

## RESEARCH ARTICLE

# Fiord populations of *Astrobrachion constrictum* (Ophiuroidea: Asteroschematidae) show little genetic differentiation for mitochondrial DNA

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The fiords of southwestern New Zealand may promote the existence of genetically divergent populations as a result of geological and ecological isolation. The ophiuroid *Astrobrachion constrictum* lives on black coral in these fiords, and is thought to have a relatively short pelagic larval duration that may limit dispersal among populations. Samples of *A. constrictum* were collected from seven sites (four fiords) in Fiordland. Analysis of the mitochondrial COI gene using single-stranded conformational polymorphism revealed eight haplotypes, representatives of which were sequenced. These included nine variable sites, none of which were phylogenetically informative. *G*-test and AMOVA revealed little structuring of samples within or among fiords, but there was evidence for reduced gene flow among the most geographically separated fiords. We conclude that fiords do not house relict populations from earlier glaciations, and that little differential sorting of haplotypes has occurred in the last 10,000 years.

**Keywords:** *Astrobrachion*; biogeography; brittle star; cytochrome oxidase 1; fiord; genetic differentiation; mtDNA; New Zealand; population genetics; SSCP

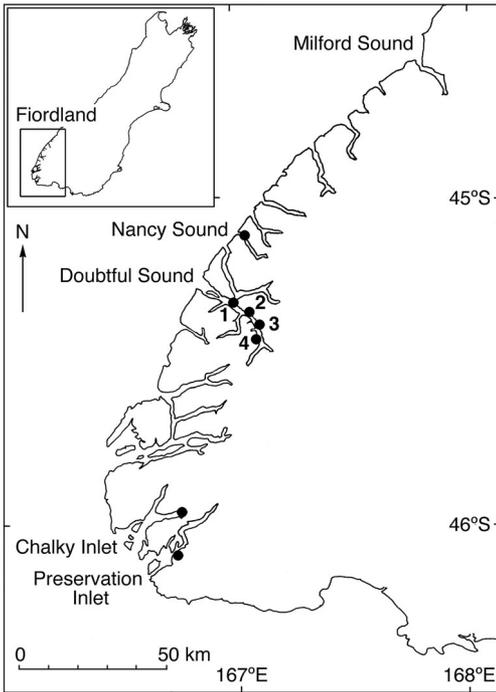
## Introduction

The southwest coast of New Zealand comprises 14 glacial fiords (Fig. 1) extending from Milford Sound in the north to Preservation Inlet c. 200 km to the south (Augustinus 1992). Although information on early glacial activity in this area is lacking due to intense erosion during subsequent stages (McKellar & Soons 1982), speleothem deposits in the Te Ana-au cave system suggest seven glacial events in the last 230 ka (Williams 1996). The mountainous nature of Fiordland and prevailing westerly weather systems result in high regional rainfall (Stanton & Pickard 1982). The resulting freshwater run-off forms a low-salinity surface layer in fiords (Stanton & Pickard 1981), creating an estuarine-like circulation system in the upper layers. The freshwater

surface layer flows seaward entraining water of higher salinity beneath, driving this lower layer towards the head of the fiord (Garner 1964; Stanton 1978; Stanton & Pickard 1981; Stanton 1986). Sea-water below sill depth tends to stay in fiords, with limited displacement from above when incoming coastal water is denser than the deep water of the fiords (Stanton & Pickard 1981; Stanton 1986). High concentrations of chromophoric dissolved organic matter in the low salinity surface layer (Peake & Mosley 2004) reduce light penetration to deeper water in the fiord (Grange et al. 1981). Other deep-water conditions such as low current and little wave action are also promoted within the fiords due to their sheltered nature (Grange et al. 1981). These factors

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**Figure 1** Fiordland region of South Island, New Zealand, showing collection locations for the snake star *Astrobrachion constrictum*. Doubtful Sound sites are: 1, Espinosa Point; 2, Tricky Cove; 3, Oz; 4, Crowded House.

combine to allow species that normally occupy deep offshore habitats, such as the snake star *Astrobrachion constrictum*, to exist within the fiords (Grange et al. 1981). These populations have the potential to be genetically distinct from each other as a result of the geological age and hydrological disconnectedness of fiords (Turan et al. 1998; Wallis & Trewick 2009).

*Astrobrachion constrictum* lives exclusively on the antipatharian black coral *Antipathes fiordensis* (Stewart & Mladenov 1997) in a mutualistic relationship (Vasques 1997). The apparent lecithotrophic larval phase of *A. constrictum* (Strathmann & Rumrill 1987; Stewart & Mladenov 1993; Stewart 1995) suggests that its dispersal potential is relatively low, further increasing the opportunity for genetic differentiation among fiords. Alternatively, gene flow through offshore populations could maintain genetic connectedness of fiord populations.

This study was undertaken to assess the level of genetic differentiation of populations of *A.*

*constrictum* among fiords with three possible scenarios in mind (Graham 2008; Wallis & Trewick 2009): 1. genetic differentiation consistent with initial glacial activity and fiord formation (high; 2.7 Ma); 2. differentiation indicative of the end of the last glacial period (low; 0.1 Ma); and 3. differentiation consistent with recent or ongoing gene flow within the last interglacial (none; 0 Ma). We include multiple population samples within one fiord to contrast levels of differentiation within and among fiords.

### Materials and methods

Specimens of *A. constrictum* were collected by SCUBA from four sites (Espinosa Point, Tricky Cove, Oz, Crowded House) in Doubtful Sound, and one site in Nancy Sound, Chalky Inlet and Preservation Inlet (Fig. 1). Several collecting trips were made between October 1996 and September 1998. Due to the patchy distribution of this species (Stewart & Mladenov 1997), sample numbers vary considerably among sites. At all sites except Crowded House and Oz, animals were collected from several different host coral colonies within the dive site. All animals ( $n = 117$ ) were kept alive in seawater for transport to the laboratory, where they were immediately stored at  $-80^{\circ}\text{C}$ . No samples were collected from the continental shelf outside the fiords as *A. constrictum* lives beyond SCUBA depth and the only collecting method is dredging. Dredging was not a viable option as the black coral on which *A. constrictum* lives is a protected species.

Approximately 50 mg of gonad tissue was dissected from the upper arm of each specimen and macerated with 50  $\mu\text{l}$  of ultrapure water. Samples were placed in a boiling water bath for c. 10 min then centrifuged at 15,000 g for 2 min. The supernatant was transferred to a clean tube and DNA precipitated with 1/20 volume of 5 M NaCl and 2 $\times$  volume of cold 100% ethanol. Tubes were centrifuged at 15,000 g for 10 min, ethanol removed and the dried DNA dissolved in 30  $\mu\text{l}$  of ultrapure water. This DNA solution was used directly for polymerase chain reaction (PCR).

Two mitochondrial gene regions were amplified (Simon et al. 1994): a 473-bp fragment of the

cytochrome c oxidase subunit I (COI) gene using primers C1-J-1718 and C1-N-2191, and a 511-bp fragment of the large ribosomal subunit (16S) using primers LR-J-12887 and LR-N-13398. Polymerase chain reactions were carried out using 3.5 mM MgCl<sub>2</sub>, 200 µM each dNTP, 0.25 µM each primer, 0.025 units of Qiagen<sup>®</sup> *Taq* polymerase, 10× Qiagen<sup>®</sup> buffer in a total volume of 10 µl (1 µl of template) for single-stranded conformational polymorphism (SSCP) and 25 µl (2 µl of template) for sequencing and RFLP analysis. The following thermocycling protocol was used for amplification: initial denaturation at 94 °C for 60 s; 40 cycles of denaturation at 94 °C for 15 s, annealing at 50 °C for 30 s and extension at 72 °C for 60 s; final extension at 72 °C for 3 min.

Sequence variation in COI and 16S amplicons was surveyed across individuals using SSCP (Trewick et al. 2000). We sequenced at least one representative of each COI haplotype to ascertain underlying sequence differences. Twenty-five micro-litre PCR products and a 1-kb Plus DNA ladder (Gibco BRL, Grand Island, NY) were electrophoresed in 2% agarose gels stained with EtBr. Products of expected length were excised and purified using Prep-A-Gene<sup>®</sup> (BioRad, Hercules, CA), and quantified by electrophoresis with 1-kb Plus DNA ladder. Cycle sequencing was carried out with BigDye<sup>™</sup> chemistry according to the manufacturer's recommendations (Applied Biosystems, Carlsbad, CA). The product was precipitated using EtOH/NaAc and the sequence read on an ABI377.

Sequences were aligned by eye and nucleotide differences noted using SeqEd v1.03 (ABI, Perkin Elmer, Foster City, CA). A minimum spanning network showing relationships between haplotypes and their frequencies across sites was drawn using PopArt (Population Analysis with Reticulate Trees; <http://popart.otago.ac.nz/index.shtml>). Haplotype frequencies were analysed using *G*-tests (Sokal & Rohlf 1981) and sequence data by AMOVA (Excoffier et al. 1992).

## Results

Using SSCP, 114 individuals were scored for COI and 19 individuals (representing at least one

example of every COI haplotype present in each fiord) were scored for 16S. Eight COI haplotypes (A–H; Table 1; Fig. 2) and a single 16S haplotype were identified from the SSCP gels. Four individuals of COI haplotype A, two of haplotypes D and E, and one each of haplotypes B, C, F, G and H were sequenced (haplotypes A–H; Genbank Accession #KR140027–34). One individual was heteroplasmic for two haplotypes (A/D, Steel et al. 2000). Because only one 16S haplotype was identified from the SSCP gels, no individuals were sequenced for 16S. All SSCP-resolved haplotypes could be distinguished from each other at the sequence level, and no additional 'hidden' variation was found. That is, there was a one-to-one matching of SSCP band to haplotype.

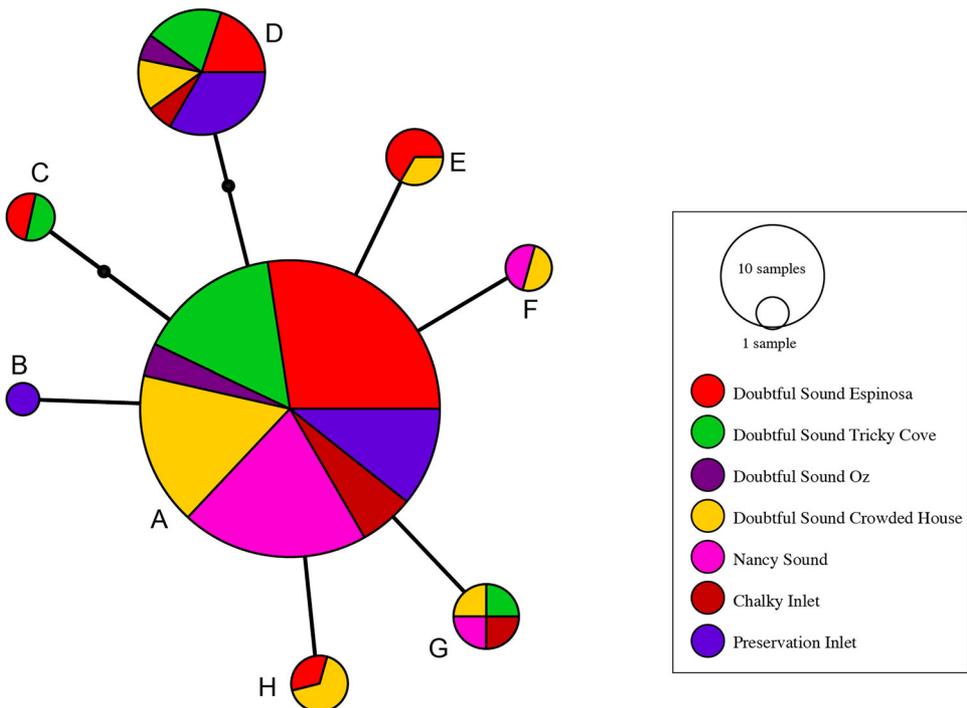
Of the 473-bp of COI sequenced, there were nine variable nucleotide sites, all representing synonymous polymorphisms (one in the first position and eight in the third position of the codon). The transition:transversion ratio was 7:2. All haplotypes appear to derive from the most common haplotype A by one (B, E–H) or two (C–D) substitutions (Fig. 3).

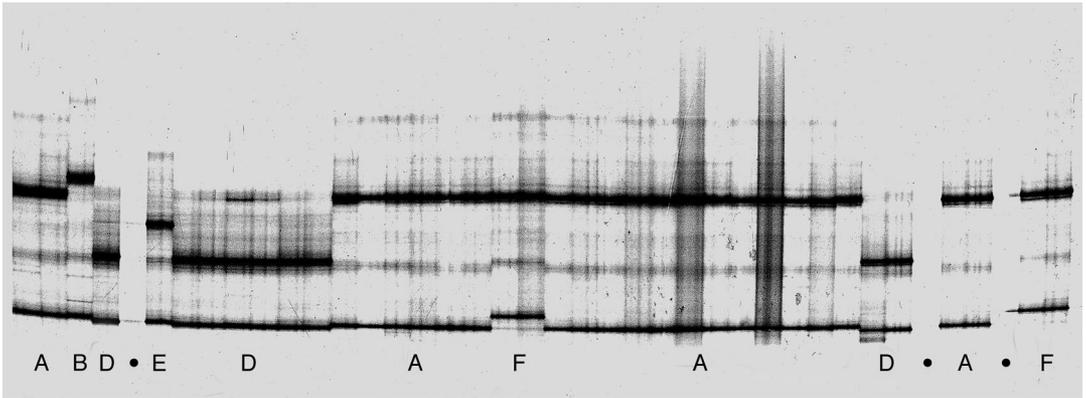
*G*-test analysis (Sokal & Rohlf 1981) showed no significant difference in haplotype frequencies among all seven sampling sites ( $G_{[36]} = 37.9$ ,  $P > 0.2$ ), among the four sites in Doubtful Sound alone ( $G_{[21]} = 13.1$ ,  $P > 0.9$ ), or among the four fiords after pooling Doubtful Sound sites ( $G_{[21]} = 24.8$ ,  $P > 0.2$ ). Due to the large number of haplotypes with low or zero frequency at some locations, *G*-test analysis was repeated having pooled low-frequency haplotypes (i.e. A, D, others). The results of these three tests were similarly not significant ( $G_{[12]} = 13.7$ ,  $P > 0.3$ ;  $G_{[6]} = 3.49$ ,  $P > 0.7$ ;  $G_{[6]} = 10.21$ ,  $P > 0.1$ ). However, the two geographically most distant fiords (Preservation Inlet and Nancy Sound) do show a significant difference ( $G_{[4]} = 13.1$ ,  $P < 0.02$ ), further enhanced by pooling of rare haplotypes ( $G_{[2]} = 9.3$ ,  $P < 0.01$ ). This result reflects a higher frequency of D haplotypes to the south. AMOVA analysis (Excoffier et al. 1992) showed no significant difference among fiords ( $F_{ST} = 0.013$ ,  $P > 0.05$ ) or among sites within Doubtful Sound ( $F_{SC} = -0.037$ ,  $P > 0.05$ ). These results suggest that nucleotide variation among sites or fiords is not significantly greater than variation within a sample.

**Table 1** Summary of COI SSCP haplotype data for snake stars from Nancy Sound, Chalky Inlet and Preservation Inlet, and four sites within Doubtful Sound (Espinosa Point, Tricky Cove, Oz, Crowded House).

Haplotype	Nancy	Doubtful				Chalky	Preservation
		EP	TC	Oz	CH		
A	17	23	13	3	14	5	9
B	–	–	–	–	–	–	1
C	–	1	1	–	–	–	–
D	–	3	3	1	2	1	5
E	–	2	–	–	1	–	–
F	1	–	–	–	1	–	–
G	1	–	1	–	1	1	–
H	–	1	–	–	2	–	–
<i>n</i> haplotypes	3	5	4	2	6	3	3
<i>n</i> individuals	19	30	18	4	21	7	15

CH, Crowded House; EP, Espinosa Point; TC, Tricky Cove.

**Figure 2** Minimum spanning network for eight snake star COI haplotypes (A–H; [Table 1](#)) constructed from sequence data. Black dots on the C and D lineages indicate hypothetical intermediates. Sample size (as assessed by SSCP) is reflected by area of each circle.



**Figure 3** SSCP gel showing a variety of snake star COI haplotypes (blank lanes indicated by dots).

### Discussion

Analysis of the genetic structure of seven population samples of *Astrobrachion constrictum* showed no significant overall differentiation among samples as a whole within, or among, fiords. Within Doubtful Sound, there is no evidence for heterogeneity among samples whatsoever ( $P > 0.9$ ). The lack of deep genetic subdivision among fiords is consistent with recolonisation since the most recent glaciation, as has been described for Chilean pycnogonids (Weis & Melzer 2012) and limpets (Gonzalez-Wevar et al. 2012). The relatively recent development of New Zealand fiords has probably not allowed sufficient time for differentiation to accrue. Associated with the end of the last glacial period 10,000–18,000 years ago (Pillans et al. 1992) was a rise in sea level of 110–120 m, stabilising at today's level around 6500 years ago (Pickrill et al. 1992). The timing of marine incursions into each fiord would be dependent on the height of the entrance sill; fiords such as Preservation Inlet with high sills would have been flooded later than fiords with low sills. Therefore, Fiordland as it is today has only been in existence for a maximum of 10,000 years (Pickrill et al. 1992; Graham 2008). Although this time is long enough to detect a correlation between age of fiord and species richness (Smith 2001), it is too short for detectable DNA substitutions to have occurred in situ (Hickerson et al. 2006).

Although these results are inconsistent with the first of our hypotheses (high differentiation), some

weak genetic subdivision among fiords might, however, still be expected over this time frame. If effective population sizes were restricted, differential sorting of haplotypes from an original heterogeneous population would be likely. There is some evidence for subdivision at the level of haplotype frequency differences among samples of *A. constrictum* for Nancy Sound and Preservation Inlet, the most geographically separated fiords. Thus, although our sample sizes are small, there is evidence for restricted gene flow among fiords. The nature of the differentiation is best exemplified as a clinal increase in frequency of haplotype D from north to south: Nancy 0/19 (0%); Doubtful 9/73 (12%); Chalky 1/7 (14%); and Preservation 5/15 (33%). This finding is consistent with strong currents flowing southwest (Stanton 1986; Graham 2008): once a haplotype is lost to the north, it is hard for it to be reintroduced. Our findings are therefore probably best characterised by our second hypothesis of low differentiation.

Other research reports similar findings. The Fiordland asteroid *Coscinasterias muricata* shows two main haplotypes differing by a single substitution, also with a north–south cline in frequency (Perrin et al. 2004). Genetic differentiation through random genetic drift is also reported among fiord populations of black coral *A. fiordensis* (Miller 1997) and *C. muricata* (Skold et al. 2003) using allozymes. Despite prevailing ocean current towards the southwest, Mladenov et al. (1997) showed some differentiation between coastal and Doubtful Sound

populations of sea urchin (*Evechinus chloroticus*), suggesting some limitation to gene flow. *Evechinus chloroticus* has a long pelagic larval duration and thus high dispersal potential (Dix 1969; Walker 1984), and also inhabits shallow coastal waters between fiords (Dix 1970). These two factors should make gene flow among fiords more likely for this echinoid than for deepwater *A. constrictum*.

It is worth mentioning that COI is often highly variable within populations (Muths et al. 2006; Richards et al. 2007) and has been useful in assessing connectedness between populations of marine invertebrates (Burton & Lee 1994; Duran et al. 2004; Richards et al. 2007; Christensen et al. 2008; Hunter & Halanych 2008). Indeed, the overall lack of divergence in our samples (Fig. 3) is striking, and (on the basis of this single marker) argues for recent ancestry of populations. Although we cannot rule out some gene flow occurring among fiords (our third hypothesis), our results are more consistent with recent origins of fiord populations without invoking ongoing gene flow. Further finescale assessment of gene flow among populations of fiord echinoderms using microsatellite analysis would be useful, though even these markers have shown little differentiation in some other studies (Jorde et al. 2007).

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## Disclosure statement

No potential conflict of interest was reported by the authors.

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