STOCK IDENTIFICATION AND DISCRIMINATION IN SNAPPER (*Pagrus auratus*) IN SOUTHERN AUSTRALIA

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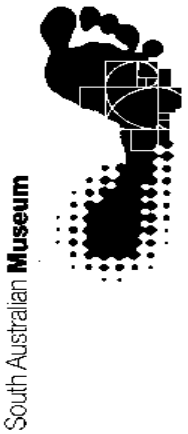

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ISBN 0 7308 5808 1

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NON-TECHNICAL SUMMARY

94/168 Stock discrimination and identification of snapper (*Pagrus auratus*) in southern Australia.

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OBJECTIVE

To use molecular genetic techniques to identify stock structuring in snapper in South Australian waters; and discriminate between any stocks that may be present.

SUMMARY

The snapper (*Pagrus auratus*) fishery in South Australia is currently managed as one stock, although recent changes to the regional management of the recreational fishery have been based in part on an assumption of a multi-stock population structure. Tagging and genetic studies in other States have shown that different stocks do occur over relatively small geographical areas. This prompted an interest in identifying the existence or otherwise of separate stocks in South Australian snapper. An urgency to identify the appropriate spatial management units for this species has been prompted by the decline of the commercial catch to its lowest levels since 1968.

Studies of genetic variation in the proteins and DNA of snapper were carried out at the South Australian Museum to determine the presence of genetically isolated breeding stocks. Fish from each of the three commercially fished areas within the state and from Victoria and Western Australia were sampled over a 5 year period with assistance from commercial and recreational fishers.

The present genetic study provided no evidence of the existence of more than one stock in the commercially fished waters in S.A. and south-western W.A. This is consistent with tagging studies carried out in South Australia over the past 19 years. In contrast, both genetic and tagging studies have shown that fish from Port Phillip Bay, Victoria represent a different stock which extends into the south-east of S.A.

The significance of these results is that the snapper fishery in S.A. should be managed as a single genetic stock. Ultimately, final management of the fishery will incorporate other scientific and economic parameters, but will have a stronger biological basis because of the availability of these genetic data.

KEYWORDS

Pagrus auratus; mitochondrial DNA; allozyme; stock structure

BACKGROUND

The snapper (*Pagrus auratus*) fishery in South Australia is currently managed as one stock, although current recreational bag limits have assumed a multi-stock population structure. Tagging and genetic studies in other States have shown that sub-stocks do occur over relatively small geographical areas (e.g. Sanders 1974; Johnson *et al*. 1986). Thus there is an urgent need to identify the existence or otherwise of sub-stocks in South Australian snapper. Furthermore, evidence derived from catch data indicates that the Gulf of St. Vincent may be currently over-exploited as catch rates in this area over the past six years have declined (Rohan *et al*. 1991). Jones (1987) and Rohan *et al*. (1991) have cautioned that the fishery may also be exploited to near-maximum levels in the Spencer Gulf .

To date, limited mark-recapture studies (Jones 1984) and allozyme electrophoretic analyses (MacDonald 1980) have produced ambiguous evidence of stock structure of snapper in South Australian waters west of the Murray River mouth. The tagging studies of Jones (1984) suggest that fish from Spencer Gulf and Gulf of St Vincent constitute a single stock. MacDonald (1980) screened variation in seven polymorphic allozyme loci in samples from the upper Spencer Gulf and the Investigator Strait. Two loci, *Adh* and *Est*, were informative indicating that Spencer Gulf snapper constitute a discrete stock. However, there is evidence that the *Est* locus is subject to selection in snapper and therefore may not be a reliable indicator of population structure (Smith *et al*. 1978, Smith 1979). In a similar study, Johnson *et al*. (1986) used allozyme electrophoresis to examine population structure of snapper stocks in Shark Bay, Western Australia which represents a smaller area than Spencer Gulf, but has a similar physical environment (ie. salinity, water temperature, geography) to the South Australian gulfs (Nunes and Lennon 1986). They found four informative polymorphic loci that indicated the presence of three separate stocks within the bay. The limited studies to date on South Australian snapper stock structure and the presence of multiple stocks in Shark Bay points to a need for a more comprehensive and robust study of stock structure of the South Australian snapper fishery using molecular genetic techniques.

It is desirable to test for the presence of population substructuring with data from more than one locus, because the effects of selection may go undetected from the analysis of a single locus. Because mitochondrial DNA (mtDNA), a widely used marker for population structure studies, is effectively a single locus due to the mitochondrial genome's non-recombining, haploid nature, nuclear loci, for example single-copy nuclear sequence, microsatellites or allozymes should also be assayed (Avise, 1994; Li and Graur, 1991). Furthermore, a number of studies of phylogeographic structure have revealed contrasting results between mitochondrial and nuclear gene data sets due to the disparity between individual gene trees and species trees (Degnan, 1993; Palumbi and Baker, 1994) or because of divergent migratory behaviour of males and females (e.g. Norman *et al*. 1994).

In the present study, we examined variation in mtDNA and allozyme markers to address the question of whether more than one stock is present in the three main snapper-fishing areas of South Australia, i.e., Spencer Gulf, the Gulf of St. Vincent and the west coast (ie eastern Great Australian Bight). Firstly, we sequenced part of the control region, the major non-coding region of the vertebrate mitochondrial genome, from four or more individuals from five representative areas. Secondly, we screened a larger number ($\geq$30) individuals for variation at informative restriction sites identified from the sequences using a restriction fragment length polymorphism analysis of a polymerase chain reaction (PCR-RFLP) amplified portion of the control region. The former approach provides a phylogenetic perspective relevant mostly to long-term population processes and management issues, while the latter approach generates allele frequency data that mainly address recent population processes and short-term management issues. Thirdly, we extended MacDonald's (1980) allozyme survey was screening for variation at fifty-two loci in South Australian samples and by sampling fish from each of the three commercially fished areas. The study was carried out under

a null hypothesis of panmixia, ie., South Australian snapper constitute a single, randomly interbreeding population.

NEED

Our present knowledge of the stock structure of snapper is insufficient for the present and future management of this fishery. Information on the number of stocks, their genetic identities and geographical distributions is essential, not only for estimating the relative contribution of the different stocks to the fishery (thereby ensuring the rational allocation of effort to each stock), but also ensuring genetic integrity of the stocks if re-seeding of depleted stocks takes place.

OBJECTIVES

The objective of this proposal is to use molecular genetic techniques to identify stock structuring in snapper in South Australian waters; and discriminate between any stocks that may be present.

METHODS

Mitochondrial DNA

Tissue Samples

Snapper liver was collected from 18 locations in Western Australia, South Australia and Victoria (Table 1, Fig. 1). Most liver tissue was frozen in liquid nitrogen at the site of capture and subsequently kept at -70ºC until analysis. The Western Australian tissues were stored in a solution of 1:1 ethanol:saline (0.85%) until used for DNA extraction. Liver tissue from a black bream, *Acanthopagrus butcheri* (Family Sparidae), collected from the Todd River near Port Lincoln, S.A. was used as an outgroup.

DNA Extraction

Total cellular DNA was extracted using either a standard phenol/chloroform technique (Sambrook et al. 1989) or a salt-extraction protocol (Integrated Sciences). Alcohol-stored tissue was washed several times with Tris buffer (pH 8.0) prior to homogenisation and extracted as above.

PCR Amplification and DNA Sequencing

Aliquots of 50 to 100ngms of the extracted total genomic DNA solutions were added to a 50µl reaction mixture containing 4mM $MgCl_2$, 1X reaction buffer (supplied by manufacturer of polymerase), 0.8mM dNTPs, 10pM of each primer, 1 unit of either Promega *Taq* or Biotech *Tth* plus DNA polymerase. Initially control region sequences from a snapper were obtained with primers, L15926 and H16498 designed by Kocher et al. (1989) and Meyer et al. (1990) respectively . These sequences were used to design nested primers, 5'-CGG AAT TCG GTT CTT ACT GCA TAG TTA TT-3' (heavy strand) and 5'-GTA CGT ACC TAC ATT AGA CTA TTC TTT CAT AGT-3' (light strand), for amplifying part of the control region of snapper and black bream, the outgroup for the phylogenetic analysis. Amplifications, carried out on a Corbett FTS-320 Thermal Sequencer, comprised denaturation for 2.25 min at 94ºC, and 30 cycles of 45 s at 94ºC, 45 s at 55ºC and 1 min at 72ºC and ended with a single extension step of 6 min at 72ºC.

Products were purified for sequencing using a Bresa-Clean DNA Purification Kit (Bresatec), following the manufacturer's protocol for DNA extraction from solutions. Products were

cycle-sequenced following the manufacturer's instructions on a Corbett FTS-1 Thermal Sequencer using the Applied Biosystems PRISM Ready Reaction DyeDeoxy Terminator Cycle sequencing kit. PCR products were directly sequenced from both strands using the original PCR primers. The sequencing program comprised 25 cycles of 30 s at 94ºC, 15 s at 50ºC and 4 min at 60ºC. Reaction products were analysed by electrophoresis on an Applied Biosystems Model 373A Sequencing System. Sequences were edited using the Applied Biosystems SeqEd program and were aligned using the CLUSTAL V program (Higgins *et al*. 1991).

A test for the presence of nuclear paralogues of the snapper control region was made a comparison of sequences obtained by PCR and direct sequencing from total cellular DNA and purified mtDNA from an individual from Cape Jervis (CJ). Mitochondrial DNA was purified from frozen liver through a caesium-chloride gradient following the method of Dowling *et al*. (1990).

Phylogeographic analysis

Parsimony analysis was performed on the aligned nucleotide sequences using the heuristic search option in PAUP v. 3.1.1 (Swofford 1993). Trees were rooted with the sequence from the black bream. Trees were also constructed with the neighbour-joining (NJ) algorithm from distance matrices, implemented in MEGA (Kumar *et al*. 1993. Bootstrap and standard error tests were used to test the reliability of the trees.

Restriction Fragment Length Polymorphism Typing

Single restriction endonuclease digests, using the enzymes *Acc* I, *Mse* I and *Ssp* I were performed directly on PCR products in a 10µl reaction mixture, using 9µl of PCR reaction mix following the manufacturers (New England Biolabs) instructions. Digestion products were electrophoresed on either 6% non-denaturing polyacrylamide or 2.5% agarose gels, stained with ethidium bromide and viewed under UV light. Haplotypes were defined by the composite restriction fragment pattern across the three enzymes typed.

Statistics

Measures of the proportion of overall genetic variation due to population subdivision were obtained using the G_{st} statistic (Takahata and Palumbi 1985). G_{st} values were calculated with the MacMiracle program (supplied by S. Palumbi) from restriction site presence or absence data (Table 4). Effective long-term average migratory exchange of females between populations per generation, N_m, was obtained using the approximation $G_{st} = 1/(1 + 2 N_m)$, assuming an island model of population structure (Takahata and Palumbi 1985). Heterogeneity of RFLP haplotype frequency distribution among samples was tested with the Monte-Carlo χ^2 test (Roff and Bentzen 1989). Monte-Carlo values were obtained using REAP (McElroy et al. 1991). For each Monte-Carlo test, 1000 bootstrap replicates were performed.

Allozymes

Tissue Samples

Between November 1991 and January 1996, seven samples of *Pagrus auratus* comprising 541 individuals were obtained from each of two areas in the Gulf of St Vincent and Spencer Gulf and from one area on the west coast of South Australia (Fig. 1, Table 1). The two adjacent samples (CED and STR) from the West Coast were pooled to form a single sample (WC) for analysis. In total, six samples (WC, DR, WHY, USG, CJ, ADE) were considered for analysis (Table 1, Fig. 1). Liver and muscle was dissected from fresh specimens and stored at -70°C until use. The

standard length of fish was recorded. Individual fish examined in the mtDNA study were all included in the allozyme samples.

Homogenates of liver were electrophoresed on sheets of cellulose acetate (Cellogel: Chemetron) as described by Richardson *et al.* (1986). An initial pilot study of 50 individuals drawn from across the species range in South Australia was used to identify variable loci. Homogenates were assayed for 41 enzymes which were encoded by 52 presumptive loci. The enzymes stained, E.C. numbers and abbreviations (Murphy *et al.* 1990) are: aspartate aminotransferase (AAT, E.C. 2.6.1.1), aconitate hydratase (ACOH, E.C. 4.2.1.3), acid phosphatase (ACP, EC 3.1.3.2), adenosine deaminase (ADA, EC 3.5.4.4), alcohol dehydrogenase (ADH, EC 1.1.1.1), aldehyde dehydrogenase (ALDH, EC 1.2.1.5), adenylate kinase (AK E.C. 2.7.4.3), carbonic hydratase (CA E.C. 4.2.1.1), leucine aminopeptidase (CAP, E.C. 3.4.11.1), enolase (ENO, E.C. 4.2.1.11), esterase (EST, EC 3.1.1.?), fructose-diphosphatase (FBP, E.C. 3.1.3.11), fumarate hydratase (FUMH, E.C.4.2.1.2), glyceraldehyde-phosphate dehydrogenase (GAPDH, E.C. 1.2.1.2), (S)-2-hydroxy-acid oxidase (GOX, EC 1.1.3.15), glutamate dehydrogenase (GTDH E.C. 1.4.1.3), glycerol-3-phosphate dehydrogenase (G3PDH, E.C. 1.1.1.8), glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49), glucose-phosphate isomerase (GPI, E.C. 5.3.1.9), alanine aminotransferase (GPT, EC 2.6.1.2), glutathione peroxidase (GPX, EC 1.11.1.9), glutathione reductase (GSR, EC 1.6.4.2), beta-glucuronidase (GUS, EC 3.2.1.31), 3-hydroxybutyrate dehydrogenase (HBDH, E.C. 1.1.1.30), L-iditol dehydrogenase (IDDH, E.C. 1.1.1.14), isocitrate dehydrogenase (IDH, E.C. 1.1.1.42), lactate dehydrogenase (LDH, E.C. 1.1.1.27), lactoyl-glutathione lyase (LGL, E.C. 4.4.1.5), malate dehydrogenase (MDH, E.C. 1.1.1.37), mannose-phosphate isomerase (MPI, E.C. 5.3.1.8), nucleoside-diphosphate kinase (NDPK, EC 2.7.4.6), purine-nucleoside phosphorylase (NP, EC 2.4.2.1), nucleoside-triphosphate-adenylate kinase (NTAK, EC 2.7.4.10), peptidases (PEP, E.C. 3.4.11 or 13.*), phosphoglycerate mutase (PGAM, EC 5.4.2.1), 6-phosphogluconate dehydrogenase (PGDH, E.C. 1.1.1.44), phosphoglycerate kinase (PGK E.C. 2.7.2.3), phosphoglucomutase (PGM, E.C. 2.7.5.1), superoxide dismutase (SOD E.C. 1.15.1.1), and triose-phosphate isomerase (TPI, E.C. 5.3.1.1). Alleles were identified by comparison with samples that were repeatedly included on each gel (internal controls) and through critical side-by-side comparisons (line-ups; see Richardson *et al.*, 1986). Buffers used and running conditions for those loci finally typed are presented in Table 3.

Statistical analysis

Calculations of allele frequencies, and tests of conformance to Hardy-Weinberg genotypic proportions were performed with BIOSYS-1 (Swofford and Selander 1981). To avoid statistical difficulties associated with small expected values for some genotypic classes tests for conformance to Hardy-Weinberg expectations were made with pooled homozygous and pooled heterozygous classes such that the expected numbers of genotypes in a given class were always at least one. Heterogeneity among areas was tested by χ^2 contingency test of allele numbers. When multiple tests were carried out, the Bonferroni correction was used to adjust significance levels. Foe each locus, the usual biological significance level (0.05) was divided by the number of tests to obtain a corrected significance level.

Standardised variance in allelic frequencies, F_{ST} (Wright 1978) was calculated for each of the nine loci, as an average F_{ST} of the alleles at that locus, weighted by their frequencies. Five hundred bootstrap pseudoreplications were performed across loci to estimate the confidence intervals on the F_{ST} estimates. These procedures were implemented in GDA- Genetic Data Analysis (Lewis and Zaykin 1996).

DETAILED RESULTS

Mitochondrial DNA

A total of 441 aligned sites were available from the 37 individuals (Fig. 2a,b). Sixty-three sites were variable and 55 sites were informative under the parsimony criterion (Fig. 2b). Sequences from the total genomic and purified mtDNA templates from the one individual tested were identical. Of the 36 *Pagrus* sequences, 29 had unique sequences. Three PCR product length variants were observed (Fig. 2a). Length variants were defined by insertion or deletion of tandem imperfect repeats. The longest length variant, referred to as the "long variant" had in order from 5' to 3', single 50bp, 55bp, 53bp repeats and a 33bp truncated version of the repeat. It was observed in a single individual, ADE1 from Gulf St Vincent. The most frequently encountered length variant, referred to as the "intermediate variant" was missing the 50bp repeat and was observed in all sampled locations. The shortest product, referred to as the "short variant", was missing the 50bp and 55bp repeats and was found in two individuals, ARD2 from Gulf St Vincent and BLA1 from the Spencer Gulf. The black bream individual had the "intermediate variant". Among the snapper haplotypes, maximum uncorrected sequence divergence was 8.05%.

For the snapper control region data, values up to 0.083 were found for Jukes Cantor distances between haplotypes and transition/transversion ratios were high (>>2). Under these conditions, Kumar et al. (1993) recommend analysis using the Kimura distance or the gamma distances for Kimura's 2-parameter model. Fig. 3 shows a phylogenetic tree of these sequences constructed with neighbour-joining algorithm from Kimura-2-parameter distances between haplotypes. This tree was rooted with the black bream sequence. Two major lineages are apparent among the snapper sequences, henceforth referred to as the "A" and "B" lineages. Both lineages included fish from all sampled locations except the Gulf of St. Vincent samples (ADE, ARD, CJ) which were present only in the "A" lineage. The individuals with the "short" and "long" length variants were present in the "A" lineage. Bootstrap and standard error tests provided strong support (>90%) for the two major lineages but little support for any well-resolved phylogenetic structure within either major lineage, apart from resolution of several terminal lineages of two to three taxa (Fig. 3). Maximum parsimony analysis of these sequences produced 192 equally most parsimonious trees of length 122 steps. In a strict consensus tree, which summarised the nodes common to all of the equally most parsimonious trees, the "A" and "B" lineages also were present.

The absence of individuals from the Gulf of St. Vincent in the "B" lineage was the only evidence for phylogeographic structure in these data. Rather than sequence more individuals to determine the frequencies of the two major lineages among further samples, we designed a RFLP-PCR assay to detect the "A" or "B" lineages. MacClade 3.0 (Maddison and Maddison 1992) was used to identify phylogenetically informative nucleotide substitutions, i.e. synapomorphic substitutions defining the "A" and "B" lineages. Three restriction enzymes, *Acc* I, *Mse* I and *Ssp* I, were selected which detected two out of the eight and three out of the four of the synapomorphies that defined the "A" and "B" lineages respectively (Fig. 2). *Mse* I also cut at three sites that were not synapomorphies (Table 4). PCR products of previously sequenced individuals were digested with these restriction enzymes to test that samples could be unambiguously assigned to one or the other lineage. Subsequently, snapper samples from Western Australia (WA), the west coast of South Australia (WC), Spencer Gulf, (DR and USG) the Gulf of St. Vincent (ADE and CJ) and Port Phillip Bay, Victoria (HOB) were PCR-amplified and RFLP-typed (Fig. 1, Table 3). Note that the sample USG for PCR-RFLP typing is a composite of individuals collected from three adjacent localities in the upper Spencer Gulf (Table 3).

A total of 11 haplotypes were observed among the 235 individuals typed with the three restriction enzymes (Table 2). Representatives of the nine haplotypes that were not predicted from the sequence analysis were sequenced and included in the phylogenetic analysis to determine if they represented lineages additional to the "A" and "B" lineages (Fig. 3). In all cases additional haplotypes were descended from within either the "A" or "B" lineages. The frequencies of the "A" and "B" lineages at each of the seven sampled locations are presented

in Table 3. The "A" lineage predominated in all samples except for HOB. Haplotype A1 is the most common haplotype in all samples (overall mean 65%) with the exception of the HOB sample, which consisted of only 6.7% A1. Excluding the HOB sample, the overall mean frequency for haplotype A1 is 73%, with a range among samples of 61-80%. The most common alternative haplotype is B1 (overall mean 12%), which occurs in all sample sites, and is the most common haplotype in the HOB sample, comprising 40% of that sample. Haplotype B2 is the third most common haplotype (overall mean 8.5%) and occurs in all samples except WC and CJ. Haplotype A3 is found only in South Australian samples (overall mean 6.0%). Haplotypes B3 and B6 are specific to WA, B4 and B5 are specific to HOB, A5 is restricted to Gulf of St Vincent (ADE and CJ), A4 is present in Gulf of St Vincent and WC, but not in either sample from Spencer Gulf.

χ^2 tests for heterogeneity of allele frequencies (Table 6) showed significant heterogeneity (χ^2 = 163.41, $p << 0.0001$) among all sites sampled. The analysis, repeated excluding the Victorian sample, showed marginally significant heterogeneity (χ^2 = 53.23, p = 0.05), probably due to mainly to the WA sample, as subsequent analysis of the South Australian samples only did not show significant heterogeneity (χ^2 = 33.34, $p > 0.09$).

G_{st} analysis (Table 6) did not show significant mtDNA control region restriction site variation due to population subdivision either among all South Australian samples or among the samples from the two South Australian gulfs. G_{st} values were negligible (-0.0004 and 0.0001, respectively), indicating that snapper populations in South Australian waters were unlikely to be fully or partially reproductively isolated. Similarly, comparison of the WA sample and South Australian samples WC, USG and ADE yielded an insignificant result (G_{st} = -0.0011). Nearly 11% of molecular variance among the two South Australian gulfs and the Victorian sample could be accounted for by population subdivision (G_{st} = 0.1074; $p < 0.001$). The resulting effective long-term average rate of migration, N_m, of females between these populations is equivalent to 4.16 females per generation.

Allozymes

Twelve enzymes encoded by 13 loci showed variation in the initial screening panel drawn from across the species range in South Australia. Three loci, *Aldh-1, Est* and *Pgk*, could not be reliably scored and a single heterozygote was observed at the fourth locus, *Aat-3*, during the main phase of the study. The following nine loci gave sufficient resolution to be typed on all specimens, *Aat-2*, *Acon*, *Ada*, *Adh*, *Gpi*, *Iddh*, *Idh*, *Mpi*, and *Pgm-2*.

Allele frequencies in fish from the six samples are presented in Table 7. The frequency of the most common allele was less than 0.95 for five loci and less than 0.90 for three loci. Sexes of fish were not available, so no testing of variation in genotypes frequencies between the sexes was possible. However Johnson *et al.* (1986) did not detect significant differences in genotype frequencies between the sexes in snapper from Shark Bay, Western Australia.

Tests of conformance to Hardy-Weinberg genotypic proportions within samples indicated disequilibria ($P < 0.05$) in 6 out of 34 valid (expected frequencies $\geq$ 1.0 per cell) comparisons. One had a probability of 0.038 and the remainder had probabilities less than 0.008. The probabilities for all except two, *Iddh* for CJ (0.001) and *Pgm* for ADE (0.001), were greater than the Bonferroni-adjusted significance level of 0.001 (0.05/34).

Contingency χ^2 analysis of allele frequencies showed significant differences at six of the 78 valid tests. Five of these were at the *Pgm* locus (probabilities ranging from 0.005 to 0.025) and the sixth at *Idh*, probability 0.025). All of these probabilities were considerably greater than the Bonferonni-adjusted significance level of 0.0006 (0.05/78). Samples from each of the two gulfs were pooled such that a second contingency χ^2 analysis was conducted with three samples: WC,

SG (DR+WHY+USG) and GSV (CJ+ADE). Only one test, which involved *Pgm*, of the 27 valid tests was significant, but the probability was greater than the Bonferroni-adjusted significance level of 0.002 (0.05/27).

Among all six samples the average F_{ST} was 0.006264, and the bootstrapped (500 replicates) 95% confidence limits were 0.011751 to -0.001316, consistent with the lack of between sample differentiation observed in the contingency χ^2 analysis.

In both the present study and MacDonald (1980), *Adh* was typed on cellogel medium but with different buffers, a Tris-maleate buffer in the former and a Tris-EDTA-maleate $MgCl_2$ buffer in the latter. However, the results of each study appear comparable as four alleles with roughly similar relative frequencies were detected among the South Australian samples in both studies. Contingency χ^2 analysis of allele frequencies at the *Adh* locus were used to compare the two data sets (Table 8). MacDonald's two Cowled Landing samples were significantly different from the USG, WHY and ADE samples and one of the Cowled Landing samples was also significantly different from the DR sample. However, all of these probabilities were considerably greater than the Bonferroni-adjusted significance level of 0.0017 (0.05/30).

DISCUSSION

In the present study, analysis of variation in the mitochondrial control region and variation at nine allozyme loci for the same samples has not produced evidence of substructuring of snapper populations in South Australian waters west of the River Murray mouth. However, both of these data sets contrast with the allozyme study of MacDonald (1980) in which evidence of substructuring within the South Australian gulf was found at two allozyme loci, *Adh* and *Est*. Although we were unable to adequately resolve variation at *Est* in the present study, we were able to type *Adh*. The geographic distribution of the Adh^c allele (Adh^{125} in MacDonalds terminology) is different between the two studies. In the present study it was found in all samples at a frequency of 0.01, but in MacDonald's study it was observed only in the three samples from Spencer Gulf at frequencies ranging from 0.01 to 0.09. The pattern of differences among samples is informative as significant differences were found not only between MacDonalds' Cowled Landing, upper Spencer Gulf samples and ADE but also with the samples from the upper Spencer Gulf (USG and WHY) typed in the present study. Samples from the upper Spencer Gulf were collected 15 years apart, MacDonald's in 1978-9 and in 1991 for the present study. These samples have acted effectively as temporal replicates, giving a comparison of turnover in allele frequencies between generations in the one geographic area and thus putting into perspective the biological relevance of population differentiation at the *Adh* locus observed by MacDonald (1980).

However, as rates of demographic and ecological change are high relative to rates of molecular change, it is possible that stock structure may exist, but the molecular genetic analysis may not reveal it. If population subdivision has occurred relatively recently, this may not yet be reflected in the DNA, ie., sequences may not have had time to diverge to a sufficient extent to allow recent population subdivision to be seen in DNA data (Palumbi *et al.* 1991, Moritz 1994). Additionally, insufficient time may have passed for sequences or mtDNA haplotypes from different subpopulations to be lost due to the effects of genetic drift, so that most haplotypes may be represented in all recently diverged populations (Avise 1994), especially if standing population sizes are large, as they are likely to be in marine fish. It is emphasised, therefore, that absence of significant genetic differences across a geographical area is not proof of a panmictic population across that area (Palumbi *et al*. 1991). Additionally, relatively low rates of female migration are sufficient to maintain relatively homogeneous mtDNA haplotype frequencies (Wright 1931, Slatkin 1985) independent of population size (Slatkin 1987). Because such rates of migration may be negligible compared to growth and mortality rates, separate subpopulations may exist without exhibiting significant genetic differentiation (Baker *et al.* 1994).

The analysis revealed a number of RFLP haplotypes that were restricted to single samples. Such 'private' haplotypes can be evidence for reduced gene flow between populations (Slatkin 1985), though in the present case their frequencies were too low to be statistically significant (Carr et al. 1995). Analysis of many more individuals would be required to obtain a robust estimate of their frequencies, but this is impractical because sample sizes would be so large that the cost and time required for analysis would be prohibitive. Alternatively, the approach of Norman et al. (1994) in which the common haplotypes were screened by denaturing gradient gel electrophoresis (DGGE) for locality-specific cryptic variants may be more profitable. Norman et al. (1994) observed three and four cryptic variants of the two common haplotypes in the green turtle (*Chelonia mydas*) which revealed geographic structuring not apparent from their original PCR-RFLP analysis. Similarly, the sequencing of a number of common haplotype (A1) individuals from each South Australian sample and subsequent phylogenetic analysis may also reveal phylogeographic structure not apparent from the RFLP data.

Direct sequencing of the 5' end of the mitochondrial control region among snapper from southern Australian waters revealed a large amount of polymorphism (29 haplotypes among 36 snapper). In contrast, RFLP analysis of the same control region product for 235 individuals yielded only 11 unique haplotypes, one of which (A1) was the dominant haplotype in all but the HOB sample. This is a similar finding to the white sturgeon (*Acipenser transmontanus*) study of Brown et al. (1993) and the swordfish (*Xiphias gladius*) study of Rosel and Block (1996). Although the amount of nucleotide variation able to be detected is sacrificed somewhat in the RFLP screening compared to direct sequence analysis, the ability of the analysis to detect population structure is not. The variable sites screened by restriction enzymes are known to be phylogenetically informative from the initial sequencing phase. Where the phylogenetic analysis of control region sequences allows insight into long-term population processes on an evolutionary scale, the PCR-RFLP screening potentially provides information into more recent population processes, i.e., on an ecological time scale (Moritz 1994). This approach has been used to successfully detect intra-species population structure in marine turtles (Norman *et al*. 1994) and bats (Worthington Wilmer *et al*. 1994).

Genetic and tagging data together provide a reasonably robust test of the null hypothesis that snapper in South Australia west of the River Murray mouth constitute a single stock. Both the mitochondrial DNA data (Barclay *et al*. submitted) and the present allozyme analysis are consistent in not detecting significant differences in allele frequencies among the two gulfs and the west coast. The limited tagging data of Jones (1984) detected movements of adult snapper from Spencer Gulf into the Investigator Strait. The situation in the South Australian gulfs contrasts with the strongly structured stocks found in Shark Bay, Western Australia (Johnson *et al*. 1986). However, Shark bay has two features, a very low rate of exchange of waters between the inner and outer portions of the bay and steep salinity gradients, that may impose selection against migration between different areas of the bay. In Spencer Gulf, a steep salinity gradient is present in the upper part of the gulf. The middle portion of the gulf also has a higher salinity than oceanic waters as it acts as a reservoir for the high salinity outflow from the upper gulf. However the middle protion of the gulf flushes its high salinity load to shelf waters during winter. Importantly, the Shark Bay system and Spencer Gulf may differ in the rate of water exchange between the inner and outer portions and/or the seasonal stability of salinity gradients.

It is important to point out that snapper do show strong evidence of stock structure in South Australian waters. Indeed, allozymes (MacDonald 1980), mitochondrial DNA (Barclay *et al.* submitted) and tagging studies (Sanders 1974) are consistent in showing a stock division between Victorian and South Australian waters west of the River Murray mouth. The exact nature of the stock differentiation is not established, but in the absence of a commercial fishery along the south-eastern coast of South Australia, resolving the situation is not a priority at present. The mitochondrial DNA data add a further perspective. Although the amount of molecular variance

in mtDNA that could be accounted for by population subdivision was moderate (almost 11%), this result was highly significant. However, the resultant estimate of long term average migration rate, or effective gene flow, of 4.16 females per generation is theoretically large enough to prevent genetic differentiation of populations by drift alone (Slatkin 1985). Given that snapper populations are likely to be relatively large (>>1000 individuals), the observed N_m will be negligible relative to rates of population growth and mortality (Baker *et al*. 1994). This, combined with the observed significant χ^2 and G_{st} results and MacDonald's (1980) allozyme analysis indicates that there is a strong stock partition between South Australia and Victoria. Despite a marginally significant Monte-Carlo χ^2 value when South Australian and Western Australia samples were compared, the corresponding G_{st} value (-0.0011) could not reject the null hypothesis of no population structuring.

CONCLUSION

Although our results fail to show evidence of separate stocks of snapper in South Australian waters, the current decline in both size and number of snapper being caught by professional fishers is a real phenomenon. Whether this due to over-exploitation or a natural cycle of decline, caution must be exercised in the management of the South Australian snapper fishery. Localised over-fishing within an area that experiences a relatively large amount of migration and interbreeding between geographic locations is likely to have minimal evolutionary effect as opposed to potentially significant ecological effects. Localised extinctions can result from over-exploitation in an area where fully or partially genetically isolated populations exist, between which rate of migration and interbreeding are low (Ovenden *et al.* 1989).

BENEFITS

The beneficiaries of the study were primarily identified as the Marine Scalefish Fishery of South Australia (commercial and recreational sectors) via a more comprehensive understanding of the biology of the species. This in turn leads to more confident management of the snapper fishery in terms of the appropriate spatial management unit to apply. The results of the study have confirmed that management of snapper as a single stock is appropriate in terms of genetic structure. It highlights the need to monitor changes to fishery practices in regional areas with the knowledge that these may adversely affect the fishery in other areas.

INTELLECTUAL PROPERTY / VALUABLE INFORMATION

The study used existing standard molecular biological techniques and, as such, has not produced intellectual property of a commercial nature.

FURTHER DEVELOPMENT

An attempt to provide finer scale resolution of stock substructuring by analysis of micro-satellites was unsuccessful due to technical difficulties in the isolation of individual micro-satellite loci. However, the results of both allozyme and mtDNA analyses, together with tagging studies, suggest that further development of this aspect is not warranted for the commercially fished areas of South Australia.

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FINAL COST

The total cost of the project is estimated at $102,996. The contribution from FRDC totalled $52,340 and was entirely expended on the Scientific Officer salary and laboratory consumables. The contribution by the SA Museum is estimated at $41,156 and includes the supervision of the Scientific Officer and laboratory technicians by the Principal Investigator as well as direct involvement in analyses and report writing. Employment of the Scientific Officer was continued after the conclusion of the funded project to complete final analyses and manuscript preparation. The employment costs were borne by the SA Museum and totalled $1,900. Additionally, the employment costs of Tanya Hollfelder and Carolyn Horne were borne by the SA Museum and totalled $2,600.

The contribution by SARDI is estimated at $5,000 and includes the collection of all South Australian tissue samples.

Acknowledgment

We thank Phil Bowey, Bob and Annette Clark, Peter Doley, Keith Evans, Martine Kinloch, Murray MacDonald, Owen Macintosh, Brian Mundy, Alan Suter, Lianos Triantofillos and Len Williams for collecting tissue samples, and Mark Adams, Michael Gardiner, Tanja Hollfelder, Carolyn Horne, Sally Sims and Paul Monis for assistance in the laboratory and computing. We thank Keith Jones for his support in the early stages of this project. The project was supported by an FRDC grant (Number 94/168) and a Friends of the South Australian Museum Summer Vacation scholarship**.**

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Table 1 Collection location details of *Pagrus auratus* sampled for mitochondrial DNA analysis.

Collection Location	Map Code	RFLP Sample Code	Collection Date	Latitude	Longitude
Wilson Inlet	WA	WA	17/4/94	35º02' S	117º24' E
Ceduna	CED	WC	3-4/1/92	32º11.8' S	133º34.0' E
Streaky Bay	STR	WC	3-4/3/94,11/95-1/96	32º41.0' S	134º03.0' E
Dangerous Reef	DR	DR	14/2/94	34º49.0' S	136º12.0' E
Illusion Wreck	ILL	USG	17/11/91	33º29.0' S	137º32.6' E
Port Pirie	PIR	USG	16/11/91	33º07.5' S	137º56.5' E
Point Lowly	LOW	USG	15-21/11/91	32º59.7' S	137º46.9' E
Blanche Harbour	BLA	USG	21-22/11/91	32º58.1' S	137º47.5' E
Cape Jervis	CJ	CJ	21/2/94	35º41.0' S	138º04.0' E
Adelaide	ADE	ADE	13/2/93-24/3/93	34º37' S	138º17' E
Ardrossan	ARD	-	9/4/92	34º25' S	138º00' E
Marsden Point, 5' N	MAR	-	1/10/92	35º?8' S	137o?8' E
Investigator Strait	INV	-	7/11/92	35º33.4' S	137º39.6' E
Beaumaris	BEA	-	-/3/92	38º00' S	145º01' E
Geelong	GEE	-	-/3/92	38º12' S	144º30' E
Hobsons Bay	HOB	HOB	-/3/92	37º52' S	144º55' E
Mornington	MOR	-	-/3/92	38º20' S	145º01' E
St Leonards	STL	-	-/3/92	38º11' S	144º43' E

Table 2. Collection details of samples of *Pagrus auratus* for allozyme analysis.

Abbreviations in parentheses represent samples treated as individual populations. N = sample size.

Location	N	Collection Date	Location details
Gulf of St Vincent			
Adelaide (ADE)	100	13/2/93-24/3/93	34°37'S,138°17'E
Cape Jervis (CJ)	94	21/2/94	35°41.0'S,138°04.0'E
Spencer Gulf			
Upper Spencer Gulf (USG)	111	15-21/11/91	33°10’S, 137°45’E
Whyalla (WHY)	97	6/3/94	32°59.7’S, 137°46.9’E
Dangerous Reef (DR)	94	14/2/94	34°49.0'S,136°12.0'E
West Coast (WC)			
Ceduna (CED)	7	3-4/1/92	31°11.8'S,133°34.0'E
Streaky Bay (STR)	44	3-4/3/94,11/95-1/96	32°41.0'S,134°03.0'E

Table 3. Variable loci screened, buffers used and gel running conditions
Full details of buffers are presented in Richardson *et al.* (1986).

Locus	Buffer	Running Conditions
Aat-2	0.05 Tris-maleate pH 7.8	2.5hrs, 200V
Acon	0.02 Tris-glycine pH8.5	2hrs, 200V
Ada	0.02 Tris-glycine pH8.5	1.75hrs, 200V
Adh	0.05 Tris-maleate pH 7.8	2hrs, 200V
Gpi	0.02 Tris-EDTA-citrate pH 7.5	2hrs, 200V
Iddh	0.01 Tris-EDTA-borate $MgCl_2$ pH 7.8	2.5hrs, 200V
Idh	0.01 Citrate-phosphate pH 6.4	2hrs, 200V
Mpi	0.02 Phosphate pH 7.0	2hrs, 200V
Pgm-2	0.02 Tris-EDTA-citrate pH 7.5	2hrs, 200V

Table 4 Presence (+) or absence (-) of restriction sites for three restriction enzymes among 11 mtDNA control region RFLP haplotypes

	Restriction sites						
	Acc I	*Ssp I*	*Mse I*	*Mse I*	*Mse I*	*Mse I*	*Mse I*
Haplotype	1	2	3a	3b	3c	3d	3e
A1	+	+	+	-	+	-	-
A2	+	+	+	-	-	-	-
A3	+	+	-	-	+	-	-
A4	+	-	+	-	+	-	-
A5	-	+	+	-	+	-	-
B1	-	-	+	+	+	+	+
B2	-	+	+	+	+	+	+
B3	+	-	+	+	+	+	+
B4	-	-	+	+	+	+	-
B5	-	+	+	+	+	+	-
B6	-	-	-	+	+	+	+

Table 5 Distribution of mtDNA control region PCR-RFLP haplotypes among 7 samples from Western Australia, South Australia and Victoria.

		mtDNA RFLP haplotype											
Sample[1]	*n*	A1	A2	A3	A4	A5	B1	B2	B3	B4	B5	B6	A:B
WA	28	20	1	0	0	0	4	1	1	0	0	1	21: 7
WC	52	40	0	4	2	0	6	0	0	0	0	0	46: 6
USG	30	24	0	1	0	0	2	3	0	0	0	0	25: 5
DR	31	19	1	6	0	0	3	2	0	0	0	0	26: 5
ADE	30	20	2	1	2	1	1	3	0	0	0	0	26: 4
CJ	34	27	1	2	1	2	1	0	0	0	0	0	33: 1
HOB	30	2	0	0	0	0	12	11	0	4	1	0	2:28
Total	235	152	5	14	5	3	29	20	1	4	1	1	179:56

[1] See Table 1 for explanation of sample codes.

Table 6 Monte Carlo χ^2 and G_{st} values and resultant estimates of long-term rates of female migration between regions per generation (N_m) for areas of the south coast of Australia.

Regional Comparison	χ^2	G_{st}	N_m
All regions	163.41**	n.a.	
South Australia only	33.34	-0.0004	n.a.
Spencer Gulf and Gulf St Vincent	n.a.	0.0001	n.a.
South Australia and Victoria	n.a.	0.1074#	4.16
Western Aust. and South Australia	53.23*	-0.0011	n.a.

** denotes highly significant heterogeneity among sample regions

* denotes marginally significant heterogeneity among sample regions

Significance of G_{st} coefficient tested by 1000 bootstraps. Values not exceeded in any bootstraps noted by #

G_{st} was not calculated for "All regions" due to software constraints. "South Australia only" includes samples WC, DR, USG, ADE and CJ; "Spencer Gulf and Gulf St Vincent" includes samples DR, USG, ADE and CJ; "South Australia and Victoria" includes samples DR, USG, ADE, CJ and HOB; "Western Aust.and South Australia" includes all samples except HOB for chi-squared value and samples WA, WC, USG and ADE for G_{st} value.

Table 7 Allele frequencies, expressed as a percentage, at 9 loci in *Pagrus auratus* from three areas in South Australia. Alleles are designated alphabetically in order of increasing cathodal migration. Where enzymes were encoded by more than one locus, each locus is numbered in order of increasing electrophoretic mobility. Sample size is in parentheses at the head of each column, D is the coefficient of heterozygote deficiency or excess.

Locus	allele	WC (51)	DR (94)	WHY (97)	USG (111)	CJ (94)	ADE (100)
Aat-2	b	1	1	1	1	1	
	a	99	99	99	99	99	100
	D	0.00	0.00	0.00	0.01	0.00	0.00
Acon	c	2	4	6	7	7	4
	b	97	95	91	92	93	95
	a	1	1	3	1		1
	D	0.01	0.05	0.08	0.08	0.06	-0.17
Ada	c	83	89	84	85	85	89
	b	17	10	16	15	15	11
	a		1				
	D	-0.22	-0.06	-0.06	-0.05	0.00	-0.09
Adh	d	1	1			1	
	c	1	1	1	1	1	1
	b	97	96	97	97	96	97
	a	1	2	2	2	2	2
	D	0.01	0.02	0.02	0.02	-0.12	0.01
Gpi	c	1	1	1	1		
	b	96	96	95	97	98	97
	a	3	3	4	2	2	3
	D	0.02	0.02	0.04	-0.13	0.01	0.02
Iddh	c	1				1	
	b	4	2	3	2	2	2
	a	95	98	97	98	97	98
	D	0.03	-0.66	0.03	0.02	-0.32	0.02
Idh	c	16	22	28	20	25	17
	b	76	70	67	72	66	74
	a	8	8	5	8	9	9
	N =	46	92	89	105	93	100
	D	-0.17	-0.00	-0.11	0.04	-0.02	-0.04
Mpi	d	1	5	5	6	4	6
	c	97	93	93	93	96	93
	b	2	2	1	1		1
	a			1			
	D	0.01	0.05	0.06	-0.08	0.04	0.07
Pgm	d	10	10	5	3	4	3
	c						1
	b	90	90	93	96	93	94
	a			2	1	3	2
	D	0.10	-0.26	-0.10	-0.20	-0.13	-0.25

Table 8. Contingency χ^2 tests of allele frequencies for the *Adh* locus for the present study and the data of MacDonald (1980). B-1, B-2: Backstairs Passage; R: Rosalind Shoal, lower Spencer Gulf; C-1, C-2: Cowled Landing, Upper Spencer Gulf. $*P < 0.05$, $**P < 0.01$

	B-1	B-2	R	C-1	C-2
WC	n.s.	n.s.	n.s.	n.s.	n.s.
DR	n.s.	n.s.	n.s.	*	n.s.
WHY	n.s.	n.s.	n.s.	*	*
USG	n.s.	n.s.	n.s.	**	**
CJ	n.s.	n.s.	n.s.	*	n.s.
ADE	n.s.	n.s.	n.s.	**	**

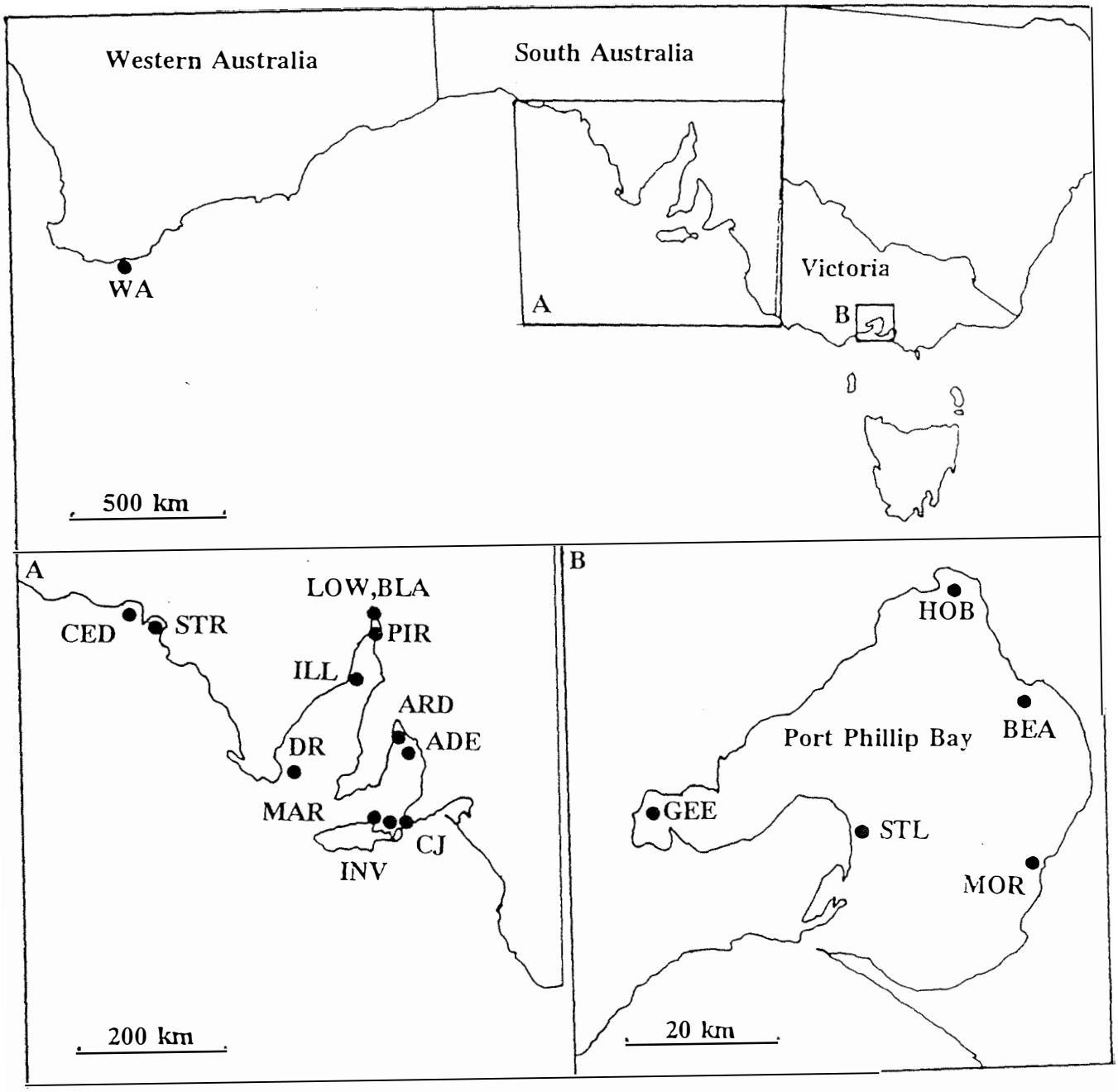


Fig. 1.Collection locations of snapper in southern Australia, - a) samples for mtDNA analysis, See the Table 1 for an explanation of location codes.

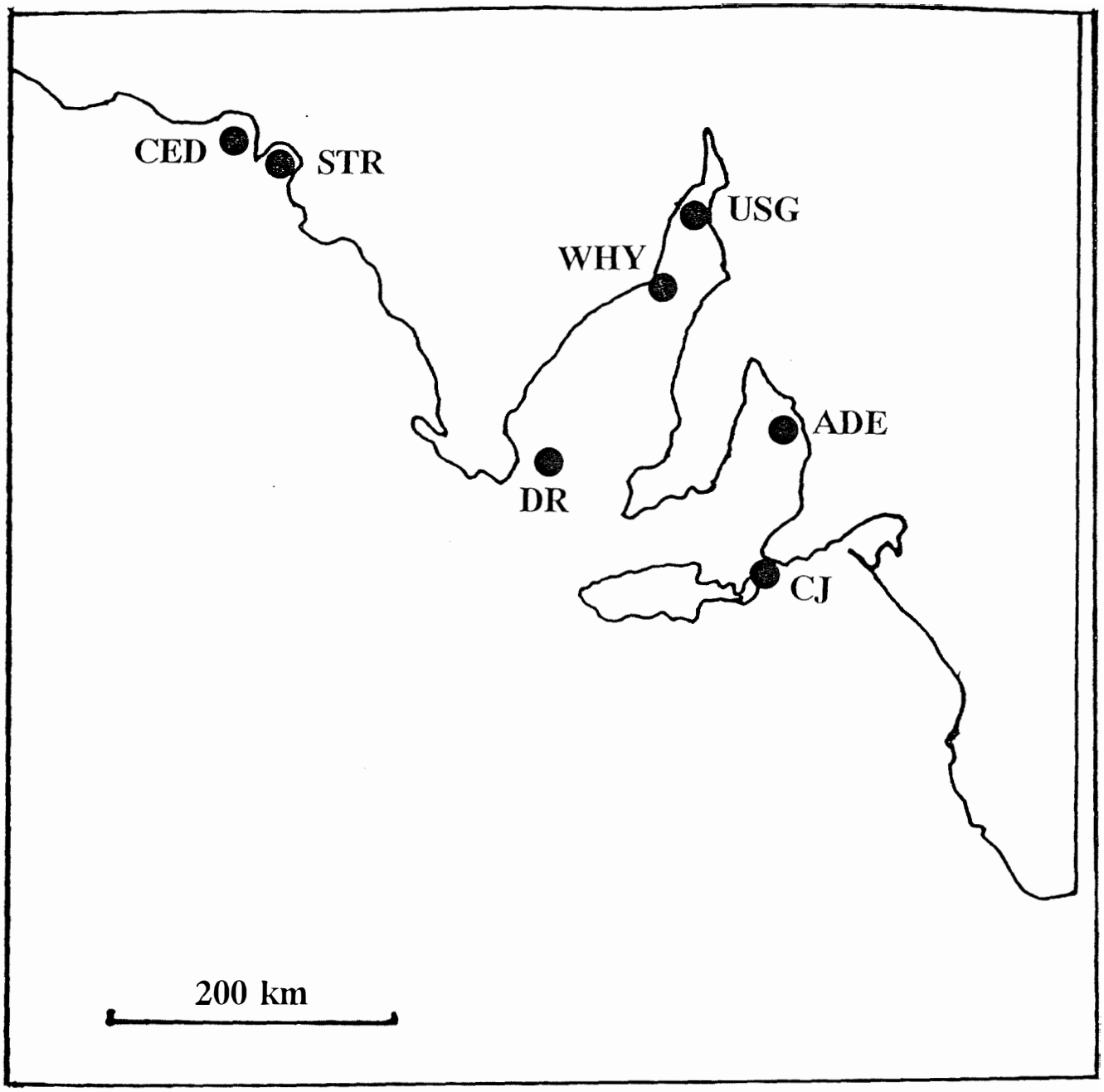
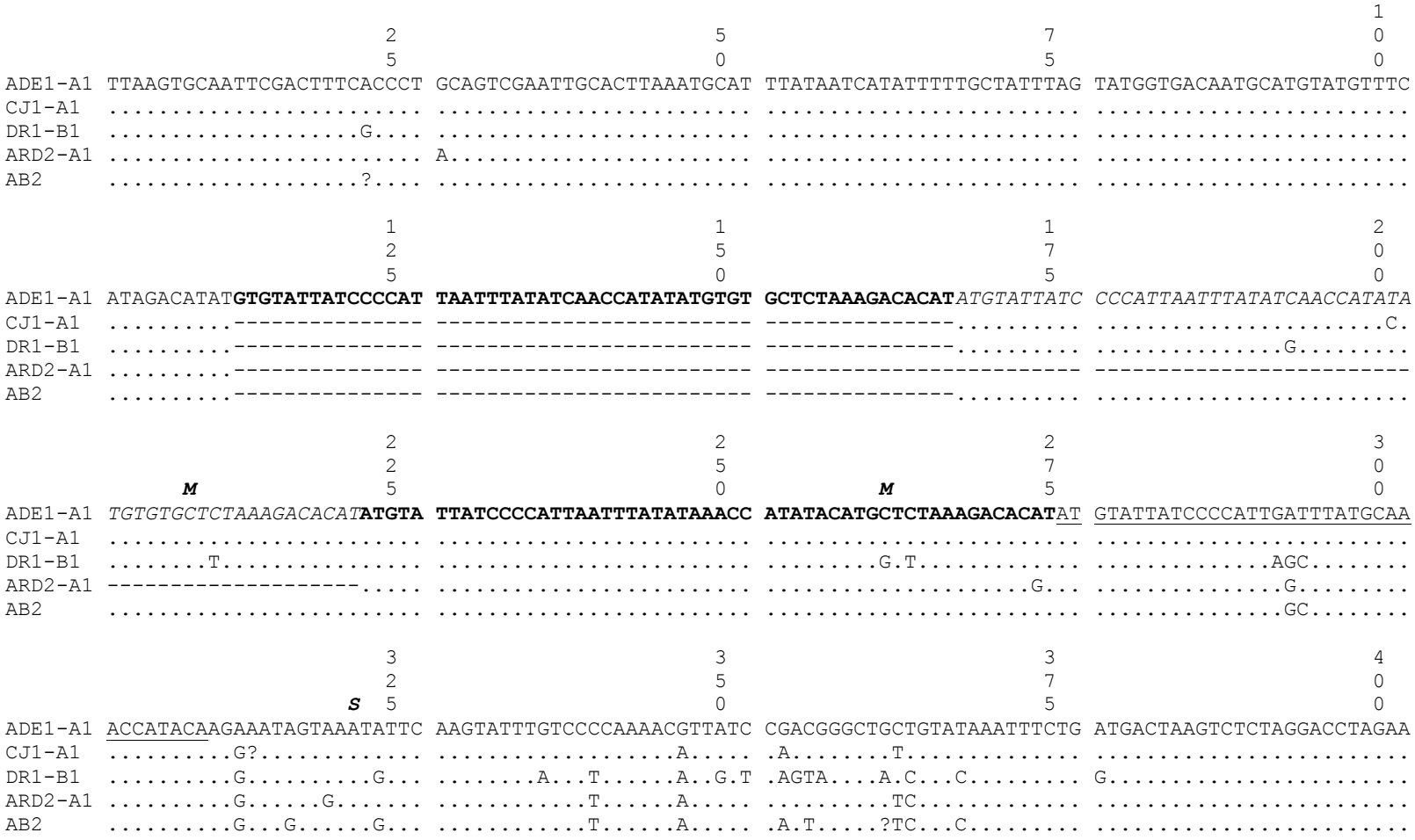


Fig. 1.Collection locations of snapper in southern Australia, - b) samples for allozyme analysis. See the Table 1 for an explanation of location codes.

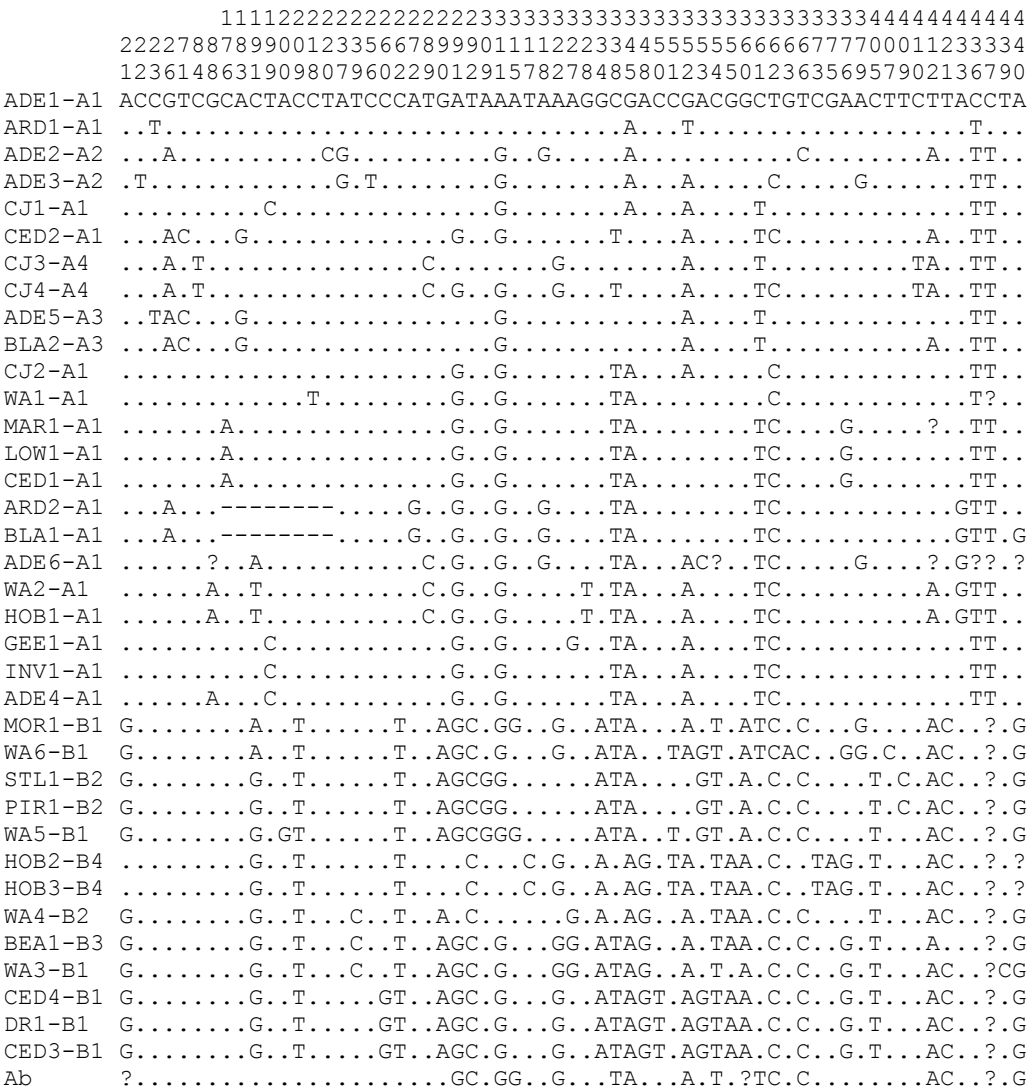
Fig. 2. Aligned snapper and black bream partial control region sequences. Dots indicate identity to top reference sequence, dashes indicate gaps inserted to improve alignment, and question marks indicate unknown bases. Numbers refer to the nucleotide's position in complete sequence. Bold letters in superscript above nucleotide positions, refer to apomorphic restriction sites defining the two haplotype lineages. ***A*** = *Acc* I, ***M*** = *Mse* I, ***S*** = *Ssp* I. Each haplotype is indicated by the collection location code (e.g. **ADE**1-A1), followed by the individuals number from that site (e.g. ADE**1**-A1) and finally by the RFLP phenotype (e.g. ADE1-**A1**). a) All sequenced sites of three length variant haplotypes and representatives of two major lineages and the outgroup. ADE-A1 is a long PCR product variant, CJ1-A1 and DR1-B1 are intermediate length variants, ARD2-A1 is a short variant. Sequences in bold, italics, bold and underlined indicate 50bp, 55bp, 53bp and 33bp tandem repeats respectively. b) Variable sites only from all sequenced individuals.

Fig. 2a


ADE1-A1 TTAAGTGCAATTCGACTTTCACCCT GCAGTCGAATTGCACTTAAATGCAT TTATAATCATATTTTTGCTATTTAG TATGGTGACAATGCATGTATGTTTC
CJ1-A1 .....
DR1-B1 .....G.... .....
ARD2-A1 ..... A..... .....
AB2 .....?.... .....
ADE1-A1 ATAGACATATGTGTATTATCCCCCATTAATTTATATCAACCATATATGTGTGCTCTAAAGACACATATGTATTATCCCCCATTAATTTATATCAACCATATA
CJ1-A1 .....---------- ---------- ----------..... .....C.
DR1-B1 .....---------- ---------- ----------..... .....G.....
ARD2-A1 .....---------- ---------- ---------- ----------
AB2 .....---------- ---------- ----------..... .....
ADE1-A1 TGTGTGCTCTAAAGACACATATGTATTATCCCCATTAATTTATATAAACCATATACATGCTCTAAAGACACATATGTATTATCCCCATTGATTTATGCAA
CJ1-A1 .....
DR1-B1 .....T..... .....G.T..... .....AGC.....
ARD2-A1 ----------..... .....G... .....G.....
AB2 ....GC.....
ADE1-A1 ACCATACAAGAAATAGTAAATATTC AAGTATTTGTCCCCAAAACGTTATC CGACGGGCTGCTGTATAAATTTCTG ATGACTAAGTCTCTAGGACCTAGAA
CJ1-A1 .....G?..... .....A..... .A.....T..... .....
DR1-B1 .....G.....G... .....A...T.....A..G.T .AGTA....A.C...C..... G.....
ARD2-A1 .....G.....G..... .....T.....A..... .....TC..... .....
AB2 .....G...G.....G... .....T.....A..... .A.T.....?TC...C..... .....

```
                                4               4
                                2               4
                              A 5               1
ADE1-A1  ATAACTTCTCGTCAAATGTCTACCA AGTATCAACACCCTAT
CJ1-A1   ......................... ..........TT....
DR1-B1   ....T......A........C.... ..........?..G.
ARD2-A1  ......................... .......G..TT....
AB2      ..........A........C.... ..........?..G.
```

Fig. 2b


111122222222222222333333333333333333333333333344444444444
222278878990012335667899901111222334455555566666777700011233334
123614863190980796022901291578278485801234501236356957902136790
ADE1-A1 ACCGTCGCACTACCTATCCCATGATAAATAAAGGCGACCGACGGCTGTCGAACTTCTTACCTA
ARD1-A1 ..T.....A...T.....T...
ADE2-A2 ...A.....CG.....G..G.....A.....C.....A..TT..
ADE3-A2 .T.....G.T.....G.....A...A.....C.....G.....TT..
CJ1-A1 .....C.....G.....A...A....T.....TT..
CED2-A1 ...AC...G.....G..G.....T....A....TC.....A..TT..
CJ3-A4 ...A.T.....C.....G.....A....T.....TA..TT..
CJ4-A4 ...A.T.....C.G..G...G...T....A....TC.....TA..TT..
ADE5-A3 ..TAC...G.....G.....A....T.....TT..
BLA2-A3 ...AC...G.....G.....A....T.....A..TT..
CJ2-A1 .....G..G.....TA...A.....C.....TT..
WA1-A1 .....T.....G..G.....TA.....C.....T?..
MAR1-A1 .....A.....G..G.....TA.....TC....G.....?..TT..
LOW1-A1 .....A.....G..G.....TA.....TC....G.....TT..
CED1-A1 .....A.....G..G.....TA.....TC....G.....TT..
ARD2-A1 ...A...--------.....G..G..G..G....TA.....TC.....GTT..
BLA1-A1 ...A...--------.....G..G..G..G....TA.....TC.....GTT.G
ADE6-A1 .....?..A.....C.G..G..G....TA...AC?..TC.....G....?.G??.?
WA2-A1 .....A..T.....C.G..G.....T.TA...A....TC.....A.GTT..
HOB1-A1 .....A..T.....C.G..G.....T.TA...A....TC.....A.GTT..
GEE1-A1 .....C.....G..G....G..TA...A....TC.....TT..
INV1-A1 .....C.....G..G.....TA...A....TC.....TT..
ADE4-A1 .....A...C.....G..G.....TA...A....TC.....TT..
MOR1-B1 G.....A..T.....T..AGC.GG..G..ATA...A.T.ATC.C...G....AC..?.G
WA6-B1 G.....A..T.....T..AGC.G...G..ATA..TAGT.ATCAC..GG.C..AC..?.G
STL1-B2 G.....G..T.....T..AGCGG.....ATA....GT.A.C.C....T.C.AC..?.G
PIR1-B2 G.....G..T.....T..AGCGG.....ATA....GT.A.C.C....T.C.AC..?.G
WA5-B1 G.....G.GT.....T..AGCGGG.....ATA..T.GT.A.C.C....T...AC..?.G
HOB2-B4 .....G..T.....T....C...C.G..A.AG.TA.TAA.C..TAG.T...AC..?.?
HOB3-B4 .....G..T.....T....C...C.G..A.AG.TA.TAA.C..TAG.T...AC..?.?
WA4-B2 G.....G..T...C..T..A.C.....G.A.AG..A.TAA.C.C....T...AC..?.G
BEA1-B3 G.....G..T...C..T..AGC.G...GG.ATAG..A.TAA.C.C..G.T...A...?.G
WA3-B1 G.....G..T...C..T..AGC.G...GG.ATAG..A.T.A.C.C..G.T...AC..?CG
CED4-B1 G.....G..T.....GT..AGC.G...G..ATAGT.AGTAA.C.C..G.T...AC..?.G
DR1-B1 G.....G..T.....GT..AGC.G...G..ATAGT.AGTAA.C.C..G.T...AC..?.G
CED3-B1 G.....G..T.....GT..AGC.G...G..ATAGT.AGTAA.C.C..G.T...AC..?.G
Ab ?.....GC.GG..G...TA...A.T.?TC.C.....AC..?.G

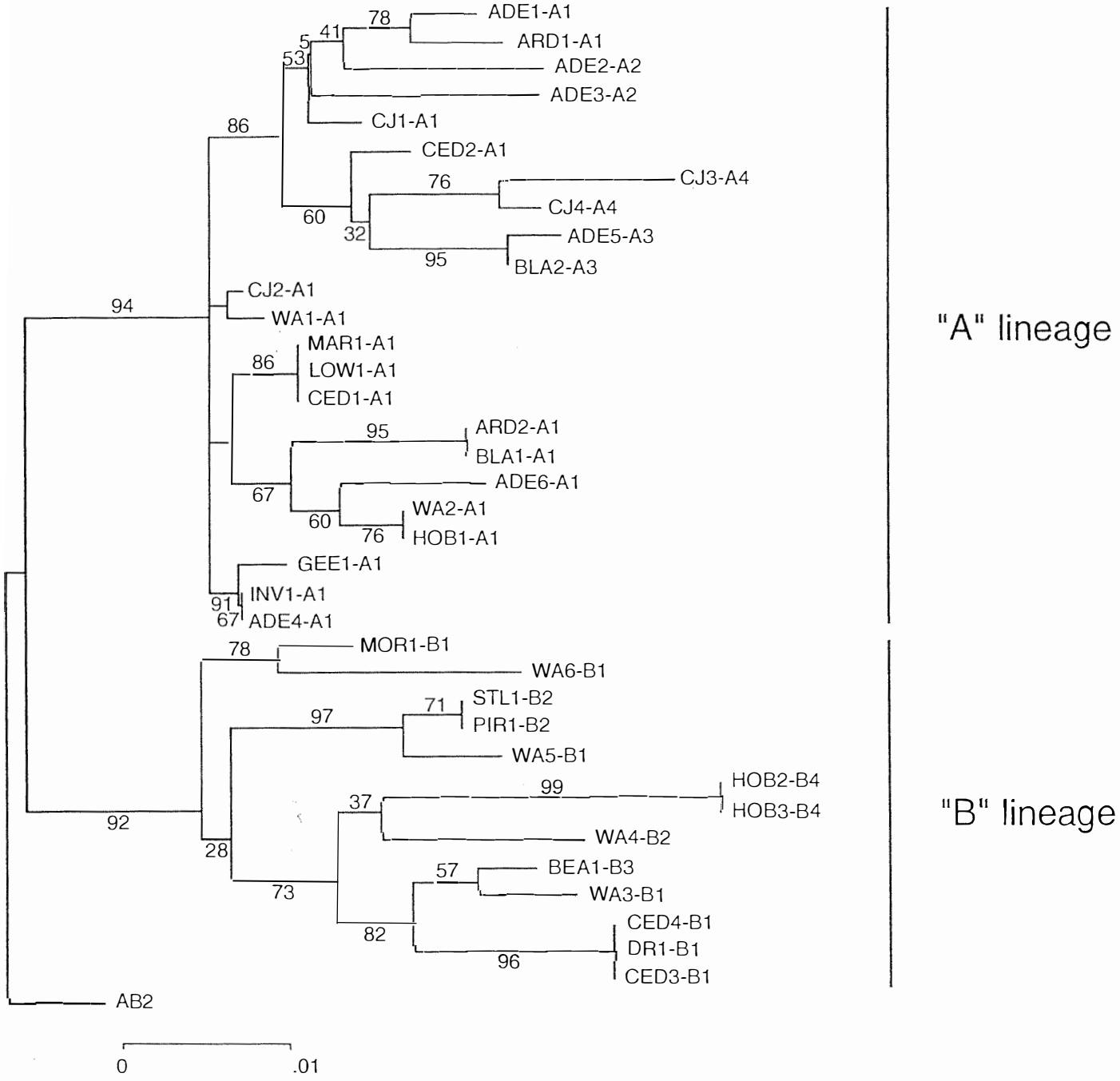


Fig. 3. Neighbour-joining tree constructed from Kimura-2-parameter distances between snapper and the outgroup, black bream, partial control region sequences. Values above branches are standard error test values, branch lengths are proportional to Kimura-2-parameter distances, the scale bar indicates Kimura-2-parameter distances.

Fig. 4. Representative *Acc* I, *Mse* I and *Ssp* I digestion profiles of the partial control region PCR products from snapper of the A1 and B1 haplotypes. M: *Hinf* I, *Rsa* I, and *Sin* I digested pGEM-3 (Promega) size marker; a,b: *Acc* I digest; c,d: *Mse* I digest; e,f: *Ssp* I digest; g: undigested PCR product. A haplotype is the left hand profile and B haplotype is the right hand profile of each pair.