and HRMC analysis can be applied in a similar way to almost any species and/or loci, with a number of different real-time PCR machines available for scoring genetic differences.

There are a number of future research possibilities for the study of carp in Australia. These include improving the accuracy and power of the research presented here by scoring more genetic markers and including more outgroup populations; investigating more fully the population genetics of the many coastal populations of carp in Australia; more accurately quantifying introgression between carp and goldfish by scoring more DNA markers in both species; and investigating the presence of crucian carp (*Carassius carassius*) in the MDB, and possible interbreeding between this species and carp and goldfish.

This research is the most comprehensive study of common carp in a single river basin to date. The quantity of samples (983 in the MDB) and collection sites (36 in the MDB) exceeds any previous study of common carp, and is not often achieved amongst other studies of freshwater fish. This is the first study in which the population history of common carp has been investigated in detail at a local level, and in which management units for this species have been proposed. A number of surprising findings have been made, namely the presence of the Prospect strain in the MDB, the extent of population genetic structuring in the Basin, the disparate distribution of the different stains as a result of human-mediated dispersal, and the cryptic introgression between goldfish and carp. It was shown here that despite being recently introduced, carp can exhibit population structuring within a single river basin,and that this structuring that is consistent with the population not yet being in mutation-drift-migration equilibrium and gene flow playing a larger role than genetic drift in shaping genetic structure. This study serves as a guide to other research groups looking to understand the population genetics of invasive freshwater fish species as a step towards their control.

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List of Abbreviations

General Abbreviations

A	**Mean Number of Alleles Per Locus**
AB	**Applied Biosystems**
ACE	**After Common Era (AD)**
ACT	**Australian Capital Territory**
AMOVA	**Analysis of Molecular Variance**
A_r	**Allele Richness**
BCE	**Before Common Era (BC)**
bp	**Base Pairs**
CR	**Control Region**
CRC	**Cooperative Research Centre**
DNA	**Deoxyribonucleic Acid**
ESU	**Evolutionary Significant Unit**
FCA	**Factorial Correspondence Analysis**
H_E	**Expected Heterozygosity**
H_O	**Observed heterozygosity**
HRMC	**High-Resolution Melt-Curve**
HVR	**Highly Variably Region**
HW	**Hardy-Weinberg**
KHV	**Koi Herpes Virus**
MCMC	**Monte Carlo Markov-Chain**
MDB	**Murray-Darling Basin**
MIA	**Murrumbidgee Irrigation Area**
mtDNA	**Mirochondrial DNA**
NSW	**New South Wales**
PCR	**Polymerase Chain Reaction**
QLD	**Queensland**
RFLP	**Restriction Fragment Length Polymorphisms**
rt	**Real-Time**
SA	**South Australia**
SMM	**Stepwise mutation model**
SSCP	**Single Strand Conformational Polymorphism**
subsp.	**Subspecies**
TAS	**Tasmania**
TGGE	**Temperature-Gradient Gel Electrophoresis**
TPM	**Two Phase Model**
VIC	**Victoria**

Carp Sample Sites

AV	**Avoca**
BD	**Burrendong Dam**
BJ	**Burrinjuck Dam**
BK	**Bourke**
BR	**Broken River**
CDM	**Condamine**
CM	**Cooma**
CN	**Coonamble**
CS	**Campaspe**
CV	**Charleville**
CW	**Lake Cargelligo**
D	**River Danube**
DB	**Dubbo**
DQ	**Deniliquin**
EC	**Echuca**
EI	**Lake Eildon**
GB	**Goulburn**
J	**Jaenschwalde, German Mirror -Scaled carp**
K	**Koi (Germany)**
Kb	**Koi (Sydney)**
KIW	**Kiewa**
KP	**Lake Keepit**
LD	**Loddon**
LH	**Lake Hume**
LL	**Lower Lakes**
MG	**Mudgee**
MR	**Moree**
NB	**Narrabri**
ND	**Narrandera**
NG	**Nyngan**
OV	**Ovens**
P	**Prospect Reservoir**
PR	**Paroo River**
R	**Ropsha**
TAS	**Tasmania**
WC	**Wilcannia**
WG	**Walgett**
WM	**Horsham**
WN	**Wellington**

WT	**Wentworth**
WY	**Wyangala Dam**

Carp Strains

B	**Boolara**
K	**Koi**
P	**Prospect strain**
Y	**Yanco**

Chapter 1: Introduction

Common carp (*Cyprinus carpio* L.) are a highly invasive species of freshwater fish. They have been introduced into Australia on a number of occasions since the late 19th Century (Koehn et al 2000), were established in the Murray-Darling Basin (MDB) by the 1920s (Clements 1988), and have been the dominant fish species in the basin since the mid-late 1970s (Harris and Gehrke 1997; Reid *et al.* 1997; Koehn *et al.* 2000; MDBC 2008b).

The presence of carp is undesirable throughout Australian waterways. Many Australians find carp unpalatable (although they are highly prized by some European and Asian migrant communities), considering them too bony and their flesh poor in taste (Koehn *et al.* 2000). Their feeding habit, in which they suck up mud, filter it through their gill rakers and expel water and fine particles through their gill opening, has been linked to increases in water turbidity (Crivelli 1981; Fletcher *et al.* 1985; Newcome and Macdonald 1991; Roberts *et al.* 1995; Driver *et al.* 1997; King *et al.* 1997; Schiller and Harris 2001; Angeler *et al.* 2002; Tapia and Zambrano 2003; Pinto *et al.* 2005), algal blooms (Breukelaar *et al.* 1994; Gehrke and Harris 1994; Williams *et al.* 2002; Pinto *et al.* 2005), damage to river banks (Wilcox and Hornbach 1991), loss of aquatic vegetation (Crivelli 1981; Hume *et al.* 1983a; Panek 1987; Roberts *et al.* 1995), alterations to the trophic cascade of ecosystems (Angeler *et al.* 2002; Khan 2003; Parkos III *et al.* 2003) and declines in native fish (Fletcher *et al.* 1985; Page and Burr 1991; Koehn *et al.* 2000). Although the effects of carp are often difficult to discern from other factors degrading waterways and affecting native fish, such as flow regulation, irrigation and land clearing (Hume *et al.* 1983a; Koehn *et al.* 2000), there is much public interest in carp control. The cost of carp management in Australia has been estimated at a total of $15.8 million annually, with $2 million spent on research, $2 million on management, and $11.8 million on remediation of environmental impacts (McLeod 2004; Gilligan and Rayner 2007). Common carp are currently considered by fisheries biologists as the worst freshwater pest fish in both Australia and New Zealand (Chadderton *et al.* 2003).

A range of physical and biological controls are in various stages of development to control invasive carp populations. These are summarised in Gilligan and Rayner (2007), and include barring carp from key breeding sites, introduction of disease, daughterless technology and various methods for removing carp from waterways.

The MDB is Australia's most important river system, covering some 1,061,469 square kilometres, equivalent to 14% of the country's total area; and containing Australia's three longest rivers, the Darling (2,740km), the Murray (2,530km) and the Murrumbidgee (1,690km). In 1992, the MDB accounted for 71.1% of the total area of irrigated crops and pastures (2,069,344 hectares), 70% of all water used for agriculture in Australia, and $10.75 billion in industry turnover (MDBC 2008a). The basin harbours an estimated 30,000 wetlands of various sizes, 46 species of native fish and 11 species of alien (non-Australian) or translocated fish (Australian but not native to the MDB) fish (Lintermans 2007). Although no fish has become extinct in the basin since European settlement, local extinctions have occurred, 26 of the 46 native species are recognised as threatened or of conservation concern, and alien species comprise 80-90 per cent of the fish biomass in parts of many rivers (Lintermans 2007). In addition, the basin hosts no fewer than 35 species of endangered birds and 16 species of endangered mammal (MDBC 2008a). Conservation of all aspects of the MDB is of great importance to Australia.

The main aims of this Ph.D. study were:

1. to characterise the population genetic structure and level of genetic diversity of carp in the MDB;
2. to discern the history of introduction and spread of carp in the MDB;
3. to identify barriers to gene flow in the MDB, and from this data propose management units for control programs.

In addition, a number of side projects were also initiated with the following aims:

4. to discern the origin of the different strains of common carp that have been introduced into Australia;
5. to investigate the population genetics of three carp populations in separate waterways on the east coast of Australia;
6. to optimise PCR of microsatellite loci in both carp and goldfish;
7. to characterise the level of introgression between feral carp and goldfish in the MDB; and
8. to develop a protocol for the screening of sequence variants in the mitochondrial control region using real-time PCR and high-resolution melt-curve analysis technology.

This Ph.D. thesis contains four research chapters (*Chapters 3-6*). *Chapters 3, 4* and *6* were written for journal publication and are in various stages of peer review at the time of thesis submission. *Chapter 5* was not written specifically for any journal, although will be rewritten for publication in the near future. Each chapter is written so that it can be read independently. The original journal formatting of *Chapters 3, 4* and *6* has been preserved where possible. However, minor changes have been made so that the formatting of the thesis is internally consistent.

The main aims of the CRC-funded research are addressed in *Chapter 3*. *Chapter 4* addresses the aims of discerning the origin of the different strains of common carp that have been introduced into Australia and of investigating the population genetics of three carp populations in separate waterways on the east coast. These chapters are highly relevant to the control of common carp in Australian waterways. Taken together, they explain the origin of carp in Australia, and the mechanisms by which it has spread to new regions following initial introduction; they suggest potential genetic factors that could account for carp being so invasive; and they make recommendations for future control programs.

In *Chapter 5*, introgression between feral carp and goldfish (*Carassius auratus*) in the MDB is characterised. This chapter is of some significance for the control of feral carp, as it identifies goldfish as a potential source of genetic variation which could allow carp to become more virulent as an invasive species, and enable carp to overcome biological controls (daughterless gene technology and introduced diseases) implemented against them. It is also of broader interest, as it explores the ongoing exchange of genetic material between related species, a process that likely has long-term evolutionary significance.

Chapter 6 details a protocol developed for the screening/genotyping of sequence variants in the mitochondrial control region using real-time PCR and high-resolution melt-curve (HRMC) analysis technology. To the knowledge of the authors of this chapter, this is the first study in which real-time PCR and HRMC analysis are used to genotype sequence variants in an aquaculture species. This combination of technologies has such applications as identifying the success of different maternal lineages in mixed stock breeding programs

and measuring the contribution of escaped domestic strains to wild populations. Although the protocol presented is specifically targeted at screening the mitochondrial DNA control region in common carp using one particular brand and model of real-time PCR machine with HRMC capacity, a range of such machines is available for the application of real-time PCR and HRMC to other loci and species.

1.1 References

Angeler DG, Álvarez-Cobelas M, Sánchez-Carrillo S, Rodrigo MA (2002) Assessment of exotic fish impacts on water quality and zooplankton in a degraded semi-arid floodplain wetland. *Aquatic Sciences,* **64,** 76-86. doi: 10.1007/s00027-002-8056-y.

Breukelaar AW, Lammens EH, Breteler K (1994) Effects of benthivorous bream (*Aramis brama*) and carp (*Cyprinus carpio*) on sediment re-suspension and concentration of nutrients and chlorophyll a. *Freshwater Biology,* **32,** 113-121. doi: 10.1111/j.1365-2427.1994.tb00871.x.

Chadderton WL, Grainger N, Dean T (2003) Appendix 1 – Prioritising control of invasive freshwater fish. *Managing invasive freshwater fish in New Zealand.* Workshop hosted by the Department of Conservation, Hamilton, New Zealand.

Clements J (1988) *Salmon at the Antipodes A History and Review of Trout, Salmon and Char and Introduced Coarse Fish in Australasia,* Ballarat, Victoria, Australia, Published by the author. ISBN: 0731637194.

Crivelli AJ (1981) The biology of common carp, *Cyprinus carpio* Linneaus in the Camargue, Southern France. *Journal of Fish Biology,* **18,** 271-290. doi: 10.1007/BF00018966.

Driver PD, Harris JH, Norris RH, Closs GP (1997) The role of the natural environment and human impacts in determining biomass densities of common carp in New South Wales. In Harris, J. H. & Gehrke, P. (Eds.) *Fish and Rivers in stress: the NSW rivers survey.* Cronulla and Canberra, Australia, NSW Fisheries Office of Conservation and Cooperative Research Center for Freshwater Ecology. ISBN: 0731094085.

Fletcher RF, Morison AK, Hume DJ (1985) Effects of carp *Cyprinus carpio* L., on communities of aquatic vegetation in the lower Goulburn River Basin. *Australian Journal of Marine and Freshwater Research,* **36,** 311-327. doi:10.1071/MF9850311.

Gehrke PC, Harris JH (1994) The role of fish in cyanobacterial blooms in Australia. *Australian Journal of Marine and Freshwater Research,* **45,** 905-915. doi:10.1071/MF9940905.

Gilligan D, Rayner T (2007) The distribution, spread, ecological impacts, and potential control of carp in the upper Murray River. NSW Department of Primary Industries, Sydney.

Harris JH, Gehrke PC (1997) Fish and rivers in stress - the NSW rivers survey. NSW Fisheries Office of Conservation & the Cooperative Research Centre for Freshwater Ecology, Cronulla and Canberra.

Hume DH, Fletcher AR, Morsion AK (1983) Final Report. Carp Program Report No. 10. Fisheries and Wildlife Division, Victorian Ministry for Conservation,

Khan TA (2003) Dietary studies on exotic carp (*Cyprinus carpio*) from two lakes in Western Victoria, Australia. *Aquatic Sciences,* **65,** 272-286. doi: 10.1007/s00027-003-0658-5.

King AJ, Rovertson AI, Healy MR (1997) Experimental manipulations of the biomass of introduced carp (*Cyprinus carpio*) in billabongs. 1. Impacts on water column properties. *Marine and Freshwater Research,* **48,** 435-443. doi:10.1071/MF97031.

Koehn J, Brumley B, Gehrke P (2000) *Managing the Impacts of Carp,* Canberra, Australia, Bureau of Rural Sciences (Department of Agriculture, Fisheries and Forestry). ISBN 0644292407 (set) 0642732019.

Lintermans M (2007) *Fishes of the Murray-Darling Basin,* Canberra, Australia, Murray-Darling Basin Commission. ISBN: 1 921257 20 2.

McLeod R (2004) Counting the cost: impact of invasive animals in Australia. Cooperative Centre for Pest Animal Control, Canberra.

MDBC (2008a). Murray Darling Basin Commission, Basin Statistics: http://www.mdbc.gov.au/about/basin_statistics

MDBC (2008b) Sustainable rivers audit report. Murray-Darling Basin Commission,

Newcome CP, Macdonald DD (1991) Effects of suspended sediments on aquatic ecosystems. *North American Journal of Fisheries Management,* **11,** 72-82. Doi: 10.1577/1548-8675(1991)011.

Page LM, Burr BM (1991) *A field guide to freshwater fishes: North America North of Mexico*, Boston, USA, Houghton Miffin Company. ISBN: 0395910919.

Panek FM (1987) Biology and ecology of carp. In Cooper, E. L. (Ed.) *Carp in North America.* Maryland, USA, American Fisheries Society. ISBN: 9780913235447.

Parkos III JJ, Santucci VJ, Wahl DJ (2003) Effect of adult common carp (*Cyprinus carpio*) on multiple trophic levels in shallow mesocosms. *Canadian Journal of Fisheries and Aquatic Sciences,* **60,** 182-192.

Pinto L, Chandrasena N, Pera J, Hawkins P, Eccles D, Sim R (2005) Managing invasive carp (*Cyprinus carpio* L.) for habitat enhancement in at Botany Bay, Australia. *Aquatic Conservation: Marine and Freshwater Ecosystems,* **15,** 447-462. doi: 10.1002/aqc.684.

Reid DD, Harris JH, Chapman DJ (1997) NSW Inland Commercial Fishery Data Analysis. Fisheries Research & Development Corporation, NSW Fisheries, Cooperative Research Centre for Freshwater Ecology, Sydney, Australia.

Roberts J, Chick A, Oswald L, Thompson P (1995) Effects of carp, *Cyprinus carpio* L., an exotic benthivorous fish on aquatic and water quality in experimental ponds. *Marine and Freshwater Research,* **46**. doi:10.1071/MF9951171.

Schiller C, Harris JH (2001) Native and alien fish. In Young, W. J. (Ed.) *Rivers as ecological systems: the Murray-Darling Basin.* Canberra, Australia, Murray-Darling Basin Commission. ISBN: 1876830034.

Tapia M, Zambrano L (2003) From Aquaculture Goals to Real Social and Ecological Impacts: Carp Introduction in Rural Central Mexico. *Ambio,* **32,** 252-557.

Wilcox TP, Hornbach DJ (1991) Macrobenthic community response to carp (*Cyprinus carpio*) foraging. *Journal of Freshwater Ecology*, **6,** 170-183.

Williams AE, Moss W, Eaton J (2002) Fish induced macrophyte loss in shallow lakes: top-down and bottom-up processes in mesocosm experiments. *Freshwater Biology*, **47,** 2216-2232. doi: 10.1046/j.1365-2427.2002.00963.x.

Chapter 2: Literature Review

2.1. A brief introduction to the study of population genetics

Population genetics can be defined as the study of changes in allele or gene frequencies in space and time. Population genetic studies address such questions as identifying of population structure (i.e. the presence of subpopulations), quantifying genetic differences between subpopulations, estimating effective population sizes and effective migration rates (i.e. gene flow), and making phylogenetic inferences.

The field of population genetics was pioneered by such scientists as Sewall Wright (1889-1988), John Haldane (1892–1964) and Sir Ronald Fisher (1890–1962), who developed the theoretical foundation upon which many of the analytical methods used today are based (e.g. Haldane 1924; Fisher 1930; Wright 1931; 1951). It has been widely recognised, however, that the early models of population structure, migration, demographics and evolution are unrealistic, as they rely on assumptions that do not accurately reflect the real biological world, such as constant population size and migration rates, and equilibrium between mutation, migration and genetic drift (Whitlock and McCauley 1999; Pearse and Crandall 2004).

The F_{ST} statistic (Wright 1951), which quantifies the difference in allele frequencies between subpopulations relative to the overall population, has featured heavily in population genetic studies since its inception (Pearse and Crandall 2004). F_{ST} has been widely used to estimate migration rates between subpopulations under the equation $F_{ST}=1/(4Nm)$ ($2Nm$ for haploid loci), where Nm is the migration rate (Wright 1951). Numerous analogues of Wright's (1951) original statistic have been devised to analyse different types of genetic data or to operate under different population genetic models with different assumptions, some of which are detailed in Table 2.1. The model of population dynamics under which F_{ST} was built, however, is far too simplistic to reflect the complexity of real biological scenarios (Whitlock and McCauley 1999). F_{ST}-based estimates of migration and population structure can therefore be highly inaccurate (Whitlock and McCauley 1999; Pearse and Crandall 2004), with F_{ST} analogues suffering from the similar limitations as the original estimator (Pearse and Crandall 2004). F_{ST} and its analogues are, however, still very useful as comparative benchmarks between studies and as a basic descriptors of population subdivision (Pearse and Crandall 2004).

Table 2.1. Analogues and modified version of Wright's (1951) F_{ST} statistic

Statistic	Description and References
G_{ST}	Devised for use with data for multiple alleles at diploid, co-dominant loci (Nei 1973).
θ	F_{ST} analogue that is for "all intents and purposes" equivalent to Wright's (1951) original F_{ST} (Weir and Cockerham 1984). Weir and Cockerham (1984) detail the use of a weighting procedure to combine information across all alleles and loci.
F_{ST} (no special signifier)	F_{ST} can be estimated from sequence data by treating each polymorphic site as a separate locus and each polymorphism as a separate allele (Hudson *et al.* 1992).
N_{ST}	Devised for use with restriction fragment length polymorphism (RFLP) data sets (Lynch and Crease 1990).
Analysis of molecular variation (AMOVA)	Hierarchical partitioning genetic variation within and between different levels of population subdivisions. Originally devised for haploid sequence data (Excoffier *et al.* 1992), and adapted for use with co-dominant, diploid data sets .
R_{ST}	Designed specifically for use with diploid microsatellite data sets; attempts to take into account they way in which microsatellite alleles most commonly mutate (i.e. with the addition or subtraction of a units of the repeat motif), by using the stepwise mutation model (SMM) rather than the infinite allele model (IAM) (Slatkin 1995).
$(\delta\mu)^2$	Designed specifically for use with diploid microsatellite data sets under the SMM (Goldstein *et al.* 1995).
Φ_{ST}	Designed specifically for use with diploid microsatellite data sets under the SMM (Michalakis and Excoffier 1996).
ρ_{ST}	Designed specifically for use with diploid microsatellite data sets under the SMM (Rousset 1996).
D_R	Genetic distance measure for use with diploid microsatellite data sets under the SMM, incorporating mutational constraints on allele sizes (Zhivotovsky 1999).
Slatkin's F_{ST}	Follows a coalescence-based model of population subdivision (Slatkin 1991).
Reynolds' F_{ST}	Derived from the co-ancestry-based genetic distance of Reynolds *et al.* (1983) and implemented in the computer program Arequin (Excoffier *et al.* 2005).

In the last 10-15 years, advances in computing power coupled with the increasing ability to generate large genetic data sets have led to the development of a wide array of new analysis techniques. Rather then rely on summary statistics, such as F_{ST}, many of these new analyses involve computationally-intensive procedures that simultaneously estimate several parameters to find the overall set of parameters that best fits the data. More information can therefore be extracted from genetic data, with it now being possible to make inferences about past demography, identify genetic loci/regions under selection, quantify genetic diversity, estimate the number of populations, estimate the rate of gene

flow, detect asymmetrical gene flow, discern the relative effects of migration and random genetic drift on population structure, and make inferences about current and historical effective population sizes (Pearse and Crandall 2004; Excoffier and Heckel 2006).

An extensive list of programs written for population genetic analysis is given by Excoffier and Heckel (2006) and Pearse and Crandall (2004). These make an excellent starting point for researchers engaged in population genetic analysis. However, as new programs are constantly being written to either address new hypotheses or improve on current methods to investigate existing hypotheses, a literature search for new programs is highly recommended. In particular, such journals as *Molecular Ecology Resources* (previously called *Molecular Ecology Notes*) and *Bioinformatics* are a good place to search for new population genetic analysis programs.

Of particular relevance to this Ph.D. research are (1) analyses which employ Bayesian statistics to make population genetic inferences, and (2) populations which are not in equilibrium. These are discussed briefly in the sections below.

2.1.1. Bayesian statistics

Bayesian statistics are inference frameworks, based on the work of Thomas Bayes (1702-1761), in which the posterior (post-analysis) probability of a parameter depends explicitly on its prior (pre-analysis) probability (Excoffier and Heckel 2006). Bayesian analyses are frequently used in conjuncture with Markov-chain Monte-Carlo (MCMC) techniques. MCMC analysis makes it possible to estimate the joint posterior distribution (i.e. the probability distribution of all possible combinations of parameter values, when a model is defined by more than one parameter) of a set of parameters for a given data set under a given model. MCMC techniques do this by exploring the parameter space (i.e. fitting the data to different combinations of the parameters) one ‘step’ at a time. After enough steps, the parameter space with the highest likelihood can be found (Excoffier and Heckel 2006). MCMC methods bypass the computationally prohibitive measure of characterising the entire parameter space (i.e. every possible combination of parameters for the data) and have hence made it possible to address a range of biological questions for the first time. Bayesian analyses coupled with MCMC techniques have therefore added greatly to the study of population genetics.

A range of computer programs are now available that use Bayesian analyses to make population genetic inferences. A number of these programs are described in Table 2.2. Many implement different types of assignment test, where individuals are assigned to populations based on their genotypes. By far the most popular assignment test is the one developed by Pritchard *et al.* (2000) and implemented in their program Structure. This method uses a clustering algorithm to assign individuals into a predefined number of populations, *K*. The correct value of *K* (i.e. the actual number of populations) can then be inferred using the ΔK statistic of Evanno *et al*. (2005). Although it can be computationally heavy, Structure has the advantage of (1) assigning individuals rather than groups to a population, and (2) this assignment being independent of where the individuals where samples from (i.e. individuals can be assigned solely on the basis of their genotype). Numerous improvements have been added since its inception (Falush *et al.* 2003; 2007). Structure assumes that each population is in Hardy-Weinberg and linkage equilibrium, and can be subsequently unsuitable for species where genetic differentiation follows a cline, rather than a set of discrete subpopulations.

In addition to population assignment, Bayesian analyses can be used to infer detailed demographic history of populations by combining information from both genetic and non-genetic data. Estuope *et al*. (2001; 2003), for example, investigated the demographic history of introduced populations of cane toads (*Bufo marinus*) in Australia and a species of silveye bird (*Zosterops lateralis lateralis*), which naturally introduced itself from Australia into New Zealand, Norfolk Island and Chatham Island in the Pacific Ocean. In both studies, a large amount of historical and demographic information was available for each species, such as the origin and date of introduced, when range expansions occurred, generation time, and the size of a migrating flock (for silvereyes). This demographic data was used to inform the Bayesian analyses (which included MCMC techniques) and allowed many demographic parameters to be estimated for the first time. For cane toads, number of introduced individuals, effective population size after the demographic boom (the population explosion that occurred after the species was first introduced into Australia), duration of the population boom, and the effective population size after stabilisation was inferred. As cane toads were originally sourced from two different places in South America, the length of time that these two source populations had been separated and level of admixture between them was also estimated. For silvereyes, the number of

founding individuals in each island, the duration of the population bottleneck following initial introduction, and the stable effective population size on each island were estimated. Both these scenarios are too complex to be addressed using traditional statistical methods, such as F_{ST}. One limitation of such inferences, however, is that they require specialised programming skills, which many biologists lack.

Table 2.2. Computer programs for population genetic analyses that employ Bayesian analyses

Program	Description and References
Structure	Assignment program that employs a clustering algorithm to assign individuals into predefined number of populations (*K*). Uses genotype data from co-dominant, unlinked, diploid genetic loci, and assumes Hardy-Weinberg equilibrium. No prior information about where individuals were collected from is required. Individuals can be assigned completely to a single population, or to more than one population (i.e. intercrossed individuals) (Pritchard *et al.* 2000; Falush *et al.* 2003; 2007).
Partition	Similar to Structure, Partition employs a Bayesian model to identify genetic subdivisions and to assign individuals into populations. Uses genotype data from co-dominant, unlinked, diploid genetic loci, and assumes Hardy-Weinberg equilibrium. Assumes that all individuals are of pure ancestry, i.e. does not allow for the presence of admixed individuals (Dawson and Belkhir 2001).
Geneland	Package in R that detects population subdivision. Uses genotype data from co-dominant, unlinked, diploid genetic loci, and assumes Hardy-Weinberg equilibrium. Takes into account the spatial position of samples when determining the most likely number of population subdivisions, and outputs a graphical distribution of the population subdivisions (Guillot *et al.* 2005a; Guillot *et al.* 2005b).
BAPS	Assignment program that estimates the number of populations and assigns individuals into them. Uses genotype data from co-dominant, unlinked, diploid genetic loci, and assumes Hardy-Weinberg equilibrium. Like Structure, individuals can belong entirely to one population, or their genotype can be partitioned into multiple populations. BAPS is different from Structure in that the analysis is performed at the level of predefined population units rather than at the level of the individual, and prior information about the geographic sampling design is used to inform the analysis (Corander *et al.* 2004).
NewHybrids	Specifically tests for and categorises (F1, F2, or backcrossed) recently admixed individuals. Uses genotype data from co-dominant, unlinked, diploid genetic loci, and assumes Hardy-Weinberg equilibrium in parent populations (Anderson and Thompson 2002).
BayesAss+	Estimates recent migration rates between populations. Uses genotype data from co-dominant, unlinked, diploid genetic loci, and assumes Hardy-Weinberg equilibrium. Requires all source populations of migrants to be sampled, and estimates each individual's immigrant ancestry, the generation in which immigration occurred and inbreeding levels within populations (Wilson and Rannala 2003).

2.1.2. Non-equilibrium populations

Many population genetic analyses, including F_{ST}, assume that the populations under investigation are in equilibrium between random genetic drift, mutation and migration. Such assumptions about equilibrium are often not met in the biological world. For invasive species, populations may have undergone a recent population bottleneck (e.g. Puillandre *et al.* 2008) and/or range expansions (e.g. Estoup *et al.* 2001), been sourced from multiple sub-populations (e.g. Kolbe *et al.* 2004) and/or be under new selection regimes (e.g. Carroll 2007). Populations of endangered species may have undergone a rapid population reduction and populations may have become recently fragmented (Pearse and Crandall 2004). Even in well established species there can be a lack of regional or local equilibrium due to such factors as unequal migration or gene flow between regions, sporadic gene flow, meta-population (sub-populations subject to local extinction and re-colonisations events) dynamics and insufficient time having past since ancient range expansion or contractions (e.g. from the contraction or growth of glaciers) for equilibrium to have become established (Crispo and Hendry 2006; Bay *et al.* 2008). There is therefore a need for analyses that are not strongly dependent on population equilibrium to be accurate.

Bayesian and Maximum likelihood (the latter estimates the parameters of a model that maximise the probability of the data under that model, Excoffier and Heckel 2006) analyses are especially useful in investigating populations which may not be in equilibrium. As such measures depend on few assumptions and estimate all parameters simultaneously, they can be very robust or even independent of assumptions about population equilibrium (Pearse and Crandall 2004; Hänfling and Weetman 2006). The population genetic analysis introduced cane toads and silvereye birds of Estoup *et al*. (2001; 2003), for example, were specifically designed to not rely on assumptions about population equilibrium.

Population equilibrium can occur on different scales. While equilibrium may have been reached on a regional scale, the small subpopulations that make up the larger population can still show departure from mutation-drift-migration equilibrium. One example is includes the fish *Acanthochtomis polycanthus* in the Great Barrier Reef in Australia, which shows isolation-by-distance type genetic structuring on the regional (i.e. continental shelf) scale, consistent with overall population equilibrium (Hutchinson and Templeton 1999); but shows unequal migration rates, strong population structure and variation in genetic

diversities, consistent with meta-population type population dynamics (i.e. local subpopulations not in equilibrium) on the scale of the individual reefs (Bay *et al.* 2008). Equally, Hänfling and Weetman's (2006) investigation of river sculpin fish (*Cottus gobio*), in the River Rye in Europe, found regional equilibrium, demonstrated by isolation-by-distance type population structure (Hutchinson and Templeton 1999). Evidence was also found, however, that the populations at river headwaters showed signs of population bottlenecks. The authors therefore postulated that headwater populations may be prone to cycles of decline and recovery and hence may never obtain equilibrium. Conversely, a species could be in equilibrium on the local scale but not at the regional scale. Subpopulation of the European alpine plant *Arabis alpina*, for example, were shown to be in mutation-drift equilibrium in some regions, while the overall population showed strong departure from equilibrium (Ansell *et al.* 2008). The findings of wide-scale population genetic studies should be therefore interpreted with caution, as population equilibrium could be present at one scale but not another.

2.2. A brief introduction to invasive species

For the purpose of this chapter, an invasive species can be defined as any species that has been translocated from its indigenous environment to a new environment and successfully established a self-sustaining population. Such translocations can result from natural processes, such as long-distance dispersal events (e.g. silver-eye birds, *Zosterops lateralis*, colonised New Zealand from Australia in 1830 (Estoup and Clegg 2003)) or the formation of dispersal pathways between previously isolated environments (e.g. the formation of land bridges between previously isolated continents or islands; hydrological rearrangement river catchments). The exchange of organisms between regions has undoubtedly played an enormous role in the shaping the evolution of life on this planet.

Humans have always taken with them a host of organisms as they travelled across the planet. These organisms include parasites (e.g. tapeworms and lice), scavengers (e.g. rats and mice) and useful organisms that were translocated intentionally (e.g. pets, livestock and crops). In recent times, the rate of such anthropomorphic translocation of species has rapidly increased to the extent that invasive species are now recognised as having a negative effect on the world's biodiversity that is second only to habitat destruction and habitat fragmentation (Sakai *et al.* 2001; Allendorf and Lundquist 2003; Zanden 2005).

There is therefore much interest in the study and control of invasive species. Invasive species also offer the opportunity to study evolution in action, as both the invasive species must evolve to meet the challenges of their new environment, and organisms in the invaded environment must evolve to survive the impact of the newly established species.

The invasion of an environment by a new species typically has three phases: the initial introduction; a lag period where the species remains localised and is either evolving to meet the challenges of its new novel environment and/or building up it numbers; and finally range expansion, where the species becomes truly invasive and starts colonising new regions. These phases are described in detail in Sakai *et al.* (2001) and Allendorf and Lundquist (2003) and will not be discussed further here.

There are two paradoxes that commonly arise in the study of invasive species. Firstly, how can any species become invasive when it must compete with indigenous species that have had a much longer time to adapt to local conditions? Secondly, how is it possible that invasive species manage to thrive and evolve in a new environment, when the process of introduction likely includes a genetic bottleneck (i.e. a small number of individuals are translocated, which carry only a random sub-sample of the species' overall genetic diversity), which should leave the species genetically depauperate and prone to inbreeding depression? These two paradoxes are addressed in the sections below.

2.2.1. Paradox 1: How does any species manage to invade a new environment that already appears to be occupied by well-adapted indigenous species?

The success of an introduced species in a new environment tends to be idiosyncratic and context-dependant, with very few general traits that characterise good invaders, or environments that are vulnerable to invasion (Colautti *et al.* 2006; Moles *et al.* 2008). Never-the-less, a number of traits have been shown to be significantly correlated with the invasiveness of introduced species and with the invasibility of an environment. Invasiveness traits including propagule pressure (i.e. many individuals released into the new environment), the invasive species being commensal with human activities (e.g. introduced livestock or crops), high germination rate (in invasive plants), high reproductive output, and the ability of the invasive species to specialise in using an ecological niche that is not being exploited fully in the invaded environment (Cassey *et al.* 2005; Colautti *et al.* 2006). Predictors of invasibility of an environment include high propagule pressure of the

introduced species, the habitat being disturbed (generally by human activities), and there being high resource availability (Colautti *et al.* 2006). Consistent with this, Moles *et al.* (2008) theoretically and empirically demonstrated that a strong predictor of invasiveness and invasibility is where invasive species can occupy an ecological niche in the invaded environment that is not being used by indigenous species, as commonly occurs in environments that have been disturbed by human activities.

Invasive species have also been shown to benefit from escaping their native parasites, predators or competitors (Allendorf and Lundquist 2003; Frankham 2005). In addition, some invasive species may simply be more competitive than the indigenous species. Examples include the replacement of all thylacines (*Thlacinus cynocephalus*) and Tasmanian devils (*Sarcophilus harrisii*) on mainland Australia by dingoes (*Canis familiaris dingo*) that were introduced from New Guinea around three thousand years ago (Paddle 2000; Savolainen *et al.* 2004), and the replacement of practically all indigenous marsupials in South America with ecologically equivalent North American placental mammals when a land bridge formed between the two continents around three million years ago (Flannery 2001). This greater competitiveness could stem from the invasive species evolving in a more competitive environment (Callaway and Aschehoug 2000).

2.2.2. Paradox 2: How can invasive species survive and evolve in a new environment, after the genetic bottleneck of the introduction process?

Pulliandre *et al*. (2008) identified three types of introduction: continuous expansion after the permanent removal of a natural barrier, several discrete introductions from several native populations, and a single introduction from a single source. For the first two types of introduction, there are no problems with reduced genetic diversity and subsequent inbreeding depression and lack of evolvability. If a natural barrier is removed, by the opening of a canal, for example, a continuous stream of the introduced species will be able to disperse into the new environment and the colonising species will subsequently have similar levels of genetic diversity to their founding population. Examples include two rabbit fishes (*Siganus luridus* Rüppell and *Siganus rivulatus* Forsskål) and one goat fish (*Upeneus moluccensis* Bleeker) that invaded the Mediterranean Sea from the Red Sea after the opening of the Suez Canal, and show no signs of reduced genetic variability and no significant genetic differentiation from their source populations (Hassan *et al.* 2003; Hassan and Bonhomme 2005; Hänfling 2007). When several introductions from several

source populations occur, levels of genetic diversity can be similar or even greater than in the species native range. The green anole lizard, for example, was repeatedly introduced to Florida from a number of different source populations in its native range in Cuba, and subsequently shows greater genetic diversity in Florida than in any one location in Cuba (Kolbe *et al.* 2004). Most introductions, however, come from a single source population and a single introduction event and show reduced levels of genetic diversity relative to their source populations (Puillandre *et al.* 2008).

There are a host of reason why species sourced from a single population in a single or small number of introduction events can still become invasive. These are summarised below:

1. Inbreeding depression is a stochastic probability, not a certainty. Introduced species with reduced genetic variability can subsequently still be genetically viable in their new environment.
2. Asexual and self-fertilising plants are often not vulnerable to inbreeding depression (Frankham 2005)
3. Rapid population growth following introduction can minimise the subsequent loss of genetic diversity. An example of this is the introduction of rabbits into Australia (Zenger *et al.* 2003).
4. A genetic bottleneck can actually increase the genetic variation in a population, through the epistatic interactions between loci, and through increased frequency of recessive alleles that were rare in the parent population but more frequent in the introduced population (Hänfling 2007). Such an increase in genetic variation has been observed in introduced populations of invasive guppy fish (*Poecilia reticulata*) in Queensland (Lindholm *et al.* 2005).
5. The genetic drift associated with a population bottleneck can allow alleles that are advantageous in the new environment to become fixed, possible even ‘jumping’ across fitness valleys (Hänfling 2007). A good example of this chance fixation of advantageous alleles is the introduced fire ants (*Linepithema humile*) in Chile, Bermuda, and the United States. The loss of genetic diversity in these is associated with reduced inter-colony aggression and subsequently ecological success (Tsutsui *et al.* 2000).

2.2.3. Hybridisation and invasive species

The success of some invasive species is closely linked with hybridisation. This hybridisation can occur between closely related species or between divergent lineages within this same species; and can occur between introduced and native species/lineages, and between two introduced species/lineages. Hybridisation can lead to the formation of invasive lineages by creating new, novel combinations of alleles for selection to act upon (Hänfling 2007). An example of this is an invasive hybrid lineage of sculpins (*Cottus* sp.) in the River Rhine. These sculpins show novel habitat adaptations and life-history characteristics which allow them to colonise downstream river habitats that are not suitable for either parental taxa. They likely formed through secondary contact and interbreeding between lineages that were previously geographically separate (allopatric) (Nolte *et al.* 2005). Even if early-generation hybrids have low fitness, hybridisation can still prove beneficial as it can allow advantageous alleles (e.g. alleles associated with resistance to local diseases) to be incorporated into the gene pools of invasive populations (possible from interbreeding with related, indigenous species) without the large-scale mixing of genomes (Hänfling 2007).

Hybridisation can also lead to changes in chromosome number (ploidy), creating lineages that have one or two complete sets of chromosomes from two separate species (allopolyploid lineages). These lineages can be capable of, or limited to, asexual reproduction (parthenogenesis). They can subsequently have greatly elevated levels of reproductive output, as all individuals are capable of producing offspring (c.f. only the females in diploid, sexually reproducing species) (Hänfling 2007). Polyploidy lineages can also have high levels of genetic diversity upon which natural selection can act, even if they are limited to asexual reproduction, as they carry the full genomes of two separate species (Hänfling 2007). An example of an invasive, allopolyploid lineage is the gibel carp (*Carassius auratus gibelio* Bloch). This species colonised Europe from the Far East in the early 20th Century and has since been progressively expanding westward (Zhou and Gui 2002; Hänfling 2007).

2.3. What are Common Carp?

The word ‘carp’ is applied to many species of freshwater fish. Examples include the grass carp (*Ctenopharyngodon molitorella*), the silver carp (*Hypopthalmichthys molitrix*), the black carp (*Mylopharyngodon piceus*), the Indian major carps (*Cirrhinus mrigala, Catla*

catla, *Labeo rohita*), European crucian carp (*Carassius carassius*), Japanese crucian carp (*Carassius cuvieri*) and the Prussian carp (*Carassius auratus gibelio*). The name 'common carp,' however, refers to *Cyprinus carpio* L. All uses of the word carp in this review refer to the common carp, unless otherwise stated.

Common carp belong to the Order Cypriniformes and the family Cyprinidae. The family Cyprinidae (cyprinids) is one of the most speciose families of freshwater teleost fish in the world, containing seven subfamilies, 220 genera and approximately 20,000 described species. Examples include carps, barbs, minnows, roaches, rudds, daces, bitterlings, rasboras, danios and gudgeons (Howes 1991).

Common carp are characterized by the following traits: forked tail (caudal fin); no teeth in the mouth; three rows of pharyngeal teeth on the lower element of the last gill arch, the outer two rows of these pharyngeal teeth each having one tooth and the inner row having three teeth (1,1,3:3,1,1 arrangement, which separates common carp from many other Cyprinid species); 33–40 lateral-line scales; and two pairs of fleshy whiskers (barbels) on either side of the mouth, with the posterior pair being longer than the anterior pair (Koehn *et al*., 2000). These features (bar the toothless mouth) are illustrated in Figure 2.1, along with some other basic features of common carp anatomy. Common carp are typically full-scaled and coloured silvery-black/grey, olive-green or yellow-brownish on their backs, softening to pale yellow or cream on their bellies (Kirpichnikov 1981; Balon 1995; Lintermans 2007), although many colour and scale variants occur in both wild and cultivated populations (see section *2.5. Morphological Variation*).

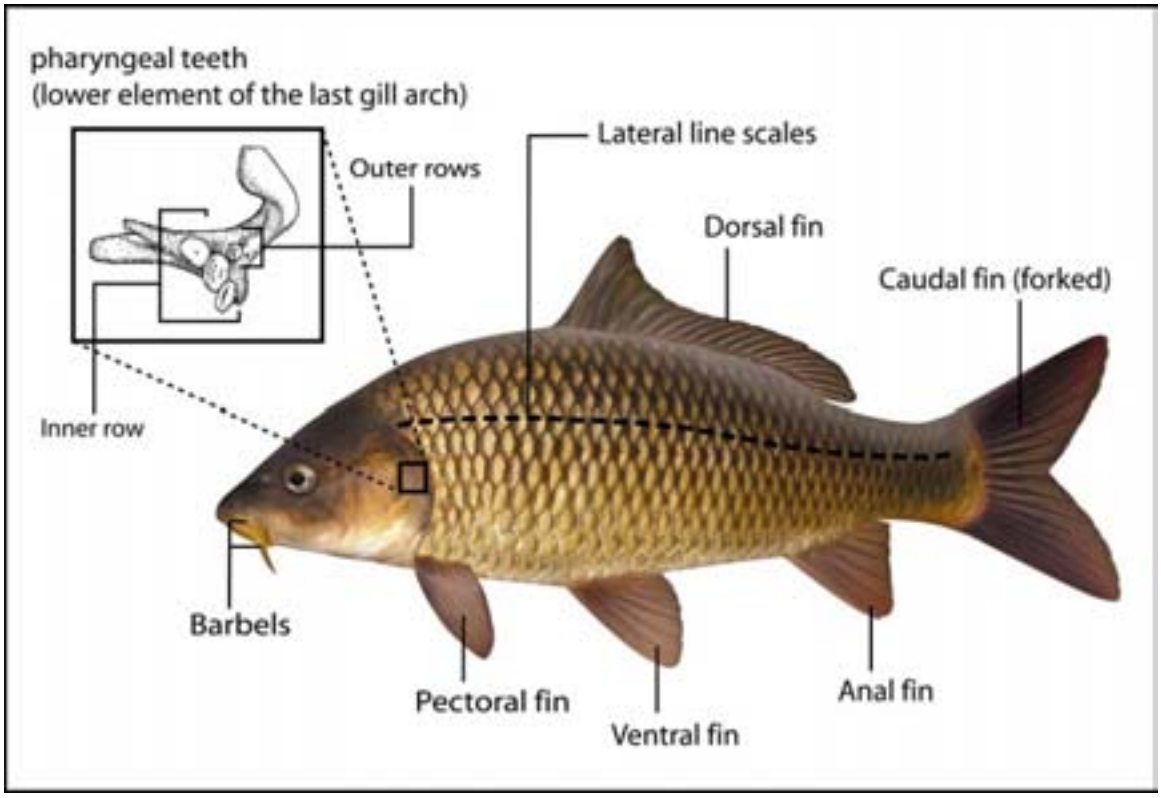


Figure 2.1. Key morphological features of common carp. Image supplied by D.M. Gilligan.

2.4. Biology of Common Carp

Carp are ecological generalists. They are tolerant to oxygen levels as low levels as 7 per cent saturation, high levels of turbidity, moderate salinity (14%), a wide range of temperatures (2-40.6°C) and high levels of toxicants (Koehn 2004). They prefer mid-latitude, low-altitude, slow-flowing rivers and standing waters (lakes, dams, billabongs etc.) and are less common in cool, swift-flowing streams (Koehn *et al.* 2000). In Australia, carp are generally rare at altitudes greater than 500 metres above sea level in NSW (Koehn *et al.* 2000; Gilligan and Rayner 2007), although a large carp population is present in the upper reaches of the Murrumbidgee catchment around the township of Cooma, at an elevation of approximately 798 metres; and they occur at other sites in NSW as high as 900 metres (Gilligan, unpublished data).

Carp are bottom feeders, sucking sediments into their mouths and expelling indigestible particles through their gill openings (Koehn *et al.* 2000). Their diet varies depending on what foods are available, but they are known to eat microcrustaceans, aquatic insect larvae,

molluscs, swimming and terrestrial insects and seeds and other plant matter (Hume *et al.* 1983a; Koehn *et al.* 2000; Khan 2003).

In Australia, common carp have been observed to spawn in waters that show seasonal temperatures of between 17-29°C (Hume *et al.* 1983b; P. Gehrke, unpublished data, cited by Koehn *et al.* 2000). While spawning normally occurs in spring through to autumn in Australia (Smith 2005), year-round spawning has been observed in the invasive carp population in the Botany Wetlands in Sydney, Australia (Pinto *et al.* 2005). Carp migrate to and from appropriate spawning grounds during breeding season, sometimes travelling hundreds of kilometres (Balon 1995; Stuart and Jones 2006). Eggs are sticky and are laid on submerged vegetation (Balon 1995; Koehn *et al.* 2000; Horváth *et al.* 2002). This stickiness has been hypothesised as facilitating carp dispersal, as eggs can stick to the feet of water fowl and be subsequently transported between waterways (Gilligan and Rayner 2007). Flood conditions are especially favourable to carp spawning, as they provide abundant food resources for adults and abundant vegetation for the attachment of eggs and result in plankton blooms to provide food resources for growing larvae and juveniles.

2.5. Common carp as an invasive species

The native range of common carp extends from Japan (Mabuchi *et al.* 2005) to the River Danube in Eastern Europe (Balon 1995). Human activities associated with the cultivation and domestication of carp for food and for ornamental characteristics, however, have introduced common carp into many new waterways throughout Asia, Africa, the Americas, Oceania, Australia and New Zealand (Koehn 2004). Climate and habitat-matching studies indicate that carp have great potential to further expand their range in Australia (Koehn 2004) and the Americas (Zambrano *et al.* 2006), and to a limited extent in Africa (Costa-Pierce *et al.* 1993)..

In Australia, common carp are a highly invasive species. They are established in all states and territories, except the Northern Territory (Koehn 2004). They are the dominant species in the MDB, being present in practically all parts of the basin (Lintermans 2007), except where colonisation is limited by unsuitable habitat (e.g. upper Murray) (Gilligan and Rayner 2007), weirs or waterfalls (Koehn *et al.* 2000; Graham *et al.* 2005); reaching densities of up to 11,316 individuals per ha (7,700 individuals ≤100mm in length, 3 616 individuals >100mm) in some regions (Reid *et al.* 1997); and constituting 85.9% of total

fish biomass in the Murrumbidgee drainage (Gilligan 2005). Carp are also common in many coastal waterways (Koehn 2004). A small population is present in the interconnected Lakes Crescent and Sorell in Tasmanina, although extensive effort has been made to eradicate or control these populations and they will likely be extirpated in the near future (Inland Fisheries Service 2007). The range of carp in Australia is illustrated in Figure 2.2.

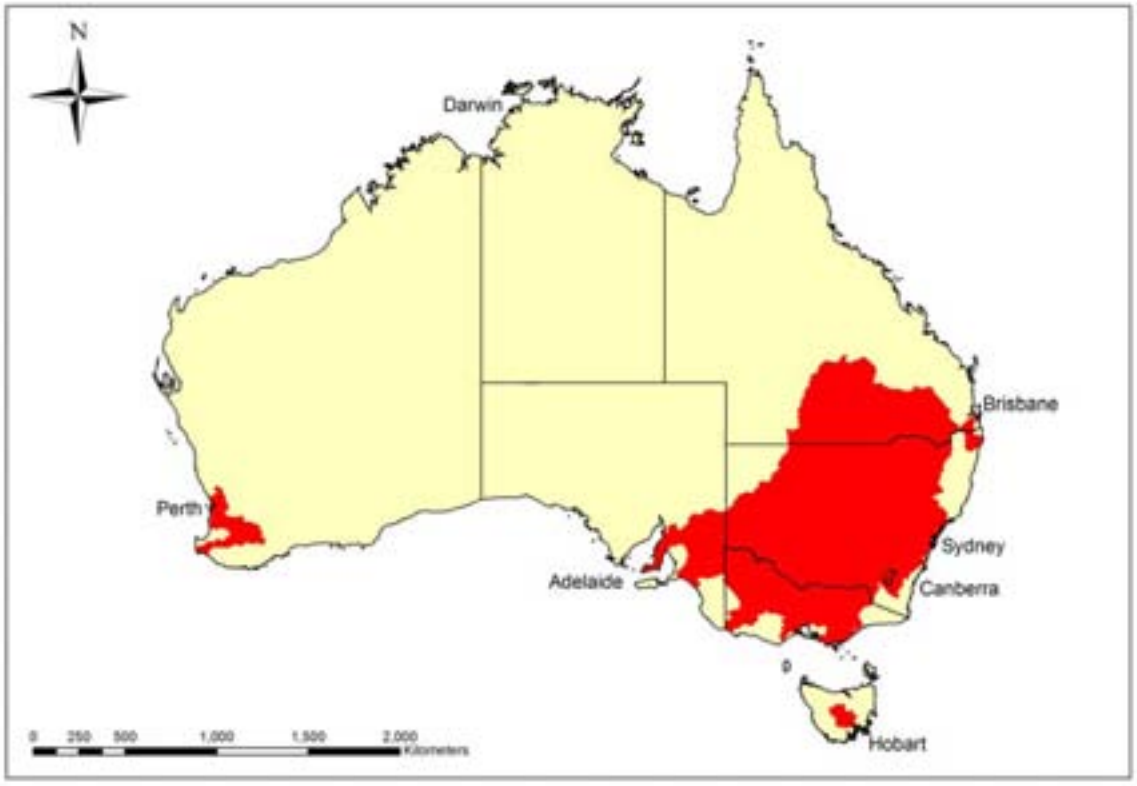


Figure 2.2. Australian catchments where introduced carp have established self-sustaining populations (map supplied by D.M. Gilligan, 2008).

The only other freshwater fish species with comparable invasiveness in Australia is the goldfish. This species is even more widely distributed than common carp, being present in most of MDB (Lintermans 2007), around urban centres (Brumley 1991), in the Lake Eyre drainage (in which carp are not established) (Koehn 2004) and in many carp-free rivers in Western Australia (Morgan *et al.* 2004). As millions of goldfish are imported annually for the aquarium industry, range expansion via the release of individuals and greater invasiveness through the addition of genetic variation is likely (Brumley 1991). Fortunately, despite their widespread distribution, goldfish are far less destructive than carp, and comprise only a small percentage of the total number of fish in the MDB

(Faragher and Lintermans 1997), and only 0.5% of the biomass in the Murrumbidgee catchment (Gilligan 2005).

The success of carp in the MDB can be explained, at least in part, by the heavy modification of the basin by human activities since European settlement. As described previously in section 2.1, Moles *et al*. (2008) demonstrated that in habitats recently modified by human activities, new ecological niches are created that are not fully occupied by indigenous species, and introduced species have a much greater change of becoming invasive if they are preadapted to utilising these newly created niches. The development of water resources for agriculture, hydroelectricity, flood mitigation, and domestic use in the MDB has required the construction of many dams, weirs, reservoirs and irrigation canals, and has made some wetlands more permanent. These still-water environments are ideal habitats for carp. In addition, human activities have increased the levels of pollution, salinity and nutrient runoff, all of which carp are tolerant of (Koehn *et al.* 2000). These same modifications have been largely detrimental to native fish species, because the natural flow regimes of the rivers to which the native species are adapted have been drastically altered; the stability of the human-controlled environment favours only a small number of native species; cold water released from the lower levels of dams inhibits native species' ability to spawn; dams and weirs prevent migration; and native fish are largely intolerant of high levels of pollution, salinity and nutrient runoff (Koehn *et al.* 2000). Carp in the MDB are, in effect, occupying newly created ecological niches that the native fish have not evolved to utilise. This is confirmed by Koehn (2004), who compared 13 species-specific attributes in carp and in abundant native fish species, and found that carp in the MDB differed clearly from native species in their behaviour, resource use and population dynamics.

In addition to occupying ecological niches not occupied by native species, carp may simply be a 'good' invasive species. While invasions success of an introduced species can be difficult to predict and highly idiosyncratic (Colautti *et al.* 2006; Moles *et al.* 2008), Koehn (2004) compared the ecological, behavioural, life history and genetic characteristics of Australian common carp with those of other invasive fish species and identified eleven characteristics that common carp share with these other invasive fish. These characteristics are summarised in Table 2.3. Taken individually, any one of these traits would be unlikely

to predict invasiveness in common carp. Taken together, however, these 11 traits make a strong case that carp are fundamentally well adapted to invading new environments.

Table 2.3. Attributes of carp as an invasive species; modified from Koehn (2004).

Attribute	Details
History of invading many areas	Introduced and successfully established throughout Europe, Asia, Africa, North America, South and Central America, Australia, New Zealand, Papua New Guinea and some islands in Oceania
Wide environmental tolerances	High environmental tolerances, with temperature tolerance ranging from 2 to 40.6 °C, salinity tolerances up to about 14 % (40% the salinity of seawater) and pH from 5.0 to 10.5, oxygen levels as low as 7% saturation.
High genetic variability	At least four strains have been introduced into Australia: Yanco, Prospect, Boolara, and Japanese koi (see section *2.11.3. Population genetics of common carp in Australia*)
Early sexual maturity	Males as early as 1 year, females as early as 2 years
Short generation time	2-4 years
Rapid growth	Hatching of eggs is rapid (2 days at 25 °C) and newly hatched carp grow very quickly
High reproductive capacity	They are highly fecund broadcast-spawners with egg counts as high as 2 million per female
Broad diet	Omnivore/ detritivore
Gregariousness	Carp, like many other invasive fish species, form schools
Possessing natural mechanisms of dispersal	A mobile species with fish moving between schools. Dispersal can also occur with the downstream drift of larvae. Rates of transfer can be increased by conditions such as flooding
Commensal with human activity	Bred as an ornamental and aquaculture species, used as bait and sought by some anglers

2.6. Domestication

Common carp have a long history of domestication. They have been reared in ponds in China as early as the 5th century B.C.E. (Horváth *et al.* 2002), and in Europe by monks as early as the Middle Ages (Balon 1995). Balon (1995) argues for an even older domestication of common carp in Europe, suggesting that carp were first domesticated by the Romans in the 1st and 2nd centuries C.E. This argument is based on evidence that the Romans maintained ponds for freshwater fish, that carp were an important food source in Roman settlements along the River Danube, and that common carp are sufficiently robust

to survive being transported from the River Danube to man-made ponds in Western Europe, provided that they are wrapped in wet moss. There is, however, no direct evidence to support this theory. Balon (1995) also disputes early domestication of common carp in China, arguing that the fish stocked in early Chinese ponds could have been other carp species, such as grass carp, silver carp or the Indian major carps, rather than *Cyprinus carpio*. While it is difficult to verify exactly which species were reared in ancient China, there is currently a wealth of aquaculture carp strains in China that have been derived from indigenous wild populations and have a long history of cultivation (Kohlmann *et al.* 2003; Zhou *et al.* 2004a; Zhou *et al.* 2004b; Kohlmann *et al.* 2005). Xingguo red carp, for example, have been cultivated for approximately 1,300 years (Zhou *et al.* 2004a). Even if common carp domestication in China does not date back to the B.C.E. period, it has been in practice in this region for over 1,000 years.

Today common carp are a globally important species. Being fecund and robust, they are ideal for aquaculture and as such are farmed extensively throughout Eurasia, and to a lesser extent in North and South America (Zambrano *et al.* 2006) and Africa (Costa-Pierce *et al.* 1993). In Asia they are typically grown in polyculture ponds with 3-5 other fish species, each exploiting a different ecological niche in the pond (Koehn *et al.* 2000). The harvesting of common carp for food, both from the wild and from aquaculture, has been growing steadily since the late 1970s, surpassing the production of all salmonoid species combined in 1997, and was estimated to be in excess of 3 million tons in 2006 (FAO 2008). Common carp are subsequently an important source of protein and income for many people. The trade in ornamental Japanese koi carp is also worth millions of dollars annually (Balon 1995).

Due to their enormous natural range and long history of domestication, carp exhibit much morphological variation (see section 2.7 below). They can be divided into at least four naturally occurring subspecies and innumerable domestic strains and evolutionary significant units (ESUs) (see sections 2.8 and 2.9).

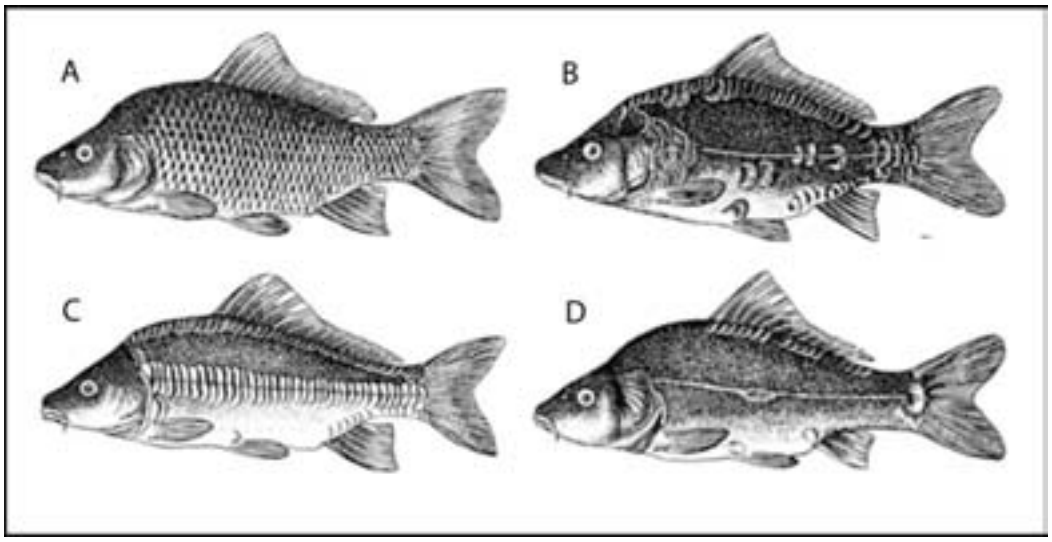
2.7. Morphological variation

Wild carp are typically torpedo-shaped, full-scaled, and coloured silvery-black/grey, olive-green or yellow-brownish on the dorsal surface, softening to pale yellow or cream on the ventral surface and flanks (Kirpichnikov 1981; Balon 1995; Lintermans 2007). Variations

in scale morphology, colour and body shape, however, are common in both wild populations and domestic strains.

Domestic carp are typically rounder and plumper-bodied than wild carp (Michaels 1998). Feral population of domestic carp, however, revert to a wild-type body shape soon after establishment (Balon 1995). Traits such as dwarfism, the absence of ventral fins, the presence of an additional fin, elongated fins and a dolphin-like head have also been reported in both wild and domestic populations (Kirpichnikov 1981; Wang and Li 2004).

'Mirror' scales are a common feature of domestic carp strains. These scales are larger and shinier than ordinary scales, and usually do not cover the entire body (Kirpichnikov 1981). The absence of normal scales has been favoured by artificial selected in domestic fish to make them easier to de-scale for cooking (Michaels 1998). According to Kirpichnikov (1981), the inheritance of mirror scales is controlled by two loci, *S* and *N*. Depending on genotype at the two loci, a carp may have scattered mirror scales ('scatter scale' phenotype), a single line of mirror scales running along its flanks ('linear mirror' phenotype), no scales or almost no scales ('nude' or 'leather' phenotype), or a full cover of normal scales (wild-type phenotype) (Kirpichnikov, 1981). The scale phenotypes and genotypes described by Kirpichnikov (1981) are summarised in Figure 2.3. The extent of scale covering in mirror-scaled individuals is not entirely governed by these two loci. Nicolescu (2004), for example, observed nude phenotype individuals in the absence of the *N* allele, presumably as an extreme variant of the scatter scale phenotype (*ssnn*). Mirror-scaled carp are found in wild and domestic populations of European and Asian carp (Kirpichnikov 1981). About five per cent of Australian feral carp have mirror-scale phenotypes (Koehn *et al.* 2000), with both the scattered and linear phenotypes being observed (personal observations).



A.	Full covering of normal scales (wild-type)	*SSnn, Ssnn*
B.	Scattered mirror scales	*ssnn*
C.	Linear mirror scales	*SSNn, SsNn*
D.	Nude or leather	*ssNn*

Figure 2.3. Phenotypes and hypothesised genotypes of scale morphs in common carp, as controlled by the genes *S* and *N*. The genotypes have been inferred from extensive multi-generational breeding experiments. Individuals with genotypes *SSNN, SsNN* or *ssNN* are presumed to be non-viable embryos. The large, shiny scales in phenotypes B-D are referred to as "mirror scales." Note that much variation in the location and number of mirror scales occurs, and that the illustrations here only represent 'ideal' scattered mirror, linear mirror and nude common carp. Information and illustrations taken from Kirpichnikov (1981).

Reported colour variations include golden, red, blue, orange, steel, green, albino, yellow, lemon-yellow, green, violet and brown. These variants are reported in both wild and domestic populations (Kirpichnikov 1981; Bialowas 2004; Wang and Li 2004). In particular, red, golden and orange individuals are found amongst domestic and wild populations in both Europe and Asia (Kirpichnikov 1981; Balon 1995). Selective breeding for individuals for these novel colourations has led to the production of fancy carp, or koi, in Japan. Koi have been bred in Japan for at least 190 years, although the beginning of koi

farming might actually be far older (Balon 1995). Koi are now available in a wide range of colours, colour patterns, scale morphologies and body shapes.

2.8. Subspecies of common carp

The division of a species into subspecies is not always clear-cut. Biological systems rarely consist of discrete units beyond the level of the individual. Rather, they exist as a continuum of gene flow through space and time. Common carp have an enormous natural range and show much regional variation. Dividing the most divergent groups into subspecies is therefore a natural extension of their taxonomic classification, although there is no definitive way to decide where regional variation ends and subspecies status begins.

Common carp are frequently separated into two subspecies: the central-Asian/European *C. carpio carpio* and the east-Asian subspecies *C. carpio haematopterus*. This separation is well supported by microsatellite and mitochondrial genetic data (Kohlmann *et al.* 2003; Zhou *et al.* 2003; Zhou *et al.* 2004b; Kohlmann *et al.* 2005). The separation of south-east-Asian carp into an additional subspecies, *C. carpio viridiviolaceus/ rubrofuscus*, on the basis of mitochondrial sequence and morphological differences, has been suggested by some researchers (e.g. Kirpichnikov 1981; Zhou *et al.* 2004b). A central-Asian subspecies, *C. carpio aralensis*, was proposed by Kirpitchnikov (1967, cited by Balon, 1995). However, Kohlmann *et al.* (2003; 2005) and Memiş and Kohlmann (2006) demonstrated that European and central-Asian carp are closely related, with the latter comprising a subset of the genetic diversity of the former. The authors subsequently classified both European and central-Asian carp as subsp. *carpio*.

A unique Japanese subspecies may also exist. Mabuchi *et al.* (2005; 2008) investigated mitochondrial control region and cytochrome b sequences from a morphologically distinct lineage of carp indigenous to Lake Biwa, Japan. Phylogenetic analysis placed this Lake Biwa (LBW) strain basal to all other carp strains investigated, indicating its ancient origin. Although not suggested by the authors, the uniqueness of this lineage could warrant it being classified as a subspecies or even a separate species.

The natural distribution of common carp subspecies is illustrated in Figure 2.4.

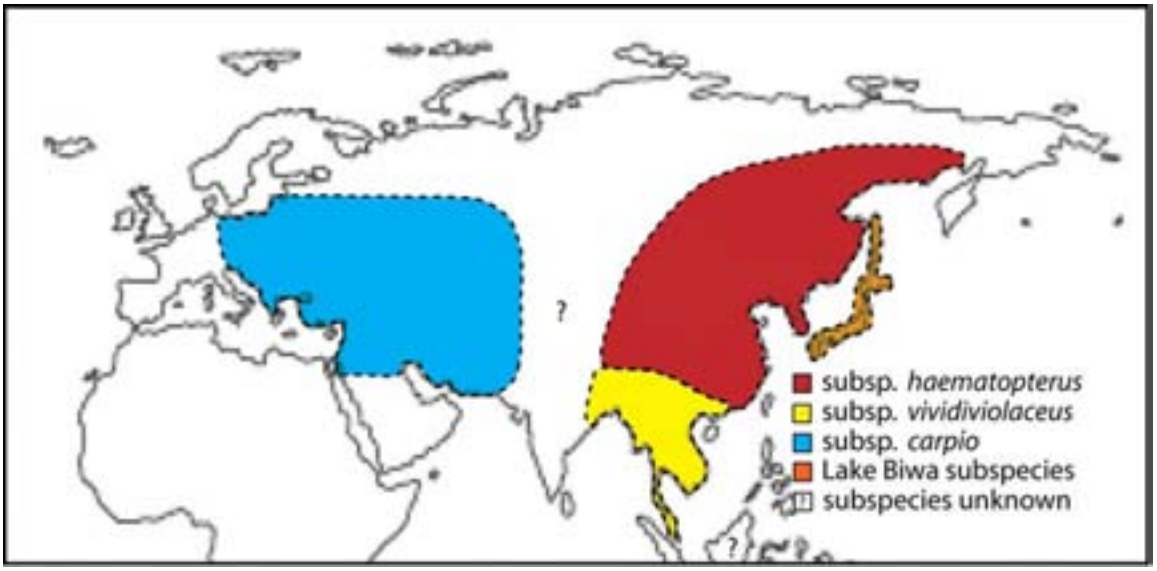


Figure 2.4. Natural distribution of common carp subspecies

2.9. Aquaculture strains and evolutionary significant units

The domestication of common carp has led to the development of innumerable aquaculture strains. These strains are typically selected for rapid growth and the ability to survive in the resource-limited and sometimes crowded conditions in fish ponds. Some strains are also selected for survival and growth under specific conditions or for ornamental characteristics. Examples include the Ropsha strain, which was developed in western Russia by the crossing of local domestic strains with wild carp from the River Amur in east Russia and selection of progeny for cold-tolerance (Zonova and Kirpichnikov 1968); the Xigguo red and purse red carp, which have been traditionally reared as food carp in China for centuries (Zhou *et al.* 2004a); the Oujiang colour carp and koi carp, which are bred for ornamental colouration in China and Japan, respectively (Wang and Li 2004); and the Bac Kan strain from Vietnam, which is specifically adapted to conditions in rice paddies (Edwards *et al.* 2000). These strains are important resources, providing a wealth of genetic diversity for aquaculture, research and the evolutionary potential of the species. Live "gene banks", where carp of different strains are maintained in separate ponds, have been established to maintain the genetic diversity and unique characteristics of these many strains (e.g. Gorda *et al.* 1995; Flajšhans *et al.* 1999; Bakos and Gorda 2001).

In addition to the four subspecies described above, common carp can be further divided into naturally occurring evolutionary significant units (ESUs). ESUs are populations which

are (1) substantially reproductively isolated from other conspecific units and (2) represent an important component of the evolutionary legacy of the species (Waples 1991). While delimiting ESUs is at least partially subjective, Moritz (1994) suggests that that ESUs can be identified by the presence of reciprocally monophyletic mitochondrial lineages among areas, coupled with a corresponding divergence in nuclear allele frequencies. To date, I know of no comprehensive study addressing the total number of wild carp ESUs around the world for conservation. However, the genetic structuring detected between natural populations (Kohlmann *et al.* 2005) indicates that at least some must exist beyond the four subspecies. At a minimum, one naturally occurring population from each major river basin in carp's natural range could be proposed as ESUs. The genetic integrity of many carp ESUs is threatened by the release of aquaculture strains into waterways (Balon 1995; 2004; Mabuchi *et al.* 2005; Mabuchi *et al.* 2008). Like aquaculture strains, though, representatives of wild carp populations – that could be ESUs - are also maintained in live gene banks.

2.10. Population Genetics of the Common Carp

There have been numerous population genetic studies on common carp throughout the world. These studies have utilised morphological markers, microsatellites, allozymes and mitochondrial DNA, and combinations of such genetic markers. Key studies are listed in Table 2.4.

The majority of population genetic studies have been performed at a local level, comparing a small number of populations that are geographically close together (e.g. Desvignes *et al.* 2001). More recently, however, large-scale studies have been performed that compare multiple populations/strains of carp from across Europe and Asia (Froufe *et al.* 2002; Kohlmann *et al.* 2003; Kohlmann *et al.* 2005; Thai *et al.* 2005). These studies have provided fresh insights into the taxonomy, evolutionary origin, demographic history and the genetic variation and structure of common carp populations. The taxonomy of carp is addressed in sections *2.1*, *2.6* and *2.7*. The evolutionary origin and demographic history and the genetic variation and structure are summarised in the following sections.

Table 2.4. Population genetic studies of common carp

Regions sampled	Genetic markers used	Reference
Europe, Asia	Morphology	Svetovidov (1933) and Mišík (1958), cited by Balon (1995)
Australia (feral carp)	Allozymes, morphology	Shearer and Mulley (1978)
Japan	Allozymes	Macaranas *et al*. (1986)
Italy	Allozymes	Catuadella *et al*. (1987)
Indonesia	Allozymes	Sumantadinata and Taniguchi (1990)
Estonia	Allozymes	Paaver and Gross (1991)
Hungary	Allozymes	Csizmadia *et al*. (1995)
Poland	Allozymes	Anjum (1995)
Germany	Allozymes	Kohlmann and Kersten (1999)
Australia (feral carp)	Allozymes, mitochondrial RFLP	Davis *et al*. (1999)
Israel	Allozymes	Ben-Dom *et al*. (2000)
France, Czech Republic	Allozymes, microsatellites	Desvignes *et al*. (2001)
Czech Republic	Allozymes	Slechtova *et al*. (2002)
Europe, East Asia	mitochondrial PCR-RFLP	Gross *et al*. (2002)
Austria and Hungary (River Danube), Japan (koi), East Russia (River Amur)	Mitochondrial sequences	Froufe *et al*. (2002)
Uzbekistan	Allozymes	Murakaeva *et al*. (2003)
Europe, Middle East, Central Asia, East Asia, South East Asia	Allozymes, microsatellites, mitochondrial PCR-RFLP	Kohlmann *et al*. (2003)
Europe, Asia	Mitochondrial PCR-RFLP	Zhou *et al*. (2003)
East Asia, Eastern Europe	Mitochondrial sequences	Zhou *et al*. (2004b)
East Asia (China)	Microsatellites	Zhou *et al*. (2004a)
East Asia, Europe, South East Asia, Indonesia, India	Mitochondrial sequences	Thai *et al*, (2005)
East Asia	Mitochondrial sequences, RAPD analysis	Wang and Li (2004)
Europe, Central Asia, East Asia, South East Asia	Microsatellites	Kohlmann *et al*. (2005)
Japan	Mitochondrial sequences	Mabuchi *et al*. (2005)
Vietnam	Mitochondrial sequences, mitochondrial PCR-SSCP	Thai *et al*. (Thai *et al*. 2006)
Turkey	Microsatellites, mitochondrial PCR-RFLP	Memiş and Kohlmann (2006)
Japan	Mitochondrial sequences	Mabuchi *et al*. (2008)
Europe and East Asia	Mitochondrial sequences	Wang and Li (submitted)

2.10.1. Evolution and demographic history of common carp

Carp most likely evolved from an ancestral species in east-Asia between 0.85 and 3.0 million years ago (Froufe *et al.* 2002). Evidence for this includes the presence of basal mitochondrial lineages in east-Asia (Froufe *et al.* 2002; Mabuchi *et al.* 2005), and the higher prevalence of private microsatellite alleles in east-Asia compared to Europe and central-Asia (Kohlmann *et al.* 2005). While it is possible that carp evolved in central-Asian or Europe and lost much genetic diversity in a severe bottleneck, this is a far less likely scenario, as these regions lack the basal lineages detected in east-Asia. Carp have unusually shallow levels of mitochondrial sequence divergence relative to other freshwater

fishes (Thai *et al.* 2006), indicating that their split from an ancestral species has been relatively recent, or that they have undergone an extensive selective sweep.

From east-Asia, carp spread to central-Asia. From central-Asia, they colonised the European catchments, most likely after the last glacial maximum (~19,000 years ago), when fish from the Caspian Basin entered the Danube Basin (Kohlmann *et al.* 2003). Although many authors assume that this colonisation was a natural event (e.g. Balon 1995), Froufe *et al.* (2002) speculates that the colonisation of the Danube Basin could have been human-mediated.

Carp underwent a severe bottleneck when colonising Europe. European carp hence show less mitochondrial diversity than Asian populations. Froufe *et al*. (2002) detected no polymorphism when sequencing the control region of 21 wild carp from the River Danube. Kohlmann *et al*. (2003) detected only two composite haplotypes (H1 and H3) in 227 European carp sampled from 11 locations, when screening for polymorphisms of the ND-3/4 and ND-5/6 loci using PCR-RFLP. One of these haplotypes (H3) was more likely a result of contamination of local fish stocks with Asian carp rather than naturally occurring variant in the European population. Further PCR-RFLP for ND-3/4 and ND-5/6 of carp in Turkey by Memiş and Kohlmann (2006) revealed four additional haplotypes that differed from haplotype H1 by only one or two restriction sites. Wang and Li (submitted) identified some additional control region sequences in European carp, but these differed from the sequences of Froufe *et al*. (2002) at only one or two sites.

2.10.2. Genetic variation and structure

Despite their relatively short evolutionary history, common carp show strong regional variation (i.e. population structure). Almost all genetic studies to date have detected significant differentiation (departure from panmixia, non-zero genetic distances and F_{ST} values) between carp from different rivers and aquaculture stocks (e.g. Desvignes *et al.* 2001; Kohlmann *et al.* 2005). Kohlmann *et al*. (2005) detected one notable exception to this among the many carp populations they analysed, with pairwise comparisons between four wild central-Asian populations not revealing significant F_{ST} values. A wider study including more free-living wild and feral populations of carp in adjacent waterways could help resolve this. Generally, domestic populations have less genetic diversity and are more genetically differentiated than wild populations (Kohlmann *et al.* 2003; 2005; Memiş and

Kohlmann 2006), which likely stems from domestic populations undergoing repeated founder effects (leading to smaller effective population sizes) and having less dispersal ability than wild populations.

2.10.3. Limitations of the population genetic studies of common carp

There are four main limitations to population genetics studies of common carp: sample size, human-mediated movement, the apparent effect of domestication on genetic variability, and the variability in markers used in different studies.

Insufficient sample sizes can lead to inaccurate representations of allele, genotype and haplotype frequencies. Studies on simulated data by Kalinowski (2004), however, indicate that small sample sizes can be compensated for by using a greater number of loci.

Human-mediated movement of carp can confound historical natural patterns of genetic variation. Balon (1995), for example, described the wild "large, torpedo-shaped, fully-scaled and gold-coloured carp" in the River Danube as "endangered… because of rampant introduction of the domesticated form… a pure wild form may not exist anymore." Kohlmann *et al.* (2003) detected Asian mitochondrial haplotypes (haplotype H3) present in the Danube (near Straubing, Germany). The active release of foreign strains into Vietnamese ponds, mentioned by Thai *et al.* (2006), will no doubt affect the genetic composition of the wild population, as escaped domestic fish find their way into the local waterways and breed. Mabuchi *et al*. (2008) detected high levels of carp introduced from Eurasia in Japanese waterways, stating that "almost half or more of the haplotypes in all of the locations studied originate from domestic strains introduced from Eurasia." Many modern carp population are now composed of a mixture of the local strain of carp and of escaped domestic carp, which can be derived from almost anywhere in the world. Population genetic studies of common carp in the wild will increasingly reflect recent patterns of human-mediated dispersal, rather than historical patterns of genetic variation.

There are some limitations to comparing free-living carp populations to domestic populations. Differences in genetic diversity between regions can be used to work out colonisation routes, and to detect ancient and recent bottlenecks. Results from population genetic studies must be interpreted cautiously when comparisons are made between wild

and domestic populations, as the genetic diversity in the domestic populations will likely be more reflective of modern breeding regimes than of long-term historical processes.

The fourth limitation of population genetic studies of common carp is that different studies often use different combinations of loci and marker types, which presents a challenge if one wishes to combine data. Furthermore, allozyme and microsatellite data from different studies cannot be readily combined, even when the same loci are used, because scoring of alleles is not consistent between the apparatus (e.g. gel rigs, sequencing machines) used by different research groups, even for equipment of the same make and model. In addition, different mitochondrial loci are favoured by different research groups, so the results of many mitochondrial studies also cannot be combined for further analysis (see Table 2.5).

Inter-study comparisons are possible, though. Results concerning genetic diversity (e.g. nucleotide diversity, mean allele diversity, allele richness, observed and expected heterozygosity) or distance (e.g. F_{ST}, Nei's genetic distances, Cavalli-Sforza & Edwards distance) are comparable, even when different loci are analysed. Most studies report a number of such measures, further facilitating such inter-study comparisons. Sequence and RFLP data are also comparable, provided that the same loci are scored using the same methods (region sequenced, enzymes used in the RFLP, etc.). Microsatellite and allozyme data sets can also be combined, provided that the same loci are used, researchers are willing collaborate to share allele scoring data (which is generally not published with the original research article), and representative samples are made available from which to calibrate allele callings (see *2.8.4 Future work* below).

Table 2.5. Mitochondrial loci and analysis methods used in different studies of common carp.

Study	Mitochondrial Loci	Analysis method
Memiş and Kohlmann (2006), Kohlmann *et al*. (2003), Gross *et al*. (2002)	ND-3/4, ND-5/6	RFLP
Thai *et al*. (2006)	control region	sequencing, SSCP
Mabuchi *et al.* (2005)	control region cytochrome b	Sequencing
Thai *et al.* (2005)	MTATPase6/ MTATPhase8 control region	Sequencing
Zhou *et al*. (2004a; 2004b)	control region cytochrome b	Sequencing
Zhou *et al*. (2003)	ND-5/6 control region	RFLP (ND-5/6), sequencing (control region)
Froufe *et al*. (2002)	control region	Sequencing
Davis *et al*. (1999) Davis (1996)	complete mitochondrial genomes	RFLP

2.10.4. Future Work

Future studies of the population genetics of common carp could aim to 1) identify genetic units for conservation of wild and aquaculture populations, or control of carp where it is an invasive species; and 2) learn more about the evolution and history of common carp. Such studies would require a large-scale sampling regime that covers the entire range of carp. In addition to neutral genetic markers, information about the morphology and ecology of the carp under investigation could also be included, as some ESUs or strains may be specifically adapted to local conditions without showing strong genetic differentiation from neighbouring populations at neutral markers. Once population units have been determined, recommendations can be made to government and industry bodies for the conservation of wild and aquaculture strains, and for the control of feral populations. Inferences about the population dynamics, history and evolution could also be made. These could also help further inform conservation or control programs, by refining the delimiting of population

units, and by identifying aspects of carp biology relevant to their conservation or control (e.g. recruitment dynamics).

Future work on common carp would be facilitated if a consistent suite of markers were used between studies. Yue *et al.* (2004) list 21 unlinked microsatellite markers reported from different studies, with the recommendation that they be adopted by different research groups for the sake of consistency. Nineteen of these microsatellites, however, are dinucleotides (two different base pairs repeated in tandem, e.g. $(TA)_n$). Dinucleotides can be difficult to score accurately as slippage of the DNA polymerase enzyme during PCR can lead to the insertion or deletion of nucleotide repeats in the PCR products, creating products of different size from the original template. These inaccurately replicated products create a 'stutter' pattern which can be difficult to discern from the true alleles. Tri- and tetranucleotide microsatellites (three and four base pairs repeated in tandem) are much less prone to stuttering and are hence preferable for use in genetic studies over dinucleotides. At least 17 tri- and tetranucleotides have been reported for common carp and related species (Naish and Skibinski 1998; David *et al.* 2001; McConnell *et al.* 2001; Yue *et al.* 2004). Nine of these were reported by Yue *et al.* (2004), of which 6 had comparable levels of genetic diversity (4-11 alleles) to the 21 recommended loci (4-17 alleles). It would therefore have been useful if You *et al.* (2004) had included more tri- and tetranucleotides in their list of 21 microsatellites. This may not have been practical, however, as Yue *et al.* (2004) may not have been confident that these markers were all unlinked.

As mentioned in the previous section, merely using the same microsatellite markers does not make it possible to combine data from different studies, as even machines of the same make and model will give slightly different results in different laboratories. Combining data from different studies requires collaboration between different research groups. Such groups need to exchange samples with known genotypes, and use these samples to calibrate their microsatellite allele calling.

Future work with mitochondrial markers should ideally employ sequencing of whole loci. I recommend using both the control region, as this locus is highly variable, and the cytochrome b locus, as this gene is used as a universal barcode for living organisms (Hebert *et al.* 2003). The control region and the cytochrome b sequences can be combined

into composite haplotypes for analysis. The time and expense required for the sequencing of whole loci, however, could make this impractical for some research groups or projects.

2.11. Common carp in Australia

2.11.1. Introduction of carp

Carp were first introduced to Australia by acclimatization societies trying to establish food resources and recreational fisheries (Brumley 1991). In addition to common carp, such societies were successful in introducing other species of Cyprinid, namely goldfish (*Carassius auratus*), crucian carp (*Carassius carassius*), tench (*Tinca tinca*) and roach (*Rutilus rutilus*). Exactly which species were and are present has been a matter of confusion since colonial times. Stead (1929) noted that introduced fish were frequently misclassified by aquaculturalists, with goldfish, common carp and crucian carp being frequently confused with each other. The presence of crucian carp in the MDB was reported by Whitley (1951), and was later refuted by museum curators in 1980 (Clements 1988), before being recently confirmed in the Campaspe River (a tributary to the Murray River) in eastern Victoria (MDBC 2008b). Tench and roach are more distinctive in appearance than common carp, crucian carp and goldfish, and therefore are not subject to the same confusion as the latter three. The history of known common carp introductions is summarised in Table 2.6.

The earliest known introductions occurred in Hobart, Sydney and Melbourne. Carp, most likely from England, were introduced to Hobart in 1858. In Melbourne, introductions (possibly from Hobart) occurred from 1859 to 1876, but did not give rise to self-sustaining populations in the wild. A large population of red-orange-yellow colourful carp, however, was established in the Melbourne Botanical Gardens, where it remained until 1962 when it was eradicated by the state government after the Noxious Fish Act was passed (Clements 1988; Koehn *et al.* 2000). In Sydney, cyprinids of unknown origin, which may have included common carp, were released into ponds around Government House around 1865 and were distributed to local waterways (Koehn *et al.* 2000). In 1907-08, David Stead, an employee of NSW Fisheries, purchased 17 fingerlings of unknown origin from a Sydney pet store, 14 of which were grown and bred in a fish farm in Prospect, a suburb of Sydney (Stead 1929). The descendants of these fingerlings were used to seed other populations around the Sydney Basin and were eventually released into Prospect Reservoir (Clements

1988), where they have persisted till the present day. These are referred to as the Prospect strain in the literature (Shearer and Mulley 1978; Davis *et al.* 1999) (see *2.9.3. Population genetics of carp in Australia*). Whether they are solely descended from the fingerlings purchased by Joseph Stead, or also have some ancestry with the cyprinids released in 1865, is unknown.

Carp have been present in the MDB at least as early as the 1920s. Correspondence between the NSW Fisheries Department and the Victorian Fisheries and Games Department in 1929 describes low numbers of *C. carpio* being caught in the MDB in both states (Clements 1988). While I know of no precise record of the events surrounding the introduction of these carp, they were possibly sourced from Sydney. Rolls (1969) mentions that cyprinids from the ponds around Government House in Sydney were frequently transported to and released into the MDB prior to the 1920s. By the 1960s, carp were "widespread but only common in irrigation canals and some other sluggish waters in New South Wales" (Weatherly and Lake 1967).

In the Murrumbidgee Irrigation Area (MIA), an extensively irrigated region of the MDB in central-southern NSW, a distinctive orange-coloured strain of carp became established. It is unclear when these carp were first introduced. Brown (1996) states that the strain was introduced in the 1950s. Koehn *et al*. (2000), however, suggest that introduction occurred in the 1930s or 1940s, when large numbers of fish were released by acclimatisation societies (Clements 1988). Gilligan (pers comm. 2008) suggests that these fish were introduced after work began on the region's irrigation systems in 1912. Shearer and Mulley (1978) describe these carp as the Yanco strain (see *2.9.3. Population genetics of carp in Australia*).

In the late 1950s and early 1960s, Boolara strain carp (see *2.9.3 Population genetics of carp in Australia*) bred by Boolara Fish Farms Pty. Ltd., in Gippsland, Victoria, were distributed to farm dams throughout Victoria. Despite eradication attempts, these carp spread to the La Trobe River and Lake Wellington in south-eastern Victoria by 1962. They entered the Murray River via Lake Hawthorn in 1968 (Clements 1988; Koehn *et al.* 2000).

More recently, ornamental Japanese koi carp have been released into Australia by irresponsible pet owners. These colourful fish are now often sighted in urban waterways

(pers. obs.; Figure 2.5.). Koehn *et al*. (2000) reports the presence of koi in coastal rivers near Perth, Lake Burley Griffin in the ACT, and Lakes Crescent and Sorell in Tasmania. Additionally, Graham *et al.* (2005) report the presence of koi in the Richmond, Bellinger, Hastings, Karuah and Towamba catchments in coastal NSW. Most recently, a koi carp was collected from the Macleay catchment (coastal NSW) in February 2008 (D.M. Gilligan pers. comm.).

Figure 2.5. Colourful koi carp in urban waterways. Picture taken in Lake Northam, Victoria Park, Sydney (33°35’6.08”S 151°11’36.26”E) by Gwilym Haynes.

Table 2.6. History of introduction of common carp in Australia. Unrecorded introductions are also likely to have occurred. Further details are given in *2.11.3 Population genetics of common carp in Australia*.

Origin	Introduction Date	Location	Strain	Current distribution	Reference
Europe, probably England	1858	Hobart, Tasmania	Unknown	Did not establish	Clements (1988)
Unknown; possibly from Tasmania	1859-1876	Melbourne, Victoria	Unknown	Did not establish in the wild; persisted in the Botanic Gardens till 1962	Clements (1988); Anon (1862) and Hume *et al.* (1983b) cited by Koehn *et al.* (2000).
Unknown	1865-1866, 1907-08	Multiple locations in Sydney Basin, most notably Prospect Reservoir	Prospect	Present in Sydney Basin; possibly introduced into MDB	Stead (1929); Koehn *et al.* (2000)
Unknown. Suggested as being Singapore koi, or from Melbourne Botanical Gardens	Unknown. Suggested as 1920-30s, or 1950s.	MIA, NSW	Yanco	Originally restricted to MIA; may have spread to other parts of the MDB.	Shearer and Mulley (1978); Davis *et al*. (1999); Brown (1996); Koehn *et al*. (2000)
Boolara Fish Farms Pty. Ltd.	1962	Multiple farm dams and lakes in Victoria, includingLake Hawthorn	Boolara	Widely distributed throughout MDB and Melbourne Basin	Clement (1988); Shearer and Mulley (1978)
Unknown	Between 1970 and 1977	Torrens catchment, Adelaide, South Australia	Unknown	Present in Torrens catchment. May have spread to other sites in SA.	Koehn *et al*. (2000)
Unknown	Between 1977 and 1998	Glenelg and Barwon Rivers, Victoria	Unknown	Still established. May have spread to adjacent water bodies.	Koehn *et al*. (2000)
Japan*	1976	Lake Burley Griffin, ACT	Japanese koi	Urban waterways in ACT; may have reached MIA.	Koehn *et al*. (2000), Davis *et al*. (1999)
Japan*	1990s	Lakes Crescent and Sorell, Tasmania	Japanese koi	Restricted	Koehn *et al.* (2000)
Japan*	1990s	Coastal rivers near Perth, WA	Japanese koi	Restricted	Koehn *et al.* (2000)
Japan*	2004	Sydney Basin	Japanese koi	Restricted	Personal observation
Japan*	Before 2005	Multiple NSW coastal waterways	Japanese koi	Restricted	Graham *et al*. (2005)
Japan*	2008	Macleay catchment	Japanese koi	Restricted	Gilligan (pers. comm.)

* These carp were not necessarily sourced directly from Japan. Although a Japanese strain, they could have been bred in local fish farms from Japanese ancestors prior to release.

2.11.2. Population growth and spread of carp in Australia

In the Sydney Basin, the carp established in Prospect Reservoir remained localised despite being introduced to multiple locations around Sydney (Koehn *et al.*, 2000). This localisation was not likely a result of any genetic limitations, as there are early reports of Sydney carp reproducing "at an alarming rate" (Clements, 1988). More likely, the introduced carp were physically constrained by the Great Dividing Range and the Tasman Sea, and hence had little opportunity to expand their range outside of the Hawkesbury-Nepean and Port Jackson (Sydney) catchments without human-mediated dispersal.

In the MDB, carp populations were either localized or at low density prior to the 1970s (Clements 1988; Koehn *et al.* 2000). These carp may have lacked the genetic variation necessary to become widespread, or may have been in the lag phase of their invasion (Sakai *et al.* 2001; Allendorf and Lundquist 2003). During the 1970s, carp numbers began to rise rapidly and carp began colonising regions from which they had previously been absent (Koehn *et al.* 2000). By the fiscal year 1971/72, carp were sufficiently abundant to become part of the commercial fish harvest from the MDB. In 1977/78, carp numbers peaked, with 548 tonnes being caught by commercial fisheries. Carp abundance subsequently declined and stabilised, with approximately 150 tonnes harvested each fiscal year from 1986/87 to 1995/96 (Reid *et al.* 1997). The sudden rise in carp numbers and the expansion of their range in the MDB corresponds to widespread flooding in 1974 and 1975 and the introduction of the Boolara strain. The 1974-75 floods were likely essential to carp attaining their current dominance of the MDB, as they provided abundant habitat for food and spawning and gave carp access to a plethora of new waterways by filling dry creek beds and drowning out weirs. The expansion of carp in the MDB was also facilitated by additional flooding in 1993 (Koehn *et al.* 2000).

The introduction of the Boolara strain is also frequently cited as being responsible for the dominance of carp in the MDB (e.g. Shearer and Mulley 1978; Koehn *et al.* 2000). It has been speculated that either the Boolara strain was already pre-adapted to flourishing in the MDB environment, or the Boolara carp inter-bred with the strains already present in the basin, which could have resulted in heterosis and produced more-invasive intercrossed progeny (Brown 1980a, cited by Davis 1996).

In addition to flooding and release of the Boolara strain, human-mediated dispersal has played a large role in facilitating the range expansion of carp after their initial introduction. Carp have been spread through the accidental contamination of artificially stocked native fish with carp fry, the use of carp fry as live bait, accidental or intentional release of koi, and intentional introduction by people trying to establish recreational fisheries (Koehn *et al.* 2000). The large Keepit, Burrendong, Wyangala, Burrinjuck, Hume and Eildon Dams at river headwaters all contain carp populations. As these dams are too large to be drowned out by floods, they were in all likelihood seeded by human activities, although introduction via the movement of eggs on the feet of waterfowl cannot be excluded.

2.11.3. Population genetics of common carp in Australia

As discussed previously in section *2.11.1 Introduction of carp*, common carp have been introduced into Australia on a number of occasions and from a number of different source populations. The exact number of successful introductions will probably never be known. The research of Shearer and Mulley (1978) and Davis *et al*. (1999), however, shows that at least four strains of carp have been introduced successfully into Australia: the Prospect, Yanco, Boolara and Japanese koi strains. These papers are discussed below. A fifth group, the Burrinjuck strain, is identified in the Ph.D. study reported in this thesis. This is detailed in *Chapter 3*, and will not be further discussed here.

Shearer and Mulley (1978) investigated carp from Prospect Reservoir, in the Sydney Basin, and Yanco and Narrandera in the MIA. The carp from Prospect were assumed to have been the descendants of the carp released by David Stead in 1907 and 1908 (Stead 1929). The carp at Yanco were assumed to have been present in the MDB before the carp from Boolara Fish Farms, as carp were mentioned in this region by Weatherly and Lake (1967). The carp at Narrandera were assumed to have been descended from the stocks released by Boolara Fish Farms. The aim of this study was to work out if the carp from the three regions belonged to three different strains, by identifying diagnostic allozyme alleles and/or morphological characters.

Shearer and Mulley (1978) scored the carp in their study for allozymes (20 loci), morphological measurements (15 traits) and colour. Of the 20 allozymes, *G-6-pd*, *Pgm* and *Pt-3* had alleles diagnostic of each sample group. Of the 15 morphological measurements, the Yanco carp could be separated from the Prospect and Boolara carp by the number of

dorsal fin rays, but none of the other measurements was diagnostic. Colour was found to be a useful defining traits, with the Yanco carp being bright orange-red-yellow, and the Boolara and Prospect carp being differing shades of silver, white, black and bronze. Intriguingly, some carp at Yanco were even excluded from analysis because they clearly had Boolara-type colouration and were hence regarded as vagrant Boolara carp rather than resident Yanco carp. The diagnostic allozymes and colours are summarised in Table 2.7. The ability of Shearer and Mulley (1978) to distinguish the carp from the three regions led the authors to conclude that the carp represented three genetically distinct populations from three separate introduction events. These were dubbed the Yanco, Boolara and Prospect strains.

Table 2.7. Allozyme and colour traits diagnostic of the Yanco, Boolara and Prospect strains of common carp. Data from Shearer and Mulley (1978).

Sample Site	**Allozymes**			**Colouration**		
	G-6-pd	***Pgm***	***Pt***	**Colour of head and dorsal surface**	**Colour of ventral surface**	**Colour of caudal fin**
Yanco	Allele a	Allele b, c	Three bands	Red to orange	Yellow	Red to orange
Boolara	Allele a	Allele a	Four bands	Silver grey	Cream	Silver grey on dorsal lobes, red of ventral lobes
Prospect	Allele b	Allele a	Four bands	Bronze to black	White	Bronze to black

Shearer and Mulley (1978) found no evidence of interbreeding between the Yanco, Boolara and Prospect strains, despite the range of the Yanco and Boolara carp having "recently begun to overlap." In a follow-up paper, however, Mulley and Shearer (1980) investigated a number of unusually coloured individuals in the MIA. Using the same morphological measurements and allozymes as Shearer and Mulley (1978), they concluded that these were F1 hybrids between the Yanco and Boolara strains.

The distinctive red-orange-yellow colouration of Yanco carp is no longer common in the MIA (Bell, pers. comm. 2007). Either the Yanco strain has died-out, has bred extensively with Boolara (or other) strain carp, or the frequencies of the alleles conferring red-orange-yellow colouration have been decreased by selection and/or chance.

Davis *et al*. (1999) conducted a wider-ranging study than Shearer and Mulley (1978), sampling carp from 14 locations in south-eastern Australia. These carp were scored for seven polymorphic allozyme loci and RFLP of whole mitochondrial genomes. The results of this study are generally consistent with those of Shearer and Mulley (1978) and Mulley and Shearer (1980). Carp from the samples sites in the MIA showed the most genetic diversity (number of allozyme alleles per locus), consistent with the presence of interbreeding of the Yanco and Boolara strains in this regions. The Boolara and Prospect strains could not be distinguished, however, because the allozyme that was diagnostic for the Prospect carp in Shearer and Mulley (1978)'s study, *G-6-pd*, was monomorphic in the Davis *et al*. (1999) study; and none of the other polymorphic loci in their study were diagnostic.

The mitochondrial data of Davis *et al*. (1999) indicated that descendants of the Yanco strain had spread beyond the MIA. Three haplotypes were detected, dubbed Haplotype 1, 2 and 3. Haplotype 2 was found only at Narrandera, in the MIA, and at Pooncarie, on the Darling River. The presence of this rare haplotype at Narrandera is consistent with it being indicative of Yanco strain maternal ancestry. The detection of Haplotype 2 in the Darling River is therefore consistent with Yanco-descended carp having migrated out of the MIA.

Davis *et al*. (1999) found evidence for the dissemination of Japanese koi carp at some sites in Australia. Seventeen koi from a fish farm in Bringelly, Sydney, were included in the study, all of which had Haplotype 3. Haplotype 3 may therefore be diagnostic of koi maternal ancestry, and was detected in carp from Tasmania and from Lake Burley Griffin in the ACT.

Davis *et al*. (1999) also provided some fresh insights into the population genetic structure of common carp in Australia. Analysis of allozyme allele and mitochondrial haplotype frequencies found that carp were genetically structured both within the MDB and across south eastern Australia as a whole. However, Davis *et al*. (1999) could find no clear pattern behind this structuring.

There is some evidence that the Boolara and Prospect carp may have predominantly European ancestry. The mirror-scale phenotype is found among both strains. Stead (1929) noted that five of the fourteen fingerlings he used to found the Prospect strain of carp had

mirror-scales. Mirror-scaled carp were noted amongst the original carp released by Boolara Fish Farms (Davis 1996), and are found in the MDB today, sometimes in high numbers (Koehn *et al.* 2000; pers. obs.; Bell, pers. comm. 2007). Neither Stead (1929), Davis (1996) nor Koehn *et al*. (2000) make any distinction between linear or scattered mirror scales, although I have personally observed both forms in the MDB. Although mirror scales are not unknown amongst Asian carp, they are actively selected for in many domestic European carp breeds (see section *2.5.Morphological Variation*). There is also testimonial evidence that the carp from Boolara Fish Farms were illegally imported from Germany (Clements 1988).

The origin of the Yanco strain remains a mystery. The bright colouration suggests that it is a feral strain of koi carp. However, such colourations also occasionally occur in European carp (see *2.5. Morphological Variation*), so European ancestry cannot be eliminated. Shearer and Mulley (1978) suggested that the Yanco strain was an escaped Singapore strain, based on mention of Singapore carp in Taronga Zoo, Sydney, by Whitley (1951). The Singapore carp was described as "a small eyed, pale-coloured variety" of carp. Shearer and Mulley (1978) noted that the Yanco carp had small eyes. However, there is no other evidence that the Yanco carp were from Singapore, especially as they were not simply "pale," but were coloured a distinct red-orange-yellow. Another hypothesis about the origin of the Yanco strain was put forward by Clements (1988). He suggests that the Yanco strain is descended from the coloured carp that were maintained in the Melbourne Botanical Gardens until 1962 (Table 2.6). Many of these carp had colourations similar to those described in Yanco, and some individuals were also small-eyed. This hypothesis is not mutually exclusive to Shearer and Mulley's (1978) suggestion of Singapore origin, as the coloured carp in Melbourne could possibly have been sourced from Singapore.

2.11.4. Interbreeding between common carp and goldfish

Hybridisation is common between closely related species of Cyprinid (Howes, 1991). Common carp and goldfish are no exception. Hybrids between carp and goldfish have been reported in all locations where the two species occur in Australia (Brumley, 1991). Hybrids have intermediate morphology between their parent species. They can be tentatively identified in the field by having a rounded body and face (personal observation), and reduced or absent barbels (Hume *et al.*, 1983b; Koehn *et al.* 2000).

It is likely that all carp strain in Australia have some potential to hybridise with goldfish. In Australia, goldfish-Boolara strain carp hybrids were reported by Hume *et al*. (1983b) and goldfish-Yanco strain carp hybrids were reported by Shearer and Mulley (1978). In New Zealand, goldfish-koi carp hybrids have been confirmed in genetic studies (Pullan and Smith 1987). In the UK, hybridisation between goldfish and local carp strains has also been confirmed genetically (Hänfling *et al.* 2005).There is no reason to suspect that Prospect strain carp can not hybridise with goldfish also.

The fertility of carp-goldfish hybrids is questionable. Putative F1 individuals are observed to be healthy and to produce eggs and milt in the wild (pers. obs.; Hume *et al.* 1983b). Hybrids have been reported to be either sterile (Hubbs 1955) or to be able to back-cross frequently (Trautman 1957, cited by Hume, 1983b; Aduma-bossman 1971, cited by Hänfling *et al*., 2005). In an analysis of 34 hybrids (identified on the basis of five meristic traits) from 14 different sites, Hume *et al.* (1983b) identified two subsets of hybrids: those with only one pair of barbels, and those with two pairs of reduced barbels. The observation that these groups also differed significantly for three morphological characters (ratio of length of lower barbel to standard length, number of lateral-line scales and arrangement of pharyngeal teeth) was interpreted as indicating that the two hybrid groups represented different generations of intercrossing or backcrossing. More recently, backcrossed carp-goldfish hybrids were detected in English waterways using microsatellite markers (Hänfling *et al.* 2005). It can therefore be concluded that although fertility may be reduced in carp-goldfish crosses, reproductive isolation is not complete and successful backcrossing does occur.

Brumley (1991) noted that millions of goldfish are imported into Australia each year for the aquarium industry, and that some of these imports are inevitably released into water bodies. As goldfish and carp can hybridise, and their hybrids have some potential to backcross with carp, goldfish likely act as a reservoir of genetic diversity for invasive common carp in Australia. Even if hybridisation is rare and hybrid individuals have reduced fitness, introgression between the two species could still allow the exchanging of advantageous alleles between species (Hänfling 2007) and hence facilitate invasiveness. The extent of introgression between carp and goldfish in any part of Australia has yet to be quantified.

2.11.5. Summary

To summarise, carp have been present in Australia since the late 19th Century. Previous studies have revealed that least four strains exist: Yanco, Japanese koi, Boolara and Prospect. There is some evidence that the Prospect and Boolara strains are European in origin. The Japanese koi, of course, has Asian origin. The origin of the Yanco strain is unknown, although its colouration suggests that it is a feral form of koi. Of the strains, interbreeding has been recorded between the Yanco and Boolara strain, and there is no reason to believe that it cannot occur between the other carp strains also. The current scarcity of Yanco-coloured carp in the MIA suggests that the Yanco strain has bred with Boolara strain carp extensively. Some genetic structuring of carp within the MDB has been detected, although no clear pattern to this structuring was discernable. Hybridisation between carp and goldfish has been detected in the MDB, but the level of introgression between the two species has never previously been quantified.

2.12. Scope of this project

There are many gaps in our knowledge of the population genetics of common carp in the MDB. Still unknown when this project was initiated was the number and distribution of strains, the extent and pattern of genetic structuring between populations in different river basins, the history of introduction and dispersal of the different strains and the level of interbreeding between the strains of carp and between carp and feral goldfish. Previous studies were unable to address these issues comprehensively, due to limited sampling schemes, the types of genetic markers used and the population-genetics analysis tools available.

In this Ph.D. study, the population genetics of common carp in the MDB was comprehensively investigated and the gaps in our knowledge left by previous studies addressed in accordance with the project aims described in *Chapter 1*. This was possible because:

(1) A comprehensive sampling regime was implemented, with carp being sampled from all major river catchments in the MDB and every effort being made to collect at least thirty individuals per sample site. In river catchments with large dams, carp were sampled from above and below these impoundments. In addition, feral goldfish and

carp from two of the four known strains (Prospect and koi) and carp from overseas populations (Europe and Russia) were also sampled.

(2) Microsatellite markers were used predominantly in the project. These are highly polymorphic and robust to score, and are hence far more informative than the allozymes, morphological characters and mitochondrial RFLP that previous studies had to rely upon.

(3) A new range of population-genetics analyses and programs have been developed since the last Australian carp project (Davis 1996) was completed. These have made it possible to quickly calculate such useful measures as pairwise genetic differences between regions/sample groups and the probability of departure of genotype frequencies from expectation under Hardy-Weinberg Equilibrium; a process that once had to be performed manually. Most importantly, a range of assignment tests have been developed (Paetkau *et al.* 1995; Rannala *et al.* 1997; Pritchard *et al.* 2000; Baudouin and Lebrun 2001; Anderson and Thompson 2002; Falush *et al.* 2003; 2007), making it possible to investigate the distribution and interbreeding of different genetic groups with greater precision than ever before.

2.13. References

Allendorf, Lundquist (2003) Introduction: population biology, evolution, and control of invasive species. *Conservation Biology*, **17,** 24-30. doi: 10.1046/j.1523-1739.2003.02365.x.

Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160,** 1217-1229.

Anjum R (1995) Biochemical and Chromosomal Genetic Characteristic of Several Breeding Populations of Common Carp, *Cyprinus carpio* (L.). *Faculty of Water Protection and Freshwater Fisheries.* Olsztyn, Poland, Olsztyn University of Agriculture and Technology.

Ansell SW, Grundmann M, Russell SJ, Schneider H, Vogel JC (2008) Genetic discontinuity, breeding-system change and population history of Arabis alpina in the Italian Peninsula and adjacent Alps. *Molecular Ecology*, **19,** 2245-2257. doi: 10.1111/j.1365-294X.2008.03739.x.

Bakos J, Gorda S (2001) *FAO Fisheries Technical Paper 417: Genetic Resources of Common Carp at the Fish Culture Research Institute, Szarvas, Hungary,* Rome, Food and Agriculture Organization of the United Nations. ISBN: 9789251046586.

Balon EK (1995) Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman Gourmets to the swimming flower. *Aquaculture,* **129,** 3-48. doi: 10.1016/0044-8486(94)00227-F

Balon EK (2004) About the oldest domestication among fishes. *Journal of Fish Biology,* **65 (Supplement A),** 1-27. doi: 10.1111/j.1095-8649.2004.00563.x.

Baudouin L, Lebrun P (2001) An operational bayesian approach for the identification of sexually reproduced cross-fertilized populations using molecular markers. In Doré, C., Dosba, F. & Baril, C. (Eds.) *Proceedings of the International Symposium on Molecular Markers for Characterizing Genotypes and Identifying Cultivars in Horticulture.* Montpellier, France

Bay LK, Caley MJM, Crozier RH (2008) Meta-population structure in a coral reef fish demonstrated by genetic data on patterns of migration, extinction and re-colonisation. *BMC Evolutionary Biology,* **8,** 248. doi:10.1186/1471-2148-8-248.

Ben-Dom N, Cherfas NB, Gomelsky B, Hu;ata G (2000) Genetic stability of Israeli common carp stocks inferred from electrophoretic analysis of transferrin, phosphoglucomutase and glucose-6-phosphate isomerase. *Israeli Journal of Aquaculture,* **52,** 30-35.

Bialowas H (2004) Inheritance of orange pigmentation and scale patterns in common carp (*Cyprinus carpio* L.). *Archives of Polish Fisheries,* **12,** 145-150.

Brown P (1996) Fish Facts 4 Carp in Australia. NSW Fisheries (now part of the NSW Department of Primary Industries), Narrandera, Australia.

Brumley AR (1991) Cyprinids of Australasia. *Cyprinid fishes systematics, biology and exploitation.* Suffolk, United Kingdom, St. Edumdsbury Press. ISBN: 92-5-103027-8.

Callaway RM, Aschehoug ET (2000) Invasive plant versus their new and old neighbors: a mechanism for exotic invasion. *Science,* **290,** 81-94. doi: A66888386.

Carroll SP (2007) Brave new world: the epistatic foundation of natives adapting to invaders. *Genetica,* **129,** 193-204. doi: 10.1007/s10709-006-9014-8.

Cassey P, Blackburn TM, Duncan RP, Lockwood JL (2005) Lessons from the establishment of exotic species: a meta-analytical case study using birds. *Journal of Animal Ecology*, **74,** 250-258. doi: 10.1111/j.1365-2656.2004.00918.x.

Cataudella S, Sola L, Corti M, Arcangeli R, La Rosa G, Mattoccia M, Coboldi S, Sbordoni V (1987) Cytogenetic, genetic and morphometric characterization of groups of common carp, *Cyprinus carpio*. In: Tiews, K (Ed.), Proc. World Symp. on

Selection, Hybridization, and Genetic Engineering in Aquaculture, Bordeaux 27-30 May 1986. Schriften der Bundesanstalt fur Fischerei Hamburg vol. 1, pp 113-129. Berlin.

Clements J (1988) *Salmon at the Antipodes A History and Review of Trout, Salmon and Char and Introduced Coarse Fish in Australasia,* Ballarat, Victoria, Australia, Published by the author. ISBN: 0731637194.

Colautti RI, Grigorovich IA, MacIsaac HG (2006) Propagule pressure: a null model for biological invasions. *Biological Invasions,* **8,** 1023-1037. doi: 10.1007/s10530-005-3735-y.

Corander J, Waldmann P, Marttinen P, Sillanpaa MJ (2004) BAPS 2: enhanced possibilities for the analysis of genetic population structure. *Bioinformatics,* **20,** 2363-2369. doi: 10.1093/bioinformatics/bth250.

Costa-Pierce BA, Moreau J, Pullin RSV (1993) New introductions of common carp (*Cyprinus carpio* L.) and their impact on indigenous species in sub-Saharan Africa. *Discovery and Innovation,* **5,** 211-221.

Crispo E, Hendry AP (2006) Does time since colonization influence isolation by distance? A meta-analysis. *Conservation Genetics,* **6,** 1572-9737. doi: 10.1007/s10592-005-9026-4.

Csizmadia C, Jeney Z, Szerencses I, Gorda S (1995) Transferrin polymorphism of some races in a live gene bank of common carp. *Aquaculture,* **129,** 193-198. doi:10.1016/0044-8486(94)00247-L.

David L, Rajasekaran P, Fang J, Hillel J, Lavi U (2001) Polymorphisms in ornamental and common carp strains (*Cyprinus carpio* L.) as revealed by AFLP analysis and a new set of microsatellite markers. *Molecular Genetics and Genomics,* **266,** 353-362. doi: 10.1007/s004380100569.

Davis KM (1996) Investigation into the genetic variation of carp (*Cyprinus carpio*) in Southeastern Australia. Sydney, Australia, University of NSW.

Davis KM, Dixon PI, Harris JH (1999) Allozyme and mitochondrial DNA analysis of carp, *Cyprinus carpio* L., from south-eastern Australia. *Marine and Freshwater Resources,* **50,** 253-60. doi: 10.1071/MF97256

Dawson KJ, Belkhir K (2001) A Bayesian approach to the identification of panmictic populations and the assignment of individuals. *Genetic Resources,* **78,** 59-77. doi:10.1017/S001667230100502X.

Desvignes JF, Laroche J, Durand JD, Bouvet Y (2001) Genetic variability in reared stocks of common carp (*Cyprinus carpio* L.) based on allozymes and microsatellites. *Aquaculture,* **194,** 291-301. doi:10.1016/S0044-8486(00)00534-2.

Edwards P, Hiep DD, Anh PM, Mair G (2000) Traditional culture of indigenous common carp in rice fields in northern Vietnam. *World Aquaculture,* **31,** 34-40.

Estoup A, Clegg SM (2003) Bayesian inferences on the recent island colonization history by the bird *Zosterops lateralis lateralis*. *Molecular Ecology,* **12,** 657 - 674. doi: 10.1046/j.1365-294X.2003.01761.x.

Estoup A, Wilson IJ, Sullivan C, Cornuet J-M, Moritz C (2001) Inferring population history from microsatellite and enzyme data in serially introduced cane toads, *Bufo marinus*. *Genetics,* **159,** 1671-1687.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the sortware STRUCTURE: a simulation study. *Molecular Ecology,* **14,** 2611-2620. doi: 10.1111/j.1365-294X.2005.02553.x.

Excoffier L, Heckel G (2006) Computer programs for population genetics data analysis: a survival guide. *Nature Reviews,* **7,** 745-758. doi: 10.1038/nrg1904.

Excoffier L, Laval G, Schneider S (2005) Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1,** 47-50.

Excoffier L, Smouse PE, Quattro JM (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics,* **131,** 479-491.

Falush D, Stephens M, Pritchard JK (2003) Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies *Genetics,* **164,** 1567-1587.

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes,* **7,** 574–578. doi: 10.1111/j.1471-8286.2007.01758.x.

FAO (2008). Yearbook of Fisheries Statistics Summary Table. Available at: http://www.fao.org/fishery/statistics/global-production/en.

Faragher RA, Lintermans M (1997) Alien fish species from the New South Wales rivers survey. *Fish and rivers in stress: the NSW rivers survey.* Cronulla, Australia, NSW Fisheries (now NSW Department of Primary Industries). ISBN: 0731094085.

Fisher RA (1930) *The genetical theory of natural selection,* Oxford, Clarendon Press.

Flajšhans M, Linhart O, Šlechtová V, Šlechta V (1999) Genetic resources of commercially important fish species in the Czech Republic: present state and future strategy. *Aquaculture,* **172,** 471–483. doi: 10.1016/S0044-8486(98)00477-3.

Flannery T (2001) *The eternal frontier : an ecological history of North America,* London, Heinemann. ISBN: 9781877008566.

Frankham R (2005) Resolving the genetic paradox in invasive species. *Heredity,* **94,** 385. doi: 10.1038/sj.hdy.6800634.

Froufe E, Magyary I, Lehoczky I, Weiss S (2002) mtDNA sequence data supports an Asian ancestry and single introduction of the common carp into the Danube Basin. *Journal of Fish Biology,* **61,** 301-304. doi:10.1006/jfbi.2002.2046.

Gilligan D (2005) Fish communities of the Murrumbidgee catchment: status and trends. NSW Department of Primary Industries, Narrandera, NSW.

Gilligan D, Rayner T (2007) The distribution, spread, ecological impacts, and potential control of carp in the upper Murray River. NSW Department of Primary Industries, Sydney.

Goldstein DB, Linares AR, Cavalli-Sforza LL, Feldman MW (1995) Genetic absolute dating based on microsatellites and the origin of modern humans. *Proceedings of the National Academy of Science of the United States of America,* **92,** 6723-6727.

Gorda S, Bakos J, Liska J, Kakuk C (1995) Live gene bank of common carp strains at the Fish Culture Research Institute, Szarvas. *Aquaculture,* **129,** 199-202. doi:10.1016/0044-8486(94)00248-M.

Graham KJ, Lowry MB, Walford TR (2005) Carp in NSW: assessment of distribution, fishery and fishing methods. NSW Department of Primary Industries, Cronulla, Australia.

Gross R, Kohlmann K, Kersten P (2002) PCR-RFLP analysis of the mitochondrial ND-3/4 and ND-5/6 gene polymorphisms in the European and East Asian subspecies of common carp (*Cyprinus carpio* L.). *Aquaculture,* **204,** 507-516. doi:10.1016/S0044-8486(01)00836-5.

Guillot G, Estoup A, Mortier F, Cosson JF (2005a) A spatial statistical model for landscape genetics *Genetics,* **170,** 1261-1280. doi:10.1534/genetics.104.033803.

Guillot G, Mortier F, Estoup A (2005b) Geneland: a computer package for landscape genetics *Molecular Ecology Notes,* **5,** 712-715. doi: 10.1111/j.1471-8286.2005.01031.x.

Haldane JBS (1924) A mathematical theory of natural and artificial selection" Part 1 (1924) Transactions of the Camrbidge philosophical society. *Transactions of the Camrbidge philosophical society,* **23,** 19-41.

Hänfling B (2007) Understanding the establishment success of non-indigenous fishes: lessons from population genetics. *Journal of Fish Biology,* **71 (Supplement D),** 115-135. doi: 10.1111/j.1095-8649.2007.01685.x.

Hänfling B, Bolton P, Harley M, Carvalho GR (2005) A molecular approach to detect hybridisation between crucian carp (*Carassis carassius*) and non-indigenous carp species (*Carassisus* cpp. and *Cyprinus carpio*). *Freshwater Biology,* **50,** 403-417. doi:10.1111/j.1365-2427.2004.01330.x.

Hänfling B, Weetman D (2006) Concordant genetic estimators of migration reveal structural anthropogenically enhanced source-sink population structure in the river sculpin, *Cottus gobio*. *Genetics,* **173**.

Hassan M, Bonhomme F (2005) No reduction in neutral variability of mitochondrial and nuclear genes for a Lessepsian migrant, *Upeneus moluccensis*. *Journal of Fish Biology,* **66,** 865-870. doi: 10.1111/j.0022-1112.2005.00643.x.

Hassan M, Harmelin-Vivien M, Bonhomme F (2003) Lessepsian invasion without bottleneck: example of two rabbitfish species (*Siganus rivulatus* and *Siganus luridus*) *Journal of Experimental Marine Biology and Ecology,* **291,** 219-232. doi:10.1016/S0022-0981(03)00139-4.

Hebert PDN, Cywinska A, Ball SL, deWaard JR (2003) Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B,* **270,** 313-321.

Horváth L, Tamás G, Seagrave C (2002) *Carp and pond fish culture second edition,* Oxford, United Kingdom, Blackwell Science. ISBN: 0852381794.

Howes GJ (1991) Chapter 1 Systematics and biogeography: an overview. In Winfield, I. J. & Nelson, J. S. (Eds.) *Cyprinid fishes systematics, biology and exploitation.* Bury St Edmunds, United Kingdom, St. Edumdsbury Press. ISBN: 92-5-103027-8.

Hubbs CL (1955) Hybridization between fish species in nature. *Systematic Zoology* **4,** 1-20.

Hudson RR, Slatkin M, Maddison WP (1992) Estimating levels of gene flow from DNA sequence data. *Genetics,* **132,** 583-589.

Hume DH, Fletcher AR, Morsion AK (1983a) Final Report. Carp Program Report No. 10. Fisheries and Wildlife Division, Victorian Ministry for Conservation,

Hume JD, Fletcher AR, Morison AK (1983b) Interspecific hybridization between carp (*Cyprinus carpio* L.) and goldfish (*Carassius auratus* L.) from Victorian waters. *Australian Journal of Marine and Freshwater Research,* **34,** 915-919. doi:10.1071/MF9830915.

Hutchinson DW, Templeton AR (1999) Correlations of pairwise genetic distance and geographic distance measures: inferring the relarive influences of gene flow and drift on the distribution of genetic variability. *Evolution,* **53,** 1898-1914.

Inland Fisheries Service (2007) Carp Management Program Annual Report 2006-07. Hobart, Tasmania.

Kalinowski ST (2004) Do polymorphic loci require large sample sizes to estimate genetic distances? *Heredity,* **94,** 33-36. doi:10.1038/sj.hdy.6800548

Khan TA (2003) Dietary studies on exotic carp (*Cyprinus carpio*) from two lakes in Western Victoria, Australia. *Aquatic Sciences,* **65,** 272-286. doi: 10.1007/s00027-003-0658-5.

Kirpichnikov VS (1981) *Genetic Bases of Fish Selection,* Berlin and Heidelberg, Germany, New York, USA, Springer-Verlag. ISBN: 0387109110.

Koehn J (2004) Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. *Freshwater Biology,* **49,** 882-894. doi: 10.1111/j.1365-2427.2004.01232.x.

Koehn J, Brumley B, Gehrke P (2000) *Managing the Impacts of Carp,* Canberra, Australia, Bureau of Rural Sciences (Department of Agriculture, Fisheries and Forestry). ISBN 0644292407 (set) 0642732019.

Kohlmann K, Gross R, Murakaeva A, Kersten P (2003) Genetic variability and structure of common carp (*Cyprinus carpio*) populations throughout the distribution range inferred from allozyme, microsatellite and mitochondrial DNA markers. *Aquatic Living Resources,* **16,** 421-431. doi:10.1016/S0990-7440(03)00082-2.

Kohlmann K, Kersten P (1999) Genetic variability of German and foreign common carp (*Cyprinus carpio* L.) populations. *Aquaculture,* **173,** 435-445. doi:10.1016/S0044-8486(98)00474-8.

Kohlmann K, Kersten P, Flajshans M (2005) Microsatellite-based genetic variability and differentiation of domesticated, wild and feral common carp (*Cyprinus carpio* L.) populations. *Aquaculture,* **247,** 253-256. doi:10.1016/j.aquaculture.2005.02.024.

Kolbe JJ, Glor RE, Schettino LR, Lara AC, Larson A, Losos JB (2004) Genetic variation increases during biological invasion by a Cuban lizard. *Nature,* **431,** 177-181. doi: 10.1038/nature02807.

Lindholm AK, Brenden F, Alexander HJ, Chan W-K, Thakurta SG, Brooks R (2005) Invasion success and genetic diversity of introduced populations of guppies *Poecilia reticulata* in Australia. *Molecular Ecology,* **14,** 3671-3682. doi: 10.1111/j.1365-294X.2005.02697.x.

Lintermans M (2007) *Fishes of the Murray-Darling Basin,* Canberra, Australia, Murray-Darling Basin Commission. ISBN: 1 921257 20 2.

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

Mabuchi K, Senou H, Nishida M (2008) Mitochondrial DNA analysis reveals cryptic large-scale invasion of non-native genotypes of common carp (*Cyprinus carpio*) in Japan. *Molecular Ecology,* **17,** 796-809. doi:10.1111/j.1365-294X.2007.03626.x.

Mabuchi K, Senou H, Suzuki T, Nishida M (2005) Discovery of an ancient lineage of *Cyprinus carpio* from Lake Biwa, central Japan, based on mtDNA sequence data, with reference to possible multiple origins of koi. *Journal of Fish Biology,* **66,** 1516-1528. doi:10.1111/j.0022-1112.2005.00676.x.

Macarnes JM, Sato J, Fujio Y (1986) Genetic characterization of cultured populations of Japanese common carp. *Agricultural Research,* **218,** 153-166.

McConnell SK, Leamon J, Skibinski DOF, Mair GC (2001) Microsatellite markers from the Indian major carp species, *Catla catla*. *Molecular Ecology Notes,* **1,** 115-116. doi: 10.1046/j.1471-8278.2000.00025.x.

MDBC (2008) Sustainable rivers audit report. Murray-Darling Basin Commission,

Memiş D, Kohlmann K (2006) Genetic characterization of wild common carp (*Cyprinus carpio* L.) from Turkey. *Aquaculture,* **258,** 257-262. doi:10.1016/j.aquaculture.2006.03.041.

Michaels VJ (1998) *Carp Farming,* Farnham, United Kingdom, The Dorset Press. ISBN: 0852381530.

Michalakis Y, Excoffier L (1996) A generic estimator of population subdivision using distances between alleles with special reference for microsatellite loci. *Genetics,* **142,** 1061-1064.

Moles AT, Gruber MAM, Bonser SP (2008) A new framework for predictinginvasive plant species. *Journal of Ecology,* **96,** 13-17. doi: 10.1111/j.1365-2745.2007.01332.x.

Morgan D, Gill HS, Maddern MG, Beatty S (2004) Distribution and impacts of introduced freshwater fishes in Western Australia. *New Zealand Journal of Marine and Freshwater Research,* **38,** 511-523. doi: 0028–8330/04/3803–0511.

Moritz C (1994) Defining evolutionary significant units for conservation. *Trends in Ecology and Evolution,* **9,** 373-375. doi: 10.1016/0169-5347(94)90057-4.

Mulley JC, Shearer KD (1980) Identification of natural 'Yanco' × 'Boolara' hybrids of the carp, Cyprinus carpio. *Australian Journal of Marine and Freshwater Research,* **31,** 409-411. doi:10.1071/MF9800409

Murakaeva A, Kohlmann K, Kersten P, Kamilov B, Khabibullin D (2003) Genetic characterisation of wild and domestic common carp (*Cyprinus carpio* L.) populations from Uzbekistan. *Aquaculture,* **218,** 153-166. doi:10.1016/S0044-8486(03)00005-X.

Naish KA, Skibinski DOF (1998) Tetranucleotide microsatellite loci for Indian major carp. *Journal of Fish Biology,* **53,** 886-889. doi:10.1111/j.1095-8649.1998.tb01841.x.

Nei M (1973) Analysis of gene diversity in subdivided populations. *Proceedings of the National Academy of Science of the United States of America,* **70,** 3321-3323.

Nicolescu C (2004) The phenotypical expression of the common carp's scale cover pattern in accordance with the genotypic structure and the gene dose. *Buletinul Universitatii de Stiinte Agricole si Medicina Veterinara Cluj-Napoca, Seria Zootehnie si Biotehnologii* **60,** 78-83.

Nolte AW, Freyhof J, Stemshorn KC, Tautz D (2005) An invasive lineage of sculpins, *Cottus sp* (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogeographic groups. *Proceedings of the Royal Society B,* **272,** 2379-2387. doi: 10.1038/rspb.2005.3231.

Paaver T, Gross R (1991) Genetic variability of *Cyprinus carpio* L. stocks reared in Estonia. *Soviet Genetics***,** 839-846 (translated from Genetika 26 (1990), 1269-1278).

Paddle R (2000) *The last of the Tasmanian tiger, the history of the extinction of the thylacine,* Cambridge, UK, Cambridge University Press. ISBN: 0-521-53154-3.

Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology,* **3,** 347-354. doi:10.1111/j.1365-294X.1995.tb00227.x.

Pearse DE, Crandall KA (2004) Beyond F_{ST}: Analysis of population genetic data for conservation. *Conservation Genetics,* **5,** 585-602. doi: 10.1007/s10592-003-1863-4.

Pinto L, Chandrasena N, Pera J, Hawkins P, Eccles D, Sim R (2005) Managing invasive carp (*Cyprinus carpio* L.) for habitat enhancement in at Botany Bay, Australia.

Aquatic Conservation: Marine and Freshwater Ecosystems, **15,** 447-462. doi: 10.1002/aqc.684.

Pritchard JK, Stefens M, Donelly P (2000) Inference of population structure using multilocus genotype data. *Genetics,* **155,** 945-959.

Puillandre N, Dupas S, Dangles O, Zeddam J-L, Capdevielle-Dulac C, Barbin K, Torres-Leguizamon M, Silvain J-F (2008) Genetic bottleneck in invasive species: the potato tuber moth adds to the list. *Biological Invasions,* **10,** 319-333. doi: 10.1007/s10530-007-9132-y.

Pullan S, Smith PJ (1987) Identification of hybrids between koi (*Cyprinus carpio*) and goldfish (*Carassius auratus*). *New Zealand Journal Of Marine And Freshwater Research,* **21,** 41-46.

Rannala B, Mountain JLDibu, 9197-9221. mgPNASU (1997) Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Science of the United States of America,* **94,** 9197-9221.

Reid DD, Harris JH, Chapman DJ (1997) NSW Inland Commercial Fishery Data Analysis. Fisheries Research & Development Corporation, NSW Fisheries, Cooperative Research Centre for Freshwater Ecology, Sydney, Australia.

Reynolds J, Weir BS, Cockerham CC (1983) Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics*, **105,** 767-779.

Rolls EC (1969) *They all ran wild : the story of pests on the land in Australia*, London, United Kingdom, Angus & Robertson Publishers. ISBN: 020713491X.

Rousset F (1996) Equilibrium values of measures of population subdivision from stepwise mutation processes. *Genetics,* **142,** 1357-1362.

Sakai AK, Allendorf FW, Holt JS, Lodge DM, Molofsky J, With KA, Baughman S, Cabin RJ, Cohen JE, Ellstrand NC, McCauley DE, O'Neil P, Parker IM, Thompson JN, Weller SG (2001) The Population Biology of Invasive Species. *Annual Review of Ecology and Systematics (now the Annual Review of Ecology, Evolution and Systematics),* **32,** 305-332. doi: 0066-4162/01/1215.

Savolainen P, Leitner T, Wilton AN, Matisoo-Smith E, Lundeberg J (2004) A detailed picture of the origin of the dingo, obtained from the study of mitochondrial DNA. *Proceedings of the National Academy of Science of the United States of America,* **101,** 12387-12390. doi: 10.1073/pnas.0401814101.

Shearer KD, Mulley JC (1978) The introduction and distribution of the carp, *Cyprinus carpio* Linnaeus, in Australia. *Australian Journal of Marine and Freshwater Research,* **29,** 661-563. doi:10.1071/MF9780551

Slatkin M (1991) Inbreeding coefficients and coalescence times. *Genetical Research, Cambridge* **58,** 167-175.

Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics,* **139,** 457-462.

Slechtova V, Slechta V, Flajshans M, Gela D (2002) Protein variability in common carp (*Cyprinus carpio*) breeds in the Czech Republic. *Aquaculture,* **204,** 241-271.

Smith B (2005) The state of the art: a synopsis of information on common carp (*Cyprinus carpio*) in Australia. Final Technical Report, SARDI Aquatic Sciences Publication No. RD04/0064-2. South Australian Research and Development (SARDI), Adelaide, Australia.

Stead DG (1929) Introduction of the great carp *Cyprinus carpio* into waters of New South Wales. *Australian Zoologist,* **6,** 100-102.

Stuart I, Jones M (2006) Large, regulated forest floodplain is an ideal recruitment zone for non-native common carp (*Cyprinus carpio* L.). *Marine and Freshwater Research,* **57,** 333–347. doi: 10.1071/MF05035.

Sumantadinata K, Taniguchi N (1990) Comparison of electrophoretic allele frequencies and genetic variability of common carp stocks from Indonesia and Japan. *Aquaculture,* **88,** 263-271. doi:10.1016/0044-8486(90)90153-E

Thai BT, Burridge CP, Pham TA, Austin CM (2005) Using mitochondrial nucleotide sequences to investigate diversity and genealogical relationships within common carp (*Cyprinus carpio* L.) *Animal Genetics*, **36,** 23-28. doi: 10.1111/j.1365-2052.2004.01215.x.

Thai BT, Pham TA, Austin CM (2006) Genetic diversity of common carp in Vietnam using direct sequencing and SSCP analysis of the mitochondrial DNA control region. *Aquaculture,* **258,** 228-240. doi:10.1016/j.aquaculture.2006.03.025.

Tsutsui ND, Suarez AV, Holway DA, Case TJ (2000) Reduced genetic variation and the success of an invasive species. *Proceedings of the National Academy of Science of the United States of America,* **97,** 5948-5953. doi: 10.1073/pnas.100110397.

Wang C, Li S (2004) Phylogenetic relationships of ornamental (koi) carp, Oujiang color carp and long-fin carp revealed by mitochondrial DNA COII gene sequences and RAPD analysis. *Aquaculture,* **231,** 83-91. doi:10.1016/j.aquaculture.2003.10.040.

Wang C, Li S (submitted) Genetic structure and phylogeny of Chinese and Hungarian common carp (Cyprinus carpio L.) strains based on mitochondrial DNA sequence analysis, with reference to the origin of European common carps.

Waples RS (1991) Pacific salmon, *Oncorhynchus spp*., and the definition of "species" under the endangered species act *Marine Fisheries Review,* **53,** 11.

Weatherly AH, Lake JS (1967) Introduced Fish Species in Australian Inland Waters. In Weatherly, A. H. (Ed.) *Australian Inland Waters and Their Fauna.* Canberra, Australia, A.N.U. Press. ISBN: 1131973461.

Weir BS, Cockerham CC (1984) Estimating F-statistics for the analysis of population structure. *Evolution,* **38,** 1358-1370.

Whitley GP (1951) Introduced Fishes-II. *The Australian Museum Magazine,* **10,** 234-238.

Whitlock M, McCauley DE (1999) Indirect estimates of gene flow and migration: $F_{ST} \neq 1/(4Nm + 1)$. *Heredity,* **82,** 117-125. doi:10.1038/sj.hdy.6884960.

Wilson GA, Rannala B (2003) Bayesian inferences of recent migration rates using multilocus genotypes. *Genetics,* **163,** 1177-1191.

Wright S (1931) Evolution in Mendelian populations. *Genetics,* **16,** 97-159.

Wright S (1951) The genetic structure of populations. *Annals of Eugenics,* **15,** 97-159.

Yue GH, Ho MY, Orban L, Komen J (2004) Microsatellites within genes and ESTs of common carp and their applicability in silver crucian carp. *Aquaculture,* **234,** 85-98. doi:10.1016/j.aquaculture.2003.12.021.

Zambrano L, Martinez-Meyer E, Menezes N, Peterson AT (2006) Invasive potential of common carp (*Cyprinus carpio*) and Nile tilapia (*Oreochromis niloticus*) in American freshwater systems. *Canadian Journal of Fisheries and Aquatic Sciences,* **63,** 1903-1910. doi: 10.1139/F06-088.

Zanden MJV (2005) The success of animal invaders. *Proceedings of the National Academy of Science USA,* **102**. doi: 10.1073/pnas.0502549102.

Zenger KR, Richardson BJ, Vachot-Griffin A-M (2003) A rapid population expansion retains genetic diversity within European rabbits in Australia. *Molecular Ecology,* **12,** 789-794. doi:10.1046/j.1365-294X.2003.01759.x.

Zhivotovsky LA (1999) A new genetic distance with application to constrained variation at microsatellite loci. *Molecular Biology and Evolution,* **16,** 467-471.

Zhou J, Wu Q, Wang Z, Ye Y (2004a) Genetic variation within and among six varieties of common carp (*Cyprinus carpio* L.) in China using microsatellite markers. *Russian Journal of Genetics,* **40,** 1144-1148. doi: 10.1023/B:RUGE.0000044758.51875.25.

Zhou JF, Wu Q, Wang Z, Ye Y (2004b) Molecular phylogeny of three subspecies of common carp *Cyprinus carpio*, based on sequence analysis of cytochrome *b* and control region mtDNA. *Journal of Zoological Systematics and Evolutionary Research,* **42,** 266-269. doi: 10.1111/j.1439-0469.2004.00266.x.

Zhou JF, Wu QJ, Ye YZ, Tong JG (2003) Genetic divergence between *Cyprinus carpio carpio* and *Cyprinus carpio haematopterus* as assessed by mitochondrial DNA analysis, with emphasis on the origin of European domestic carp. *Genetica,* **119,** 93-97.

Zhou L, Gui JF (2002) Karyotypic Diversity in Polyploid Gibel Carp, *Carassius Auratus Gibelio* Bloch *Genetica,* **115,** 223-232. doi: 10.1023/A:1020102409270.

Zonova AS, Kirpichnikov VS (1968) Selection of Ropsha carp. *Seminar/study tour in the U.S.S.R. on genetic selection and hybridization of cultivated fishes.* FAO Corporate Document Repository http://www.fao.org/documents.

Chapter 3: Population genetics and management units of invasive common carp (*Cyprinus carpio* L.) in the Murray-Darling Basin, Australia

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3.1. Abstract

Common carp (*Cyprinus carpio* L.) were introduced into Australia on several occasions and are now the dominant fish in the Murray-Darling Basin (MDB), the continent's largest river system. In this study, variability at fourteen microsatellite loci was examined in carp (n = 1037) from 34 sites throughout the major rivers in the MDB, from 3 cultured populations, from Prospect Reservoir in the Sydney Basin and from Lake Sorrell in Tasmania. Consistent with previous studies, assignment testing indicated that the Boolara, Yanco and koi strains of carp are present in the MDB. Unique to this study, however, the Prospect strain was widely distributed throughout the MDB. Significant genetic structuring of populations (Fisher's exact test, AMOVA and distribution of the different strains) amongst the MDB sub-drainages was detected, and was strongly associated with contemporary barriers to dispersal and population history. The distributions of the strains were used to infer the history of introduction and spread of carp in the MDB. Populations in 15 management units, proposed for control programmes, have high levels of genetic

diversity, contain multiple interbreeding strains and show no evidence of founder effects or recent population bottlenecks.

Key words: Boolara; freshwater fish; invasive species; koi carp; Prospect Reservoir; Yanco

3.2. Introduction

Common carp (*Cyprinus carpio* L.) are a highly invasive species of freshwater fish. Native to Eurasia, they have been successfully introduced to parts of the Americas, Oceania, Africa, Asia, Europe and Australia (2004). Carp have been introduced into Australian rivers several times since the late 19th century (Anderson 1920; Clements 1988; Koehn *et al.* 2000) and have spread from introduction sites through natural range expansions and through intentional and accidental releases (Koehn *et al.* 2000). They have been in the Murray-Darling Basin (MDB), Australia's largest river system, since at least 1917 (Anderson 1920; Clements 1988). After extensive flooding in 1974–1975, carp numbers increased sharply, and carp became the dominant species in the MDB (Harris and Gehrke 1997; Koehn *et al.* 2000). There is much interest in carp control, because carp have a detrimental effect on the aquatic environment and are considered a pest in most Australian states (Koehn *et al.* 2000). The extent of population sub-structure in the MDB can be identified with a population genetic assessment, which can be a useful guide for implementing pest-management strategies. Molecular population markers can be used to determine where there are multiple, independent subpopulations or a single panmictic unit.

Previous genetic studies indicated the presence of at least four common carp strains in Australia: Prospect, Yanco, Boolara and koi (Shearer and Mulley 1978; Davis *et al.* 1999). The Prospect strain was founded in Sydney from 14 fingerlings of unknown origin in 1907–1908 (Stead 1929) and was used to seed several waterways in the Sydney Basin (Clements 1988). The Yanco strain was introduced into the MDB between 1910–1950 (Brown 1996). Individuals of this strain was originally a distinctive orange colour (Shearer and Mulley 1978), a trait which is now rarely observed in the MDB carp (K. Bell, pers. comm.). Interbreeding with other strains, and possibly natural selection, has presumably led to the replacement of this colouration with the wild-type phenotype in contemporary populations. The Boolara strain was likely illegally imported from Germany in the late 1950s and was deliberately spread throughout Victoria. It invaded the Murray River in

1968 (Clements 1988; Koehn *et al.* 2000). Koi are an ornamental strain of carp from Japan (Balon 1995), sometimes illegally released into waterways (Koehn *et al.* 2000; Graham *et al.* 2005). Previous studies detected Yanco carp at two sites and koi at one site in the MDB, the Boolara strain throughout the MDB, and the Prospect strain only in the Sydney Basin (Shearer and Mulley 1978; Davis *et al.* 1999). The introduction history of these strains may provide insights into the contemporary genetic structuring of carp in the MDB.

In the present study, repeat-length variability in fourteen microsatellite loci was surveyed to determine the distributions of the various strains, to estimate the extent of genetic structuring between sub-drainages, and to assess levels of genetic diversity within the MDB. The distribution of the different strains is interpreted in conjunction with historical and demographic data to infer the history of colonization and expansion of carp in the MDB since their introduction. In addition, the microsatellite variability between sub-drainages was used to identify barriers to migration which, when considered with the geography of the region, is used to define management units that can inform strategies for control programs.

3.3 Materials and Methods

3.3.1. Sample Collection

Common carp were collected by electro-fishing from March 2004 to October 2006. A fin-clip was taken from each individual and immediately placed in 70% ethanol. Effort was made to collect at least 30 fish from each major river catchment in the MDB. Samples were collected upstream and downstream of major dams to assess the effect of the dams on migration. Carp were also sampled from Lake Sorell, Tasmania, where they were first reported in 1995 (Koehn *et al.* 2000). Prospect strain carp were collected from Prospect Reservoir in the Sydney catchment, and koi were obtained from two fish breeders, one in Germany and one in Sydney. Mirror-scale domestic carp were obtained from a fish farm in Jaenschwalde, Germany, to represent ‘pure’ European carp that have not interbred with non-European strains. Sample site names and coordinates, and sample sizes, appear in Table 3.1. Sample site locations are given in Figure 3.1.

Table 3.1. Collection sites for common carp. Sample site names and locations, sample size (N), and *p*-value for overall Hardy-Weinberg (HW) equilibrium adjusted for multiple testing using the BH method. Significant *p*-values (<0.05) for departure of allele frequencies from expectations under HW equilibrium are in bold.

Sample Site	**River Collected**	**State**	**Identifier**	**Location**		**N**	**HW equilibrium *P*-Value**
				Latitude (S)	**Longitude (E)**		
Walgett	Barwon River	NSW	WG	-30.017	148.100	30	0.738
Nyngan	Bogan River	NSW	NG	-31.555	147.169	30	0.800
Coonamble	Castlereagh River	NSW	CN	-30.967	148.381	30	0.229
Mudgee	Cudgegong River	NSW	MG	-32.340	149.350	30	**0.043**
Bourke	Darling River	NSW	BK	-30.089	145.938	9	0.841
Wilcannia	Darling River	NSW	WC	-31.550	143.367	28	0.435
Deniliquin	Edwards River	NSW	DQ	-35.516	144.959	31	0.919
Moree	Gwydir River	NSW	MR	-29.465	149.844	33	0.140
Lake Cargelligo	Lachlan River	NSW	CW	-33.267	146.400	30	0.468
Wyangala Dam	Lachlan River	NSW	WY	-33.950	148.933	30	**0.020**
Burrendong Dam	Macquarie River	NSW	BD	-32.650	149.100	30	0.897
Dubbo	Macquarie River	NSW	DB	-32.233	148.600	25	0.921
Wellington	Macquarie River	NSW	WN	-32.533	148.933	30	0.201
Echuca	Murray River	NSW	EC	-36.116	144.805	30	0.900
Lake Hume	Murray River	NSW	LH	-35.967	147.067	31	**0.000**
Wentworth	Murray River	NSW	WT	-34.105	141.912	30	0.242
Burrinjuck Dam	Murrumbidgee	NSW	BJ	-34.983	148.583	30	**0.026**
Cooma	Murrumbidgee	NSW	CM	-36.237	149.125	30	0.212
Narrandera	Murrumbidgee	NSW	ND	-34.733	146.550	30	**0.000**
Lake Keepit	Namoi River	NSW	KP	-30.850	150.500	30	**0.006**
Narrabri	Namoi River	NSW	NB	-30.360	149.831	31	0.067

Table 3.1. (Continued…)

Sample Site	River Collected	State	Identifier	Location		N	HW equilibrium
				Latitude (S)	Longitude (E)		*P*-Value
Walgett	Barwon River	NSW	WG	-30.017	148.100	30	0.738
Condamine	Condamine	QLD	CDM	-27.901	148.637	30	0.751
Paroo River	Paroo River	QLD	PR	-28.056	145.368	30	0.914
Charleville	Warrego River	QLD	CV	-26.402	146.938	30	0.436
Lower Lakes	Murray River	SA	LL	-35.507	138.956	30	0.387
Avoca	Avoca River	VIC	AV	-37.087	143.463	25	0.393
Broken River	Broken River	VIC	BR	-36.551	145.966	30	0.948
Campaspe	Campaspe River	VIC	CS	-36.481	144.613	30	0.213
Goulburn	Goulburn River	VIC	GB	-36.718	145.165	30	0.711
Kiewa	Kiewa River	VIC	KIW	-36.119	146.940	30	0.681
Lake Eildon	Lake Eildon	VIC	EI	-37.208	146.035	30	0.909
Loddon	Loddon River	VIC	LD	-37.082	144.013	30	0.890
Ovens	Ovens River	VIC	OV	-36.056	146.187	25	0.777
Horsham	Wimmera River	VIC	WM	-36.718	142.184	25	0.940
Tasmania*	Lake Sorell	TAS	TAS	-42.100	147.167	24	0.517
Prospect *	Prospect Reservoir	NSW	P	-38.815	150.901	24	**0.001**
Jaenschwalde*	na	na	J	na	na	30	0.868
Koi (Sydney)*	na	na	Kb	na	na	46	**0.000**
Koi (Germany)*	na	na	K	na	na	30	**0.001**

* Not part of the MDB

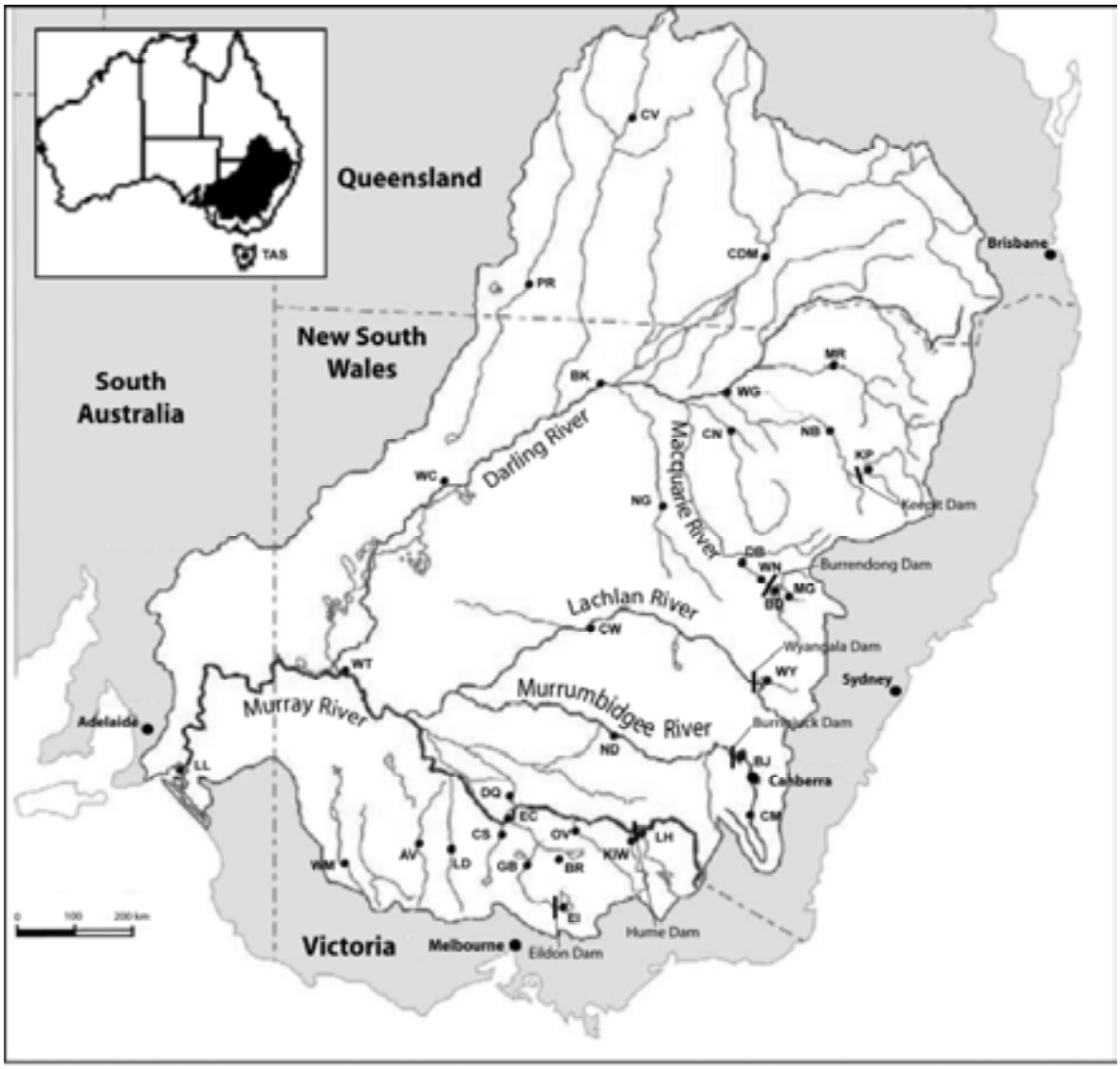

Figure 3.1. Collection sites for common carp. Murray-Darling Basin is indicated in white, the rest of Australia in grey. Sample site coordinates and full names are in Table 3.1

3.3.2. PCR and genotyping

DNA was extracted according to Wasko *et al*. (2003), and samples were genotyped for fourteen di-, tri- or tetranucleotide microsatellite loci, including Cca02, Cca07, Cca09, Cca17, Cca19, Cca65, Cca67, Cca72, GF1, Koi5-6, Koi29-30, Koi41-42, MFW6 and MFW26 (Crooijmans *et al.* 1997; David *et al.* 2001; Yue *et al.* 2004). Microsatellite DNA was amplified using the polymerase chain reaction (PCR) in 8 single-locus and 3 multiplex reactions. Primers for Cca65, Cca09, Cca07, Cca17, Koi5-6, Koi29-30 and Koi41-42 were redesigned to anneal at higher temperatures and to change the size of the PCR products to facilitate multiplexing. Primer sequences appear in Appendix 3.1. Optimal conditions for each PCR consisted of 1 μl (10–100 ng) total genomic DNA, 1x PCR Buffer (Fisher

Biotech), 200 μM each dNTP, 1 unit *Taq* DNA polymerase, primer and $MgCl_2$ concentrations (Appendix 3.1), and sterile water to 15 μl total volume. PCR amplifications were made with touch-down protocols (Appendix 3.1 & 3.2). PCR products were pooled into two groups and genotyped using an AB 3730 DNA Analyzer. Genotypes were scored with GeneMapper 3.1^{TM} and checked by eye by at least two individuals.

3.3.3. Statistical analysis

Allelic diversity

Allelic size ranges and numbers at each locus were summarized using GenAlEx 6.0 (Peakall and Smouse 2006). Departures of genotype frequencies from Hardy-Weinberg (HW) proportions were tested in GENEPOP 4.0 (Raymond and Rousset 1995). As a large number of sites were tested, the HW *P*-values were adjusted for multiple tests using the Benjamini-Hochberg (BH) method (Benjamini and Hochberg 1995), which has been demonstrated to be robust and effective at minimising type 1 errors (Reiner *et al.* 2003).

Assignment tests

Assignment tests were made with a Bayesian algorithm in STRUCTURE 2.1 (Pritchard *et al.* 2000; Falush *et al.* 2003), which uses HW expectations and linkage disequilibrium to assign individuals to population groups. Analyses were run for K = 1–10 potential population groups with 500 000 burn-in steps and 1 000 000 Markov-Chain Monte-Carlo steps. The 'allele frequencies correlated' and 'use prior population information to assist clustering' models were used, as preliminary analyses indicated that these two models were best able to differentiate between the populations analyzed here. Three replicates were made for each value of K. The ΔK statistic (Evanno *et al.* 2005) was used to estimate the actual number of population groups present (i.e. the true value of K). This statistic is the change in the log probability values [LnP(D)] between successive values of K, and when plotted against K produces a sharp peak at the most likely value of K (Evanno *et al.* 2005). The Prospect, koi, and Jaenschwalde strains were included in the analysis to test how effectively STRUCTURE differentiated among isolated populations and to estimate the extent to which these strains were introduced into the MDB. The USEPOPINFO parameter was set to 1 for these samples to indicate they were learning samples and to 0 for the remaining samples. Koi from Sydney and Germany were pooled in this analysis.

Genetic structure

The *F*-statistics of Weir & Cockerham (1984) were estimated with GENEPOP 4.0 (Raymond and Rousset 1995), and analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) performed with GenAlEx 6.0 (Peakall and Smouse 2006). The significances of the AMOVA results were tested against an empirical null distribution derived from 9999 permutations. As the large dams at river headwaters likely limit carp dispersal, the AMOVA and *F*-statistic analyses were conducted on three groups: 1) all MDB samples, 2) below-dam MDB samples and 3) above-dam MDB samples. In addition, departure of allele frequencies from the null hypothesis of panmixia was tested for each pair of above-dam versus below-dam sites (KP, BD, WY, BJ, LH and EI sites against the NB, WN, CW, ND, KIW and GB sites respectively) using Fisher's exact test in GENEPOP 4.0.

To test for isolation-by-distance population structure, geographic distances between MDB sample sites were measured in Google Earth, both 'as the crow flies' and following the shortest path along river channels. Two measures of the fixation index between subpopulations, F_{ST}, were calculated between all pairs of sample sites in ARLEQUIN 3.1 (Excoffier *et al.* 2005). These were Slatkin's estimate of F_{ST} (Slatkin 1991) and Reynolds' estimate of F_{ST}, derived from the coancestry-based genetic distance of Reynolds *et al.* (1983). Correlations between geographical and genetic distances were estimated for each combination of geographic (along-river and crow-flies) and genetic (Reynolds' and Slatkin's estimates of F_{ST}) distance. Twelve combinations of sample sites were tested to account for barriers to dispersal imposed by impoundments and limited flows (Appendix 3.3). The Bourke (BK) site was excluded, because its small sample size (n = 9) could skew results. The significance of each correlation was determined using a Mantel permutation test in GenAlEx 6.0, with 9999 permutations. As tests were not independent (i.e., same sample sites used in multiple tests), *P*-values were adjusted for multiple tests using the Benjamini & Yekutieli (BY) procedure (Benjamini and Yekutieli 2001) in the MULTTEST package (Pollard *et al.* 2008) in R, rather than the BH procedure (Benjamini and Hochberg 1995) used previously, as this false discovery rate correction takes into account that *P*–values may not be independent.

Barriers to dispersal

Barriers to dispersal were identified with BARRIER 2.2 (Manni *et al.* 2004), which uses geographic and genetic distances to identify genetic discontinuities between regions. The

potential number of barriers (predefined by user) can range from 1 to the number of sample sites. Non-MDB sites were excluded from these analyses. The BK site was also excluded, because of its small sample size. BARRIER was run for each of the two measures of genetic distance (Reynolds' and Slatkin's estimate of F_{ST}). Bootstrap values for each barrier were generated by subsampling with replacement from each sample to generate 100 randomly re-sampled datasets, by computing a genetic distance matrix for each of the 100 re-sampled datasets and by analyzing these matrices with BARRIER. Bootstrap values for each apparent barrier can range from 1% (barrier detected in one of the resampled matrices) to 100% (barrier detected from each of the 100 resampled matrices). Bootstrap values were arbitrarily classed as strong (>80%), weak (40–79%) or not significant (<40%).

Defining management units

Management units can be defined as populations "connected by such low levels of gene flow that they are functionally independent", at least on the time scale relevant to short-term management, and identified by the presence of divergent allele frequencies between regions (Moritz 1994). Management units were proposed in this study based on genetic differentiation between regions implicit in the assignment tests (i.e. different population groups present in different regions), on genetic discontinuities being consistently detected by BARRIER for the two genetic distance measures, and on the known physical barriers to dispersal (primarily catchment boundaries within the MDB). As the dams at river headwaters almost certainly limit carp dispersal, the level of bootstrap support for the barriers detected by BARRIER between the above-dam and below-dam sites was used as a guide to the minimal level of bootstrap support necessary to delimit a management unit from the BARRIER results. Consistency between assignment tests and BARRIER analysis was desirable, but not strictly necessary to delimit a management unit.

Genetic diversity and population bottlenecks

Genetic diversity was estimated as allele richness (A_r), mean number of alleles per locus (A) and observed (H_O) and expected heterozygosity (H_E). These measures were calculated for the MDB as a whole, for the proposed management units (see Discussion) and for the Tasmania, Prospect, koi and Jaenschwalde samples. A_r, A and H_E were estimated with HP RARE (Kalinowski 2005), and H_O with GENEPOP 4.0 (Raymond & Rousset, 1995). For A_r, rarefaction was used to adjust for different sample sizes. As the smallest group analyzed

consisted of 24 individuals, the number of genes per locus was set to 48 for this calculation.

Departures from mutation-drift equilibrium indicative of a recent population bottleneck (inflated heterozygosity relative to heterozygosity expected at mutation-drift equilibrium) were tested using BOTTLENECK 1.2.02 (Cornuet and Luikart 1996; Pirey *et al.* 1999). A two-phase model (TPM) of mutation was used, and significance was assessed with a two-tailed Wilcoxon sign-rank test, which provides relatively large power with as few as four loci. Departures from expected values under mutation-drift equilibrium were tested for the MDB as a whole, separately for each proposed management unit and for Tasmanian, Jaenschwalde, koi and Prospect carp. Koi from Sydney and Germany were analyzed separately. *P*-values were adjusted for multiple testing using the BH method (Benjamini and Hochberg 1995), and adjusted values of $< 0{\cdot}05$ were considered significant.

3.4. Results

3.4.1. Allele Diversity

The number of detected alleles ranged from four (GF1 and Cca07) to 17 (MFW26), with allele size ranges being consistent with size ranges reported in the literature, expect in some where primers were redesigned to anneal in different regions (Table 3.2). As shown in the last column of Table 3.1, nine of the 39 sample sites showed a significant ($P < 0{\cdot}05$) overall departure from HW after adjustment for multiple testing.

3.4.2. Assignment tests

The graph of ΔK against K produced a single, distinctive peak at $K = 5$ (data not shown), indicating the presence of five population groups in the analysis. The distribution of these population groups is illustrated in Figure 3.2. Jaenschwalde and koi carp corresponded closely to population groups 2 and 3, respectively. Prospect carp correspond most strongly with group 1, although about 30% of their overall genetic variation was assigned to group 2. Population group 4 is distributed ubiquitously throughout the MDB and is the dominant group in Victoria. Population group 5 is also widely distributed and is dominant in the Murrumbidgee catchment (ND site). A large proportion (59%) of individuals from the MDB and Tasmania were allocated to more than one population group. The distribution of

the population groups suggested approximately eleven genetically different regions in the MDB.

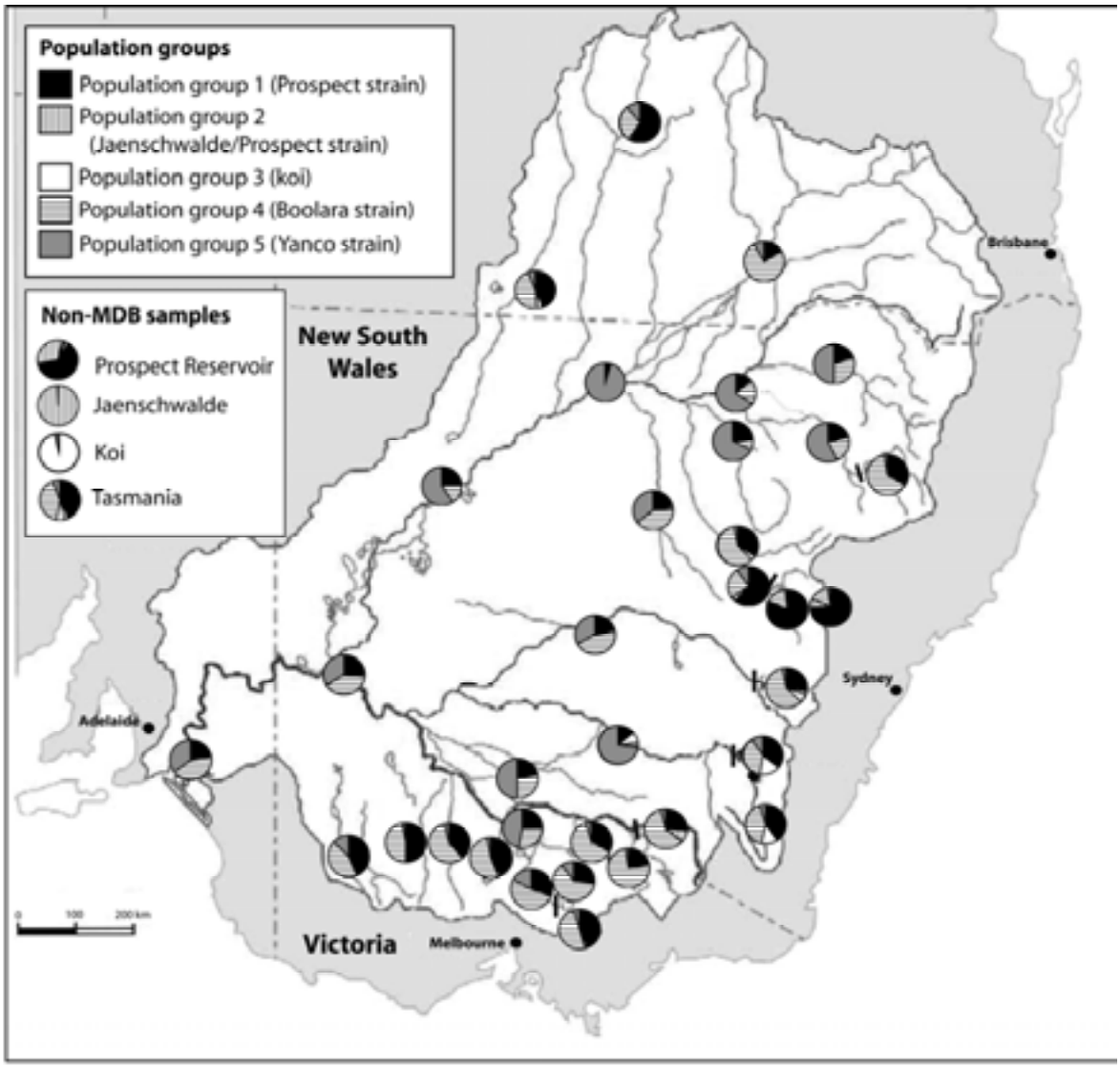

Figure 3.2. Assignment results from STRUCTURE for K=5 population groups. Pie diagrams indicate the overall proportions of each population group (1-5) to the genetic diversity of each sample site.

Table 3.2. Microsatellite alleles and allele size ranges detected

Microsatellite loci	Size Range Reported*	Detected	Number of alleles detected
Cca02	173-194	159-205	12
Cca09a	303-387	332-380	11
Cca65a	184-194	150-160	5
Cca72	244-299	237-304	12
GF1**	337-353	335-376	4
Koi41-42a	228	285-316	6
MFW26	122-150	125-170	17
MFW6	144-152	116-168	15
Cca07a	216– 245	224-236	4
Cca17a	322– 367	371-389	5
Cca19	262-370	291-299	5
Cca67	228-254	231-267	11
Koi29-30a	247	334-344	5
Koi5-6a	189	234-255	6

* References for allele size ranges are listed in Table 3.3. Reported ranges differ greatly from detected ranges in some cases because primers were redesigned to anneal in different regions. ** Additional information about size ranges was reported by Hänfling *et al*. (2005).

3.4.3. Genetic structuring

Significant allele-frequency differences were detected among sample sites. The AMOVA showed significant variation among sites (Table 3.3), with 11% of variation among sites and 89% within sites in the MDB overall. As expected, the percentage of among-site variation was smaller (7%) among below-dam samples and larger (20%) among above-dam samples. F-statistics also indicated that population structuring was greatest among above-dam samples (F_{ST} = 0·1724), lowest among the below-dam samples (F_{ST} = 0·0384) and intermediate among all samples (F_{ST} = 0·0720). All exact test comparisons between above- and below-dam samples were highly significant ($P < 0·001$).

In the plots of genetic distance against geographic distance that were generated to test for isolation-by-distance, the data points showed little scatter about the y-axis (genetic distance) (data not shown). None of the 48 correlations between geographic and genetic distance was significant after BY adjustment (Benjamini and Yekutieli 2001) for multiple testing.

Table 3.3. F-statistics (Weir and Cockerham 1984) and AMOVA results. Statistics are calculated across all 14 microsatellite loci

Analysis	*F*-Statistics			AMOVA		
	F_{ST}	F_{IS}	F_{IT}	Variation within sites	Variation among sites	*P*-value
All MDB sites	0.0720	0.0237	0.0940	89%	11%	0.010
Below-dam MDB sites	0.0384	0.0043	0.0426	93%	7%	0.010
Above-dam MDB sites	0.1724	0.0990	0.2543	79%	21%	0.001

3.4.4. Barriers to dispersal

Barriers to dispersal identified by BARRIER were similar for Slatkin's and Reynolds' F_{ST}, differing more in bootstrap values than in location (Figure 3.3). Since the population-assignment results from STRUCTURE indicated 11 regions in the MDB (Figure 3,2.), the results for the 12 barriers were used, as these allowed the detection of discontinuities along with an additional obvious boundary not identified by STRUCTURE. Strong barriers (>80% bootstrap support) were consistently detected around the Broken, Campaspe and Goulburn rivers in Victoria (sites BR, CS and GB, respectively), the Murrumbidgee catchment (ND), the Paroo and Warrego Rivers (PR and CV) and Lake Eildon (EI) and Wyangala (WY) Dams. Combinations of weak (40–79% support) and strong barriers were detected around the Macquarie River sites (DB and WN), between the Avoca (AV) and Loddon (LD) Rivers and the rest of the MDB, and Burrinjuck (BJ, CM), Burrendong (BD, MG) and Lake Keepit (KP) dams. Both F_{ST} measures indicated weak barriers around Lake Hume (LH), Burrendong Dam (BD) and the Condamine River (CDM), and between the upper (OV, KIW) and mid-Murray (EC, DQ). Slatkin's F_{ST} also detected a strong barrier between the Wimmera catchment (WM) and the rest of the MDB. Minimal bootstrap support for a barrier to delimit a management unit was set at 41%, as this was the lowest bootstrap value for a barrier detected between above-and below-dam sites (Slatkin's F_{ST}, between the LH and KIW sites).

3.4.4. Genetic diversity and population bottlenecks

No significant departures from mutation-drift equilibrium ($P < 0{\cdot}05$) were detected for any management unit by BOTTLENECK after adjustment for multiple testing (data not shown). For management units, A_r ranged from 2·1–4·0, A from 2–5, H_O from 0·179–0·467 and H_E from 0·182–0·498 (Figure 3.4). Genetic diversity was highest in the Murrumbidgee catchment, and lowest in the Wimmera catchment (A and A_r), Lake Keepit (A and A_r) and Burrendong Dam (all measures) management units. When the MDB is considered overall, A_r and A are much higher than in the individual management units, both being 8·3.

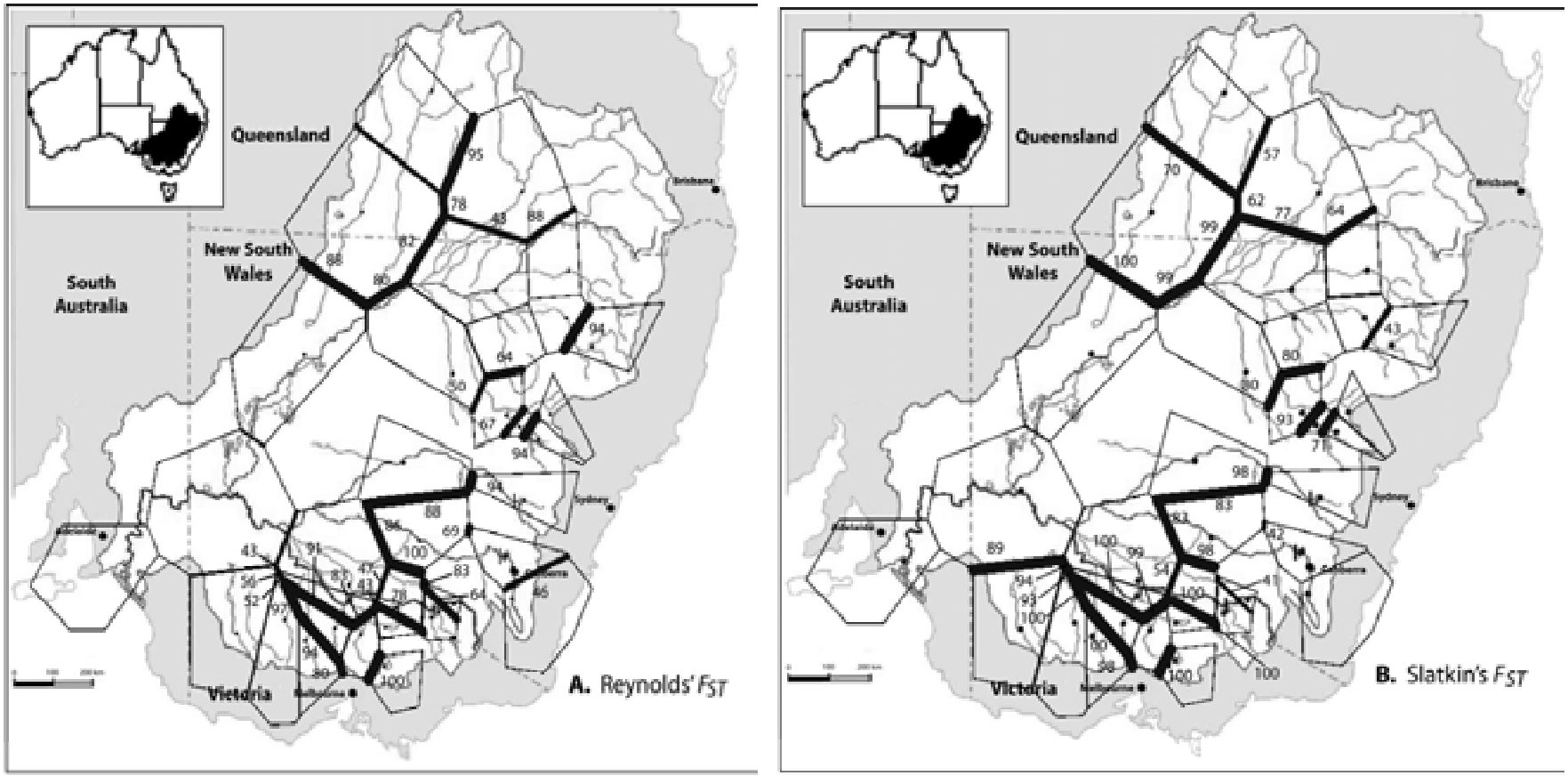

Figure 3.3. Putative barriers to dispersal calculated from A. Reynolds' estimate of FST, and B. Slatkin's estimate of FST. Polygons around each sample site represent the Voronï tessellations drawn around each sample site by BARRIER. Thickened lines represent putative barriers to dispersal. The level of bootstrap support for each barrier is indicated by both the number associated with the barrier, and the thickness of the barrier. Bootstrap values less than 40 are not shown.

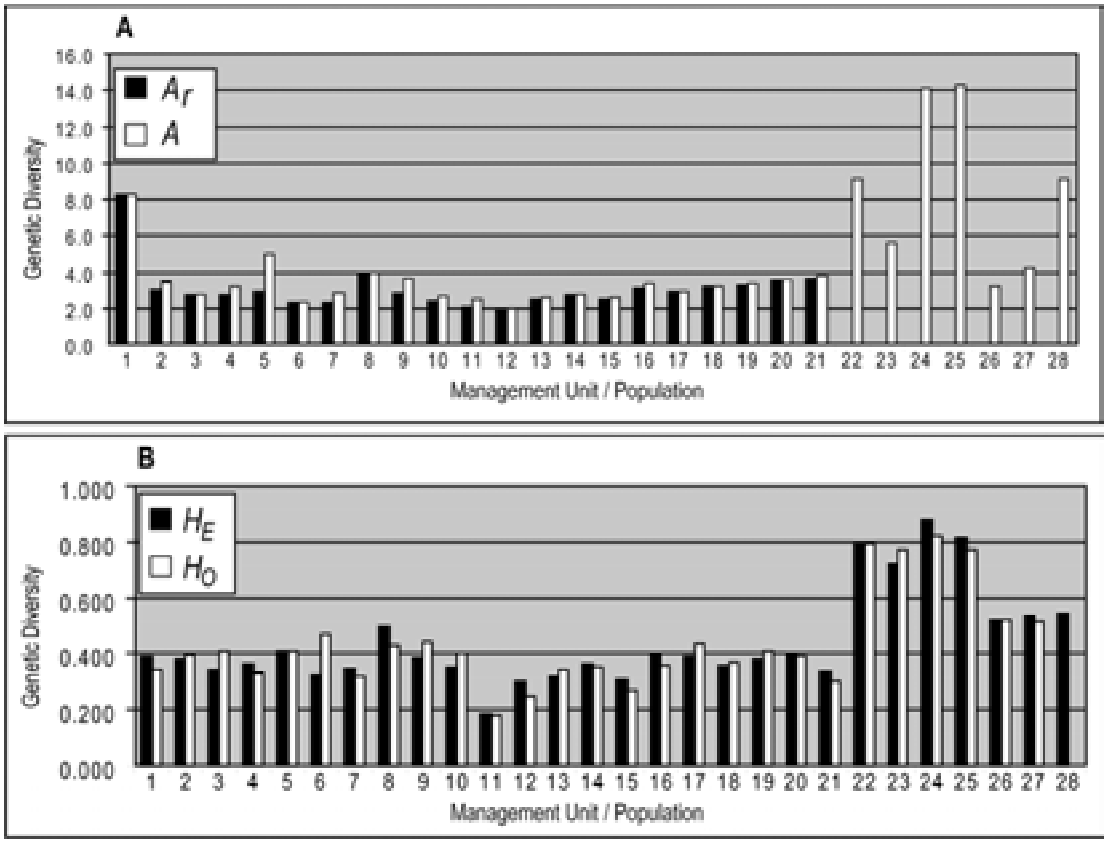

Figure 3.4. Genetic diversity in common carp in the MDB. **A.** Allele richness (A_r) and mean number of alleles per locus (A). **B.** Observed and expected heterozygosity (H_O and H_E respectively). Genetic diversity indices from this study are shown in comparison with published data from common carp in their native range, other invasive species of freshwater fish, and freshwater fish in general. Allele richness was not reported in any of the published studies, and *Ho* was not reported for freshwater fish in general. Data from carp in Australia is given for the MDB as a whole, and for each individual management unit (Figure 3.5). Numbers are: 1. all MDB, 2. Paroo-Warrego Catchments, 3. Condamine Catchment, 4. Macquarie Catchment, 5. Main MDB, 6. Wimmera Catchment, 7. Avoca-Loddon Catchments, 8. Murrumbidgee Catchment, 9. Central Victoria, 10. Upper Murray, 11. Burrendong Dam, 12. Lake Keepit, 13. Wyangala Dam, 14. Lake Eildon, 15. Lake Hume, 16. Burrinjuck Dam, 17. Tasmania, 18. Prospect Reservoir, 19. Jaenschwalde, 20. koi (Sydney fish farm), 21. koi (German fish farm), 22. *C.carpio,* European, wild*, 23. *C.carpio,* European, domestic*, 24. *C.carpio,* Central Asian, wild*, 25. *C.carpio,* East Asian, wild*, 26. *Petromyzon marinus***, 27. *Poecilia reticulata*†, 28. Freshwater fish overall††. *Kohlmann *et al*. (Kohlmann *et al.* 2005), ** Bryan *et al*. (2005), †Lindholm *et al*. (2005), ††DeWoody and Avise (2000).

3.5. Discussion

This research is the most comprehensive population genetic study of common carp in a single river basin to date. Consistent with the findings of previous Australian studies, this study confirms that the Boolara, Yanco and koi strain are present in the MDB (Shearer and Mulley 1978; Davis *et al.* 1999). The results of this study also show that the Prospect strain is widely distributed throughout the MDB. Significant genetic structuring appears across the MBD and is strongly associated with contemporary barriers to dispersal. Levels of genetic variation in the MDB were similar to those in domestic populations (koi and Jaenschwalde), indicating that carp are not genetically depauperate in Australia. A history of introduction and spread of the various carp strains in Australia is proposed below, based on the current distribution of the strains. The MBD is divided into fifteen management units for control programs, each corresponding to natural or man-made barriers to carp dispersal.

3.5.1. Strains of common carp in the Murray-Darling Basin

Five population groups of carp were identified with STRUCTURE (Figure. 3.2). Groups 1, 2 and 3 likely represent the Prospect, Jaenschwalde and koi carp, respectively, as these strains correspond most closely with these groups. The imperfect separation between groups 1 and 2 in the Prospect strain is likely a result of a smaller sample size (24) of Prospect individuals, the limited number of microsatellite loci (14) and the genetic similarity between Prospect and Jaenschwalde carp. Group 4 likely represents the Boolara strain, as it is ubiquitously distributed throughout the MDB and is the dominant group in Victoria (Davis *et al*. 1999). Group 5 likely represents the Yanco strain, as it is the dominant group at Narrandera in the Murrumbidgee Catchment (ND site), close to where Shearer and Mulley (1978) caught the Yanco-strain individuals in their study. The ability of STUCTURE to detect these strains in the MDB, despite several generations of potential interbreeding, may stem from the longevity of carp. Older individuals of ‘pure’ strain ancestry may have been caught alongside younger, intercrossed progeny, as carp over 50 years in age have been caught in the wild (P. Sorenson, pers. comm.).

3.5.2. Population genetic structure

Significant variation among sites (AMOVA) and the heterogeneous distribution of the strains indicate that carp in the MDB exhibit considerable population genetic structure. Dams play a role in limiting gene flow, as among-site variation measured by AMOVA was

greater when samples from above dams were included than when only below-dam samples were analyzed. All pairwise comparisons of allele frequencies between above- and below-dams sites showed highly significant departures from panmixia. This genetic structuring is not associated with isolation-by-distance. The lack of scatter around the y-axis (genetic distance) in the plots of genetic distance against geographic distance is similar to a scenario theoretically and empirically demonstrated by Hutchinson and Templeton (1999), in which a lack of regional equilibrium, and migration and gene flow play a larger role in shaping genetic structure than does genetic drift. The pattern of genetic structure can therefore be attributed to contemporary barriers to dispersal that limit migration and gene flow, as well as historical patterns of introduction and range expansion.

3.5.3. Genetic diversity

Although many invasive species show decreased levels of genetic diversity in their introduced range relative to their native range (e.g. Hamner *et al.* 2007), some invasives have comparable or greater levels of genetic diversity, because they originated from multiple source populations and rapid population growth following establishment minimized the loss of genetic diversity through drift (Zenger *et al.* 2003; Frankham 2005; Hänfling 2007). Common carp in the MDB generally have high levels of genetic diversity, with multiple strains detected in all regions, a large proportion (59%) of individuals showing mixed-strain ancestry and no evidence for a recent population bottleneck. Only three of the 15 management units (Burrendong Dam, Lake Keepit and the Wimmera catchment) showed greatly reduced A, A_r, H_E or H_O relative to the domestic populations (koi and Jaenschwalde carp) analyzed here. The high level of genetic diversity in the Murrumbidgee catchment management unit is consistent with the presence of a self-sustaining population of Yanco strain carp before the introductions of the Boolara and Prospect strains. Overall values of A and A_r in MDB populations are greater than in domestic populations in Europe (Kohlmann *et al.* 2005), invasive lampreys (*Petromyzon marinus* Linnaeus, 1758) in the Great Lakes of North America (Bryan *et al.* 2005), and invasive guppies (*Poecilia reticulata* Peters, 1859) in Queensland, Australia (Lindholm *et al.* 2005). Genetic diversity, however, is less than estimates for indigenous populations of wild carp reported by Kohlmann *et al*. (2005), although this may be due to the use of a different set of microsatellite loci by Kohlmann *et al*. (2005). H_E and H_O for the management units and the MDB as a whole are also lower than previous estimates for wild and domestic carp in their native range (Kohlmann *et al.* 2005), freshwater fish overall

(DeWoody and Avise 2000), and invasive lampreys and guppies, and may have resulted from the inclusion of different strains in the samples (Wahlund's effect). The high level of genetic diversity of carp in the MDB may have facilitated invasiveness and adaptation to new environments.

3.5.4. History of introduction and range expansion

The following scenarios for the introductions and spread of carps in the MDB are proposed. (1) As the Prospect strain was detected throughout the MDB, it was likely introduced early, and perhaps expanded its range during the extensive 1950s floods. (2) The widespread distribution of the Boolara strain is consistent with a range expansion during large-scale floods in 1974–5 (Reid *et al.* 1997; Koehn *et al.* 2000), perhaps aided by heterosis (hybrid vigour) resulting from mating with the already present Prospect strain. (3) The scarcity of the Yanco strain in some regions indicates a range expansion after the expansion of the Prospect and Boolara strains. Prospect and Boolara carp and their intercrossed progeny may not have entered the Murrumbidgee catchment in significant numbers until the 1974–1975 floods. Prospect and Boolara carps may have bred with the resident Yanco carps, resulting in further heterosis and providing the genetic diversity necessary for the descendents of introduced Yanco carp to lose their conspicuous orange coloration and expand their range. Descendents of Yanco carp are now scarce in some of the rivers in the Darling River catchment, because these rivers have remained partially isolated from the rest of the MDB since the 1974–1975 floods. The Yanco strain was also possibly prevented from penetrating far into the Victorian rivers and the upper reaches of the Murray River by weirs and by the abundance of adult Boolara and Prospect strain carp already present in these regions. (4) Koi carp have been released in low numbers throughout the MDB, but have contributed little to the overall population. Thirty-seven carp with 5–50% koi ancestry were detected above Burrinjuck Dam (BJ and CM sites) and seven in the sample from Tasmania, consistent with the detection by Davis *et al*. (1999) of putative koi haplotypes in Lake Burley Griffin (which is also located above Burrinjuck Dam) and in Tasmania.

The establishment of carp above six of the large dams in the MDB indicates that carp were present before the dams were constructed or were introduced by humans, as these dams are too large to be submerged by flooding. Dispersal of sticky carp eggs on the feet or plumage of waterfowl has been postulated as a mechanism of disperal (Gilligan and Rayner 2007),

although to date no empirical evidence to supports this. The following is proposed for these populations. (1) The carp above the Eildon (EI) and Hume (LH) dams were likely introduced from adjacent waterways, possibly those immediately downstream, as they have a similar strain composition to these adjacent rivers. (2) The Keepit (KP), Wyangala (WY) and Burrinjuck (BJ, CM) dam populations were likely introduced before the expansion of the Yanco strain, as these populations include the Prospect and Boolara strains. (3) The reduced levels of genetic diversity and prevalence of the Prospect strain above Burrendong Dam (BD, MG) is consistent with a founding by a small number of Prospect-strain carp, which may have been introduced from the Sydney Basin. This strain was unlikely present before the construction of Burrendong Dam in 1967, as aging data from otoliths indicate that the oldest of 300 carp caught in Burrendong Dam was spawned in 1989 (D.M. Gilligan, unpublished data). As carp can live over 50 years in the wild (P.W. Sorenson, pers. comm.), the rivers above Burrendong Dam were not likely populated with carp prior to the dam's construction. Whether these introductions are the results of accidental releases, through use of carp as live bait or contamination of stocked native fish with carp fry (Koehn *et al.* 2000), or of deliberate introductions is unknown.

3.5.5. Barriers to dispersal and management units

The presence of fifteen discrete genetic entities that could be classified as individual management units were identified by the assignment tests and BARRIER analyses, in conjunction with known dispersal barriers in the MDB. These management units are illustrated in Figure 3.5, and supporting information appears in Appendix 3.4. Each management unit corresponds with the presence of impoundments, naturally limited flows and catchment boundaries. These units should be interpreted with some caution, however, for two reasons. First, the ongoing construction of fishways (Stuart *et al.* 2008) and improved flow management may increase connectivity between populations in various regions and may render some units obsolete, although this could be minimized by the inclusion of William's carp-separation cages to reduce the movement of carp (Stuart *et al.* 2006b). Second, these units are defined over a broad area, including the whole river catchment within the MDB. As additional barriers to dispersal may be present within each unit, the fine details of the hydrology of each river system should also be considered when implementing control programs. The proposed units, however, indicate which catchments can be managed independently and which should be managed in conjunction with each other units for the effective long-term control of invasive carp.

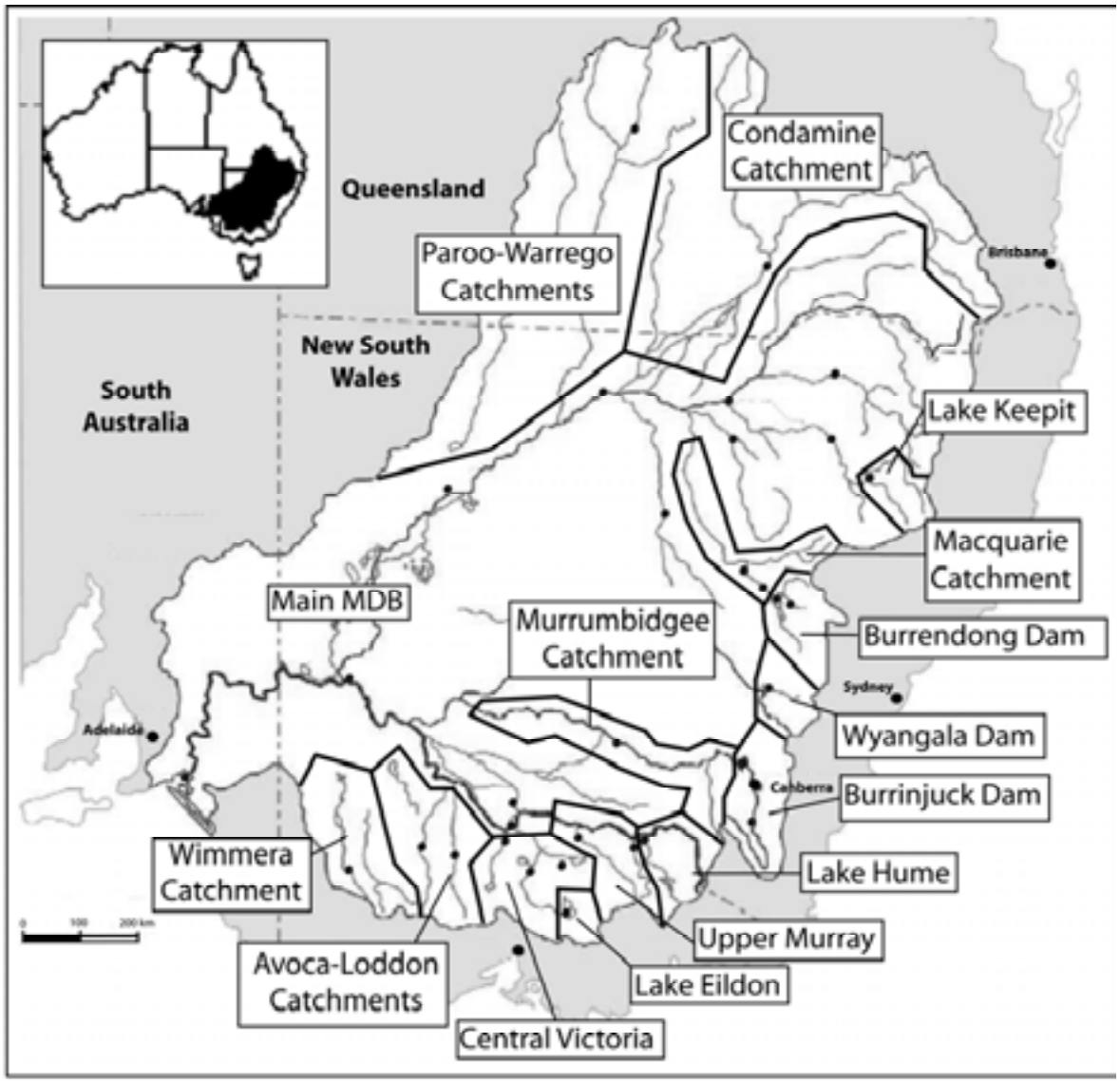

Figure 3.5. Proposed management units for common carp in the MDB. Units are based on genetic discontinuities and geographic barriers to dispersal (see Appendix 3.4).

3.6. Acknowledgements

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3.7. References

Anderson, H. K. (1920). Rescue operation on the Murrumbidgee River. *The Australian Zoologist* **1**, 157-160.

Balon, E. K. (1995). Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman Gourmets to the swimming flower. *Aquaculture* **129**, 3-48. doi: 10.1016/0044-8486(94)00227-F

Benjamini, Y. & Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society: Series B (Statistical Methodology)* **57**, 289-300.

Benjamini, Y. & Yekutieli, D. (2001). The control of the false discovery rate in multiple hypothesis testing under dependency. *Annals of Statistics* **29**, 1165-1188.

Brown, P. (1996). Fish Facts 4 Carp in Australia. Narrandera, Australia: NSW Fisheries (now part of the NSW Department of Primary Industries).

Brownstein, M. J., Carpten, J. D. & Smith, J. R. (1996). Modulation of non-templated nucleotide addition by *Taq* DNA polymerase: primer modifications the facilitate genotyping. *BioTechniques* **20**, 1004-1010.

Bryan, M. B., Zalinski, D., Filcek, K. B., Libants, S., LI, W. & Scribner, K. T. (2005). Patterns of invasion and colonization of the sea lamprey (*Petromyzon marinus*) in North America as revealed by microsatellite genotypes. *Molecular Ecology* **14**, 3757–3773. doi: 10.1111/j.1365-294X.2005.02716.x.

Clements, J. (1988). *Salmon at the Antipodes A History and Review of Trout, Salmon and Char and Introduced Coarse Fish in Australasia*. Ballarat, Victoria, Australia: Published by the author.

Cornuet, J. M. & Luikart, G. (1996). Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**, 2001-2014.

Crooijmans, R. P. M. A., Bierbooms, V. A. F., Komen, J., Van der Poel, J. J. & Groenen, M. A. M. (1997). Microsatellite markers in common carp (*Cyprinus carpio* L.). *Animal Genetics* **28**, 129-134. doi: 10.1111/j.1365-2052.1997.00097.x.

David, L., Rajasekaran, P., Fang, J., Hillel, J. & Lavi, U. (2001). Polymorphisms in ornamental and common carp strains (*Cyprinus carpio* L.) as revealed by AFLP analysis and a new set of microsatellite markers. *Molecular Genetics and Genomics* **266**, 353-362. doi: 10.1007/s004380100569.

Davis, K. M., Dixon, P. I. & Harris, J. H. (1999). Allozyme and mitochondrial DNA analysis of carp, *Cyprinus carpio* L., from south-eastern Australia. *Marine and Freshwater Resources* **50**, 253-260. doi: 10.1071/MF97256

DeWoody, J. A. & Avise, J. C. (2000). Microsatellite variation in marine, freshwater and anadromous fishes compared with other animals. *Journal of Fish Biology* **56**, 461–473. doi: 10.1111/j.1095-8649.2000.tb00748.x.

Evanno, G., Regnaut, S. & Goudet, J. (2005). Detecting the number of clusters of individuals using the sortware STRUCTURE: a simulation study. *Molecular Ecology* **14**, 2611-2620. doi: 10.1111/j.1365-294X.2005.02553.x.

Excoffier, L., Laval, G. & Schneider, S. (2005). Arlequin ver. 3.0: An integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1**, 47-50.

Excoffier, L., Smouse, P. E. & Quattro, J. M. (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**, 479-491.

Falush, D., Stephens, M. & Pritchard, J. K. (2003). Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies *Genetics* **164**, 1567-1587.

Frankham, R. (2005). Resolving the genetic paradox in invasive species. *Heredity* **94**, 385. doi: 10.1038/sj.hdy.6800634.

Gilligan, D. & Rayner, T. (2007). The distribution, spread, ecological impacts, and potential control of carp in the upper Murray River. In *NSW Department of Primary Industries - Fisheries Research Report Series* Sydney: NSW Department of Primary Industries.

Graham, K. J., Lowry, M. B. & Walford, T. R. (2005). Carp in NSW: assessment of distribution, fishery and fishing methods. In *Fisheries Final Report Series No. 72*. Cronulla, Australia: NSW Department of Primary Industries.

Hamner, R. M., Freshwater, D. W. & Whitfield, P. E. (2007). Mitochondrial cytochrome *b* analysis reveals two invasive lionfish species with strong founder effects in the western Atlantic. *Journal of Fish Biology* **71 (Supplement B)**, 214-222. doi: 10.1111/j.1095-8649.2007.01575.x.

Hänfling, B. (2007). Understanding the establishment success of non-indigenous fishes: lessons from population genetics. *Journal of Fish Biology* **71 (Supplement D)**, 115-135. doi: 10.1111/j.1095-8649.2007.01685.x.

Hänfling, B., Boton, P., Harley, M. & Carvalho, G. R. (2005). A molecular approach to detect hybridisation between crucian carp (*Carassis carassius*) and non-indigenous carp species (*Carassisus* cpp. and *Cyprinus carpio*). *Freshwater Biology* **50**, 403-417. doi:10.1111/j.1365-2427.2004.01330.x.

Harris, J. H. & Gehrke, P. C. (1997). Fish and rivers in stress - the NSW rivers survey. Cronulla and Canberra: NSW Fisheries Office of Conservation & the Cooperative Research Centre for Freshwater Ecology.

Hutchinson, D. W. & Templeton, A. R. (1999). Correlations of pairwise genetic distance and geographic distance measures: inferring the relarive influences of gene flow and drift on the distribution of genetic variability. *Evolution* **53**, 1898-1914.

Kalinowski, S. T. (2005). HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Molecular Ecology Notes* **5**, 187–189. doi: 10.1111/j.1471-8286.2004.00845.x.

Koehn, J. (2004). Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. *Freshwater Biology* **49**, 882-894. doi: 10.1111/j.1365-2427.2004.01232.x.

Koehn, J., Brumley, B. & Gehrke, P. (2000). *Managing the Impacts of Carp*. Canberra, Australia: Bureau of Rural Sciences (Department of Agriculture, Fisheries and Forestry).

Kohlmann, K., Kersten, P. & Flajshans, M. (2005). Microsatellite-based genetic variability and differentiation of domesticated, wild and feral common carp (*Cyprinus carpio* L.) populations. *Aquaculture* **247**, 253-256. doi:10.1016/j.aquaculture.2005.02.024.

Lindholm, A. K., Brenden, F., Alexander, H. J., Chan, W.-K., Thakurta, S. G. & Brooks, R. (2005). Invasion success and genetic diversity of introduced populations of guppies *Poecilia reticulata* in Australia. *Molecular Ecology* **14**, 3671-3682. doi: 10.1111/j.1365-294X.2005.02697.x.

Manni, F., Guérard, E. & Heyer, E. (2004). Geographic patterns of (genetic, morphologic, linguistic) variation: how barriers can be detected by "Monmonier's algorithm". *Human Biology* **76**, 173-190.

Moritz, C. (1994). Defining evolutionary significant units for conservation. *Trends in Ecology and Evolution* **9**, 373-375. doi: 10.1016/0169-5347(94)90057-4.

Peakall, R. & Smouse, P. E. (2006). GENALEX 6: genetic analysis in EXCEL. Population genetic software for teaching and research. *Molecular Ecology Notes* **6**, 288-295. doi: 10.1111/j.1471-8286.2005.01155.x.

Pirey, S., Luikart, G. & Cornuet, J. M. (1999). BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* **90**, 502-503.

Pollard, K. S., Dudoit, S. & van der Laan, L. J. (2008). Multiple testing procedures: R multtest package and applications to genomics *U.C. Berkeley Division of Biostatistics Working Paper Series*. Working Paper 164. http://www.bepress.com/ucbbiostat/paper164.

Pritchard, J. K., Stefens, M. & Donelly, P. (2000). Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.

Raymond, M. & Rousset, F. (1995). GENEPOP (version 1.2): population genetics software for exact tests and eumenicism. *Journal of Heredity* **86**, 248-249.

Reid, D. D., Harris, J. H. & Chapman, D. J. (1997). NSW Inland Commercial Fishery Data Analysis. In *FRDC Project No. 94/027*. Sydney, Australia: Fisheries Research & Development Corporation, NSW Fisheries, Cooperative Research Centre for Freshwater Ecology.

Reiner, A., Yekutieli, D. & Benjamini, Y. (2003). Identifying differentially expressed genes using false discovery rate controlling procedures. *Bioinformatics* **19**, 368-375.

Reynolds, J., Weir, B. S. & Cockerham, C. C. (1983). Estimation of the coancestry coefficient: basis for a short-term genetic distance. *Genetics* **105**, 767-779.

Shearer, K. D. & Mulley, J. C. (1978). The introduction and distribution of the carp, *Cyprinus carpio* Linnaeus, in Australia. *Australian Journal of Marine and Freshwater Research* **29**, 661-563. doi:10.1071/MF9780551

Shuber, A. P., Grondin, V. J. & Klinger, K. W. (1995). A simplified procedure for developing multiplex PCRs. *Genome Research* **5**, 488-493.

Slatkin, M. (1991). Inbreeding coefficients and coalescence times. *Genetical Research, Cambridge* **58**, 167-175.

Stead, D. G. (1929). Introduction of the great carp *Cyprinus carpio* into waters of New South Wales. *Australian Zoologist* **6**, 100-102.

Stuart, I. G., Williams, A., McKenzie, J. & Holt, T. (2006). Managing a migratory pest species: a selective trap for common carp. *North American Journal of Fisheries Management* **26**, 888-893.

Stuart, I. G., Zampatti, B. P. & Baumgartner, L. J. (2008). Can a low-gradient vertical-slot fishway provide passage for a lowland river fish community? *Marine and Freshwater Research* **59**, 332-346. doi: 10.1071/MF07141

Wasko, A. P., Martins, C., Oliveira, C. & Foresti, F. (2003). Non-destructive genetic sampling of fish. An improved mehtod for DNA extraction from fish fins and scales. *Hereditas* **138**, 161-165. doi:10.1034/j.1601-5223.2003.01503.x.

Weir, B. S. & Cockerham, C. C. (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**, 1358-1370.

Yue, G. H., Ho, M. Y., Orban, L. & Komen, J. (2004). Microsatellites within genes and ESTs of common carp and their applicability in silver crucian carp. *Aquaculture* **234**, 85-98. doi:10.1016/j.aquaculture.2003.12.021.

Zenger, K. R., Richardson, B. J. & Vachot-Griffin, A.-M. (2003). A rapid population expansion retains genetic diversity within European rabbits in Australia. *Molecular Ecology* **12**, 789-794. doi:10.1046/j.1365-294X.2003.01759.x.

Zheng, W., Stacey, N. E., Coffin, J. & Strobeck, C. (1995). Isolation and characterization of microsatellites loci in the goldfish *Carassius auratus*. *Molecular Ecology* **4**, 791-792.

Appendix 3.1. PCR conditions and primer sequences for microsatellite loci. PCRs A, B and C comprise multiplexes of two loci; all other PCRs amplify only a single locus. Non-template components of the primer sequences (Shuber *et al.* 1995; Brownstein *et al.* 1996) are shown in *italics*. Primer names with an 'a' suffix have been redesigned. PCR cycling protocols are presented in Appendix 3.2 and PCR product size ranges in Table 3.2.

PCR	Loci Amplified	Primers	Primer Sequence	Primer Concentration	$MgCl_2$ Concentration	PCR Protocol*
A	Cca72*	F-NED	CAGGCCAGATCTATCATCATCAA	0.2µM	2.5mM	TD5060
		R	***GTTTCTT***CTGCTGTTGGATATGCACTACATC	0.2µM		
	Cca02*	F-VIC	ATGCAGGGCTCATGTTGCTCATAG	0.2µM		
		R	***GTTTCTT***GCAGACAGACACGTTGCTCTCG	0.2µM		
B	MFW6**	F-NED	ACCTGATCAATCCCTGGCTC	0.2µM	2mM	TD6850
		R	***GTTTCTT***TTGGGACTTTTAAATCACGTTG	0.2µM		
	MFW26**	F-VIC	CCCTGAGATAGAAACCACTG	0.2µM		
		R	***GTTTCTT***CACCATGCTTGGATGCAAAAG	0.2µM		
C	Koi 41-42††	Fa-VIC	*GCGGTCCCAAAAGGGTCAGT*ATCTCTGAAAAGCCCAATATGTCAA	0.17µM	1.5mM	TD6452
		Ra	*GTTTCTTCAAAAGGGTCAGT*CTGTAAATCTTCATGGTGTGTGTCC	0.17µM		
	Cca09*	F-6FAM	*GCGGTCCCAAAAGGGTCAGT*AATGCCTATTCACATTATGAAAAT	0.2µM		
		Ra	*GTTTCTTCAAAAGGGTCAGT*AATCAGGTATAGTGGTTATATGAGTT	0.2µM		
D	GF1†	F-NED	*GCGGTCCCAAAAGGGTCAGT*ATGAAGGGTAGGAAAAGTGTGA	0.2µM	2mM	TD6452
		R	*GTTTCTTCAAAAGGGTCAGT*CAGGTTAGGGAGAAGAAGGAAT	0.2µM		
Da	Cca65*	Fa-6FAM	AAGTGAGCGGGAGACAGAGA	0.17uM	1.5mM	TD6452
		Ra	*GTTTCTTCAAAAGGGTCAGT*CAGACAAGTGTGCATGAGTGG	0.17uM		
F	Cca19*	F-HEX	*GCGGTCCCAAAAGGGTCAGT*CCTGACCCTGAAGAGAACAACTAC	0.2µM	2mM	TD6452
		R	*GTTTCTTCAAAAGGGTCAGT*TGGCCTCATCAAAGACATCAAG	0.2µM		
G	Cca67*	F-VIC	GTAGCCCCAAAAGATGTAGCA	0.2µM	1.5mM	TD6850
		R	***GTTTCTT***TGGTCAAGTTCAGAGGCTGTAT	0.2µM		
H	Koi 5-6††	Fa-NED	*GCGGTCCCAAAAGGGTCAGT*TTTGTGTTTTCTGTTGTAGGCTCTG	0.2µM	1.5mM	TD6452
		Ra	*GTTTCTTCAAAAGGGTCAGT*TTTTACTTCATCTCTCGCACTCATCT	0.2µM		
I	Koi 29-30††	Fa-NED	*GCGGTCCCAAAAGGGTCAGT*CCCTGACCCTGAAGAGAACAACTAC	0.2µM	1.5mM	TD6452
		Ra	*GTTTCTTCAAAAGGGTCAGT*GCCTCATCAAAGACATCAAG	0.2µM		
J	Cca07*	Fa-6FAM	*GCGGTCCCAAAAGGGTCAGT*CATTGCGCTGTAATATGAGGTTTCT	0.2µM	1.5mM	TD6452
		Ra	*GTTTCTTCAAAAGGGTCAGT*CTCGTTCCTTTTCTGACGCTTTT	0.2µM		
K	Cca17*	Fa-6FAM	*GCGGTCCCAAAAGGGTCAGT*CAGGTCTTGATTTACTGCTGTCTTT	0.2µM	1.5mM	TD6452
		Ra	*GTTTCTTCAAAAGGGTCAGT*GATAACTGCGTGTAGGCTCTGTATT	0.2µM		

*Yue *et al.* (2004), ** Crooijmans *et al.*(1997), †Zheng *et al.* (1995), ††David *et al.* (2001)

Appendix 3.2. PCR cycling protocols

PCR Protocol	Denaturing Step	Touch-Down Cycle		Standard Cycle		Final Extension Step
TD6850	95°C 10 min	Denaturing:	95 °C for 45 sec	Denaturing:	95°C for 45 sec	72°C 30 min
		Annealing:	68°C for 90 sec*	Annealing:	50°C for 60 sec	
		Extension:	72°C for 60 sec	Extension:	72°C for 60 sec	
		Total cycles: 9		Total cycles: 30		
TD6050	95°C 10 min	Denaturing:	95°C for 30 sec	Denaturing:	95°C for 30 sec	72°C 30 min
		Annealing:	60°C for 30 sec**	Annealing:	50°C for 30 sec	
		Extension:	72°C for 30 sec	Extension:	72°C for 30 sec	
		Total cycles: 10		Total cycles: 30		
TD6452	95°C 10 min	Denaturing:	95°C for 30 sec	Denaturing:	95°C for 30sec	72°C 30 min
		Annealing:	64°C for 60 sec**	Annealing:	52°C for 30sec	
		Extension:	72°C for 60 sec	Extension:	72°C for 30sec	
		Total cycles: 12		Total cycles: 30		

* decrease by 2°C each cycle, ** decrease by 1°C each cycle

Appendix 3.3. Samples used in isolation-by-distance analyses

Name of analysis	Samples sites
All sites	CDM,PR,CV,DB,WN,WG,NG,CN,WC,MR,NB,DQ,EC,ND,CW,LL,WT,WM,AV,BR,CS,BG,KIW,LD,OV,BD,MG,KP,WY,EI,LH, BJ,CM
Below dams	CDM,PR,CV,DB,WN,WG,NG,CN,WC,MR,NB,DQ,EC,ND,CW, LL,WT,WM,AV,BR,CS,BG,KIW,LD, OV
Main MDB management unit	WG,NG,CN,WC,MR,NB,DQ,EC,CW,LL,WT
Murray Basin	DQ,EC,ND,CW,LL,WT,WM,AV,BR,CS,GB,KIW, LD,OV
Murray River (LH included)	DQ,EC,LL,WT,KIW,OV,LH
Murray River (LH excluded)	DQ,EC,LL,WT,KIW,OV
Darling Basin - 1	CMD,PR,CV,DB,WT,WG,NG,CN,WC,MR,NB,LL,WT
Darling Basin - 2	WG,NG,CN,WC,MR,NB,LL,WT
Darling Basin - 3	CMD,PR,CV,DB,WT,WG,NG,CN,WC,MR,NB
Darling Basin - 4	WG,NG,CN,WC,MR,NB
Darling River	WG,,WC,NB,LL,WN
Murray River + Darling River	LL,WT,EC,DQ,OV,KIW,WC,WG,MR

Appendix 3.4. Management units for common carp in the MDB. A map of these units is given in Figure 3.4.

Unit	Sample sites	Reason for delimiting as a management unit
Main MDB	LL, WT, EC, DQ, CW, BK, WC, WG, MR, NG, CN, NB, WM	Multiple known barriers to dispersal, multiple genetic discontinuities detected by STRUCTURE (predominantly Prospect, Yanco and Boolara strains present) and BARRIER. Although the Yanco strain is more prevalent in the Darling catchment than in the Murray catchment, sites from both catchments are included in the management units as a genetic discontinuity was not detected by BARRIER between the two catchments.
Paroo-Warrego Catchments	PR, CV	Genetic discontinuity detected by STRUCTURE (predominantly Prospect and Boolara strain) and BARRIER; Paroo and Warrego Rivers linked by irrigation channels.
Condamine Catchment	CDM	Genetic discontinuity detected by STRUCTURE (predominantly Boolara strain) and BARRIER
Macquarie Catchment	WN, DB	Genetic discontinuity detected by STRUCTURE (predominantly Prospect and Boolara strain) and BARRIER. Both sites in the Macquarie River (WN and DB) are proposed to be part of the same management unit, despite discontinuities being consistently detected between them by BARRIER, because there are no major barriers to dispersal between the two sites. The discontinuity is likely an artefact of the predominantly Prospect strain carp in Burrendong Dam dispersing downstream and hence being more prevalent at the WN site immediately below the dam outlet than at the more distant DB site.
Murrumbidgee Catchment	ND	Genetic discontinuity detected by STRUCTURE (predominantly Yanco strain) and BARRIER
Wimmera Catchment	WM	Strongly isolated from other parts of the MDB, genetic discontinuity detected for Slatkin's F_{ST} by BARRIER
Avoca-Loddon Catchments	AV, LD	Genetic discontinuity detected by STRUCTURE (predominantly Prospect and Boolara strain) and BARRIER
Central Victoria	BR, GB, CS	Genetic discontinuity detected by STRUCTURE (predominantly Prospect and Boolara strain) and BARRIER

Appendix 3.4. Management units for common carp in the MDB (Continued)

Unit	Sample sites	Reason for delimiting as a management unit
Upper Murray	OV, KIW	Genetic discontinuity detected by STRUCTURE (predominantly Prospect and Boolara strain), weak genetic discontinuity detected for Slatkin's F_{ST} by BARRIER
Lake Keepit	KP	Large dam at river headwaters limits carp dispersal, genetic discontinuity detected by STRUCTURE (predominantly Prospect and Boolara strain) and BARRIER
Burrendong Dam	BD, MG	Large dam at river headwaters limits carp dispersal, genetic discontinuity detected by STRUCTURE (predominantly Prospect strain) and BARRIER
Wyangala Dam	WY	Large dam at river headwaters limits carp dispersal, genetic discontinuity detected by STRUCTURE (predominantly Prospect and Boolara strain) and BARRIER
Burrinjuck Dam	BJ, CM	Large dam at river headwaters limits carp dispersal, genetic discontinuity detected by STRUCTURE (greater contribution from koi carp and much lesser contribution from Yanco strain than downstream sites) and BARRIER
Lake Hume	LH	Large dam at river headwaters limits carp dispersal, genetic discontinuity detected by BARRIER
Lake Eildon	EI	Large dam at river headwaters limits carp dispersal, genetic discontinuity detected by STRUCTURE (Prospect strain more prevalent than at downstream sites) and BARRIER

Chapter 4: Invasive common carp (Cyprinus carpio L.) in Australia: origin of founding strains and population genetics of coastal waterways

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Key words: population genetics, common carp, *Cyprinus carpio*, invasive species, koi, microsatellite

Running Title: Origin of common carp in Australia

4.1. Abstract

Common carp (*Cyprinus carpio*) are a highly invasive freshwater fish species in many places around the world, including Australia. In a previous study, we confirmed the findings of earlier genetic studies that four strains of carp – Japanese koi, Prospect, Boolara and Yanco - have been introduced into Australia. In this study, the origin of the strains is investigated by comparing representatives of each strain with populations from Europe using factorial correspondence analysis (FCA). As isolated populations were not available for all strains, groups of individuals' representative of the Boolara and Yanco strains were inferred from the assignment tests of the previous study. It was found that the Prospect, Boolara and Yanco strains are descended essentially from the European/central-Asian carp subspecies *C. carpio* subsp. *carpio*. The population genetics of common carp in the east coast of Australia is also investigated. Coastal populations exhibited levels of genetic variation comparable with domestic populations (although lower than indigenous, wild populations), were non-panmictic and contained different proportions of each strain, consistent with each being an independent population founded in separate introduction events. Recommendations are made for preventing the further spread of carp in the rest of Australia.

4.2. Introduction

The common carp (*Cyprinus carpio* L.) are the oldest cultivated species of freshwater fish in the world, having been reared in ponds in China as early as the 5th century BC (Horváth *et al.* 2002), and in Europe at least as early as the Middle Ages (Balon 1995). While the native range of common carp extends from Japan to the River Danube in Eastern Europe (Balon 1995; Mabuchi *et al.* 2005), human cultivation has introduced them into many new waterways throughout Asia, Africa, the Americas, Oceania and Australia (Koehn 2004). Potential problems associated with the introduction of common carp outside their natural

range include uprooting of aquatic vegetation, undermining of river banks, increased water turbidity, competition with indigenous freshwater species, increased incidence of blue-green algal blooms and alteration of the trophic cascade of the waterway (Koehn *et al.* 2000; Angeler *et al.* 2002; Parkos III *et al.* 2003; Tapia and Zambrano 2003; Pinto *et al.* 2005). However, the extent to which these problems can be attributed to carp invasion or to anthropogenic changes to ecosystems is still unclear (Hume *et al.* 1983a; Koehn *et al.* 2000).

Common carp are frequently separated into two subspecies: the central-Asian/European *C. carpio* subsp. *carpio* and the east-Asian subspecies *C. carpio* subsp. *haematopterus*. This separation is supported by microsatellite and mitochondrial genetic data (Kohlmann *et al.* 2003; Zhou *et al.* 2003; Zhou *et al.* 2004b; Kohlmann *et al.* 2005). The division of *C. c. haematopterus* into additional southeast-Asian subspecies is also suggested by some researchers (e.g. Kirpichnikov 1981; Zhou *et al.* 2004b).

Common carp have been introduced into Australia on a number of different occasions since the late 19th century (Clements 1988; Koehn *et al.* 2000), and has spread from introduction sites through a combination of natural range expansion and intentional and accidental release (Koehn *et al.* 2000). They are now established in all states and territories, bar the Northern Territory (Koehn 2004), and are currently the dominant species in the Murray-Darling Basin (MDB) (Harris and Gehrke 1997; Koehn *et al.* 2000), Australia's largest river system.

Previous studies indicated that at least four strains of common carp have been introduced into Australia: Yanco, Boolara, Prospect, and koi (Shearer and Mulley 1978; Davis *et al.*

1999; Haynes *et al.* submitted). Koi carp originate from Japan, where they have a long history of cultivation and selective breeding for ornamental traits (Balon 1995). Koi belong essentially to the east-Asian subsp. *haematopterus*, although crossing with subsp. *carpio* does sometimes occurred in their breeding (Balon, 1995). The Prospect strain was founded from fourteen fingerlings in 1907-08, but the origin of these fingerlings in unknown (Stead 1929). The Boolara strain is alleged to have been imported illegally from Germany, but this was never been proven (Clements 1988). It has been suggested that the orange-coloured Yanco strain was sourced from Singapore (Shearer and Mulley 1978) or from colourful carp of unknown origin held in the Melbourne Botanical Gardens from the late 19th century till 1962 (Clements, 1988), but no rigorous direct comparisons have been made to confirm either of these suggestions. Assignment testing using the program Structure version 2.1 (Pritchard *et al.* 2000) in our previous study (Haynes *et al.* submitted) confirmed the existence of all four strains, and indicated that in the MDB, the Prospect and Boolara strains are distributed almost ubiquitously, the Yanco strain is also widespread, and koi make only a small contribution to the overall genetic diversity. The history of the four strains is summarised in Table 4.1.

In this study, the four strains of common carp in Australia are compared to populations from Europe to investigate whether the Prospect, Yanco, and Boolara strains are of European (subsp. *carpio*) or east-Asian (subsp. *haematopterus*) origin. The population genetics of introduced carp populations from three coastal waterways in New South Wales is also investigated. This is the first time nuclear genetic markers have been used to specifically investigate the origin of invasive common carp populations.

4.3. Materials and Methods

4.3.1. Selection of individuals to represent the strains of common carp in Australia

Samples of Prospect (P) and koi (K) strain carp that had been maintained in isolation from other carp strains were used in our previous study (Haynes *et al.* submitted), with Prospect strain individuals being collected from Prospect Reservoir in Sydney, Australia, and koi carp donated by fish farms in Sydney and Germany. These same samples were used to represent the Prospect and koi strains in this study, with the exception of a small number of koi samples that were excluded from the present study after applying a more stringent criteria for proportion of loci scoreable than Haynes *et al.* (submitted). The Australian strains investigated in this study are summarised in Table 4.1.

Individuals representative of the Yanco (Y) and Boolara (B) strains were identified for this study from the assignment test implemented in the program Structure version 2.1 (Pritchard *et al.* 2000) in our previous study (Haynes *et al.* submitted). Structure implements a Bayesian clustering analysis that assigns individuals into population clusters under the assumption of Hardy-Weinberg (HW) and linkage equilibrium. Structure has an advantage over other assignment programs, in that individuals rather than populations are assigned into population clusters, and that individual can be assigned solely on the basis of their genotype, without reference to where they were sampled. An individual can be assigned completely (100%) to a single cluster, or can be a hybrid of two or more clusters. In the analysis of Haynes *et al*. (submitted), four Structure-assigned clusters were considered highly likely to be synonymous with the four strains of common carp known to have been introduced into Australia. For the present study, individuals that had been assigned 95-100% to either of the clusters presumed to be synonymous with Boolara or Yanco strains were assumed to be a ‘pure’ representative of the relevant strain. In this way,

118 Boolara and 85 Yanco strain individuals were identified for the present study, from the 983 MDB samples analysed by Haynes *et al*. (submitted).

4.3.2. European common carp populations

Tissue samples from wild common carp from the River Danube (D) (Germany), domestic mirror-scale common carp (J) (fish farm in Jaenschwalde, Germany), and Ropsha strain common carp (R) (maintained in a live gene bank in the Czech Republic) were generously donated by Dr. Kohlmann; and samples of wild and domestic common carp from England (UK) and from River Danube (D) (Germany) donated by Dr. Hänfling. The domestic mirror-scale carp likely represent 'pure' European subsp. *carpio*, with little or no genetic contribution from non-European populations (Kohlmann, pers. comm.). The River Danube and Ropsha strain comprise a mixture of European subsp. *carpio* and east-Asian subsp. *haematopterus*, as Asian varieties have escaped and been released into the River Danube in recent times (Kohlmann, pers. comm.) and the Ropsha strain was developed by crossing domestic European subsp. *carpio* with wild subsp. *haematopterus* from the River Amur in east-Asia (Zonova and Kirpichnikov 1968). The English common carp were sourced from local waterways in Hampshire and Hertfordshire, and from Riverfield Carp Farm in Kent (Hänfling, pers comm.). These European populations are summarised in Table 4.2.

Table 4.1. Founding strains of common carp used in this study

Strain	History of introduction and spread	Location samples	N	References
Prospect (P)	Founded from 14 fingerlings in 1907-08 in Prospect Reservoir in the Sydney catchment, and used to seed multiple populations around Sydney. Suggested as having European origin. Currently form a self-sustaining population in Prospect Reservoir, Sydney. Found extensively throughout MDB.	Prospect Reservoir*	23	Stead (1929), Shearer and Mulley (1978), Davis *et al*. (1999), Haynes *et al*. (submitted).
Boolara (B)	Distributed to farm dams around Victoria in the early 1960s by Boolara Fish Farms Ltd. Escaped into the MDB in 1968 and are now widespread. Claimed by Boolara Fish Farms Ltd. to have been sourced from Prospect Reservoir. Alleged to have been illegally imported from Germany.	MDB*	29	Shearer and Mulley (1978), Clements (1988), Brown (1996), Haynes *et al*. (submitted)
Yanco (Y)	Established in MDB between 1920 and 1950. Originally orange coloured, but introgression with Prospect and Boolara strains has masked this colouration. Origin is unknown, although they have been suggested as being a feral form of Singapore koi, or as being sourced from the colourful carp that formerly present in the Melbourne Botanical gardens. Currently found throughout the MDB.	MDB*	38	Shearer and Mulley (1978), Clements (1988), Brown (1996), Haynes *et al*. (submitted)
Koi (K)	Originally selectively bred for novel colourations in Japan. Now a popular aquarium and pond variety of common carp in many places. Have made a minor contribution to genetic diversity of common carp in the MDB.	Fish farms in Sydney and Germany*	50	Balon (1995), Haynes *et al*. (submitted)

*Same samples used by Haynes *et al*. (submitted), †Same samples used by Kohlmann *et al.* (2005), ††Same samples used by Hänfling *et al.* (2005)

Table 4.2. European populations of common carp used in this study

Population	History	Location sampled	N	References
Ropsha (R)	Developed in the former USSR by crossing European domestic .*C carpio* with wild *C. carpio* from the Amur River	Live gene bank in the Czech Republic	30	Zonova and Kirpichnikov (1968)
River Danube (D)	Western most extent of *C. carpio* L. natural range.	Germany†	30	Balon (1995)
German mirror-scale carp (J)	Aquaculture strain, selectively bred for palatability. Mirror-scale phenotype makes scaling easier.	Jaenschwalde, Germany*	30	NA
English carp (UK)	Both wild and farmed varieties from the UK.	England, UK††	23	NA

*Same samples used by Haynes *et al*. (submitted), †Same samples used by Kohlmann *et al.* (2005), ††Same samples used by Hänfling *et al.* (2005)

4.3.3. Coastal samples

Three waterways on the east coast of Australia were selected for this study: the Hunter River at a site close to Clarence Town (CT), the Hawkesbury-Nepean (HN) River and the Parramatta (PM) River, the latter two of which run through urban areas of Sydney. Specimens were collected by electrofishing between November 2004 and June 2006. A fin-clip was taken from each individual and immediately placed in 70% ethanol. Effort was made to catch at least 30 individuals per site. These coastal samples are summarised in Table 4.3.

Table 4.3. Coastal populations of common carp used in this study

River collected	Sample site coordinates	N
Hawkesbury-Nepean River (HN)	-33.60297 (S) 150.80724 (E)	27
Parramatta River (PM)	-33.80727 (S) 151.00468 (E)	20
Hunter River (CT)	-32.58416 (S) 151.783503 (E)	27

4.3.4. DNA extraction and genotyping

DNA extraction and genotyping for 14 microsatellite loci in the European and coastal samples was performed according to Haynes *et al*. (submitted).

4.3.5. Data analysis

Origin of Australian common carp strains

To determine whether the Prospect, Boolara, and Yanco strains are descended primarily from subspecies *carpio* or *haematopterus*, traditional phylogenetic trees were not considered appropriate, as the history of human-induced interbreeding amongst populations in recent times cannot be suitably represented with a branching-tree diagram. Factorial correspondence analysis (FCA) (Guinand *et al.* 2003) was instead implemented to

elucidate the extent of allele sharing amongst the Australian, European and Asian carp strains, using the software Genetix version 4.05.2 (Belkhir *et al.* 2000). A two-dimensional plot was generated to represent the extent of allele sharing between individuals (i.e. each individual represented by a single data point). A second plot representing the average allele-sharing between populations or strains (i.e. each population or strain represented by a single data point) was also generated.

4.3.6. Population genetics of common carp in coastal rivers

Departure of genotype frequencies from expectations under HW equilibrium was tested in Genepop version 1.2 (Raymond and Rousset 1995). F_{ST} (Weir and Cockerham 1984) values between each pair of populations was calculated in Genepop, and analysis of molecular variance (AMOVA) (Excoffier *et al.* 1992) performed in GenAlEx 6.0 (Peakall and Smouse 2006), with an empirical null distribution derived from 9999 permutations used to test significance. Departure of genotype frequencies between each pair of coastal rivers from expectations under panmixia was tested using Fisher's exact test in Genepop.

The contribution of different common carp strains to different coastal regions was estimated in Structure 2.1 (Pritchard *et al.* 2000), with the the individuals representative of Yanco, Prospect, Boolara and koi strains used as learning samples (i.e. the USEPOPINFO parameter was set to 1 for these samples and 0 for the remaining samples). Run conditions were 500 000 burn-in steps and 1 000 000 Markov-Chain Monte-Carlo steps, under the Prior Population Information and Allele Frequencies Correlated models. The analysis was run for K = 1-10 population clusters, with 3 iterations to check for consistency between runs. The ΔK statistic (Evanno *et al.* 2005) was used to estimate the actual number of population groups present (i.e. the true value of K).

To assess the level of genetic diversity in each river, allele richness (A_r) The mean number of alleles per locus (A) was estimated in the program HP-Rare (Kalinowski 2005). For comparison, representatives of the Australian strains, the European populations, and common carp from the MDB used in Haynes *et al*. (submitted) were analysed in addition to the coastal samples. The koi from the fish farms in Sydney and Germany were analysed seperately. For A_r estimates, the rarefaction strategy was implemented to compensate for different sample sizes between the groups analysed. As the smallest population (Parramatta River) had 20 individuals, the number of 'genes per locus' was set to 40 (2 genes per diploid locus × number of individuals) for this calculation.

4.4. Results

4.4.1. Origin of carp in Australia

In the FCA, the first two axes accounted for 32% and 27% (total 59%) of variation in the data (Figure 1). The third axis, accounting for 14% of the variation, was not included in the figure as it did not significantly affect the visualisation of the results. The koi (K) and Ropsha (R) strains formed distinct clusters, while the Australian strains and other European populations grouped together.

4.4.2. Population genetics of common carp in coastal rivers

The samples from the Parramatta and Hunter rivers showed significant departure from expectations under HW equilibrium (p=0.0047 and p<0.0001 respectively), while genotype frequencies detected in the Hawkesbury-Nepean River were consistent with HW frequencies (p=0.7802). F_{ST} values between rivers were 0.0918 (CT and HN), 0.1964 (HN and PM) and 0.244 (CT and PM). The AMOVA analysis was highly significant (p=0), partitioning 76% of the genetic variation within rivers and 24% among rivers. As expected,

significant departure (p<0.0001) from panmixia was detected between the three coastal samples.

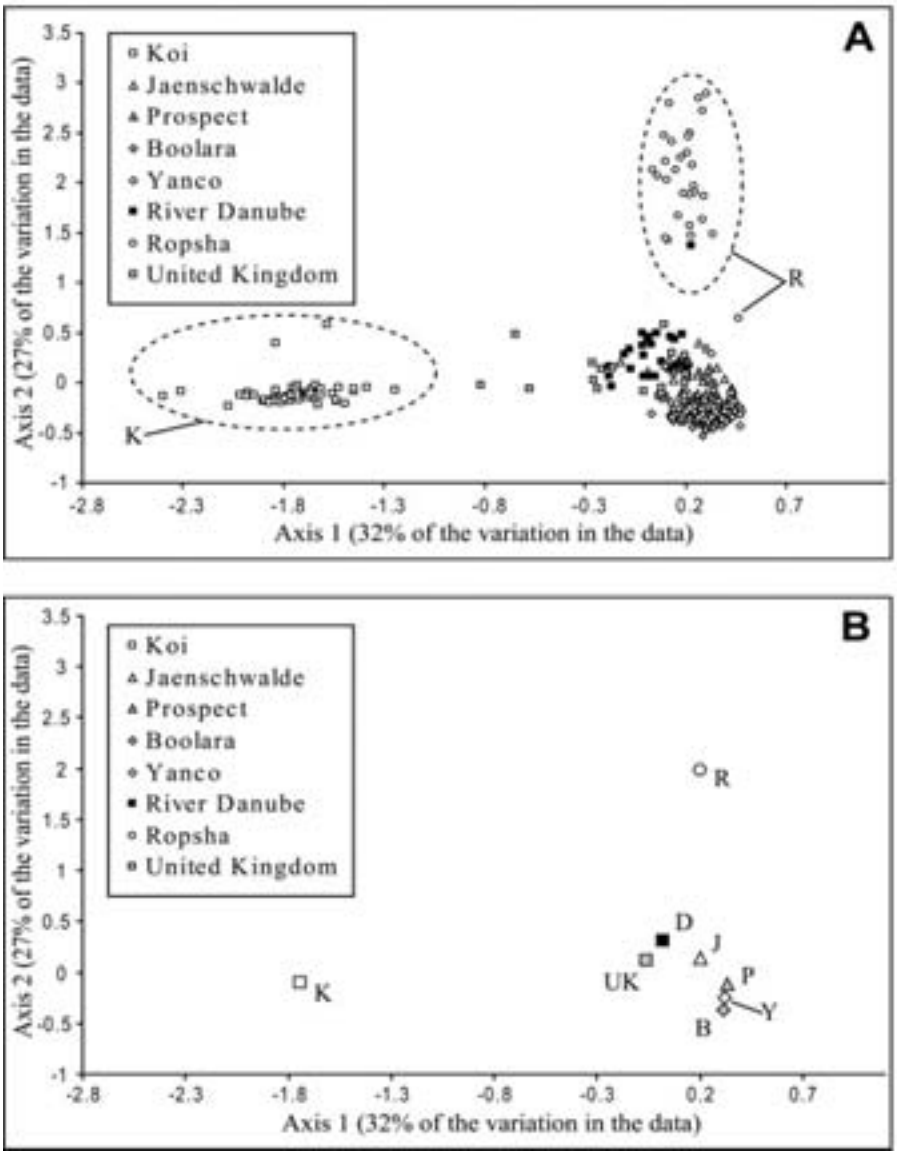

Figure 4.1. FCA illustrating the relationship between common carp strains/population: A. individuals; B. strains/populations. K = koi strain, P = Prospect strain, B = Boolara strain, Y = Yanco strain, J = Jaenschwalde (German mirror-scale) carp, D = River Danube population, R = Ropsha strain, UK = English carp population.

The ΔK statistic (Evanno *et al.* 2005) indicated the presence of four population clusters the Structure analysis. Visual inspection of the $K = 4$ results confirmed that the different runs produced very similar results. All subsequent analyses are based on the output of the first run. Consistent with expectations, the four population clusters each corresponded closely with one of the representative samples of Prospect, Boolara, Yanco and koi strain carp, and partitioned the carp from the coastal populations into these stains (Figure 4.2). Most of the genetic diversity of the Parramatta River came from the koi strain carp (69%), followed by Prospect strain (20%), with smaller contributions from the Yanco (3%) and Boolara (8%). The Hawkesbury-Nepean River samples consisted predominantly of Prospect strain carp (78%), followed the Boolara strain (11%), with very minor contributions from the Yanco and koi strains (6% and 5% respectively). The Hunter River samples were the most diverse, comprising 57% Booara strain carp, 16% koi, 13% Prospect, 14% Yanco. These results should be interpreted with caution, however, as the detection of all 4 strains at each site could be an artefact of some strains being genetically similar, and/or from having to use individuals inferred to belong to different genetic groups to represent the different strains, rather than individuals from populations with a known history of isolation from other strains.

The results of the A and A_r analysis are summarised in Figure 3. A and A_r were very similar for most samples, with the exeption of the MBD overall, where A (7.6) was much greater than A_r (3.4). The River Danube common carp were the most diverse (A=7.1 A_r=7) and the koi from the German fish farm the least (A and Ar=2.5). Amongst the coastal samples, A and A_r ranged from 3.4 (CT) to 4.4 (PM). Genetic diversity in the coastal rivers was similar to or slightly higher than the founding strains and the Jaenschwalde and Ropsha carp, and less than carp from the River Danube and the from UK.

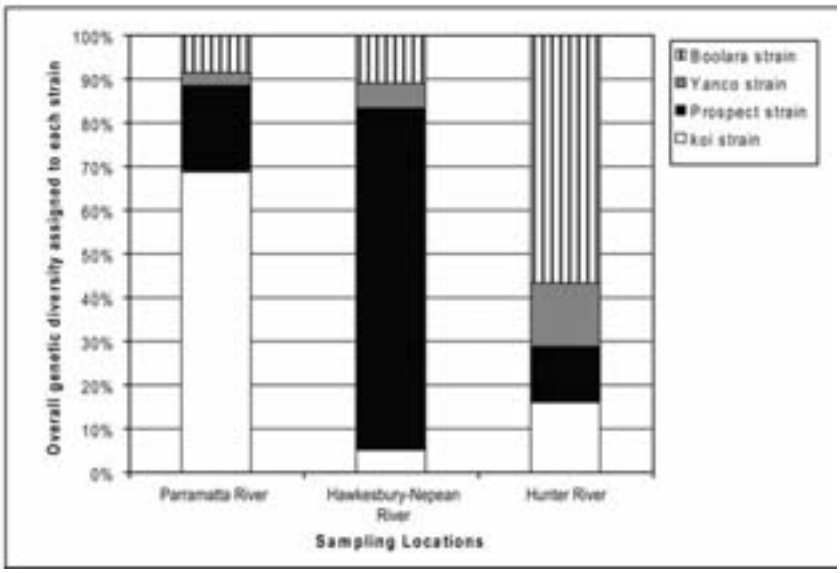

Figure 4.2. Contribution of the Prospect, Boolara, Yanco, and koi strains of common carp to the coastal waterways, as assigned by the K = 4 analysis in Structure version 2.1.

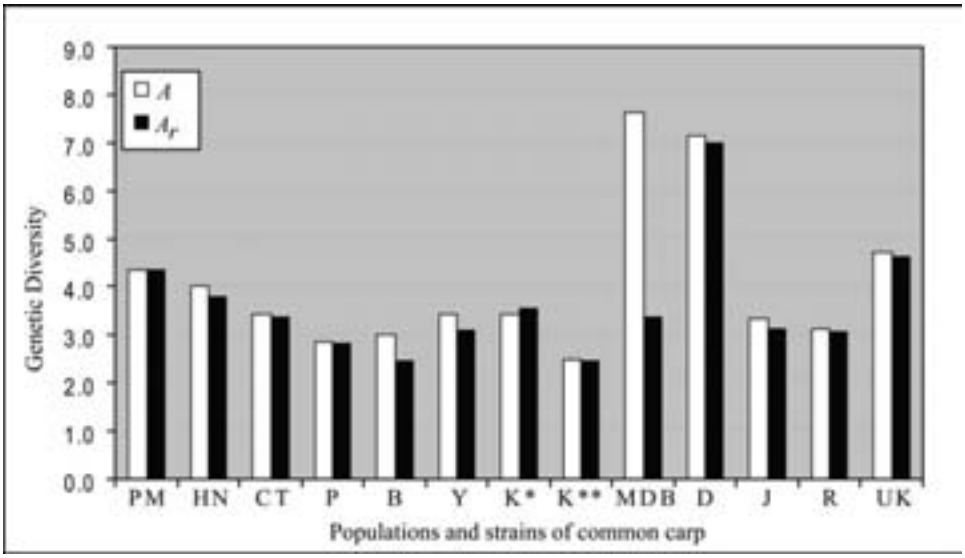

Figure 4.3. Allele richness (A_r) and mean number of alleles (A) of common carp. Population identifiers are given in Tables 4.1. and 4.2. * koi sourced from fish farm in Sydney, ** koi sourced from fish farm in Germany.

4.5. Discussion

4.5.1. Origin of and relationship between founding common carp strains

The FCA results of this study confirm Shearer and Mulley's (1978) suggestion that the Prospect and Boolara strains were derived essentially from European subsp. *carpio,* as both these strains clustered with the European carp rather than the Asian koi in this analysis. All or most of the fourteen fingerlings from which the Prospect strain was founded (Stead 1929) were likely subsp. *carpio.* The claim by Boolara Fish Farms Ltd. that the common carp they distributed were sourced solely from Prospect Reservoir (Table 4.1) (Clements 1988) is, however, refuted by this study as the Prospect strain is more similar to the Yanco strain than it is to Boolara strain.

The suggestion that the Yanco strain is a feral form of Singapore common carp (Shearer and Mulley 1978) is refuted by FCA results of this study Common carp from Singapore would be expected to belong to subsp. *haematopterus,* rather than subsp. *carpio*. In the FCA, however, the Yanco carp cluster strongly with the European carp, rather than the Asian carp. Shearer and Mulley (1978) originally described the Yanco strain as being orange coloured, a trait commonly associated with the koi strain. This colouration, however, also occurs in European common carp (Kirpichnikov 1981; Bialowas 2004), and is determined by recessive alleles at two loci (Bialowas 2004). These alleles were likely at high frequency when Shearer and Mulley (1978) collected their Yanco samples, possibly as a result of a bottleneck when the population was founded or because the initial introduction was of orange fish. Interbreeding with Boolara and Prospect strains (Haynes *et al.* submitted) would have since decreased the frequency of these alleles in areas where they were formerly common. While the true history of the introduction of the Yanco strain may never be known, it was likely introduced to establish a coarse-angling fishery

sometime after 1912, when construction began on the irrigation system from which the strain gets its name.

The European origin of the Boolara, Prospect and Yanco strains is consistent with previous studies of mitochondrial DNA of carp from Australia, where all Australian sequences analysed were shown to be of European descent (Thai *et al.* 2004b; Haynes *et al.* 2009).

4.5.2. Population genetics of common carp in coastal rivers

The heterogeneous distribution of common carp strains and lack of panmixia (Fisher's exact test and AMOVA) between the three coastal rivers is consistent each river being having an independent population of carp founded in different introduction events and with little gene flow between rivers. Consistent with historical accounts of common carp being distributed around the Sydney Basin from Prospect Reservoir in the early twentieth century (Clements 1988), Prospect strain carp contributed significantly to both the Hawkesbury-Nepean and the Parramatta Rivers. Large numbers of koi, however, have been more recently introduced into the Parramatta River, with koi now the dominant strain in this waterway. Carp were introduced into the Hunter River some time between 1980 and 1985, as extensive river surveys document that carp where absent from the Hunter catchment in 1980, but were present in 1985 (Battaglene 1985). The colonisation of the Hunter River was likely independent of the Parramatta and Hawkesbury-Nepean Rivers, with the Hunter River be seeded largely with Boolara strain carp. The lack of HW equilibrium in the Parramatta and Hunter Rivers is consistent with these rivers containing multiple strains of carp that (i.e. non-random mating), and insufficient time having passed for the equilibrium conditions to have become established (i.e. random mating between strains and death of parental individuals). The detection of koi genotypes in all three waterways indicates that further introduction is still occurring via the irresponsible disposal of unwanted koi. The

longevity of carp (carpover 50 years of age have been caught in the wild, P. Sorenson, Pers. comm.) and ongoing introduction could mean that these populations never reach equilibrium.

There is no evidence for a loss of genetic diversity associated with a population bottleneck in any of the coastal river populations, as these populations have similar levels of genetic diversity to other strains of common carp. The independent introduction of multiple strains to each coastal river possibly masked any such loss of genetic diversity. These findings should be interpreted with caution, however, as small sample sizes and the representation of two of the founding strains using individuals of inferred membership could lead to an underestimation of genetic diversity, as is apparent by the discrepancy between A and A_r results from the MDB.

4.5.3. Implications for management and control

Of major concern is the potential for future expansion of common carp into currently unoccupied waterways in Australia. While they currently occupy waterways draining only one million of Australia's seven million square kilometres of land area, climate matching indicates that they are suited to all bodies of permanent freshwater in Australia (Koehn 2004). Of particular concern are the currently uncolonised coastal rivers, especially on the east and south-east coast of the mainland; Tasmanian waterways; the drainages along the north coast; and the Lake Eyre and Bulloo-Bancannia drainages in the interior of the continent (Koehn 2004). Secondary contact between separate populations is also a concern, as mating between divergent lineages could result in increased genetic diversity and heterosis that could make populations more invasive. This process has been implicated in allowing a number of introduced species to become invasive (e.g. Kolbe *et al.* 2004; Facon *et al.* 2005), including common carp in the MDB (Haynes *et al.* submitted). The presence

of multiple strains and high levels of genetic diversity in all coastal rivers investigated, and the ongoing release of koi, will likely facilitate further invasiveness and spread of common carp in Australia.

To protect ecosystems from further degradation by introduced common carp, we make the following recommendations for control of this species : (1) monitoring of common carp-free waterways, with rapid-response removal efforts undertaken if common carp are detected; (2) keeping the public well informed about the environmental risks associated with releasing common carp; (3) consideration of making possession of all strains of common carp, including koi, illegal in all parts of Australia; (4) consistent policing and punishment of individuals releasing fish; and (5) extirpation of small, isolated populations if possible, as is currently being attempted in Tasmania (Gilligan and Rayner 2007).

4.5.4. Future work

Much can be done to further investigate the population genetics of common carp in Australia. The fourteen microsatellite loci used here and in Haynes *et al.* (submitted) can form the basis of the future studies. More loci could be included to improve the accuracy and power of statistical inferences. The accuracy of the assignment of individuals to one of the founding strains could also be improved if a greater number of individuals representing those strains were genotyped. This would require sampling of common carp from non-MDB rivers in Victoria for the Boolara strain; sampling of Potts Point Reservoir (Davis 1996) and further sampling of Prospect Reservoir for the Prospect strain; and purchasing more fish from breeders for the koi strain. We know of no isolated populations of Yanco strain, although some material may be available from museums. Obtaining samples from more pure Asian and European populations may also make it possible to ascertain a more

precise origin of the different strains. Sequencing of mitochondrial loci in Australian carp could also facilitate this.

In Australia, a project involving the more extensive sampling of coastal rivers could identify source populations and putative pathways for the spread of common carp in these regions. Equally, scoring of microsatellite and mitochondrial loci from carp around the world could identify additional historical and ancient patterns of carp dispersal that have not yet been investigated.

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4.7. References

Angeler DG, Álvarez-Cobelas M, Sánchez-Carrillo S, Rodrigo MA (2002). Assessment of exotic fish impacts on water quality and zooplankton in a degraded semi-arid floodplain wetland. *Aquat. Sci.* **64:** 76-86.

Balon EK (1995). Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman Gourmets to the swimming flower. *Aquaculture* **129:** 3-48.

Battaglene SC (1985). *Preliminary study of the fish resources of the Hunter Valley*. NSW Department of Agriculture - Division of Fisheries: Taylors Beach, Australia.

Belkhir K, Borsa P, Goudet J, Chikli L, Bonhomme F (2000). *GENETIX, logiciel sous WindowsTM pour la ge´ne´tique des populations, version 4.01*. Release University of Montpellier II, Montpellier.

Bialowas H (2004). Inheritance of orange pigmentation and scale patterns in common carp (*Cyprinus carpio* L.). *Archives of Polish Fisheries* **12:** 145-150.

Brown P (1996). *Fish Facts 4 Carp in Australia*. NSW Fisheries (now part of the NSW Department of Primary Industries): Narrandera, Australia.

Clements J (1988). Salmon at the Antipodes A History and Review of Trout, Salmon and Char and Introduced Coarse Fish in Australasia Published by the author, Ballarat, Victoria, Australia.

Davis KM (1996). Investigation into the genetic variation of carp (*Cyprinus carpio*) in Southeastern Australia. Ph.D., University of NSW.

Davis KM, Dixon PI, Harris JH (1999). Allozyme and mitochondrial DNA analysis of carp, *Cyprinus carpio* L., from south-eastern Australia. *Mar. Freshw. Res.* **50:** 253-60.

Evanno G, Regnaut S, Goudet J (2005). Detecting the number of clusters of individuals using the sortware STRUCTURE: a simulation study. *Mol. Ecol.* **14:** 2611-2620.

Excoffier L, Smouse PE, Quattro JM (1992). Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131:** 479-491.

Facon B, Jarne P, Pointier JP, David P (2005). Hybridization and invasiveness in the freshwater snail Melanoides tuberculata: hybrid vigour is more important than increase in genetic variance. *J. Evolution Biol.* **18:** 524-535.

Gilligan D, Rayner T (2007). *The distribution, spread, ecological impacts, and potential control of carp in the upper Murray River*. NSW Department of Primary Industries: Sydney.

Guinand B, Parisseaux B, Lebreton J, Bonhomme F (2003). *Estimating population genetics 'F-statistics' using factorial correspondence analysis with respect to instrumental variables*. International Conference on Correspondence Analysis and Related Methods, Barcelona.

Hänfling B, Bolton P, Harley M, Carvalho GR (2005). A molecular approach to detect hybridisation between crucian carp (*Carassis carassius*) and non-indigenous carp species (*Carassisus* cpp. and *Cyprinus carpio*). *Freshwater Biol.* **50:** 403-417.

Harris JH, Gehrke PC (1997). *Fish and rivers in stress - the NSW rivers survey*. NSW Fisheries Office of Conservation & the Cooperative Research Centre for Freshwater Ecology: Cronulla and Canberra.

Haynes GD, Gilligan DM, Grewe P, Nicholas FW (submitted). Population genetics and management units of invasive common carp (*Cyprinus carpio* L.), in the Murray-Darling Basin, Australia. *J. Fish. Biol.* **000:** 000.

Haynes GD, Gongora J, Nicholas FW, Zenger KR (2009). Rapid identification of maternal lineages in common carp (*Cyprinus carpio* L.) using real-time PCR and high resolution melt-curve analysis. *Aquaculture* **287:** 59-66.

Horváth L, Tamás G, Seagrave C (2002). Carp and pond fish culture second edition Blackwell Science, Oxford, United Kingdom.

Hume DH, Fletcher AR, Morsion AK (1983). *Final Report. Carp Program Report No. 10*. Fisheries and Wildlife Division, Victorian Ministry for Conservation:

Kalinowski ST (2005). HP-RARE 1.0: a computer program for performing rarefaction on measures of allelic richness. *Mol. Ecol. Notes* **5:** 187–189.

Kirpichnikov VS (1981). Genetic Bases of Fish Selection Springer-Verlag, Berlin and Heidelberg, Germany, New York, USA.

Koehn J (2004). Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. *Freshw. Biol.* **49:** 882-894.

Koehn J, Brumley B, Gehrke P (2000). Managing the Impacts of Carp Bureau of Rural Sciences (Department of Agriculture, Fisheries and Forestry), Canberra, Australia.

Kohlmann K, Gross R, Murakaeva A, Kersten P (2003). Genetic variability and structure of common carp (*Cyprinus carpio*) populations throughout the distribution range inferred from allozyme, microsatellite and mitochondrial DNA markers. *Aquat. Living Resour.* **16:** 421-431.

Kohlmann K, Kersten P, Flajshans M (2005). Microsatellite-based genetic variability and differentiation of domesticated, wild and feral common carp (*Cyprinus carpio* L.) populations. *Aquaculture* **247:** 253-256.

Kolbe JJ, Glor RE, Schettino LR, Lara AC, Larson A, Losos JB (2004). Genetic variation increases during biological invasion by a Cuban lizard. *Nature* **431:** 177-181.

Mabuchi K, Senou H, Suzuki T, Nishida M (2005). Discovery of an ancient lineage of *Cyprinus carpio* from Lake Biwa, central Japan, based on mtDNA sequence data, with reference to possible multiple origins of koi. *J. Fish. Biol.* **66:** 1516-1528.

Parkos III JJ, Santucci VJ, Wahl DJ (2003). Effect of adult common carp (*Cyprinus carpio*) on multiple trophic levels in shallow mesocosms. *Can. J. Fish. Aquat. Sci.* **60:** 182-192.

Peakall R, Smouse PE (2006). GENALEX 6: genetic analysis in EXCEL. Population genetic software for teaching and research. *Mol. Ecol. Notes* **6:** 288-295.

Pinto L, Chandrasena N, Pera J, Hawkins P, Eccles D, Sim R (2005). Managing invasive carp (*Cyprinus carpio* L.) for habitat enhancement in at Botany Bay, Australia. *Aquat. Conserv.* **15:** 447-462.

Pritchard JK, Stefens M, Donelly P (2000). Inference of population structure using multilocus genotype data. *Genetics* **155:** 945-959.

Raymond M, Rousset F (1995). GENEPOP (version 1.2): population genetics software for exact tests and eumenicism. *J. Hered.* **86:** 248-249.

Shearer KD, Mulley JC (1978). The introduction and distribution of the carp, *Cyprinus carpio* Linnaeus, in Australia. *Aust. J. Mar. Fresh. Res.* **29:** 661-563.

Stead DG (1929). Introduction of the great carp *Cyprinus carpio* into waters of New South Wales. *Aust. J. Zool.* **6:** 100-102.

Tapia M, Zambrano L (2003). From Aquaculture Goals to Real Social and Ecological Impacts: Carp Introduction in Rural Central Mexico. *Ambio* **32:** 252-557.

Thai BT, Burridge CP, Pham TA, Austin CM (2004). Using mitochondrial nucleotide sequences to investigate diversity and genealogical relationships within common carp (*Cyprinus carpio* L.). *Anim. Genet.* **36:** 22-28.

Weir BS, Cockerham CC (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38:** 1358-1370.

Zhou JF, Wu Q, Wang Z, Ye Y (2004). Molecular phylogeny of three subspecies of common carp *Cyprinus carpio*, based on sequence analysis of cytochrome *b* and control region mtDNA. *J. Zoo. Syst. Evol.* **42:** 266-269.

Zhou JF, Wu QJ, Ye YZ, Tong JG (2003). Genetic divergence between *Cyprinus carpio carpio* and *Cyprinus carpio haematopterus* as assessed by mitochondrial DNA analysis, with emphasis on the origin of European domestic carp. *Genetica* **119:** 93-97.

Zonova AS, Kirpichnikov VS (1968). *Selection of Ropsha carp*. Seminar/study tour in the U.S.S.R. on genetic selection and hybridization of cultivated fishes, FAO Corporate Document Repository http://www.fao.org/documents.

Chapter 5: Invasive Cyprinid species *Cyprinus carpio* and *Carassius auratus* in Australia: cryptic hybridisation and introgression

5.1. Introduction

Hybridisation between divergent lineages is increasingly recognised as playing an important role in introduced species becoming invasive. Two non-exclusive hypotheses used to explain this are (1) heterosis (hybrid vigour) occurring in the cross-bred progeny and/or (2) novel allele combinations extending the phenotypic range beyond that of the parent populations, and consequently increasing the potential for survival and reproduction in the new environment (Hänfling 2007).

Within-species hybridisation between divergent lineages is commonly reported in invasive populations. Examples include the green anole lizard in Florida and Hawaii (Kolbe *et al.* 2004) and the European rabbit in Australia being sourced from multiple parent populations (Zenger *et al.* 2003); and invasive European green crabs in North American and the amphipod *Gammarus tigrinis* in Europe undergoing range expansion following admixture between divergent lineages (Kelly *et al.* 2006; Roman 2006).

Hybridisation between different species can also lead to the formation of invasive lineages. Nolte *et al.* (2005), for example, reported an invasive lineage of sculpin fish, *Cottus* sp., in the River Rhine that displayed novel habitat adaptations and life-history characteristics and was likely generated by the interbreeding of two previously allopatric species. Changes in ploidy resulting from between-species hybridisation have been responsible for the generation of new, reproductively isolated species. The Gibel carp (*Carassius auratus gibelio* Bloch), for example, is a polyploidy hybrid species, and has been progressively expanding its range in Europe since the early 20th century (Hänfling 2007). Even when hybrid fitness is low, inter-specific hybridisation can still facilitate the invasiveness of introduced species by allowing them to obtain advantageous alleles from related species (Hänfling 2007).

Two well known species from the Family Cyprinidae, common carp (*Cyprinus carpio*) and goldfish (*Carassius auratus*), have a long history of sympatry. Both are native to east-Asia (Balon 2004) and have been introduced to waterways outside their native range by human

activities (Brumley 1991; Koehn *et al.* 2000; Koehn 2004). Both species have a similar number of chromosomes. An exhaustive study of 249 metaphase cells by Al-Sabati (1986) concluded that 2n=98 for carp, while Ruiguang and Zheng (1980) found that 2n=100 for both species, and Ohno *et al*. (1967) reported 2n=104 or less of both species. The two species are known to hybridise in the wild (Taylor and Mahon 1977; Shearer and Mulley 1978; Hume *et al.* 1983b; Pullan and Smith 1987; Brumley 1991; Hänfling *et al.* 2005). Hybrids can be readily identified in the field using a range of morphological and morphometric characters (Taylor and Mahon 1977; Hume *et al.* 1983b), the easiest to score being the fleshy barbels (whiskers) around the mouth. Carp have two such pairs of barbels, goldfish have no barbels, and hybrids of the two species have aberrant barbels that are often truncated and reduced to a single pair. The number of lateral-line scales has been claimed to be diagnostic, with one study reporting carp as having 34-37 such scales, goldfish 26-29, and their hybrids having an intermediate 29-35 scales (Hume *et al.* 1983b), but the overlap in distributions limits the utility of this trait.

The fertility of carp-goldfish hybrids is questionable. Putative F1 individuals are observed to be healthy and to produce eggs and milt in the wild (Hume *et al.* 1983b). Hybrids have been reported to be either sterile (Hubbs, 1955) or to be able to back-cross frequently (Trautman 1957, cited by Hume *et al.* 1983; Aduma-bossman 1971, cited by Hänfling *et al.* 2005). In an analysis of 34 hybrids (identified on the basis of five meristic traits) from 14 different sites, Hume *et al.* (1983b) identified two subsets of hybrids: those with only one pair of barbels, and those with two pairs of reduced barbels. The observation that these groups also differed significantly for three morphological characters (ratio of length of lower barbel to standard length, number of lateral-line scales and arrangement of pharyngeal teeth) was interpreted as indicating that the two hybrid groups represented different generations of intercrossing or backcrossing. More recently, Hänfling *et al.* (2005) detected backcrossed carp-goldfish hybrids in English waterways using microsatellite markers. It can therefore be concluded that although fertility may be reduced in the progeny of carp and goldfish crosses, reproductive isolation is not complete and intercrossing does sometimes occur.

In this study, introgression between naturalised carp and goldfish populations in Australia is investigated. Carp were collected extensively from the Murray-Darling Basin (MDB), Australia's largest river system. Goldfish and putative hybrids were collected

opportunistically along with the carp. Additionally, three domestic populations of common carp were collected from breeders, and domestic goldfish from pet stores. The aims of this study were to (1) identify microsatellite loci that can be PCR-amplified reliably in both species; (2) investigate whether individuals classed as hybrids on the basis of having aberrant barbels are indeed true hybrids; (3) identify and quantify cryptic introgression; and (4) investigate the direction of hybridisation. This study offers a guide to researchers looking to investigate the phenomenon of introgression between sympatric populations where there is a lack of good samples representative of parental populations.

5.2. Materials and Methods

5.2.1. Sample collection

Naturalised carp were collected by electrofishing from the MDB and from Prospect Reservoir in the Sydney catchment between March 2004 and October 2006. Effort was made to collect at least 30 carp from every major river catchment in the MDB. For MDB rivers with major dams, fish were collected both upstream and downstream of the dam. Goldfish (readily identified from morphology) and putative carp-goldfish hybrids (identified by having aberrant barbels) were collected opportunistically with carp. A fin-clip was taken from each individual and immediately placed in 70% ethanol. In addition, a fin clip was taken from a single live tench (*Tinca tinca*) collected from Lake Eildon, Goulburn River catchment, Victoria, Australia. Samples from domestic populations of carp were kindly provided by Dr. Klaus Kohlmann from Germany (ornamental koi and mirror-scale carp) and from a live "gene bank" in the Czech Republic (Russian Ropsha strain carp); and by a local fish breeder (ornamental koi carp). A small number of domestic goldfish were donated by a pet store in Sydney. The samples used in this study are summarised in Table 5.1, and the sample sites from which fish were collected in Australia are illustrated in Figure 5.1.

5.2.2. DNA extraction

DNA was extracted using the TNES protocol of Wasko *et al.* (2003).

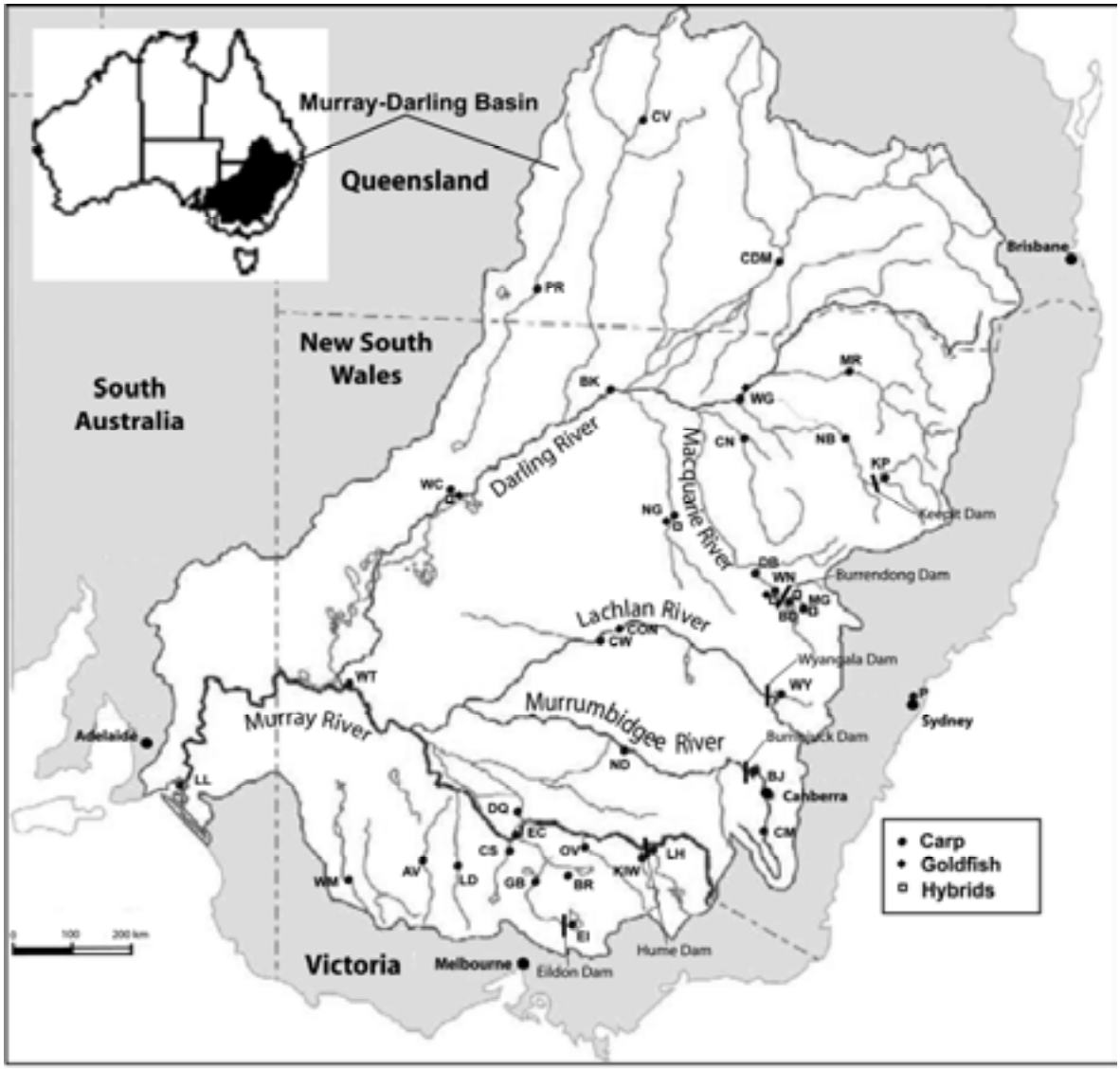

Figure 5.1. Collection sites for carp, goldfish and putative hybrids in the Murray-Darling Basin. Site identifiers are given in Table 5.1.

Table 5.1. Carp, goldfish and suspected hybrids investigated in this study

Sample name	Collected from	Identifier	Location		N		
			Latitude (S)	Longitude (E)	*Carp*	*Goldfish*	*Hybrids*
Domestic fish populations							
Mirror-scale carp	Fish farm, Jaenschwalde, Germany	J	NA	NA	30	0	0
Japanese ornamental koi carp	Fish breeders in Germany and Australia	K	NA	NA	66	0	0
Ropsha strain carp	Live genebank, Czech Republic	R	NA	NA	30	0	0
Domestic goldfish	Pet store, Sydney	PS	NA	NA	0	14	0
Naturalised fish populations							
Prospect	Prospect Reservoir, Sydney	P	-38.8	150.9	25	0	0
Walgett	Barwon River	WG	-30.0	148.1	30	9	0
Nyngan	Bogan River	NG	-31.6	147.2	30	42	1
Coonamble	Castlereagh River	CN	-31.0	148.4	30	0	0
Mudgee	Cudgegong River	MG	-32.3	149.4	26	3	14
Bourke	Darling River	BK	-30.1	145.9	9	0	0
Wilcannia	Darling River	WC	-31.6	143.4	28	5	3
Deniliquin	Edwards River	DQ	-35.5	145.0	29	0	0
Moree	Gwydir River	MR	-29.5	149.8	29	0	0
Lake Cargelligo	Lachlan River	CW	-33.3	146.4	29	0	0
Condobolin	Lachlan River	CON	33.1	147.2	0	3	0
Wyangala Dam	Lachlan River	WY	-34.0	148.9	29	0	0
Burrendong Dam	Macquarie River	BD	-32.7	149.1	28	0	4
Dubbo	Macquarie River	DB	-32.2	148.6	26	0	0
Wellington	Macquarie River	WN	-32.5	148.9	28	2	1

Table 5.1. Carp, goldfish and suspected hybrids investigated in this study (continued)

Sample name	Collected from	Identifier	Location		N		
			Latitude (S)	Longitude (E)	*Carp*	*Goldfish*	*Hybrids*
Echuca	Murray River	EC	-36.1	144.8	28	0	0
Lake Hume	Murray River	LH	-36.0	147.1	25	0	0
Wentworth	Murray River	WT	-34.1	141.9	29	0	0
Burrinjuck Dam	Murrumbidgee	BJ	-35.0	148.6	26	0	0
Cooma	Murrumbidgee	CM	-36.2	149.1	30	0	0
Narrandera	Murrumbidgee	ND	-34.7	146.6	24	0	0
Lake Keepit	Namoi River	KP	-30.9	150.5	30	0	0
Narrabri	Namoi River	NB	-30.4	149.8	31	0	0
Condamine	Condamine	CDM	-27.9	148.6	24	0	0
Paroo River	Paroo River	PR	-28.1	145.4	21	0	0
Charleville	Warrego River	CV	-26.4	146.9	28	0	0
Lower Lakes	Murray River	LL	-35.5	139.0	29	0	0
Avoca	Avoca River	AV	-37.1	143.5	25	0	0
Broken River	Broken River	BR	-36.6	146.0	30	0	0
Campaspe	Campaspe River	CS	-36.5	144.6	29	0	0
Goulburn	Goulburn River	GB	-36.7	145.2	30	0	0
Kiewa	Kiewa River	KIW	-36.1	146.9	30	0	0
Lake Eildon	Lake Eildon	EI	-37.2	146.0	29	0	0
Loddon	Loddon River	LD	-37.1	144.0	22	0	0
Ovens	Ovens River	OV	-36.1	146.2	24	0	0
Horsham	Wimmera River	WM	-36.7	142.2	22	0	0
				Total:	**1068**	**78**	**23**

5.2.3. Microsatellite cross-species PCR amplification and assessment

Cross-species PCR amplification was attempted with the fourteen microsatellite loci used successfully on common carp by Haynes *et al.* (submitted): Cca02, Cca07, Cca09, Cca17, Cca19, Cca65, Cca67, Cca72 (Yue *et al.* 2004), MFW6, MFW26 (Crooijmans *et al.* 1997), GF1 (Zheng *et al.* 1995), Koi5-6, Koi29-30, and Koi41-42 (David *et al.* 2001). PCR was performed on genomic DNA from three goldfish using the same primers and touch-down cycling protocols as Haynes *et al*. (submitted), and PCR products were separated by electrophoresis on 1.5% agarose gels to assess the quantity and accuracy of amplification. Cross-species amplification of loci GF1 and MFW6 was previously attempted by Hänfling *et al*. (2005), who were successful in cross-species amplification of locus GF1, but were not successful with MFW6. Eight loci (Cca09, Cca17, Cca65, Cca67, GF1, Koi5-6, Koi29-30, and Koi41-42) amplified consistently in goldfish and were considered for further analysis.

The eight loci were scored according to Haynes *et al*. (submitted) in the 42 goldfish collected from Nyngan (Table 5.1, Figure 5.1). These goldfish were all collected from the same weir pool and expressed the wild-type phenotype of olive-grey colouration, consistent with them coming from a single breeding population rather than from a number of recent, independent introductions. For each locus, the per cent of missing data (PCR failure or unscorable alleles) was calculated. Two loci (Cca09 and Cca67) had exceptionally high levels of missing data (35.7% and 26.2%, respectively; Table 5.2), and were not considered further. The remaining loci were tested for departure of genotype frequencies from expectations under Hardy-Weinberg Equilibrium (HWE), using Genepop (Raymond and Rousset 1995) under suboptions 1.1 (H_1=locus not in HWE), 1.2 (H_1= heterozygote excess) and 1.3 (H_1=homozygote excess). Locus Koi41-42a showed significant (p-value < 0.05) departure from HWE and homozygote excess and was consequently deemed unreliable and not used further. The five remaining loci were PCR amplified and scored in all remaining goldfish and putative hybrids.

Table 5.2. Missing data and departure from Hardy-Weinberg Equilibrium (HWE) in goldfish from Nyngan (n=42). Dashes (-) indicate loci that were monomorphic in this population**.**

Locus	**%**	**HWE *p*-values**		
	Missing data	***HWE***	***Heterozygote excess***	***Homozygote excess***
Cca09a*	35.7	NA	NA	NA
Cca17a	1.2	1	0.8819	1
Cca65a	1.2	-	-	-
Cca67*	26.2	NA	NA	NA
GF1	0.0	0.7262	0.72022	1
Koi29-30a	2.4	-	-	-
Koi41-42a*	0.0	0.0105	1	0.0121
Koi5-6a	0.0	-	-	-

* Loci deemed unreliable and not used further in this study.

5.2.4. Statistical analysis of microsatellite data

Clustering algorithms employing Markov-Chain Monte-Carlo simulations were implemented in the programs STRUCTURE version 2.1 (Pritchard *et al.* 2000; Falush *et al.* 2003; 2007) and NEWHYBRIDS version 1.1 (Anderson and Thompson 2002) to analyse the data from the five microsatellite scored in all fish. Both programs can be run without *a priori* information about where samples were collected, do not require pure representatives of each group, and do not require that the different groups being analysed have diagnostic alleles. STRUCTURE assigns individuals to one or more of a predefined number of clusters, *K*, under the assumptions of HWE and linkage equilibrium. For each individual, the estimated proportion of ancestry from a particular cluster (*K*) is given by the statistic q_K. In accordance with the strategy of Vähä and Primmer (2006), individuals assigned 90-100% to a single cluster (q_K = 0.9-1.0) were considered to be representative of the strain most associated with that cluster (i.e. 'purely' descended from that group). Individuals with q_K = 0.35-0.65 were considered F1-generation hybrids, and individuals with $0.1 < q_K < 0.35$ for one cluster, and the balance from the other cluster, were considered F2-generation or backcross. NEWHYBRIDS estimates the posterior probability (*p*-value) for each individual belonging to each specific category (Parental, F1, F2 or backcross).

The analysis in STRUCTURE was run for *K*=2 clusters (corresponding to carp and goldfish) under the admixture and allele-frequencies-correlated models with 50,000 burn-in steps, 500,000 Markov-Chain Monte-Carlo steps, and three replicates to check for consistency between runs. To ensure that burn-in and run lengths were adequate, convergence of the summary statistics alpha (α), P(D) and likelihood were checked by eye.

The NEWHYBRIDS analysis was run with 50 000 burn-in steps and 100 000 MCMC sweeps after burn-in. The analysis was repeated 10 times, and final *p*-values for each hybrid category were calculated as the average over these replicates.

The program GENALEX (Peakall and Smouse 2006) was used to calculate allele frequencies and identify private alleles in carp and goldfish. The individuals identified phenotypically as hybrids, and any carp or goldfish identified as having mixed ancestry in the NEWHYBRIDS or STRUCTURE analyses, were excluded from the identification of private alleles. Microsatellite profiles of the 23 putative hybrids were inspected to identify species-specific and shared alleles in each individual, to explain the assignments made by STRUCTURE and NEWHYBRIDS.

Factorial correspondence analysis (FCA) (Guinand *et al.* 2003) was implemented to elucidate the extent of allele sharing amongst carp, goldfish and hybrids using the software Genetix version 4.05.2 (Belkhir *et al.* 2000).

5.2.5. Mitochondrial DNA analysis

To determine of the putative hybrids had carp or goldfish maternal ancestry, the mitochondrial control region was sequenced in all 23 putative carp-goldfish hybrids, five carp, one goldfish and one tench. The control region was PCR amplified according to (Zhou *et al.* 2003). PCR products were purified using UltraClean GelSpin DNA Purification Kits (Mo Bio, Solana Beach) and sequenced with BigDye Terminator Version 3.1 chemistry (AB, Foster) using the forward (LD) primer to initiate transcription. The first 600 base pairs of each sequence were aligned in CLUSTALW (Thompson *et al.* 1994) against published sequences of three carp, two goldfish, one Japanese crucian carp (*Carassius cuvieri*) and one tench sequence from the NCBI database (http://www.ncbi.nlm.nih.gov/). Both the central-Asian/European (*C. carpio carpio*) and

east Asian (*C. carpio haematopterus*) carp subspecies were represented in the alignment. The sequences used are listed in Table 5.3. Unweighted Pair Group Method with Arithmetic mean (UPGMA) and Nwighbour-Joining phylogenetic trees were constructed in MEGA version 3.0 (Kumar *et al.* 2004) under default settings with 1000 bootstraps to test the significance of each node.

Table 5.3. Mitochondrial sequences used in this study.

Species and location	Identifier[1]	Accession Number	Reference
Putative Carp-goldfish hybrids			
Australia, Bogan River, Nyngan	NG/H1	EU780045	This study
Australia, Mudgee River	MG/H1-14	EU780026-39	This study
Australia, Darling River, Wilcannia	WC/H1-3	EU780046-48	This study
Australia, Macquarie River, Burrendong Dam	BD/H1-4	EU780040-43	This study
Australia	WN/H1	EU780044	This study
Carp			
Australia, Prospect Reservoir	P1	EU780023	This study
Australia, Macquarie River, Dubbo	DB1	EU754007	This study
Australia, Murrumbidgee River, Narrandera	ND01	EU780022	This study
Australia, Cudgegong River, Mudgee	MG19	EU754008	This study
Japanese koi, fish farm, Germany	K1*	EU754016	This study
Yangtze River, China	YR*	AY345334	Zhou *et al*. (2003)
Volga River, Russia	VR**	AY345339	Zhou *et al*. (2003)
Lake Biwa wild carp, Japan	LB†	AB158809	Mabuchi *et al.* (2005)
Goldfish			
Australia, Bogan River, Nyngan	NG/G2	EU780024	This study
Unknown origin	G1	AY786072	Yang *et al*. (unpublished)
Unknown origin	G2	AY786072	Yang *et al*. (unpublished)
Japanese crucian carp			
Unknown origin	JCC	AY786075	Yang *et al*. (unpublished)
Tench			
Australia, Lake Eildon, Victoria	T1	EU780025	This study
Saone River, France	T2	AB218686	Saitoh *et al.* (2006)

[1] Hybrids and goldfish are indicated as XX/Yn, where XX is the sampling location identifier listed in Table 5.1, Y = H (hybrid) or G (goldfish) and n indicates sample number(s)

* East-Asian subspecies *haematopterus*; ** central-Asian/European subspecies *carpio*; † basal lineage of carp, possible a separate subspecies.

5. 3. Results

5.3.1. Microsatellite data

In the analysis in STRUCTURE, visual inspection of the program outputs of the statistics α, P(D) and likelihood showed that these measures all converged, indicating that a sufficient number of burn-in and run steps were used. Visual inspection also indicated that the partitioning of samples into *K*=2 genetic clusters was consistent between runs. All

subsequent analyses and discussions are based on the first such run. The results for the domestic carp and goldfish samples confirmed that the two clusters closely correspond (average q_K=0.99) to the two species. However, three of the 66 koi samples were classed as F2 or backcross, having only 77%-86% of their ancestry from the carp cluster (q_c=0.77-0.86). Of the naturalised fish, four individuals identified phenotypically as carp were classed as F1-generation hybrids (q_c=0.52-0.55); and 11 individuals identified phenotypically as carp and one as goldfish were classed as F2-generation or backcross (q_c=0.66-0.79 and q_g= 0.83, respectively). Amongst the 23 putative hybrids, 20 were classified as F1-generation (q_c=0.36—0.51) and three as F2 or backcross (q_c=0.77-0.78). These results are summarised in Tables 5.4 and 5.5. Overall, 1.6% of the genetic diversity of naturalised carp was assigned to the goldfish cluster; and 1% of the genetic diversity of naturalised goldfish was assigned to the carp cluster.

In the classification of fish in NEWHYBRIDS, two of the three koi carp identified as F2 or backcross in the STRUCTURE analysis were assigned a highest probability of being carp backcross (*p*-value=0.72). Of the naturalised fish, 3 individuals identified as carp in the field were assigned the highest probability (*p*-value=0.48-0.71) of being carp backcrosses, and eight as F2 generation crosses (*p*-value=0.0.43-0.57). No individuals were classified as F1. Four individuals classified as F2 or backcrossed in STRUCTURE were classed as parental carp (*p*-value=0.8-0.82). Amongst the 23 putative hybrids, 20 had highest probability of being F1 generation (*p*-value=0.65-0.91) and three of being carp backcross (*p*-value=0.72-73)..These results are summarised in Tables 5.4 and 5.5.

Allele frequencies differed between species and strains/populations, and 18 carp-specific and six goldfish-specific alleles were identified (Appendix 5.1). Inspection of the microsatellite profiles of the putative hybrids (Table 5.6) indicated that species-specific alleles were not present for loci Cca65 and Koi5-6; that a carp-specific allele (335) was present at locus GF1, and both carp-specific and goldfish-specific alleles were present at locus Cca17 (alleles 371 and 374 for carp; and alleles 387 and 389 for goldfish), and a goldfish-specific allele was present at locus Koi29-30 (322).

Table 5.4. Assignment analysis of putative F1 carp-goldfish hybrids. *c* = carp, *g* = goldfish, P = parental, and BX = backcross; q_K = proportion of ancestry from cluster *K*. The NEWHYBRIDS classification with the highest probability is marked in bold.

	STRUCTURE			NEWHYBRIDS (*p*-value)					
	q_c	q_g	Classification	P.*c*	P.*g*	F1	F2	*c* BX	*g* BX
BD/H01	0.50	0.50	F1	0.00	0.00	**0.84**	0.07	0.09	0.00
BD/H02	0.50	0.50	F1	0.00	0.00	**0.84**	0.07	0.09	0.00
BD/H03	0.36	0.64	F1	0.00	0.00	**0.65**	0.28	0.04	0.03
BD/H04	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
MG/H01	0.50	0.50	F1	0.00	0.00	**0.91**	0.04	0.05	0.00
MG/H02	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
MG/H03	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
MG/H04	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
MG/H05	0.78	0.22	F2 or BX	0.05	0.00	0.00	0.23	**0.72**	0.00
MG/H06	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
MG/H07	0.37	0.63	F1	0.00	0.00	**0.73**	0.20	0.04	0.02
MG/H08	0.37	0.63	F1	0.00	0.00	**0.73**	0.20	0.04	0.02
MG/H09	0.37	0.63	F1	0.00	0.00	**0.73**	0.20	0.04	0.02
MG/H10	0.77	0.23	F2 or BX	0.04	0.00	0.00	0.24	**0.73**	0.00
MG/H11	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
MG/H12	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
MG/H13	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
MG/H14	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
NG/H01	0.50	0.50	F1	0.00	0.00	**0.91**	0.04	0.05	0.00
WC/H01	0.50	0.50	F1	0.00	0.00	**0.91**	0.04	0.05	0.00
WC/H02	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
WC/H03	0.51	0.49	F1	0.00	0.00	**0.90**	0.04	0.05	0.00
WN/H01	0.78	0.22	F2 or BX	0.05	0.00	0.00	0.23	**0.72**	0.00

Table 5.5. Assignment analysis of individuals showing inter-species ancestry. c = carp, g = goldfish, P = parental, and BX = backcross; qK = proportion of ancestry from genetic cluster K. NewHybrid classification with the highest probability is marked in bold**.**

	STRUCTURE			NEWHYBRIDS (*p*-value)					
	q_c	q_g	Classification	P.*c*	P.*g*	F1	F2	*c* BX	*g* BX
Domestic carp									
K31	0.77	0.77	F2 or BX	0.04	0.00	0.00	0.24	**0.72**	0.00
K39	0.86	0.86	F2 or BX	**1.00**	0.00	0.00	0.00	0.00	0.00
K43	0.77	0.77	F2 or BX	0.04	0.00	0.00	0.24	**0.72**	0.00
Naturalised carp									
AV05	0.68	0.32	F2 or BX	0.01	0.00	0.12	0.30	**0.57**	0.00
BJ25	0.66	0.34	F2 or BX	0.21	0.00	0.01	**0.43**	0.36	0.00
Cs26	0.55	0.45	F2 or BX	0.39	0.00	0.00	**0.53**	0.08	0.00
Cs27	0.55	0.45	F2 or BX	0.39	0.00	0.00	**0.53**	0.08	0.00
Cs28	0.75	0.25	F2 or BX	**0.80**	0.00	0.00	0.13	0.07	0.00
Cs29	0.75	0.25	F2 or BX	**0.80**	0.00	0.00	0.13	0.07	0.00
Cs30	0.75	0.25	F2 or BX	**0.80**	0.00	0.00	0.13	0.07	0.00
Ei05	0.65	0.35	F2 or BX	0.01	0.00	0.01	**0.52**	0.47	0.00
Ei11	0.66	0.34	F2 or BX	0.21	0.00	0.01	**0.43**	0.36	0.00
Ei13	0.52	0.48	F1	0.00	0.00	0.10	**0.57**	0.33	0.00
Ei19	0.52	0.48	F1	0.03	0.00	0.10	**0.55**	0.32	0.00
Ei20	0.76	0.24	F2 or BX	0.82	0.00	0.00	0.11	0.08	0.00
Ei28	0.65	0.35	F2 or BX	0.01	0.00	0.01	**0.52**	0.47	0.00
MR25	0.79	0.21	F2 or BX	0.37	0.00	0.01	0.14	**0.48**	0.00
Naturalised goldfish									
WGG5	0.17	0.83	F2 or BX	0	**0.55**	0	0.4	0	0.05

Table 5.6. Microsatellite alleles in the 23 carp-goldfish hybrids identified by phenotype. Orange = allele shared by both species; blue = carp-specific allele; yellow = goldfish-specific allele (see Appendix 5.1). "0" designates missing data**.**

Individual	Microsatellite Locus									
	Cca65		GF1		Cca17		Koi29-30		Koi5-6	
BD/H01	152	158	335	349	0	0	322	340	237	237
BD/H02	152	158	335	349	0	0	322	340	237	237
BD/H03	152	158	349	351	371	387	322	340	237	237
BD/H04	152	158	335	351	374	389	322	340	237	237
MG/H01	152	158	335	349	374	389	322	340	237	237
MG/H02	152	158	335	351	374	389	322	340	237	237
MG/H03	152	158	335	351	374	389	322	340	237	237
MG/H04	152	158	335	351	374	389	322	336	237	237
MG/H05	152	152	335	351	371	374	322	340	237	237
MG/H06	152	158	335	351	374	389	322	340	237	237
MG/H07	152	158	351	351	374	389	322	340	237	237
MG/H08	152	158	351	351	374	389	322	340	237	237
MG/H09	152	158	351	351	374	389	322	340	237	237
MG/H10	152	158	335	335	374	374	322	340	237	237
MG/H11	152	158	335	351	374	389	322	340	237	237
MG/H12	152	158	335	351	374	389	322	340	237	237
MG/H13	152	158	335	351	374	389	322	340	237	237
MG/H14	152	158	335	351	374	389	322	340	237	237
NG/H01	152	158	335	349	374	389	322	336	237	237
WC/H01	152	158	335	349	374	389	322	340	237	237
WC/H02	152	158	335	351	371	387	322	336	237	237
WC/H03	152	158	335	351	374	389	322	340	237	237
WN/H01	152	152	335	351	371	374	322	340	237	237

A two-dimensional representation of the results of the FCA is shown in Figure 5.2, with the first two axes/dimensions accounting for 45% and 20% respectively of the variation in the data. A third axis/dimension, accounting for an additional 19% of the genetic variation, was excluded as it did not significantly affect the positioning of the data points relative to one another. The MDB, mirror-scale (J) and Prospect Reservoir (P) carp cluster together, and the koi (K) and Ropsha (R) carp form distinctly separate clusters. The Ropsha carp have a very diffuse cluster, with some individuals being close to European mirror-scale carp, and others being highly differentiated. This is consistent with these carp being derived from crossing and backcrossing of European domestic carp with wild carp from the River Amur in eastern Russia (Zonova and Kirpichnikov 1968). The goldfish were distinct from carp, with the fish from the pet store being widely spread, consistent with them

coming from a wide range of domestic breeding lineages. The hybrids clustered between the carp and goldfish data points.

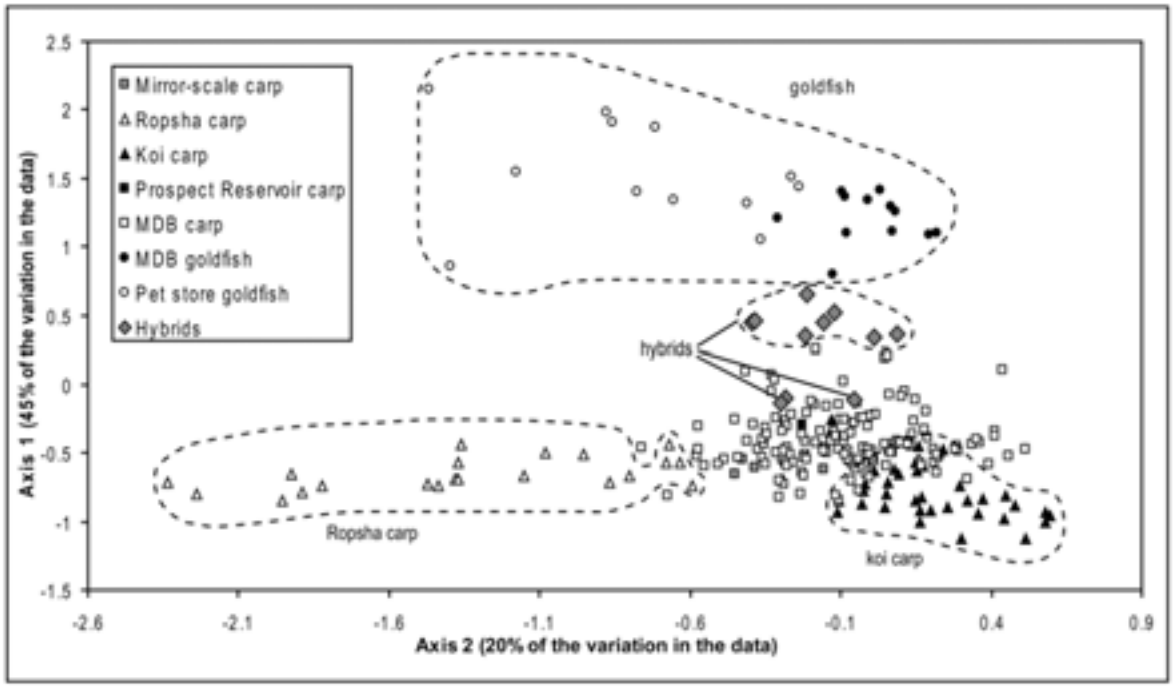

Figure 5.2. FCA of the genetic relatedness between carp, goldfish and putative carp-goldfish hybrids

5.3.2. Mitochondrial sequence data

The UPGMA and Neighbour-Joining phylogenetic trees were highly consistent, differing only in the level of bootstrap support given to some nodes (Figure 5.3), and separating each species into a well supported (86 - 100% bootstrap value) monophyletic clade. As the purpose of the two trees was to identify DNA sequences as having come from common carp or goldfish, they were considered sufficient for this study, although more sophisticated and accurate tree-building methods, such as maximum likelihood (Hasegawa and Yano 1984) and maximum parsimony (Sober 1988), would be necessary for a more detailed phylogenetic study. As expected, the *Cyprinus carpio* sequence from Lake Biwa, Japan (AB158809), was placed basal to all other carp sequences, consistent with the findings of Mabuchi *et al*. (2005). Previous studies have detected European ancestry of naturalised carp in Australia (Shearer and Mulley 1978; Thai *et al.* 2004a; Haynes *et al.* 2009). Consistent with these studies, the carp sequences from the MDB and Prospect Reservoir clustered with the carp from the Volga River, which belongs to the European/central-Asian

subspecies *C. carpio carpio* (Zhou *et al.* 2003). Of the hybrids, 21 were placed in the goldfish clade, and two in the carp clade.

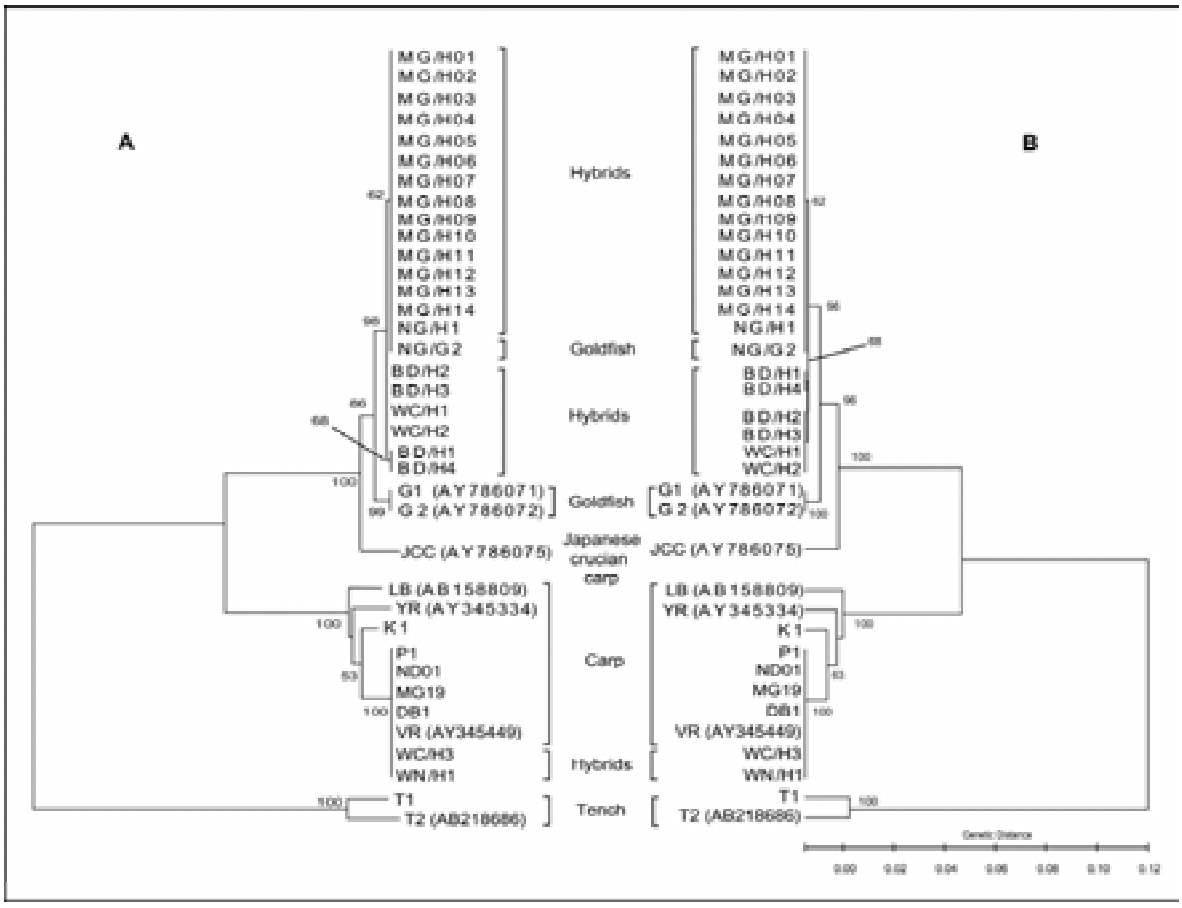

Figure 5.3. Phylogenetic relationship of the first 600bp of the mitochondrial control region in carp, goldfish, Japanese crucian carp, tench and the putative carp-goldfish hybrids from the MDB. Individual identifiers are given in Table 5.3. A. Neighbour-Joining tree, B. UPGMA tree. Bootstrap values less than 50 are not shown**.**

5.4. Discussion

In this study, the cross-species PCR amplification of five loci made it possible to confirm that hybridisation is occurring between carp and goldfish and to detect cryptic inter-species hybridisation. Some evidence was found that hybridisation was biased in favour of male carp. The implications of these and other findings are discussed below.

5.4.1 Cross-Species amplification of microsatellite loci

To facilitate future studies of introgression between carp and goldfish, cross-species PCR was optimised for four additional microsatellite loci in this study: Cca09, Cca17, Cca65

and Cca67 (Yue *et al.* 2004). Combined with the six loci that were amplified in both carp and goldfish by Hänfling *et al*. (2005), namely GF1, GF17, GF29, MFW2, MFW7, MFW17 (Croojimans *et al.* 1997; Zheng *et al*, 1995), 10 such loci are now available for cross-species studies. PCR conditions are given for these loci by Hänfling *et al*. (2005) and Haynes *et al.* (submitted). Locus GF1 was used in both studies, and can be PCR-amplified under either protocol. While Vähä and Primmer (2006) recommend using no fewer than 12 loci for assignment testing to fully resolve the level of hybridisation (F1, F2, or backcross) in a given individual, the loci used here and by Hänfling *et al*. (2005) have been shown to be highly effective at detecting cross-species introgression, so optimising cross-species PCR in for additional loci may not be necessary.

5.4.2. Hybridisation between carp and goldfish

This study confirms that interbreeding is occurring between invasive carp and goldfish in Australia, with mixed-species ancestry confirmed genetically in all 23 phenotypically identified hybrids. Of these hybrids, both the NEWHYBIRDS and STRUCTURE assignments identified 20 as being most likely to be F1 generation, and three as carp-backcrosses. The presence of backcrossed individuals confirms that hybrids are not sterile (although they may show reduced levels of fertility), and there is likely some gene flow between naturalised populations of carp and goldfish. The presence of aberrant barbels around the mouth is clearly a strong indicator fish having inter-species ancestry.

5.4.3. Cryptic introgression between carp and goldfish

This study has successfully detected cryptic introgression between invasive carp and goldfish in Australia, with 16 individuals from the MDB identified phenotypically as carp and one individual identified phenotypically as goldfish showing putative inter-species ancestry. The classification of these individuals as F1, F2 or backcrossed is less clear than for the phenotypically identified hybrids, as results differ more between the STRUCTURE and NEWHYBRIDS assignments. As NEWHYBRIDS specifically tests for the level of hybridisation in each individual, however, the results from NEWHYBRIDS analyses are likely more accurate than STRUCTURE. It is clear that cross-species introgression is very rare, with mixed ancestry detected in only approximately 1.3% of carp and 1.6% of goldfish; and with 1.63% of the overall genetic diversity of naturalised carp likely to have been sourced from goldfish, and 1% of the genetic diversity of naturalised goldfish

assigned to carp. These numbers may be understated, however, as hybrid individuals comprised only a small fraction of the total samples analysed; and this has been demonstrated to reduce the power of both STRUCTURE and NEWHYBRIDS to detect such hybrids (2006).

Interestingly, some of the koi used as controls also showed mixed ancestry. Possibly the ancestors of these fish were crossed with domestic goldfish to introduce novel alleles desirable for the selective breeding of ornamental traits.

5.4.4. Direction of hybridisation

Prior to beginning this study, it was speculated that the hybridisation between carp and goldfish would be biased in favour of male carp, as the larger carp males could potentially exclude the smaller goldfish males from spawning aggregations (D.M. Gilligan, per. comm.). The detection of goldfish mitochondrial DNA in 21 of the 23 hybrids, and carp mitochondria in only two, suggests that interspecies hybridisation is a male-carp biased. The small number of hybrids and microsatellite loci analysed makes this far from certain, however, and further research is needed to fully resolve this issue.

5.4.5. Implications for Conservation

The introgression detected in this study is of some concern for the conservation of Australian freshwater ecosystems. Even through the level of introgression was low, it indicates that goldfish could act as a reservoir of genetic diversity for highly invasive and destructive carp. Large numbers of goldfish are imported each year for the aquarium industry (Brumley 1991), and inevitable some individuals find their way into waterways and survive and reproduce. Even if the fertility of inter-species hybrids is low, interbreeding coupled with natural selection could result in the spread of adaptive alleles into carp populations (Hänfling 2007).

Two forms of biological control of carp are being developed in Australia. These are the daughterless gene technology, and the introduction of diseases. Both of these controls could potentially be compromised by introgression with goldfish (Bax and Thresher 2003; Gilligan and Rayner 2007).

Daughterless gene technology involves releasing large number of carp that have been genetically modified to produce only male progeny. Continuous release of such fish has been predicted to skew sex ratios and ultimately reduce carp numbers (Thresher and Bax 2003). Goldfish, however, could act as a reservoir of functional copies of the modified gene(s), and so undermine attempts at control.

Koi herpes virus (KHV) is currently being assessed for use in controlling carp numbers in Australia (Gilligan and Rayner 2007). It is especially desirable, as related species of Cyprinid such as goldfish and grass carp (*Ctenopharyngodon idella*) are not affected by the virus and do not act as carriers (Perelberg *et al.* 2003; Ronen *et al.* 2003). Were the virus to be widely released, however, individuals carrying genes for KHV resistance inherited from goldfish ancestors could gain a selective advantage. KHV resistance genes could hence move rapidly through the population and negate any long-term impact of the disease. Goldfish are resistant to a number of other viruses to which carp are susceptible (e.g. Hedrick *et al.* 2006), and could act as a genetic reservoir of resistance to a whole range of potential disease controls.

5.5. Acknowledgements

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5.6. References

Al-Sabti K (1986) Karyotypes of Cyprinus carpio and Leuciscus cephalus. *Cytobios,* **47,** 19-25.

Anderson EC, Thompson EA (2002) A model-based method for identifying species hybrids using multilocus genetic data. *Genetics* **160,** 1217-1229.

Balon EK (2004) About the oldest domestication among fishes. *Journal of Fish Biology,* **65 (Supplement A),** 1-27. doi: 10.1111/j.1095-8649.2004.00563.x.

Bax N, Thresher R (2003) The science of producing daughterless technology; possibilities for population control using daughterless technology; maximising the impact of carp control. *Proceedings of the National Carp Control Workshop.* Canberra, Australia.

Belkhir K, Borsa P, Goudet J, Chikli L, Bonhomme F (2000) GENETIX, logiciel sous WindowsTM pour la ge´ne´tique des populations, version 4.01. Montpellier, University of Montpellier II.

Brumley AR (1991) Cyprinids of Australasia. *Cyprinid fishes systematics, biology and exploitation.* Suffolk, United Kingdom, St. Edumdsbury Press. ISBN: 92-5-103027-8.

Crooijmans RPMA, Bierbooms VAF, Komen J, Van der Poel JJ, Groenen MAM (1997) Microsatellite markers in common carp (*Cyprinus carpio* L.). *Animal Genetics,* **28,** 129-134. doi: 10.1111/j.1365-2052.1997.00097.x.

David L, Rajasekaran P, Fang J, Hillel J, Lavi U (2001) Polymorphisms in ornamental and common carp strains (*Cyprinus carpio* L.) as revealed by AFLP analysis and a new set of microsatellite markers. *Molecular Genetics and Genomics,* **266,** 353-362. doi: 10.1007/s004380100569.

Falush D, Stephens M, Pritchard JK (2003) Inference of Population Structure Using Multilocus Genotype Data: Linked Loci and Correlated Allele Frequencies *Genetics,* **164,** 1567-1587.

Falush D, Stephens M, Pritchard JK (2007) Inference of population structure using multilocus genotype data: dominant markers and null alleles. *Molecular Ecology Notes,* **7,** 574–578. doi: 10.1111/j.1471-8286.2007.01758.x.

Gilligan D, Rayner T (2007) The distribution, spread, ecological impacts, and potential control of carp in the upper Murray River. NSW Department of Primary Industries, Sydney.

Guinand B, Parisseaux B, Lebreton J, Bonhomme F (2003) Estimating population genetics 'F-statistics' using factorial correspondence analysis with respect to instrumental variables. *International Conference on Correspondence Analysis and Related Methods.* Barcelona, Spain.

Hänfling B (2007) Understanding the establishment success of non-indigenous fishes: lessons from population genetics. *Journal of Fish Biology,* **71 (Supplement D),** 115-135. doi: 10.1111/j.1095-8649.2007.01685.x.

Hänfling B, Bolton P, Harley M, Carvalho GR (2005) A molecular approach to detect hybridisation between crucian carp (*Carassis carassius*) and non-indigenous carp species (*Carassisus* cpp. and *Cyprinus carpio*). *Freshwater Biology,* **50,** 403-417. doi:10.1111/j.1365-2427.2004.01330.x.

Hasegawa M, Yano T (1984) Maximum likelihood method of phylogenetic inference from DNA sequence data. *Bulletin of the Biometric Society of Japan,* **5,** 1-7.

Haynes GD, Gilligan DM, Grewe P, Nicholas FW (submitted) Population genetics and management units of invasive common carp (*Cyprinus carpio* L.), in the Murray-Darling Basin, Australia. *Journal of Fish Biology,* **000,** 000.

Haynes GD, Gongora J, Nicholas FW, Zenger KR (2009) Rapid identification of maternal lineages in common carp (*Cyprinus carpio* L.) using real-time PCR and high resolution melt-curve analysis. *Aquaculture,* **287,** 59-66. doi:10.1016/j.aquaculture.2008.10.035.

Hedrick RP, Waltzek TB, McDowell TS (2006) Susceptibility of koi carp, common carp, goldfish and goldfish x common carp hybrids to cyprinid herpesvirus-2 and herpesvirus-3. *Journal of Aquatic Animal Health,* **18,** 26-34.

Hume JD, Fletcher AR, Morison AK (1983) Interspecific hybridization between carp (*Cyprinus carpio* L.) and goldfish (*Carassius auratus* L.) from Victorian waters. *Australian Journal of Marine and Freshwater Research,* **34,** 915-919. doi:10.1071/MF9830915.

Kelly DW, Muirhead JR, Heath DD, Macisaac HJ (2006) Contrasting patterns in genetic diversity following multiple invasions of fresh and brackish waters. *Molecular Ecology,* **15,** 3641–3653. doi: 10.1111/j.1365-294X.2006.03012.x.

Koehn J (2004) Carp (*Cyprinus carpio*) as a powerful invader in Australian waterways. *Freshwater Biology,* **49,** 882-894. doi: 10.1111/j.1365-2427.2004.01232.x.

Koehn J, Brumley B, Gehrke P (2000) *Managing the Impacts of Carp,* Canberra, Australia, Bureau of Rural Sciences (Department of Agriculture, Fisheries and Forestry). ISBN 0644292407 (set) 0642732019.

Kolbe JJ, Glor RE, Schettino LR, Lara AC, Larson A, Losos JB (2004) Genetic variation increases during biological invasion by a Cuban lizard. *Nature,* **431,** 177-181. doi: 10.1038/nature02807.

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. *Briefings in Bioinformatics,* **5,** 150-163. doi:10.1093/bib/5.2.150.

Mabuchi K, Senou H, Suzuki T, Nishida M (2005) Discovery of an ancient lineage of *Cyprinus carpio* from Lake Biwa, central Japan, based on mtDNA sequence data, with reference to possible multiple origins of koi. *Journal of Fish Biology,* **66,** 1516-1528. doi:10.1111/j.0022-1112.2005.00676.x.

Nolte AW, Freyhof J, Stemshorn KC, Tautz D (2005) An invasive lineage of sculpins, *Cottus sp* (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogeographic groups. *Proceedings of the Royal Society B,* **272,** 2379-2387. doi: 10.1038/rspb.2005.3231.

Ohno S, Muramoto J, Christian L, Atkin NB (1967) Diploid-tetraploid relationship among Old-World members of the Fish Family *Cyprinidae*. *Chromosoma (Berlin),* **23,** 1-9. doi: 10.1007/BF00293307.

Peakall R, Smouse PE (2006) GENALEX 6: genetic analysis in EXCEL. Population genetic software for teaching and research. *Molecular Ecology Notes,* **6,** 288-295. doi: 10.1111/j.1471-8286.2005.01155.x.

Perelberg A, Smirnov M, Hutoran M, Diamant A, Bejerano Y, Kotler M (2003) Epidemiological description of a new viral disease afflicting cultured Cyprinus carpio in Israel. *The Israeli Journal of Aquaculture,* **55,** 5-12.

Pritchard JK, Stefens M, Donelly P (2000) Inference of population structure using multilocus genotype data. *Genetics,* **155,** 945-959.

Pullan S, Smith PJ (1987) Identification of hybrids between koi (*Cyprinus carpio*) and goldfish (*Carassius auratus*). *New Zealand Journal Of Marine And Freshwater Research,* **21,** 41-46.

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and eumenicism. *Journal of Heredity,* **86,** 248-249.

Roman J (2006) Diluting the founder effect: cryptic invasions expand a marine invader's range. *Proceedings of the Royal Society B,* **273,** 2453-2459. doi: 10.1098/rspb.2006.3597.

Ronen A, Perelberg A, Abramovitz J, Mutoran M, Tinman S, Bejerano I, Steinitz M, Kotler M (2003) Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine,* **21,** 4677-4684. doi:10.1016/S0264-410X(03)00523-1.

Ruiguang Z, Zheng S (1980) Analysis and comparison between the karyotypes of *Cyprinus carpio* and *Carassius auratus* as well as *Aristichthys nobilis* and *Hypophthalmichthys molitrix*. *Acta Genetica Sinca,* **7,** 72-77.

Saitoh K, Sado T, Mayden RL, Hanzawa N, Nakamura K, Nishida M, Miya M (2006) Mitogenomic evolution and interrelationships of the Cypriniformes (Actinopterygii:)stariophysi): the first evidence toward resolution of higher-level relationships of the world's largest freshwater fish clade based on 59 whole mitogenome sequences. *Journal of Molecular Evolution,* **63,** 826-841 doi: 10.1007/s00239-005-0293-y.

Shearer KD, Mulley JC (1978) The introduction and distribution of the carp, *Cyprinus carpio* Linnaeus, in Australia. *Australian Journal of Marine and Freshwater Research,* **29,** 661-563. doi:10.1071/MF9780551

Sober E (1988) *Reconstructing the past: parsimony, evolution, and inference,* Cambridge, Massachusetts, MIT Press. ISBN: 026219273x

Taylor J, Mahon R (1977) Hybridization of *Cyprinus carpio* and *Carassius auratus*, the first two exotic species in the lower Laurentian Great Lakes *Environmental Biology of Fishes,* **1,** 205-208. doi:10.1007/BF00000412.

Thai BT, Burridge CP, Pham TA, Austin CM (2004) Using mitochondrial nucleotide sequences to investigate diversity and genealogical relationships within common carp (*Cyprinus carpio* L.) *Animal Genetics,* **36,** 23-28.

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research,* **22,** 4673-4680.

Thresher R, Bax N (2003) The science of producing daughterless technology; possibilities for population control using daughterless technology; maximising the impact of carp control. In Lapidge, K. L. (Ed.) *Proceedings of the National Carp Control Workshop, March 2003, Canberra.* Cooperative Research Center for Pest Animal Control, Canberra, Australia.

Vähä JP, Primmer CR (2006) Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology,* **15,** 63–72. doi:10.1111/j.1365-294X.2005.02773.x

Wasko AP, Martins C, Oliveira C, Foresti F (2003) Non-destructive genetic sampling of fish. An improved mehtod for DNA extraction from fish fins and scales. *Hereditas,* **138,** 161-165. doi:10.1034/j.1601-5223.2003.01503.x.

Yue GH, Ho MY, Orban L, Komen J (2004) Microsatellites within genes and ESTs of common carp and their applicability in silver crucian carp. *Aquaculture,* **234,** 85-98. doi:10.1016/j.aquaculture.2003.12.021.

Zenger KR, Richardson BJ, Vachot-Griffin A-M (2003) A rapid population expansion retains genetic diversity within European rabbits in Australia. *Molecular Ecology,* **12,** 789-794. doi:10.1046/j.1365-294X.2003.01759.x.

Zheng W, Stacey NE, Coffin J, Strobeck C (1995) Isolation and characterization of microsatellites loci in the goldfish *Carassius auratus*. *Molecular Ecology,* **4,** 791-792.

Zhou JF, Wu QJ, Ye YZ, Tong JG (2003) Genetic divergence between *Cyprinus carpio carpio* and *Cyprinus carpio haematopterus* as assessed by mitochondrial DNA analysis, with emphasis on the origin of European domestic carp. *Genetica,* **119,** 93-97.

Zonova AS, Kirpichnikov VS (1968) Selection of Ropsha carp. *Seminar/study tour in the U.S.S.R. on genetic selection and hybridization of cultivated fishes.* FAO Corporate Document Repository http://www.fao.org/documents.

Appendix 5.1. Allele frequencies and private alleles by population. Phenotypically identified hybrids and individuals identified as having mixed ancestry in the STRUCTURE and HEWHYBRIDS analyses were excluded from the estimation of private alleles**.**

Locus	Allele	Carp				Goldfish		Hybrids[2]
		Mirror-scale	Koi	Ropsha	Feral[1]	Feral	Pet store	
Cca65a	**150***	0.017	0.484	0.155	0.065	0.000	0.000	0.000
	152	0.983	0.452	0.845	0.873	0.033	0.036	0.543
	156*	0.000	0.000	0.000	0.001	0.000	0.000	0.000
	158	0.000	0.065	0.000	0.061	0.959	0.964	0.457
	160*	0.000	0.000	0.000	0.001	0.000	0.000	0.000
	166†	0.000	0.000	0.000	0.000	0.008	0.000	0.000
GF1	**335***	1.000	0.984	1.000	0.935	0.000	0.000	0.435
	339†	0.000	0.000	0.000	0.000	0.000	0.143	0.000
	349	0.000	0.000	0.000	0.003	0.190	0.679	0.130
	351	0.000	0.016	0.000	0.061	0.810	0.179	0.435
Cca17a	**371**	0.583	0.175	0.433	0.128	0.000	0.000	0.095
	374*	0.417	0.825	0.400	0.867	0.000	0.000	0.476
	377*	0.000	0.000	0.000	0.006	0.000	0.000	0.000
	381	0.000	0.000	0.167	0.000	0.000	0.107	0.000
	387†	0.000	0.000	0.000	0.000	0.081	0.143	0.048
	389†	0.000	0.000	0.000	0.000	0.919	0.750	0.381
Koi29-30a	**309***	0.000	0.024	0.000	0.000	0.000	0.000	0.000
	322†	0.000	0.000	0.000	0.000	0.984	1.000	0.000
	332*	0.000	0.000	0.300	0.000	0.000	0.000	0.000
	334*	0.000	0.016	0.000	0.001	0.000	0.000	0.000
	336	0.133	0.806	0.000	0.346	0.008	0.000	0.152
	338*	0.000	0.000	0.000	0.047	0.000	0.000	0.000
	340	0.867	0.000	0.650	0.607	0.008	0.000	0.848
	344*	0.000	0.153	0.000	0.000	0.000	0.000	0.000
	348*	0.000	0.000	0.050	0.000	0.000	0.000	0.000
Koi5-6a	**234***	0.000	0.026	0.000	0.000	0.000	0.000	0.000
	237	0.833	0.077	0.414	0.988	1.000	0.429	1.000
	243*	0.000	0.026	0.000	0.000	0.000	0.000	0.000
	249	0.000	0.000	0.000	0.003	0.000	0.071	0.000
	252*	0.167	0.269	0.103	0.009	0.000	0.000	0.000
	255*	0.000	0.551	0.000	0.001	0.000	0.000	0.000
	263*	0.000	0.051	0.000	0.000	0.000	0.000	0.000
	267	0.000	0.000	0.483	0.000	0.000	0.143	0.000
	273†	0.000	0.000	0.000	0.000	0.000	0.357	0.000

* Carp-specific alleles, † goldfish-specific alleles.

[1] Carp from the MDB and Prospect Reservoir

[2] Identified phenotypically by the presence of aberrant barbels around the mouth

Chapter 6: Rapid identification of maternal lineages in common carp (*Cyprinus carpio* L.) using real-time PCR and high resolution melt-curve analysis

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6.1. Abstract

This study presents a protocol for using real-time PCR and high-resolution melt-curve (HRMC) analysis to score polymorphisms in the mitochondrial DNA control region of common carp. This is the first time HRMC analysis has been used in an aquacultural species. The technique is accurate, robust and rapid to apply. It has a number of advantages over other existing techniques for rapidly scoring DNA polymorphisms, namely it is rapid, taking less than three hours from start to finish; all procedures take place in closed PCR tubes, reducing the risk of contamination and human error; cycling conditions in the Rotorgene 6000 PCR machine used in the methodology are more homogenous than in traditional block-based PCR machines; and the progress and success of each individual PCR is monitored real-time. The primers were designed to score a greater number of polymorphic sites than in previous studies, and specifically target a section of the control region that is polymorphic amongst European carp races, which otherwise have very little mitochondrial DNA variation. The technique was used to accurately identify three common carp and one goldfish haplotype, with no haplotypes incorrectly identified. Although the method outlined here is optimised for scoring common carp mitochondrial haplotypes using the Rotorgene 6000 machine, real-time PCR and HRMC analysis can be applied in a similar way to almost any species and/or loci, with a number of different real-time PCR machines available for scoring genetic differences.

Key words: real-time PCR, high resolution melt-curve analysis, common carp, control region

6.2. Introduction

Common carp (*Cyprinus carpio* L.) are one of the most commercially important species of freshwater fish in the world. The trade in ornamental varieties of common carp (koi) forms a thriving global industry. The harvesting of common carp for food, both from the wild and from aquaculture, has been growing steadily since the late 1970s. It surpassed the production of all salmonoid species combined in 1997, and was estimated to be in excess of 3 million tons in 2005 (FAO 2007). In some regions, introduced common carp are regarded as invasive pests, and measures are undertaken to control their numbers and to limit their negative impact on the environment (Koehn *et al.* 2000). Management of carp, both in the wild and in aquaculture, has been aided in recent times by advances in genetic

technologies, which have assisted population monitoring and species identification studies (e.g. Hänfling *et al.* 2005; Thai *et al.* 2006).

The genotyping and comparison of maternally inherited mitochondrial DNA (mtDNA) sequences have been utilised in a range of evolutionary and population investigations in common carp. These include phylogeographic studies (Kohlmann *et al.* 2003); monitoring the success of different carp strains in aquaculture (Thai *et al.* 2006); identifying the source of invasive populations (Davis *et al.* 1999; Mabuchi *et al.* 2008); and identifying unique, local common carp strains for conservation (Mabuchi *et al.* 2005; Thai *et al.* 2006). Traditionally, mtDNA polymorphisms are identified either through direct sequencing (e.g. Mabuchi et al., 2005), using restriction fragment length polymorphisms (RFLP) analysis (e.g. Davis *et al.* 1999; Gross *et al.* 2002) or single-strand conformational polymorphisms (SSCP) (e.g. Thai et al., 2006).

The current study presents a novel technique for the rapid identification of mtDNA haplotypes in common carp using high-resolution melt curve (HRMC) analysis. HRMC analysis takes advantage of different DNA sequences having slightly different melting temperatures, even if they differ by only a single base pair. Polymorphisms in PCR-amplified DNA sequences are identified by their unique melt-curve profiles. This technique is similar in principal to temperature-gradient gel electrophoresis (TGGE) (Riesner *et al.* 1989), but is carried out using a real-time (rt) PCR machine with high-resolution melt-curve analysis capabilities. The benefit of this technique is the relative speed and cost of the procedure, with it being possible to analyse hundreds of samples in just a few hours for little more than the cost of a PCR reaction for each sample. HRMC has been shown to be highly accurate (Reed *et al.* 2007; Vandersteen *et al.* 2007), and has been used extensively in clinical studies to identify different viral and bacterial species and strains (Cheng *et al.* 2006; Dames *et al.* 2007; Fortini *et al.* 2007; Nakagawa *et al.* 2008), to identify affected and carrier individuals for genetic disorders (McKinney *et al.* 2004; Zhou *et al.* 2004d) and to match organ donors with compatible recipients (Zhou *et al.* 2004c). To our knowledge, this is the first time a HRMC protocol has been developed for the purpose of investigating evolutionary and population relationships of wild or aquacultural populations. Although the protocol presented here is optimised for the mitochondrial control region of common carp, HRMC analysis can readily be applied to other loci and other species.

6.3. Materials and Methods

6.3.1. Identification of mtDNA polymorphisms

Mitochondrial DNA polymorphisms specific to *C. carpio* were identified from comparisons among published mtDNA control region (CR) sequence (Table 6.1) and from the sequence obtained from 11 individuals in this study (Table 6.2), comprising Japanese koi (n=2), domestic mirror-scaled carp from Germany (n=3), wild carp from the river Danube (n=1), Russian Ropsha strain carp (n=3), and feral carp from Australia (n=2). Sequences from feral goldfish (*Carassius auratus*) (n=3) from the Cudgegong River, Australia, were also obtained for use as outgroups in the HRMC analysis.

Table 6.1. Common carp mitochondrial control-region sequences from Genbank (http://www.ncbi.nlm.nih.gov/)

Origin	Strain	Haplotype in Figure 6.2.*	Accession Number	Reference
Unknown	Unknown	na	AY597943	Thai *et al*. (2004)
Unknown	Unknown	na	AY597942	Thai *et al*. (2004)
Unknown	Unknown	na	AY597944	Thai *et al*. (2004)
Asia	Japanese food carp	21	AB158811	Mabuchi *et al.* (2005)
Asia	Koi carp	10	AY347298	Zhou *et al*. (2004)
Asia	Koi carp	10	AY347299	Zhou *et al*. (2004)
Asia	Koi carp	15	AB158812	Mabuchi *et al.* (2005)
Asia	Koi carp	3	AF508933	Froufe *et al.* (2002)
Asia	Wild, Lake Biwa	20	AB158809	Mabuchi *et al.* (2005)
Asia	Wild, Lake Biwa	19	AB158808	Mabuchi *et al.* (2005)
Asia	Wild, Lake Biwa	18	AB158810	Mabuchi *et al.* (2005)
Asia	Big belly carp	14	AY347304	Zhou *et al*. (2004)
Asia	Purse red carp	16	AY347301	Zhou *et al*. (2004)
Asia	Purse red carp	17	AY347300	Zhou *et al*. (2004)
Asia	Qingtian carp	7	AY347296	Zhou *et al*. (2004)
Asia	Xingguo carp	9	AY345332	Zhou *et al*. (2004)
Asia	Xingguo carp	9	AY345335	Zhou *et al*. (2004)
Asia	Wild, Yangtze R.	6	AY345334	Zhou *et al*. (2004)
Asia	Vietnam, domestic	na	AY597968	Thai *et al*. (2006)
Middle-East	Israel food carp	na	AY597982	Thai *et al*. (2006)
Middle-East	Israel food carp	na	AY597981	Thai *et al*. (2006)
Europe	German carp	3	AY345337	Zhou *et al*. (2004)
Europe	German carp	3	AY345338	Zhou *et al*. (2004)
Europe	Leather carp	3	AF508931	Froufe *et al.* (2002)
Europe	Mirror carp	3	AF508932	Froufe *et al.* (2002)
Europe	Wild, R. Danube	3	AF508929	Froufe *et al.* (2002)
Europe	Wild, Hungary	3	EU259966	Wang and Li (unpublished)

Table 6.1. (continued).

Origin	Strain	Haplotype in Figure 6.2.*	Accession Number	Reference
Europe	Wild, Hungary	3	EU259962	Wang and Li (unpublished)
Europe	Wild, Hungary	3	EU259953	Wang and Li (unpublished)
Europe	Wild, Hungary	3	EU259964	Wang and Li (unpublished)
Europe	Wild, Hungary	2	EU259954	Wang and Li (unpublished)
Europe	Wild, Hungary	1	EU259952	Wang and Li (unpublished)
Europe	Wild, Volga R.	3	AY345339	Zhou *et al*. (2004)
Europe	Wild, Volga R.	5	AY345340	Zhou *et al*. (2004)
Russia	Russian carp	8	AY345333	Zhou *et al*. (2004)
Russia	Russian carp	8	AY345336	Zhou *et al*. (2004)
Russia	Wild, R. Amur	4	AF508935	Froufe *et al.* (2002)
Russia	Wild, R. Amur	11	AF508938	Froufe *et al.* (2002)
Russia	Wild, R. Amur	13	AF508937	Froufe *et al.* (2002)
Russia	Wild, R. Amur	12	AF508936	Froufe *et al.* (2002)
Australia	Feral carp	na	AY597977	Thai *et al*. (2004)

* Haplotypes are based only on the first 510 base pairs of the control region

Table 6.2. Mitochondrial control-region sequences obtained in this study.

Name	Origin	Accession Number	Haplotype*
AU01	Australian feral carp	EU754008	A
AU02	Australian feral carp	EU754007	A
R01	Ropsha strain Russian carp	EU754009	A
R02	Ropsha strain Russian carp	EU754010	A
R03	Ropsha strain Russian carp	EU754011	A
D01	Wild carp, River Danube	EU754012	A
GR01	German mirror-scale carp	EU754013	B
GR02	German mirror-scale carp	EU754014	B
GR03	German mirror-scale carp	EU754015	B
K01	Ornamental koi carp, Japan	EU754016	C
K02	Ornamental koi carp, Japan	EU754017	C
MGG6	Australian feral goldfish	EU754018	G
MGG7	Australian feral goldfish	EU754019	G
MGG8	Australian feral goldfish	EU754020	G

*DNA sequence polymorphisms characteristic of haplotypes A, B and C are summarised in Table 6.3.

To generate PCR products for sequencing, mtDNA CR was amplified using the light strand (LD) and heavy strand (HD) primers of Zhou et al. (2003), which anneal in the two genes (tRNA-Pro and tRNA-Phe) that flank the control region (Figure 6.1). Each PCR (total of 15 µl) consisted of 10-100ng DNA template, 1x PCR buffer, 1.5mM $MgCl_2$, 0.2mM

dNTPs, 0.2µM each primer and 0.4 units of Platinum *Taq*-DNA polymerase (Invitrogen, Carlsbad). The PCR amplification cycle consisted of an initial denaturation of 95°C for 6 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min, then a final extension at 72°C for 20 min. PCR products were purified with UltraClean GelSpin DNA Purification Kits (Mo Bio, Solana Beach). Sequencing was performed using BigDye Terminator Version 3.1 chemistry (AB, Foster). Separate sequencing reactions were run using the LD and the HD primers. Consensus sequences for each individual were generated in Sequencher Version 4.7^{TM}. Forward and reverse sequences were consistent with each other and had few difficult-to-call bases. There was no evidence for either point or length heteroplasmy in the individuals sequenced, as secondary peaks greater than 10% of the height of the primary peaks were not detected (see Brandstätter *et al.* 2004).

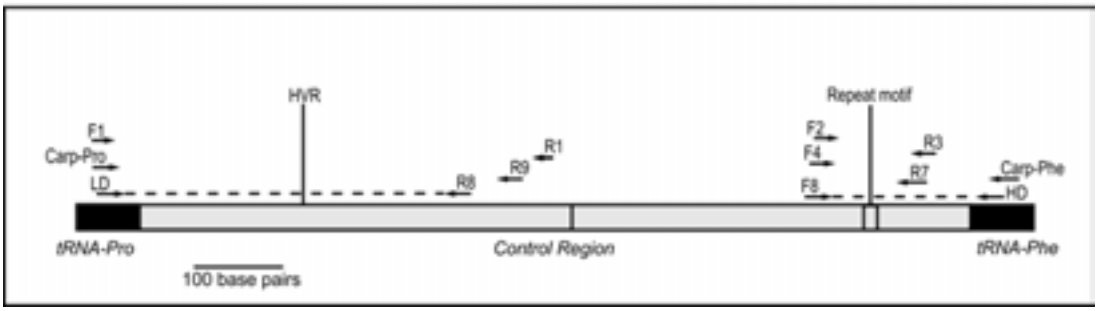

Figure 6.1. Primer positions in the mitochondrial control region of common carp. Primer sequences are listed in Table 6.4. Dashed lines indicate regions PCR amplified in this study and subject to HRMC analysis.

Complete and partial mtDNA CR sequences from common carp were located in the NCBI website (http://www.ncbi.nlm.nih.gov/) through BLAST searching with the carp sequences AU01 and K01 (Table 6.2). Forty-one of these sequences, representing the worldwide geographical range of common carp (Table 6.1), plus the 11 sequences obtained in this study (Table 6.2), were aligned in CLUSTALW (Thompson *et al.* 1994), and conserved and polymorphic regions were identified by eye (Table 6.3). A median-joining network was constructed in Network version 4.5 (www.fluxus-engineering.com) (Bandelt *et al.* 1995) to illustrate the relationship between these sequences and individuals sequenced in this project. Only the first 510bp were used in this analysis, as the 3' region of this locus was not available for many sequences. Sequences AY597944, AY597943, AY597942,

AY597982, AY597981, AY597977 and AY597968 were excluded because parts of the 5' region were missing for these individuals.

A total of 55 polymorphic sites were identified within the common carp mtDNA CR sequences: a $(TA)_{8-14}$ repeat motif at the 3' end of the CR, and 54 other polymorphic sites. These are summarised in Table 3. Two regions were selected for further analysis. These were a highly variable region (HVR) containing 40 polymorphic sites at the 5' end of the control region (positions 1-545), and the $(TA)_{8-14}$ repeat motif (positions 812-854) and the 3' end.

Table 6.3. Polymorphisms detected in the mitochondrial DNA control region

Source	Strain	Identifier (Haplotype)	23	35	59	64	68	110	126	141	153	169	172	192	198	238	239	242	243	263	266	289	290	291
This study	German mirror carp	GR01, GR02, GR03 (B)	T	T	T	A	A	T	G	T	C	A	T	C	A	C	C	T	C	A	G	G	G	C
This study	Ropsha strain, R. Danube, Australian feral carp	R01, R02, R03, D01, AU01, AU02 (A)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
This study	Koi carp	K01, K02 (C)	.	.	.	.	.	.	.	C	.	.	.	.	:	T	T	.	T	.	.	.	.	.
Genbank	Unknown	AY597944	-	-	-	-	-	.	.	.	.	.	C	.	:	T	.	.	T	.	.	.	.	T
Genbank	Unknown	AY597943, AY597942	-	-	-	-	-	.	A	.	.	.	C	.	:	T	.	.	.	.	.	.	.	T
Genbank	Big belly carp	AY347304 (18)	.	.	.	.	.	.	.	.	.	G	.	.	:	T	.	.	T	.	.	.	.	T
Genbank	Japanese food carp	AB158811 (21)	.	.	.	.	.	.	.	C	.	.	.	.	:	T	T	.	T	.	.	.	.	
Genbank	Koi carp	AY347298, AY347299 (10)	.	.	.	G	.	.	A	.	.	.	C	.	:	T	.	.	.	.	.	.	.	T
Genbank	Koi carp	AB158812 (15)	.	.	.	.	.	.	.	.	.	G	.	.	:	T	.	.	T	.	.	.	.	T
Genbank	Lake Biwa wild carp	AB158808 (19)	.	.	C	.	.	.	A	.	.	.	.	.	:	T	T	.	T	.	A	.	A	T
Genbank	Lake Biwa wild carp	AB158810 (18)	.	.	C	.	.	.	A	.	.	.	.	.	:	T	T	.	T	.	A	.	A	T
Genbank	Lake Biwa wild carp	AB158809 (20)	.	.	C	.	.	.	A	.	.	.	.	.	.	T	T	.	T	.	A	.	A	T
Genbank	Purse red carp	AY347301 (16)	.	.	C	.	.	.	.	.	.	.	C	.	:	T	.	C	T	.	.	.	.	T
Genbank	Purse red carp	AY347300 (17)	.	:	C	.	.	:	.	.	.	.	C	.	:	T	.	C	T	G	.	.	.	T
Genbank	Qingtian carp	AY347296 (7)	.	.	.	G	.	.	A	.	.	.	C	.	:	T	.	.	.	.	.	.	.	T
Genbank	Xiniggo carp	AY345332, AY345335 (9)	.	.	.	G	.	.	A	.	.	.	C	.	:	T	.	.	.	.	.	.	.	T
Genbank	Yantze River wild carp	AY345334 (6)	C	.	C	G	.	.	A	.	.	.	C	.	:	T	.	.	.	.	.	.	.	T
Genbank	R. Amur wild carp	AF508936 (12)	.	.	.	.	.	.	.	.	.	G	.	T	:	T	.	.	.	.	.	.	.	T
Genbank	R. Amur wild carp	AF508937 (13)	.	.	.	.	.	.	.	.	.	G	.	.	:	T	.	.	T	.	.	.	.	T
Genbank	R. Amur wild carp	AF508938 (11)	.	.	.	.	C	.	.	.	.	.	.	.	:	T	.	.	T	.	.	A	.	T
Genbank	R. Amur wild carp	AF508935 (4)	.	.	.	G	.	.	.	.	.	.	.	.	:	.	.	.	.	.	.	.	.	.
Genbank	Russian carp	AY345333, AY345336 (8)	.	.	.	G	.	.	A	.	.	.	C	.	:	T	.	.	.	.	.	.	.	T
Genbank	Leather carp, mirror carp, R. Danube wild carp, koi	AF508931, AF508932, AF508929, AF508933 (3)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Genbank	Israel, Israel, Australia, Vietnam	AY597982, AY597981, AY597977, AY597968	-	-	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Genbank	Hungary	EU259966, EU259962, EU259953, EU259964 (3)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Genbank	Hungary	EU259954 (2)	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.
Genbank	Hungary	EU259952 (1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Genbank	Germany	AY345338 (3)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Genbank	German carp, Volga R. wild carp	AY345337, AY345339 (3)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Genbank	Volga River	AY345340 (5)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Table 6.3. (Continued).

Identifier	299	321	328	334	376	397	416	419	434	442	445	463	467	484	485	490	492	518	549	553	646	674	723	765	777	812-839	854	863	867	883	884	897	909
GR01, GR02, GR03	A	G	G	A	G	A	C	C	:	T	T	T	G	A	G	T	T	T	:	T	G	T	A	T	A	$(TA)_{14}$	T	C	C	:	:	.	.
R01, R02, R03, D01, AU01, AU02	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	$(TA)_{10}$	.	.	.	:	:	.	.
K01, K02	.	A	.	.	.	.	.	.	.	.	.	G	.	G	.	C	C	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AY597944	.	A	A	G	.	.	.	.	.	C	.	G	A	.	.	.	.	.	.	.	.	G	C	C	.	$(TA)_{9}$	.						
AY597943, AY597942	.	A	A	.	.	.	.	.	.	.	.	G	A	.	A	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.						
AY347304	.	A	A	.	.	.	.	.	A	C	.	G	A	.	.	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AB158811	.	A	.	.	.	.	.	.	.	.	.	G	.	G		C	C	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AY347298, AY347299	.	A	A	.	.	.	.	.	.	.	.	G	A	.	A	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AB158812	.	A	A	.	.	.	.	.	A	C	.	G	A	.	.	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AB158808	C	A	.	.	.	.	.	.	.	C	C	G	A	G	.	C	.	C	.	.	A	.	C	.	G	$(TA)_{9}$	:	T	T	:	:	C	A
AB158810	C	A	.	.	.	.	.	T	.	C	C	G	A	G	.	C	.	C	.	.	A	.	C	.	G	$(TA)_{9}$	:	T	T	:	:	C	A
AB158809	C	A	.	.	.	.	.	.	.	C	C	G	A	G	.	C	.	C	.	.	A	.	C	.	G	$(TA)_{10}$	:	T	T	:	:	C	A
AY347301	.	A	A	G	.	.	.	.	.	C	.	G	A	.	.	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AY347300	.	A	A	G	.	.	.	.	.	C	.	G	A	.	.	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AY347296	.	A	A	.	.	.	.	.	.	.	.	G	A	.	.	.	.	.	.	.	.	.	C	C	.	$(TA)_{10}$	.	.	.	:	:	.	.
AY345332, AY345335	.	A	A	.	.	.	.	.	.	.	.	G	A	.	A	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AY345334	.	A	A	.	A	.	.	.	.	.	.	G	A	.	A	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AF508936	.	A	.	.	.	.	.	.	.	C	.	G	A	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AF508937	.	A	A	.	.	.	.	.	A	C	.	G	A	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AF508938	.	A	.	G	.	.	.	.	.	C	.	G	A	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AF508935	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AY345333, AY345336	.	A	A	.	.	.	.	.	.	.	.	G	A	.	A	.	.	.	.	.	.	.	C	C	.	$(TA)_{9}$	.	.	.	:	:	.	.
AF508931, AF508932, AF508929, AF508933	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AY597982, AY597981, AY597977, AY597968	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	$(TA)_{10}$	.	-	-	-	-	-	-
EU259966, EU259962, EU259953, EU259964	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	$(TA)_{10}$	-	-	-	-	-	-	-
EU259954	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	$(TA)_{10}$	-	-	-	-	-	-	-
EU259952	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	$(TA)_{10}$	-	-	-	-	-	-	-
AY345338	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	$(TA)_{10}$	.	.	.	G	C	.	.
AY345337, AY345339	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	$(TA)_{10}$	.	.	.	:	:	.	.
AY345340	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	A	:	.	.	C	.	.	$(TA)_{10}$	.	.	.	:	:	.	.

Numbers indicate position of polymorphic bases based on the longest CR sequence (haplotype B, individuals GR01, GR02, GR03). Identity with the first sequence is denoted with dots (.), insertion-deletions that are not repeat motifs are denoted by colons (:), and missing data by dashes (-). The names of the mtDNA CR haplotypes identified in this study are listed in Table 6.2.

6.3.2. Primer design and PCR optimisation for HRMC analysis

A number of primers were designed to amplify the repeat motif and most of the HVR in the mtDNA CR in common carp. Primer positions are given in Figure 6.1, and sequences in Table 6.4. PCR was performed under a range of annealing temperatures, DNA and $MgCl_2$ concentrations and primer combinations, and PCR products were run on a 1.5%

agarose gel to assess quality and quantity. The primer pairs that gave the clearest PCR product for the two regions of interest were used in the subsequent rt PCR and HRMC analysis. For the HVR, the combination of the LD forward primer and the R8 reverse primer yielded the clearest and strongest PCR product. This product is 416-417bp in length and contains 25 identified polymorphic sites. The repeat motif at the 3' end of the mtDNA CR were best amplified with the F8 forward and HD reverse primers, to produce PCR products 234-245bp in length. Both products are shown in Figure 6.1.

Table 6.4. Primer sequences for the 5' highly variable region (HVR) and the 3' repeat motif region of the mtDNA CR locus**.** The location of the primer binding sites is on Figure 6.1.

5' hypervariable region		3' repeat motif $(TA)_{8-14}$	
Forward Primers		*Forward Primers*	
F1	AACTCTCACCCCTGGCTCC	F2	CCAAGGAGGACTCAAGAACG
LD*	TCACCCCTGGCTCCCAAAGC	F4	CGAAACCAAGGAGGACTCAA
Carp-Pro**	AACTCTCACCCCTGGCTACCAAA	F8	CAAACCCCGAAACCAAGGAGGAC
Reverse Primers		*Reverse Primers*	
R1	TCGGCATGTGGGGTA	HD*	CATCTTAGCATCTTCAGTG
R8	AAATAGGAACCAGATGCCAGTAA	Carp-Phe**	CTAGGACTCATCTTAGCATCTTCAG
R9	CACCATTAATCAGATGCCAGT	R3	TTTGGGGATTTTTGGTAGGG
		R7	TGGTAGGGACTTTTAGGTAAGTGG

* Zhou *et al* . (2004), ** Thai *et al.* (2004)

6.3.3. High-Resolution Melt-Curve analysis

Rt PCR and HMRC analysis was performed using a Rotorgene 6000 Real-Time PCR machine. PCR conditions for both regions of interest consisted of 1x PCR buffer (Invitrogen, Carlsbad), 1x LCGreen^{+} (Idaho Technology, Salt Lake City), 1.5mM $MgCl_2$, 0.2mM dNTPs, 0.2µM each primer, 5% DMSO, 25µg/ml BSA, 0.4 Units Platinum *Taq*-DNA polymerase (Invitrogen, Carlsbad), ~120ng genomic DNA and sterile water to 10µl total volume. Optimal cycling conditions were 4 min initial denaturation at 94°C, followed by 30 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 40 sec, then a final extension step of 10 min at 72°C, followed by HRMC. In the HRMC analysis, the temperature of the PCR products was raised from 75-85°C in 0.1°C increments, with a two-second hold at each increment.

The high-resolution melt curves were analysed using Rotor-gene series 6000 software version 1.7. In each analysis, the melt curve of a representative of each identified haplotype (A, B, C and G, see Results) was used as a control (Table 6.5).

The remaining melt curves (unknowns) were then compared with the control haplotypes. It is important to note that only a small subset of the haplotypes in Table 6.3 were analysed in the present study, and hence are included in Table 5. In this comparison, melt-curves for all products from a single PCR were first normalised, so that all curves from a single PCR had identical levels of fluorescence at the start (75°C) and at the end (85°C) of the HRMC analysis. A melt-curve profile for each unknown sample was then generated by graphing this normalised fluorescence against temperature. Following this, a confidence parameter (*C*) was calculated as:

$C = 1.05^{-0.02 \times S}$,

where $S = \sum_{i=T_s}^{T_e} (Unknown_i - Control_i)^2$, with T_s = start temperature, T_e = end temperature, and $Unknown_i$ and $Control_i$ being the height of the respective curves at temperature *i*.

If the highest *C* for an unknown with respect to a control was ≥ a given threshold, this unknown was allocated to the haplotype corresponding to that control. If the highest *C* for an unknown was < the given threshold in relation to any control, the unknown was not assigned to a haplotype (Speller, personal communication). After some preliminary testing, the threshold chosen for the present analysis was *C* = 80%.

For the HVR, carps AU01 and K01 and goldfish MGG8 were randomly chosen from within their relevant haplotype groups to be the controls for haplotypes A/B, C and G, respectively (see Table 6.5). Two replicates of each of the carps D01, R01 and GR01, and a second sample of carps AU01 and K01, were genotyped via PCR-HRMC analysis, by comparing their melt curves (unknown) with each of the control melt curves. Two unreplicated goldfish with identical mtDNA CR sequences (MGG6 and MGG7) were also genotyped by PCR-HRMC analysis, to check that carp and goldfish sequences could be distinguished, and that identical goldfish sequences also produced the same melt curves.

Individuals were genotyped for the 3' repeat motif in the same way as for the HVR, only with the addition of two replicates of individual GR02 (haplotype B), and with the first replicate of carp GR01 set as a control for haplotype B. GR02 was included to check that haplotype B melt-curves were consistently identified when amplified from different individuals.

6.4. Results

6.4.1. Identification of mitochondrial DNA polymorphisms

The mtDNA CR sequence polymorphisms identified in this study are summarised in Table 6.3, alongside those from the published literature. The individuals sequenced in this study revealed four previously unidentified mtDNA CR haplotypes: A, B, C and G (Table 6.2, Table 6.3). Haplotype A is present in the Australian feral carp (AU01, AU02), the Russian Ropsha strain carp (R01, R02, R03) and the wild carp from the River Danube (D01). Haplotype B was found in the German mirror scale carp (individuals GR01, GR02 and GR03). The Japanese koi carp (K01 and K02) have haplotype C. The three goldfish have haplotype G. None of the detected haplotypes is identical to any complete CR sequence known to be reported in the literature, although the 5' region of haplotypes A and B are identical to 15 of the partial sequences (Table 6.3).

Haplotypes A and B differ by their number of repeat motifs in the 3' end of the mtDNA CR (haplotype A is $(TA)_{10}$ and haplotype B $(TA)_{14}$) and at a SNP [A/C] at sequence position 723. Both haplotypes are very similar to sequences reported from European carp by Froufe et al. (2002) and Zhou et al. (2004b) and Wang and Li (unpublished) and to sequences reported from carp of European descent in Australia, Israel and Vietnam by Thai et al. (2006). The presence of European-type haplotypes in the Ropsha and Australian carp suggests that these carp have European maternal ancestry. Not surprisingly, the Asian haplotype C is very different from the European haplotypes A and B, whereby it is $(TA)_9$ at the 3' repeat motif and differs from A and B at 12 other polymorphic sites. Haplotype G is highly divergent from the two carp haplotypes, differing from haplotype A at a total of 109 sites, haplotype B at 108 sites, haplotype C at 109 sites, and being $(TA)_8$ at the 3' repeat motif.

The relationship between haplotypes A, B, and C and the mtDNA CR sequences from Genbank is illustrated in a median-joining network in Figure 6.2. Haplotypes A and B are

grouped with European carp (haplotypes 1-5) and haplotype C with Japanese food carp (haplotype 21). This is consistent with Thai et al. (2004a) where mtDNA sequences from Australian carp were identical to those of European carp. Also consistent with previous studies, the Japanese Lake Biwa carp form a lineage basal to all other carp strains (Mabuchi *et al.* 2005), and one of the koi (AF508933) and one of the Russian carp (AF508935) cluster with the European carp, indicative of those particular fish having a European maternal ancestor (Froufe *et al.* 2002).

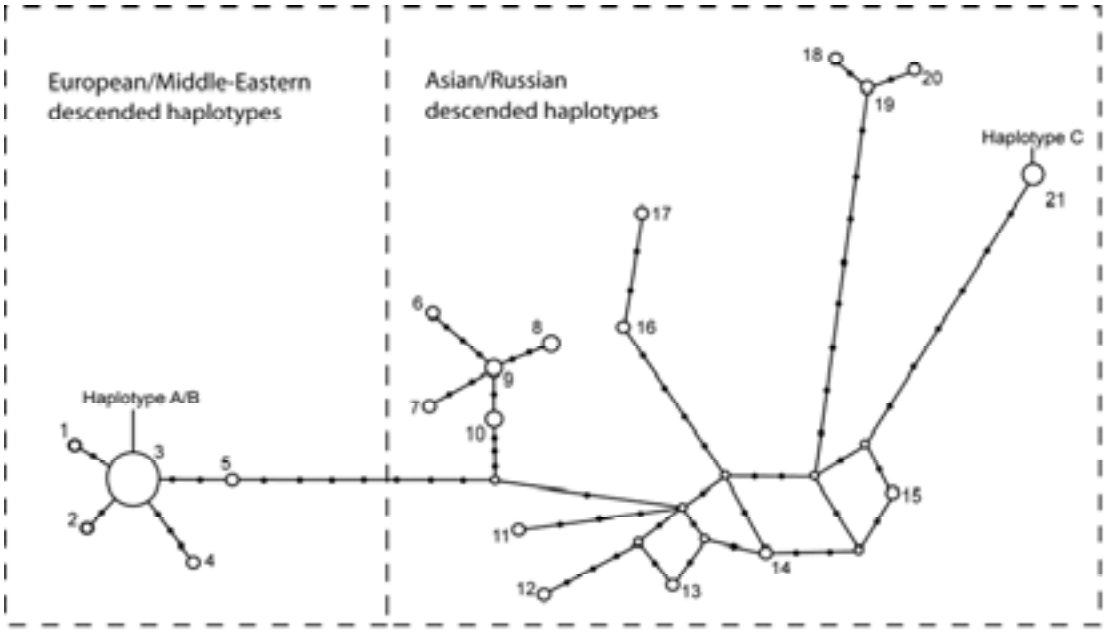

Figure 6.2. Median-joining network of the first 510 base pairs of the mtDNA CR in common carp. Numbered circles represent haplotypes listed in Table 6.1. Small, black, unlabelled circles represent putative mutation events, and small, white, unlabelled circles represent theoretical ancestral haplotypes.

6.4.2. High-Resolution Melt Curve Analysis

The HRMC analysis was able to identify each of the four mtDNA CR haplotypes (A, B, C and G) described and investigated in this study. On the basis of pre-determined sequence, it is evident that no unknown was misassigned across the two regions amplified. Only one individual (MGG6) could not be assigned with greater than 80% confidence to any of the haplotypes used as controls, and this was likely a result of poor PCR amplification. In other words, amongst all the unknowns that could be assigned, each was correctly assigned to its haplotype. Amongst those unknowns that could be assigned a haplotype, the average

confidence was 95.8% (range 93.9%-99.4%) for replicates of the control animals, and 95.0% (range 80.6% – 100.0%) for samples from other animals. The melt curve generated from either the HVR or the repeat motif was sufficient to correctly identify haplotypes C and G. Haplotypes A and B, however, have identical HVR sequences, and hence could be accurately identified only if the repeat motif was analysed. HRMC results are summarised in Table 6.5, and the two different melt-curve profiles (one for each region) are illustrated in Figure 6.3.

Table 6.5. Mitochondrial haplotypes (HVR region and 3'repeat motif) detected by the high-resolution melt curve analysis.

Individual	**replicate**	**HVR**			**3' repeat motif**	
		Known Haplotype	Detected Haplotype[†]	*C* (%)	Detected Haplotype	*C* (%)
AU01	1	A	A/B*	100.0	A*	100.0
AU01	2	A	A/B	99.4	A	95.6
D01	1	A	A/B	99.9	A	94.6
D01	2	A	A/B	98.1	A	91.2
R01	1	A	A/B	97.0	A	92.8
R01	2	A	A/B	94.5	A	80.6
GR01	1	B	A/B	96.0	B*	100.0
GR01	2	B	A/B	97.0	B	93.9
GR02	1	B	-	-	B	100.0
GR02	2	B	-	-	B	98.7
K01	1	C	C*	100.0	C*	100.0
K01	2	C	C	95.5	C	94.3
MGG6	1	G	Not assigned	-	G	97.6
MGG7	1	G	G	82.3	G	96.3
MGG8	1	G	G*	100.0	G*	100.0

[†] Haplotypes A and B are indistinguishable at the HVR

* Set as controls (hence the 100% C).

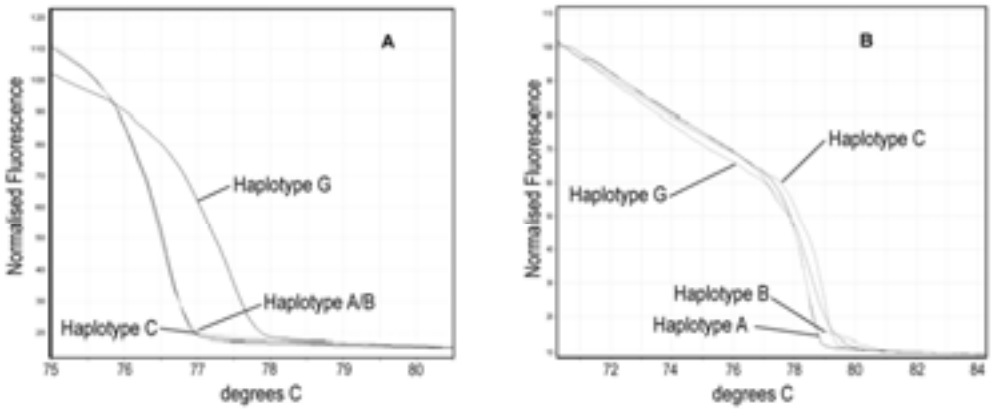

Figure 6.3. Melt curve profiles of mitochondrial control region haplotypes A, B, C and G. **A**. HVR, **B**. 3' repeat motif loci.

As expected, there is some consistency between the differences in the curves in Figure 6.3 and the sequence differences in the haplotypes. In the HVR (Figure 6.3A), the melt curve for haplotype G is markedly different from the other melt curves, consistent with its sequence being highly divergent in this region. In contrast, the curves for haplotypes A/B and C are much more similar, consistent with their differing at only 5 sites in the same region. In the 3' repeat-motif region (Figure 6.3B), the differences between the curves are far less marked, consistent with the haplotypes differing only in total length by a very small number of bases. Even though differences as small as this are difficult to discern by eye in Figure 6.3, the photometric analysis was able to distinguish between them with a high degree of confidence.

6.5. Discussion

6.5.1. HRMC Analysis and haplotype identification

In the protocol presented here, HRMC analysis is used to rapidly and accurately identify different mtDNA CR haplotypes in common carp. Two regions of the mtDNA CR locus are amplified by rtPCR, and HRMC analysis is performed on the PCR products. Different sequences are identified by their unique melt-curve profiles. Although DNA sequencing is the most accurate way to identify different haplotypes, HRMC offers a faster and cheaper alternative, and has been shown here to be able to distinguish haplotypes that differ by as few as two bases.

HRMC analysis has a number of advantages over other methods for DNA polymorphism screening. These are (1) rapidity, taking less than three hours from start to finish; (2) reduced risk of contamination and human error as all procedures take place in closed PCR tubes; (3) homogeneity of PCR thermal cycling conditions in the Rotorgene 6000 between different PCR tubes, unlike traditional block-based PCR machines; and (4) the progress and success of each individual PCR is monitored in real-time.

The HRMC analysis also has some limitations. When optimising PCR conditions on the Rotorgene 6000, we found that the PCR was very sensitive to DNA quality and concentration, with the PCRs failing if too much DNA or degraded DNA was used. DNA template must therefore be of high quality and must be accurately quantified to avoid overloading.

6.5.2. Choice of regions

The primers used in the present study were specifically designed to capture as much genetic variation as possible, with the two regions of the CR analysed comprising around 650bp and containing a total of 35 sites known to be polymorphic amongst carp, including a 3' $(TA)_{8-14}$ repeat motif, which makes it possible to distinguish between otherwise invariable European carp mtDNA CR haplotypes. By comparison, the smaller 230 bp fragment of the mtDNA CR screened by Thai et al. (2006) contains only 17 polymorphic sites, none of which is known to vary amongst European carp.

6.5.3. Other methods for rapid scoring of mitochondrial haplotypes

A number of other methods have been devised for the rapid scoring of mitochondrial DNA in common carp. All such methodologies forgo the accuracy of directly sequencing the locus/region of interest in favour of being able to score the locus/region in a large number of individuals in a faster and more cost-effective manner. These techniques are discussed below.

Davis et al. (1999) digested whole mitochondrial genomes, extracted from muscle and gonad tissue, with three restriction enzymes. Three composite haplotypes were identified and used to infer where different strains of common carp had become established or were introduced in Australia. The requirement to extract whole mitochondrial genomes makes

this technique impractical for studies where large amounts of high quality tissue are not available.

Gross et al. (2002) used PCR-RFLP to analyse the mitochondrial ND-3/4 and ND-5/6 genes. The loci were amplified in two separate PCR reactions, and PCR products were digested with ten restriction enzymes. Composite haplotypes were inferred from the presence and absence of restriction sites. This technique was used in both the Gross et al. (2002) study and in subsequent population genetic studies (Kohlmann *et al.* 2003; Memiş and Kohlmann 2006) to infer relatedness between carp populations from different regions. This technique has many strengths. The use of PCR allows it to be applied even when only small amounts of DNA are available, and digestion of DNA with restriction enzymes is generally robust and reproducible. The choice of loci made it possible to detect polymorphisms amongst the mitochondrial DNA of European carp populations (Gross *et al.* 2002). The technique is limited, however, in that multiple restriction enzymes must be purchased, and that all DNA digestions (20 in this case) have to be performed in separate tubes and analysed individually.

More recently, Thai et al. (2006) developed an SSCP protocol to screen genetic variation in the carp mtDNA CR. Fourteen SSCP haplotypes, out of 25 determined by sequencing, were resolved using this method. Upon close inspection of the sequences used by Thai et al. (2006), six different sequences were all identified as SSCP haplotype C, and two as haplotype J. SSCP techniques also can be difficult to reproduce in different laboratories, and are likely less accurate than HRMC analysis as conditions in the closed PCR tubes used in HRMC analysis are more controlled and more homogeneous than conditions in the polyacrylamide gels used in SSCP, and HRMC information is read with a laser instead of by eye.

6.5.4. Recommendations for using HRMC analysis

The following recommendations are made for researchers wishing to use real-time PCR and HRMC analysis to score mtDNA haplotypes in common carp. (1) DNA should of the highest quality and extracted using appropriate methodologies, to remove PCR inhibitors, RNA and degraded DNA template. (2) DNA should be accurately quantified, using a NanoPhotometer™ or equivalent high-accuracy technology. (3) Several known representatives of each haplotype should be used as controls. (4) After the HRMC run, the

haplotypes of a sample of unassigned individuals should be sequenced. (5) If new haplotypes are identified, it is then a simple matter to reanalyse the existing melt-curve analysis data (i.e. without doing another HRMC run), with one or two individuals from each of the new haplotypes set as controls. This will automatically identify all samples with the new haplotypes.

Based on current prices, the total cost of the HRMC methodology is significantly less than the cost of directly sequencing large numbers of individuals at commercial sequencing rates. This is especially the case, when the number of haplotypes identified is much smaller than the number of samples under investigation.

6.5.5. Uses and future direction

The rapid identification of mitochondrial DNA haplotypes using real-time PCR and HRMC analysis has a wide range of applications. These include screening of wild and domestic common carp populations to identify unique or rare strains for conservation or selective breeding; measuring the contribution of escaped domestic carp strains to wild populations; investigating the direction (sex or species bias) of introgression in regions where multiple species of closely related cyprinids co-occur; and investigating interrelatedness between common carp populations. Hänfling et al. (2005) used six nuclear microsatellites to investigate interbreeding between the related cyprinids common carp, goldfish (*C. auratus*) and gibel carp (*Carassius* spp.) in the UK, and detected F1 hybrids and backcrossed individuals. The HRMC protocol outlined here could build upon this research by scoring mtDNA CR of the fish from that study. This information could in turn by used to infer whether the introgression was sex or species biased, and to what extent mitochondrial lineages characteristic of one species are persisting in another. The technique easily distinguishes between the mtDNA CR haplotypes of common carp and goldfish, and would in all likelihood be able to distinguish gibel carp haplotypes also.

Previous studies of European carp have found very little mitochondrial DNA diversity (Froufe *et al.* 2002; Mabuchi *et al.* 2005). Only Gross et al. (2002), Li and Wang (unpublished) and this study have detected mitochondrial variation amongst European carp races. Kohlmann et al. (2003) also detected mitochondrial variation within European populations, but attributed it to the release of Asian carp into the River Danube. The relatively low variation has been attributed to carp undergoing a series of bottlenecks as

they expanded their distribution from East Asia, where they originated, to Europe (Kohlmann et al., 2003). Additional work to identify more polymorphisms amongst European carp races for screening with HRMC would therefore be useful.

Although the method outlined here is optimised for scoring common carp mitochondrial haplotypes, rtPCR and HRMC analysis can be applied in a similar way to almost any species and/or loci to score genetic differences. A range of real-time PCR machines are available for this in addition to the Rotorgene 6000, such as the HR-1™ System (Idaho Technology Inc.) and the LightCycler ® (Roche). PCR-HRMC analysis has already been used effectively in a diverse range of studies to identify sequence differences in both diploid (McKinney *et al.* 2004; Zhou *et al.* 2004c; Zhou *et al.* 2004d) and haploid (Cheng *et al.* 2006; Dames *et al.* 2007; Fortini *et al.* 2007; Nakagawa *et al.* 2008) genomes; has been shown to be highly accurate, both here and in previous studies (Reed *et al.* 2007; Vandersteen *et al.* 2007); and has much to offer the study of aquacultural species.

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6.7. References

Bandelt, H.-J., Forster, P., Sykes, B.C., Richards, M.B., 1995. Mitochondrial portraits of human populations. Genetics 141, 743-753

Brandstätter, A., Niederstätter, H., Parson, W., 2004. Monitoring the inheritance of heteroplasmy by computer-assisted detection of mixed basecalls in the entire human mitochondrial DNA control region. Int J Legal Med 118, 47-54.

Cheng, J.C., Huang, C.L., Lin, C.C., Chen, C.C., Chang, Y.C., Chang, S.S., Tseng, C.P., 2006. Rapid detection and identification of clinically important bacteria by high-

resolution melting analysis after broad-range ribosomal RNA real-time PCR. Clinical Chemistry 52, 1997-2004.

Dames, S., Pattison, D.C., Bromley, L.K., Wittwer, C.T., Voelkerding, K.V., 2007. Unlabeled probes for the detection and typing of herpes simplex virus. Clinical Chemistry 53, 1847-1854.

Davis, K.M., Dixon, P.I., Harris, J.H., 1999. Allozyme and mitochondrial DNA analysis of carp, *Cyprinus carpio* L., from south-eastern Australia. Marine and Freshwater Resources 50, 253-260.

FAO, 2007. Yearbook of Fisheries Statistics Summary Table. Available at: http://www.fao.org/fi/statist/summtab/default.asp.

Fortini, D., Ciammaruconi, A., De Santis, R., Fasanella, A., Battisti, A., D'Amelio, R., Lista, F., Cassone, A., Carattoli, A., 2007. Optimization of high-resolution melting analysis for low-cost and rapid screening of allelic variants of *Bacillus anthracis* by multiple-locus variable-number tandem repeat analysis. Clinical Chemistry 53, 1377-1380.

Froufe, E., Magyary, I., Lehoczky, I., Weiss, S., 2002. mtDNA sequence data supports an Asian ancestry and single introduction of the common carp into the Danube Basin. Journal of Fish Biology 61, 301-304.

Gross, R., Kohlmann, K., Kersten, P., 2002. PCR-RFLP analysis of the mitochondrial ND-3/4 and ND-5/6 gene polymorphisms in the European and East Asian subspecies of common carp (*Cyprinus carpio* L.). Aquaculture 204, 507-516.

Hänfling, B., Boton, P., Harley, M., Carvalho, G.R., 2005. A molecular approach to detect hybridisation between crucian carp (*Carassis carassius*) and non-indigenous carp species (*Carassisus* cpp. and *Cyprinus carpio*). Freshwater Biology 50, 403-417.

Koehn, J., Brumley, B., Gehrke, P., 2000. Managing the Impacts of Carp. Bureau of Rural Sciences (Department of Agriculture, Fisheries and Forestry - Australia), Canberra.

Kohlmann, K., Gross, R., Murakaeva, A., Kersten, P., 2003. Genetic variability and structure of common carp (*Cyprinus carpio*) populations throughout the distribution range inferred from allozyme, microsatellite and mitochondrial DNA markers. Aquatic Living Resources 16, 421-431.

Mabuchi, K., Senou, H., Nishida, M., 2008. Mitochondrial DNA analysis reveals cryptic large-scale invasion of non-native genotypes of common carp (*Cyprinus carpio*) in Japan. Molecular Ecology 17, 796-809.

Mabuchi, K., Senou, H., Suzuki, T., Nishida, M., 2005. Discovery of an ancient lineage of *Cyprinus carpio* from Lake Biwa, central Japan, based on mtDNA sequence data, with reference to possible multiple origins of koi. Journal of Fish Biology 66, 1516-1528.

McKinney, J.T., Longo, N., Hahn, S.H., Matern, D., Rinaldo, P., Strauss, A.W., Dobrowlski, S.F., 2004. Rapid, comprehensive screening of the human medium chain acyl-CoA dehydrogenase gene. Molecular Genetics and Metabolism 82, 112-120.

Memiş, D., Kohlmann, K., 2006. Genetic characterization of wild common carp (*Cyprinus carpio* L.) from Turkey. Aquaculture 258, 257-262.

Nakagawa, T., Higashi, N., Nakagawa, N., 2008. Detection of antigenic variants of the influenza B virus by melting curve analysis with LCGreen. Journal of Virological Methods 148, 296-299.

Reed, G.H., Kent, J.O., Wittwer, C.T., 2007. High-resolution DNA melting analysis for simple and efficient diagnostics. Pharmacogenomics 8, 597-608.

Riesner, D., Steger, G., Zimmat, R., Owens, R.A., Wagenhöfer, M., Hillen, W., Vollbach, S., Henco, K., 1989. Temperature-gradient gel electrophoresis of nucleic acids: Analysis of conformational transitions, sequence variations, and protein-nucleic acid interactions. Electrophoresis 10, 377-379.

Thai, B.T., Pham, T.A., Austin, C.M., 2006. Genetic diversity of common carp in Vietnam using direct sequencing and SSCP analysis of the mitochondrial DNA control region. Aquaculture 258, 228-240.

Thai, B.T., Burridge, C.P., Pham, T.A., Austin, C.M., 2004. Using mitochondrial nucleotide sequences to investigate diversity and genealogical relationships within common carp (*Cyprinus carpio* L.) Animal Genetics 36, 23-28.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Research 22, 4673-4680.

Vandersteen, J.G., Bayrak-Toydemir, P., Palais, R.A., Wittwer, C.T., 2007. Identifying common genetic variants by high-resolution melting. Clinical Chemistry 53, 1191-1198.

Zhou, J.F., Wu, Q.J., Ye, Y.Z., Tong, J.G., 2003. Genetic divergence between *Cyprinus carpio carpio* and *Cyprinus carpio haematopterus* as assessed by mitochondrial

DNA analysis, with emphasis on the origin of European domestic carp. Genetica 119.

Zhou, J.F., Wu, Q., Wang, Z., Ye, Y., 2004a. Molecular phylogeny of three subspecies of common carp *Cyprinus carpio*, based on sequence analysis of cytochrome *b* and control region mtDNA. Journal of Zoological Systematics and Evolutionary Research 42, 266-269.

Zhou, L., Vandersteen, J., Wang, L., Fuller, T., Taylor, M., Palais, B., Wittwer, C.T., 2004b. High-resolution DNA melting curve analysis to establish LHA genotypic identity. Tissue Antigens 64, 156-164.

Zhou, L.M., Myers, A.N., Vandersteen, J.G., Wang, J.G., Wittwer, C.T., 2004c. Closed-tube genotyping with unlabeled oligonucleotide probes and a saturated DNA dye. Clinical Chemistry 5, 1328-1335.

Chapter 7: General discussion and conclusions

7.1. Summary of findings

The population genetics of common carp in the MDB has been comprehensively investigated in this Ph.D. The findings of this project are listed below.

1. No fewer than four strains of carp exist in Australia: Boolara, Prospect, Yanco and Koi.
2. The origin of the Yanco, Boolara and Prospect strains has been confirmed as European (*C. carpio carpio*). The Koi carp, as expected, belong to the Asian subspecies (*C. carpio haematopterus*).
3. The four strains are differently distributed throughout the MDB (Table 7.1).
4. The carp population in the MDB is somewhat structured, owing in part to the differential distribution of the different strains, and in part to man-made and natural barriers to dispersal that prevent the population(s) becoming panmictic. The natural longevity of carp may also have a role in slowing the cap population in the MDB becoming panmictic. While the younger carp are the result of multiple generations of inter-strain breeding and presumably are becoming more homogeneous with each generation, the older individuals are likely similar to the original founding strains. As the older carp are not evenly distributed throughout the basin, genetic structuring is detected when these fish are collected and analysed with their cross-bred progeny. The genetic structuring of carp in the MDB does not follow an isolation-by-distance type pattern, consistent with a lack of regional equilibrium, and migration and gene flow play a larger role in shaping genetic structure than genetic drift.
5. A history of the introduction and spread of carp in the MDB is proposed, based on demographic, historical and genetic data (see *Chapter 3* for details).
6. The MDB can be divided into 15 management units.
7. The three east-coast carp populations assessed show strong genetic structuring and consist of different proportions of the four carp strains (Table 7.1).
8. Of the 14 microsatellite loci used in the project, five can be amplified reliably in goldfish and are hence suitable for cross-species studies.
9. Low levels of introgression occur between feral carp and goldfish in the MBD. Goldfish may therefore act as a reservoir of genetic diversity for common carp and vice versa.

Table 7.1. Distribution of the different strains of common carp in Australia (see *Chapters 3 and 4*).

Strain	Range		
	MDB	**Coastal waterways**	**Tasmania**
Prospect	Ubiquitous, dominant above Burrendong Dam.	All carp in Prospect Reservoir belong to this strain. Dominant in Hawkesbury-Nepean River, smaller contribution to Parramatta and Hunter Rivers.	Major contribution
Boolara	Ubiquitous below major dams; rare above Wyangala, Burrendong and Burrinjuck Dams.	Minor contribution to Parramatta, Hawkesbury-Nepean Rivers; major contribution to the Hunter River.	Major contribution
Yanco	Dominant in the Murrumbidgee River and several other NSW rivers. Rare in the Macquarie River and the rivers in Victoria and Queensland.	Minor contribution to Parramatta, Hawkesbury-Nepean and Hunter Rivers.	Very minor contribution or absent
Koi	Rare.	Dominant in Parramatta River, minor contribution to Hawkesbury-Nepean and Hunter Rivers.	Very minor contribution or absent

7.2. Reinterpretation of previous population genetic studies of carp in Australia

There are two key findings in this study that affect how the results of the previous studies by Davis *et al.* (1999) and Shearer and Mulley (1978) are interpreted. These are (1) the widespread distribution of Prospect-strain carp in the MDB, and (2) the differential distribution of the different carp strains throughout the MDB.

The abundance of the Prospect-strain individuals in the MDB explains why Davis *et al.* (1999) could not differentiate between carp in the MDB that were thought to be Boolara strain and carp in Potts Point Reservoir which were thought to be Prospect strain. This also negates the commonly held view (Koehn *et al.* 2000) that dominance of carp in the MDB is due solely to the release of the Boolara strain. Additionally, it puts into doubt the accuracy of measures used by Shearer and Mulley (1978) to differentiate between Yanco, Prospect and Boolara carp. While Yanco carp could be readily identified by their orange

colouration, the individuals used by Shearer and Mulley (1978) to represent the Boolara carp in this study may have in fact been a mixture of Boolara and Prospect-strain individuals. This does not put into doubt, however, the existence of the two strains themselves, as there is abundant historical and genetic evidence to support their distinctiveness.

The inability of Davis *et al*. (1999) to discern a clear pattern from the genetic structuring of carp in the MDB can be explained by the differential distribution of the different strains detected in this study. The genetic structuring is partially derived from these strains being present at different proportions in different regions. Without the knowledge that four strains were present, had been distributed by human activities and were at different proportions in different regions, the distribution of different alleles and significant genetic differences between sample sites would appear puzzling.

7.3. Implications of this research

A range of control strategies are currently being used or considered for the control of carp in Australian waters. These are detailed in Gilligan and Rayner (2007) and can be divided into two groups: physical controls and biological controls.

Physical controls are the methods that involve physical removal of carp from waterways, or physical exclusion of carp from breeding sites. They include:

1. Financial subsidisation of commercial carp fisheries.
2. The inclusion of William's carp separation cages (Stuart *et al.* 2006a) in fishways. These cages exploit the tendency of common carp to jump, and fish indigenous to Australia not to jump, over obstacles while swimming. These cages temporarily trap migrating fish in the fishways. The carp subsequently jump into a specifically designated holding cage from which they are later removed, while the native fish remain in the fishway. The carp separation cage is periodically opened to let the native fish pass.
3. The use of a Judas fish involves the release of a radio-tagged fish (the 'Judas' fish) into waterways infested with carp. As carp are a schooling species, this fish will locate and school with other carp. The school of carp can then be located via the radio tag and fished from waterways.

4. Physical exclusion of carp from breeding sites either using mesh too fine for adult carp to pass through, or manipulation of water levels so that ideal breeding sites remain dry throughout the carp breeding season.

Gilligan and Rayner (2007) suggest three possible biological controls for the management of carp in Australia. These are the release of the koi herpes virus, the release of carp pox and the use of daughterless carp gene technology. The CSIRO Livestock Industries' Australian Health Laboratory is currently assessing koi herpes virus as a potential biological control agent, while the daughterless technology is still under development in CSIRO Marine and Atmospheric Research Laboratories. Carp pox is not currently under consideration as a biological control, but may be considered in future to be used in conjunction with other biological control agents. It may be difficult to gain approval for the release of disease against carp, as these diseases could impact the trade in ornamental koi and goldfish, and could pose a risk to native species.

The most important achievement of this research has been to devise management units for control of carp in the MDB. These 15 units can assist in the targeting of the control programs described above, in such ways as identifying areas where physical removal will be most effective (smaller management units with limited immigration), identifying regions from which areas could be recolonized after control (upstream management units or within the same management unit), and predicting which regions will be affected by the release of biological controls (downstream management units).

The introgression detected between carp and goldfish in *Chapter 5* could affect the implementation of biological controls. If diseases to which goldfish are immune are released to control carp, individual alleles conveying immunity carried by goldfish could spread rapidly through the carp population and negate the effect of the disease. In addition, the goldfish could act as a reservoir of genetic diversity that undermines the effect of the daughterless-gene technology.

Ultimately, control of carp in Australia requires an integrated approach that incorporates physical controls, biological controls, public education and remediation of MDB habitats and flow regimes to make habitats more suitable for native species and less so for common carp.

7.4. Future research

There are a range of future research possibilities for the study of carp in Australia. These include improving the accuracy and resolution of this study, further investigating the population genetics of the many coastal populations of carp, further study of introgression between carp and goldfish, and investigating the presence of crucian carp (*Carassius carassius*).

7.4.1. Improving accuracy and power of this study

In all genetic studies, the accuracy and power of statistical analysis is proportional to the number of genetic markers used. More loci means more accuracy and power, and hence more confidence in the findings. The inclusion of more microsatellite or other types of DNA markers would therefore increase the resolution of the research reported in this thesis.

The inclusion of additional outgroups would also improve the resolution of future studies. Such outgroups could include:

- 1-2 strains representative of the south-east Asia carp subspecies *C carpio. viridiviolaceus* (Kirpichnikov 1981; Zhou *et al.* 2004b) ;
- 1-2 Chinese strains, which would be better representative of *C. carpio. haematopterus* than Koi;
- 1-2 additional European strains;
- a population of Boolara carp that has not been interbred with other carp varieties (available from Gippsland, Victoria); and
- additional Prospect strain carp, caught from Prospect Reservoir and/or Potts Point Reservoir (Davis 1996).

Application of the RT-PCR and HRMC analysis protocol detailed in *Chapter 5* to all the carp samples analysed in this Ph.D. would also add to the value of future studies, as novel mitochondrial variants could be identified and the extent of Asian carp and goldfish maternal introgression into the MDB carp population could be quantified (this was not done in this study due to time and budgeting constraints).

Improving this study could possibly alter the recommendations made for management. New management units could be delimited and/or previous units could be found to be undifferentiated. In addition, the history of introduction and range expansion proposed in *Chapter 3* could be altered. However, a comprehensive sampling regime and a relatively large number of microsatellite loci were used in this study, to minimise this possibility. Such improvements would therefore be unlikely to indicate required changes to management strategy.

7.4.2. Study of coastal populations

A detailed population genetic study of the coastal populations of common carp in Australia would be useful for control of carp in these regions, for the same reasons that the present study is useful for control of carp in the MDB. Emphasis would be placed, however, on genetic structuring between catchments, rather than between regions within the same catchment, as was the main focus of this Ph.D. research.

7.4.3. Further study of carp-goldfish introgression

In this study, only five loci were used to quantify introgression between carp and goldfish. Such a small number of loci have limited power and accuracy. However, cross-species amplification has been optimised for another five loci by Hänfling et al. (2005), making a total ten such loci available. Simulations by Vähä and Primmer (2006) indicate that at least 12 loci are required for accurate assignment in cross-species studies, so it might be optimal to optimise cross-species amplification in at least another two microsatellite loci. More cross-species PCR of microsatellite loci could also be made possible by sequencing published microsatellites and their flanking regions in both species, identifying conserved regions and designing primers to anneal in those conserved regions. However, as, the loci used here and by Hänfling *et al*. (2005) have been shown to be highly effective at detecting cross-species introgression, so optimising cross-species PCR in for additional loci may not be necessary.

7.4.4. Investigating the presence of crucian carp

The presence of crucian carp in the MDB was reported by Whitley (1951), was later refuted by museum curators in 1980 (Clements 1988), and has recently been confirmed in the Campaspe River (a tributary to the Murray River) in eastern Victoria, based on morphological characteristics (MDBC 2008b). Crucian carp are very similar to goldfish in

appearance, and the two species can be easily confused. Confirming the presence of crucian carp with genetics would therefore be worthwhile.

Confirming the presence of crucian carp would require genetic markers that amplify in carp, goldfish and crucian carp, and a reference sample of crucian carp. The six genetic markers used by Hänfling *et al*. (2005) were shown to be effective at distinguishing between the three species. In addition, mitochondrial sequencing (or screening using the real-time PCR and HRMC analysis protocol detailed in *Chapter 6*) would also likely separate the three species. A reference sample of crucian carp could also be obtained from the UK in the same manner as described by Hänfling *et al*. (2005).

Once the genetic markers and reference samples were available, putative crucian carp could readily be identified by assignment testing (*Chapter 5*), and inter-species introgression could be quantified as described in *Chapter 5*.

7.5. References

Clements J (1988) *Salmon at the Antipodes A History and Review of Trout, Salmon and Char and Introduced Coarse Fish in Australasia,* Ballarat, Victoria, Australia, Published by the author. ISBN: 0731637194.

Davis KM (1996) Investigation into the genetic variation of carp (*Cyprinus carpio*) in Southeastern Australia. Sydney, Australia, University of NSW.

Davis KM, Dixon PI, Harris JH (1999) Allozyme and mitochondrial DNA analysis of carp, *Cyprinus carpio* L., from south-eastern Australia. *Marine and Freshwater Resources,* **50,** 253-60. doi: 10.1071/MF97256

Gilligan D, Rayner T (2007) The distribution, spread, ecological impacts, and potential control of carp in the upper Murray River. NSW Department of Primary Industries, Sydney.

Hänfling B, Bolton P, Harley M, Carvalho GR (2005) A molecular approach to detect hybridisation between crucian carp (*Carassis carassius*) and non-indigenous carp species (*Carassisus* cpp. and *Cyprinus carpio*). *Freshwater Biology*, **50,** 403-417. doi:10.1111/j.1365-2427.2004.01330.x.

Kirpichnikov VS (1981) *Genetic Bases of Fish Selection,* Berlin and Heidelberg, Germany, New York, USA, Springer-Verlag. ISBN: 0387109110.

Koehn J, Brumley B, Gehrke P (2000) *Managing the Impacts of Carp,* Canberra, Australia, Bureau of Rural Sciences (Department of Agriculture, Fisheries and Forestry). ISBN 0644292407 (set) 0642732019.

MDBC (2008) Sustainable rivers audit report. Murray-Darling Basin Commission,

Shearer KD, Mulley JC (1978) The introduction and distribution of the carp, *Cyprinus carpio* Linnaeus, in Australia. *Australian Journal of Marine and Freshwater Research,* **29,** 661-563. doi:10.1071/MF9780551

Stuart I, Williams A, McKenzie J, Holt T (2006) The Williams' cage: a key tool for carp management in Murray-Darling Basin fishways. Arthur Rylah Institute for Environmental Research, Department of Sustainability and Environment, Heidelberg, Victoria.

Vähä JP, Primmer CR (2006) Efficiency of model-based Bayesian methods for detecting hybrid individuals under different hybridization scenarios and with different numbers of loci. *Molecular Ecology,* **15,** 63–72. doi:10.1111/j.1365-294X.2005.02773.x

Whitley GP (1951) Introduced Fishes-II. *The Australian Museum Magazine,* **10,** 234-238.

Zhou JF, Wu Q, Wang Z, Ye Y (2004) Molecular phylogeny of three subspecies of common carp *Cyprinus carpio*, based on sequence analysis of cytochrome *b* and control region mtDNA. *Journal of Zoological Systematics and Evolutionary Research,* **42,** 266-269. doi: 10.1111/j.1439-0469.2004.00266.x.