The evolutionary significance of Balston's Pygmy Perch and Mud Minnow populations in the Blackwood River

Prepared for the Department of Water
June 2007

Centre for Fish and Fisheries Research

MURDOCH UNIVERSITY
PERTH, WESTERN AUSTRALIA

The evolutionary significance of Balston's Pygmy Perch and Mud Minnow populations in the Blackwood River

Report to:
Department of Water
June 2007

Prepared by:
N. Phillips, J. Chaplin, D. Morgan & S. Beatty
Centre for Fish & Fisheries Research
Murdoch University

Acknowledgements:
This work was funded by the Department of Water and we appreciate the project co-ordination by Robert Donohue.

Cover page: Milyeannup Pool on the Blackwood River and Balston's Pygmy Perch (inset left) and the Mud Minnow (inset right).

Executive Summary

The aims of this study were to use genetic (mtDNA) data to assess: (1) the evolutionary significance of the populations of the Balston's Pygmy Perch (*Nannatherina balstoni*) in the Blackwood River; and (2) the connectivity among populations of the Mud Minnow (*Galaxiella munda*) in different tributaries in the Blackwood River. This work was commissioned by the Department of Water in view of the potential vulnerability of the populations of these threatened species due to historical and future environmental change.

The following specimens of Balston's Pygmy Perch were included in the study. Twenty individuals from Milyeannup Brook in the Blackwood River, 4 individuals from McAtee Brook, also in the Blackwood River, as well as 21 individuals from the Gardner River watershed, 5 individuals from Margaret River, 11 individuals from the Shannon River, 13 individuals from Deep River, 2 individuals from the Angove River and 1 individual from pools on Deeside Coast Road (Shannon River). Genetic data were ultimately obtained for all specimens, except the five from Margaret River. The samples from outside of the Blackwood River were used to assess the distinctiveness of samples from within this river system.

The following specimens of the Mud Minnow were included in the study. (i) 65 individuals from five sites within the Blackwood River, comprising 18 individuals from St John Brook, 18 individuals from Red Gully, 14 individuals from Milyeannup Brook, 13 individuals from Rosa Brook, and 2 individuals from McAtee Brook; and (ii) 27 individuals from three sites outside of the Blackwood River, comprising 10 individuals from Margaret River, 9 individuals from Deep River and 8 individuals from pools on the Deeside Coast Road (Shannon River). Genetic data were obtained for all of these individuals. The samples from outside of the Blackwood River were used to assist in interpreting the relationships among those from within the Blackwood River.

The methodological and analytical approaches used for both species study were similar. Both sets of results are based on nucleotide sequence variation in a portion of the control region of the mitochondrial DNA (mtDNA). This type of approach was selected because it provides a relatively inexpensive means of generating information about the distribution of genetic variation within and between the populations of a species. Polymerase chain reaction (PCR) was used to amplify the target portion of the control region from each fish specimen, while automated sequencing methods were used to determine the nucleotide composition of the amplification product. The resultant partial control region sequences of the specimens are called "haplotypes". A range of analytical approaches was used to assess the extent and patterns of genetic diversity within and between samples of each species. These included estimates of haplotype diversity, nucleotide diversity and standardised genetic variation (F_{ST}), as well as an analysis of molecular variance (AMOVA). In addition, haplotype networks, which illustrate the relationships among the different haplotypes of each species, were constructed.

The amplified portion of the control region was 389-bp in length in all sampled individuals of Balston's Pygmy Perch. Over all samples eight of the 389 sites were variable and a total of eight different haplotypes were present. The levels of genetic diversity in the samples of Balston's Pygmy Perch, including those from Milyeannup Brook and McAtee Brook in the Blackwood River, were low. Most samples had only one common haplotype, although multiple haplotypes were usually present. In addition, the eight different haplotypes were all relatively similar to each other, differing from each other at between only one to five mutational steps. The overall amount of genetic variation between the samples of Balston's Pygmy Perch from the different sites was large (57.87% of the total) and statistically significant. The haplotype compositions of the samples from the Milyeannup and McAtee Brooks were very similar to each other, but different to those of all other samples. The haplotype compositions of the samples from outside of the

Blackwood River were also different to each other. Only two haplotypes were found in the Blackwood River samples. One of these haplotypes (haplotype 3) appears to be widespread, while the other (haplotype 1) was found only in the sample from Milyeannup Brook. Haplotype 1 appears to be a recent derivative of haplotype 3 and may have recently evolved within the Blackwood River Catchment. To a certain extent, the significance of these findings about Balston's Pygmy Perch is limited by the small size of some samples, including that from McAtee Brook. Nevertheless, the results strongly suggest that the populations of Balston's Pygmy Perch in the Blackwood River are demographically isolated from those outside of the Blackwood River and should therefore be managed separately from these other populations. The results also indicate that Balston's Pygmy Perch exhibits a limited amount of evolutionary divergence in the study.

The aligned portion of the control region amplified from the Mud Minnow was 426-bp in length. Over all samples 29 of the 426 sites were variable and seven of these sites included indels (deletions or insertions). The total number of haplotypes present was eleven. The samples of the Mud Minnow from St John Brook, Red Gully and Milyeannup Brook in the Blackwood River contained only one or two haplotypes and had low levels of genetic diversity. In contrast the sample from Rosa Brook, also in the Blackwood River, had five different haplotypes and noticeably higher levels of genetic diversity. The levels of genetic diversity in the samples of this species from outside the Blackwood River also varied from low (Margaret River and pools on the Deeside Coast Rd) to relatively high (Deep River). The overall amount of genetic variation between the samples of the Mud Minnow from the different sites was large (63.74% of the total) and statistically significant. The haplotype compositions of the samples of the Mud Minnow from St John Brook, Red Gully and Milyeannup Brook in the Blackwood River were similar to each other but very different to all other samples. The samples from Red Gully and Milyeannup Brook, in particular, contained the same two haplotypes (haplotypes 1 and 7) in approximately equal proportions, while only haplotype 1 was present in the sample from St. John Brook. The small sample from McAtee Brook (N = 2) also contained haplotypes 1 and 7. The haplotype composition of the sample from Rosa Brook was very distinctive and included a group of haplotypes that differed from the other Blackwood haplotypes by at least eight mutational steps. The haplotype compositions of the samples from Margaret River, Deep River and the pools on the Deeside Çoast Rd were each distinctive and included haplotypes that were not found elsewhere. One haplotype (haplotype 7) was widespread in the Blackwood River samples, including the one from Rosa Brook, but was not found in samples from outside of the Blackwood River.

The genetic differences between the samples of the Mud Minnow from different tributaries in the Blackwood River were usually statistically significant. This suggests that the contemporary connections among populations of the Mud Minnow in the sampled tributaries of the Blackwood River are typically weak and implies that there are barriers to gene flow in the Mud Minnow in the Blackwood River. However, the patterns of haplotype sharing indicate that there have been historical connections (or is very occasional gene flow) between populations that are now essentially isolated from each other. The connections between the populations in Red Gully, Milyeannup Brook and St John's Brook have likely been relatively recent (or relatively strong) compared to those between these populations and the one in Rosa Brook. Similarly, the connections among all of the sampled Blackwood River populations have likely been relatively recent (or relatively strong) compared to those between these populations and those outside of the Blackwood River. The relationships among the different control region haplotypes of the Mud Minnow indicate that Blackwood River populations are the source of the populations in near-by catchments or have retained more of the genetic characteristics of a common ancestral population. In conclusion, the genetic data indicate that the populations in the different tributaries of the Blackwood River should be managed as demographically independent units and with regard to the fact that a large amount of the genetic variation present in this species in south-western Australia is present in these populations.

Table of Contents

Executive Summary.....3
Table of Contents.....5
Background.....6
Aims of the study.....7
Methodology.....7
Balston's Pygmy Perch.....7
Sampling.....7
Genetic methods.....8
Overview.....8
DNA extraction.....9
PCR amplification.....9
Sequencing.....9
Data analyses.....9
Genetic diversity.....9
Population genetic differentiation.....10
Evolutionary relationships.....11
Mud Minnow.....11
Sampling.....11
Genetic methods.....11
Overview.....11
DNA extraction.....11
PCR amplification.....11
Sequencing.....11
Data analyses.....12
Results & Discussion.....13
Balston's Pygmy Perch.....13
General.....13
Genetic diversity.....14
Population differentiation.....15
Evolutionary relationships among haplotypes.....18
Mud Minnow.....20
General.....20
Genetic diversity.....22
Population differentiation.....23
Evolutionary relationships among haplotypes.....27
Conclusions and Management Implications.....29
References.....30
Appendix 1: Samples.....32
Appendix 2: DNA extraction.....33
Appendix 3: DNA amplification with PCR.....34
Appendix 4: DNA sequencing.....35

Background

Since European settlement of south-western Australia, the quality and volume of freshwater habitats have become greatly reduced. As a consequence, many freshwater habitats no longer support some of the region's freshwater fishes. Considering that 80% of the native freshwater fishes of the South-west Drainage Division are endemic to the region, and that many of these have undergone recent reductions in range (Morgan *et al*. 1998), it is important to validate the evolutionary significance of each of the remaining populations so as to aid in their conservation. This will allow the prioritisation of conservation efforts to remnant and/or genetic isolated populations.

The decline in both the range and number of populations of the fishes of south-western Australia has recently been highlighted by two of the region's freshwater fishes being Federally listed under the EPBC Act 1999 in 2006. This includes Balston's Pygmy Perch (*Nannatherina balstoni*), which is listed as *VULNERABLE* and the Trout Minnow (*Galaxias truttaceus*), which is listed as *CRITICALLY ENDANGERED*. These species are also listed in State regulations under Schedule 1 of the Wildlife Conservation Act 1950 (which is for fauna that is rare or likely to become extinct or are declared fauna that is in need of special protection). A further species, the Mud Minnow (*Galaxiella munda*) is also listed as Schedule 1 under State regulations.

While the Trout Minnow is extremely rare and is known from three small catchments, Balston's Pygmy Perch and the Mud Minnow are more widespread but the populations have become widely fragmented or lost (Figure 1) (Morgan *et al*. 1998, 2002). For example, the population of Balston's Pygmy Perch from the Moore River catchment has not been seen for a quarter of a century, despite a fairly intensive survey effort.

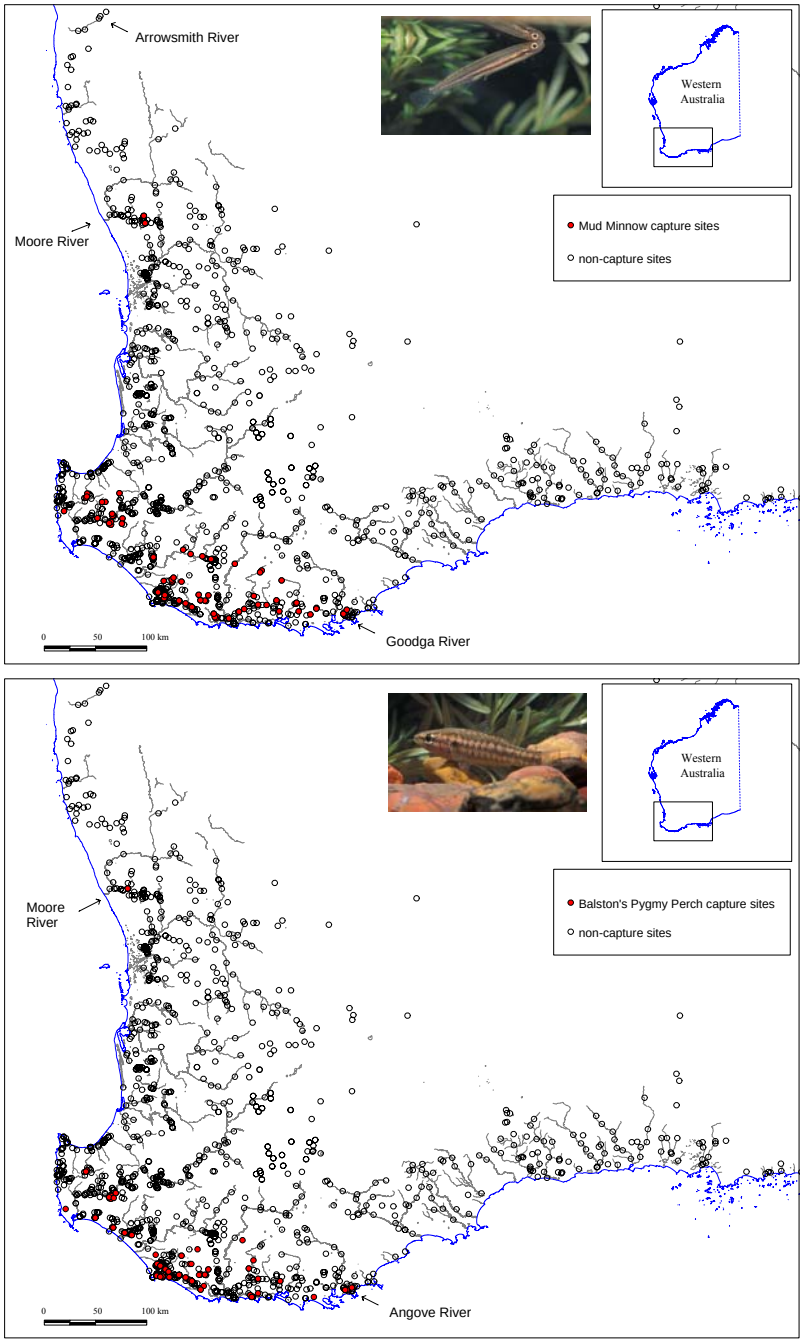


Figure 1 The known distribution of the Mud Minnow (top) and Balston's Pygmy Perch (bottom). Includes sites sampled where each species has not been recorded (source: Morgan and Beatty unpublished data).

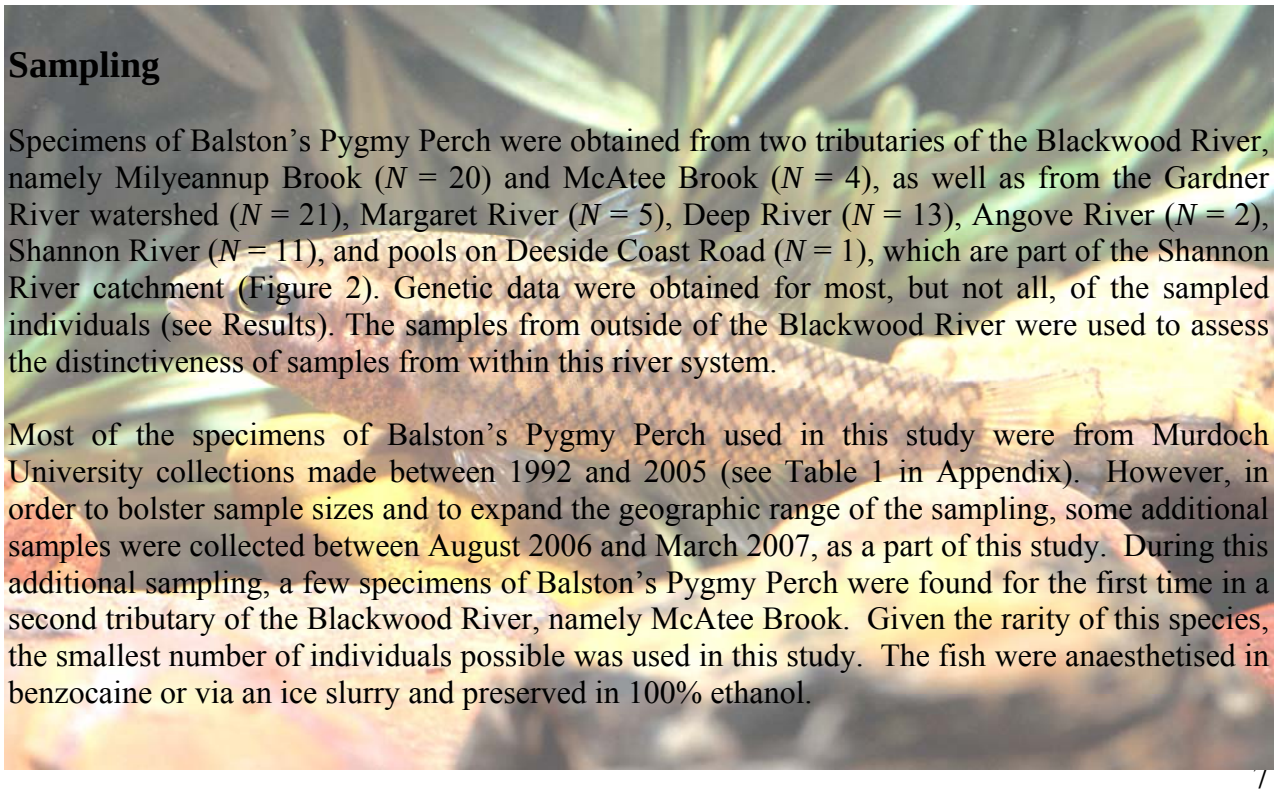
Within the Blackwood River catchment, which along with many of the region's rivers has been severely affected by salinisation, Balston's Pygmy Perch is essentially restricted to a single tributary (Milyeannup Brook) (Morgan *et al*. 2003; Beatty *et al*. 2006), although a small number of individuals were recently found in the nearby McAtee Brook during this study. Similarly, within this catchment, the Mud Minnow is now found in only a few tributaries, including St John Brook, Milyeannup Brook, Red Gully, Rosa Brook and McAtee Brook. The explanation for their restricted occurrence within this system may be simply that salinisation of the main channel has limited their ability to migrate between tributaries and that when a tributary becomes dry the 'population' is lost. It may however be a far more complex relationship with their environment in that the tributaries that continue to maintain populations of the species are intrinsically linked to groundwater discharge; in these cases either the Yarragadee or Leederville Aquifers. Most likely it is a combination of the above. Groundwater intrusion into streams, and particularly into smaller discharge tributaries, is often an important mechanism for buffering habitats against extreme conditions, such as extreme temperatures and low oxygen levels, while also maintaining minimum habitats and migratory routes. Thus, such habitats often have fauna that can be deemed relictual or remnant. This is the case for many of the ichthyologically important tributaries of the Blackwood catchment, with salinities in the main channel probably exceeding the tolerance of these principle freshwater species at times (Beatty *et al*. 2006).

Aims of the study

There were two overriding aims in this study. The first is to use genetic data to assess the evolutionary significance of the population of Balston's Pygmy Perch in Milyeannup Brook in the Blackwood River. The second is to use genetic data to assess the connectivity of assemblages of the highly restricted Mud Minnow in different parts of the Blackwood River. This will allow us to assess whether there is migration between tributaries.

Methodology

Balston's Pygmy Perch



Sampling

Specimens of Balston's Pygmy Perch were obtained from two tributaries of the Blackwood River, namely Milyeannup Brook (N = 20) and McAtee Brook (N = 4), as well as from the Gardner River watershed (N = 21), Margaret River (N = 5), Deep River (N = 13), Angove River (N = 2), Shannon River (N = 11), and pools on Deeside Coast Road (N = 1), which are part of the Shannon River catchment (Figure 2). Genetic data were obtained for most, but not all, of the sampled individuals (see Results). The samples from outside of the Blackwood River were used to assess the distinctiveness of samples from within this river system.

Most of the specimens of Balston's Pygmy Perch used in this study were from Murdoch University collections made between 1992 and 2005 (see Table 1 in Appendix). However, in order to bolster sample sizes and to expand the geographic range of the sampling, some additional samples were collected between August 2006 and March 2007, as a part of this study. During this additional sampling, a few specimens of Balston's Pygmy Perch were found for the first time in a second tributary of the Blackwood River, namely McAtee Brook. Given the rarity of this species, the smallest number of individuals possible was used in this study. The fish were anaesthetised in benzocaine or via an ice slurry and preserved in 100% ethanol.

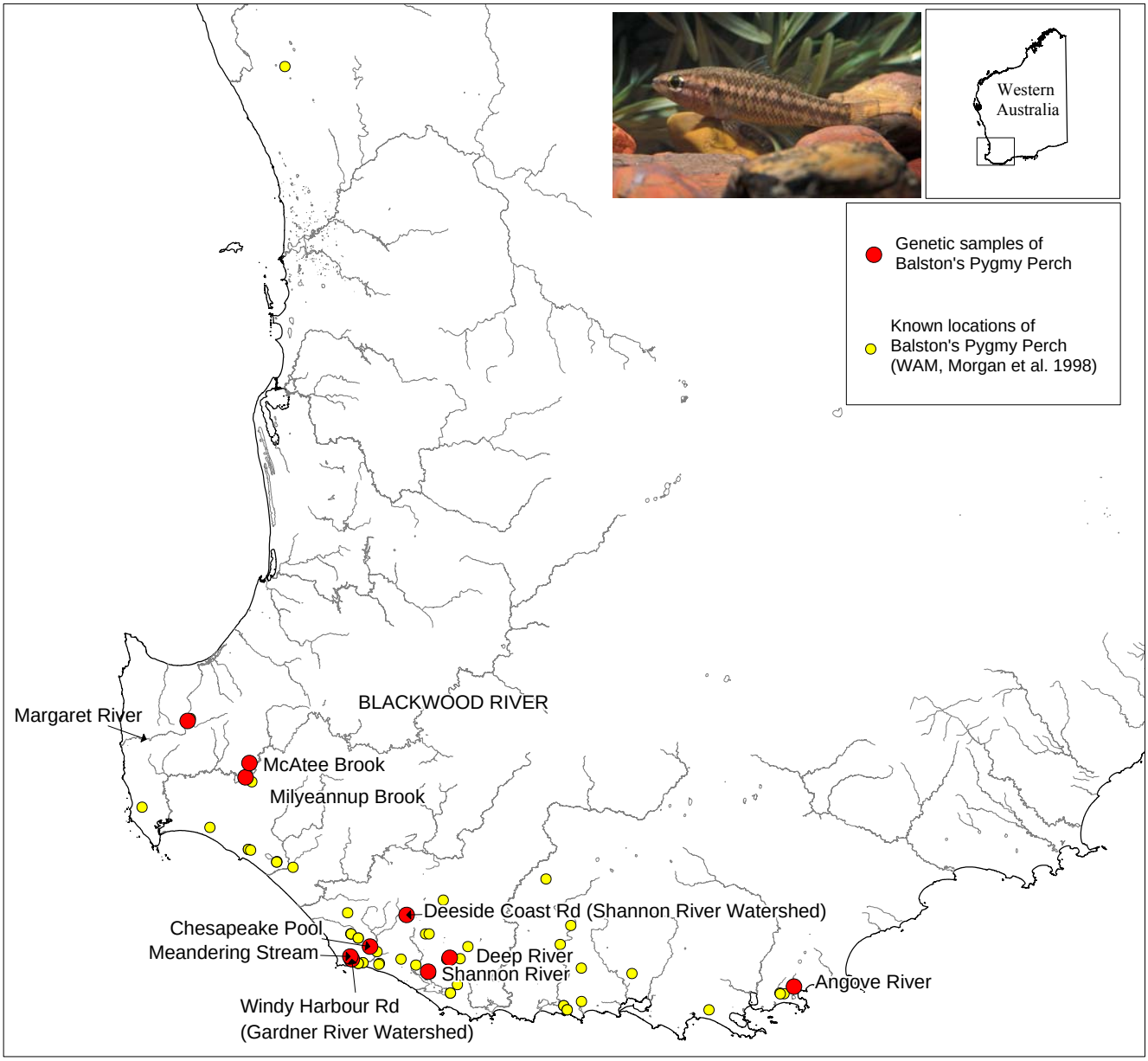


Figure 2 The known distribution of Balston's Pygmy Perch and locations utilised during this study. N.B. Samples from Chesapeake Pool and Meandering Stream failed to yield useable DNA.

Genetic methods

Overview

The results for Balston's Pygmy Perch are based on nucleotide sequence variation in a portion of the control region of the mitochondrial DNA (mtDNA). This type of approach was selected because it provides a relatively inexpensive means of generating information about the distribution of genetic variation within and between the populations of a species. The methods used to generate the control region data are described below.

DNA extraction

Total genomic DNA was extracted from approximately 5 mg of muscle tissue of each fish using a Masterpure™ (Epicentre Technologies, Sydney) DNA extraction kit.

PCR amplification

The target portion of the control region was amplified from the extracted DNA of each individual using polymerase chain reaction (PCR), with the universal fish primers H16498: 5'CCTGAAGTAGGAACCAGATG 3' (Meyer *et al.* 1990) and L15926: 5'AACTCTCACCCCTAGCTCCCAAAG 3' (Kocher *et al.* 1989).

PCR amplification was performed in a reaction mixture containing approximately 10 ng of DNA template, 10 mM TAQ buffer with 1.5 mM $MgCl_2$ (Roche), 0.1 mM of each of the dNTPs (Promega), 0.5 U of *Taq* DNA polymerase (Roche), 20 µmol of each primer, and adjusted to a final volume of 50 µl with PCR-grade water. The amplification conditions consisted of an initial 5 minute denaturation phase at 94ºC, followed by 30 amplification cycles, with each cycle consisting of 30 seconds of denaturation at 94ºC, 30 seconds of annealing at 60ºC, and 30 seconds of extension at 72ºC, followed by a final 7 minute extension at 72ºC.

Sequencing

Prior to sequencing, the PCR products were cleaned using Qiaquick columns (Qiagen) according to the manufacturer's protocol.

The sequencing was carried out using the dye terminator cycle sequencing method. Each sequencing reaction was prepared using approximately 30 ng of clean PCR product, 3.2 pmol of the forward or reverse primer and a Big Dye 3.1 terminator cycle sequencing ready reaction kit following the instructions of the manufacturer (Applied Biosystems Inc. 2001), except that all sequencing was done using 'half' reactions. The sequencing products were electrophoresed, and the raw data chromatograms generated, using an Applied Biosystems 3230 DNA Analyzer automated sequencer.

Data analyses

A partial sequence of both the heavy and light strands of the control region of the mtDNA was determined for each individual of Balston's Pygmy Perch. Both strands were then used to construct a single consensus sequence for the heavy strand for each individual. The consensus sequences of all individuals were aligned using the software GeneTool™ Lite 1.0 (BioTools Inc. 2000). The length of this sequence was the same in all sampled individuals and the nucleotide composition at each position was resolved for all these individuals. Thus, there were no indels (*i.e.,* insertions or deletions) or missing data in the data set. The partial control region sequence of an individual is termed a 'haplotype'.

Genetic diversity

The level of genetic diversity within each sample and within the total (*i.e.,* all samples pooled) was estimated in terms of haplotype diversity (h) and nucleotide diversity (π).

Haplotype diversity (h) represents the probability that two randomly selected individuals exhibit different haplotypes (Nei 1987). Thus, values of h range from 0 (all individuals have the same haplotype) to 1 (all individuals have different haplotypes). The estimates of haplotype diversity were calculated according to equation 8.5 in Nei (1987) using the software ARLEQUIN version 3.0 (Excoffier *et al.* 2005). The standard errors of these estimates were derived using this same software, with the variance formula presented in the Arlequin manual.

Nucleotide diversity (π) is the probability that two randomly selected homologous nucleotides are different (Nei 1987). Thus, it provides a measure of the extent of genetic differences between individuals; the greater the genetic differences, the higher the value. Nucleotide diversities were calculated according to equation 10.5 in Nei (1987) using pairwise differences and a gamma correction of 0.02. The gamma correction is used to correct for variation in the rate of substitution among nucleotide sites (Uzzell & Corbin 1971). The appropriate value of the correction for the control region haplotypes of Balston's Pygmy Perch was empirically determined using the maximum likelihood method in TREE-PUZZLE 5.2 (Schmidt *et al*. 2002). The estimates of π were calculated using the software ARLEQUIN version 3.0 (Excoffier *et al.* 2005). The standard errors of these estimates were derived using this same software, with the variance formula presented in the Arlequin manual.

Population genetic differentiation

Overall population genetic subdivision

Analysis of molecular variance (AMOVA) (Excoffier *et al.* 2002) was used to assess how genetic variation was partitioned within and among samples of Balston's Pygmy Perch. The statistical significance of the extent of among-sample variation was assessed using a nonparametric permutation approach, in which sequences were randomly permutated among samples (see Excoffier *et al.* 2005). These analyses were conducted using the software ARLEQUIN version 3.0 (Excoffier *et al.* 2005).

Pair-wise comparison of populations

Exact tests were used to ascertain whether the haplotype frequency distributions of pairs of samples were significantly different to each other. The results of this analysis are presented in terms of the exact probability of a Type I error (*P* values), *i.e.,* the probability of rejecting the null hypothesis (no significant genetic difference between a particular pair of samples) when it is true. The probability values were estimated using the Markov chain method (Raymond & Rousset 1995), with 10,000 steps in the Markov chain, as implemented by the software ARLEQUIN version 3.0 (Excoffier *et al.* 2005). Exact probability tests were used because they are not biased by small sample sizes or low haplotype frequencies (see Raymond & Rousset 1995). Since multiple tests were conducted as a part of this analysis, a sequential Bonferroni procedure, which controls for group-wide Type I error rates (Rice 1989), was used to assess the statistical significance of the probability values.

In order to resolve the patterns of genetic differentiation among samples, the multi-dimensional scaling method (*e.g.* see Clark & Warwick 2001) was used to map the 'genetic distance' between each pair of samples in two-dimensional space. The 'genetic distance' between each pair of samples was estimated as the standardised value of F_{ST}. F_{ST} provides a measure of the extent of allele (haplotype) frequency variation between samples (Weir & Cockerham 1984). The raw values of F_{ST} were standardised to minimise the bias introduced by variation in the amount of underlying genetic polymorphism between samples (see Meirmans 2006). The standardised value of F_{ST} equals the raw value of F_{ST} divided by the maximum possible value of F_{ST}, which is calculated by recoding the raw data such that no alleles are shared between samples (Meirmans 2006). The multi-dimensional scaling was conducted using the software PRIMER version 6 (Clark & Warwick 2001). The raw and maximum values of F_{ST} were calculated using the software FSTAT version 2.9.3.2 available at http://www2.unil.ch/popgen/softwares/fstat.htm (see Goudet 1996), while the raw data were recoded such that all alleles were sample specific using the software RecodeData version 0.1, available at:

http://www.bentleydrummer.nl/software/software/Other%20software.html

Evolutionary relationships

The evolutionary relationships among haplotypes were estimated by constructing a haplotype network using the parsimony method of Templeton *et al*. (1992). This method estimates the maximum number of substitutions required to connect any two haplotypes parsimoniously (with 95% confidence) and builds the network by firstly linking sequences with the smaller number of differences. This analysis was performed using the software TCS version 1.21 (Clement *et al*. 2000).

Mud Minnow

Sampling

Specimens of the Mud Minnow were obtained from five tributaries of the Blackwood River, namely St John Brook (N = 18), Red Gully (N = 18), Rosa Brook (N = 13), Milyeannup Brook (N = 14), and McAtee Brook (N = 2), as well as from Margaret River (N = 10), Deep River (N = 9), and watershed pools on Deeside Coast Road (N = 8) (Figure 3). The samples from outside of the Blackwood River were used to assist in the assessment of the relationships among the samples from within this river.

Most of the specimens of the Mud Minnow used in this study were from Murdoch University collections made between 2003 and 2005 (see Table 2 in Appendix). However, in order to bolster sample sizes and to increase the geographic range of the sampling, some additional samples were collected between July 2006 and December 2006, as a part of this study. Given the rarity of this species, the smallest number of individuals possible was used in this study. The fish were anaesthetised in benzocaine or via an ice slurry and preserved in 100% ethanol.

Genetic methods

Overview

As for Balston's Pygmy Perch, the results for the Mud Minnow are based on nucleotide sequence variation in a portion of the control region of the mitochondrial DNA (mtDNA). The methods used to generate this information are described below.

DNA extraction

Total genomic DNA was extracted from approximately 5 mg of muscle tissue of each Mud Minnow using a Masterpure™ (Epicentre Technologies, Sydney) DNA extraction kit.

PCR amplification

The PCR amplification primers and protocols used for the Mud Minnow were identical to those for Balston's Pygmy Perch, except that the thermal cycling consisted of an initial 5 minute denaturation phase at 94ºC, followed by 33 amplification cycles, with each cycle consisting of 30 seconds of denaturation at 94ºC, 30 seconds of annealing at 58ºC, and 30 seconds of extension at 72ºC, followed by a final 7 minute extension at 72ºC.

Sequencing

The sequencing protocol for the Mud Minnow was identical to that described for Balston's Pygmy Perch.

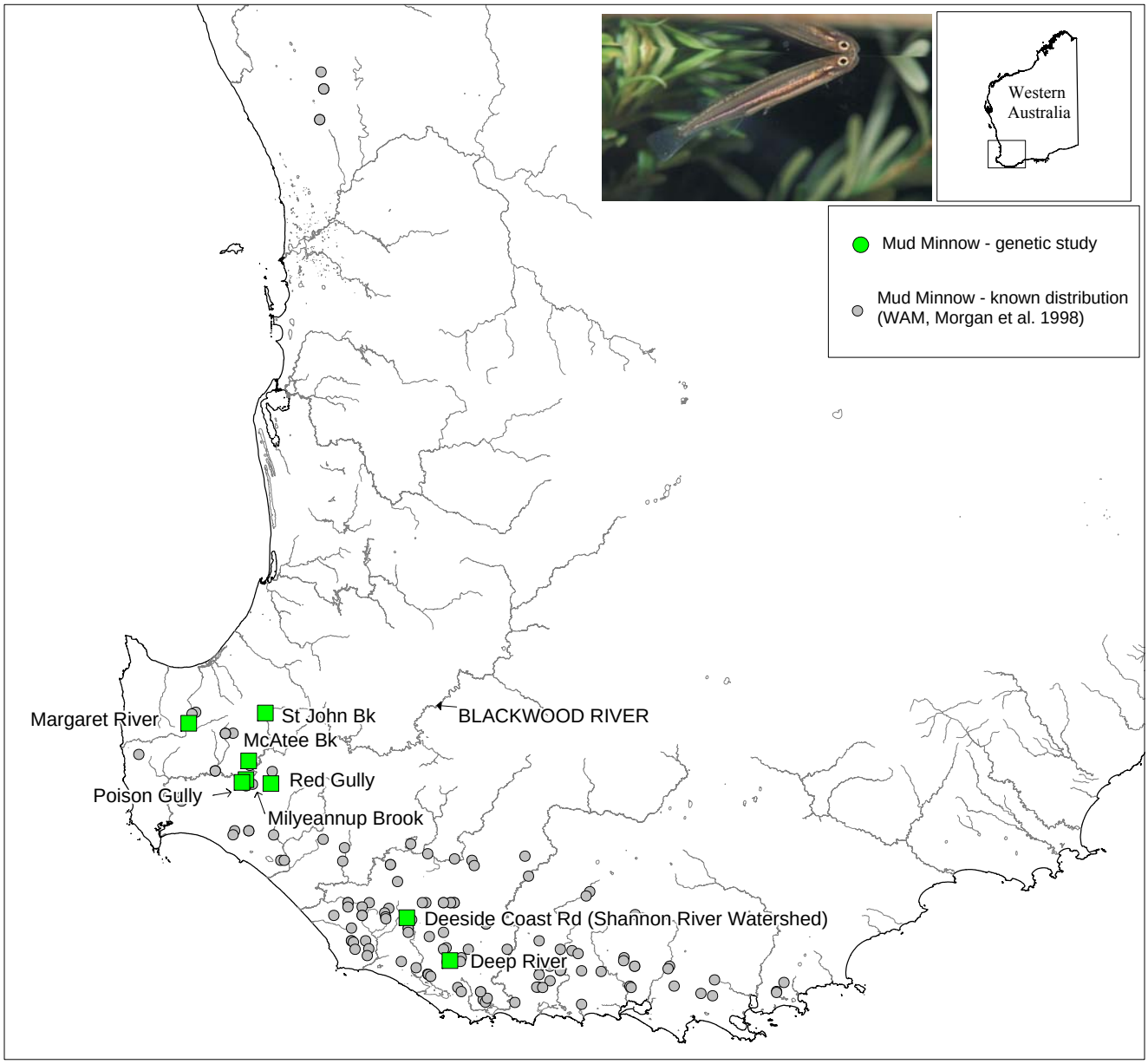


Figure 3 The known distribution of the Mud Minnow and locations utilised during this study. N.B. The Poison Gully samples failed to yield useable DNA.

Data analyses

The data analyses used for the Mud Minnow were identical to those used for Balston's Pygmy Perch, with two minor exceptions as follows. (1) The gamma correction, used in the calculation of nucleotide diversity, was 0.13. This value was empirically determined, as described for Balston's Pygmy Perch. (2) Indels were detected among the control regions haplotypes of the Mud Minnow. The indels were included as variable sites in the analyses of the patterns of genetic variation within and among populations and assigned as a 5th state when constructing the haplotype network using TCS version 1.21 (Clement *et al*. 2000).

Balston's Pygmy Perch

General

A 389-bp portion of the control region was successfully sequenced for 72 (of 77) individuals of Balston's Pygmy Perch from seven locations (Figure 2). The average base composition of these sequences was: A–33.20%; T–33.53%; C–14.99%; and G–18.28%. Thus, the base composition was biased towards adenine and thymine (66.73%), as has been found for the mtDNA of other teleost fishes (*e.g.* Meyer *et al.* 1990; McMillan & Palumbi 1997).

Although total genomic DNA was extracted and PCR products obtained, it was not possible to determine the nucleotide sequence of the PCR product for any of the five specimens of Balston's Pygmy Perch from Margaret River, even after five attempts. Instead, twin peaks (rather than a single clear peak) were present at nucleotide sites in the chromatograms of both the heavy and light strand sequences for all five individuals. The presence of multiple peaks in a sequence chromatogram usually results from a PCR product that contains divergent DNA regions rather than a single region (Mirol *et al*. 2000). Regardless of the reason for the multiple peaks, it was not possible to generate any sequence data for individuals of the Balston's Pygmy Perch from Margaret River. Thus, ultimately, nucleotide sequence data were obtained for 72 individuals from six sites, comprising 20 individuals from Milyeannup Brook and 4 individuals from McAtee Brook in the Blackwood River, 21 individuals from the Gardner River watershed, 13 individuals from the Deep River, 2 individuals from the Angove River, 11 individuals from the Shannon River, and 1 individual from pools on the Deeside Coast Road (Shannon R.). However, the samples from Angove River and pools on Deeside Coast Road were excluded from all frequency-based analyses, *i.e.,* haplotype diversity, nucleotide diversity, AMOVA, exact tests and the F_{ST}-based distance estimates, due to the small number of individuals contained in these samples.

Eight of the 389 sites in the partial sequence of the control region varied among the 72 individuals of Balston's Pygmy Perch (Table 1). The eight variable positions revealed a total of eight different control region haplotypes (Table 1). The number of haplotypes per sample varied from one in the small samples from McAtee Brook and the Deeside Road Pools to three in the samples from the Gardner River watershed and Deep River (Table 2). The sample from Milyeannup Brook contained two haplotypes, namely haplotypes 1 and 3.

Table 1 The location and distribution of eight variable positions among eight haplotypes for a 389-bp portion of the control region in the mtDNA of the sampled individuals of Balston's Pygmy Perch. Dots represent matches with nucleotides present in haplotype 1. Numbers refer to position of base pairs from the start of the fragment.

Haplotype number	10	35	171	323	349	350	371	383
1	G	T	T	G	T	T	G	A
2	.	.	.	A	C	A	A	G
3	.	.	.	.	.	.	A	.
4	.	.	.	A	.	.	A	.
5	.	C	.	.	.	.	A	.
6	.	.	.	A	.	.	.	.
7	.	.	A	.	.	.	A	.
8	A	.	.	.	.	.	A	.

Genetic diversity

The level of haplotype diversity in the sample of Balston's Pygmy Perch from Milyeannup Brook was low (Table 2). In fact, the levels of haplotype diversity in most of the samples of this species were low (Table 2). The generally low levels of haplotype diversity reflects the fact that most samples were dominated by a single haplotype, although two or three haplotypes were usually present (Table 3). The highest level of haplotype diversity was in the sample from the Gardner River watershed, which had three haplotypes, including two abundant ones (Table 3). The overall level of haplotype diversity, *i.e.,* for all samples pooled, was 0.7728 ± 0.0199, which is higher than that of the individual samples, largely reflecting the fact that different haplotypes tend to dominate in different samples (Table 3).

The level of nucleotide diversity in the sample of Balston's Pygmy Perch from Milyeannup Brook, as in most of the other samples, was also low (Table 2). This partly reflects the fact that most samples were dominated by a single haplotype and partly the fact that, when multiple haplotypes were present in a sample, they usually differed from each other by only one or two base substitutions (Tables 2 and 3). Haplotype 2 was the most divergent, differing from the other seven haplotypes by three or more base substitutions (Table 1). Accordingly, the level of nucleotide diversity was highest in the sample from the Gardner River watershed, in which haplotype 2 and another haplotype (#3) were both common (Table 3). The level of nucleotide diversity in the pooled sample (0.0057 (± 0.0035)) was higher than that in most individual samples, but nevertheless relatively low, largely reflecting the fact that most of the haplotypes were very similar to each other (Table 1).

The control region is the most variable part of the mitochondrial genome in most vertebrates (*e.g.* Gaffney *et al.* 2007). Thus, unlike some more slowly evolving molecular markers, the control region allows for a relatively sensitive assay of the amount of genetic variation within a species. The low levels of genetic (control region) diversity indicate that the effective sizes (*i.e.,* N_e) of the

assemblages of Balston's Pygmy Perch in Milyeannup Brook and at most the other sampling locations are low.

Table 2: The sample sizes and a summary of levels of diversity in a 389-bp portion of the mtDNA control region in samples of Balston's Pygmy Perch. Haplotype and nucleotide diversities were not calculated for the samples from the Angove River and Deeside Coast Road because of the small number of individuals contained in these samples.

Sample locations	Sample size	No. of haplotypes	No. of polymorphic sites	Haplotype diversity (± SE)	Nucleotide diversity (± SE)
Milyeannup Brook	20	2	1	0.268 (±0.113)	0.00069 (±0.001)
McAtee Brook	4	1	0	0.000 (±0.000)	0.00000 (±0.000)
Gardner River watershed	21	3	5	0.610 (±0.057)	0.00575 (±0.004)
Shannon River	11	2	4	0.182 (±0.144)	0.00187 (±0.002)
Deep River	13	3	2	0.410 (±0.154)	0.00112 (±0.001)
Angove River	2	2	2	N/A	N/A
Deeside Coast Rd.	1	1	2	N/A	N/A

Population differentiation

The results of the AMOVA indicate that a large and statistically significant amount of the control region variation found in Balston's Pygmy Perch was due to variation among samples (57.87%; $P = 0.000$). The remaining 42.13% of variation was within samples.

The haplotype composition of the sample of Balston's Pygmy Perch from Milyeannup Brook in the Blackwood River was distinct from those of samples from other river systems (Tables 3 and 4). For example, the results of the exact tests indicate that the haplotype frequency distribution in the Milyeannup Brook samples are significantly different to those in samples from outside of the Blackwood River (Table 4). The haplotype composition of the sample from McAtee Brook in the Blackwood River seemed to be similar to that from Milyeannup Brook and different from those of the other samples (Tables 3 and 4), but the veracity of the results concerning this sample are limited by its small size ($N = 4$). The haplotype compositions of the samples from the Gardner River watershed and Deep and Shannon rivers were also significantly different from each other (Tables 3 and 4).

The distinctiveness of the samples from Milyeannup Brook and McAtee Brook relative to the other samples was largely due to the fact that haplotype 1 was relatively common in these samples, but was not found elsewhere (Table 3). Haplotype 3 also occurred in the sample from Milyeannup Brook. This haplotype was relatively widespread and, in fact, was absent only from those samples with very few individuals ($N \leq 4$) (Table 3). The remaining six haplotypes were

not found in samples from the Blackwood River; they were restricted to one or two of the other samples rather than being widespread (Table 3).

The relationships among the samples of Balton's Pygmy Perch are displayed in the MDS plot of the standardised values of F_{ST} between pairs of samples (Figure 4). This plot highlights the similarity of the samples from Milyeannup and McAtee Brook relative to those from outside of the Blackwood River and the distinctiveness of each of the samples from outside of the Blackwood River (Figure 4).

In summary, Balston's Pygmy Perch in the south-western corner of Western Australia is typical of freshwater fishes in that it shows a high level of genetic variation among populations from different river systems but a low level of variation within populations (*e.g.* Avise 2000; Hughes & Hillyer 2006). This pattern is presumably due, in part, to the improbability of individuals of Balston's Pygmy Perch (and of many other freshwater fishes) dispersing between disjunct waterways.

Table 3: The distribution and abundance of the eight mtDNA control region haplotypes found in 72 individuals of Balston's Pygmy Perch.

	Sample locations						
Hap. #	Milyeannup Brook	McAtee Brook	Gardner River Watershed	Shannon River	Deep River	Deeside Coast Rd	Angove River
1	17	4	-	-	-	-	-
2	-	-	9	10	-	-	-
3	3	-	10	1	1	-	1
4	-	-	-	-	10	-	-
5	-	-	2	-	-	-	-
6	-	-	-	-	2	-	-
7	-	-	-	-	-	-	1
8	-	-	-	-	-	1	-

Table 4: Comparisons of the frequency distributions of control region haplotypes between pairs of samples of Balston's Pygmy Perch. The outcomes of these comparisons are expressed in terms of the exact probability of rejecting the null hypothesis (no genetic difference) when it is true. *P* values that were significant after a sequential Bonferroni correction was applied are indicated in bold. The samples from the Angove River and Deeside Coast Road pools are not included in this analysis because they contained only one or two individuals.

Sample locations	Milyeannup Brook	McAtee Brook	Gardner River watershed	Shannon River	Deep River
Milyeannup Brook	---				
McAtee Brook	*P* = 1.000	---			
Gardner River watershed	***P* = 0.000**	***P* = 0.001**	---		
Shannon River	***P* = 0.000**	***P* = 0.001**	***P* = 0.024**	---	
Deep River	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	---

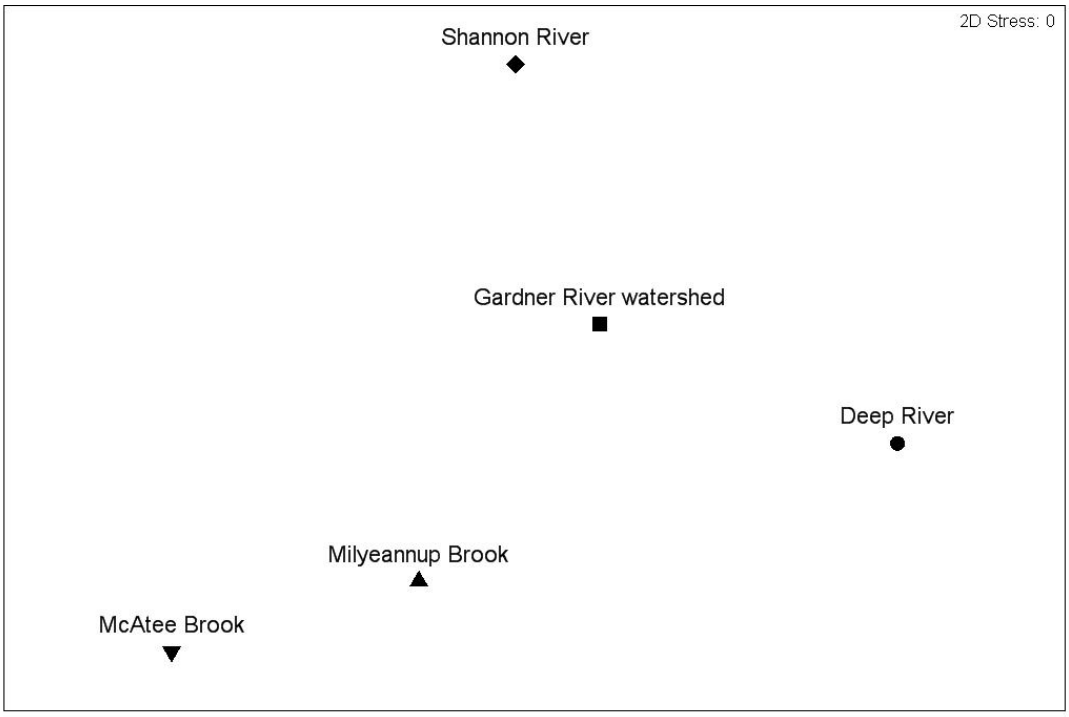


Figure 4 A two-dimensional ordination of standardised values of F_{ST}, based on variation in a 389-bp portion of the control region of the mtDNA, between pairs of samples of Balston's Pygmy Perch. The stress value provides an indication of how accurately the variation in the underlying data set is portrayed in the MDS. A stress value of 0 indicates that the ordination provides a reliable representation of the relationships among the samples (see Clarke & Warwick 2001). The samples from the Angove River and Deeside Coast Road were not included in this analysis because of the small number of individuals contained in these samples.

Evolutionary relationships among haplotypes

The haplotype network indicates the following about the relationships among the eight haplotypes found in the samples of Balston's Pygmy Perch. (1) The haplotypes found in the samples from the Blackwood River (haplotypes 1 and 3) differed from the other haplotypes by only a small number of mutational steps (between 1 and 5) (Figure 5). (2) Haplotype 3 appears to be an ancestral halpotype because it is located internally in the network and is geographically widespread (Figure 5). (3) Most of the remaining haplotypes are only one or two mutational steps from haplotype 3 and so appear to be recent derivatives of haplotype 3 (Figure 5). Haplotype 1, which was unique to the samples from the Blackwood River, is included in this group. The apparently limited geographic distribution of this haplotype raises the possibility that it evolved *in situ* in the Blackwood River region, although more intensive sampling is required to confirm (or otherwise) this suggestion. In general, the network is shallow, for example the maximum number of mutational steps between any two haplotypes was five. This suggests that the amount of evolutionary divergence in Balston's Pygmy Perch in the Blackwood River and near-by catchments is small. While we are unable to exclude the possibility that more divergent forms of this species exist in the study area but were not captured during sampling, with genetic information from a total of 72 individuals from 6 sites, it seems likely that this study has at least captured the common forms of this species in this general area. The apparently limited divergence in Balston's Pygmy Perch in this area suggests a common recent ancestry for all of the individuals of this species in this area.

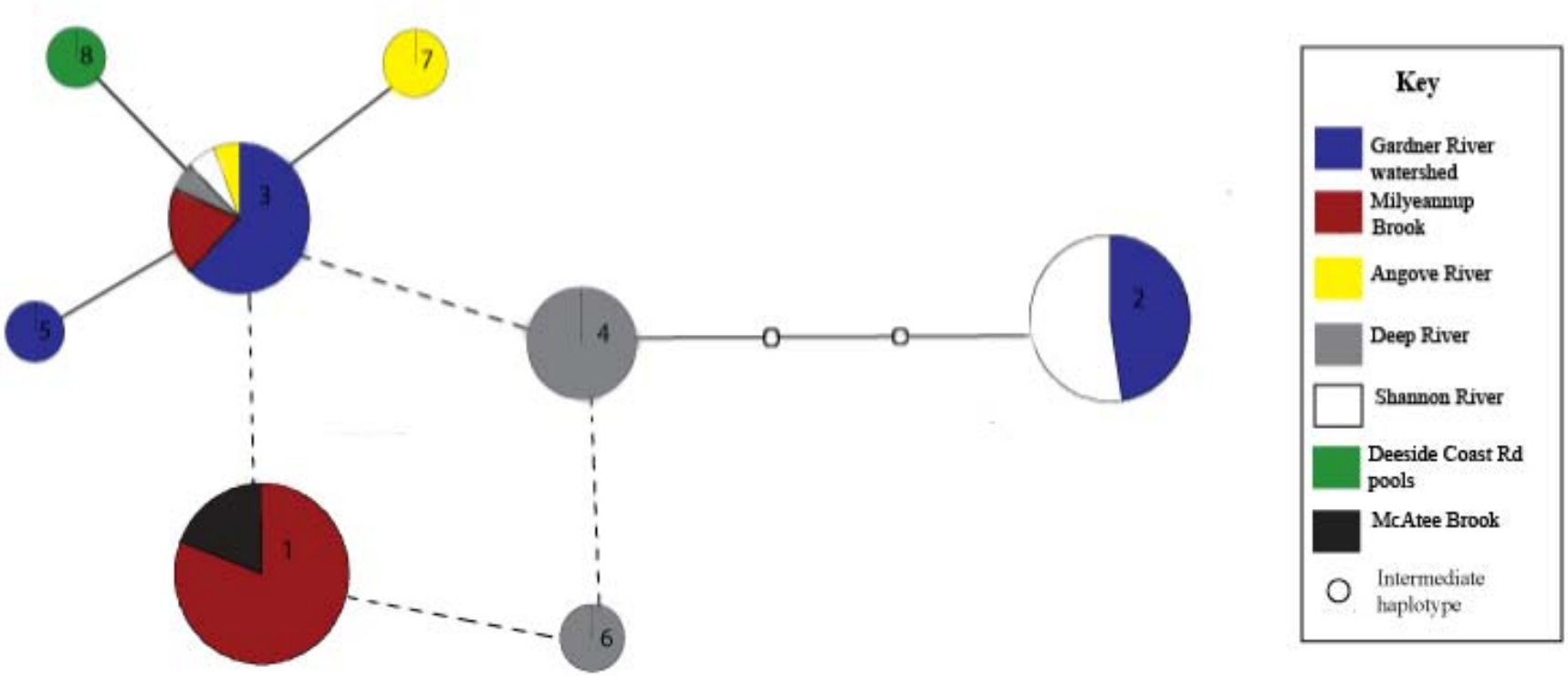


Figure 5 Haplotype network showing the relationships among the eight control region haplotypes of Balston's Pygmy Perch. Each line in the network shows a single mutational change regardless of the length of the line. A numbered circle is used to represent each haplotype. The surface area of each circle is proportional to the total number of individuals with that haplotype. The coloured regions of the circles are used to represent the abundances of individuals with each haplotype at each sampling site (see key). These missing haplotypes are necessary to link all observed haplotypes present in the network. Dashed lines show the ambiguities present in the haplotype network.

Mud Minnow

General

A 426-bp portion of the control region was sequenced for a total of 92 individuals of the Mud Minnow from a total of eight sites. The average base composition of these sequences was A-27.44%; T-32.42%; C-18.69%; and G-21.45%. Thus, the results indicate that the sequence composition is biased in favour of adenine and thymine (59.86%), as is the case for the mtDNA of Balston's Pygmy Perch and other teleost fishes (Meyer *at al.* 1990; McMillan & Palumbi 1997).

The individuals of the Mud Minnow for which sequence data were obtained were as follows: (i) 65 individuals from five sites within the Blackwood River, comprising 18 individuals from St John Brook, 18 individuals from Red Gully, 14 individuals from Milyeannup Brook, 13 individuals from Rosa Brook, and 2 individuals from McAtee Brook; and (ii) 27 individuals from three sites outside of the Blackwood River, comprising 10 individuals from Margaret River, 9 individuals from Deep River and 8 individuals from pools on the Deeside Coast Road. The McAtee Brook sample was excluded from all frequency-based analyses, *i.e.,* haplotype diversity, nucleotide diversity, AMOVA, exact tests and the F_{ST}-based distance estimates, because it contained only two individuals.

Twenty-nine of the 426 positions in the partial sequences of the control region from the 92 individuals of the Mud Minnow were variable; 7 of the 29 included indels (*i.e.,* insertions or deletions) (Table 5). The 29 variable positions revealed a total of 11 different control region haplotypes (Table 5). The number of haplotypes in the samples from the Blackwood River ranged from one in the sample from St John Brook to five in the sample from Rosa Brook (Tables 6 and 7). Similarly, the number of haplotypes in the samples from outside the Blackwood River ranged from one in the samples from Margaret River and the pools on the Deeside Coast Road to four in the sample from Deep River (Tables 6 and 7).

Table 5: The location and distribution of 29 variable positions among 11 haplotypes for a 426-bp portion of the control region in the mtDNA of the sampled individuals of the Mud Minnow. Dots represent matches with nucleotides present in haplotype 1. Numbers refer to position of base pairs from the start of the fragment. Dashes indicate indels.

Haplotye number	8	15	17	23	79	112	129	184	189	196	203	217	219	298	299	307	321	323	325	326	328	329	332	333	334	338	343	344	393
1	T	C	A	G	T	T	A	T	C	A	T	--	G	T	G	A	G	G	--	T	T	--	C	G	--	A	C	G	A
2	A	T	C	T	G	C	G	A	.	T	C	C	--	.	.	G	C	G	.	.	.	T.	.	C	.	G	T	A	.
3	A	.	.	.	.	C	.	.	A	.	.	.	.	C	A	.	.	.	A	.	--	.	.	.	G	.	.	.	.
4	.	T	C	T	G	C	G	A	.	T	C	C	--	.	G	G	C	A	.	.	.	T	.	C	.	G	T	A	.
5	.	T	C	T	.	C	G	A	.	T	C	C	--	.	.	G	C	A	.	.	.	T	.	.	.	G	T	A	G
6	.	T	C	T	.	C	G	A	.	.	C	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
7	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
8	.	.	.	.	.	C	.	.	A	.	.	.	.	C	A	.	.	.	.	A	.	.	.	.	G	.	.	.	.
9	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	A	.	.	.	--	.	.	.	.	.	.
10	.	.	.	.	.	C	.	.	A	.	.	.	.	C	A	.	.	.	A	.	--	.	.	.	G	.	.	.	.
11	.	T	C	T	G	C	G	A	.	T	C	C	--	.	.	.	C	A	--	.	.	T	.	.	.	G	.	.	.

Genetic diversity

With the exception of those from Rosa Brook and Deep River, the levels of haplotype diversity in the samples of the Mud Minnow from inside and outside of the Blackwood River were low (Table 6), reflecting the fact that each of these samples contained only one haplotype or only one abundant haplotype (Table 7). The samples from Rosa Brook in the Blackwood River and Deep River had noticeably higher levels of haplotype diversity (Table 6), reflecting the fact that these samples contained a relatively large number of different haplotypes (Table 7). The overall level of haplotype diversity, *i.e.,* for all samples pooled, was 0.7291 ±0.0439 and so higher than that in most of the individual samples, largely reflecting the fact that some samples had different dominant haplotypes (Table 7).

The results for nucleotide diversity tend to parallel those for haplotype diversity. Thus, while the levels of nucleotide diversity in most samples were low, those in the samples from Deep River and especially Rosa Brook were noticeably higher (Table 6). The level of nucleotide diversity in the pooled sample (0.0201 ± 0.0104) was fairly high. This partly reflects the fact that a diverse range of haplotypes was present in the combined sample (*e.g.* see Figure 7).

The low levels of genetic diversity in most of the samples of the Mud Minnow from sites inside and outside of the Blackwood River suggest that the effective sizes of the assemblages of this species in this general area are generally low (see above discussion about genetic diversity in Balston's Pygmy Perch). However, Rosa Brook and Deep River may support larger populations (because the samples from these sites had higher levels of genetic diversity). This view is consistent with the fact that the habitat in Rosa Brook, at least, is relatively large compared to that in the other sampled tributaries of the Blackwood River; it also retains more water throughout the drier months (Beatty *et al*. 2006). Prior to the study by Morgan & Beatty (2005), Mud Minnows within the Blackwood catchment were thought to be restricted to Rosa Brook, the headwaters of which receive perennial flows from the Leederville Aquifer; which, similar to the perennial input of discharge from the Yarragadee Aquifer into Milyeannup Brook and Poison Gully, is considered to be critical in maintaining the species in these tributaries (see Beatty *et al*. 2006).

Table 6: The sample sizes and a summary of the levels of diversity in a 426-bp portion of the control region in the mtDNA in the eight samples of the Mud Minnow. Haplotype and nucleotide diversities were not estimated from the sample from McAtee Brook because of the small size of this sample.

Sample locations	Sample size	No. of haplotypes	No. of polymorphic sites	Haplotype diversity (± SE)	Nucleotide diversity (± SE)
St John Brook	18	1	0	0.000 (±0.000)	0.00000 (±0.000)
Red Gully	18	2	1	0.425 (±0.099)	0.00101 (±0.001)
Milyeannup Brook	14	2	1	0.264 (±0.137)	0.00063 (±0.001)
McAtee Brook	2	2	1	N/A	N/A
Rosa Brook	13	5	19	0.628 (±0.143)	0.01070 (±0.001)
Margaret River	10	1	0	0.000 (±0.000)	0.00000 (±0.000)
Deep River	9	4	9	0.750 (±0.112)	0.00708 (±0.004)
Deeside Coast Rd	8	1	0	0.000 (±0.000)	0.00000 (±0.000)

Population differentiation

The results of the AMOVA indicate that a large and statistically significant amount of the control region variation found in the Mud Minnow was due to variation among samples (63.74%; P = 0.000). The remaining 36.26% of variation was within samples.

The haplotype compositions of the samples of the Mud Minnow from St John Brook, Red Gully and Milyeannup Brook in the Blackwood River were relatively similar to each other, but very different from that of the sample from Rosa Brook, also in the Blackwood River (Table 8; Figure 6). The samples from Red Gully and Milyeannup Brook were effectively identical to each other (Table 8); both comprised haplotypes 1 and 7 in approximately equal proportions, with the former haplotype being more common (Table 7). The sample from St. John Brook contained haplotype 1 alone (Table 7). Haplotype 1 was also found in the small sample from McAtee Brook, as well as the sample from Deep River (Table 7). Haplotype 7 was found at all of the Blackwood River sites except St John Brook, but not outside of the Blackwood River (Table 7). The differences in haplotype composition between the sample from St John Brook and those from Red Gully and Milyeannup Brook were statistically significant before, but not after, the Bonferroni correction was

applied (Table 7). It is likely that the haplotype composition of the assemblage in St John Brook is slightly different to those of the assemblages in Red Gully and Milyeannup, but larger sample sizes are required to improve the power of the statistical analysis.

The haplotype composition of the sample from Rosa Brook was distinctive compared to those of all other samples (Table 8; Figure 6). All but one of the haplotypes in the Rosa Brook sample was unique to this sample; the remaining haplotype (#7) was restricted to the Blackwood River samples, as noted above (Table 7).

The haplotype compositions of the samples from outside the Blackwood River, *i.e.,* from Margaret River, Deep River and Deeside Coast Road pools, were significantly different to all of the samples from Blackwood River, as well as to each other (Table 8; Figure 6). In fact, with the exception of an individual with haplotype 1 in the Deep River sample, all of the haplotypes in each of these samples were not found in any other sample (Table 7).

The above results indicate that the amount of gene flow in the Mud Minnow between the tributaries of the Blackwood River is typically negligible (because the samples from the different tributaries were usually genetically different). This finding is consistent with the view that the relatively high salinity of the main channel of the Blackwood River restricts the movements of the Mud Minnow between the different tributaries (Beatty *et al*. 2006). Environmental differences between the different tributaries (e.g. Morgan & Beatty 2005, Beatty *et al*. 2006) may reduce the fitness of any individuals that do manage to move between tributaries, which could have also contributed to the genetic differentiation of the Mud Minnow within the Blackwood River. The similarity between the samples from Red Gully and Milyeannup Brook (the only combination of samples that showed no evidence of genetic differences) could be due to historical and/or contemporary genetic connections between these two sites. In general, the patterns of haplotype sharing among the samples of the Mud Minnow indicate that there were historical connections (or is very occasional gene flow) between populations that are now essentially isolated from each other. The connections between Red Gully, Milyeannup Brook and St John Brook assemblages are likely to be relatively recent (or relatively strong) compared to those between these assemblages and the one in Rosa Brook. Similarly, the connections among all of the sampled Blackwood River populations are likely to be relatively recent (or relatively strong) compared to those between these populations and those outside of the Blackwood River. This general pattern is similar to that in many other species of freshwater fish, where the genetic compositions of populations within a drainage system tend to be more similar to each other than to those of populations from different drainages (*e.g.* Avise 2000; Hughes & Hillyer 2006), although there are some exceptions.

Table 7: The distribution and abundance of the eleven mtDNA, control region haplotypes found in 92 individuals of the Mud Minnow.

	Sampling locations							
Hap. #	St John Brook	Red Gully	Milyeannup Brook	Rosa Brook	McAtee Brook	Margaret River	Deep River	Deeside Coast Rd
1	18	13	12	-	1	-	1	-
2	-	-	-	2	-	-	-	-
3	-	-	-	-	-	-	1	-
4	-	-	-	8	-	-	-	-
5	-	-	-	-	-	10	-	-
6	-	-	-	1	-	-	-	-
7	-	5	2	1	1	-	-	-
8	-	-	-	-	-	-	4	-
9	-	-	-	-	-	-	-	8
10	-	-	-	-	-	-	3	-
11	-	-	-	1	-	-	-	-

Table 8: Comparisons of the frequency distributions of control region haplotypes between pairs of samples of Mud Minnow. The outcomes of these comparisons are expressed in terms of the exact probability (*P* values) of rejecting the null hypothesis (no genetic difference) when it is true. *P* values that were significant after a sequential Bonferroni correction was applied are indicated in bold. The sample from the McAtee Brook was not included in this analysis because it contained only one or two individuals.

Sample locations	St John Brook	Red Gully	Milyeannup Brook	Rosa Brook	Margaret River	Deep River	Deeside Coast Rd
St John Brook	---						
Red Gully	*P* =0.045	---					
Milyeannup Brook	*P* = 0.174	*P* = 0.429	---				
Rosa Brook	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	---			
Margaret River	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	---		
Deep River	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	--	
Deeside Coast Rd	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	***P* = 0.000**	--

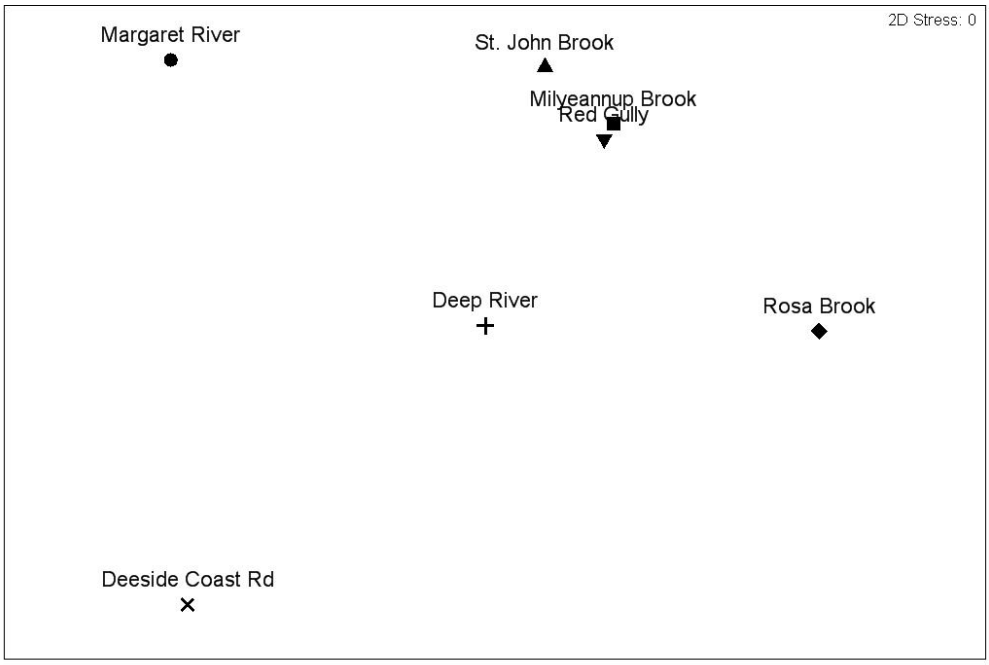


Figure 6 A two-dimensional ordination of standardised F_{ST} values, based on variation in a 426-bp portion of the control region of the mtDNA, between pairs of samples of the Mud Minnow. The stress value provides an indication of how accurately the variation in the underlying data set is portrayed in the MDS. A stress value of zero indicates that the ordination provides an accurate representation of the relationships among the samples (see Clarke & Warwick 2001). The sample from the McAtee Brook was not included in this analysis because it contained only one or two individuals.

Evolutionary relationships among haplotypes

The haplotype network indicates the following about the relationships among the eleven haplotypes in the samples of the Mud Minnow. (1) Some of the haplotypes are separated by a relatively large number of mutational steps (Figure 7). Related to this, haplotypes 6, 11, 4 and 2 in the Rosa Brook sample were very divergent from those found elsewhere in the Blackwood River (Figure 7). Thus, the Mud Minnow exhibits a significant amount of evolutionary divergence in south-western corner of Australia in general and in the Blackwood River in particular (2). The haplotypes that were found in the Blackwood River tend to be internal in the network, while those from other sites tend to be terminal (Figure 6). This raises the possibility that the other assemblages are derivatives of Blackwood River stocks or that the Blackwood River assemblages have retained more of the characteristics of a common ancestral population.

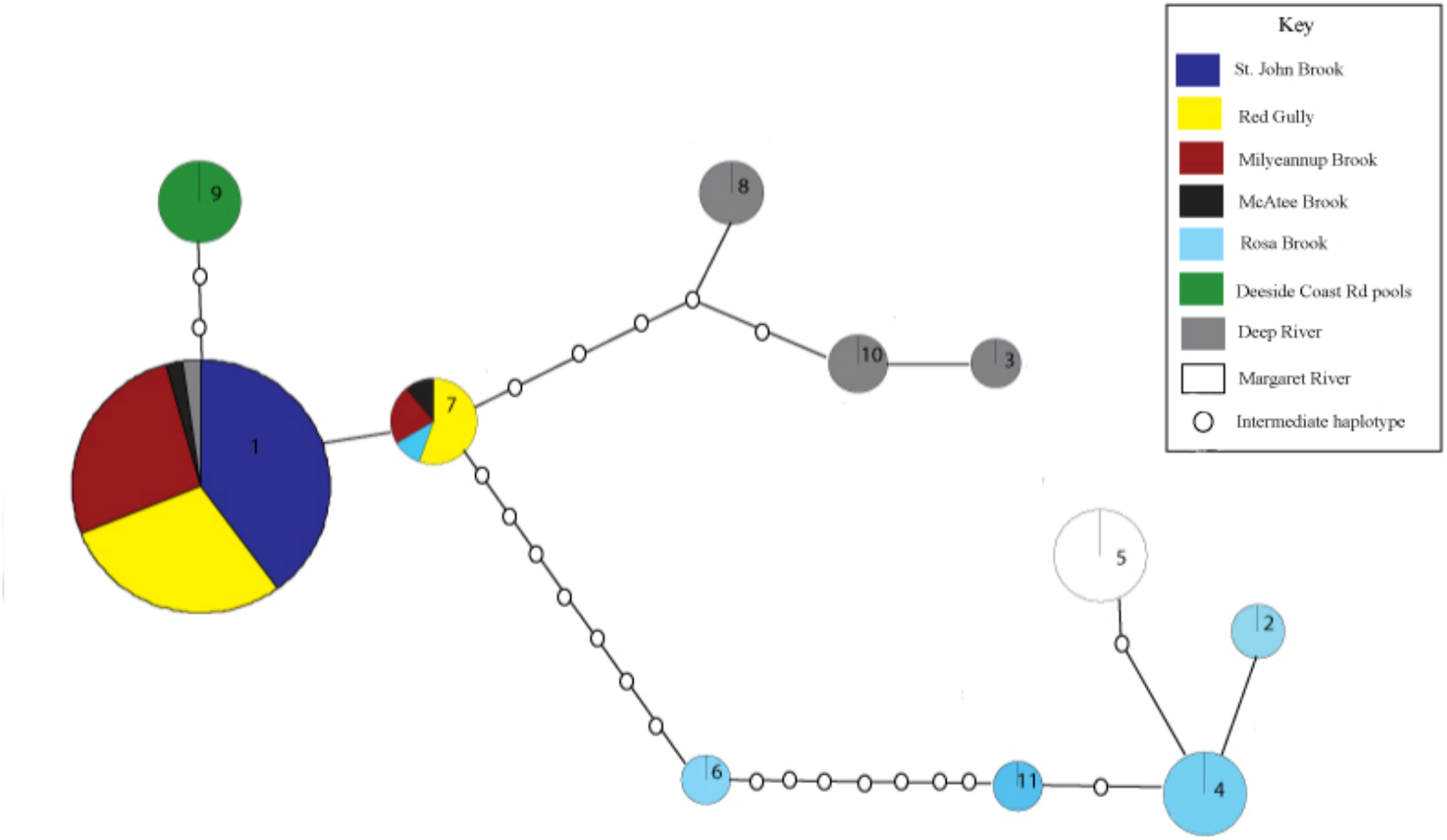


Figure 7 Haplotype network showing the relationships among the eleven control region haplotypes of the Mud Minnow. Each line in the network shows a single mutational change regardless of the length of the line. A numbered circle is used to represent each haplotype. The surface area of the circle is proportional to the total number of individuals with that haplotype. The coloured regions of the circles are used to indicate the abundances of individuals with each haplotype at each sampling site (see key). Empty circles indicate missing intermediate haplotypes. These missing haplotypes are necessary to link all observed haplotypes present in the network.

Conclusions and Management Implications

Balston's Pygmy Perch

The results of this study indicate that the assemblage of Balston's Pygmy Perch in Milyeannup Brook in the Blackwood River contains a low level of genetic diversity and is genetically differentiated from assemblages of this species at other near-by sites (*e.g.* Deep River, Shannon River and the Gardner River catchment). The low level of genetic diversity in this assemblage is mostly likely due to a small effective population size, while its genetic distinctiveness suggests that the amount of gene exchange (≡ dispersal) in Balston's Pygmy Perch between the Blackwood River and elsewhere is negligible. The genetic composition of the assemblage of Balston's Pygmy Perch in McAtee Brook in the Blackwood River appears to be similar to that of the assemblage in Milyeannup Brook, although larger sample sizes are required to confirm (or otherwise) this. Overall, the findings suggest that the assemblages of Balston's Pygmy Perch in the Blackwood River: (i) constitute a separate management unit(s) relative to assemblages outside of the Blackwood River; and (ii) are unlikely to be re-established via dispersal from outside sites if they were to become extinct. In general, the majority of genetic variation in Balston's Pygmy Perch in the southern-western corner of Western Australia appears to occur between (rather than within) assemblages of this species. Thus, if the preservation of genetic diversity in Balston's Pygmy Perch is a priority, assemblages of this species at as a many different locations as possible should be maintained. Indeed, the results indicate that most locations, including Milyeannup Brook, contain unique control region haplotypes, *i.e.,* haplotypes that are restricted to a single location, although additional sampling is required to confirm (or otherwise) this. However, the results also indicate that the extent of evolutionary divergence in Balston's Pygmy Perch in the south-western corner of Australia is limited. This apparently limited divergence suggests a recent (in an evolutionary context) common ancestry for all of the individuals of this species in this area.

Mud Minnow

The results of this study indicate that the genetic compositions of the assemblages of the Mud Minnow in different tributaries in the Blackwood River are often, although not invariably, differentiated from each other. This suggests that the amount of gene flow (≡ dispersal) in this species between the different tributaries of the Blackwood River is typically negligible, although it is possible that some individuals are exchanged between certain tributaries. The most likely explanation of this finding is that the main channel of the Blackwood River restricts dispersal in the Mud Minnow. Environmental differences between the tributaries could also limit the success of any individuals that do manage to move between tributaries. On this basis, the assemblages of the Mud Minnow in the different tributaries of the Blackwood River should be regarded as demographically independent and so managed as separate units. It should also be recognised that the loss of the Mud Minnow from a particular tributary is likely to be irreversible. In general, the results of this study suggest that the levels of genetic variation in the Mud Minnow are typically relatively low within sites but relatively high between sites. Thus, as was the case for Balston's Pygmy Perch, if the preservation of genetic diversity in the Mud Minnow is a priority, assemblages of this species at as a many different locations as possible should be maintained. The Mud Minnow exhibits relatively high levels of genetic divergence in the south-western Australia and thus the potential for the loss of significant amounts of genetic diversity seems to be greater for this species than for Balston's Pygmy Perch. Furthermore, certain assemblages of the Mud Minnow, such as the ones in Rosa Brook and Deep River, appear to contain diverse and unusual suites of haplotypes and so might warrant a special status in the development of conservation priorities. Since the increased genetic diversity at these sites is likely to be linked to larger effective population sizes, the size of the assemblages that are to be maintained at these sites (and other sites) is an important issue.

References

Applied Biosystems, Incorporated (2001). Seq Ed version 1.0.3.

Avise, J.C. (2000) *Phylogeography – The History and Formation of Species.* Harvard University Press, Massachusetts.

Beatty, S.J., McAleer, F.J., Morgan, D.L., Koenders, A., & Horwitz, P.H.J. (2006). *Fish and crayfish communities of the Blackwood River: migrations, ecology, and influence of surface and groundwater*. Centre for Fish & Fisheries Research (Murdoch University), Centre for Ecosystem Management (Edith Cowan University) report to Department of Water and South West Catchments Council.

Clarke, K.R. & Warwick, R.M. (2001). *Change in marine communities: an approach to statistical analysis and interpretation 2nd edition.* Primer-E Ltd., Plymouth, UK.

Clement, M., Posada, D., & Crandall, K.A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9** (10): 1657-1659.

Excoffier, L., Laval, G., & Schneider, S. (2005). Arelquin version 3.0: An integrated software package for populations 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: applications to human mitochondrial DNA restriction data. *Genetics* **131**: 479-491.

Fu, Y.X. (1997). Statistical tests of neutrality against population growth, hitchhiking and background selection. *Genetics* **147**: 915-925.

Gaffney, P.M., Rupnow, J., & Domeier M.L. (2007) Genetic similarity of disjunct populations of the giant sea bass *Stereolepis gigas*. *Journal of Fish Biology* **70 (Supplement A)**: 111-124.

GeneTool Lite v 1.0 (2000) BioTools Incorporated, http://www.biotools.com/products/genetool.html

Goudet, J. (1996). FSTAT (Version 1.2): A computer program to calculate *F*-statistics. *Journal of Heredity* **86**: 485-486.

Hughes, J.M. & Hillyer, M.J. (2006). Mitochondrial DNA and allozymes reveal high dispersal abilities and historical movement across drainage boundaries in two species of freshwater fishes from inland rivers in Queensland, Australia. *Journal of Fish Biology* **68 (Supplement B):** 270-291.

Kocher, T.D., Thomas, W.K., Meyer, A., Edwards, S.V., Paabo, S., Villablanca, F.X., & Wilson, A.C. (1989). Dynamics of mitochondrial DNA evolution in animals: Amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences USA* **86**: 6196-6200.

McMillan, W.O. & Palumbi, S.R. (1997). Rapid rate of control region evolution in Pacific butterflyfishes (Chaetodontidae). *Journal of Molecular Evolution* **45**: 473-484.

Meirmans, P.G. (2006). Using the AMOVA framework to estimate a standardised genetic differentiation measure. *Evolution.* **60** (11): 2399-2402.

Meyer, A., Kocher, T.D., Basasibwaki, P., & Wilson, A.C. (1990). Monophyletic origin of Lake Victoria cichlid fishes suggested by mitochondrial DNA sequences. *Nature* **347**: 550-553.

Mirol, P.M., Mascheretti, S., & Searle, J.B. (2000). Multiple nuclear pseudogenes of mitochondrial cytochrome b in *Ctenomys* (Caviomorpha, rodentia) with either great similarity to or high divergence from the true mitochondrial sequence. *Heredity* **84**, 538-547.

Morgan, D.L. & Beatty, S.J. (2005). *Baseline study on the fish and freshwater crayfish fauna in the Blackwood River and its tributaries receiving discharge from the Yarragadee Aquifer*. Report to the Department of Environment, Government of Western Australia.

Morgan, D.L., Gill, H.S. & Potter, I.C. (1998). Distribution, identification and biology of freshwater fishes in south-western Australia. *Records of the Western Australian Museum Supplement No. 56:* 97 pp.

Morgan, D.L., Hambleton, S.J., Gill, H.S. & Beatty, S.J. (2002). Distribution, biology and likely impacts of the introduced redfin perch (*Perca fluviatilis*) (Percidae) in Western Australia. *Marine & Freshwater Research* **53**: 1211-1221.

Morgan, D.L., Thorburn. D.C. & Gill, H.S. (2003). Salinization of south-western Western Australian rivers and the implications for the inland fish fauna – the Blackwood River, a case study. *Pacific Conservation Biology* **9**: 161-171.

Nei, M. (1987). *Molecular Evolutionary Genetics.* Columbia University Press, New York.

Raymond, M. & Rousset, F. (1995). An exact test for population differentiation. *Evolution* **49**: 1280-1283.

Rice, W.R. (1989). Analysing tables of statistical significance. *Evolution* **43**: 223-225.

Schmidt, H.A., Strimmer, K., Vingron, M., & von Haeseler, A. (2002). TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18**:502-504.

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123**: 585-595.

Templeton, A.R., Crandall, K.A., & Sing, C.F. (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics* **132**: 619-633.

Uzzell, T. & Corbin, K.W. (1971). Fitting discrete probability distributions to evolutionary events. *Science* **172**: 1089-1096.

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

Appendix 1: Samples

The dates and locations of samples of Balston's Pygmy Perch and the Mud Minnow used in this study (Table 9 and 10).

Table 9: The locations and collection dates of specimens of Balston's Pygmy Perch used in this study. Each fish was given a number for identification purposes. The samples from Windy Harbour, Chesapeake Road, and Meandering Stream did not yield DNA, most likely due to the age of the samples. Fish # 1, 16, 21, 22, 25, 27, and 28 from Gardner River watershed also did not yield DNA, and thus were not included in analyses. GPS coordinates of locations are available from the authors upon request.

Location	Dates of collection	Fish identification numbers
Milyeannup Brook	26-27/10/2005	1-20
McAtee Brook	04/08/2006	1-4
Margaret River	24/03/2007	1-5
Deep River	13/12/2006	1-13
Shannon River	13/12/2006	1-11
Gardner River watershed	21/08/1992 22/10/1992 24/10/1992	1-15; 17 18; 21-26; 28-30 16, 27
Pools on Deeside Coast Road	13/12/2006	1
Angove River	13/12/2006	1-2
Windy Harbour	21/04/1994	1-11
Chesapeake Road	26/03/1992	1-5
Meandering Stream	18/05/1994	1-64

Table 10: The locations and collections date of specimens of the Mud Minnows used in this study. Each fish was given a number for identification purposes. Sample numbers from Rosa Brook 7, 10-12, and 14 did not yield DNA, and were therefore not used in analyses. GPS coordinates of locations available from the authors upon request.

Location	Dates of collection	Fish identification numbers
St. John Brook	11/04/2005	1-18
Milyeannup Brook	28/10/2005 25-26/10/2006 26-27/10/2005 02/02/2006	4 5,6 7-10 1-3; 11-14
McAtee Brook	13/09/2006	1-2
Red Gully	07/04/2005	1-18
Rosa Brook	23/09/2003 10/12/2003 11/11/2003 22/10/2003	1-6 7-13 14-15 16-18
Margaret River	28/07/2006	1-10
Deep River	13/12/2006	1-10
Pools on Deeside Coast Road	13/12/2006	1-11

Appendix 2: DNA extraction

An example of an agarose gel used to determine the quality and quantity of DNA extracted from tissue samples (Figure 8). The presence of dark bands (and/or smears) indicates the extraction has successfully extracted DNA (*i.e.,* lanes 2 - 7) whereas an empty lane indicates that the extraction did not yield DNA (*i.e.,* lane 1). The relative concentrations of the DNA sample can be estimated by comparing the intensity of the associated band to that of the λ marker (lanes 8 and 16), which is of a known concentration (5µg/ml).

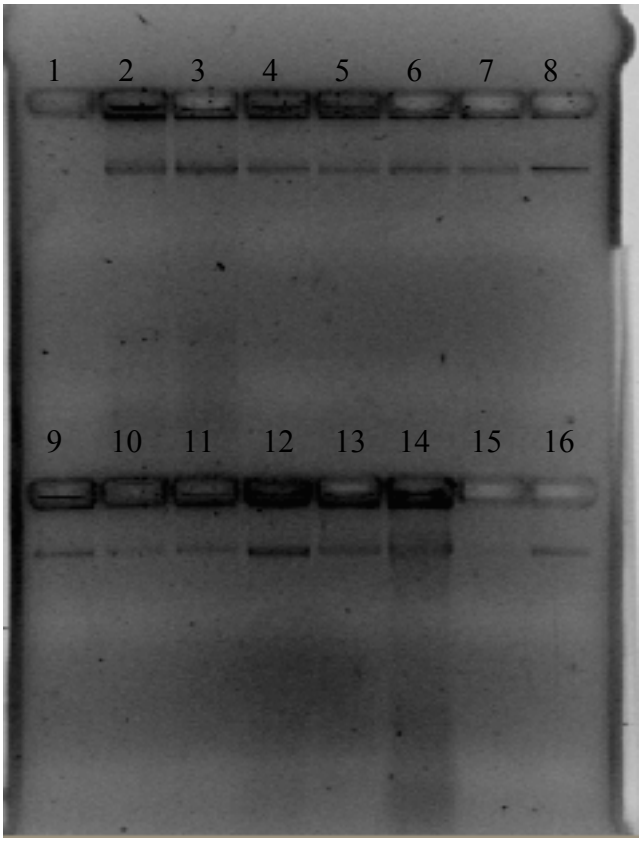


Figure 8 Agarose gel of DNA extracted from Balston's Pygmy Perch. Sharp single bands indicate high quality, non-degraded DNA, whereas smears are indicative of degraded DNA and empty lanes indicate that no DNA was yielded from the extraction. Samples were electrophoresed in a 2% agarose gel for 30 minutes at 46 mA. Lanes 1-7 and 9-15 tissue extractions and Lanes 8 and 16 are the λ marker (5µg/ml) used to determine the relative concentration of DNA extracts.

Appendix 3: DNA amplification with PCR

An example of an agarose gel used to determine whether the target portion of the control region was successful amplified, via PCR, from the DNA extracts (Figure 9). The presence of a single band without streaking indicates that the PCR product is of sufficient quality for sequencing. The relative concentrations of a PCR product can be estimated by comparing the intensity of the associated band to that of the *phi x* 174 marker (lane 8), which is of a known concentration (10μg/ml). The size of the PCR product can also be determined by using the standard weight marker *phi x* 174 as a guide.

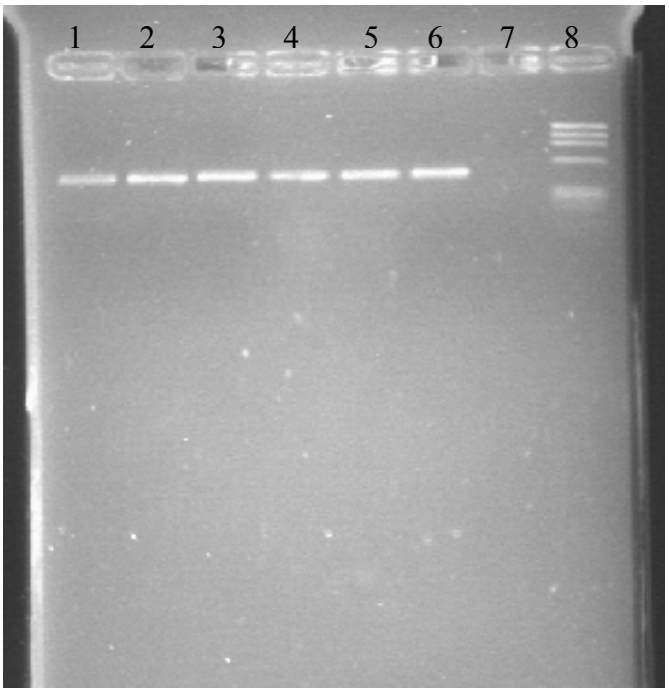


Figure 9 Agarose gel image demonstrating successful PCR amplification of the control region (lanes 1-6) of Balston's Pygmy Perch. A negative control (lane 7) and a molecular weight marker, *phi x* 174 (10μg/ml) (lane 8) were included on each gel. The molecular marker is used to estimate the size and relative quantity of the PCR products.

Appendix 4: DNA sequencing

Figure 10 is an example of a DNA sequence for Balston's Pygmy Perch. Each peak in the chromatograph corresponds to a base (A, C, G, or T). A quality sequence has only a single, strong peak at each position, as shown in Figure 10.

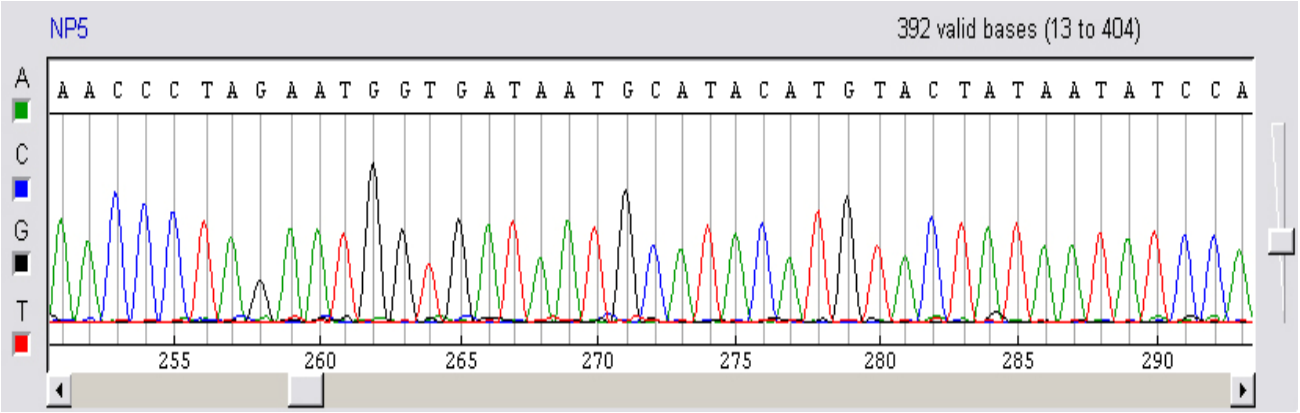


Figure 10 Sequence data for a portion of the control region in the mtDNA of Balston's Pygmy Perch. Each peak codes for a base (A, C, G, or T).