Population structure of the Patagonian toothfish (*Dissostichus eleginoides*) in Australian waters

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OBJECTIVES:

1. To use microsatellite variation to resolve the connectivity of different spatial and temporal toothfish samples collected from Macquarie Island and Heard and McDonald Islands.
2. To compare genetic and tagging data from Macquarie and Heard and McDonald Islands regions to maximise toothfish stock structure knowledge in these regions.
3. To report on the outputs from the research and the resultant management response.

1. NON-TECHNICAL SUMMARY:

OUTCOMES ACHIEVED:

Molecular genetic studies using mitochondrial DNA and microsatellites of toothfish from Australian fishing waters showed that Macquarie Island toothfish were differentiated from Heard and McDonald Islands toothfish, and that both were differentiated from toothfish from Shag Rocks/South Georgia. No temporal or spatial genetic heterogeneity within these three fishing locations was observed. Geographic isolation by distance (due to deep water basins and non-migratory species) and low levels of ocean drift of young pelagic stages over large distances are factors that may contribute to the restriction on gene flow among the fishing locations. Tagging data show that adult fish move very little, and thus genetic homogeneity within locations may be more attributable to pelagic egg or larval drift in these areas than to adult movements. The project results will contribute to the more effective management of commercial fisheries for Patagonian toothfish in Australian fishing waters and indeed more globally. The findings support current management practise of considering the Heard and McDonald Islands fish as a single stock. At Macquarie Island, the new genetic data do not support earlier suggestions from more limited data of genetic differences between the two major fishing grounds; rather, the hypothesis of a single Macquarie stock could not be rejected. However, the conservative approach of managing the two grounds as separate entities will remain at least until the behaviour of these two fisheries is better understood.

The Patagonian toothfish (*Dissostichus eleginoides* Smitt, 1897) is a bentho-pelagic shelf species of the sub-Antarctic, and is one of only three exploited finfish species within the CCAMLR statistical area. It has a high landed value of around $9 to $12/kg and as such is a very valuable fishery. Although the fishery has been utilised commercially only in the last 17 years, there is already unsustainable fishing pressure in some locations. This is due mainly to major problems with illegal, unreported and unregulated fishing.

Little is known about the stock structure of toothfish within the Southern Ocean, nor about the amount of linkage via pelagic egg/larval drift or adult migration. Such knowledge

is required for effective and sustainable management. Stock structure can be assessed in multiple ways, no one of which can give a complete picture. We have used a genetic approach. Mitochondrial DNA (mtDNA) and microsatellite loci were examined in Patagonian toothfish from two Australian fishing locations in the Southern Ocean: Macquarie Island, five collections (southwest Pacific sector), and Heard and McDonald Islands, four collections (CCAMLR area 58.5.2, western Indian sector). A small sample from the Shag Rocks/South Georgia location (CCAMLR area 48.3, southwest Atlantic sector) was also examined.

Spatial and temporal collections within the same fishing location showed no evidence of mtDNA heterogeneity, but extensive and highly significant heterogeneity among the three fishing locations was recorded. The microsatellite data revealed minor and inconsistent heterogeneity at several loci among the ten collections. MtDNA is more sensitive to factors leading to genetic drift than nuclear (microsatellite) DNA, and in toothfish was a better indicator of population structure. The mtDNA heterogeneity indicates that gene flow between the three fishing locations is severely restricted.

Tagging data from Macquarie Island and Heard and McDonald Islands showed that the great majority of recaptured toothfish had moved 15 nautical miles or less, with very little movement between grounds within locations. The genetic homogeneity of grounds within locations might thus be more likely attributed to pelagic egg or larval drift than adult movements. No fish have been recorded as moving between Macquarie and Heard and McDonald Islands. The substantial genetic differentiation between these locations (and the even more distant Shag Rocks/South Georgia) is consistent with their geographical isolation; gene flow from ocean drift of young pelagic stages over such large distances appears to be trivial. However, it is interesting to note that one (of 719 recaptured) tagged fish from Heard and McDonald Islands was recaptured at Kerguelen Islands (c. 200 miles), and two were recaptured at Crozet Islands (c. 1 000 nautical miles). No genetic studies of toothfish from these locations have been carried out, but they are planned.

Genetic, tagging and other data show that separate Patagonian toothfish populations exist among the Australian fishing locations and, more globally, across the Southern Ocean.

Finally, the utility of mtDNA for successful species identification of Patagonian and Antarctic toothfish was also demonstrated.

KEYWORDS: **Patagonian toothfish, *Dissostichus eleginoides*, stock structure, microsatellite, mitochondrial DNA**

2. ACKNOWLEDGEMENTS

We thank the Australian Fisheries Research and Development Corporation (FRDC) for funding this work (Project No. 2000/108). We appreciate the hard work undertaken by the observers onboard the *Austral Leader* and *Southern Champ*ion in collecting the toothfish samples and conducting the tagging programme. We also thank Tim Lamb for managing the fish database and providing summaries of the sample data, and Anne Reilly for development of the CMR microsatellite markers.

3. BACKGROUND

Antarctica and the Southern Ocean

The Southern Ocean, which surrounds the Antarctic continent, is not formed from a single distinct basin-like feature like other oceans, but is composed of the southern parts of the Atlantic, Indian and Pacific basins delimited to the south by the Antarctic Continent and to the north by the oceanographic feature of the Antarctic Polar Front (APF) (Kock, 1992; Eastman and McCune, 2000). The northern boundary of the statistical reporting area of the Commission for the Convention on the Conservation of Antarctic Marine Living Resources (CCAMLR) largely coincides with the APF (Kock, 1992).

After separation from the Australian continent (about 37 million years ago) and the deepening of the Drake Passage (about 25 million years ago) between Cape Horn and the Antarctic Peninsula, Antarctica was isolated from other continents (Eastman and Clarke, 1998; Bargelloni *et al*., 2000). The APF was formed from deep water between South America and the Antarctic continent (Kennett, 1982) and is located where the cold Antarctic waters meet warmer waters to the north. The front extends to depths of approximately 1 000 m at approximately 47-63^{0}S (Kock, 1992).

The Southern Ocean consists of systems of deep water basins separated by three ridges: the Macquarie Ridge south of New Zealand and Tasmania, the Kerguelen-Gaussberg Ridge at about 80^{0}E, and the Scotia Ridge which extends from the Patagonian shelf to the South Shetland Islands and the Antarctic Peninsula (Kock, 1992). The Antarctic shelf within the Southern Ocean is not contiguous with other shelf areas of the Southern Hemisphere, and is mainly isolated from other landmasses by deep water (Eastman and McCune, 2000). Within the Southern Ocean, the circum-continental current system, West Wind Drift, influences most of the peri-Antarctic islands (White, 1998), while a system of currents, particularly the clockwise Circumpolar Current, aids dispersal of planktonic organisms south of the APF (Bargelloni *et al*., 2000).

Toothfish in the Southern Ocean

The Patagonian toothfish (*Dissostichus eleginoides* Smitt, 1897) is a bentho-pelagic shelf species of the sub-Antarctic and is the largest member of the family Nototheniidae (the 'Antarctic cods') (Evseenko *et al*., 1995). The species is widely distributed from the slope waters off Chile and Argentina south of 30-35^{0}S to the peri-Antarctic islands and shelf areas in sub-Antarctic waters of the Atlantic, Indian and Pacific Ocean sectors of the Southern

Ocean (Kock *et al*., 1985; DeWitt *et al*., 1990; Kock, 1992). Both the Patagonian toothfish and its sister species the Antarctic toothfish (*D. mawsoni*) occur in CCAMLR waters. While Patagonian toothfish are caught both inside and outside of CCAMLR waters, Antarctic toothfish are found only within CCAMLR waters (Kock, 1992)

Patagonian toothfish have a wide distribution in the sub-Antarctic zone, on sea mounts and submarine ridges around the east and west coasts of South America, South Georgia and Shag Rocks, South Sandwich Islands, Kerguelen Plateau, Crozet Island, Ob and Lena Banks, the South African Shelf, Macquarie Island and the Campbell Plateau south of New Zealand (SC-CAMLR XIV, 1995). These areas are separated by large basins, which may inhibit the interchange of fish (Fishery Status Reports, 1998). The southernmost area of toothfish distribution has been recorded at South Orkney Island (Efremenko, 1979) and the South Sandwich Islands (SC-CAMLR XII, 1993) and they have been found in waters down to 2 500 m (Evseenko *et al*., 1995; Bargelloni *et al*., 2000). Since the APF extends to only 1 000 m depth, it may not pose a hydrological barrier to toothfish movement.

Toothfish are large active predators and grow to lengths of between 1.5 and 2.0 m and up to 80 kg (Fisheries Status Reports, 1998). They are a long-lived finfish species with a life span of 35-50 years. Amongst the notothenioids, toothfish fecundity is considered relatively high, with number of eggs produced ranging from 48 000-500 000 per female, varying with fish length and locality (Kock *et al*., 1985; Chikov and Melnikov, 1990). It has been suggested that the species spawns over the various continental slopes within the Southern Ocean at around 1 500 m, probably between March and August (Kock, 1993; Evseenko *et al*., 1995; Des Clers *et al*., 1996). Pelagic eggs hatch to larvae between August and November (Kellerman, 1990) and juveniles probably remain pelagic for another year before becoming demersal (Des Clers *et al*., 1996). Apart from this, little is known of spawning grounds within the Southern Ocean.

Up until recently, toothfish were one of only two finfish species exploited within the CCAMLR statistical area, the other being the mackerel icefish, *Champsocephalus gunnari* (Constable *et al*., 2000). The Antarctic toothfish, *Dissostichus mawsoni* is now also being exploited, albeit at only low levels (specifically the Ross Sea, about 400-500 t per annum). The Commission for CCAMLR first met in 1982 after the Convention was established in 1980, with one of its main responsibilities the conservation of fisheries resources in the Southern Ocean (Lack and Sant, 2001). Fishing for toothfish species began in the 1970s when small tonnages were taken as by-catch in the mixed bottom trawl fishery around South Georgia Island and Shag Rocks in the southwest Atlantic sector of the Southern Ocean

(Constable *et al.*, 2000). In the mid 1980s, aggregations were discovered near Kerguelen Island and a trawl fishery developed there. In the late 1980s, the introduction of long-lining enabled the exploitation of older and larger demersal toothfish in the South Georgia and Kerguelen areas (Constable *et al.*, 2000). Long-lining is now the principal method of fishing within the CCAMLR statistical reporting area, although trawl fishing, which takes smaller, juvenile fish, occurs around Heard, Kerguelen and Crozet Islands (CCAMLR area) and around Macquarie Island (Australian national waters). Within CCAMLR reporting zones, most of the legal toothfish catch is taken in waters around the islands of Kerguelen and Crozet, South Georgia, and Heard and McDonald Islands. The total legal catch reported to FAO for 1999 was 41 045 t (FAO 1999). The fish has a high landed value of around $9 to $12/kg making this a very valuable fishery.

Although the fishery has been utilised commercially only in the last 17 years, there is already unsustainable fishing pressure in some locations. This is due largely to major problems with illegal, unreported and unregulated fishing (IUU) (CCAMLR XVII, 1998; Constable *et al.*, 2000; Lack and Sant, 2001).

Toothfish Fisheries in Australian Waters, Macquarie Island

In 1996, an Australian deep-sea trawl fishery targeting toothfish developed in the Australian Exclusive Economic Zone (EEZ) around Macquarie Island (Macquarie) after two seasons of exploratory fishing (Fishery Status Reports, 1998). Macquarie Island is a sub-Antarctic island about 1 300 km south of Tasmania. It is surrounded by a very narrow continental shelf, which extends less than 10 nautical miles from the island. The island lies within the Australian Fishing Zone (AFZ), but is outside CCAMLR jurisdiction, however the Australian Fishing Management Authority (AFMA) manages this fishery in a manner consistent with CCAMLR advice. Initial catch records from the exploratory fishing in 1994 to 1996 by the *Austral Leader* showed approximately 1 000 t were taken (Fishery Status Reports, 1998). The developmental fishery has been restricted to trawl fishing methods only and the Sub-Antarctic Fisheries Assessment Group (SAFAG) has recommended to AFMA that a conservative and precautionary catch be allowed around Macquarie Island. Currently, the *Austral Leader* is the only boat licensed to fish in the Macquarie Island area.

Toothfish catch rates in an area known as Aurora Trough at Macquarie have been very variable and catches within Colgate Valley, Grand Canyon and Beer Garden (collectively known as the Northern Valleys) have come previously from large aggregations encountered from time to time (Fishery Status Reports, 1998). In 1997, AFMA decided to undertake

separate stock assessments for Aurora Trough and Northern Valleys. In 1998, toothfish biomass in Aurora Trough was estimated at 1 122 t. Based on previous estimates in 1997, fish biomass in the Northern Valleys was determined to be 36 600 t, but in 1998 this estimate had dropped drastically to 2 222 t (Fishery Status Report, 2000). It is not known if the fish, which comprised the large aggregation, migrated out of the region or if they just became unavailable to the trawl (for example by moving deeper) and acoustic equipment used for fishing and biomass estimation (Fishery Status Report, 2000).

The Aurora Trough toothfish are now believed to be below target levels and it is not known if the large aggregations of toothfish observed in 1997 outside the Aurora Trough were part of a resident population or transients (Fishery Status Report, 2000). Accordingly, in the Macquarie Island fishery for 2001, the Aurora Trough was closed to commercial fishing with a total allowable catch (TAC) outside the trough area set at only 420 t. Unless large aggregations of toothfish are rediscovered in the Northern Valleys, fishing in this area will also be limited (Fishery Status Report, 2000).

Preliminary results from genetic studies (Reilly *et al.*, 1998; Reilly and Ward, 1999) combined with limited tag information has suggested little interchange between fish in the Aurora Trough and the Northern Valleys areas even though these two areas are only separated by 40 nautical miles (Fishery Status Report, 2000). In the Approved Management Policy of November 1996, fishery managers assumed that fish within the Aurora Trough are a separate stock to those in other waters of Macquarie Island (Fishery Status Report, 1998).

Toothfish Fisheries in Australian Waters, Heard and McDonald Islands

Heard and McDonald Islands (HIMI) are part of Australia's external territories and are located in the southern Indian Ocean sector of the Southern Ocean about 4 000 km south west of Perth. Both HIMI and the territorial sea around the islands (to 12 nautical miles) are a Wilderness Reserve that is managed by the Australian Antarctic Division (AAD). This area is closed to fishing. Waters surrounding HIMI from 12 out to 200 nautical miles are part of the Australian Fishing Zone (AFZ), which is managed by AFMA.

The islands lie south of the APF and the HIMI AFZ falls under the management jurisdiction of CCAMLR, of which Australia is one of 24 international members. The HIMI Exploratory Fishery refers to the portion of Australia's AFZ which falls within CCAMLR statistical area 58.5.2. The HIMI fishery began in March 1997 and since then catches have been between 3 000 and 3 500 t per annum. Australia collects fishery data (AAD and AFMA), carries out biological research on the principal commercial species (mainly at AAD

and CSIRO), contributes to the CCAMLR stock assessment process, and adopts CCAMLR total allowable catches (TACs) for 58.5.2. At the most recent CCAMLR XX commission meeting, the TAC for HIMI was set at 2 815 t (from 1/12/01-30/11/02) (SC-CAMLR XX, 2001). Currently two boats fish the HIMI region, the *Austral Leader* and the *Southern Champion*.

At both Macquarie and HIMI islands, fishing takes place at well-defined grounds separated from each other by distances of at least 40 nautical miles. Biological data have been gathered since the beginning of the fisheries. Tagging studies reveal that nearly all recaptured fish are caught at the ground where they were tagged.

Toothfish Stock Structure in the Southern Ocean

Little is known about the actual stock structure or degree of stock separation of this fish (CCAMLR XIV, 1995; Evseenko *et al*., 1995) within the Southern Ocean. The amount of linkage via possible pelagic larval drift or by migration is also unknown. Only when these relationships are known, can fully effective management plans be introduced, taking into account the degree of isolation of fish between the relatively small and widely spaced fishing locations.

Although there is minimal information on toothfish stock structure within the Southern Ocean, earlier morphological, otolith, enzymatic and preliminary genetic studies have indicated that there are separate stocks of toothfish present.

Morphological evidence has shown that toothfish populations around the Falkland Islands and South Georgia are distinct from each other (Kock *et al*., 1985). However, Des Clers *et al*. (1996) suggested that the mainly adult toothfish in the Falkland Islands waters are replenished from nursery areas in the sub-Antarctic.

An analysis of chemical data from cores of toothfish otoliths collected from fisheries off Chile, Falkland Islands, HIMI, Kerguelen Islands, Macquarie Island, Prince Edward Island and South Georgia suggests at least four stocks within the Southern Ocean (Kalish and Timmis, 2000). These are a South American group, a Falkland Islands group, Macquarie Island, and an Indian Ocean island group (Kalish and Timmis, 2000).

Toothfish collections from Crozet and Kerguelen Islands appeared to show slight differences in K_m values for the enzyme acid phosphatase (Diano, 1989), but the data as presented cannot be assessed for statistical significance. The results were, however, interpreted as suggesting the possibility of two populations, and the differences attributed to sensitive adaptation mechanisms to different local conditions (Diano, 1989).

Direct genetic evidence for population sub-structuring has come from nuclear DNA analysis. Allozyme and microsatellite analysis in toothfish populations from the Atlantic, Indian and Pacific Ocean sectors of the Southern Ocean revealed small but significant spatial heterogeneity for alleles at microsatellite loci but not at allozyme loci (Smith and McVeagh, 2000). Smith and McVeagh (2000) concluded that different fishing grounds may support differentiated stocks, and that there was likely to be restricted gene flow throughout the Southern Ocean. Preliminary microsatellite data led Reilly and Ward (1999) to suggest that two samples of toothfish from sites 40 nautical miles apart off Macquarie Island may not be genetically homogenous; sample sizes used were, however, very small (n=15-17 individuals).

Movement data from sub-adult and adult fish tagged at Macquarie and HIMI are also available (R. Williams, unpubl.; Williams *et al.*, 2001). With very few exceptions, these show that tagged fish are recaptured at the fishing ground they were released at, having swum 15 nautical miles or less from the point of release. No movements between Macquarie and HIMI have been recorded, although one fish tagged at HIMI was recaptured at Kerguelen (200 nautical miles away) and two at Crozet (1 000 nautical miles away) islands. The overriding conclusion from the tagging studies is that, with very rare exceptions, adult toothfish are quite sedentary. Therefore, geographically isolated populations will usually be quite isolated from one another, at least with respect to adult movements.

Current Toothfish Study

From a fisheries management perspective, the identification of any genetic population structure is vital for long-term sustainable management. Uncertainty regarding stock structure restricts the ability to make both regional and more global stock assessments.

The number of genetically distinct populations for a species can depend on environmental factors and life-history traits (Avise, 1994; Bargelloni *et al.*, 2000). Environmental factors, including glaciations, formation of land-bridges, sea level changes, thermoclines and deep water ridges and basins can affect or help to maintain separate marine fish populations (Sinclair and Iles, 1989). In marine fish, life-history stages such as pelagic larvae or large population sizes can play a role in determining genetic differentiation (Gyllensten, 1985; Waples, 1988; Ward *et al.*, 1994). Marine species with larval stages that are carried by the ocean currents across large distances or marine populations that have few geographic barriers to dispersal will probably show little stock structure (Ward *et al.*, 1994; Bargelloni *et al.*, 2000). For Antarctic fish species, gene flow is likely to be restricted by the geographical isolation of populations (caused by deep water basins or gyres), reliance on shelf habitats for spawning, low fecundity and delayed maturation. On the other hand, gene flow

may be promoted by prolonged pelagic developmental stages and current systems (White, 1998).

Patagonian toothfish is considered a valuable resource in the Australian AFZ and key issues for the sustainable management of this fishery are concerned with the effects of fishing pressure and identification of the distribution, movement and abundance of toothfish. Development of any ecologically sustainable toothfish fishery requires a stock assessment model, which in turn requires information on stock structure. Basic biological information about toothfish is scarce. However, a recently completed major FRDC project (FRDC 97/122) aimed to provide this information for Macquarie fish. The focus on Macquarie Island arises from the need to be seen to protect the conservation values of this special area while developing a sustainable fishery. There are also management concerns about the nature and extent of HIMI toothfish stocks, and their relationship to Macquarie.

As a step towards understanding the stock structure of toothfish within the AFZ, the present project undertook an investigation of both mtDNA and microsatellite loci in samples from the Macquarie and HIMI areas. Analyses investigated both temporal and spatial genetic structure of various collections sampled from both these areas.

MtDNA is a small, closed circular genome found in the mitochondria of cells. It shows variation between individuals on both an intra and inter-species level, and has proven to be an effective genetic marker for population structure analysis (Avise *et al.*, 1987; Ovenden, 1990; Billington and Hebert, 1991). Owing to its haploid nature and maternal mode of inheritance, the effective population size of mtDNA is one quarter that of nuclear DNA (given equal numbers of males and females). MtDNA is therefore more sensitive to bottlenecks in population size than nuclear genes, and more subject to genetic drift and population differentiation (Nei and Tajima, 1981; Wilson *et al.*, 1985; Moritz *et al.*, 1987; Billington and Hebert, 1991). In addition, mtDNA has a comparatively rapid rate of evolution, which assists in the definition of recently diverged populations (Wilson *et al.*, 1985; Avise *et al.*, 1987).

Microsatellites are tandem repeats of short sequence motifs that are distributed throughout the nuclear DNA genome and believed to be abundant in all eukaryotes (Tautz, 1989). Microsatellites are fast evolving and a high proportion of microsatellite loci surveyed in fish are polymorphic (O'Connell and Wright, 1997; DeWoody and Avise, 2000). Microsatellites show high levels of genetic variation and high mutation rates, meaning that populations are likely to diverge not only by genetic drift but by mutation as well. Since they

reflect variation in non-coding sequences, they are thought to be neutral markers (unaffected by selection).

Use of both mtDNA and microsatellite analyses is more powerful than either single approach, and a joint approach was employed here. We also aimed to put the genetic results into the context of tagging results.

4. NEED

The resolution of stock structure of toothfish in Australian and other waters is required. Tagging experiments, while important, cannot by themselves give a complete picture of stock structure. If genetic differences between areas are detected, then gene flow between areas must be restricted and more than one stock can be assumed. If differences are not detected, then the hypothesis of a single genetic stock cannot be rejected. However, this hypothesis may be rejected on other grounds, such as a lack of evidence from tagging experiments of fish movement between areas. Therefore, resolution of stock structure is better managed by a combination of approaches than by any single approach in isolation. Tagging experiments are under way; we proposed to carry out genetic analyses, and then consider both sets of data jointly to define likely stock structures.

Genetic evidence already collected, albeit based on very small sample sizes from two areas of Macquarie (Reilly and Ward, 1999), suggested that the amount of movement between fishing areas might be very limited. A broader-scale genetic survey suggested restricted gene flow was likely between different Southern Ocean populations of toothfish (Smith and McVeagh, 2000). If these conclusions of both fine-scale and broad-scale structure can be verified, then careful management of the fishery will be needed, as depletion of one area is unlikely to be quickly replaced by immigration from another.

The work proposed will examine the question of whether or not there is fine-scale genetic separation of the Macquarie Island areas. It will also investigate whether there are separable sub-stocks of toothfish around HIMI, and whether the HIMI stock(s) are separable from the Macquarie Islands stock(s).

These issues have to be resolved for sustainable management of toothfish fisheries by Australian management (AFMA). Furthermore, the information gathered will also be very useful to CCAMLR and to the management authorities of other nations controlling toothfish fisheries. Recent meetings of CCAMLR (1998 & 1999) recognised the urgent requirement of further work on stock structure and stock separation in *D. eleginoides*.

The results of our research will add to the very limited knowledge of genetic stock structure of toothfish in the Southern Ocean. While there have been two microsatellite studies of toothfish published (Reilly and Ward, 1999; Smith and McVeagh, 2000), there have been no population mtDNA or temporal microsatellite studies on toothfish published to date.

5. OBJECTIVES

1. To use microsatellite and mtDNA variation to resolve the connectivity of different spatial and temporal toothfish samples collected from Macquarie Island and Heard and McDonald Islands.
2. To compare genetic and tagging data from Macquarie and Heard and McDonald Islands to maximise toothfish stock structure knowledge in these regions.
3. To report on the outputs from the research and the resultant management response.

Genetic data will also be compared to that obtained from a small collection of toothfish from South Georgia/Shag Rocks (located within the southwest Atlantic region of the Southern Ocean). If evidence of stock structuring within the Southern Ocean is evident, then a more global study will be proposed to investigate the number and degree of relatedness of different toothfish stocks within the Southern Ocean. Additionally, samples were also used to develop a diagnostic species test for differentiation between Patagonian and Antarctic toothfish.

6. METHODS

6.1. Toothfish sampling

Samples from within the AFZ were collected around islands in the western Indian Ocean (Heard and McDonald Islands) (HIMI) and southwest Pacific Ocean (Macquarie Island) (Macquarie) sectors of the Southern Ocean from 1997 to 1999 (Figure 1). Fish from the southwest Atlantic Ocean sector (Shag Rocks and South Georgia) were also used for comparative study and these were collected in 1999.

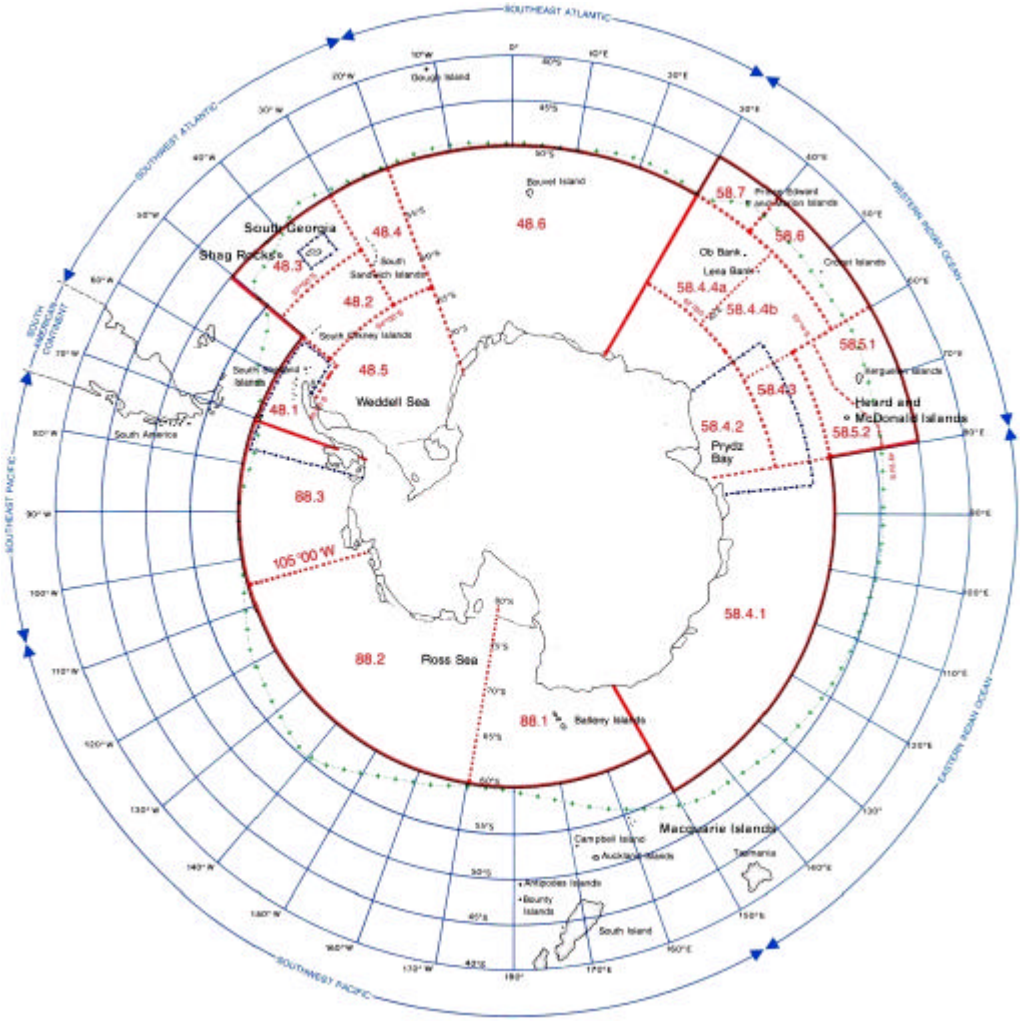

Figure 1. Approximate locations of Macquarie Island, Heard and McDonald Islands and Shag Rocks/South Georgia toothfish collections sampled in this study (shown in larger font). Map given is that of the CCAMLR Boundaries of the Statistical Reporting Areas in the Southern Ocean, sourced with thanks from CCAMLR.

All samples were collected from commercial fishing boats fishing legally for toothfish (Table 1). Exact fishing locations cannot be given due to commercial confidentiality restrictions. Samples at HIMI were collected from each of the three commercial fishing

grounds, separated by approximately 100 to 200 nautical miles, by observers on board the *Austral Leader* or *Southern Champion.* Samples from two fishing grounds at Macquarie approximately 40 nautical miles apart were collected by observers on board the *Austral Leader.* The samples from Shag Rocks and South Georgia were collected by Jim Taylor on board the *Tierra Del Fuego*.

Table 1 *Dissostichus eleginoides* samples, lengths, sampling locations and sex ratios.

Fishing Location	Ground	Collection	Date of Sampling	Sample Size	Average Length cm (s.e)
Macquarie	A	M-A97	11.97	11	479.09 (14.84)
Macquarie	B	M-B98	1.98	60	686.78 (11.41)
Macquarie	A	M-A98	1.98	56	746.73 (17.68)
HIMI	B	H-B98	7.98	126	656.97 (7.12)
HIMI	A	H-A98	7.98	58	874.62 (35.92)
HIMI	C	H-C98	9.98	73	634.30 (15.34)
Macquarie	B	M-B99	1.99	78	581.19 (15.51)
Macquarie	A	M-A99	1.99	60	660.27 (26.52)
HIMI	B	H-B99	4.99	56	619.30 (18.58)
Shag Rocks	-	SRG-99	6.99	24	655.50 (1.44)
South Georgia	-	SRG-99	6.99	24	679.50 (1.43)

Samples from the fishing grounds at both HIMI (H-B99) and Macquarie (M-A99 and M-B99) can be further divided into smaller sub-sampling locations or ‘suburbs’ (Table 2). Additionally, fish sampled from the Macquarie fishing grounds in 1999 were also divided into small and large fish (Table 2).

Table 2 Toothfish sampled from various fishing locations and 'suburbs' (different locations within fishing grounds) at HIMI and Macquarie Islands fishing grounds, together with dates, sample sizes, and mean lengths.

Fishing Location	Ground	Suburb	Date of Sampling	Sample Size	Average Length cm (s.e)
HIMI	B	SA	7.98	25	669.52 (14.88)
HIMI	B	ES	7.98	76	651.37 (9.58)
HIMI	B	HE	7.98	16	689.88 (13.22)
HIMI	B	FH	7.98	9	610.89 (28.44)
HIMI	A	BD	7.98	58	874.62 (35.92)
HIMI	C	KC	9.98	73	634.30 (15.34)
HIMI	B	HG	4.99	25	504.52 (12.82)
HIMI	B	FH	4.99	31	711.87 (19.93)
Macquarie	A	AT	11.97	11	479.09 (14.84)
Macquarie	B	BG	1.98	60	686.78 (11.41)
Macquarie	A	AT	1.98	56	746.73 (17.68)
Macquarie	B	CV	1.99	16	440.25 (7.08), small
Macquarie	A	AT	1.99	20	857.25 (14.60), large
Macquarie	B	BG	1.99	23	482.57 (7.18), small
Macquarie	A	YL	1.99	20	722.35 (22.03), large
Macquarie	B	BG	1.99	39	697.18 (15.15), large
Macquarie	A	AT	1.99	20	401.20 (8.71), small

Samples consisted of pieces of white muscle dissected from whole fish and stored frozen in alcohol preserving solutions at -80^0C until DNA was extracted. Frozen white muscle tissue of several samples of *D. mawsoni* (source: Australian Antarctic Division collections from near Casey Station, $66^0$17'S:$110^0$32'E) was used to develop diagnostic tests for the two toothfish species.

Genetic analysis involved the use of mtDNA for haplotype differentiation of populations using restriction fragment length polymorphism (RFLP) analyses and for development of a species diagnostic test between *D. eleginoides* and *D. mawsoni*. Nuclear DNA was used in microsatellite analyses for population discrimination.

6.2. DNA extraction

For both mtDNA and nuclear DNA approaches, total genomic DNA was extracted from approximately 50 mg of tissue from each individual using a modified CTAB (hexadecyltrimethylammonium bromide) extraction protocol (Doyle and Doyle, 1987; modified as in Appleyard, 1998). Genomic DNA was precipitated with isopropanol and ethanol. DNA pellets were then resuspended in 100-200 μl of deionized H_2O and stored at

4^0C. Stock DNA from the collections was usually diluted 1:10 with distilled water for both mtDNA and microsatellite applications. In some instances, undiluted genomic DNA was used, particularly for mtDNA amplifications.

6.3. MtDNA PCR Amplification

Restriction fragment length polymorphism (RFLP) analysis on mtDNA from the various toothfish populations was examined through restriction digestion of two regions (ND2 and BCL). MtDNA variation for species identification was examined through digestion of the 16S ribosomal RNA gene.

The ND2 fragment contains the NADH dehydrogenase subunit 2 gene and was amplified using the forward primer t-Met of Park *et al*. (1993) and the reverse primer (Mt-76) of Smith *et al*. (2001) which targeted the $tRNA^{Trp}$ gene (Table 3). Amplified fragments were approximately 1.1 kilobase pairs (kbp) in size, as in Smith *et al*. (2001).

The more variable BCL fragment contains the control region or D-loop of the mtDNA and is flanked by the transfer RNA proline gene and the 12S rRNA gene. This fragment was amplified using primers 12SAR-H (Palumbi *et al*., 1991) and L16498 (following Smith *et al*., 2001, without the GC clamp) (Table 3). Amplified fragments were approximately 1.3 kbp (as in Smith *et al*., 2001).

A fragment of approximately 630 bp from the 16S ribosomal RNA gene was amplified using the universal primers 16SAR and 16SBR (Palumbi *et al*., 1991), as in Smith *et al*. (2001) (Table 3).

Table 3 Sequence of mtDNA primers used in toothfish study.

Primer	Sequence	Region
t-Met	5’ AAG CTA TCG GGC CCA TAC CC 3’	ND2 - NADH dehydrogenase subunit 2
Mt-76	5’ CCG CTT AGY GCT TTG AAG GC 3’	
12SARH	5’ ATA GTG GGG TAT CTA ATC CCA GTT 3’	BCL - transfer RNA proline & 12S rRNA
L16498	5’ ATC TGG TTC CTA CTT CAG G 3’	
16SAR	5’ CGC CTG TTT ATC AAA AAC AT 3’	16S - 16S ribosomal RNA
16SBR	5’ CCG GTC TGA ACT CAG ATC ACG T 3’	

The same PCR conditions (except annealing temperatures for primers, see below) were used for all fragments. PCR amplifications were performed in a PE-Applied Biosystems 9600 thermocycler in a total volume of 50 μl. Individual amplifications consisted of 200 μM dNTP’s, 10 mM Tris HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 μM of forward and

reverse primer, 0.025U/μl Amplitaq Gold (Perkin Elmer, USA) and 2-10 μl of 1/10 or undiluted genomic DNA. After an initial cycle of 93^0C $\times$ 10 minutes, samples were subjected to 93^0C $\times$ 30 seconds, 50^0C $\times$ 1 minute (BCL and 16S primers) or 55^0C $\times$ 1 minute (ND2 primers) then 72^0C $\times$ 2 minutes for 40 cycles with a final extension step of 72^0C $\times$ 10 minutes.

6.3.1. Restriction digestion of mtDNA fragments

Seven and 14 restriction enzymes were initially trialed on the PCR products for ND2 and BCL respectively in an attempt to identify mtDNA polymorphisms. Coupled with this, and using prior investigations in toothfish mtDNA by P. Gaffney (unpubl), PCR products for ND2 and BCL were then subjected routinely to restriction endonuclease digestion with the enzymes *NlaIII* (New England Biolabs, 10 000U/mL) and *BstN1* (New England Biolabs, 10 000U/ml) respectively to determine haplotype differences between populations. For *NlaIII*, 10 μl of PCR product was added to 1.6 μl buffer, 1.5 μl BSA (bovine serum albumin), 1.5 μl water and 0.4 μl of the enzyme and incubated at 37^0C for 120 minutes. For *BstNI*, 10 μl of PCR product was added to 1.6 μl buffer, 1.5 μl BSA, 1.5 μl water and 0.4 μl of the enzyme and incubated at 60^0C for 120 minutes.

For 16S, partial sequence data available from GenBank (Accessions AF145410; *D. eleginoides* and Z32726; *D. mawsoni*) suggested that at least *AluI* and *TaqI* would provide species-specific restriction patterns. Smith *et al.* (2001) describe *Taq1* variation. In our case, for testing species differentiation between *D. eleginoides* and *D. mawsoni*, resulting 16S PCR products were subjected to a digest with either *TaqI or AluI* (New England Biolabs, 20 000 & 8 000 U/mL respectively). For the *AluI* digest, 8 μl of PCR product was added to 1.5 μl buffer, 5 μl water buffer and 0.5 μl of enzyme and incubated at 37^0C for 120 minutes. For the *TaqI* digest, 8 μl of PCR product was added to 1.5 μl buffer, 1.5 μl BSA, 3.6 μl water and 0.4 μl of enzyme and incubated at 65^0C for 120 minutes.

The products from each restriction digest were run separately on a 2.5% 1X TBE agarose gel (containing ethidium bromide) at 140 volts for 40-45 minutes. A 100 base pair ladder (Promega) was loaded on each gel to enable sizing of various fragments. Resulting fragments were visualised under UV light and photographed using a digital camera.

6.4. DNA microsatellite markers

Five DNA microsatellite loci previously isolated from toothfish and developed in our laboratory were used (Reilly and Ward, 1999) (Table 4). A further two unpublished loci from the laboratory of Peter Smith (Smith and Moon, unpubl.) were also used (Table 4). Oligonucleotides were synthesised by GeneWorks (Adelaide, South Australia) with one of the primer pairs end-labelled with a fluorescent tag; FAM, TET or HEX. The seven loci are designated *cmrDe2*, *cmrDe9*, *cmrDe30*, *cmrDe4*, *cmrDe13* (*cmr*=CSIRO Marine Research, *De=Dissostichus eleginoides*), *To2* and *To5* (Smith and Moon, unpubl.).

Table 4 Microsatellite motif, primer sequences, number of alleles observed and allele size of toothfish microsatellite loci. The motif listed is that obtained from the original toothfish sequence used to generate amplification primers.

Locus	Motif	Primer sequences	No.of alleles	Allele size range[a]
To5	$(CA)_n$	5'-CTCTGAAGATGAATTGGTGGATGC-3'	6	157-171
		5'-CATCATGTCACCCTGTCTTTAACG-3'		
To2	$(CA)_n$	5'-CACAGACCAGCACTACAACCCAAGG-3'	13	123-147
		5'-AAGTGTAGTAATCCAAATGCACGC-3'		
cmrDe30	$(CA)_{14}$	5'-CACTGACCTTTAACCTGCG-3'	17	161-199
		5'-CCAGCCAAAAAACCTCAC-3'		
cmrDe2	$(CA)_{29}$	5'-GAGACCTCTGACAGGGTAG-3'	21	119-159
		5'-TGACAGATGTTTTCTGATTAAG-3'		
cmrDe4	$(CAA)_8$	5'-GCCTTCCCAAACCTGAGC-3'	14	264-303
		5'-ACCCCCTCATCCCAACAC-3'		
cmrDe13	$(CAA)_7$	5'-GAGAGAAGACAGGATAAACAC-3'	8	171-192
		5'-TGGCTAAAGCCTTTTTAAC-3'		
cmrDe9	$(CA)_{32}$	5'-TGAGGAGCATCCTAATAC-3'	36	210-282
		5'-AACCAATAGAATCCAGAG-3'		

[a]sizing in base pairs

6.4.1. Microsatellite analysis

In the development phase, individual microsatellite loci were amplified separately in a sub-sample of individuals. These were then run on an ABI Prism 377 DNA sequencer (PE Applied Biosystems) for three hours to check for overlapping allele sizes and to select colours. Four loci (*cmrDe2*, *cmrDe30*, *To5* and *To2*) were optimised for use in the first multiplex reaction where all four pairs of primers were added to a single PCR reaction. The remaining loci (*cmrDe9*, *cmrDe4*, *cmrDe13*) were optimised for use in another PCR reaction. PCR amplifications were performed in a PE-Applied Biosystems 9600 thermocycler in a total volume of 25 μl. Individual amplifications consisted of 100 μM dNTP's, 10 mM Tris HCl pH

8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.12-0.32 μM for each forward and reverse primer (varies according to primer), 0.05U/μl Amplitaq Gold (Perkin Elmer) and 10 μl of 1/10 genomic DNA. After an initial cycle of $93^0C \times 10$ minutes, samples were subjected to $93^0C \times 30$ seconds, $54^0C \times 1$ minute and $72^0C \times 2$ minutes for 40 cycles with a final extension step of $72^0C \times 10$ minutes.

Amplified products were used undiluted (2 μl of PCR product from the first multiplex was added to 3 μl of product from the second multiplex) and mixed with 2 μl of formamide loading dye containing ABI Prism GeneScan 500 Tamra internal lane size standards (PE Applied Biosystems) and blue dextran loading dye. This was then denatured at $94^0C \times 2$ minutes, and immediately placed on ice. 1.3 μl of the denatured sample was stagger loaded into a 4.8% 6 M urea denaturing polyacrylamide gel and run using 1X TBE buffer on the ABI Prism 377 DNA sequencer (PE Applied Biosystems) for two and half hours.

PCR products for each locus were analysed using GENESCAN™ 3.1 collection software (PE Applied Biosystems) and local southern size calling method. Microsatellite allele sizes were determined in relation to the GeneScan350 internal standard. Genotyping at each locus was completed using GENOTYPER™ 1.1.1 software (PE Applied Biosystems) which enabled the formation of approximately two (for the dinucleotide repeats) and three (for the trinucleotide repeats) base pair bins for each locus. Bin widths thus generally corresponded to a repeat unit.

6.5. Statistical analysis

In all instances when population data were available from the various toothfish suburbs (see Table 2), these were first tested within fishing grounds for genetic homogeneity. If these groupings were not significantly different, the data were tested according to fishing grounds and then for fishing locations. If temporal collections from the various fishing grounds were available, these were also tested for genetic homogeneity.

6.5.1. MtDNA haplotypes

The genetic structuring among haplotypes was analysed by calculating the mean number of substitutions (d values) from restriction fragment site data. This was done using the D program in the REAP package (McElroy *et al*., 1992). Levels of variation within each population were estimated by calculating the unbiased haplotype diversity (*h*) and nucleotide diversity (π) (following Nei, 1987). Haplotype diversity ranges from zero (all individuals share a common haplotype) to one (all individuals have different haplotypes) and nucleotide

diversity estimates the average number of nucleotide substitutions for a pair of haplotypes randomly drawn from the population. To analyse genetic structuring among the collections, a pairwise matrix of haplotype and nucleotide diversity (Nei, 1987) was computed using the program DA from the REAP package (McElroy *et al*., 1992). For nucleotide divergence, total nucleotide diversity between two collections was estimated and the component of this diversity not explained by within-collection polymorphism was extracted. Nucleotide divergence (Nei and Tajima, 1981) among collections was also calculated, corrected for within collection variation.

To further test for genetic structure among the collections, each fragment pattern from the ND2 and BCL regions formed a composite haplotype. Both temporal and spatial variation in mtDNA composite haplotype frequencies among toothfish collections was assessed using standard Monte-Carlo chi-square approaches (Roff and Bentzen, 1989) in the program CHIRXC (Zaykin and Pudovkin, 1993), with 10 000 randomisations of the data used to estimate *P* values.

Genetic differentiation among collections was quantified by Analysis of Molecular Variance (AMOVA, Excoffier *et al.* (1992)) in the program ARLEQUIN vers 2.00 (Schneider *et al*., 2000). ϕ_{ST} (an analogue of F_{ST}) was obtained as the estimated variance component resulting from differences among collections divided by the estimated total variance (as in Michalakis and Excoffier, 1996). Equal genetic distances among haplotypes were assumed based on the presence/absence of restriction sites and significance values were calculated after 16 000 permutations. F_{ST} values for all pair-wise comparisons of collections and exact tests of differentiation were also undertaken using ARLEQUIN vers 2.00 (Schneider *et al*., 2000). Significance of values was based on 100 000 Markov chain lengths and 16 000 permutations respectively.

In all cases with multiple tests, significance levels were adjusted using a sequential Bonferroni procedure (Rice, 1987). *P* values had to be equal to or less than this adjusted value (0.05 divided by the rank of the *P* value in the multiple tests) to be deemed significant.

6.5.2. Microsatellite genotypes

Genetic diversity for each locus per collection was estimated by the number of alleles per locus and by the observed (H_{obs}) and Hardy-Weinberg expected (H_{exp}) heterozygosity. H_{obs}, H_{exp} and tests for deviations from Hardy-Weinberg Equilibrium (HWE) within samples were estimated using ARLEQUIN vers. 2.00 (Schneider *et al*., 2000). Significance levels for deviations from HWE were based on 100 000 iteration steps of a Markov chain procedure.

GENEPOP vers 3.2 (Raymond and Roussett, 2000) was used to test for linkage disequilibrium between all possible pairs of microsatellite loci. Significance of departure from all equilibrium levels was tested by a Markov chain procedure, with significance levels determined after 500 batches of 5000 iterations each.

The significance of allele frequency differences at each locus among toothfish collections (both temporal and spatial) was assessed using exact tests in GENEPOP vers. 3.2 (Raymond and Roussett, 2000). The null hypothesis of allele distributions being identical across collections was estimated with an unbiased estimate of the *P* value; significance levels were determined after 500 batches of 5000 iterations each of a Markov chain.

The computation of estimates of F-statistics (F_{IS}, F_{IT} and F_{ST}) per locus was done in GENEPOP vers. 3.2 (Raymond and Roussett, 2000). These are all types of inbreeding coefficients but differ in respect to their reference population (Hartl, 1988). F_{IS} values estimate the reduction in heterozygosity of an individual due to non-random mating within its subpopulation (Hartl, 1988). The overall inbreeding coefficient of an individual, F_{IT}, measures the reduction of heterozygosity of an individual relative to the total population. The effects of population subdivision are measured by the fixation index, F_{ST}, which is the reduction in heterozygosity of a subpopulation relative to the total population of which they are a part, due to random genetic drift (Hartl, 1988). F_{ST} values can be used to estimate overall population differentiation. F_{ST} values in the current study were estimated by a weighted analysis of variance (Cockerham, 1973; Weir and Cockerham, 1984) and multilocus estimates were computed as in Weir and Cockerham (1984). These F estimates are related by: $1- F_{IS} = (1- F_{IT})(1- F_{ST})$.

AMOVA in the program ARLEQUIN vers. 2.00 (Schneider *et al*., 2000) was also used to partition the genetic variance of toothfish collection structure. ϕ_{ST} was obtained as the estimated variance components resulting from differences among collections divided by the estimated total variance. As in the mtDNA analysis, ϕ_{ST} values were calculated assuming equal genetic distances among alleles. The significance of the variance component associated with ϕ_{ST} was tested using non-parametric permutation procedures (Excoffier *et al*., 1992) based on 16 000 re-sampling trials.

Global exact tests of collection differentiation and F_{ST} comparisons (for pairwise comparisons of collections) based on the seven microsatellite loci (loci considered jointly) were also undertaken in ARLEQUIN vers. 2.00 (Schneider *et al*., 2000) to test the hypothesis of random distribution of individuals between pairs of collections (Raymond and Rousset,

1995; Goudet *et al.*, 1996). Significance levels were based on 100 000 steps of a Markov chain procedure.

In all cases with multiple tests, significance levels were again adjusted using a sequential Bonferroni procedure (Rice, 1987).

7. RESULTS

7.1. MtDNA for species identification

The 16S ribosomal RNA fragment exhibited species-specific banding patterns for *AluI* and *TaqI* digestions. In the Patagonian toothfish, *D. eleginoides*, five major bands were produced when digested with *AluI*. In the Antarctic toothfish, *D. mawsoni*, four major bands were observed (Table 5) (Figure 2). *TaqI* also produced species-specific banding patterns (Table 5) (Figure 3) (as in Smith *et al*., 2001)

Table 5 Sizes (base pairs) of restriction fragments for the amplified 16S RNA gene of toothfish mtDNA. The *Alu1* sizes are from sequence data.

D. eleginoides		*D. mawsoni*	
AluI	*TaqI*	*AluI*	*TaqI*
	550		
			450
		238	
167		167	
132			
106			(100)
87		87	
50		50	

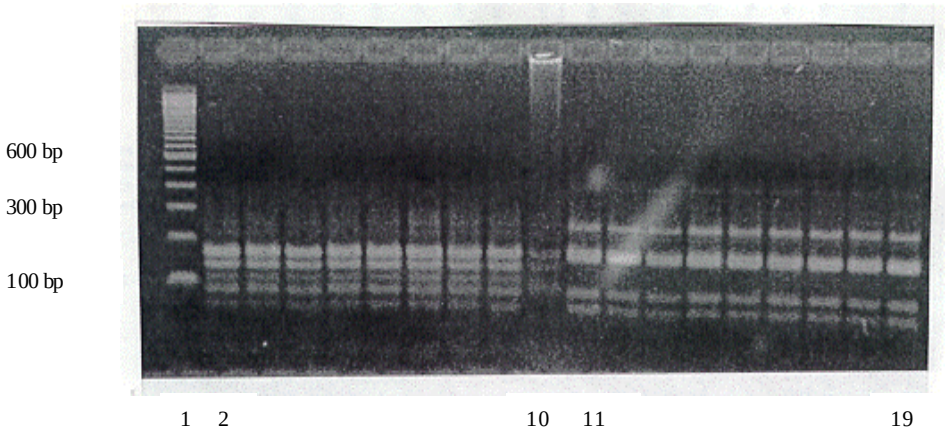

Figure 2 16S ribosomal RNA exhibited species-specific banding patterns for *AluI* digestions. Lanes 1, 100 bp marker (Promega); 2-10, *D. eleginoides*; 11-19, *D. mawsoni*. In *D. eleginoides*, five major bands were produced when digested with *AluI* and four major bands observed in *D. mawsoni*.

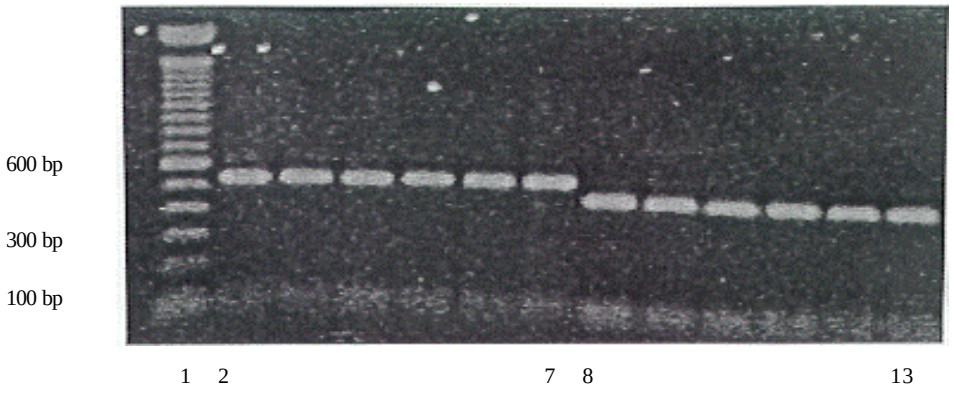

Figure 3 16S ribosomal RNA exhibited species-specific banding patterns for *TaqI* digestions. Lanes 1, 100 bp marker (Promega); 2-7, *D. eleginoides*; 8-13, *D. mawsoni*.

Amplification of the ND2 fragment and digestion with *NlaIII* produced two haplotypes in *D. eleginoides* (Table 6) (as in Smith *et al*., 20001) and a single species-specific haplotype in *D. mawsoni* (Antarctic toothfish) (bands of approximately 700 bp & 450 bp) (as in Smith *et al*., 2001).

7.2. MtDNA for sample differentiation in Patagonian toothfish

All Patagonian toothfish sampled for this study were examined for variation of the BCL and ND2 regions to determine haplotype identity. Digestion of the ND2 region produced two haplotypes; digestion of the BCL fragment with *BstNI* produced seven haplotypes (Table 6, Figures 4 & 5 respectively).

Table 6 Estimated approximate sizes (base pairs) of major restriction fragments for the amplified ND2 and BCL fragments of toothfish mtDNA. Variant restriction patterns are designated by capital letters. Fragments smaller than 100 bp not shown.

ND2	*NlaIII*			BCL	*BstNI*			
A	B	A	B	C	D	E	F	G
900							1300	
	500							950
	400		900					
200	200	600		600		600		
					500			
		340	340	340	340	340		340
		300			300			
				280				
						210		
					130			

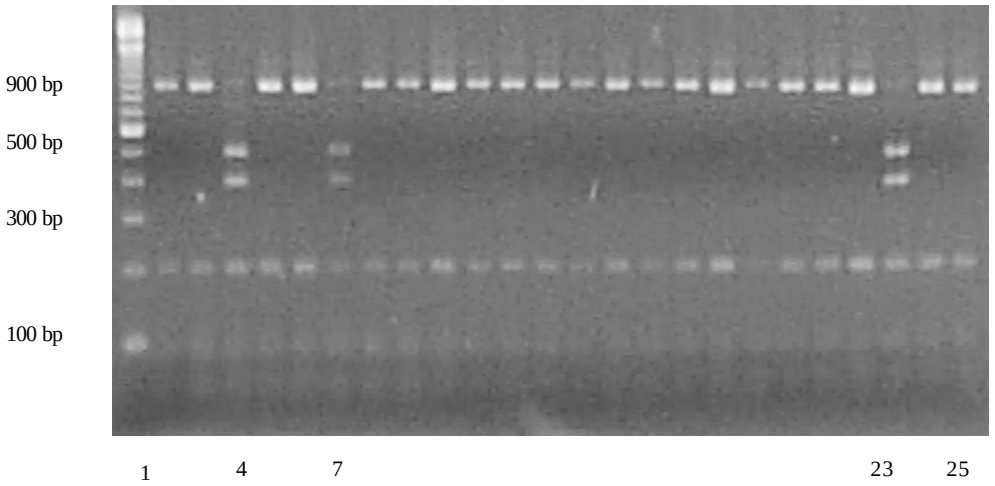

Figure 4 Haplotypes produced when the ND2 mtDNA region of *D. eleginoides* is digested with the restriction enzyme *NlaIII*. Lane 1, 100 bp marker (Promega); individuals in lanes 4, 7 & 23 display the B haplotype; individuals in all other lanes display the A haplotype.

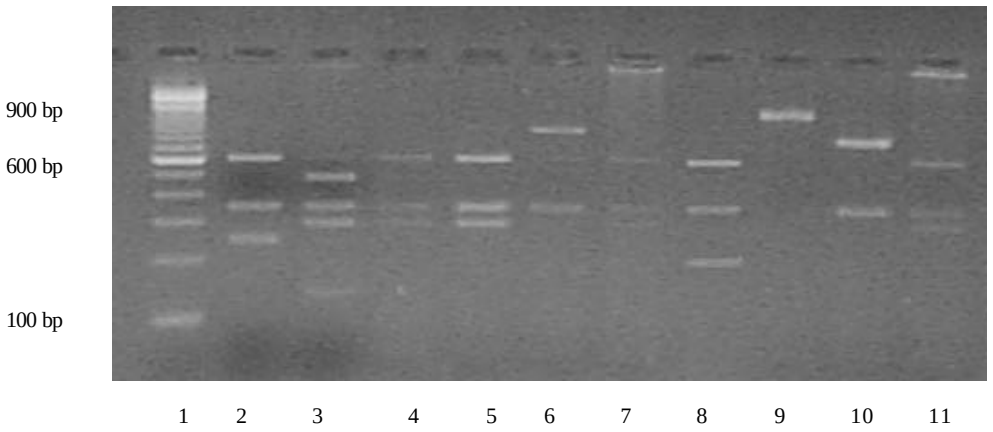

Figure 5 Haplotypes produced when the BCL mtDNA region of *D. eleginoides* is digested with the restriction enzyme *BstNI* (major bands only). Lane 1, 100 bp marker (Promega); lane 2, haplotype C; lane 3, haplotype D; lanes 4 & 5, haplotype A; lane 6, haplotype G; lane 7, haplotype A; lane 8, haplotype E; lane 9, haplotype F; lane 10, haplotype B; lane 11, haplotype A.

The majority of individuals amplified successfully for the ND2 fragment, however amplification of the BCL fragment was more difficult (possibly due to PCR inhibition from some primer-template combinations and use of the L16498 primer without the GC clamp). As the mtDNA genome is considered a single genotype that does not undergo recombination (Wilson *et al*., 1985), composite ND2 and BCL haplotypes were used for subsequent mtDNA analysis for population differentiation. Only those individuals that were scored for both ND2 and BCL haplotypes were used.

7.2.1. Suburb and size mtDNA tests for genetic homogeneity

Each of the suburbs in the various fishing grounds within years (as available), and the size specific samples from two suburbs in Macquarie Island (1999), were tested for genetic homogeneity (using Monte Carlo methods) based on composite haplotypes from the ND2 and BCL fragments (Table 7) and using 10 000 randomisations of the data.

Table 7 Homogeneity χ^2 analysis for comparisons of composite mtDNA haplotype frequencies in suburb and size-specific (small fish collections v. large fish collections) toothfish collections, *P* values are probability of H_0.

Test	Sampling	Collection (n)	Collection (n)	Collection (n)	Collection (n)	χ^2	*P*
Suburb	Macq Island 1999	AT (38)	YL (19)			8.400	0.052
	Macq. Island 1999	CV (14)	BG (63)			5.409	0.473
	HIMI 1998	SA (25)	ES (71)	HE (15)	FH (9)	15.081	0.601
	HIMI 1999	HG (25)	FH (29)			8.337	0.314
Size	Macq. AT 1999	Small (18)	Large (20)			0.062	1.000
	Macq. BG 1999	Small (39)	Large (24)			3.129	0.872

As can be seen from Table 7, there was no significant mtDNA heterogeneity evident between the various suburbs at Macquarie or HIMI, nor between fish collections categorised as either small or large from the Macquarie Island 1999 sampling. Thus the suburbs that formed the sampling events for HIMI Ground B 1998 were pooled as H-B98, and the suburbs sampled for HIMI Ground B 1999 were pooled as H-B99. Similarly, the suburb collections from Macquarie Island formed a M-B99 and M-A99 collection. The size collections from Macquarie AT 1999 were pooled as part of M-A99, and those from Macquarie BG 1999 pooled as part of M-B99.

Due to small sample sizes, individuals from the Shag Rocks and South Georgia populations were pooled together after chi-square homogeneity tests ($\chi^2 = 0.578$, $P = 1.000$) also detected no significant differences between the collections in the distribution of the composite haplotypes. This combined population was referred to as SRG-99.

After the pooling described above, a total of ten collections was formed, each being identified by the fishing location, fishing ground, and year collected: M-A97 (Macquarie, area A, 1997), M-A98 (Macquarie, area A, 1998), M-B98 (Macquarie, area B, 1998), M-A99 (Macquarie, area A, 1999), M-B99 (Macquarie, area B, 1999), H-A98 (HIMI, area A, 1998), H-B98 (HIMI, area B, 1998), H-C98 (HIMI, area C, 1998), H-B99 (HIMI, area B, 1999), SRG-99 (Shag Rocks/South Georgia, 1999).

7.2.2. Fishing ground mtDNA haplotype tests for genetic homogeneity

Eleven different composite haplotypes were identified among the 557 individuals examined. As can be seen from Table 8, there were two main composite haplotypes, AA and FA. The distribution of these two main haplotypes was clearly not homogenous among collections. FA was common in the Macquarie Island collections but rare at HIMI and Shag Rocks collections. AA was common at HIMI and BA was common at Shag Rocks.

Table 8 Haplotype frequencies at combined BCL and ND2 genes, sample sizes (n), number of haplotypes (A), haplotype diversity (*h*) and nucleotide diversity (π) for mtDNA in toothfish collections within the AFZ and CCAMLR 48.3 region.

Haplotype	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
AA	0.636	0.250	0.275	0.333	0.356	0.858	0.759	0.750	0.666	0.133
AB	-	0.036	0.025	0.013	0.036	0.058	0.190	0.125	0.129	-
BA	-	0.089	0.050	-	0.018	0.034	-	0.055	0.055	0.800
CA	-	-	-	-	-	0.008	-	-	-	-
DA	-	0.018	-	0.013	-	0.017	0.017	0.028	0.037	-
GA	-	-	0.025	0.013	0.036	0.017	0.017	0.028	0.019	-
GB	-	-	-	-	-	0.008	-	-	-	-
BB	-	-	-	-	-	-	-	-	0.019	-
EA	-	-	-	-	-	-	-	-	0.055	-
FA	0.364	0.571	0.575	0.600	0.554	-	0.017	0.014	-	0.067
FB	-	0.036	0.050	0.028	-	-	-	-	-	-
Parameter										
n	11	56	40	75	56	120	58	72	54	15
A	2	6	6	6	5	7	5	6	8	3
h	0.645	0.665	0.617	0.643	0.650	0.575	0.607	0.621	0.638	0.531
π	0.026	0.025	0.023	0.021	0.024	0.036	0.049	0.042	0.039	0.024

Based on the ten toothfish collections as outlined in Table 8, the levels of Nei's (1987) within-population haplotype diversity ranged from 0.531 to 0.665 with an average *h* of 0.619 $\pm$ 0.013. Nucleotide diversity ranged from 0.021 to 0.049 with an average π of 0.031 $\pm$ 0.003.

Corrected nucleotide divergence among the ten samples varied between –0.054% to 0.647% suggesting that population structuring was evident among the three fishing locations (HIMI, Macquarie, Shag Rocks/South Georgia).

Pairwise tests for homogeneity of composite haplotype frequency between collections (Table 9) failed to reveal significant differentiation (after Bonferroni correction) for any comparisons within fishing locations (10 such for Macquarie, 6 for HIMI), but did show significant differentiation between Macquarie and HIMI collections (18 of 20 comparisons

significant, the two non-significant comparisons both involved M-A97 which had a small sample size), between Macquarie and SRG (4 of 5 comparisons, again the exception involved M-A97), and between HIMI and SRG (4 of 4 comparisons).

Table 9 Pairwise tests for homogeneity of mtDNA composite haplotype frequencies between toothfish sampled from the three fishing locations. χ^2 values below diagonal, probabilities (estimated after 10 000 randomisations of the data) above diagonal. Significant values (after sequential Bonferroni correction) are shown in bold.

Collection	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
M-A97	-----	0.232	0.453	0.386	0.455	<0.001	<0.001	0.004	0.021	<0.001
M-B98	7.082	-----	0.851	0.503	0.419	<0.001	<0.001	<0.001	<0.001	<0.001
M-A98	5.570	3.401	-----	0.845	0.561	<0.001	<0.001	<0.001	<0.001	<0.001
M-B99	4.414	5.621	3.401	-----	0.413	<0.001	<0.001	<0.001	<0.001	<0.001
M-A99	3.396	6.309	4.249	6.309	-----	<0.001	<0.001	<0.001	<0.001	<0.001
H-B98	**45.786**	**98.702**	**91.661**	**89.332**	**83.332**	-----	0.058	0.333	0.023	<0.001
H-A98	**17.963**	**58.852**	**51.739**	**61.870**	**45.668**	12.109	-----	0.466	0.253	<0.001
H-C98	22.519	**61.375**	**55.801**	**65.148**	**50.844**	7.757	4.900	-----	0.554	<0.001
H-B99	17.625	**49.392**	**45.079**	**55.063**	**42.785**	17.213	8.891	6.291	-----	<0.001
SRG-99	**16.349**	**33.498**	**44.075**	**53.673**	**48.776**	**84.761**	**58.219**	**49.608**	**40.818**	-----

The pairwise tests (Table 9) did not reveal any significant differences between fishing grounds within fishing locations, nor between years within fishing locations. This lack of heterogeneity between collections within locations was also apparent from tests that compared multiple samples within locations simultaneously (Table 10).

Table 10 Homogeneity χ^2 analysis for comparisons of composite mtDNA haplotype frequencies in toothfish from the various fishing ground and temporal collections, *P* values are probability of H_0 calculated from 10 000 Monte Carlo runs.

Fishing Location	Collection (n)					χ^2	*P*
Macquarie	M-A97 (11)	M-A98 (40)	M-A99 (57)			9.345	0.488
Macquarie	M-A97 (11)	M-A98 (40)	M-B98 (56)	M-A99 (57)	M-B99 (73)	13.512	0.800
HIMI	H-A98 (58)	H-B98 (120)	H-C98 (73)			15.600	0.331
HIMI	H-A98 (58)	H-B98 (120)	H-C98 (73)	H-B99 (54)		37.684	0.053

If the four collections from HIMI are pooled and compared with the five-pooled collections from Macquarie, highly significant spatial differentiation in composite haplotype frequency is evident, as expected (χ^2=240.620, *P*<0.001). Additionally, an overall global investigation of collection differentiation based on haplotype frequencies resulted in significant heterogeneity across HIMI, Macquarie and Shag Rocks collections (χ^2=393.432,

P<0.001) collections. Clearly, the heterogeneity in mtDNA haplotype frequencies is a result of spatial differences between the three fishing locations in the Southern Ocean.

Pairwise F_{ST} comparisons among collections demonstrated very similar results. In all cases, except for the small M-A97 sample, HIMI collections were significantly different from Macquarie Island collections, and the SRG-99 collection was significantly different to HIMI and Macquarie (Table 11). There were no significant F_{ST} values evident from pairwise comparisons of collections from the same fishing location (i.e, between HIMI or Macquarie).

Table 11 Pairwise tests of mtDNA composite haplotype frequencies between toothfish collections. F_{ST} values below diagonal, probabilities (estimated after 100 000 randomisations of the data) above diagonal. Significant F_{ST} values (after sequential Bonferroni correction) shown in bold.

	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
M-A97	-----	0.060	0.074	0.063	0.266	0.003	0.002	0.010	0.030	<0.001
M-B98	0.089	-----	0.975	0.518	0.336	<0.001	<0.001	<0.001	<0.001	<0.001
M-A98	0.087	-0.019	-----	0.752	0.518	<0.001	<0.001	<0.001	<0.001	<0.001
M-B99	0.088	-0.005	-0.013	-----	0.542	<0.001	<0.001	<0.001	<0.001	<0.001
M-A99	0.028	0.000	-0.007	-0.007	-----	<0.001	<0.001	<0.001	<0.001	<0.001
H-B98	**0.241**	**0.480**	**0.424**	**0.484**	**0.423**	-----	0.020	0.290	0.025	<0.001
H-A98	**0.221**	**0.423**	**0.434**	**0.444**	**0.379**	0.036	-----	0.282	0.114	<0.001
H-C98	**0.153**	**0.383**	**0.393**	**0.401**	**0.333**	0.002	0.004	-----	0.601	<0.001
H-B99	0.100	**0.314**	**0.319**	**0.342**	**0.271**	0.029	0.016	-0.006	-----	<0.001
SRG-99	**0.519**	**0.385**	**0.422**	**0.471**	**0.435**	**0.671**	**0.625**	**0.557**	**0.462**	-----

A hierarchical AMOVA analysis (Table 12) revealed a high overall ϕ_{ST} value of 0.410 (*P*<0.001), which was almost entirely due to differences between the three localities, differences among collections within localities being trivial and non-significant.

Table 12 AMOVA mtDNA analysis among Macquarie, HIMI and Shag Rocks toothfish collections.

Source of Variation	d.f	Sums of Squares	Variance Components	% of Variation	*P*
Among localities	2	99.856	0.3408	40.49	<0.001
Among collections within localities	7	5.171	0.0042	0.50	0.143
Within collections	547	271.702	0.4967	59.01	
Total	556	376.729	0.8418		

Likewise, when just the HIMI and Macquarie collections were considered, significant variance components were attributed to among island fishing location differences (ϕ_{ST} =0.382, *P*=0.001).

7.3. Microsatellite loci for sample differentiation in Patagonian toothfish

The seven microsatellite loci used were a mixture of perfect (CA) and imperfect or mixed repeat motifs. Allele frequencies at the seven microsatellite loci in Patagonian toothfish are given in Appendix 3. Six (locus *To5*) to 36 (locus *cmrDe9*) alleles were detected at the seven loci. Loci *To5, cmrDe13* and *cmrDe4* (the latter two loci being the two tri-nucleotide repeat microsatellites used) produced relatively "clean" banding patterns, generally free of sub-banding or "stuttering". Locus *cmrDe30* was characterised by slight stuttering in the banding pattern while loci *cmrDe9, cmrDe2* and *To2* produced quite severe stutter bands. Dinucleotide repeats are often characterised by stuttering. Stuttering may be caused by slipped strand mis-pairing during PCR (Tautz, 1989). This laddering of bands can result in difficulties in allele scoring and while minimised using fluorescent-labelled primers and analysis on the ABI377 DNA sequencer, was still sometimes a problem in the current study.

7.3.1. Suburb microsatellite tests for genetic homogeneity

Each of the suburbs in the various 'fishing grounds' was tested for genetic spatial homogeneity (using Monte Carlo methods). These tests (Table 13) were based on genotypes at each of the seven microsatellite loci and significance of the data was tested using 10 000 randomisations of the data. No significant microsatellite heterogeneity was evident between any of the various suburbs at the seven loci (after Bonferroni correction). Samples (including those from Shag Rocks and South Georgia) were pooled into the 10 collections described in the mtDNA analyses.

Table 13 Homogeneity χ^2 analysis for comparisons of microsatellite allele frequencies in 'suburban' populations, *P* values are probability of H_0 calculated from 10 000 Monte Carlo runs.

Locus	Fishing Location	Ground	Year of Sampling	Suburb	Suburb	Suburb	Suburb	χ^2	*P*
To5	HIMI	B	1998	SA	ES	HE	FH	16.196	0.189
	HIMI	B	1999	HG	FH			7.948	0.013
	Mac. Is	B	1999	CV	BG			6.467	0.035
	Mac. Is	A	1999	AT	YL			5.188	0.080
To2	HIMI	B	1998	SA	ES	HE	FH	42.461	0.583
	HIMI	B	1999	HG	FH			9.439	0.619
	Mac. Is	B	1999	CV	BG			21.079	0.070
	Mac. Is	A	1999	AT	YL			17.711	0.142
cmrDe30	HIMI	B	1998	SA	ES	HE	FH	34.068	0.897
	HIMI	B	1999	HG	FH			11.359	0.429
	Mac. Is	B	1999	CV	BG			27.592	0.014
	Mac. Is	A	1999	AT	YL			23.378	0.296
cmrDe2	HIMI	B	1998	SA	ES	HE	FH	76.683	0.339
	HIMI	B	1999	HG	FH			25.790	0.180
	Mac. Is	B	1999	CV	BG			33.192	0.071
	Mac. Is	A	1999	AT	YL			20.646	0.234
cmrDe13	HIMI	B	1998	SA	ES	HE	FH	16.195	0.660
	HIMI	B	1999	HG	FH			7.419	0.273
	Mac. Is	B	1999	CV	BG			9.445	0.148
	Mac. Is	A	1999	AT	YL			8.957	0.109
cmrDe4	HIMI	B	1998	SA	ES	HE	FH	30.560	0.428
	HIMI	B	1999	HG	FH			15.394	0.100
	Mac. Is	B	1999	CV	BG			18.0845	0.055
	Mac. Is	A	1999	AT	YL			7.907	0.764
cmrDe9	HIMI	B	1998	SA	ES	HE	FH	50.575	0.400
	HIMI	B	1999	HG	FH			24.209	0.717
	Mac. Is	B	1999	CV	BG			46.790	0.031
	Mac. Is	A	1999	AT	YL			32.820	0.102

7.3.2. Microsatellite diversity levels

Genetic diversity statistics for the toothfish collections were estimated by the numbers of alleles per locus and observed and Hardy-Weinberg expected heterozygosity per locus and per collection (Table 14). Locus *cmrDe9* had the highest number of alleles present in all collections (mean of 24.6 alleles per collection) while locus *To5* showed the lowest number of alleles (mean of 2.4 alleles). Total numbers of alleles per locus per collection ranged from 1 to 31 (M-A97 locus *To5* to M-B98 locus *cmrDe9*) (Table 14). Allele frequencies per locus per population are tabulated in Appendix 3. Loci *cmrDe2*, *To2*, *cmrDe4* and *cmrDe9* demonstrated the highest mean observed heterozygosities across all collections (0.921, 0.837, 0.819 and 0.806 respectively) while locus *To5* had by far the lowest mean observed heterozygosity (0.028).

Table 14 Summary of microsatellite variability data per locus in each toothfish collection.

Collection		Loci						
		To5	*To2*	*cmrDe30*	*cmrDe2*	*cmrDe13*	*cmrDe4*	*cmrDe9*
M-A97	N	11	11	11	11	10	11	7
	Nallele	1	8	6	15	5	8	8
	H_{obs}	---	0.727	0.636	1.000	0.700	0.818	0.571
	H_{exp}	---	0.861	0.684	0.965	0.716	0.879	0.967
M-B98	N	60	60	60	60	55	57	54
	Nallele	2	11	14	18	7	11	31
	H_{obs}	0.033	0.833	0.683	0.933	0.581	0.772	0.852
	H_{exp}	0.049	0.867	0.752	0.937	0.691	0.879	0.951
M-A98	N	56	56	56	56	54	56	51
	Nallele	2	12	15	19	8	12	30
	H_{obs}	0.036	0.788	0.750	0.893	0.593	0.875	0.941
	H_{exp}	0.053	0.813	0.827	0.946	0.691	0.886	0.955
M-B99	N	79	79	79	79	74	79	74
	Nallele	2	12	11	19	7	11	30
	H_{obs}	0.013	0.861	0.722	0.861	0.500*	0.759	0.824
	H_{exp}	0.025	0.854	0.757	0.934	0.660	0.876	0.958
M-A99	N	60	60	60	60	56	59	53
	Nallele	2	12	15	17	6	12	25
	H_{obs}	0.033	0.817	0.800	0.850	0.589*	0.763	0.850
	H_{exp}	0.082	0.824	0.761	0.941	0.670	0.864	0.951
H-B98	N	122	106	122	112	82	111	41
	Nallele	4	13	14	20	8	11	25
	H_{obs}	0.041	0.830	0.746	0.929	0.476	0.892	0.683*
	H_{exp}	0.049	0.837	0.678	0.927	0.531	0.845	0.944
H-A98	N	58	58	58	58	52	58	58
	Nallele	3	12	10	18	6	11	24
	H_{obs}	0.034	0.931	0.707	0.931	0.615	0.844	0.759*
	H_{exp}	0.085	0.859	0.752	0.936	0.683	0.879	0.964
H-C98	N	73	72	73	72	47	72	36
	Nallele	3	12	12	18	6	10	24
	H_{obs}	0.027	0.875	0.726	0.958	0.553	0.778	0.833
	H_{exp}	0.051	0.838	0.716	0.935	0.584	0.853	0.945
H-B99	N	56	55	56	54	46	53	40
	Nallele	2	11	12	19	7	11	28
	H_{obs}	0.018	0.855	0.714	0.963	0.500	0.887	0.825*
	H_{exp}	0.036	0.851	0.660	0.937	0.619	0.868	0.964
SRG-99	N	48	48	47	47	28	45	25
	Nallele	3	12	13	19	6	13	21
	H_{obs}	0.042	0.854	0.787	0.893	0.535	0.800	0.920
	H_{exp}	0.062	0.880	0.837	0.935	0.635	0.869	0.949
Mean[b]	N	62.3	60.5	69.3	60.9	50.4	60.1	43.9
	Nallele	2.4	11.5	12.2	18.2	6.6	11.0	24.6
	H_{obs}	0.028	0.837	0.727	0.921	0.564	0.819	0.806
	H_{exp}	0.049	0.848	0.748	0.939	0.648	0.870	0.955

N=total number of fish, Nallele=number of alleles
H_{obs}=observed heterozygosity, H_{exp}= expected heterozygosity under Hardy-Weinberg expectations (Nei, 1978)
*significant deviation from Hardy-Weinberg equilibrium after sequential Bonferroni correction per locus

Nei's (1978) unbiased estimate of expected heterozygosity per locus (under Hardy-Weinberg expectations) for each of the collections ranged from 0.025 (M-B99, *To5*) to 0.967 (M-A97, *cmrDe9*) (Table 14). Genotype proportions in each collection for each locus were tested for goodness-of-fit to Hardy-Weinberg expectations (Table 14). There were ten tests of goodness-of-fit for each locus except *To5*, which had nine. Using sequential Bonferroni

correction per locus, locus *cmrDe13* in M-B99 and M-A99, and locus *cmrDe9* in H-A98 and H-B99 demonstrated significant deviations from expectations, in each case with heterozygote deficiencies.

Overall, there was also evidence of a heterozygote deficit (χ^2=18.983, df=1, P<0.01). One possible cause of heterozygote deficiencies is the presence of null or non-amplifying alleles. Estimating gene frequencies using the EM algorithm of Dempster *et al*. (1977), essentially a test for null alleles, showed that null alleles might exist for *cmrDe13* (in M-B99 and M-A99) and *cmrDe9* (in H-B98 and H-A98). Estimated null allele frequencies were around 0.10 in these cases. However, we have no proof that such alleles do exist in our toothfish samples – the Hardy-Weinberg deviations could arise from other causes – and we have not considered null alleles in the following analyses. As only five Hardy-Weinberg tests were significant, in general it was considered that the collections accorded well with Hardy-Weinberg expectations.

Linkage tests between pairs of microsatellite loci demonstrated no significant linkage disequilibrium (after Bonferroni correction) in any collection except between loci *cmrDe2* and *cmrDe9* in M-A99. As this association was not observed in any other collection, and the level of observed linkage across all collections was only 0.51%, it was concluded that the seven loci were not linked and that they represented independent genetic markers.

7.3.3. Temporal and spatial microsatellite homogeneity across collections

Exact tests of heterogeneity of allele frequencies at the seven loci in the ten toothfish collections were undertaken. There was no evidence for temporal heterogeneity of allele frequencies at any locus among the collections (after correction for multiple tests, data not shown).

An overall exact test of collection differentiation (Markov chain, significance determined after 100 000 steps), based on allele frequencies at all loci considered together, showed no significant allele frequency differences among the Southern Ocean collections (P=1.000). Additionally, no significant pairwise comparisons of collections (exact tests, all loci are considered together) were obtained (all with P=1.000).

In accordance with this, F_{ST} values of individual loci indicated very low levels of genetic differentiation over different geographic locations (Table 15). F_{ST} values per locus ranged from essentially 0 to 0.6% with an average of only 0.03%. However, despite this very low level differentiation, exact tests indicated five loci showed significant allele frequency differences among locations: *To2*, *cmrDe30*, *cmrDe2*, *cmrDe4* and *cmrDe9* (Table 15).

Table 15 F statistics at seven microsatellite loci in ten toothfish collections. F_{ST} significant values (after sequential Bonferroni correction) based on exact tests of allelic differentiation shown in bold.

Locus	No. of fish	F_{IS}[a]	F_{IT}[a]	F_{ST}[a]	*P*
To5	623	0.166	0.165	-0.001	0.168
To2	605	0.000	0.006	0.005	<**0.001**
cmrDe30	693	0.000	0.006	0.006	**0.002**
cmrDe2	609	0.022	0.023	0.000	**0.015**
cmrDe13	504	0.138	0.142	0.005	0.026
cmrDe4	601	0.049	0.055	0.005	<**0.001**
cmrDe9	439	0.135	0.137	0.003	**0.002**

[a]F statistics are estimated as in Weir and Cockerham (1984)

Exact tests of heterogeneity considering microsatellite loci individually further revealed some instances of significant (all $P<0.001$) spatial differentiation of pairwise collection comparisons after Bonferroni correction (*To2*: H-B98 & M-B99; *cmrDe30*: H-B98 & M-A98, H-B99 & M-A98; *cmrDe2*: H-B98 & M-B99; *cmrDe13*: H-B98 & M-A99; *cmrDe4*: H-B98 & M-B98, H-B98 & M-A98, H-C98 & M-B98, H-C98 & M-A98). The differences were not consistent across loci or between the fishing ground collections. However, it is interesting to note that these nine significant results (out of 315 tests) were all between fishing localities; none were comparisons within localities.

Pairwise comparisons among the ten collections based on F_{ST} values (loci considered jointly, data not shown) produced just two significant ($P=0.05$) comparisons: M-B98 & M-A99 ($F_{ST}=0.0084$) and M-A98 & M-A99 ($F_{ST}=0.007$), but neither remained significant after correction for multiple tests.

An overall hierarchical AMOVA test (Table 16), of all loci in all collections, showed that ϕ_{ST} within the ten collections was non-significant (-0.021, $P = 1.000$), among the three localities was a non-significant –0.009 ($P = 0.785$), and among collections within localities was a similarly non-significant –0.012 ($P = 1.000$).

Table 16 AMOVA microsatellite analysis among Macquarie, HIMI and Shag Rocks toothfish collections.

Source of Variation	d.f	Sums of Squares	Variance Components	% of Variation	*P*
Among localities	2	-14.882	-0.01825	-0.89	0.785
Among collections within localities	7	-6.468	-0.02475	-1.20	1.000
Within collections	1242	2604.528	2.09704	102.09	
Total	1251	2583.177	2.05405		

8. DISCUSSION

8.1. Patagonian toothfish differentiation in the Southern Ocean

In the current study, we used genetic variation in two mtDNA gene fragments and in seven microsatellite loci to investigate the population structure of toothfish, primarily from the HIMI and Macquarie Islands AFZ as well as small samples from Shag Rocks/South Georgia (SRG). Both temporal and spatial collections of toothfish from HIMI and Macquarie fishing locations were examined, along with size-separated samples at Macquarie.

RFLP analyses of the ND2 and BCL fragment of the mtDNA genome in toothfish from the Southern Ocean revealed moderate levels of variation within the region; 11 composite haplotypes were detected. Significant heterogeneity between HIMI, Macquarie and SRG fishing localities was detected, with about 40% of all variation ascribable to differences between these three locations (F_{ST}=0.405, P<0.001). No significant temporal, spatial or fish-size differences were observed among collections from within the same fishing location.

High levels of microsatellite variation were detected in toothfish collections with up to 36 alleles detected at locus *cmrDe9*. A few deviations from Hardy-Weinberg expectations were observed at two loci with some evidence of a small heterozygote deficit. These deficits might reflect the presence of null alleles, or might reflect band-scoring difficulties of loci that produce stutter bands. No linkage disequilibrium was detected between pairs of microsatellite loci in each of the collections, and it was considered that the microsatellite loci represented independent markers.

Over all loci, no significant microsatellite heterogeneity between HIMI, Macquarie and SRG fishing localities was detected (F_{ST}=-0.009, P=0.785). When loci were considered separately, evidence of small but significant allelic differentiation among collections was found for most loci. These instances of small but significant differences were all between collections from different fishing localities, but these differences disappeared in the overall microsatellite analysis. There was no significant differentiation between collections from the same fishing locality. This more extensive data analysis does therefore not support preliminary results on microsatellite loci that found evidence of small differences between different fishing sites at Macquarie Island (Reilly *et al*., 1998). Sample sizes were much larger in the current study and temporal data from the same fishing ground were also available.

The mtDNA evidence, therefore, strongly rejects the null hypothesis of a single panmictic toothfish stock in Southern Ocean, while in contrast, the microsatellite evidence does not clearly reject this hypothesis.

Smith and McVeagh (2000) used the same suite of microsatellite loci plus one more (*To3*, but, like *To2*, this locus is almost invariant) on 30 to 50 fish from each of Macquarie Island, Heard Island, Prince Edward Island and the Falkland Islands. Small but significant overall differentiation was detected for most loci and the overall F_{ST} was likewise small but significant (F_{ST}=0.028, P<0.001). Microsatellites indicated genetic patchiness with regional differentiation rather than a relationship between genetic diversity and geographic separation of the samples. Our microsatellite data suggest rather less differentiation. While five of the seven loci did show small but significant allelic differentiation, our mean F_{ST} across the ten collections (estimated from the F_{ST} values of the seven individual loci considered individually) was only 0.003 and the overall ϕ_{ST} (estimated from a pooled analysis of all loci) was a negative –0.021. However, we did observe some scattered instances of significant inter-locality differences for particular loci. Our total samples were appreciably larger than Smith and McVeagh's (our sample sizes per locus ranged from 439 to 623, Smith and McVeagh's from 196 to 230). In Smith and McVeagh's analysis, the Falkland Island sample showed the most differences; this was the only sample north of the Antarctic Convergence. Smith and McVeagh (2000) also assessed allozyme variation. These showed little variation within samples and less genetic differentiation between samples (F_{ST}=0.019, P=0.08) than their microsatellite data.

Combining these various genetic data sets, the overall picture appears to be one of large and striking mtDNA differentiation within the Southern Ocean, but little nuclear DNA differentiation.

This difference in results from mtDNA and nuclear markers has also been observed in bigeye tuna. Chow *et al*. (2000), Alvarado-Bremer *et al*. (1998) and Grewe *et al*. (2000) demonstrated significant mtDNA differentiation between bigeye tuna found in the Atlantic Ocean and those in the Indo-Pacific Oceans, while microsatellites showed no inter-ocean differentiation (Grewe *et al*., 2000).

Differentiation observed in (maternally inherited) mtDNA haplotypes but not in (biparentally inherited) nuclear DNA markers such as microsatellites may reflect females returning to their place of origin for reproduction. This could be an explanation of our data. However, this explanation of the contrast between our mtDNA and nDNA data requires that females are strongly more philopatric than males, and we are not aware of any data that

suggests this might be so. There have been a very few recorded instances of long-distance dispersal of Patagonian toothfish (see later); unfortunately, the sexes of these fish are unknown (R. Williams, unpubl.). However, sex biased dispersal in marine mammals has been used to explain significant mtDNA heterogeneity observed between ocean basins (Palumbi and Baker, 1994; Berbube *et al*., 1998; Lyrholm *et al*., 1999; Rosel *et al*., 1999).

Another, and perhaps more likely explanation, is that of population bottlenecks. MtDNA is more sensitive to genetic drift and population bottlenecks than nuclear DNA, as it has an effective population size only one quarter that of nuclear DNA due to its haploid nature and maternal-only inheritance (Wilson *et al*., 1985). If the toothfish populations in the various fishing grounds had ever been reduced to small numbers of individuals (or indeed had been founded from small numbers of individuals), then genetic drift would have accentuated the mtDNA differences more than the nuclear DNA differences.

Whatever the explanation for the high levels of mtDNA differentiation, there must be a severe restriction on gene flow between toothfish populations at Macquarie, HIMI and Shag Rocks/South Georgia.

Information about movement of individual fish can be gained from tagging studies. These have been carried out for both Macquarie and HIMI fish. At Macquarie Island, from December 1995 to January 2001, a total of 5 191 fish were tagged and released in the Aurora/Caroline Trough, the Northern Valleys, and some other locations (R. Williams, unpubl.). 537 recaptures were made, all in the grounds at which they were released except for one moving from Colgate Valley to Aurora Trough, and one moving from Aurora Trough to Colgate Valley. Apart from these two fish, which had moved 35 to 40 n miles, fish were recaptured within 15 n miles of their release position. At HIMI, most fishing occurs at three well-defined grounds on the slopes of the Heard Island Plateau (grounds A, B and C). From May 1998 to October 2001, a total of 5 064 fish were tagged and released at HIMI, mostly on those three grounds (Williams *et al.*, 2001). 719 were recaptured, all but five within 15 miles of the release points. Two fish had moved about 30 miles, between ground B and a nearby shallow plateau. No fish moved between the three fishing grounds (which are separated by a minimum of about 120 miles). Three fish made long distance movements. One was recaptured at Kerguelen (c. 210 nautical miles), and two at Crozet (c. 1 030 nautical miles); these three fish had been at liberty for three to three and a half years.

According to the General Bathymetric Chart of the Oceans (GEBCO, 1997), the shelf area between HIMI and Kerguelen Island is nowhere deeper than 750 m. Such depths would not inhibit toothfish movements in this area as the fish are known to travel to 2 500 m depth.

Between the Kerguelen-Heard Plateau and Crozet there is a trough that reaches over 4 500m depth and is at least 390 nautical miles wide between the 2 000 m isobaths; the Crozet recaptures would have had to have traversed this trough. No Macquarie tagged fish have been recovered at HIMI, or vice-versa. These island groups are separated by about 3 000 miles.

The tagging data show that generally toothfish do not move more than about 15 miles from their tagging point, even after several years, and very rarely move between grounds. Adult toothfish are largely demersal, and clearly not highly migratory (Gon and Heemstra, 1990). It had been thought that deep water basins might prevent toothfish mixing between fishing locations; however, the two HIMI tagged fish recaptured at Crozet show that under some circumstances toothfish are (if rarely) capable of wide movement across deep ocean waters.

In the case of HIMI, toothfish appear to settle on the shallow plateau (<500 m deep) and move into the fishing grounds on the peripheral slopes when about four years old (Constable *et al*., 2001). Spawning grounds are unknown (Evseenko *et al*., 1995), but are thought to be in deep water (~1 500 m). The pelagic eggs may remain in the water column for up to three months (Kellerman, 1990; Evseenko *et al*., 1995) but the duration of the pelagic phase of toothfish larvae is unknown. Larvae maybe transported in the ocean currents but may be geographically restricted to local grounds if they become trapped in local gyres (Orsi *et al*., 1995).

How does this information on dispersal abilities correlate with the genetic data? The genetic data indicate that fishing grounds at particular fishing locations (such as Macquarie Island, or HIMI) are genetically homogeneous, at least with the sample sizes used. Earlier suggestions of possible genetic heterogeneity at Macquarie Island (Reilly and Ward, 1999), based on small sample sizes, could not be confirmed in this larger-scale study. Thus at present the null hypothesis of genetic homogeneity or panmixia within locations cannot be rejected. How is this apparent homogeneity maintained? Adult fish rarely move between grounds within locations, but a (very) few such movements have been recorded. These adult movements may themselves be just sufficient to maintain the genetic homogeneity, but more likely the homogeneity is maintained by gene flow in younger stages, such as pelagic egg and larval drift. The striking genetic heterogeneity observed for mtDNA between locations suggests that gene flow between locations is likely to be very low. This is in accord with the tagging information that failed to indicate any adult fish movements between Macquarie and

HIMI (about 3000 miles). The genetic data also indicate that any pelagic egg or larval drift between Macquarie and HIMI (and Shag Rocks/South Georgia) is likely to be minimal.

It would of course be very interesting to examine toothfish from Kerguelen and Crozet Islands in order to determine the extent of genetic differentiation between HIMI and these sites, given the occasional migrant shown by tagging. Note though, that the presence of a migrant fish at a distant location does not necessarily mean that that migrant will contribute to the gene pool at that location; it might be in too poor a condition. Such genetic studies, especially of mtDNA, are planned, and should help to resolve further gene flow issues among toothfish stocks within the western Indian Ocean sector.

Recent studies on the population structure of toothfish using otolith characteristics have also indicated the likelihood of several discrete populations of toothfish being present within the Southern Ocean. Otolith shape and chemistry were used to discriminate among putative stocks of toothfish in the Southern Ocean from fisheries around Chile, Falkland Islands, Heard and McDonald Islands, Kerguelen Island, Macquarie Island, Prince Edward Islands and South Georgia. Chemical data from the otolith cores suggested at least four stocks of toothfish in the Southern Ocean – a South American group, Falkland Islands group, Macquarie Island group and an Indian Ocean group comprising toothfish from Prince Edward, Kerguelen and Heard,McDonald Islands (Kalish and Timmiss, 2000). Kalish and Timmiss (2000) suggest that at least four distinct spawning areas or nursery grounds for Patagonian toothfish are found within the Southern Ocean and dispersal is very limited between these areas. Our findings are consistent with this conclusion.

8.2. Resultant management response based on outputs

The results of this work confirm the intuitively reasonable belief that stocks of Patagonian toothfish at Macquarie Island and Heard and McDonald Islands are genetically separate and should continue to be considered as separate stocks for management purposes. This work removes any doubt that was present as a result of the possibility that populations are at least to some extent maintained by influx of pelagic larvae and juveniles from sites 'upstream' from their adult location (in this case larvae and juveniles from Heard Island settling at Macquarie Island).

The confirmation that there are no significant genetic differences between fishing grounds at the same island group (location) has somewhat different implications at each location. At Heard Island, this finding supports current management practice of considering the population as one stock and setting TAC by estimating the number of recruits (at age 4) over the whole plateau and projecting these numbers forward to estimate the spawning stock

biomass. It is assumed that each fishing ground acquires recruits from the same population pool on the plateau, so that once the TAC is set, it can be taken at any of the grounds. At Macquarie Island the situation is less clear. Early results suggested that the fish from the two main fishing grounds could be genetically distant (Reilly and Ward, 1999), which agreed with observations that the behaviour and trends of the populations differed at the two grounds. The current findings with a larger dataset that there are no significant genetic differences indicates that we are likely to be dealing with a single genetic population but does not clarify why the fisheries have behaved in such different ways in the two fishing grounds at Macquarie Island. In the case of Macquarie Island therefore, the conservative approach of managing the two grounds as separate entities will remain at least until the behaviour of the two fisheries at these grounds is better understood.

The recent discovery of three tagged fish moving from Heard Island to Kerguelen or Crozet Islands highlights the need to extend this genetic work to other sub-Antarctic island groups in the Indian Ocean. If such work reveals that the fish on some or all of these islands are not genetically different, then the possibility of managing toothfish stocks across national boundaries and isolated submarine features will have to be considered.

8.3. Studies from other Antarctic species also suggest reduced gene flow in the Southern Ocean

Other studies of Antarctic species have also indicated strong barriers to gene flow within the Southern Ocean. From allozyme studies, Antarctic octopus (*Pareledone turqueti*) populations around South Georgia were found to be panmictic but gene flow between South Georgia and Shag Rocks was very limited (Allcock *et al*., 1997). It was suggested that tracts of deep ocean present major physical barriers to octopus movement which results in reduced gene flow between some populations.

Population genetic structure of krill (*Euphausia superba*) was investigated using sequence analysis of the ND1 mtDNA fragment in four population samples from around the Antarctic continent (Zane *et al.*, 1998). Significant differentiation between samples was detected with oceanographic barriers (such as the Weddell gyre and or the Weddell-Scotia Confluence) suggested as restricting gene flow between the Weddell Sea and South Georgia (Zane *et al*., 1998). Results from 35 allozyme loci studied in *E. superba* (from the Atlantic sector of the Southern Ocean and the western coast of the Antarctic Peninsula in the Pacific Ocean sector) suggested that the samples were from quite separate populations (Fevolden and Ayala, 1981). Gyres were present at numerous localities and the authors suggested that these

act to restrict gene flow resulting in geographically isolated populations (Fevolden and Ayala, 1981).

Reduced gene flow between samples of Antarctic squid has also been observed. Four samples of squid, *Martialia hyadesi* from the Patagonian shelf and Antarctic Polar Frontal zone (over 1 000km apart) were investigated using allozyme electrophoresis. One of four samples showed fixed allele differences at 16 loci, indicating the existence of a cryptic species, but the other three samples showed small but significant allele frequency differentiation. It was concluded that the species fails to maintain effective panmixia across its geographical range (Brierley *et al.*, 1993).

MtDNA intraspecific variation in two other notothenioid taxa, *Pleuragramma antarcticum* and *Chionodraco hamatus* confirm that despite the potential for dispersal, gene flow might be reduced between populations of these species (in Bargelloni *et al.*, 2000).

A final example comes from the Patagonian toothfish's sibling species, the Antarctic toothfish, *D. mawsoni*. Twenty-one fish were sampled from each of two sites about 3 000 n miles apart, and assessed for nuclear DNA variation using the RAPD approach (randomly amplified polymorphic DNA) (Parker *et al.*, 2002). Substantial differentiation was recorded, with the F_{ST} value being a high 0.297 ($P<0.001$). This is somewhat similar to our mtDNA value for F_{ST} in the Patagonian toothfish, and much higher than F_{ST} values recorded for Patagonian toothfish nuclear DNA by ourselves (effectively zero), or Smith and McVeagh (2000) (0.02 - 0.03). The high differentiation in Antarctic toothfish was ascribed to the likelihood of currents and especially gyres retaining fish in bays; possible site fidelity to natal coastal grounds was also speculated.

More generally, White (1998) and Eastman and McCune (2000) state that despite the potential for extensive dispersal because of the opportunities for distribution of pelagic life stages on currents, endemism in Antarctic fish species is high. Each peri-Antarctic island group has a discrete species composition and therefore constraints to dispersal must prevail (White, 1998). The peri-Antarctic islands are separated by deep water basins and great distances and are influenced by the circum-continental West Wind drift. White (1998) suggests that gene flow among Antarctic populations is likely to be restricted by geographical isolation, dependence on shelf habitats, low fecundity, annual spawning and potential losses of pelagic eggs.

8.4. Patagonian and Antarctic toothfish identification

The current study has also demonstrated the utility of mtDNA for successful species identification of Patagonian and Antarctic toothfish (as in Smith *et al*., 2001 with the additional use of *AluI*). Although not outlined here, microsatellite profiles at the seven loci were also different between *D. eleginoides* and *D. mawsoni*. The mtDNA tests, based on PCR methodologies, enable the two species to be identified successfully from only a small amount of tissue. This is an important development for the toothfish fishery more widely as these tests can be used to help discourage the trade in IUU Patagonian toothfish.

8.5. Use of different genetic techniques

Stock structure in the current study was assessed using both nuclear and mitochondrial DNA markers. The variability of these markers differed markedly with 11 composite mtDNA haplotypes observed in the conserved mtDNA fragment and from 6 to 36 alleles at microsatellite loci. The issue of the number of alleles observed at a locus is important. Ferguson and Danzmann (1998) suggest that genetic marker systems such as microsatellites which are characterised by large numbers of alleles may not be suitable for detecting significant differences between genetically similar populations, at least with the sample sizes typically employed in such studies. Three of the seven microsatellite loci in toothfish have more than 15 alleles segregating, with some very low allelic frequencies. The large number of alleles at these microsatellite loci suggests that a larger sample size (>200) may be needed to confirm or identify any small but significant levels of genetic differentiation. While a high proportion of microsatellite loci screened in fish are polymorphic (O'Reilly and Wright, 1995; Nielsen *et al*., 1997; Bagley *et al*., 1999; Takagi *et al*., 1999), loci with only a few alleles tend to be more suitable for population studies while those with greater numbers of alleles are best suited for parentage and linkage studies (Carvalho and Hauser, 1994; O'Reilly and Wright, 1995). Additionally, the concern with microsatellites is that mutation rate may be so high (Goldstein *et al*., 1995; Slatkin, 1995; Shaw *et al*., 1999) that population differences brought about by restricted gene flow are obscured.

This study revealed that there is major reproductive isolation between collections in the ocean sectors of the Southern Oceans. MtDNA shows striking differentiation between the three fishing areas. The microsatellite analyses revealed however very little evidence of collection differentiation within the Southern Ocean. As discussed earlier, this apparent discrepancy might be related to the greater sensitivity of mtDNA to genetic drift than nuclear DNA, or possibly to biased sex dispersal. It has sometimes been proposed that the higher

mutation rates of microsatellites (and other nuclear non-coding markers) than mtDNA markers can result in increased powers of microsatellites for testing population differentiation (Rousset and Raymond, 1995; Goudet *et al.*, 1996); however, we found this not to be the case in the current study.

What is abundantly clear from this and studies of other species, is that it is far better to combine the use of mitochondrial and nuclear DNA analyses than rely on any one type of analysis alone. In studies of the population structure of some species, nuclear DNA analysis has proven more useful; in our study, without mtDNA analyses, the striking genetic differentiation between the toothfish fishing grounds would not have been evident.

9. BENEFITS

The major achievements of this research are:-

- a number of Southern Ocean toothfish collections were examined with both mitochondrial DNA and nuclear DNA markers, and fishing location differences were observed. Based on mtDNA haplotype analyses, Macquarie, HIMI and Shag Rocks toothfish are significantly different from each other. There was no evidence of localised genetic differentiation within Macquarie, HIMI or Shag Rocks/South Georgia fishing locations.
- microsatellites showed only small (but significant) differences among collections at different fishing locations.
- results confirm the reasonable belief that stocks of toothfish at Macquarie and HIMI are genetically separate, and should continue to be considered as separate stocks for management purposes.
- on a global scale, toothfish appear to be divided into at least 3 genetically well-separated stocks (at least based on the samples studied herein); one within the Pacific Ocean sector, the Indian Ocean sector and the Atlantic Ocean sector of the Southern Ocean.
- routine genetic tests for toothfish species differentiation based on mtDNA RFLP tests have been optimised.

MtDNA analyses demonstrated very significant differentiation among the three fishing localities examined, indicating very restricted gene flow among these regions. MtDNA was a more powerful indicator of population structure than microsatellite DNA. This may be because mtDNA has a smaller effective population size (being haploid and only maternally inherited) than nuclear DNA, and is therefore more sensitive to population bottleneck and drift effects. The routine use for population studies of a combination of genetic analysis tools is recommended, particularly combining data from both nuclear and mitochondrial genomes. While microsatellites provide the researcher with highly variable loci, the large numbers of alleles commonly observed at these loci may limit the ability to detect small but perhaps significant differences between genetically similar collections.

These data can now be used to refine conservation and management plans for these areas. Uncertainty regarding toothfish population structure has until now seriously restricted the ability of fisheries managers to make confident statements about the future sustainability. In addition, the combining of genetic data with catch statistics and morphological measurements will enhance the power of stock structure investigations.

10. FURTHER DEVELOPMENT

Seminars based on this work have been presented (by SA) at a public meeting of Sub-Antarctic Management Advisory Committee (SouthMac) (Hobart, 20th November 2001) and as part of the CSIRO Marine Research seminar series (Hobart, 23rd November 2001). The findings of this study were also presented at the Working Group on Fish Stock Assessment of CCAMLR XX (Appleyard *et al*., 2001).

Copies of this report will be forwarded to the AFMA (SouthMac/SAFAG), FRDC, CCAMLR and the industry contributor Austral Fisheries. A scientific paper from this work will also be submitted to an appropriate international journal.

While the majority of benefits of this study will be applicable to the Commonwealth fisheries at Macquarie Island and Heard and McDonald Islands, the finding of significant genetic heterogeneity among toothfish locations will also be of interest to the member nations of CCAMLR and managers of toothfish fisheries more globally.

The research indicates the high probability of different toothfish stocks throughout the Southern Ocean. Further research into toothfish stock structure particularly within the Indian Ocean sector of the Southern Ocean is highly recommended. Several CCAMLR member nations (Australia, South Africa, France) have toothfish fisheries within this sector and each is currently managed under a conservative separate TAC. Further investigation into toothfish stock structure should help to resolve gene flow issues among toothfish stocks within this sector. If there are separate stocks within this sector, the continuance of separate TACs would be justified as depletion of one area is unlikely to be quickly replaced by immigration from another area. Future genetic analysis within this sector must include mtDNA analysis.

Additionally, toothfish managers more globally are interested in the development of a quicker species identification test and a Patagonian toothfish location test for on-ground testing at inspection points. This could be coupled with the current Catch Documentation System (CDS) employed by CCAMLR to help reduce IUU fishing ("Catch Documentation Scheme for *Dissostichus* spp. (as contained in Conservation Measure 170/XIX) became binding upon all CCAMLR Members on 7 May 2000. The Scheme is designed to track the landings and trade flows of toothfish caught in the Convention Area and, where possible, adjacent waters. This will enable the Commission to identify the origin of toothfish entering the markets of all Parties to the Scheme, and help determine whether toothfish taken in the Convention Area were caught in a manner consistent with CCAMLR's conservation Measures" (CCAMLR web site, 9th November 2001)).

11. PLANNED OUTCOMES

The genetic data outputs from this project, coupled with an examination of tagging data, have achieved the primary desired outcome of a better understanding and knowledge of toothfish stock structure in the Australian fishing waters of the Southern Ocean. Toothfish populations around the fishing locations at Macquarie Island and at Heard and McDonald Islands have been identified as distinct and separable from each other. No temporal or spatial genetic heterogeneity within these two fishing locations was observed.

This knowledge will assist AFMA in determining the management boundaries for toothfish within the AFZ (no fish were studied from Williams Ridge and therefore we can not ascertain if fish from Williams Ridge are part of a straddling stock extending into the Australian Exclusive Economic Zone). Careful management of the various toothfish fisheries within the AFZ (and indeed more globally) is required, as depletion of one fishing location is unlikely to be quickly replaced by immigration from another area.

The project results will also contribute to the more effective and sustainable management of commercial fisheries for Patagonian toothfish more globally, as results indicated the presence of several genetically differentiated toothfish populations from fishing locations across the Southern Ocean.

12. CONCLUSION

The objectives of this project were:-

1. To use microsatellite variation to resolve the connectivity of different spatial and temporal toothfish samples collected from Macquarie Island and Heard and McDonald Islands.
2. To compare genetic and tagging data from Macquarie and Heard and McDonald Islands regions to maximise toothfish stock structure knowledge in these regions.
3. To report on the outputs from the research and the resultant management response.

These objectives were met.

MtDNA and microsatellite loci were used to investigate the population structure of Patagonian toothfish at two Australian fishing locations (Macquarie Island, five collections; Heard and McDonald Islands (CCAMLR area 58.5.2), four collections) in the Southern Ocean. Small samples of toothfish from the Shag Rocks/South Georgia fishing location (CCAMLR area 48.3) were also examined. Striking mtDNA heterogeneity was detected among the three fishing locations; spatial and temporal collections within the same fishing location were not significantly different. There was weak and inconsistent heterogeneity at several microsatellite loci among the ten collections, but no overall microsatellite differentiation among the three fishing locations. The mtDNA heterogeneity indicates that gene flow between the two Australian fishing locations and more generally among the three locations within the Southern Ocean is restricted, and that stocks from these areas should be considered as independent units.

The findings support current management practise of considering the Heard and McDonald fish as a single stock. At Macquarie Island, the new genetic data do not support earlier suggestions from more limited data of genetic differences between the two major fishing grounds; rather, the hypothesis of a single Macquarie stock could not be rejected. However, the conservative approach of managing the two grounds as separate entities will remain at least until the behaviour of these two fisheries is better understood. Finally, the current study also demonstrated the utility of mtDNA for successful species identification of Patagonian and Antarctic toothfish.

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APPENDIX 1

INTELLECTUAL PROPERTY

The intellectual data arising from this research are:

- Genotype, haplotype and allele frequency data.
- Copyright in this report.

APPENDIX 2

STAFF

Dr Robert Ward	CSOF7	principal investigator, genetics supervisor, report preparation
Dr Sharon Appleyard	CSOF3	genetic typing and statistical analysis of all mtDNA and microsatellite data, report preparation, collation and writing
Mr Dick Williams	SRS	biological and tagging data of toothfish

APPENDIX 3

ALLELE FREQUENCIES AT SEVEN MICROSATELLITE LOCI IN PATAGONIAN TOOTHFISH COLLECTIONS FROM THE SOUTHERN OCEAN

To5

Allele	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
157	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.007	0.000	0.010
159	0.000	0.000	0.000	0.000	0.033	0.004	0.026	0.000	0.009	0.010
161	1.000	0.983	0.982	0.994	0.967	0.980	0.966	0.986	0.991	0.979
165	0.000	0.008	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000
167	0.000	0.008	0.018	0.000	0.000	0.008	0.000	0.007	0.000	0.000
171	0.000	0.000	0.000	0.006	0.000	0.000	0.000	0.000	0.000	0.000
6 alleles	22	120	112	158	120	244	116	146	112	96

To2

Allele	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
123	0.136	0.058	0.080	0.120	0.100	0.024	0.052	0.063	0.027	0.083
125	0.227	0.142	0.116	0.089	0.092	0.038	0.026	0.049	0.027	0.063
127	0.045	0.025	0.045	0.082	0.033	0.038	0.043	0.063	0.045	0.104
129	0.091	0.092	0.036	0.103	0.117	0.090	0.078	0.104	0.127	0.115
131	0.318	0.200	0.348	0.291	0.358	0.325	0.276	0.319	0.273	0.250
133	0.091	0.217	0.205	0.158	0.108	0.151	0.147	0.153	0.200	0.125
135	0.045	0.100	0.071	0.070	0.083	0.104	0.138	0.035	0.091	0.073
137	0.000	0.067	0.036	0.019	0.017	0.085	0.129	0.132	0.100	0.083
139	0.000	0.075	0.036	0.044	0.058	0.090	0.043	0.056	0.045	0.052
141	0.000	0.000	0.009	0.013	0.008	0.028	0.026	0.014	0.036	0.021
143	0.045	0.017	0.000	0.006	0.000	0.014	0.026	0.007	0.000	0.000
145	0.000	0.008	0.009	0.000	0.017	0.005	0.000	0.000	0.000	0.010
147	0.000	0.000	0.009	0.006	0.008	0.009	0.017	0.007	0.027	0.021
16 alleles	22	120	112	158	120	212	116	144	110	96

cmrDe30

Allele	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
161	0.182	0.033	0.036	0.051	0.142	0.111	0.121	0.116	0.125	0.096
165	0.000	0.050	0.063	0.089	0.058	0.016	0.017	0.048	0.036	0.032
167	0.000	0.008	0.009	0.013	0.008	0.004	0.009	0.000	0.009	0.011
169	0.045	0.042	0.036	0.057	0.058	0.037	0.034	0.027	0.036	0.085
171	0.091	0.050	0.054	0.051	0.008	0.061	0.095	0.021	0.045	0.117
173	0.545	0.458	0.357	0.468	0.450	0.541	0.466	0.507	0.517	0.351
175	0.045	0.167	0.152	0.095	0.083	0.102	0.103	0.062	0.071	0.074
177	0.091	0.075	0.098	0.070	0.083	0.053	0.086	0.082	0.063	0.106
179	0.000	0.025	0.089	0.032	0.025	0.016	0.026	0.048	0.009	0.032
181	0.000	0.025	0.009	0.032	0.008	0.025	0.000	0.034	0.018	0.032
183	0.000	0.008	0.000	0.000	0.008	0.004	0.000	0.007	0.000	0.011
185	0.000	0.008	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.011
187	0.000	0.008	0.009	0.000	0.008	0.000	0.000	0.000	0.000	0.000
193	0.000	0.000	0.018	0.000	0.008	0.004	0.000	0.000	0.000	0.000
195	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.009	0.000
197	0.000	0.000	0.009	0.000	0.000	0.004	0.000	0.014	0.000	0.000
199	0.000	0.042	0.045	0.044	0.042	0.020	0.043	0.034	0.009	0.032
17 alleles	22	120	112	158	120	244	116	146	112	94

cmrDe2

Allele	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
119	0.091	0.075	0.071	0.095	0.058	0.071	0.052	0.090	0.056	0.138
121	0.045	0.017	0.027	0.082	0.100	0.040	0.043	0.028	0.028	0.021
123	0.000	0.092	0.045	0.013	0.075	0.103	0.017	0.104	0.046	0.074
125	0.091	0.058	0.089	0.044	0.075	0.054	0.069	0.076	0.065	0.085
127	0.136	0.075	0.071	0.044	0.067	0.063	0.103	0.090	0.037	0.011
129	0.045	0.058	0.054	0.070	0.067	0.116	0.086	0.111	0.111	0.096
131	0.091	0.033	0.071	0.051	0.058	0.036	0.078	0.049	0.093	0.043
133	0.091	0.100	0.054	0.076	0.100	0.094	0.069	0.069	0.102	0.074
135	0.045	0.058	0.071	0.063	0.042	0.103	0.121	0.063	0.093	0.106
137	0.045	0.100	0.054	0.101	0.092	0.076	0.078	0.063	0.056	0.074
139	0.045	0.092	0.098	0.120	0.067	0.067	0.052	0.049	0.083	0.064
141	0.091	0.050	0.089	0.063	0.050	0.085	0.086	0.063	0.065	0.053
143	0.000	0.075	0.045	0.051	0.017	0.027	0.000	0.035	0.028	0.021
145	0.000	0.033	0.054	0.051	0.058	0.022	0.052	0.000	0.065	0.021
147	0.045	0.025	0.036	0.032	0.033	0.009	0.043	0.035	0.019	0.043
149	0.000	0.017	0.027	0.006	0.033	0.009	0.009	0.028	0.000	0.011
151	0.045	0.000	0.009	0.025	0.008	0.013	0.026	0.014	0.009	0.011
153	0.045	0.025	0.018	0.006	0.000	0.004	0.009	0.000	0.019	0.021
155	0.045	0.000	0.018	0.000	0.000	0.004	0.000	0.000	0.009	0.032
157	0.000	0.017	0.000	0.000	0.000	0.004	0.000	0.028	0.019	0.000
159	0.000	0.000	0.000	0.006	0.000	0.000	0.009	0.007	0.000	0.000
21 alleles	22	120	112	158	120	224	116	144	108	94

cmrDe13

Allele	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
171	0.050	0.018	0.028	0.034	0.063	0.018	0.029	0.021	0.022	0.000
174	0.150	0.073	0.028	0.122	0.161	0.043	0.135	0.074	0.054	0.089
177	0.550	0.464	0.491	0.541	0.509	0.652	0.490	0.617	0.543	0.536
180	0.200	0.309	0.241	0.160	0.152	0.213	0.240	0.213	0.293	0.304
183	0.000	0.073	0.083	0.061	0.071	0.018	0.038	0.053	0.033	0.018
186	0.050	0.055	0.083	0.041	0.045	0.037	0.067	0.021	0.043	0.036
189	0.000	0.009	0.019	0.007	0.000	0.012	0.000	0.000	0.011	0.000
192	0.000	0.000	0.028	0.000	0.000	0.006	0.000	0.000	0.000	0.018
8 alleles	20	110	108	148	112	164	104	94	92	56

cmrDe4

Allele	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
264	0.045	0.000	0.000	0.025	0.017	0.023	0.000	0.014	0.009	0.000
267	0.000	0.008	0.018	0.044	0.034	0.050	0.017	0.042	0.047	0.056
270	0.136	0.079	0.107	0.177	0.178	0.221	0.172	0.243	0.217	0.267
273	0.091	0.096	0.089	0.120	0.178	0.234	0.121	0.174	0.151	0.144
276	0.318	0.167	0.152	0.177	0.194	0.149	0.121	0.132	0.179	0.078
279	0.091	0.193	0.179	0.185	0.161	0.126	0.190	0.146	0.104	0.122
282	0.136	0.158	0.134	0.089	0.085	0.113	0.155	0.132	0.132	0.156
285	0.136	0.114	0.089	0.076	0.068	0.045	0.095	0.063	0.066	0.044
288	0.000	0.087	0.125	0.070	0.042	0.018	0.052	0.021	0.047	0.044
291	0.045	0.053	0.071	0.019	0.017	0.009	0.043	0.035	0.038	0.044
294	0.000	0.042	0.018	0.000	0.008	0.014	0.009	0.000	0.009	0.011
297	0.000	0.000	0.009	0.013	0.000	0.000	0.026	0.000	0.000	0.011
300	0.000	0.018	0.009	0.000	0.017	0.000	0.000	0.000	0.000	0.0111
303	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.0111
14 alleles	22	114	112	158	118	222	116	144	106	90

cmrDe9

Allele	M-A97	M-B98	M-A98	M-B99	M-A99	H-B98	H-A98	H-C98	H-B99	SRG-99
210	0.071	0.009	0.010	0.020	0.019	0.000	0.026	0.028	0.025	0.000
212	0.000	0.009	0.000	0.020	0.000	0.012	0.009	0.000	0.000	0.000
214	0.000	0.185	0.010	0.014	0.000	0.000	0.000	0.028	0.088	0.020
216	0.000	0.009	0.020	0.014	0.000	0.024	0.017	0.042	0.025	0.000
218	0.000	0.185	0.010	0.014	0.000	0.049	0.026	0.014	0.013	0.020
220	0.000	0.000	0.000	0.014	0.057	0.037	0.009	0.028	0.075	0.020
222	0.000	0.028	0.000	0.027	0.038	0.049	0.043	0.042	0.013	0.020
224	0.143	0.019	0.049	0.054	0.047	0.110	0.060	0.083	0.050	0.040
226	0.071	0.111	0.020	0.074	0.094	0.037	0.026	0.069	0.063	0.140
228	0.143	0.130	0.078	0.061	0.113	0.134	0.060	0.181	0.075	0.060
230	0.214	0.037	0.049	0.074	0.028	0.098	0.060	0.014	0.075	0.060
232	0.000	0.065	0.108	0.088	0.057	0.049	0.034	0.042	0.062	0.100
234	0.000	0.046	0.108	0.034	0.028	0.146	0.069	0.083	0.050	0.080
236	0.000	0.074	0.029	0.044	0.057	0.012	0.094	0.014	0.050	0.060
238	0.000	0.046	0.010	0.021	0.028	0.012	0.052	0.069	0.050	0.080
240	0.000	0.009	0.039	0.088	0.113	0.012	0.060	0.028	0.025	0.040
242	0.143	0.056	0.039	0.061	0.028	0.012	0.052	0.028	0.038	0.080
244	0.000	0.065	0.059	0.034	0.038	0.024	0.034	0.000	0.013	0.000
246	0.000	0.185	0.010	0.027	0.019	0.024	0.026	0.000	0.025	0.000
248	0.000	0.009	0.029	0.020	0.019	0.012	0.017	0.000	0.013	0.040
250	0.071	0.028	0.029	0.027	0.028	0.024	0.017	0.014	0.038	0.020
252	0.143	0.019	0.020	0.007	0.000	0.024	0.009	0.028	0.000	0.020
254	0.000	0.037	0.020	0.027	0.028	0.012	0.026	0.042	0.000	0.000
256	0.000	0.009	0.020	0.020	0.019	0.037	0.026	0.000	0.013	0.000
258	0.000	0.009	0.020	0.027	0.038	0.024	0.086	0.000	0.038	0.040
260	0.000	0.028	0.020	0.034	0.028	0.000	0.043	0.014	0.013	0.000
262	0.000	0.019	0.029	0.020	0.038	0.000	0.026	0.014	0.025	0.020
264	0.000	0.019	0.069	0.000	0.009	0.000	0.009	0.028	0.013	0.000
266	0.000	0.019	0.020	0.000	0.009	0.012	0.017	0.042	0.013	0.000
268	0.000	0.000	0.029	0.007	0.000	0.000	0.017	0.028	0.013	0.020
270	0.000	0.009	0.010	0.020	0.000	0.000	0.009	0.000	0.013	0.000
272	0.000	0.000	0.020	0.000	0.000	0.000	0.000	0.000	0.000	0.000
274	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.000	0.000	0.000
276	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
278	0.000	0.019	0.000	0.000	0.019	0.012	0.009	0.000	0.000	0.000
282	0.000	0.009	0.020	0.000	0.000	0.000	0.009	0.000	0.000	0.020
36 alleles	14	108	102	148	106	82	116	72	80	80

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