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Molecular Genetic Evidence for a New Species of Bream of the Genus *Acanthopagrus* Peters (Perciformes: Sparidae)

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Abstract

Fishes of the genus *Acanthopagrus* are found throughout the coastal waters of Asia and Australia with several species being of commercial significance. In this study, genetic comparisons are made between widely disjunct populations of *Acanthopagrus australis* (Günther) from Australian and Taiwanese waters and among samples of *A. butcheri* (Munro), *A. berda*, (Forskal), *A schlegeli* (Day) and *A. latus* (Houttuyn) using mitochondrial DNA sequences obtained from the control region. The mean interspecific pairwise sequence divergence for all species is 17%, while the divergence between *A. australis* from Australia and that of Taiwan is slightly larger at 18%. These values are considerably higher than those found for intraspecific control region comparisons in some fish species. Phylogenetic analyses indicate that *A. australis* from Australia is more closely related to the Australian species *A. butcheri* than to *A. australis* from Taiwan. These findings suggest that the northern and southern hemisphere forms of *A. australis* are not monophyletic, with the former possibly representing a new undescribed species of *Acanthopagrus*.

Introduction

Fishes of the genus *Acanthopagrus* Peters, commonly known as bream or porgies, are found in estuarine and coastal waters throughout the Indo-Pacific regions of Asia, the east coast of Africa and the coast of Australia. A number of species form important commercial and recreational fisheries and there is also a high level of interest in the aquaculture of several species.

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There is considerable disagreement on the definition of generic boundaries within the Sparidae with up to nine species recognized within *Acanthopagrus* over recent times (Hayashi 1983, Lee 1983, Smith and Heemstra 1986, Grant 1991, Jean and Lee 1992, Gomon et al. 1994). Tropical members of the genus tend to have very wide distributions [e.g. *A. berda* (Forskal) and *A. latus* (Houttuyn)] whereas species occurring in subtropical or more temperate waters have more restricted distributions in either the southern hemisphere [e.g. *A. palmaris* (Whitley) and *A. butcheri* (Munro)] or the northern hemisphere [e.g. *A. cuveri* (Day), *A. schlegeli* (Day) and *A. sivicolus* (Day)]. The disjunct distribution of the yellowfin bream, *A. australis* (Günther), is therefore highly unusual for the genus as it is recorded from subtropical to temperate waters in both the southern and northern hemispheres.

Acanthopagrus australis was first described as *Chrysophrys australis* by Günther in 1859 from Australian waters and was thought to be restricted to Australia, ranging from Townsville in Queensland to the Gippsland Lakes in Victoria (Grant 1991). However Masuda et al. (1984) and Jean and Lee (1992) have also recorded this species from Japan and Taiwan respectively.

There have been a number of population genetic and phylogenetic studies of representatives of the genus *Acanthopagrus* (Rowland 1984, Sugama et al. 1989, Jean et al. 1995a,b, Farrington et al. 2000). However, none of these studies have examined phylogenetic relationships of Northern and Southern Hemisphere species or their populations.

Nucleotide sequences from the mitochondrial genome are particularly useful for reconstructing recent phylogenetic history. This is because mitochondrial DNA (mtDNA) is maternally inherited and, on the average, evolves at a faster rate than most nuclear genes (excluding microsatellite regions), which allows the identification of informative phylogenetic characters among closely related species and populations (Hillis et al. 1996, Stepien and Kocher 1997). Within the mtDNA genome, the control region is known to be highly variable and very useful for population studies. It also contains a more slowly evolving central conserved section, which contains phylogenetically reliable information for interspecific comparisons (Faber and Stepien 1997).

The primary aim of this study was to evaluate the taxonomic status of the northern and southern hemisphere *A. australis* using nucleotide sequence information. We collected sequences from the mtDNA control region for samples of *A. australis* and *A. butcheri* from southern Australia and combined these data with sequences obtained for the same gene region by Jean et al. (1995a) for samples of *A. australis, A. berda, A. latus* and *A. schlegeli* from Taiwanese waters.

Materials and Methods

Sample collection

Acanthopagrus butcheri specimens were obtained from four locations, Swan River (Western Australia), Port Adelaide River, Onkaparinga River

and Hindmarsh River (all South Australia). *Acanthopagrus australis* was collected from Port Stephens (New South Wales), and Bribie Island (Queensland). Fish were collected by angling and were either frozen whole or dissected in the field with samples of muscle tissue preserved in liquid nitrogen (Farrington et al. 2000). As no single morphometric character is a reliable diagnostic tool (Rowland 1984), *A. butcheri* and *A. australis* were identified using allozyme electrophoresis. Enzymes which allowed for taxonomic identification were ADH, LDH, MDH, NDH, 6PGD, IDDH and SOD (Farrington et al. 2000).

DNA amplification and sequencing

Total DNA was extracted from frozen muscle tissue using a modified high-salt precipitation method developed by Crandall et al. (1999), or a CTAB phenol-chloroform protocol (Doyle and Doyle 1987).

An approximately 1100 base pair (bp) segment of mtDNA including the control region, $tRNA^{phe}$, $tRNA^{pro}$ and part of the 12S rRNA gene was amplified through the polymerase chain reaction (PCR) using primers PT and PU (Jean et al. 1995a). These primers were used for amplification and sequencing; additional internal primers were constructed and used only for sequencing.

PCR was performed with a reaction volume of 100 mL, containing 10 mL of 10X reaction buffer (containing $MgCl_2$), 0.2 mL of each dNTP, 2 units of *Taq* polymerase, 0.4 mM of each primer and 2 mL of DNA extract. Reactions were carried out in a Corbett Research PC-960 Thermal Cycler using the following cycling regime: 35 cycles of 94°C for 40 seconds, 55°C for 40 seconds and 72°C for 90 seconds. Amplified mtDNA was purified using a QIAGEN QIAquick Purification Kit, and quantified against a Promega DNA/Hae III marker on a 2% agarose/TAE gel containing ethidium bromide and viewed under UV light. Samples were dried and sent to the Australian Genome Research Facility for sequencing. Sequencing reactions were performed using all four primers to allow complimentary strands to be read and base sequences to be verified.

Sequence analysis

Sequence chromatograms were viewed and edited using a combination of the EditView and SeqPup (Gilbert 1997) software. Sequences of *A. australis* and *A. butcheri* were then aligned with the sequence data obtained by Jean et al. (1995a) for the species *A. australis, A. berda, A. latus* and *A. schlegeli* collected from Taiwanese waters. Multiple alignments were performed using the program Clustal X (Thompson et al. 1997) with multiple alignment parameters of gap penalty equal to 10 to 15 and gap extension penalty equal to 3-5 and pairwise parameters of gap penalty of 3 to 5 and k-tuple of 1 to 3. Positions of uncertain alignment were excluded to produce a stable data set (Gatesy et al. 1993).

The aligned sequences were then imported into PAUP* (Swofford 1998) for phylogenetic analysis. Pairwise sequence divergences were calculated

using the Tamura-Nei method of nucleotide sequence evolution, which was implemented with the distance analysis option. Phylogenetic trees were constructed using minimum evolution, maximum parsimony and maximum likelihood methods. Maximum-parsimony analysis was performed with the heuristic search option, the minimum evolution analysis using the neighbor-joining option and maximum likelihood was performed using the quartet puzzling option. Phylogenetic confidence for parsimony and distance trees was estimated by bootstrapping with 1000 replicate data sets. Maximum likelihood trees were based on 1000 quartet puzzling replicates. All nucleotide sites and substitution classes were weighted equally, although other weightings were also tested. *Sparus sarba* control region sequences (Jean et al. 1995a) were used as an outgroup in all phylogenetic analyses.

Results

The sequence alignment (Fig. 1) after the removal of regions of ambiguous alignment yielded 876 base pairs (bp), of which 356 sites (41%) were variable and 135 sites (15%) were phylogenetically informative for maximum parsimony analysis. The total nucleotide composition (Table 1) for all species

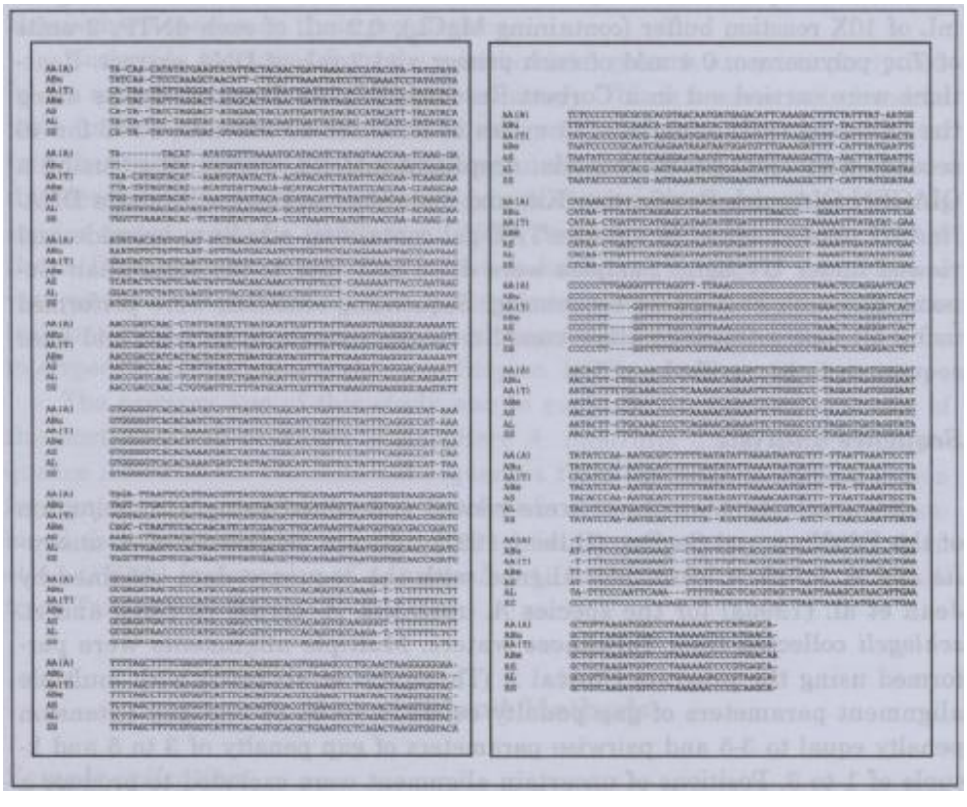

Fig. 1. Alignment of mtDNA sequences of *Acanthopagrus* species and outgroup *Sparus sarba*. AA(A) - *A australis* (Australia), Abu - *A butcheri*, AA(T) - *A australis* (Taiwan), Abe - *A berda*, AS - *A schlegeli*, AL - *A latus*, SS - *S sarba*). Regions of ambiguous alignment have not been included, actual sequence lengths range from 1120 to 1400 bp.

was A = 31%, C= 22%, G = 16% and T = 31%. This indicates that the region sequenced is A,T rich. The four *A. butcheri* samples yielded virtually identical sequences after removal of regions of ambiguous alignment, as did the two *A. australis* samples from Australia. Therefore only one individual of each species was included in the data set.

Sequence divergence between species ranged from a low of 15% between *A. schlegeli* and the Taiwanese *A. australis*, to a high of 29% between *A. butcheri* and *S. sarba* (Table 2). With the exception of *A. latus*, the distance between the outgroup, *S. sarba*, and *Acanthopagrus* species was greater than for all pairwise comparisons between *Acanthopagrus* species.

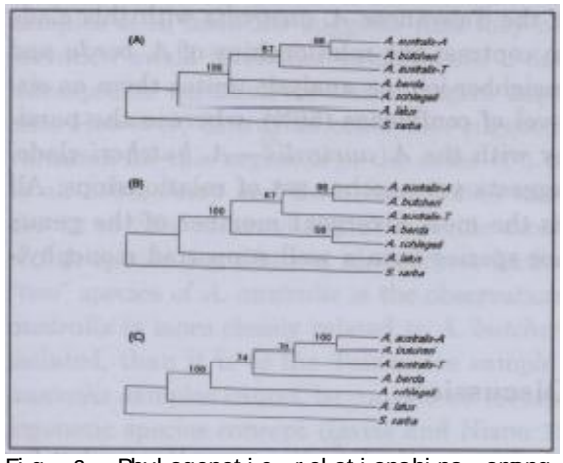

Fig. 2. Phylogenetic relationships among representatives of the genus *Acanthopagrus* derived from: (a) maximum parsimony, (b) neighbor-joining, and (c) maximum likelihood procedures. *Sparus sarba* was included as an outgroup. Bootstrap values were obtained from 1000 replications. A = Australia, T = Taiwan.

The mean distance among all Taiwanese species excluding the outgroup is 17% while the mean distance between Taiwanese species and Australian species is higher at 21%. The mean genetic distance among all *Acanthopagrus* species excluding both *A. australis* samples

Table 1. Nucleotide composition and length of sequences from the control region (regions of ambiguous alignment removed) of the 7 sparid fishes studied. *A. australis* (A) – Australia, *A. australis* (T) – Taiwan.

Taxon	A	C	G	T	# Sites
A. australis(A)	0.31	0.21	0.17	0.31	843
A. butcheri	0.31	0.21	0.15	0.33	843
A. australis(T)	0.31	0.22	0.15	0.32	847
A. berda	0.30	0.22	0.16	0.31	848
A. schlegeli	0.32	0.22	0.16	0.30	845
A. latus	0.31	0.23	0.16	0.31	847
S. sarba	0.29	0.23	0.17	0.31	845
Mean	0.31	0.22	0.16	0.31	845

Table 2. The pairwise distance matrix of aligned control region sequences among *Acanthopagrus* species and the outgroup, *S. sarba*. *A. australis* (A) – Australia, *A. australis* (T) – Taiwan.

Species	1	2	3	4	5	6	7
1. *A. australis* (A)	-						
2. *A. butcheri*	0.185	-					
3. *A. australis* (T)	0.177	0.180	-				
4. *A. berda*	0.197	0.220	0.168	-			
5. *A. schlegeli*	0.196	0.190	0.150	0.155	-		
6. *A. latus*	0.245	0.246	0.183	0.209	0.169	-	
7. *S. sarba*	0.289	0.291	0.234	0.266	0.248	0.186	-

is 20%. By comparison the distance between the two *A. australis* samples from Australia and Taiwan is similar at 18%.

The construction of phylogenetic trees using the three methods (Fig. 2) resulted in very similar topologies. All trees grouped the Australian *A. australis* with the Australian species *A. butcheri*, with this pairing in turn grouped with the Taiwanese *A. australis* sample. The close relationship of the two Australian samples was supported by high confidence levels (96 to 100%) whereas the association of the Taiwanese *A. australis* with this clade was not as strong (67 to 70%). In contrast the relationships of *A. berda* and *A. schlegeli* are unresolved. The neighbor-joining analysis unites them as sister taxa, with a relatively low level of confidence (56%), whereas the parsimony analysis gives a trichotomy with the *A. australis* – *A. butcheri* clade. Maximum likelihood analysis suggests yet another set of relationships. All analyses indicate that *A. latus* is the most divergent member of the genus under study and that all the other species form a well supported monophyletic group.

Discussion

The taxonomic value of molecular genetic data is now well accepted for the identification and delineation of species both in general (Avise 1974, Thorpe 1982, Richardson et al. 1986, Baverstock and Moritz 1996, Hillis et al. 1996) and specifically in relation to commercial fish species (Shaklee 1983, Ward and Grewe 1994). The approach to the delineation of species using molecular genetic data is largely the same as for any other type of data. Thus genetic comparisons between populations of distinct species are expected to show much greater degrees of divergence than comparison between conspecific populations. Further, the finding of genetic divergence between putative species in sympatry provides powerful indirect evidence for reproductive isolation satisfying the criterion of the biological species concept.

The four species of *Acanthopagrus* from Taiwanese waters are genetically distinct both via allozyme electrophoresis (Jean et al. 1995b) and in terms of nucleotide divergence for the gene region sequenced in this study, providing strong evidence that the four species represent biologically different species. Similarly, the degree of sequence divergence between the Australian sample of *A. australis* and *A. butcheri*, and the finding that the two species are genetically divergent with respect to allozyme electrophoresis (Rowland 1984, Farrington et al. 2000), also suggests that these two species warrant their specific status. The degree of sequence divergence between these latter two species (18%) is similar to that found for other fish species for the control region. For example, Chen et al. (1998) found 12% divergence for comparisons between *Rhinogobius* species and Sang et al. (1994) found 22% differences for comparisons between *Anguilla* species.

Dealing with the status of allopatric populations is a persistent and continuing problem for fish taxonomy (McDowall 1972, Ward and Grewe 1994) that can be often made more objective, but not eliminated using genetic

data. Generally, allopatric populations are considered to belong to distinct species if they show genetic differences of a similar order of magnitude to those observed between sympatric species as long as it is known or assumed that there are no geographically and genetically intermediate populations between the allopatric samples.

Based on levels of divergence observed among other *Acanthopagrus* species, the degree of divergence observed between the Australian and Taiwanese samples of *A. australis* suggests that they represent distinct species. This observation would be further strengthened if data were available on the degree of intraspecific variation for control region sequences within *Acanthopagrus* species. However, data from other fish suggests that the degree of intraspecific variation for this region is in the order of 1 to 3% (e.g. Sang et al. 1994, Chen et al. 1998, Jerry and Baverstock 1998) which is conspicuously smaller than the 12 to 25% observed for interspecific comparisons in this and other studies of fish species (eg. Chen et al. 1998). A further argument for the recognition of "two" species of *A. australis* is the observation that the Australian sample of *A. australis* is more closely related to *A. butcheri*, from which it is reproductively isolated, than it is to the Taiwanese sample of *A. australis*. Thus the two *A. australis* samples cannot be considered monophyletic without violating the phylogenetic species concept (Davis and Nixon 1992).

The only factor counting against the recognition of two species of "*A. australis*" is the apparent morphological similarity between the two forms. However, with the advent of molecular genetic techniques, a number of sibling or cryptic species of fish have been discovered which are genetically divergent but show minimal morphological differentiation (Shaklee 1983; Ward and Grewe 1995). There is a possibility, however, that the trees generated in this study do not mirror the true phylogenetic relationships among taxa. This is because gene trees derived from mtDNA sequence data may not always be representative of species trees (Brower et al. 1996). However the large degree of sequence divergence between the Australian and Taiwanese *A. australis* is a strong evidence that the relationships shown here truly reflect the recognition of two definite species. Further, it is quite possible that careful comparison between the Taiwanese and Australian forms of *A. australis* may reveal morphological differences which have been previously overlooked, as has been found in other molecular taxonomic studies (Hillis et al. 1996).

Conclusion

The genetic data presented in this study strongly suggests that two species of fish are confused under the name *A. australis*. As *A. australis* was originally described from specimens originating from Australia it is the northern hemisphere "form" which will need to receive a new specific epithet. More generally, this study demonstrates that sequence data from the mitochondrial control region has considerable potential for addressing systematic questions and taxonomic problems in sparid fishes.

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