

Mitochondrial 12S rRNA sequences support the existence of a third species of freshwater blackfish (Percichthyidae: *Gadopsis*) from south-eastern Australia

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Abstract

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Fish of the genus *Gadopsis* are a distinctive component of the freshwater fish fauna of south-eastern Australia. *Gadopsis marmoratus* and *G. bispinosus* are the only two species recognised within the genus, with the former of uncertain taxonomic status, as it is thought to be composed of at least two distinct geographical forms based on morphological and allozyme data. The objective of this study was to investigate DNA sequence divergence in *Gadopsis*, especially in the western portion of its distribution, using an approximately 400 base pair fragment of the mitochondrial small sub-unit 12S rRNA gene region in order to reassess the taxonomy of the genus. Individuals from 11 locations were sequenced and confirm that *G. marmoratus* and *G. bispinosus* are genetically distinct, and further that the *G. marmoratus* complex consists of two divergent clades representing the previously identified northern and southern forms. The degree of divergence between the three *Gadopsis* clades was similar (5–6% nucleotide substitutions), suggesting that they diverged from a common ancestor at approximately the same period in geological time. These results are consistent with previous allozyme studies and highlight the usefulness of mitochondrial DNA data coupled with allozyme information for clarifying taxonomic boundaries in morphologically conservative aquatic organisms.

Keywords

Mitochondrial rRNA, taxonomy, blackfish, Percichthyidae, *Gadopsis*, Australia

Introduction

Fish of the genus *Gadopsis*, commonly known as the river or freshwater blackfish, are endemic to south-eastern Australia (including Tasmania), and carry out their entire life cycle in freshwater (Jackson et al., 1996). The genus is phylogenetically distinct and its evolutionary origins remain uncertain as it may have either evolved from a marine ancestor some 15 million years ago or had a more ancient Gondwanan freshwater origin (Sanger, 1984; Jerry et al., 2001).

Gadopsis belongs to the family Percichthyidae and contains two currently recognised species, *G. marmoratus* (Richardson, 1848) and *G. bispinosus* (Sanger, 1984). Sanger (1986) suggested, based on morphological and allozyme evidence, that *G. marmoratus* potentially consists of a northern and a southern species. However, Sanger (1986) did not formally recognise the northern and southern forms of *G. marmoratus* as these putative species were not found in sympatry and because the taxonomic significance of the genetic and morphological divergence between the two forms was uncertain.

Gadopsis marmoratus has a large geographic range that includes tributaries of the Murray–Darling river system, as far north as the Condamine River in southern Queensland. The species is also found in Tasmania with endemic populations in the north and translocated populations in the Huon River and elsewhere in the south. Sanger (1986), assuming that *G. marmoratus* is in fact a complex of two species, suggested that these taxa evolved in allopatry following the isolation of Tasmania from mainland Australia. According to this scenario ancestral gadopsids are thought to have been originally widespread throughout Victoria and northern Tasmania and that the formation of the two species may have occurred during periods of raised sea levels which isolated Tasmanian from mainland populations. Subsequently, when sea levels were lower during the Pleistocene glaciation and land connections re-established with the mainland, the Tasmanian form of *G. marmoratus* invaded southern Victoria, consequently displacing the northern *G. marmoratus* (Ovenden et al., 1988).

Sanger's (1986) biogeographical hypothesis assumes that the two forms of *G. marmoratus* behave as independent

species. This hypothesis also suggests that the two forms have come into contact in the past and therefore it may still be possible to find locations where both the northern and southern forms coexist in Victoria (Koehn and O'Connor, 1990). Sanger (1986) suggested that the two forms may occur in sympatry in the state's southwest, an area encompassing the Gellibrand and Glenelg river systems. An allozyme study by Ryan et al. (in press) was unsuccessful in finding evidence supporting the existence of sympatric populations of northern and southern *G. marmoratus* in south-western Victoria. However, their findings were consistent with Sanger's (1986) results indicating genetic divergence between the two forms inhabiting adjacent river systems in this region.

In addition to Sanger's and Ryan's allozyme studies there have been several studies of blackfish using DNA-based techniques (Ovenden et al., 1988; Waters et al., 1994; Jerry et al., 2001). These studies, however, are limited by minimal sampling of the northern form of *G. marmoratus*, especially in the western portion of its distribution. This study therefore extends these studies by using Polymerase Chain Reaction (PCR) amplification of an approximately 400 base pair fragment of the mitochondrial 12S rRNA gene region coupled with direct DNA sequencing, in order to further evaluate the taxonomic status of the northern and southern forms of *G. marmoratus* with emphasis on its western distributions. This approach was chosen because mitochondrial DNA (mtDNA) has been found to be very useful for inferring phylogenetic and taxonomic relationships in groups of organisms where the protein-based techniques of allozyme electrophoresis have lacked resolution or produced ambiguous results (Hillis et al., 1996).

Methods and materials

Gadopsis samples. Tissue samples were obtained from specimens previously collected by Ryan et al. (in press). Sample selection was based on the results of Ryan et al. (in press) together with three reference sites based on previous studies by Sanger (1984) and Ovenden et al. (1988). These three sites consisted of the MacDonald River (Murray–Darling catchment, northern *G. marmoratus*), the Gellibrand River (south-west Victoria, southern *G. marmoratus*) and Cudgewa Creek (north-east Victoria, *G. bispinosus*). The other samples obtained by Ryan et al. (in press) included specimens of the *G. marmoratus* complex from eight additional sites. These sites included Darlot Creek, Brucknell Creek, the Wimmera River, and the Wannon River. In addition DNA sequences were provided by D. Jerry, James Cook University, Queensland, for samples from Stony Creek, Victoria, representing both *G. bispinosus* (GenBank accession number: AF294459) and *G. marmoratus* and Little Forester Creek (Tasmania) also representing *G. marmoratus* (AF294452). The remaining specimens were from Eight Mile Creek and Mosquito Creek (South Australia) (Fig. 1). Samples of *Maccullochella peeli peeli* (Murray cod) and *Bostockia porosa* (Western Australia nightfish) were included as outgroups (sequences derived from GenBank, accession numbers: AF295060 and AF295048).

DNA extraction and amplification of mtDNA. Total DNA was extracted from muscle tissue using an extraction protocol developed by Crandall et al. (1999). A fragment of the 12S mtDNA gene region (approximately 400 bp) was amplified via PCR using the 12S c/d primers described in Jerry et al. (2001). Double stranded PCR amplifications were performed in 50 µl volumes consisting of: 1x PCR

buffer, 2.0 µM MgCl₂, 0.2 mM dNTPs, 1 mM of each primer and 2 units *Taq* DNA polymerase. PCR amplifications were performed in a Corbett PC-960 Microplate Thermal Sequencer and consisted of 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec. An initial denaturation cycle of 3 min at 94°C was used and the program terminated with a 5 min cycle at 72°C. The PCR products were visualised in 1% agarose / TAE gels stained with ethidium bromide under UV light.

Purification and sequencing. PCR products were purified using a QIAquick PCR purification kit (QIAGEN) according to the manufacturers instructions. Purified DNA was quantified via direct comparison with DNA marker (Promega DNA / HAE III marker) of known concentration, again visualized under UV light in a 1% agarose / TAE gel containing ethidium bromide. Purified DNA was then sequenced according to Australian Genome Research Facility (AGRF), University of Queensland, protocols.

Data analysis. Sequence chromatograms were viewed using EditView and edited using SeqPup software (Gilbert, 1997). Sequences were aligned using the Clustal X program (Thomson et al., 1997) with alignment-ambiguous regions excised prior to phylogenetic reconstruction (Gatesy et al., 1993). Phylogenetic analyses were conducted using a range of approaches implemented by the PAUP* software package (Swofford, 1998). Phylogenetic signal within the data set was assessed using the *g*₁ statistic from the random tree length-frequency distribution (Hillis and Huelsenbeck, 1992). Phylogenetic relationships were estimated using maximum parsimony, neighbour-joining and maximum likelihood approaches. The most parsimonious tree was identified using a full exhaustive search with support for branches evaluated by 1,000 bootstrap replicates. Distance analysis was performed using the Tajima-Nei model of evolution and the neighbour-joining option with the number of bootstrap replicates set at 1,000. The most appropriate model of evolution for the maximum likelihood (ML) analyses was obtained via testing alternative modes of evolution using Modeltest (Posada and Crandall, 1998).

Results

Approximately 400 bp of the mitochondrial 12S rRNA coding region were sequenced for 12 individuals of *Gadopsis* from 11 locations. After sequence editing, 290 bp were used for subsequent analysis (GenBank accession numbers: AF505866 – AF505872). The random tree distribution based on the entire data set including both the outgroup taxa is significantly skewed to the left with *g*₁ = –0.899, *P* < 0.01, indicating significant phylogenetic information (Hillis and Huelsenbeck, 1992). The random tree distribution within the ingroup taxa was also significantly skewed (*g*₁ = –0.686, *P* < 0.01).

Percentage sequence divergences and the number of nucleotide substitutions among individuals (Table 1) indicate the existence of three equally distinct groups within *Gadopsis*. The individuals representing southern *G. marmoratus* (samples 9–12, Table 1) differ at 14–18 base positions (5–6% sequence divergence) in comparison with northern *G. marmoratus* individuals (samples 3–8), and 15–16 base positions (5–6% sequence divergence) compared to *G. bispinosus* (samples 1 and 2). *Gadopsis marmoratus* (northern) and *G. bispinosus* differed at 15–20 base positions (5–7% sequence divergence). In contrast, comparison between samples within each of these groups revealed much lower levels of divergence with *G. marmoratus* (southern), *G. marmoratus* (northern) and

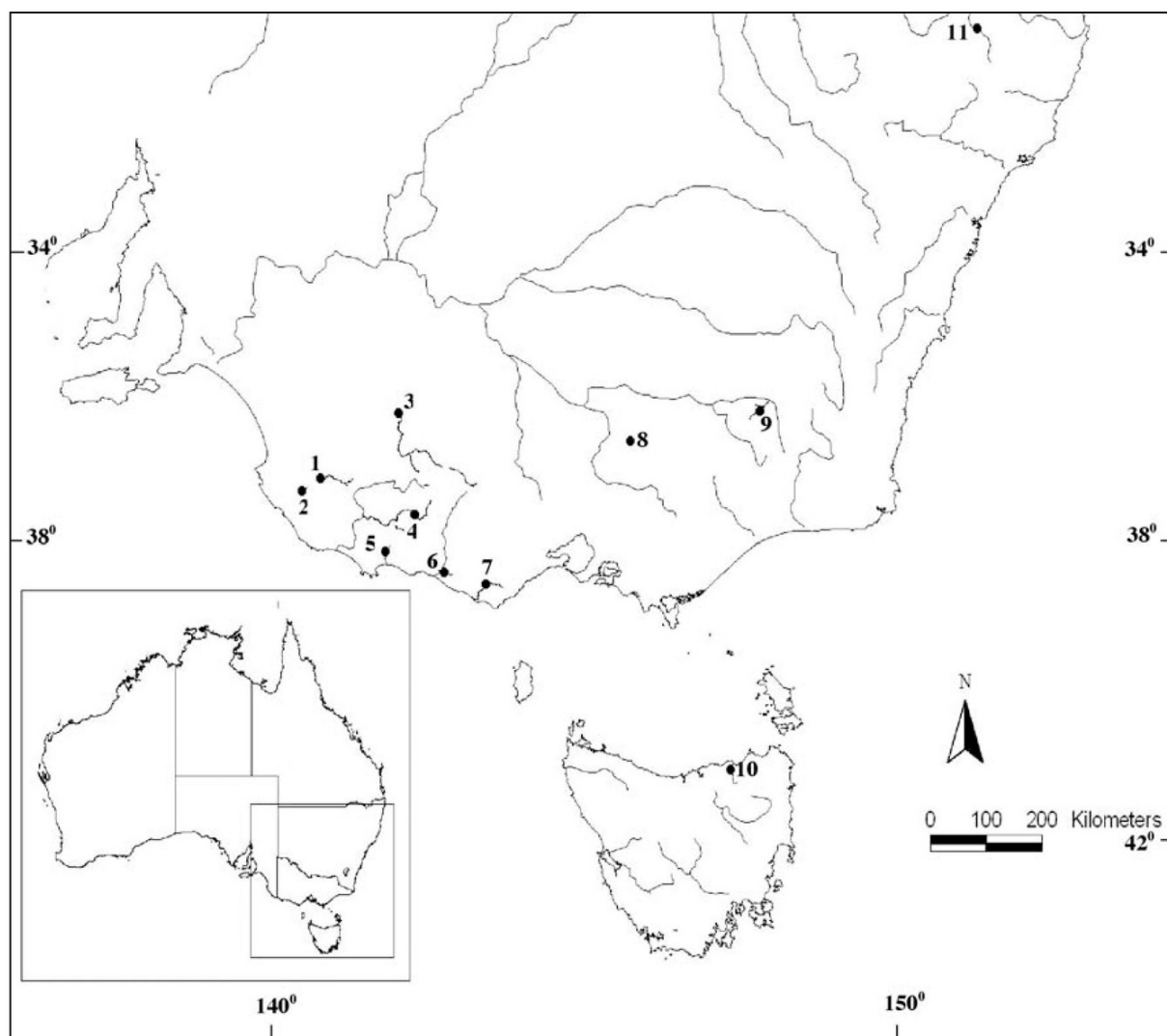


Figure 1. Sample locations: 1. Mosquito Creek, 2. Eight Mile Creek, 3. Wimmera River, 4. Wannon River, 5. Darlot Creek, 6. Brucknell Creek, 7. Gellibrand River, 8. Stony Creek, 9. Cudgewa Creek (*G. bispinosus*), 10. Little Forester Creek (Tasmania), 11. MacDonald River (New South Wales).

G. bispinosus showing differences at 0–1 bp positions (0.00–0.03%), 0–8 bp positions (0–3% sequence divergence) and 4 bp positions (1% sequence divergence) respectively. Nevertheless, geographic variation was apparent within the northern form of *G. marmoratus*. Blackfish samples from western Victoria and south-eastern South Australia (sites 1–4) differ by 7–8 base positions from the two samples from the Murray–Darling River system (sites 8–11). Variation within each of these groups was minimal with haplotypes either being identical or differing at only a single base position.

The degree of divergence between the outgroup taxa, *B. porosa* and *M. peeli peeli*, and blackfish samples was substantial ranging between 12 and 16% (34–45 bp differences). This was also similar to the difference between the two outgroup taxa (10% sequence divergence) (Fig. 2). The maximum parsimony, distance and maximum likelihood methods gave similar tree topologies. The Tamura–Nei model was chosen for the maximum likelihood analysis, involving a full heuristic

search with support for branches evaluated by 100 bootstrap replicates and the application of a gamma distribution shape parameter value equal to 0.2293 and calculated base frequency and substitutional rate matrix values. The significant feature of the trees is the clustering of *Gadopsis* into three distinct clades, representing the two putative northern and southern species of *G. marmoratus* and *G. bispinosus*. These three clades are supported by high confidence values in each method of analysis (66–100% bootstrap). Especially noteworthy is that the analyses do not necessarily indicate that the northern and southern forms are each other's closest relative. While maximum parsimony and maximum likelihood methods indicate an unresolved trichotomy for the relationship between the three clades, the distance approach suggests the northern *G. marmoratus* may in fact be more closely related to *G. bispinosus* than to the southern *G. marmoratus*, although the bootstrap support for this relationship is low. The maximum likelihood and distance analyses also highlight phylogenetic patterns within the

Table 1. Percentage sequence divergence and number of nucleotide substitutions among individuals of *Gadopsis* (specimens 1–4, southern *G. marmoratus*; 5 and 6, *G. bispinosus*; 7–12, northern *G. marmoratus*) and outgroup taxa (13, *Bostockia porosa* and 14, *Maccullochella peeli peeli*) based on 290 bp of the mitochondrial 12S rRNA gene. Below diagonal: total character differences; above diagonal: mean character differences (adjusted for missing data).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 Gellibrand R.	*	0.003	0.000	0.000	0.052	0.052	0.048	0.048	0.048	0.052	0.059	0.059	0.126	0.152
2 Brucknell Ck	1	*	0.003	0.003	0.055	0.055	0.052	0.052	0.052	0.055	0.062	0.062	0.129	0.156
3 L. Forester Ck	0	1	*	0.000	0.052	0.052	0.048	0.048	0.048	0.052	0.059	0.059	0.126	0.152
4 Darlot Ck	0	1	0	*	0.052	0.052	0.048	0.048	0.048	0.052	0.059	0.059	0.126	0.152
5 Stony Ck	15	16	15	15	*	0.014	0.052	0.052	0.052	0.055	0.069	0.069	0.136	0.149
6 Cudgewa Ck	15	16	15	15	4	*	0.052	0.052	0.052	0.005	0.062	0.062	0.133	0.145
7 Wimmera R.	14	15	14	14	15	15	*	0.000	0.000	0.003	0.024	0.024	0.147	0.138
8 Wannon R.	14	15	14	14	15	15	0	*	0.000	0.003	0.024	0.024	0.147	0.138
9 Mosquito Ck	14	15	14	14	15	15	0	0	*	0.003	0.024	0.024	0.147	0.138
10 Eight Mile Ck	15	16	15	15	16	16	1	1	1	*	0.028	0.028	0.143	0.135
11 MacDonald R.	17	18	17	17	20	18	7	7	7	8	*	0.000	0.150	0.149
12 Stony Ck	17	18	17	17	20	18	7	7	7	8	0	*	0.150	0.149
13 <i>Bostockia porosa</i>	36	37	36	36	39	38	42	42	42	41	43	43	*	0.126
14 <i>Maccullochella peeli peeli</i>	44	45	44	44	43	42	40	40	40	39	43	43	36	*

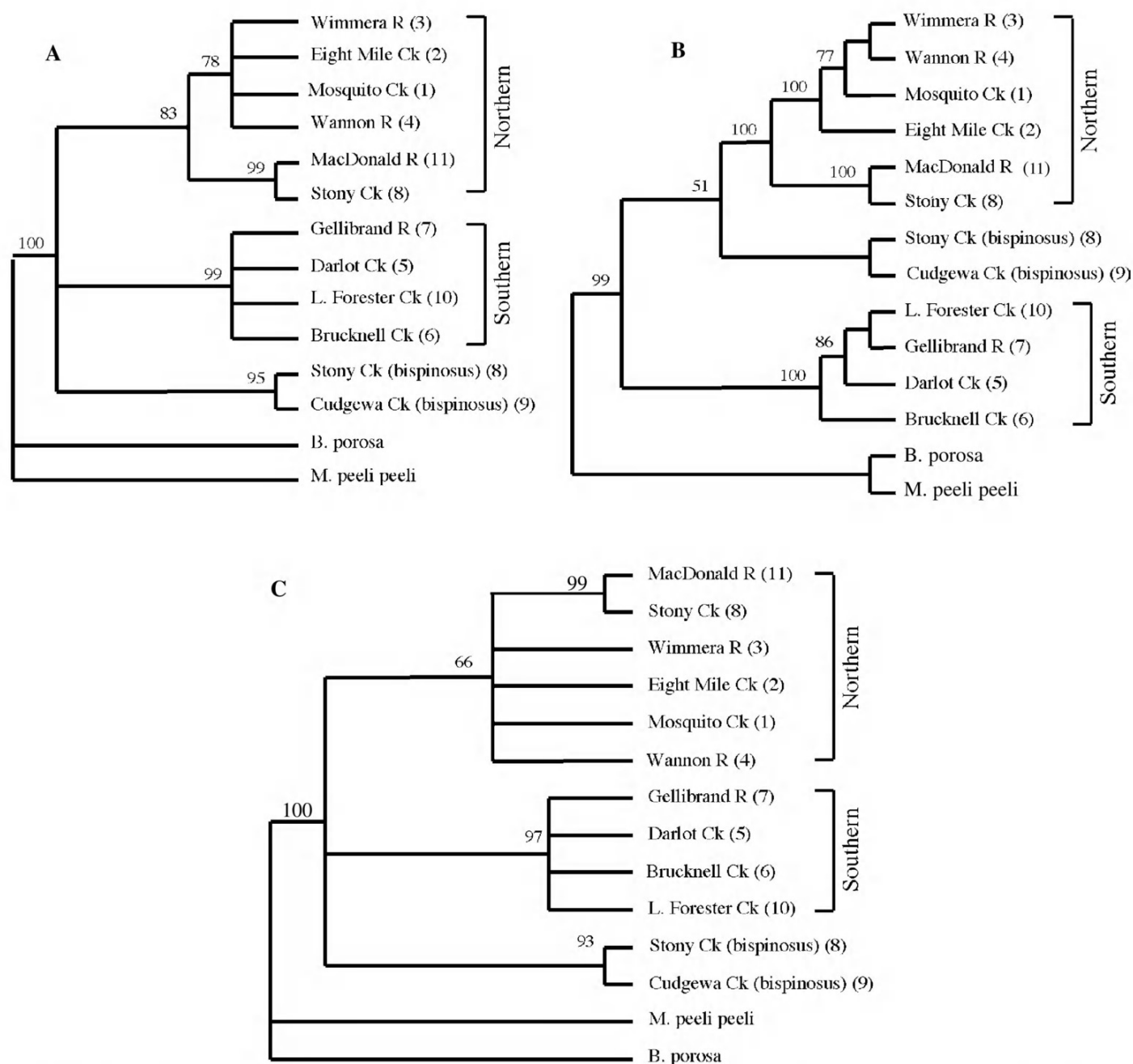


Figure 2. Phylogenetic trees, using the PAUP software package (Swofford, 1998). A, Maximum parsimony, using a full exhaustive search with 1000 bootstrap replicates. B, Distance analysis, using the neighbour-joining option, bootstrap replicates set at 1000. C, Maximum likelihood, using the Tamura-Nei model with 100 bootstrap replicates.

northern form of *G. marmoratus*. The samples from south-eastern South Australia and western Victoria (sites 1–4) form a well supported clade (78–100% bootstrap support) as do the individuals from Stony Creek and the MacDonald River from the Murray–Darling River system (99–100% bootstrap support) (Fig 2.)

Discussion

The taxonomic value of molecular genetic data is widely appreciated (Hillis et al., 1996) and they can be used in essentially

the same way as other data to address issues concerning the identification of taxonomic boundaries. Thus, finding that the degree of nucleotide divergence between *G. bispinosus* and the northern form of *G. marmoratus* is similar to that between *G. bispinosus* and the southern form (5–7%), and substantially greater than that observed within these groupings (0–3%), strongly suggests that each represents a distinct taxonomic entity.

The degree of divergence between the northern and southern forms of *G. marmoratus* and *G. bispinosus* is very similar to those reported by Jerry et al. (2001). These authors examined a

560 bp fragment of the 12S gene region for a single individual representing each of the three genetic forms of blackfish as part of phylogenetic study on Australian members of the family Percichthyidae. It is also noteworthy that Jerry et al. (2001) reported divergence levels of a similar or smaller magnitude for a number of congeneric percichthyid species that are considered good biological species. This together with the fact that *G. bispinosus* and *G. marmoratus* are known to behave as good biological species based on their maintenance of genetic differences in sympatry (Sanger 1986), suggests that the northern and southern forms of *G. marmoratus* also represent distinct biological species.

Additional support for the validity of northern and southern forms of *G. marmoratus* as discrete species derives from an examination of intra- and interspecific levels of divergence. Overall, the intraspecific comparisons average was 1.2%, compared with an average of 5.2% divergence for interspecific comparisons. The largest intraspecific comparison is 2.8% divergence between samples of northern *G. marmoratus* from Eight-Mile Creek in South Australia and the MacDonald River in New South Wales. Finding this degree of intraspecific divergence is not surprising given that the samples come from independent drainages over 1000 km apart. Conversely, finding that the Wannon River sample differs from the Darlot creek sample by 14 base positions (6%) and are less than 100 km apart strongly suggests that either a biological or geographical barrier has limited or completely impeded migration of blackfish between the two adjacent drainages. Decoupling of genetic divergence and geographic separation between samples indicates that the two forms represent good biological species and suggests that if they do come into contact they are unlikely to interbreed.

The application of the biological species concept to allopatric populations has, however, been widely criticized and is considered a persistent problem for taxonomic studies of freshwater fish (McDowell, 1972). Some authors have called for the abandonment of the biological species concept and its replacement with lineage or genealogically-based concepts such as the phylogenetic species concept (Claridge et al., 1997; Avise and Walker, 1999; Shaw, 2001). An advantage of the phylogenetic and related species concepts is that they allow recognition of species in sympatry or allopatry because genealogical relationships can be determined independently of geographical status (Shaw, 2001). While there are also operational difficulties in the application of lineage-based species concepts (Avise and Wollenberg, 1997; Sites and Crandall, 1997), the three distinct clades of blackfish identified in this study, via all three methods of phylogenetic analysis, would qualify for recognition as distinct species when applying a lineage-based species concept.

It is unwise to base the determination of species boundaries on a single source of information such as mitochondrial sequences from a single gene region. Support for the taxonomic conclusions of this study comes from studies of allozyme and morphological variation (Sanger, 1986; Ryan et al., in press) and restriction digests of the whole mitochondrial genome (Ovenden et al., 1988). Allozyme data indicated substantial differences between *G. marmoratus* and *G. bispinosus*

(22% fixed differences) and between the northern and the southern forms of *G. marmoratus* (11% fixed differences) (Ryan et al., in press). It is noteworthy that while the allozyme variation between the northern and southern forms is low relative to that between *G. marmoratus* and *G. bispinosus*, the extent of these differences are far greater than that detected between samples within the northern and southern groupings. Significantly, finding the same pattern of geographically abrupt genetic discontinuity between samples of blackfish from western Victoria in both mitochondrial DNA and allozymes (Ryan et al., in press), provides substantial support for the recognition of distinct northern and southern species. Further, results reported by Ovenden et al. (1988) are entirely consistent with the findings of this study based upon restriction digest of the whole mitochondrial genome.

An outcome of this study, which was not apparent from allozyme analyses or Ovenden's study (1988), is the finding of geographic variation in mitochondrial 12S rRNA sequences within the northern form of *G. marmoratus*. Specifically, based upon the six samples analysed, it appears that *Gadopsis* from western Victoria and south-eastern South Australia (sites 1–4) form a monophyletic group distinct from those of the Murray–Darling drainage system (sites 8 and 11). While the degree of divergence among these two groups is considerably less than that seen among the northern and southern *G. marmoratus* and *G. bispinosus*, they nevertheless represent distinct diagnosable lineages. It is noteworthy that Jackson et al. (1996), without going into any great detail expressed the view that blackfish from south-eastern South Australia may possibly be taxonomically distinct, therefore it will be important to investigate this pattern of variation in greater detail and determine if blackfish from this region may deserve taxonomic recognition.

Independent of the consideration of taxonomic status of *Gadopsis* spp., it is apparent that four evolutionary significant units (Waples, 1995) can be recognised in Victoria. If supported by additional sampling, each of these units will require the development of appropriate management strategies if the blackfish biodiversity is to be conserved and protected. In addition to loss of populations due to habitat deterioration, translocations associated with aquaculture, stocking of private water bodies and the use of *Gadopsis* as live bait, are factors that could threaten the integrity of local blackfish stocks. The genetic hazards of local translocations are well illustrated by the rapid genetic displacement of a freshwater crayfish species in the south-west of Western Australia as a result of an inadvertent translocation of a closely related species (Austin and Ryan, 2002).

The major difference between the results of this and previous allozyme studies is that the allozyme data appear to underestimate the degree of divergence between the northern and southern forms. In fact, the relationships among the three putative *Gadopsis* species remain an open question. The parsimony and maximum likelihood analyses suggest an unresolved trichotomy (see also Ovenden et al., 1988), the distance analysis suggests that the northern form of *G. marmoratus* is more closely related to *G. bispinosus*, although with poor bootstrap support, Jerry et al. (2001) supports a closer relationship

between the southern form and *G. bispinosus*, and the allozyme data suggests that the northern and southern *G. marmoratus* species are the most closely related. These inconsistencies leave the phylogenetic relationships among these species unresolved, and therefore also their possible evolution and biogeographic history (Sanger, 1986).

Given the vulnerability of *Gadopsis* from a conservation perspective, the relatively high degree of genetic diversity found in this study, and the unresolved phylogenetic relationships among the three major *Gadopsis* lineages, it becomes apparent that further research is important. Given the relatively slow evolutionary rate of the mitochondrial 12S rRNA gene region, it is suggested that genetic variation within and between *Gadopsis* populations using more rapidly evolving gene regions is determined to fully resolve geographical patterns of genetic diversity in these taxonomically distinct groups and the phylogenetic relationships among them. Further, the geographic sampling of *Gadopsis* for taxonomic and population genetic analysis needs to be expanded, especially with respect to populations in the eastern part of Victoria for which our genetic knowledge is limited.

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