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Population subdivision in the Antarctic toothfish (*Dissostichus mawsoni*) revealed by mitochondrial and nuclear single nucleotide polymorphisms (SNPs)

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Abstract: The Antarctic toothfish (*Dissostichus mawsoni*) exhibits a circumpolar distribution in coastal waters south of the Antarctic Polar Front. For a preliminary evaluation of global population structure in this species, we examined four mitochondrial regions and 13 nuclear gene fragments in samples from four CCAMLR Subareas in the Southern Ocean (Australian Antarctic Territory (Subarea 58.4.2), Ross Dependency (Subareas 88.1 and 88.2) and the South Shetland Islands (Subarea 48.1). Significant genetic differentiation within and among locations was observed for both mitochondrial and nuclear loci. The single nucleotide polymorphism (SNP) markers developed here will be useful for more extensive analyses of population structure in this species.

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Key words: dCAPS, FP-TDI, population genetics, RFLP

Introduction

The Antarctic toothfish, *Dissostichus mawsoni* (Norman, 1937), is a mesopelagic predator and endemic to the seas around Antarctica with a circumpolar distribution. It is usually caught on the bottom at depths of 88–1600 m in water temperatures of -1.8°C to 2.3°C (DeWitt *et al.* 1990). In the spring at McMurdo Sound, the Antarctic toothfish resides under heavy ice in the bottom 200 m of the water column and its blood is well fortified with antifreezes to withstand subzero temperatures (Eastman 1993). The species is reported to live over 30 years and can attain total lengths of up to 175 cm and weigh up to 80 kg (though specimens found in the stomachs of sperm whales indicate larger specimens may exist) (Yukhov 1971, DeWitt *et al.* 1990). The maximum size recorded for *D. mawsoni* in McMurdo Sound is *c.* 2 m in total length (TL) and 110 kg. On average, *D. mawsoni* is six times longer and 250 times heavier than other notothenioids in McMurdo Sound (Eastman 1993).

Tag returns suggest that long-distance movements of adult *D. mawsoni* are limited, although data are sparse due to low fishing levels outside of the Ross Sea and the short history of the commercial fishery (Smith & Gaffney 2005). A mark and recapture study in McMurdo Sound revealed that 13 out of *c.* 5000 animals tagged were recaptured after a period of one to seven years (DeVries unpublished). These animals grew an average of 2 cm yr^{-1} (Eastman 1993). Two tagging studies conducted in 2001–03 showed a recapture rate of 1.5% (30 out of 2000), with the greatest distance travelled being 73 km over a period of 695 days (Hanchet *et al.* 2001, Sullivan *et al.* 2003).

Information on reproduction in *D. mawsoni* is likewise limited. Otolith studies of the McMurdo Sound population indicate that *D. mawsoni* become sexually mature around eight years of age and total lengths of more than 95 cm (Burchett *et al.* 1984, Horn *et al.* 2003, La Mesa 2007). Everson (1984) and Yukhov (1982) suggested that *D. mawsoni* spawn in the middle to late winter/early spring. Spawning adult females have never been captured; however, Yukhov (1971) inferred that mature eggs measure 4.0–4.3 mm and are pelagic. Juvenile *D. mawsoni* up to 12 cm in length live in surface waters near the Antarctic continent and feed on adult euphausiids and larval fish (Pakhomov & Pankratov 1992, Eastman & DeVries 2000). Upon reaching total lengths of 18 cm, *D. mawsoni* assume a benthopelagic existence and descend in the water column to depths of 300–500 m (Eastman & DeVries 2000).

Antarctic toothfish remain in the coastal waters for several years until they attain sexual maturity. At this time, they migrate north to oceanic waters between the shelf break and the Antarctic Polar Front (Eastman & DeVries 2000). *Dissostichus mawsoni* make these migrations early in the summer and occupy depths between 300–500 m, with a preferred depth of 500 m (Eastman & DeVries 2000). It has been speculated that Antarctic toothfish return to coastal waters for spawning several times in their lifespan, though they may not spawn every year (Yukhov 1982, Eastman & DeVries 2000). Analysis of morphological and reproductive data from collections in CCAMLR Subarea 88.1 by Fenaughty (2006) suggested a spawning migration from the main Ross shelf to the Pacific–Antarctic Ridge to the north.

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Table I. Details of Antarctic toothfish (*Dissostichus mawsoni*) collections.

Collection code	Location (CCAMLR Subareas)	Latitude/ Longitude	Collection date(s)	Sample size
AAT1	Australian Antarctic Territory (58.4.2)	66.80°S 65°E	26–28 Jan 2001	9
AAT2	Australian Antarctic Territory (58.4.2)	67.20°S 44°E	12–13 Feb 2001	37
RD88.1A	Ross Dependency (88.1)	77°S 179°E	24–28 Jan 2001	22
RD88.1B	Ross Dependency (88.1)	73°S 171°E	11 Feb 2001	5
RD88.1C	Ross Dependency (88.1)	71°S 176°E	19 Feb 2001	10
RD88.1D	Ross Dependency (88.1)	70°S 179°E	21 Feb 2001	11
RD88.2	Ross Dependency (88.2, Set 17)	68°S 122°W	11 Jan 2005	25
RD88.2	Ross Dependency (88.2, Set 25)	68°S 123°W	15 Jan 2005	25
SS	South Shetland Islands (48.1)	N/A	2001	48
Total				192

N/A = data not available.

Recent genetic studies using both nuclear DNA and mitochondrial DNA single nucleotide polymorphisms (SNPs) of the Patagonian toothfish (*Dissostichus eleginoides* Smitt, 1898), the sister species to *D. mawsoni*, have demonstrated significant differentiation both within and among the three oceanic sectors (Kuhn 2007). Appleyard *et al.* (2002) demonstrated highly significant differences between three fishing areas (Shag Rocks / South Georgia, Heard and McDonald islands and Macquarie Island) in mtDNA restriction fragment length polymorphism (RFLP) data, but no differentiation in frequencies of seven microsatellite loci (F_{ST} = -0.009, $P = 0.785$) for Patagonian toothfish. Rogers *et al.* (2006) documented differentiation of toothfish populations from the Falkland Islands from those around South Georgia and Bouvet Island (mtDNA $F_{ST} > 0.906$, $P < 0.01$, microsatellite $F_{ST} = 0.014–0.034$, $P < 0.05$), which may be explained by hydrogeographic isolation resulting from two deep ocean fronts. These genetic studies suggest that a number of factors may limit gene flow, including large geographic distances between toothfish habitats, deep water barriers affecting adult movements, and the Polar Front affecting larval transport (Shaw *et al.* 2004).

Previous genetic work by Smith & Gaffney (2005) on the Antarctic toothfish observed limited mitochondrial sequence diversity but significant differentiation in haplotype frequencies between three fishing areas, while RFLP analysis of four nuclear loci showed no geographic differentiation. Using randomly amplified polymorphic DNA (RAPD), Parker *et al.* (2002) found significant differentiation ($F_{ST} = 0.297 \pm 0.082$, $P < 0.0001$) between McMurdo Sound and Antarctic Peninsula populations.

This study aims to expand the work of Smith & Gaffney (2005) by examining nuclear and mitochondrial single nucleotide polymorphisms (SNPs) in *D. mawsoni*. SNPs are the most prevalent form of genetic variation (Brumfield *et al.* 2003, Seddon *et al.* 2005), providing a virtually unlimited number of loci for genetic studies (Kuhn & Gaffney 2006). The simpler mutational dynamics of SNPs leads to the advantage of a lowered rate of homoplasy, with a capacity for rapid, large-scale and cost-effective genotyping (Seddon *et al.* 2005, Kuhn & Gaffney 2006). As a result, SNPs are becoming markers of choice for large-scale genotyping surveys, including fisheries applications (e.g. Elfstrom *et al.* 2006). A combination of both mitochondrial DNA (mtDNA) and nuclear DNA analyses can provide a powerful approach to population genetic structure, as these genomes have unique characteristics and different modes of inheritance (Avise 2004).

Materials and methods

Sampling of D. mawsoni

Muscle tissue samples were collected from a total of 192 Antarctic toothfish from nine collections from four CCAMLR Subareas (Table I): Australian Antarctic Territory (Subarea 58.4.1, abbreviated AAT), South Shetland Islands (Subarea 48.1, SS) and the Ross Dependency (Subareas 88.1 and 88.2, RD88.1 and RD88.2). Because the two RD88.2 collections were taken from nearby stations within a week of each other, genetic data were pooled after evaluating statistical homogeneity. Three of these Subareas (AAT, SS and RD88.1) were previously examined by Smith & Gaffney (2005). Tissue samples were stored in 95% ethanol prior to DNA extraction. DNA was extracted either using the DNeasy Tissue Kit (Qiagen Inc, Valencia, CA) or by using the Promega Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to manufacturer's protocols.

Marker development and DNA sequencing

Polymerase chain reaction (PCR) was used to amplify four mitochondrial regions (tRNA-THR/tRNA-PRO/control region (CR), cytochrome *b* (cyt *b*), 16S rRNA (16S) and NADH dehydrogenase subunit 2 (ND2)) and 14 nuclear gene fragments (bloodthirsty (*bty*), calmodulin (CaM), dystrophin 6a (Dyst6a), mixed lineage leukaemia-like protein (MLL), L-lactate dehydrogenase A introns 1 and 5 (LDHA1, LDHA5), ribosomal protein S7 (RPS7), triose phosphate isomerase (TPI), myoglobin (Mb), glucose-6-phosphate dehydrogenase (G6PD), rhodopsin (Rhod),

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Table II. *Dissostichus mawsoni* PCR primers. T_a = annealing temperature (°C).

Target region	Primer (5' → 3')	T_a	Source
Mitochondrial genes			
16S	CGCCTGTTTATCAAAAACAT CCGGTCTGAACTCAGATCACGT	52	Palumbi *et al.* 1991
cyt *b*	CGTTTTACCCTGGGGTCAAATGT GGGGAAGAAGATGAGGAAAAT	50	Smith & Gaffney 2005
tRNA/Control Region	ACATGAATTGGAGGAATACCAGT CCTGAAGTAGGAACCAGATG	55	Ishikawa *et al.* 2001 Meyer *et al.* 1990
ND2	AAGCTATCGGGCCCATACCC CCGCTTAGYGCTTTGAAGGC	50	Park *et al.* 1993
Nuclear genes			
bty	TATAAATGTCCCGAGTCAGTGTCC GGCCTGGCTTCTCTCAACAGACTC	55	Kuhn 2007
CaM	CTGACCATGATGGCCAGAAA GTTAGCTTCTCCCCCAGGTT	58	Chow 1998
CK7	CCCAAGTTYGAGGAGATCCTGAC CAGTCGGTCRGCRTTGGAGATGTC	55	Quattro & Jones 1999
Dyst6a	CAGCARACNAACAGTGAGAAGAT GCTGGAGAAGTTAACCACYTTGAC	55	Smith & Gaffney 2005
G6PD	GAGCAGACGTATTTTGTGGG GCCAGGTAGAAGAGGCGGTT	50	Chow & Nakadate 2004
LDHA1	GCAGGAACAAGGTGACGGTGGT GATCCGTGCTGCAGGTCCATGAC	50	Kuhn 2007
LDHA5	GCTATTCACAAAGAGGTGGTTGA GCCCTGGACCAGTGTTGACA	55	Kuhn & Gaffney 2006
Mb	GAGCACCCAGAAACCYTRAAGT CCCAGCTCTTTGTAGTCGG	55	Kuhn 2007
MLL	GACGAAGAACAGCAACAGCAA CTGGCCGATATGCTGCTT	55	Smith & Gaffney 2005
Rhod	CNTATGAATAYCCTCAGTACTACC CCRCAGCACARCGTGGTGATCATG	50	Kuhn 2007
RPS7	AACTCGTCTGGCTTTTCGCC TGGCCTCTTCCTTGGCCGTC	55	Chow & Hazama 1998
SEC61	CGCCACCGTCTTCGTGTTTG TTCGCTCTGCTCCTTCACAA	55	Kuhn 2007
TMO-4C4	CCTCCGGCCTTCCTAAAACCTCTC CATCGTGCTCCTGGGTGACAAAGT	55	Streelman & Karl 1997
TPI	GCATYGGGGAGAAGCTRGAT AGAACCACYTTRCTCCAGTC	50	Quattro, 2001

SEC61 (SEC61), titin-like protein (TMO-4C4) and creatine kinase intron 7 (CK7)) using primers listed in Table II. Primers for *bty*, LDHA1, Mb, Rhod, and SEC61 were designed from published GenBank sequences (AY454308, AF170027, AF547166, AY141302 and AY113841, respectively). Reactions contained 2.4 µl 25 mM MgCl (Sigma, St. Louis, MO), 3 µl 10x PCR buffer (500 mM KCl + 100 mM Tris-HCl, pH = 8.0), 0.6 µl 10 mM dNTP mix, 0.6 µl 10 mM forward primer, 0.6 µl 10 mM reverse primer, 0.3 µl JumpStart *Taq* DNA Polymerase (Sigma, St. Louis, MO) and 1 µl DNA template. PCR reactions were carried out in a RoboCycler 96 Gradient Cycler (Stratagene, La Jolla, CA) and consisted of: an initial denaturation of 2 min at 94°C, followed by 35–45 cycles of denaturing at 94°C for 45 sec, annealing at 50–62°C for 1 min, and extending at 72°C for 1 min, with a final extension of 5 min at 72°C.

PCR products were run on 2% agarose gels to check the success of amplification. PCR products were then purified for sequencing using AMPure® beads according to manufacturer's protocol (Agencourt Bioscience, Beverly, MA). Cycle sequencing reactions in both directions were run in a PTC-100 programmable thermocycler (MJ Research, Inc, Waltham, MA) under the following conditions: 50 cycles of 94°C for 15 sec, 48°C for 20 sec, and 60°C for 4 min using the ABI BigDye® Terminator v3.1 Cycle Sequencing Kit following the manufacturers instructions (Applied Biosystems Inc, Foster City, CA). Sequencing reactions were prepped for sequencing using an ethanol/EDTA/sodium acetate precipitation protocol (Applied Biosystems Inc., Foster City, CA). Sequencing reactions were read using a SpectruMedix SCE2410 capillary sequencer (SpectruMedix, State College, PA). Sequences were edited and aligned using the program SeqManII (DNASTAR Inc, Madison, WI). Varying numbers of PCR products were sequenced for SNP discovery (n = 4 to 53 for nuclear amplicons; n = 32 to 68 for mitochondrial amplicons).

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Table III. *Dissostichus mawsoni* nuclear DNA polymorphisms. Major SNPs scored in boldface. GenBank Accession numbers in parentheses. Singleton substitutions marked with a superscript. +/- = insertion/deletion polymorphism. Nucleotides in coding sequence are indicated by (c).

LDHA (EF088425)		RPS7 (AY517753)		MLL (EF088421)		TPI (EF088419)		G6PD (EF088427)		Rhod (EF088423)	
Position	SNP	Position	SNP	Position	SNP	Position	SNP	Position	SNP	Position	SNP
250	C^S/T	171	A/T	82	A/G	95...96	+/-	130	A/C	243 (c)	A/T
400	G/T	287	A/G	**95**	A/G	**127**	A/G	186...205	+/-		
870	G/T	343	A/C	311..313	+/-	135	G/T^S	222...227	+/-		
1159	G/T	**374**	G/T			170	G/T^S	224	A^S/T		
1224	A/G							260	G/T		
1254	A/C							355	C/T		
1259...1267	+/-							356	A^S/G		
1260	A/C							452	G/T^S		
1270	C/T							485	A/C^S		
1272	G/T							488	C^S/G		
1283	C/T							499	G/T		
1330	C/T^S							515...519	+/-		
1439	C/T							1085	A/C		
1454	G/T							1197	A/T		
1541	G/T							1311	A/G		
1740...1745	+/-							1363	C/T^S		
1758	A/T							1395 (c)	C/T		
1796	A^S/G										
1903	C/G										
1908	C/T										
2068 (c)	G/T										

SEC61 (EF088426)		Mb (EF088422)		CK7 (EF088419)		CaM (EF088418)		TMO-4C4 (EF088424)		Dyst6a (EF535029)	
Position	SNP	Position	SNP	Position	SNP	Position	SNP	Position	SNP	Position	SNP
33	A/G	120 (c)	A/G	85	C^S/T	141	C/T	138 (c)	C^S/T	**78**	A/T
44	C/T	164 (c)	C/T^S	**136**	G/T	173	C/T	317 (c)	A/C^S	158	C/G
196 (c)	C/T	210	G/T	220	C/T	**203**	A/C			229	A/C
315	A/C^S	**276**	G/T	**417**	C/G	241	C/T^S			414	C/T
489	A/C^S	280	C^S/T	434	A^S/T	272	A/G			416...420	+/-
493	A/G^S	**301**	A/C							985	A/T
538	A/C									1063...1067	+/-
585	A/C									1068	C/T
1245	A/T									1070	A/G
										1072	A/T
										1074	G/T
										1273	C^S/T

For two mitochondrial amplicons, overlapping sequences were assembled to yield a combined cytochrome *b*/tRNA-Thr/tRNA-Pro/control region amplicon. The most common haplotype was considered the reference sequence and submitted to GenBank (Accession number EF088420). For nuclear amplicons, reference sequences were obtained by direct sequencing of amplicons from homozygous individuals and deposited in GenBank: (EF535029 (Dyst6a), EF088418 (CaM), EF088421 (MLL), EF088423 (Rhod), EF088427 (G6PD), EF088419 (CK7), EF088417 (TPI), EF088426 (SEC61), EF088422 (Mb), EF088424 (TMO-4C4) and EF088425 (LDHA)). All sequences were examined for potentially informative SNPs, which were scored either by RFLP analysis, dCAPS or FP-TDI (see below).

SNP discovery and assay validation

In total, 14 nuclear amplicons were sequenced for SNP discovery, of which three were not used for population surveys due to low polymorphism or technical artifacts. Amplicons from 21 individuals sequenced in both directions for the titin-like protein gene (TMO-4C4) showed only two singleton mutations (Table III). Four individuals sequenced for L-lactate dehydrogenase A intron 1 showed two single nucleotide polymorphisms. The bloodthirsty (*bty*) amplicon was sequenced in both directions (613 bp) for 18 *D. mawsoni* individuals and was found to contain numerous sites at which all individuals were heterozygous. Additional sequencing of this amplicon from other notothenioid species (six *Dissostichus eleginoides* individuals, two *Notothenia coriiceps* Richardson, 1844, four *N. rossii* Richardson, 1844 and seven *Champsocephalus gunnari* Lönnberg, 1905) revealed the same pattern, suggesting the existence of a co-amplified duplicate *bty* locus in notothenioids.

The remaining 11 nuclear amplicons yielded high quality sequencing data, allowing for development of SNP scoring assays. Nine SNPs were initially scored by the FP-TDI

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Table IV. Comparison of SNP genotype calls obtained by FP-TDI (shown in parentheses) with genotypes obtained by direct sequencing, RFLP or dCAPS.

Technique(s)	SNP	Homozygote 1	Heterozygote	Homozygote 2
Sequencing and RFLP	Dyst6a 78	1 AA		2 TT
Sequencing and RFLP	CK7 417		7 CG	5 GG
Sequencing and RFLP	Mb 276	1 GG	6 GT	20 TT
Sequencing and RFLP	Mb 301	2 AA	6 AC	19 CC
Sequencing and RFLP	LDHA 1908	2 CC	7 CT	17 TT
Sequencing and RFLP	MLL 95	5 AA	10 AG	7 GG
Sequencing and dCAPS	CaM 203	1 AA	4 AC (6)	7 CC (5)
Sequencing	TPI 127		1 AG	9 GG
Sequencing	RPS7 374	5 GG	5 GT	1 TT
Sequencing	CK7 136	1 GG	9 GT	2 TT
Sequencing	LDHA 1224	14 AA (10)	8 AG (12)	4 GG
Sequencing	SEC61 44	1 CC	4 CT (8)	25 TT (21)
Sequencing	Rhod 243		3 AT (23)	27 TT (7)
Sequencing	G6PD 130	39 AA (31)	3 AC (11)	7 CC
Sequencing	G6PD 260	42 GG (37)	4 GT (9)	3 TT

method described below (LDHA:1224, SEC61:44, G6PD:130, G6PD:260, RPS7:374, TPI:127, CK7:136, CaM:203 and Rhod:243). FP-TDI analyses clustered individuals corresponding to putative genotypes. However, for six of the nine SNP loci scored, there were discrepancies between FP-TDI genotype calls and direct sequencing base calls (Table IV). Ultimately, we scored three SNPs using FP-TDI (CK7:136, RPS7:374 and TPI:127), one (CaM:203) by dCAPS and six (CK7:417, Dyst6a:78, LDHA:1908, Mb:276, Mb:301 and MLL:95) by RFLP analysis.

Template directed Dye Terminator Incorporation assay (FP-TDI)

Initial PCR reactions were carried out as described above. 7.5 µl of the PCR product was purified using 2.5 µl of an ExoAP mixture containing 0.5 µl Antarctic phosphatase (New England Biolabs, Beverly, MA), 0.5 µl dH_2O, 1 µl Antarctic phosphatase buffer and 0.5 µl of dilute Exonuclease I (Exo) (1 µl concentrated Exonuclease I into 9 µl exonuclease buffer (New England Biolabs, Beverly, MA)). Upon completion of the ExoAP digestion (37°C for 1 hour followed by 65°C for 5 min to inactivate the enzymes), the following protocol was used for the FP-TDI single-base primer extension reaction based on Chen *et al.* (1999) and Hsu *et al.* (2001). To each 10 µl sample, a total of 10 µl of the following mixture was added: 0.01 µl of each fluorescently labelled ddNTP (PerkinElmer, Waltham, MA), 0.1 µl of each unlabelled ddNTP (Amersham Biosciences, Piscataway, NJ), 5.48 µl of 5X TDI reaction buffer, 0.3 µl Therminator *Taq* polymerase

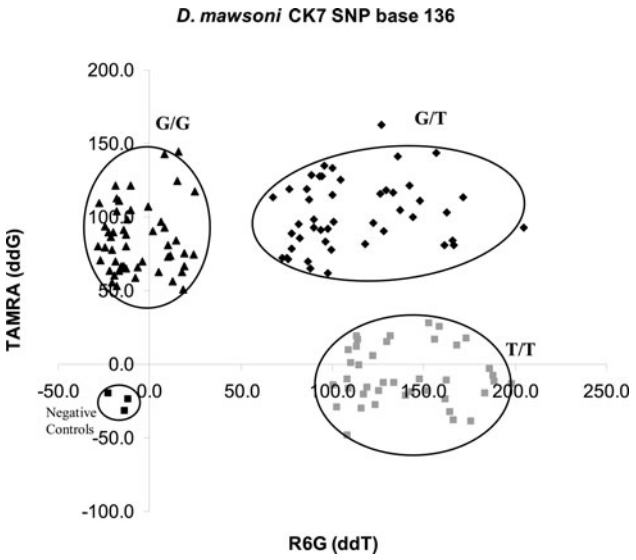

Fig. 1. FP-TDI graphical output of the CK7:136 polymorphism. Grey squares = T/T, diamonds = G/T, triangles = G/G, black squares = negative controls.

(New England Biolabs, Beverly, MA), and 4 µl of the 10 µM SNP primer (30 bp primer adjacent to the SNP site). The samples were then placed into a MWG Primus 96 thermocycler (MWG Biotech, High Point, NC) for an initial denaturation of 1 minute at 93°C followed by 35 cycles of 93°C for 10 sec and 55°C for 30 sec. Upon completion of the primer extension protocol, 10 µl of a mixture containing 0.1 µl 2.2 µg μl^{-1} single stranded binding protein (used to enhance polymerase fidelity by reducing misincorporation) (USB, Cleveland, OH), 2 µl 5X TDI buffer, and 7.9 µl dd-H_2O was added to the samples. The mixtures were then incubated at 37°C for one hour and then held at 4°C until needed. The samples were then transferred to a Costar 96-well plate for fluorescence polarization (FP-TDI) measurement with a POLARstar OPTIMA microplate reader (BMG Labtech, Durham, NC).

Because each SNP examined was bi-allelic, the reaction mixture contained two differently labelled ddNTPs. For ddCTP, a ROX-labelled dye was used (excitation filter wavelength 584 nm, emission filter wavelength 612 nm). ddATP was labelled with fluorescein dye (excitation filter wavelength 485 nm, emission filter wavelength 520 nm) and ddUTP was labelled with R6G (excitation filter wavelength 520 nm, emission filter wavelength 520 nm). ddGTP was labelled with TAMRA (excitation filter wavelength 544 nm, emission filter wavelength 590 nm). Visual separation of genotypes was achieved by plotting polarization values from the two bases scored for each locus (see Fig. 1).

Restriction Fragment Length Polymorphism (RFLP) analysis

Ten SNPs were examined with restriction enzymes (all obtained from New England Biolabs (Beverly, MA) with

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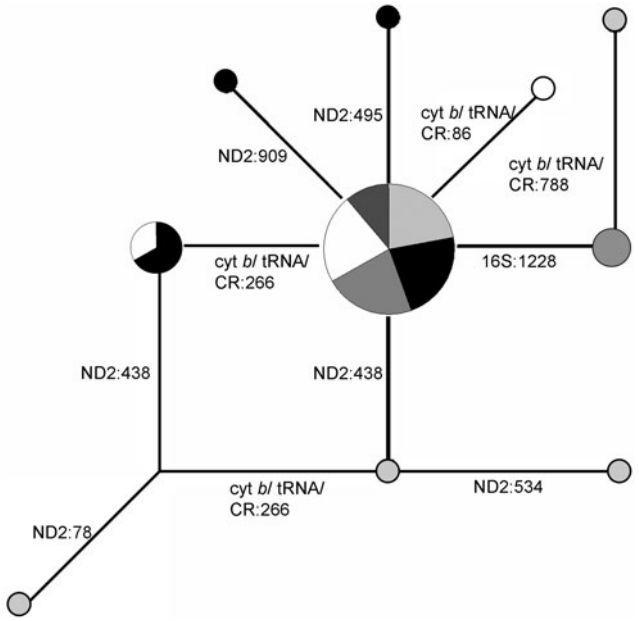

Fig. 2. Median-joining network of composite haplotypes based on the cyt *b*/tRNA/CR, ND2 and 16S amplicons from *Dissostichus mawsoni*. Geographical source of each haplotype is indicated (black = RD88.1, white = RD88.2, dark grey = AAT1, Medium grey = AAT2, Light grey = SS). Mutations separating haplotypes are indicated on the branches.

the exception of *EcoRI* (MBI Fermentas, Hanover, MD)): cyt *b*:86 (*HinfI*), cyt *b*:266 (*Sau96I*), ND2:438 (*NgoMIV*), 16S:1228 (*HaeIII*), MLL:95 (*DpnII*), Dyst6a:78 (*HinfI*), CK7:417 (*RsaI*), Mb:276 (*ApoI*), Mb:301 (*HinfI*) and LDHA:1908 (*EcoRI*). Restriction enzyme digests were performed in 12 µl volumes containing 6 µl amplified PCR product, 4.5 µl dH_2O, 1.2 µl 10X reaction buffer and 0.3 µl of enzyme (10 000 U ml^{-1}) at 37–65°C (as per manufacturer's protocol) for 2–4 hours and subsequently scored on 2% agarose gels.

Derived Cleaved Amplified Polymorphic Sequence (dCAPS)

The dCAPS method (Neff *et al.* 1998) was used to score the polymorphism at position 203 in the CaM amplicon. This method incorporates a restriction enzyme recognition site, which includes the SNP, into the PCR product by utilizing a primer with one or more deliberate mismatches to the template DNA (Neff *et al.* 1998). The PCR product is then subjected to restriction enzyme digestion and the presence or absence of the designated SNP is scored (Neff *et al.* 1998).

Each PCR reaction contained 1.4 µl 25 mM MgCl (Sigma, St. Louis, MO), 1.7 µl 10x PCR buffer (500 mM KCl + 100 mM Tris-HCl, pH = 8.0), 0.3 µl 10 mM dNTP mix, 0.3 µl 10 mM forward primer (5' CATGTGAT AGTGGTAAAGAGATGTA), 0.3 µl 10 mM reverse primer (5' TTGTGAAACCAAAAATCCTA), 0.2 µl JumpStart *Taq* DNA Polymerase (Sigma, St. Louis, MO) and 2 µl DNA template to generate a final volume of 17 µl. PCR reactions were carried out in a RoboCycler 96 Gradient Cycler (Stratagene, La Jolla, CA) and consisted of: an initial denaturation of 2 min at 94°C, followed by 35 cycles of denaturing at 94°C for 45 sec, annealing at 50°C for 45 sec and extending at 72°C for 1 min, with a final extension of 5 min at 72°C. Restriction enzyme digests were performed in 12 µl volumes containing 6 µl amplified PCR product, 4.5 µl dH_2O, 1.2 µl 10X reaction buffer and 0.3 µl of enzyme *RsaI* (10 000 U ml^{-1}) at 37°C for 2–4 hours and then subsequently scored on 10–15% precast acrylamide Criterion gels (Bio-Rad Laboratories, Hercules, CA).

Table V. *Dissostichus mawsoni* mitochondrial DNA polymorphisms. Singleton substitutions marked with a superscript. GenBank Accession numbers in parentheses. SNPs scored in all individuals in boldface.

cyt *b*/tRNA/CR (EF088420)		16S (AY520110)		ND2 (AY256561)	
Position	SNP	Position	SNP	Position	SNP
86	A/G	1228	A/G	78	A^S/G
266	A/G			**438**	A/G
788	C^S/T			495	A/G^S
				534	A/G^S
				909	C^S/G

Analysis of population structure

Analysis of molecular variance (AMOVA) was applied to both nuclear and mitochondrial data using ARLEQUIN version 3.11 (Excoffier *et al.* 2005). AMOVA evaluates patterns of molecular variation within and among populations, taking into account the sequence divergence of molecular haplotypes as well as their frequencies. The degree of population subdivision was described by F-statistics (Weir & Cockerham 1984). Nucleotide (π) and haplotype (h) diversities were calculated for mitochondrial and nuclear sequence data using the program DnaSP v. 4.10.9 (Rozas *et al.* 2003). StatXact 4.0.1 (Cytel, Cambridge, MA) provided exact R×C tests for comparison of haplotype frequencies. Nonmetric multidimensional scaling of Nei's (1978) genetic similarity values was performed using SYSTAT 11 (Systat Software, San Jose, CA). In order to minimize statistical artifacts in evaluating tables of *p*-values, we used the false discovery rate (FDR) procedure of Benjamini & Yekutieli (2001) as presented by Narum (2006).

Mitochondrial DNA

Median-joining networks are commonly used to depict relationships of closely related mitochondrial or nuclear haplotypes, for which traditional phylogenetic approaches yield multiple plausible trees (Bandelt *et al.* 1999). Median-joining networks were generated for *D. mawsoni* from mitochondrial DNA sequence data using Network

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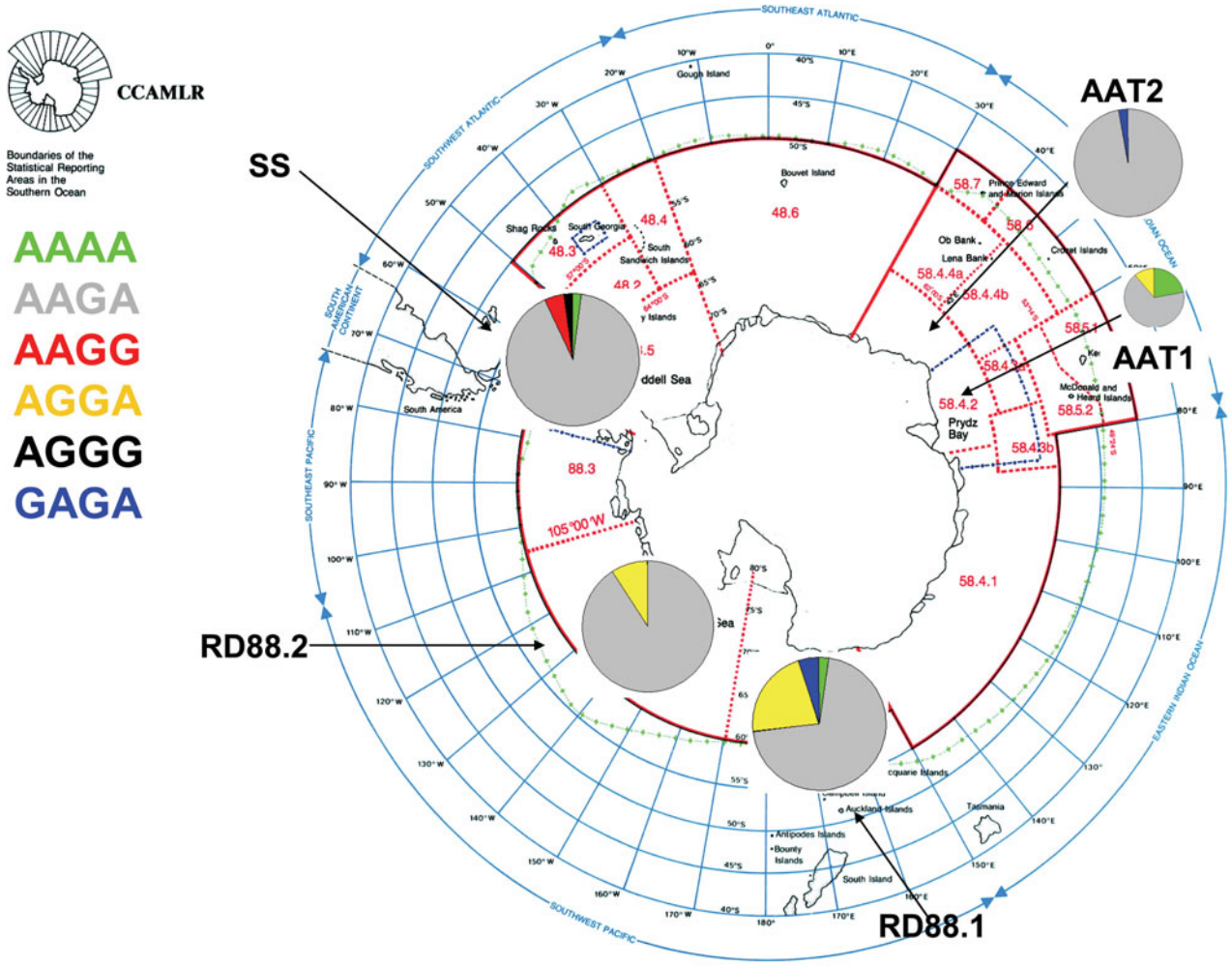

Fig. 3. CCAMLR map of *Dissostichus mawsoni* sampling locations showing major mtDNA haplotype frequencies.

v. 4.0.1.7 (www.fluxus-engineering.com). From preliminary data, a network file was generated from nine mitochondrial SNPs (Fig. 2, Table V). The four mitochondrial SNPs that were observed in multiple individuals (cyt *b*:86, cyt *b*:266, 16S:1228 and ND2:438) were used for further analyses (see Fig. 3 and Table VI).

Table VI. Mitochondrial haplotype counts in *Dissostichus mawsoni* (cyt *b*:86, cyt *b*:266, 16S:1228 and ND2:438). Collections RD88.1A–RD881.D were pooled in view of homogeneous haplotype frequencies (R×C exact $P = 0.927$).

Location	AAAA	AAGA	AAGG	AGGA	AGGG	GAGA	Total
AAT1	2	6		1			9
AAT2		36				1	37
RD88.1	1	29		9		2	41
RD88.2		40		4			44
SS	1	40	2		1		44
Total	4	151	2	14	1	3	175

Nuclear DNA

For each locus, genotypic proportions within populations were compared to Hardy-Weinberg Equilibrium (HWE) expectations using the Markov chain method (Guo & Thomson 1992) implemented in ARLEQUIN v. 3.11 (Excoffier *et al.* 2005). Analysis included resampling 10 000 iterations per batch for 100 batches. The inbreeding coefficient, *f*, was calculated for the nuclear data using the program FSTAT (Goudet 1995). Heterogeneity of allelic frequencies among sampled populations was tested by exact R×C tests (StatXact 4.0.1, Cytel). Linkage disequilibrium between nuclear SNPs was calculated using the programme in ARLEQUIN v. 3.11 (Excoffier *et al.* 2005) while genotypic disequilibrium between loci was calculated using the programme FSTAT (Goudet 1995).

For nuclear gene data, the programme PHASE Version 2.1 (http://www.stat.washington.edu/stephens/software.html) was used to infer gametic phase in individuals heterozygous

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Table VII. Haplotype (h) and nucleotide (π) diversities and number of individuals sequenced (N) for mtDNA and nDNA loci for both *Dissostichus eleginoides* and *D. mawsoni*.

	D. eleginoides			*D. mawsoni*		
Locus	N	*H*	π (%)	N	*h*	π (%)
16S	4	0.500	0.09	3	0.000	0.00
ND2	159	0.883	0.44	42	0.182	0.03
CR/tRNA	136	0.871	0.51	63	0.032	0.01
cyt *b*	110	0.663	0.20	32	0.153	0.03
mtDNA (ND2, CR & cyt *b*)	68	0.983	0.54	15	0.133	0.02
CaM	39	0.657	0.44	11	0.818	0.38
CK7	24	0.978	0.45	11	0.867	0.27
Dyst6a	10	0.773	0.49	3	1.000	0.89
G6PD	28	0.852	0.55	23	0.710	0.66
LDHA5	29	0.945	1.28	6	1.000	0.64
MLL	40	0.821	0.65	10	0.733	0.33
Mb	61	0.806	0.50	26	0.554	0.19
Rhod	24	0.663	0.35	31	0.181	0.03
RPS7	23	0.895	0.28	11	0.873	0.30
SEC61	11	1.000	0.59	11	0.873	0.33
TPI	36	0.824	1.83	9	0.545	0.64
TMO-4C4	19	0.000	0.00	16	0.242	0.06

at two or more nucleotide positions (Stephens *et al.* 2001). In all cases, certainty of correct inference of gametic phase was estimated at 100% by PHASE.

Results

Mitochondrial DNA

In total, nine variable nucleotide positions were identified in the four amplicons sequenced (Table V). Five of these were synonymous substitutions in protein-coding regions, while two were non-synonymous and two were in non-coding regions. All seven synonymous and non-synonymous substitutions were at the third position. The transition/transversion ratio was 8:1. Nucleotide diversity (π) varied among amplicons from 0.00–0.03% (mean = 0.018%) while haplotype diversity (h) ranged from 0.000–0.182 (mean = 0.094) (Table VII). For this preliminary dataset ($n = 38$ sequences), AMOVA showed no genetic differentiation among populations ($F_{ST} = 0.000$, $P = 0.44$). The genealogical relationships among the resulting haplotypes are depicted in Fig. 2.

Previously published mitochondrial DNA sequences for *D. mawsoni* differed slightly from sequences obtained in this study. For the ND2 amplicon, the common haplotype was identical to AY256561; the other published sequence, from a Ross Dependency toothfish (DQ184498), differed by a single synonymous substitution (C → T at position 732). We did not observe this haplotype in our collections. For the cyt *b* region, the sequence obtained by Sanchez *et al.* (2007, GenBank DQ498816) differed from our common haplotype by two replacement substitutions at positions 616 (Glu → Ala) and 640 (Gln → Leu). For both amino acids, the residues observed in our sequence data are universal in notothenioids and other Perciformes, whereas the substitutions presented in DQ498816 have not been previously observed in notothenioids or other Perciformes. We did not observe any variation in the control region; a specimen from Terre Adelie (DQ487352) differed by a deletion of nucleotide 878 and a C → A transition at position 1248 compared to our sequence (EF488420).

Table VIII. Pairwise mitochondrial F_{ST} estimates for Antarctic toothfish populations (below diagonal). Unadjusted p-values are shown above diagonal; comparisons significant after adjustment for multiple testing are shown in boldface.

	AAT1	AAT2	RD88.1	RD88.2	SS
AAT1		**0.011**	0.242	0.429	0.068
AAT2	**0.250**		**0.003**	0.169	0.217
RD88.1	0.035	**0.128**		0.033	**0.002**
RD88.2	0.154	0.031	0.057		0.242
SS	0.106	0.019	**0.096**	0.018	

Four common mitochondrial SNPs (cyt *b*:86, cyt *b*:266, 16S:1228 and ND2:438) were scored for the complete sample set by RFLP analysis (Table VI). AMOVA showed significant differentiation among the five groups ($F_{ST} = 0.074$, $P = 0.00148$), accounting for 7.4% of the total variation. Pairwise F_{ST} comparisons (Table VIII) showed RD88.1 to differ significantly from SS and AAT2, and AAT1 to differ from AAT2.

Smith *et al.* (2001) reported that *HaeIII* digestion of the 16S rRNA amplicon provided a diagnostic marker that distinguished *D. mawsoni* from *D. eleginoides*. Four out of 178 *D. mawsoni* individuals exhibited a *HaeIII* digestion pattern identical to that of *D. eleginoides* ($n = 42$), underscoring the desirability of multiple independent markers for reliable species identification.

Nuclear DNA

In total, 89 variable sites were identified in the 13 nuclear amplicons sequenced (excluding *bty*), eight in coding sequence and 81 in introns (Table III). Of these, nine were insertion/deletion polymorphisms, six of which were perfect or imperfect repeats of motifs two to nine base pairs in length, while the others (G6PD: 186..205, Dyst6a: 416..420 and Dyst6a: 1063..1067) were simple indels. SNPs in coding regions showed a transition/transversion ratio of 1.67, with five synonymous substitutions (all third position) and three non-synonymous substitutions (two first position and one second position). For non-coding SNPs, the transition/transversion ratio was 0.71 (not significantly different from the coding transition/transversion ratio (Fisher's exact $P = 0.159$)). Nucleotide diversity (π) varied among amplicons from 0.03–0.89% (mean = 0.35%) while haplotype diversity (h) ranged from 0.181–1.000 (mean = 0.67) (Table VII).

Comparisons with previously published sequence data were possible for three of the nuclear amplicons (Dyst6a,

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Table IX. *Dissostichus mawsoni* nuclear SNP frequencies. *N*=the number of individuals scored. *f* represents the inbreeding coefficient. Significant *f* values shown in boldface. All *f* represents the mean *f* across all loci.

	AAT1	AAT2	RD88.1A	RD88.1B	RD88.1C	RD88.1D	RD88.2	SS
CaM:203								
N	8	37	21	5	6	10	48	40
A	0.500	0.216	0.167	0.200	0.417	0.050	0.177	0.250
C	0.500	0.784	0.833	0.800	0.583	0.950	0.854	0.750
f	*0.548*	*0.159*	*-0.176*	*-0.143*	*0.273*	*0.000*	*0.340*	*-0.180*
CK7:136								
N	9	34	21	5	9	11	50	46
G	0.389	0.750	0.786	0.700	0.444	0.636	0.500	0.141
T	0.611	0.250	0.214	0.300	0.556	0.364	0.500	0.859
f	*0.351*	*0.305*	*-0.250*	*-0.333*	*0.590*	*-0.132*	*-0.032*	*0.349*
CK7:417								
N	9	37	22	5	10	11	50	48
C	0.278	0.108	0.045	0.100	0.100	0.091	0.140	0.177
G	0.722	0.892	0.955	0.900	0.900	0.909	0.660	0.823
f	*-0.333*	*0.165*	*-0.026*	*0.000*	*-0.067*	*-0.053*	*0.013*	*0.099*
Dyst6a:78								
N	9	37	20	5	10	10	41	45
A	0.722	0.784	0.875	0.800	0.850	0.800	0.817	0.738
T	0.278	0.216	0.125	0.200	0.150	0.200	0.183	0.262
f	*-0.333*	*0.244*	*0.333*	*1.000*	*-0.143*	*-0.200*	*-0.048*	*-0.220*
LDHA:1908								
N	9	37	22	5	9	11	50	44
C	0.222	0.216	0.182	0.300	0.333	0.091	0.180	0.205
T	0.778	0.784	0.818	0.700	0.667	0.909	0.820	0.795
f	*0.407*	*0.074*	*-0.212*	*-0.333*	*0.059*	*-0.530*	*0.062*	*-0.088*
Mb:276								
N	9	37	22	5	10	11	50	41
G	0.056	0.068	0.045	0.100	0.000	0.045	0.08	0.061
T	0.944	0.932	0.955	0.900	1.000	0.955	0.92	0.939
f	*0.000*	*-0.065*	*-0.026*	*0.000*	*N/A*	*0.000*	*-0.077*	*-0.054*
Mb:301								
N	9	35	21	5	2	8	50	32
A	0.111	0.114	0.262	0.200	0.250	0.250	0.240	0.188
C	0.889	0.886	0.738	0.800	0.750	0.750	0.760	0.813
f	*-0.067*	*-0.107*	***0.639***	*-0.143*	*0.000*	*0.391*	*-0.086*	*0.460*
MLL:95								
N	9	37	21	5	10	11	50	48
A	0.222	0.351	0.357	0.200	0.350	0.273	0.470	0.367
G	0.778	0.649	0.643	0.800	0.650	0.727	0.530	0.633
f	*0.407*	*-0.234*	*0.091*	*-0.143*	*0.226*	*-0.333*	*0.006*	*-0.282*
RPS7:374								
N	9	33	21	5	9	11	50	46
G	0.667	0.576	0.667	0.600	0.111	0.455	0.420	0.446
T	0.333	0.424	0.333	0.400	0.889	0.545	0.580	0.554
f	*-0.455*	***0.515***	*0.167*	*0.273*	*-0.067*	*0.310*	*0.107*	*0.249*
TPI:127								
N	9	34	21	5	9	11	50	45
A	0.368	0.574	0.738	1.000	0.778	0.591	0.680	0.611
G	0.611	0.426	0.262	0.000	0.111	0.409	0.320	0.389
f	*-0.600*	*-0.128*	*0.162*	*N/A*	*1.000*	***0.828***	*-0.001*	*0.309*
All *f*	*-0.001*	*0.112*	*0.103*	*0.030*	*0.254*	*0.148*	*0.025*	*0.074*

MLL and TMO-4C4). For Dyst6a, the published sequence (AF137119) differed from our sequences by seven substitutions and 15 indels. For MLL, the published sequence (AF137249) differed from our sequences by two nucleotide substitutions and two single-base indels. Our TMO-4C4 sequence differed from GenBank AY517803 by six nucleotides, all of which were found in the within the terminal 10 bp at either end of the latter, indicating possible sequencing errors in the previously published entry. In general, the degree of sequence divergence between the previously published sequences and those observed in our study is much higher (2.4% for Dyst6a, 2.3% for MLL, 1.5% for TMO-4C4) than the levels observed among individuals we examined (0.49% for

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Table X. Locus by locus AMOVA for *D. mawsoni* nuclear SNPs. Significant *p*-values for F_{ST} after adjustment for multiple testing are shown in boldface. *f*, the inbreeding coefficient, was calculated for loci with homogeneous allelic frequencies among populations.

Locus	Among populations % variation	Within populations % variation	F_{ST}	*p* - value	*f*
CaM 203	3.9	96.1	0.039	0.023	*0.119*
CK7 136	9.8	90.2	0.098	**< 0.0005**	
CK7 417	0.3	99.7	0.003	0.373	*0.041*
Dyst6a 78	0	100	0.000	0.681	*-0.018*
LDHA 1908	0	100	0.000	0.710	*-0.014*
Mb 276	0	100	0.000	0.941	*-0.066*
Mb 301	0.1	99.9	0.001	0.506	*0.159*
MLL 95	0.7	99.3	0.007	0.206	*-0.074*
RPS7 374	5.4	94.6	0.054	**0.013**	
TPI 127	3.2	96.8	0.032	0.048	*0.154*

Dyst6a, 0.33% for MLL, 0.06% for TMO-4C4), suggesting the presence of sequencing artifacts in the original GenBank entries.

Three tests for departure from HWE were significant after adjustment for multiple testing (Table IX). Significant among-region heterogeneity was observed for two of the 10 SNP loci (Table X). No gametic phase disequilibrium between SNPs was detected, except for two closely linked loci (Mb: 276–Mb: 301 in the South Shetland population ($P < 0.00005$). Hierarchical multilocus AMOVA showed no pattern of global differentiation (F_{ST} among Subareas = 0.0009, $P = 0.41$), but significant differentiation among collections within Subareas ($F_{ST} = 0.024$, $P = 0.032$) for the 10 nuclear SNPs analysed. Patterns of within-Subarea heterogeneity are shown by pairwise F_{ST} estimates (Table XI).

Discussion

Single nucleotide polymorphisms are the most prevalent form of genetic variation (Brumfield *et al.* 2003, Seddon *et al.* 2005), providing a virtually unlimited number of nuclear loci for genetic studies. Although the use of SNPs in non-model species is relatively recent, their potential for elucidating population structure and demographic history is great (Morin *et al.* 2004, Seddon *et al.* 2005, Kuhn & Gaffney 2006). The number of SNPs required to distinguish populations will depend upon the size of the populations studied, the genetic diversity of the populations, past and present patterns of gene flow between populations and the polymorphism of the SNPs (Seddon *et al.* 2005).

Methods for scoring SNPs are numerous. In this study we chose the FP-TDI platform on the basis of reported robustness, cost effectiveness and high throughput capabilities. Hsu *et al.* (2001) reported 100% concordance between SNP genotypes scored by FP-TDI and 5'-nuclease assays in a study that examined 90 individuals scored for 38 SNP loci. Similarly, Akula *et al.* (2002) found 100% agreement for 631 genotypes scored by FP-TDI and RFLP methods, while Helms *et al.* (2003) reported differences of 0.2–0.3% between scores from FP-TDI and Pyrosequencing for two loci. In the current investigation, although all SNP loci scored by FP-TDI yielded distinct and readily scored clusters, discrepancies between FP-TDI scores and SNP genotypes obtained by other methods (direct sequencing, RFLP or dCAPS) occurred at rates greater than 10% in six of the 15 SNP loci initially evaluated (Table IV). For all six loci, FP-TDI assays showed higher frequencies of heterozygotes than the other methods, suggesting the possibility of template contamination during the FP-TDI assays.

For the mitochondrial and nuclear genes examined in this study, the Antarctic toothfish displays greatly reduced levels of sequence diversity (π) compared to *D. eleginoides* (Table VII). Mitochondrial sequence diversity is approximately twenty-five times greater in the latter, while nuclear gene sequence diversity is roughly two and a half times greater. Allozyme surveys likewise showed higher levels of polymorphism in *D. eleginoides* ($P_{95} = 64\%$, 11 loci, Smith & McVeagh 2000) than in *D. mawsoni* ($P_{95} = 7\%$, 30 loci, McDonald *et al.* 1992), although the low estimate for the latter may be biased by low sample sizes. These striking differences in the face of similar life histories suggest a considerably greater effective population size in the Patagonian toothfish, reflecting either greater abundance or a reduced susceptibility to historical population bottlenecks.

Although the degree of population geographic structure estimated from mtDNA in the Antarctic toothfish ($F_{ST} =$

Table XI. Pairwise nuclear F_{ST} estimates for Antarctic toothfish populations (below diagonal). Unadjusted *p*-values are shown above diagonal; comparisons significant after adjustment for multiple testing are shown in boldface.

	AAT1	AAT2	RD88.1A	RD88.1B	RD88.1C	RD88.1D	RD88.2	SS
AAT1		0.030	**< 0.000005**	0.023	0.020	0.029	**0.0006**	0.150
AAT2	0.043		0.372	0.444	0.025	0.731	0.032	**0.0008**
RD88.1A	**0.103**	0.003		0.753	0.022	0.492	**0.005**	**< 0.000005**
RD88.1B	0.084	0.006	0.000		0.517	0.400	0.217	0.102
RD88.1C	0.085	0.051	0.064	0.008		0.147	0.524	0.096
RD88.1D	0.064	0.000	0.000	0.009	0.043		0.727	0.105
RD88.2	**0.068**	0.015	**0.031**	0.019	0.000	0.000		0.264
SS	0.018	**0.036**	**0.066**	0.040	0.029	0.022	0.003	

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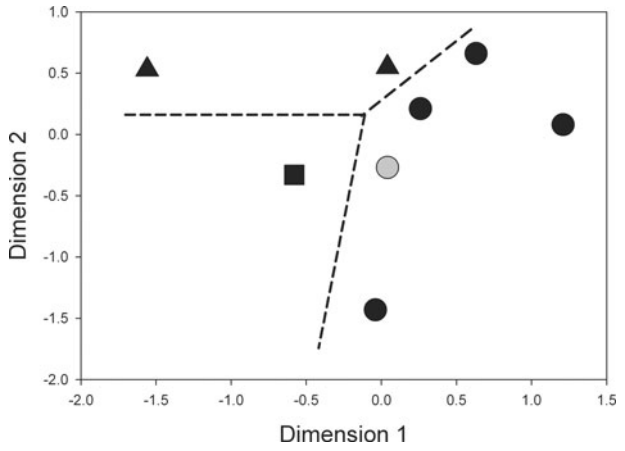

Fig. 4. Nonmetric multidimensional scaling plot of *D. mawsoni* populations based on Nei's (1978) genetic similarity estimates for nuclear SNP data. Solid triangles = Australian Antarctic Territory (Subarea 58.4.2), solid circles = Ross Dependency (Subarea 88.1), gray circle = Ross Dependency (Subarea 88.2), solid square = South Shetland Islands (Subarea 48.1).

0.074) is low compared to its sister species *D. eleginoides* (F_{ST} = 0.768, see Kuhn 2007), our results show genetically distinct populations in *D. mawsoni* in the South Shetland Islands, the Ross Dependency and the Australian Antarctic Territory (Fig. 3). The pattern of nuclear SNP differentiation is consistent with the mtDNA data, and also revealed differentiation within the Ross Dependency between CCAMLR Subareas 88.1 and 88.2 (Fig. 4). These data are concordant with the RAPD analysis conducted by Parker *et al.* (2002) which showed genetic differentiation between the Ross Dependency (McMurdo Sound) and the Antarctic Peninsula.

Genetic differentiation of *D. mawsoni* populations may be a result of separation due to geographical features in the Southern Ocean. The Weddell Sea gyre and the Ross Sea gyre, two major cyclonic clockwise circulations in the Southern Ocean, may play a role in the isolation of populations (Orsi *et al.* 1995, Smith & Gaffney 2005). Natal site fidelity has also been suggested as a factor promoting population subdivision in *D. mawsoni* (Parker *et al.* 2002).

Our results are consistent with the existence of limited gene flow among Antarctic toothfish populations within the regions sampled (Australian Antarctic Territory, Ross Dependency and the South Shetland Islands); however, additional sampling will be necessary to determine whether this species exhibits discrete stock structure or a pattern of isolation by distance across its global distribution. In any case, the evidence for population subdivision found in this and previous studies should be incorporated into fisheries management plans for the Antarctic toothfish.

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