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Larval dispersal and population genetic structure of brachiopods in the New Zealand fiords

D. Gigi Ostrow

A thesis submitted for the degree of Doctor of Philosophy in the Departments of Marine Science and Zoology at the University of Otago, Dunedin, New Zealand.

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Doubtful Sound at dusk. (Photo: H. Nollens)
Abstract

New Zealand's fourteen deep-water fiords have complex physical and hydrographic features as well as strong environmental gradients, all of which may influence the population structure of organisms that inhabit the fiords. I examined the population structure of the brachiopod *Terebratella sanguinea* over ecological and evolutionary time scales in relation to physical and hydrographic features of the fiords. To further explore the role of larval dispersal in this system, comparisons between population genetic structure of *T. sanguinea* and a brachiopod with a contrasting larval dispersal strategy (*Liothyrella neozelanica*) were made.

Aspects of the life history of the articulate brachiopod *Terebratella sanguinea* were measured. I measured density and size throughout Doubtful Sound and growth at outer (5 km from outer coast) and inner fiord sites (13.5 km from outer coast). Additionally, reproductive periodicity was measured at a single site within Doubtful Sound. *Terebratella sanguinea* occurred at significantly lower densities and was significantly smaller at the outer fiord site ($p < 0.05$), however growth rates between an inner and outer fiord site did not differ significantly. *Terebratella sanguinea* was found to have separate sexes and synchronous maturation of oocytes with spawning occurring in the austral winter. These results indicated that, on an ecological time scale, the environmental gradient of the fiords influences aspects of *T. sanguinea* population structure.

In order to determine the influence of the fiord environment on genetic population structure, patterns among *T. sanguinea* from across Fiordland were assessed using two genetic markers, and these data were compared to hydrodynamic variables. Ten sites (322 individuals) were included in a preliminary allozyme analysis, and 20 sites (358 individuals) were used for the amplified fragment length polymorphism (AFLP) analysis. Patchy genetic differentiation was revealed with both markers, and a break between Long Sound and the other Fiordland sites was detected with AFLP markers. My results suggest hydrodynamic features of this region may isolate organisms that can
disperse only during a planktonic larval phase, however this isolation is visible in genetic patterns only at the most extreme values of the hydrodynamic variables.

To better understand how the fiord environment influences population structure of organisms that disperse via planktonic larvae, I compared population genetic structure of two sympatric brachiopod species that differ in planktonic larval duration. Genetic analysis using the AFLP technique revealed population structuring corresponding to the contrasting modes of larval dispersal. AMOVA analysis indicated *Liothyrella neozelanica*, a brachiopod that broods its larvae, had more limited exchange among sites within a fiord than did *T. sanguinea*, a brachiopod that does not brood its larvae. In general, the fiord hydrographic conditions may be creating opportunities for local genetic differentiation (for example Long Sound) in organisms capable of longer distance dispersal, but organisms with lower potential for dispersal are more strongly influenced by ontogeny than by hydrography.

Understanding the population structure of some of the marine fauna of Fiordland is an important cornerstone for the developing management plan for the area. Conservation of the underwater resources of this World Heritage Area can be successful if the structure of the system and the mechanisms driving this structure are taken into account.
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Definitions

6PGD – 6-phosphogluconate dehydrogenase
AFLP – amplified fragment length polymorphism
AMOVA – analysis of molecular variance
CAF – conditional average frequency
COI – cytochrome oxidase I
CTD – conductivity, temperature, and depth survey
Cyt B – cytochrome B
EFD – effective freshwater depth

F – estimator of population genetic subdivision
H – unbiased expected heterozygosity
HK – hexokinase
K – growth rate calculated using a von Bertalanffy growth model
L – length increment for growth calculations of the von Bertalanffy model
LINZ – Land Information New Zealand
LSL – low salinity layer
MDS – non-metric multi-dimensional scaling
MPI – mannose-6-phosphate isomerase
MtDNA – mitochondrial DNA

P – the proportion of polymorphic loci
PCR – polymerase chain reaction
PGI – phosphoglucone isomerase
RFLP – restriction fragment length polymorphism
RAPD – random amplified polymorphic DNA
SL – saline layer
TFPGA – Tools for Population Genetic Analysis
UPGMA – unweighted pair group method with arithmetic mean

Φ = estimator of population subdivision based on hierarchical partitioning of variance by AMOVA analysis

θ – Weir and Cockerham’s (1984) unbiased estimator of Wright’s F
General introduction

Speciation in the marine environment
The study of speciation mechanisms is a central theme in evolutionary biology. Seminal works on the process of genetic differentiation leading to the formation of species (Dobzhansky 1937; Mayr 1954) put forth the most widely accepted model, that of allopatric speciation. Under this model, an extrinsic barrier separates a large continuous population, interrupting genetic exchange between two groups. Genetic divergence between the populations accumulates to such a point that, even if the physical barrier were removed, the two groups would remain distinct due to intrinsic barriers to reproduction.

In a terrestrial system where the physical environment presents discernible barriers to gene flow, organisms have limited dispersal potential and low fecundity, and population sizes are generally small, genetic divisions can be explained more easily under the model of allopatric speciation. In a marine system, the environment is perceived to be continuous because barriers are difficult to discern (Briggs 1974; Vermeij 1987) and rarely absolute. Furthermore, most marine organisms have high fecundity and a planktonic larval phase distributed by oceanic currents that allows organisms to move large distances from natal areas (Strathmann 1990; Palumbi 1992; Palumbi 1994; Avise 1998; Feral 2002). These life history traits result in species with high gene flow between populations, large geographic ranges, and large population sizes (Avise 1998). Such attributes would be expected to decrease levels of genetic differentiation among populations and create higher levels of genetic diversity within populations. Genetic studies of marine invertebrates have provided generally good support for the hypotheses that marine species with long larval phases disperse further, have more gene flow, and lower levels of genetic population structure (e.g. Liu et al. 1991; McMillan et al. 1992; Stickle et al. 1992; Hunt 1993; Williams and Benzie 1993; Gold et al. 2001; Goldson et al. 2001).
However, not all marine invertebrates fit the above paradigm since marine organisms exhibit variation in reproductive modes, larval types, and other life history traits that may have major evolutionary consequences. Many marine invertebrates produce directly developing juveniles or larvae that remain in the plankton for periods of only a few hours (Thorson 1950). It has been assumed a lack of a long duration pelagic phase would minimize opportunities for gene flow between populations and lead to genetic differentiation between populations. Indeed, data illustrating the genetic heterogeneity of marine invertebrates lacking a long duration planktonic phase are ubiquitous (Duffy 1993; Hunt 1993; Hellberg 1996; Hawkins et al. 2000; Goldson et al. 2001; Gutierrez-Rodriguez and Lasker 2004). But plactotrophic development does not necessarily preclude genetic differentiation of populations nor does lecithotrophic or direct development preclude genetic homogeneity (Todd et al. 1988; Sole-Cava et al. 1994; Grant and da Silva Tatley 1997; Borsa and Benzie 1996; Edmands and Potts 1997; Ayre and Hughes 2000; Porter et al. 2001). Various exceptions to the “long larval duration, low genetic differentiation” generalization have been identified, and these exceptions may be due to several non-random factors including: 1) strong selection through local adaptation (Koehn et al. 1980; Knowlton and Keller 1986; Powers et al. 1991; Schmidt and Rand 1999; Perrin 2002), 2) long distance rafting of propagules (Sponer and Roy 2002; Waters and Roy 2004a), 3) historical processes (Bert 1986; Avise 1992; McMillan and Palumbi 1995; Lavery et al. 1996; Barber et al. 2000; Uthicke and Benzie 2003), 4) currents (Shulman and Bermingham 1995; Benzie and Williams 1997; Palumbi et al. 1997; Rocha-Olivares and Vetter 1999; Stepień 1999), and 5) behavioural barriers to gene flow (Doherty et al. 1995; Burton 1997; Todd 1998; Todd et al. 1998).

Genetic partitioning in the marine environment is not well understood and may be the result of several interacting forces. It is thought to arise primarily due to ecological and geographic limitations (e.g. dispersal capability, niche partitioning, and local adaptation (Hedgcock 1986; Palumbi 1994; Bowen 1990). In the marine environment, oceanographic features such as biogeographic boundaries (Burton 1998; Wares et al. 2001; Jolly et al. 2005; Waters and Ayers 2005; Waters et al. 2005) or coastal and estuarine circulation patterns (Ayvazian et al. 1994; Yu et al. 1999; Goldson et al. 2001; Bilton et al. 2002; Waters and Roy 2004b; Watts and Johnson 2004) are thought
to play a key role in the ecological and geographic limitation of larval dispersal. Although oceanographic features will certainly influence passive planktonic dispersal, there is growing evidence that oceanographic circulation might not wholly explain the patterns of genetic structure observed among contemporary marine populations (Benzie and Williams 1997; Benzie 1999; Sotka et al. 2004). The role of barriers as a mechanism for creating genetic divergence in the marine environment remains poorly understood and is hindered by the lack of tractable natural situations in which comparative analysis can be undertaken. The New Zealand fiord system represents a series of marine habitats geographically isolated from one another. As such, this system offers an exceptional opportunity to explore the influences of barriers in a marine environment.

The New Zealand Fiords as potential barriers to gene flow

The 14 deep-water fiords spanning the southwest coast of New Zealand’s South Island (Fig. 1.1) provide an excellent environment in which to explore the process of marine speciation. The glacially scoured, near-vertical, granite faces that rise more than 1000m from the water’s surface and extend down to deep-water basins (450 m at the deepest point) serve as extensive habitat for rich and diverse subtidal sessile invertebrate assemblages that do not occur elsewhere along the coast of New Zealand or in fiords in other parts of the world (Grange et al. 1981). The glaciers that carved the long (10-44 km) and narrow (0.7-2.3 km) valleys receded ca. 18,000 years ago, leaving behind oligotrophic freshwater lakes separated from the open ocean by sills (Pickrill et al. 1992). As sea levels rose, the entrance sills were overtopped. The influx of seawater over sills of different heights created marine habitats of different ages (see Pickrill et al. 1992). This process would have allowed species from the offshore pool to colonize the new habitat at varying times on the scale of 1000’s of years (Smith 2001). The diverse assemblage of sessile invertebrates in the New Zealand fiords allows us to construct a community level synthesis of how historical and environmental factors can influence the genetic population structure of organisms that can disperse only as passive larvae.
A potential hydrographic barrier to gene flow

Unlike most other marine systems, the New Zealand fiords have high potential to isolate planktonic marine organisms. Rivers, glaciers, and substantial amounts of rainfall (up to 7,000 mm per year) create a high freshwater runoff into the fiords. This produces a low-salinity surface layer (LSL) typically 5-10 meters thick and that is separated from the saline layer (SL) below it by a prominent halocline (Stanton and Pickard 1981) (Fig. 1.2). Mixing of the LSL with the denser SL below induces a weak gravitational estuarine circulation pattern; the barotropically driven surface layer flows seaward, entraining water from the saline layer below. Outgoing saline water is replaced by a slow injection of seawater that moves back up the fiord (Stanton and Pickard 1981). The two-layer estuarine circulation pattern is of variable strength in different fiords depending (in great part) on the amount of freshwater entering the fiord (Gibbs 2001). Currents within the LSL are substantially higher (up to 40-60 cm/s; J.D. Whitman and F. Smith, unpublished data reported in Smith (2001)) than the tidally driven currents in the saline layer (mean spring tidal current of 3 cm/s (Stanton 1978)). Therefore it might be suggested that planktonic organisms would be more likely to exit fiords than enter them. However, it is generally assumed that osmotic stress would be too great for most marine organisms to survive within the LSL. Therefore, the estuarine circulation pattern characteristic of the fiords tends to retain larvae within the fiords (Lamare 1998). Depending on the strength of the oceanic inflow of the estuarine circulation, the height of the sills separating fiords from the open ocean (which can potentially restrict movement of saline water), and the depth of the LSL, incoming seawater may carry larvae and other plankton into the fiords. However, given the relatively slow rates of water movement in the saline layer, the exchange mediated by tidal currents may not be great enough to maintain genetic connectivity amongst populations of sessile marine invertebrates with short larval periods.
Fig. 1.1. Fourteen deep-water fiords indent the coastline of the Fiordland region that spans the southwest corner of New Zealand’s South Island (the eleven fiords sampled in this research are labelled). (Figure adapted from Stanton and Picard (1981)).

Fig. 1.2. Schematic illustrating estuarine circulation in two layers of a hypothetical two-dimensional fiord.
In contrast, storm events may have a less predictable effect on larval transport. During strong onshore wind events, the surface flow opposes the down-fiord directed flow in the LSL (Gibbs et al. 2000; Gibbs 2001), and larval transport direction may shift. These strong wind events may influence the fiords as often as two to three times a week and last one to three days. Furthermore, when strong southwest winds buffet the outer coast, water may be pushed into the fiords, creating a storm surge (Gibbs et al. 2000; Gibbs 2001). Such an event would induce vertical mixing between the LSL and SL, causing mortality amongst larvae intolerant of low salinities. For organisms with a long larval phase, larvae may experience several strong wind events during development, however the greatest influence on dispersal is likely to be estuarine circulation. Conversely, for organisms with a short larval duration, stochastic storm events may have a profound effect, transporting larvae in a seaward direction or even killing a proportion of the larval pool in a given year should spawning coincide with a storm event and a deepening of the LSL.

**A potential environmental barrier to gene flow**
The New Zealand fiords demonstrate not only vertical stratification of the water column, but also strong horizontal zonation of habitat along the main axis of a fiord. Sites near the entrance of a fiord are dominated by diverse macroalgal assemblages and are exposed to an open coastal advective environment influenced by strong westerly and southwesterly storms, tidal mixing, and coastally trapped waves. Inner sites are more sheltered from winds and coastal waves. Within the fiords, there is a direct relationship between light attenuation and the amount of freshwater (Goebel et al. 2005). Miller et al. (2006) found levels of photosynthetically available radiation were twice as high at an outer fiord site as an inner fiord site and that these correlated to differences in morphological and physiological characteristics of the macrualgae *Ecklonia radiata* (Miller et al. 2006). The LSL is high in tannins and other organic material leached from the soil by the prodigious rainfall, and these compounds substantially reduce the light penetration into the shallow subtidal (Goebel 2001; Goebel et al. 2005; Miller et al. 2006). At the mouth of the fiords, up to 50% of the habitat is made up of turf and kelp dominated substratum; this value declines to low values at inner fiord sites (Wing and Vasques 2001). Conversely, the percentage of
bare rock - coralline pavement habitat increases from approximately 20% at the mouth to nearly 100% at the inner fiord sites (Wing and Vasques 2001). The dramatic shifts in the marine community within the fiords may affect post-settlement survival. Should a planktonic larval organism move away from suitable habitat, post-settlement survival may be affected by such factors as an increase in inhospitable physical conditions, high levels of interspecific competition, or a decrease in food availability.

The effect of “small, local” barriers on the Fiordland community
Is it justifiable, in this system, to expect divergence of the magnitude necessary for eventual species formation? The proposed barrier to larval dispersal in Fiordland is of a smaller geographic scale than barriers implicated in marine species formation, such as the closing of the Isthmus of Panama (Bermingham and Lessios 1993; Knowlton et al. 1993; Palumbi 1994; Okazaki et al. 1996; Knowlton and Weigt 1998; Boury-Esnault et al. 1999; Lessios et al. 1999; Bowen et al. 2001; Lessios et al. 2003; Bernardi et al. 2004; Harrison 2004; Alejandrino and Valdes 2006) the wide expanse of open ocean in the eastern Pacific Ocean (East Pacific Barrier) (Briggs 1961; Grigg and Hey 1992; Collin 2003; Alejandrino and Valdes 2006) or the intense Benguela upwelling in the Southern Atlantic Ocean (Bowen et al. 2001; Lessios et al. 2003). Furthermore, the colonization of the marine environment in Fiordland followed the last glacial maximum, some 10,000 years ago. By comparison, the closing of the Panamanian Isthmus occurred 3 Mya. However, large geographic barriers are not imperative for speciation (Collin 2003), and hydrographic features considerably smaller than those listed above can limit larval exchange (Ayers and Waters 2005). While allopatric speciation generally occurs over millions of years (c. 2.7 Myr in allopatric Drosophila populations (Coyne and Orr 2004)), the process of speciation can proceed rapidly; the silverside Odontesthes perugiae complex in southern Brazil was found to have undergone a rapid radiation where some divergence times were less than 5,000 years.

Although exchange between the fiords and the open ocean may be limited by hydrography or environmental barriers that could cause genetic divergence and
eventual speciation, it is not likely that the divergence necessary for species formation has occurred. More likely, the geologically young populations in the fiords have not reached drift-migration equilibrium. Under such a scenario, past genetic connectivity may play a large role in shaping genetic patterns, potentially obscuring any current restriction of gene flow (Poisant et al. 2005).

The comparison of a number of species with different evolutionary and ecological backgrounds (e.g. Wares and Cunningham 2001) can help separate the confounding roles of historical forces. Integration of ecological, genetic, geological, and oceanographic information can reveal patterns that might have been less evident in data from a single source.

In the Northwestern Atlantic, comparisons of information from multiple species as well as background information about the abiotic environment have been used to decipher historical influences on organisms (Wares 2002). This manner of studying the genetics of a dynamic marine community is one way of looking at the how ecological interactions and historical events have combined to create the patterns of diversity we see today. By comparing genetic data from a broad range of marine organisms in the Fiordland community, we can understand the life history traits and historical events that influence organisms in this area. This sort of community level genetic comparison could prove particularly useful in the geologically young Fiordland system where history may confound current patterns of exchange.

**Previous population genetic studies in the New Zealand fiords**

Previous studies have found that the New Zealand fiords likely exert a range of effects on the marine invertebrate community. Whereas Mladenov et al. (1997) illustrated that a population of the sea urchin *Evechinus chloroticus* located inside the fiord system was genetically differentiated from coastal populations, a comparison of allozyme variation for *E. chloroticus* in several of the fiords revealed genetic homogeneity among populations (Wing, unpublished data). A microsatellite analysis throughout
Fiordland illustrated that *E. chloroticus* from inner fiord and outer fiord sites differed significantly (Perrin 2002). For the sea star *Coscinasterias muricata*, allosyme analysis (Skold *et al.* 2003) and a mitochondrial D-loop analysis (Perrin 2002; Perrin *et al.* 2004) of populations in the fiords did not follow a strict isolation by distance model, suggesting hydrographic features of the fiords may interrupt large-scale gene flow, but that the fiords are not closed. Whereas the larvae of *E. chloroticus* and *C. muricata* have long planktonic phases (1-2 mos.), the larval phases of many sessile invertebrates are shorter (measured in days), which should reduce the exchange of migrants between and, therefore, increase genetic structuring among populations. Fiord populations of two organisms with shorter planktonic periods, the black coral (*Antipathes fiordensis*) and the snake star (*Astrobrachion constrictum*), also have been the focus of genetic studies. Miller (1997) found that *A. fiordensis*, a taxon with non-feeding, negatively buoyant, short lived (approximately 10 days) planulae larvae (Miller 1996) as well as long generation times, displayed genetic differentiation between sites within fiords, but genetic structuring among fiords was not evident. No genetic structuring, either within a fiord or among fiords, was uncovered using mitochondrial RFLP data or allosyme data for the snake star *A. constrictum* (Steel 1999) (a taxon thought to have short-lived, lecithotrophic larvae (planktonic dispersing larvae that live off the yolk supplied via the egg) (Stewart and Mladenov 1995)). The current study compliments the previous studies by exploring the genetic differentiation within and between populations of two articulate brachiopod species using a novel genetic technique (AFLP).

**The articulate brachiopods Terebratella sanguinea (Leach, 1814) and Liothyrella neozelanica (Thomson, 1918)**

Brachiopods constitute one of the oldest lineages of organisms still in existence today. Common in the fossil record worldwide, brachiopods have survived for some 600 million years with no basic change in form. Articulate brachiopods were once the dominant suspension feeding taxon in the marine environment (Curry *et al.* 1989), but their current distribution is limited largely to cryptic environments, fiords, polar seas, and deep oceans (Peck 1996). New Zealand, however, has a greater diversity of
shallow water brachiopods than any other comparable region in the world. New Zealand is renowned for the abundance and diversity of living brachiopods, and the 30 brachiopod species recorded in New Zealand make up approximately 10 percent of the total recent brachiopod fauna worldwide (Kelleher et al. 1995). With only a single exception (*Thecidellina maxilla*, which has a subtropical/tropical southern Pacific distribution), all of these shallow water brachiopods are endemic to the New Zealand biogeographic region. Both of the focal species of this dissertation belong to genera that have southern Pacific-subantarctic distributions.

In order to test the effect of the fiords as potential barriers to gene flow, I examined the population genetic structure of two articulate brachiopod species with differing larval development patterns. Articulate (hinged shell) brachiopods may be either hermaphroditic or gonochoristic, however in both cases, lecithotrophic (non-feeding) larvae are produced (Williams et al. 1997). Larvae either develop in the water column and have a brief planktonic state (hours to a few days) or are brooded until just before settlement (Jablonski and Lutz 1983; Williams et al. 1997).

**Terebratella sanguinea**
The articulate brachiopod *Terebratella sanguinea* (*Brachiopoda: Terebratellidae*) is found exclusively in subtidal waters (Richardson 1981) along the southernmost part of New Zealand’s North Island, as well as along parts of the South Island, and around Stewart Island and the Auckland Islands (Foster 1974). Animals have been collected from depths of 275 m, though animals from the Fiordland region are found in as little as 4 m of water (Dawson 1992). *T. sanguinea* may be found either attached or free lying, but in the Fiordland region, this taxon attaches to rock surfaces as well as to molluscan, worm, and brachiopod shells, and to crustose algae (Richardson 1981). The adult phase of the life cycle is sessile, and it is only during the larval phase that animals are capable of dispersal. Few accurate accounts of brachiopod reproductive cycles exist (but see Rokop 1977), and spawning has rarely been witnessed (but see Long and Stricker 1991; James et al. 1992; Williams et al. 1997 for reviews). Consequently, the reproductive cycle of *T. sanguinea* is not well understood, and the duration of the planktonic larval period is not known.
Tortell (1981) reported preliminary observations indicating that *T. sanguinea* is gonochoristic, and he found no evidence of larval brooding (but see Chapter 2 for further evidence of this). Therefore, it can be assumed that the free-swimming stage lasts for a few days, similar to that of other non-brooding articulate brachiopods (*e.g.* *Hemithiris psittacea*: 5.3 days (Reed 1987), *Terebratulina retusa*: 1.75 - 2 days (James *et al.* 1992), and *Calloria inconspicua*: 1-1.25 days (Percival 1944)). Because *T. sanguinea* larvae are in the plankton for a relatively short amount of time, it is likely that dispersal is limited. Therefore, I expected this species to demonstrate genetic structuring of populations at the scale of 10’s to 100’s of kilometres, similar to other marine invertebrates with similar planktonic durations (McMillan *et al.* 1992; Palumbi 1995).

In addition to ontogeny, other factors may influence the population structure of *T. sanguinea* in Fiordland. Aldridge (1981) reported that *T. sanguinea* from two habits (free-lying versus attached) and from different geographic areas (Marlborough Sounds, Fiordland, and Stewart Island) differed in size and shape. These results suggest that oceanographic conditions of different habits and sites may have a significant effect on the phenotypes (and maybe the genotypes) of these animals.

*Liothyrella neozelanica*

*Liothyrella neozelanica* (Brachiopoda: Terebratulidae) is also an articulate brachiopod endemic to the subtidal waters of New Zealand. The species has been reported in depths up to 805 m (Foster 1974), but like *T. sanguinea*, *L. neozelanica* occurs at shallower depths in the Fiordland region, found as shallow as 28 m (Foster 1974). *Liothyrella neozelanica* attaches to rock surfaces as well as to molluscan, worm, and brachiopod shells and crustose algae (Richardson 1981). Animals are often found in highly aggregated clusters (Richardson 1981) located under overhangs or in caves (Wing, pers. com.). Female *L. neozelanica* brood their larvae in the lophophore (Chuang 1994). Larvae are lecithotrophic, and larval life is short (Chuang 1994). Because *L. neozelanica* has brooded larvae and is found in more restricted habitat, it is likely that populations are more isolated in this species than in *T. sanguinea*, and I expected this species to show more genetic structuring of populations.
Chapter 1: General introduction

Using genetic markers to uncover gene flow
Due to the high fecundity and small larval size of marine invertebrates, direct
assessment of larval dispersal presents a challenge (Hedgecock 1986; Waples 1998;
Thorrold et al. 2002). Molecular markers provide a useful tool for estimating the gene
flow between populations (Avise 1994), which can help us understand the patterns of
larval dispersal. Previous studies have addressed questions of population genetic
structure in the fiords using allozymes (Miller 1997; Mladenov et al. 1997; Steel 1999;
Skold et al. 2003). In order to gain an understanding of the genetic patterns of
brachiopods in Fiordland, a preliminary allozyme survey was undertaken. To resolve
finer spatial scale differentiation among populations, Amplified Fragment Length
Polymorphism (AFLP, (Vos et al. 1995)) markers were used for comparisons across the
Fiordland region. Initial attempts to add mitochondrial marker data to the whole-
genome AFLP marker data were abandoned due to problems associated with isolating
an appropriate region of the mitochondrial genome.

Allozymes
Allozymes, variants of polypeptides that represent different allelic alternatives of the
same gene locus, provide information about a number of nuclear loci by measuring
variation in gene products (Avise 1994). Once the basic procedures have been
optimized for a given species, allozyme data are relatively inexpensive and
straightforward to obtain (Murphy et al. 1996; Sunnucks 2000). Most allozymes
represent codominant Mendelian loci. Therefore, data are comparable across studies,
and standard statistical procedures can be used for the detection of genetic sub-
structuring of populations at different hierarchical levels (Parker et al. 1998).
However, allozymes generally illustrate limited levels of polymorphism; on average,
less than half of all loci are polymorphic and loci with \(>3\) alleles are uncommon (Parker
et al. 1998). Furthermore, allozymes represent only a small fraction of the total
genome such that if all the proteins of an organism were subjected to electrophoresis,
only 0.1% of the total possible nucleotide substitutions would be detected (Powell
1975). Potential differences in metabolic function of some allozymes may also be a
limiting factor (Powell 1975). Statistical models in population genetics assume that
variation at allozyme loci is selectively neutral, although exceptions have been well documented (Mitton and Koehn 1975; Koehn et al. 1976; Koehn et al. 1980; Garton et al. 1984; Hilbish and Koehn 1985; Burton 1986; Gaffney et al. 1990; Mitton 1997; Storz and Nachman 2003; Van Oosterhout et al. 2004). In general, however, multilocus allozyme studies are considered to be robust, and there is substantial evidence that the majority of allozyme variation is selectively neutral (Kimura 1983) and that protein polymorphism data conform to neutral mutation theory (Nei and Graur 1984). Allozyme data may be best utilized in conjunction with a genetic marker that represents non-coding DNA rather than solely relying upon a gene product that may be exposed to selective processes.

**Amplified Fragment Length Polymorphism**

The Amplified Fragment Length Polymorphism (AFLP) technique is a powerful technique originally designed to allow the construction of very high-density DNA marker maps for genome research and positional cloning of genes (Vos and Kuiper 1997). Because the technique rapidly generates hundreds of highly replicable markers (see Chapter 3) without prior knowledge of the DNA sequence of an organism, AFLP has become a useful method for screening levels of genetic diversity at the intraspecific level (Mueller and Wolfenberger 1999). The method is a multi-step technique that combines both Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) technologies (Appendix 1). The AFLP approach is particularly useful because many different DNA regions distributed randomly throughout the genome can be screened simultaneously.

Molecular markers typically used to address genetic differentiation at the intraspecific level have included allozymes, RFLP’s, RAPD’s, microsatellites, and mtDNA sequence variation (Lambert and Millar 1995; Neigel 1997; Silva and Russo 2000; Hellberg et al. 2002). The AFLP technique has obvious benefits when compared with these other methods of detecting intraspecific genetic diversity (Campbell et al. 2003). Allozyme surveys are straightforward and inexpensive, but variation between populations may not be detected due to evolutionary constraints on protein polymorphisms (Powell 1975), because variation is either non-coding or silent (Hartl and Clark 1989), or because selective forces may be acting on allozyme loci. RFLP’s,
microsatellites, and sequencing studies require more time and expense to develop (Jordan et al. 2002), which limits the number of loci that can be assayed. RAPD’s generate large numbers of markers, but problems exist with reproducibility and PCR artefacts (see Hadrys et al. 1992; Grosberg et al. 1996, for full review). Using AFLP’s one can overcome the disadvantages of other techniques by producing a large number of highly reproducible (see chapter 3) DNA markers with minimal development. However, two areas of concern have been identified with AFLP markers. First, even though some AFLP loci may segregate as codominant markers, it is necessary to analyse AFLP loci as dominant markers because inheritance patterns of the fragments are difficult to resolve when multiple loci are genotyped on a single gel. Therefore, it is necessary to assume AFLP fragments segregate according to Mendelian expectations and that populations conform to Hardy-Weinberg expectations. Second, unless all bands are sequenced, it is necessary to assume that amplified fragments (dominant alleles) of the same size are identical in state in all samples and, similarly, that unamplified fragments (recessive alleles) of a locus are identical. Additionally, AFLP’s require high quality and large quantities of DNA. Despite these issues, AFLP’s are considered a useful method for detecting genetic differentiation within a species given the quantity of information generated, the replicability, resolution, ease of use, and cost efficiency of this marker (Mueller and Wolfenberger 1999). Although the non-Mendelian inheritance of AFLP markers can make the data difficult to interpret, AFLP’s are a relatively cheap method that can reveal polymorphism when alternatives cannot.

Mitochondrial DNA
Mitochondrial DNA (mtDNA) can be a useful tool for examining levels of genetic differentiation between and among populations because it exhibits considerable variation. MtDNA has been shown to have higher rates of nucleotide substitution than coding regions of the nuclear DNA (Avise et al. 1987; Moritz et al. 1987), allowing for the detection of recent genetic divergences between closely related populations. Additionally, mtDNA is effectively a single locus with haploid maternal inheritance, resulting in fourfold lower effective population size than nuclear markers. Due to this lower effective population size, mtDNA is more sensitive to the effects of genetic drift, founder events, and bottlenecks (Harrison 1989). Furthermore, because mtDNA is
inherited maternally, phylogeny is not affected by recombination events and female lineages may be traced (an especially important factor in organisms with sex-biased dispersal) (Harrison 1989). Finally, animal mtDNA typically includes a highly polymorphic non-coding region known as the Displacement Loop (D-loop) that serves as the origin of replication for the mitochondrial genome. This region is often much more variable than the rest of the mitochondrial genome and, therefore, is often a useful marker for the study of very recently divergent populations (Parker et al. 1998).

To date, the complete mitochondrial genome of three articulate brachiopods has been sequenced (Terebratalia transversa (Helfenstein et al. 2001), Laqueus rubellus (Noguchi et al. 2000), and Terebratulina retusa (Stechmann and Schlegel 1999)). In both T. transversa and L. rubellus, a non-coding D-loop region was not identified. Instead, short, non-coding regions were found dispersed throughout the mitochondrial genome (the longest of which is 69 nucleotides in T. transversa and 54 nucleotides in L. rubellus). In T. retusa, an unassigned region of 794 nucleotides was implicated as a potential origin of replication. However, this remains a speculative assumption. Furthermore, T. sanguinea is more closely related to Terebratalia and Laqueus based on nuclear-encoded small subunit RNA gene sequences (Cohen et al. 1998). Of those two, Terebratalia is more closely related to T. sanguinea (Cohen et al. 1998).

Therefore, the longest non-coding region in the Terebratalia genome was targeted as a region of the mtDNA that might uncover genetic differentiation (in T. sanguinea) at the population level. Primers were designed using the T. transversa mtDNA sequence, and universal primers for the cytochrome oxidase I (COI) and cytochrome b (Cyt b) regions were used in combination because this non-coding region is located between the COI and Cyt b regions of the mtDNA (Appendix II). Finally, the COI region was amplified and sequenced, and the Cyt b region was amplified and screened using Single Strand Conformation Polymorphism (Orita et al. 1989) in order to look for polymorphism (Appendix II). However, an unambiguous and polymorphic mitochondrial marker was not identified from this work. Because an mtDNA marker was not identified for T. sanguinea, pursuing an mtDNA marker for the second species of interest in this study, L. neozealanica, was not considered cost and time effective.
Chapter 1: General introduction

Using genetic markers for conservation and management

When populations are small and isolated, theory predicts that the force of selection will decrease and random genetic drift will increase, allowing the accumulation of deleterious mutations (the so-called mutation load associated with inbreeding depression). Populations with a small effective population size are highly vulnerable to extinction via this mutational meltdown (Lynch et al. 1995). Furthermore, metapopulation structure, habitat loss or fragmentation, and environmental stochasticity can be expected to greatly accelerate the accumulation of mildly deleterious mutations, further lowering the genetic effective size of a population (Higgins and Lynch 2001). Empirical evidence suggests that even a small number of migrants can have beneficial fitness effects for recently fragmented populations (Newman and Tallmon 2001).

Because genetics plays a large role in population viability, it is an important factor to consider in conservation and management. As such, the growing field of genetics provides a valuable tool for conservation and management of natural resources. Among marine fishes, the use of genetics has been of invaluable importance when defining stock structure (for recent examples see: Waldman et al. 2002; Graves and McDowell 2003; Garoia et al. 2004; Lage et al. 2004; Beacham et al. 2005, Beacham et al. 2005; Voloch and Sole-Cava 2005). Knowledge of the extent to which populations are connected via genetic exchange has been useful for defining conservation units for many organisms (birds: Boulet et al. 2005; Friesen et al. 2005; Friesen et al. 2006; butterflies: Vila et al. 2006; plants: Xiao and Gung 2006; trout: Antunes et al. 2001; Perrin et al. 2004; salamanders: Miller et al. 2005; shrimp: Voloch and Sole-Cava 2005; frogs: Chiari et al. 2006). For organisms where conservation strategies include restoration, genetics plays an important role in identifying appropriate source populations for re-introduction (Ovenden and Street 2003; Hare et al. 2006; Ramp et al. 2006).

Although the focus of this study was to better understand the connectivity between fiord populations, the genetic information compiled during this study also can be applied to management issues of the Fiordland World Heritage Area. Determining the connectivity of marine populations using genetics can be an important part of a
conservation or management plan (Sweijd et al. 2000; Feral 2002; Carr et al. 2003; Palumbi 2003). Populations harbouring high levels of diversity or rare alleles may be a focus of special protection. Small isolated populations might also merit special conservation status since the depletion of such a population would be irreversible (Parker et al. 2002). Furthermore, what might appear a single, widely dispersed taxon may indeed be a compilation of cryptic species (France and Kocher 1996; Colborn et al. 2001; Sponer 2002) that should be managed individually rather than as a single entity. Molecular biology can play a role in uncovering aspects of population demography pertinent to decisions regarding conservation and management of marine species (Bowen and Avise 1990; Avise 1992; Avise 2000).

**Objectives**
The general aim of this thesis was to assess the effects of the fiord environment on larval dispersal at a meso-geographic scale (tens to hundreds of kilometres). The specific objectives of this study were fourfold: 1) to develop a better understanding of the ecology of *T. sanguinea*, a species that represents sessile organisms with limited planktonic larval dispersal, and to use patterns visible on an ecological scale to gain insight into processes that might result in population subdivision (see Chapter 2); 2) to examine the putative physical barrier to gene flow resulting from the physically fragmented and hydrographically complex environment of the fiords (see Chapter 3); 3) to determine the genetic consequences of different larval dispersal capabilities of two species of brachiopods (see Chapter 4). The unique species assemblages within the New Zealand fiords coupled with the distinct physical and environmental characteristics provide an opportunity to assess the importance of barriers to gene flow and local adaptation on genetic differentiation of brachiopods. Furthermore, the 14 fiords can be considered natural replicate populations; animals within the fiords experience similar selective pressures, however the effectiveness of the putative barrier to larval dispersal differs among the fiords.
Chapter 1: General introduction

Thesis outline

This thesis was written as a series of papers to be submitted for publication. As a result, each chapter is formatted as a stand-alone entity. Redundancy of methodologies between chapters was avoided as much as possible. Within chapters, however, data were explored in a number of manners for comparison, and these results are presented to make the reader aware of the compliment of analyses performed. Furthermore, each chapter is followed by a listing of the literature cited, and in some cases this list overlaps among chapters. Aspects of the thesis that are not suitable for publication have been included as appendices so that the reader is familiar with additional aspects of the project.

Chapter 1: This introduction.

Chapter 2: The population structure of *T. sanguinea* was first examined on an ecological time scale to determine how the fiord environment structures the current populations in Doubtful Sound. The influence of an environmental gradient within a fiord was investigated. Previous to this study, very little was known about the life history of *T. sanguinea*, and the ecological data (reproduction, growth, and density) was used to generate hypotheses about patterns in the fiords.

Chapter 3: The population structure of *T. sanguinea* was then examined on an evolutionary time scale. A comprehensive analysis of *T. sanguinea* population genetic structure across Fiordland is presented. A preliminary survey of population structure at four allozyme loci is reported. *Terebratella sanguinea* population genetic structure was compared for ten populations located across Fiordland. Animals from twenty sites were used to further analyse population structure for two AFLP primer combinations. The effects of historical and physical characteristics of the fiords on dispersal of *T. sanguinea* are assessed by comparing the degree of genetic differentiation among populations to: 1) a proxy for time of colonization, 2) a proxy for estuarine circulation, and 3) a measurement of physical distance separating populations.

Chapter 4: The effects of larval dispersal are further explored by comparing the genetic patterns among *T. sanguinea* populations to those among populations of a
sympatric brachiopod with different larval duration. Animals from two sites within each of three fiords where *T. sanguinea* and *L. neozenica* both occur were used for AFLP analysis. The effects of the hydrographic environment of the fiords were interpreted by comparing the genetic structure of the planktonic larval disperser to that of the brooding brachiopod.

**Chapter 5:** The interface between ecological and genetic data of *T. sanguinea* in the New Zealand fiords is discussed. The general influence of fiord environments on genetic patterns is examined, and the application of genetic data to conservation and management is explored.

**Appendices:** Additional information about the AFLP technique, allozyme data, and a publication from the preliminary allozyme survey are provided. In addition this section includes detailed information regarding DNA extraction and mtDNA analyses.
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32
Chapter 1: General introduction


Natural history of *Terebratella sanguinea* in Doubtful Sound, New Zealand

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**Abstract**

A requirement for understanding natural communities, their composition, and their organization is in depth knowledge of life history. To better understand the life history of the articulate brachiopod *Terebratella sanguinea* in response to an environmental gradient, density and size structure of populations throughout Doubtful Sound and growth at outer and inner fiord sites were measured. Additionally, reproductive periodicity was measured at a single site within Doubtful Sound. Density of *T. sanguinea* differed significantly across the two main arms of the fiord \((p < 0.05)\). However, growth rates \((K)\) did not differ significantly between outer and inner fiord sites. *Terebratella sanguinea* was found to have separate sexes and synchronous maturation of oocytes with spawning occurring in the austral autumn (May).

**Introduction**

The Brachiopoda arose over 550 million years ago. They were the dominant benthic filter-feeding marine invertebrates from the early Cambrian until the end of the Permian period. Since the Permo-Triassic mass extinction event, they have not returned to a position of dominance. Despite this, brachiopods are present in all of the world’s oceans at depths from the intertidal to 6000m.

Because brachiopods are seldom seen in living communities, and are important neither as edible shellfish nor as destructive pests, they have received little attention from marine zoologists. The ecology of a number of living genera has been investigated (*Argyrotheca*: (Logan 1975); *Calloria*: (Percival 1944; Rudwick 1962; Rickwood 1977; Doherty 1979; Aldridge 1981; Stewart 1981; Lee 1990); *Crania*: (Tunnicliffe and
Wilson 1988); *Glottidia* (Paine 1963); *Gyrothyris* (Lee 1990); *Hemithiris* (Thayer 1977); *Laqueus* (Thayer 1975; Tunnicliffe and Wilson 1988); *Lingula* (Kenchington and Hammond 1978; Park *et al*. 2000); *Magellania* (McCammon 1973; Lee 1990; Brey *et al*. 1995); *Liothyrella* (Lee 1990); *Neorhynchia* (Barnes and Peck 1997); *Neothyris* (Rudwick 1962; Neall 1970; Stewart 1981; Lee 1990); *Notosaria* (Rudwick 1962; Lee 1990); *Pumilus* (Rudwick 1962; Rickwood 1968); *Neocrania* (Lee 1990); *Terebratalia* (Paine 1969; Thayer 1975; Thayer 1977); *Terebratella* (Rudwick 1962; Aldridge 1981; Stewart 1981; Lee 1990); *Terebratulina* (Thayer 1975; Noble *et al*. 1976; Curry 1982; Witman and Cooper 1983; Tunnicliffe and Wilson 1988; Collins 1991)), but in only a few species have studies of population dynamics extended beyond size-frequency distributions and specifically included growth estimates. Data on growth has been collected for the intertidal articulate brachiopod *Terebratalia transversa* (Thayer 1977), the subtidal articulates *Terebratella inconspicua* (Doherty 1979) and *Terebratalia transversa* (Strickler and Reed 1985), the deep water articulate *Terebratulina retusa* (Curry 1982; Collins 1991), the shallow water inarticulate *Glottidia pyramidata* (Paine 1963), the intertidal inarticulate *Lingula unguis* (Park *et al*. 2000), and the Antarctic brachiopods *Magellania fragilis* (Brey *et al*. 1995), *Liothyrella uva* (Peck *et al*. 1997), and *Neorhynchia strebeli* (Barnes and Peck 1997). Of these studies, only two investigated changes in size of tagged individuals; the first found no growth in all tagged animals (N = 14) over a 248-day period (Doherty 1979) while the second (N = 324) found shell growth was slow and highly seasonal (Peck *et al*. 1997). The data from Peck *et al*. (1997) suggest environment plays a role in growth rates.

Doubtful Sound (45° 18' 00" S, 166° 58' 45" E) is one of the largest of a series of fiords indenting the southwest coast of New Zealand's South Island (Fig. 2.1). The main channel measures approximately 40 km long, and there are five secondary arms. The fiord has two openings to the Tasman Sea; the primary entrance (Doubtful Sound) is approximately 2 km wide, and the secondary entrance (Thompson Sound) is approximately 1 km wide. Shallow sills separate both entrances from the open ocean. Doubtful Sound has large natural and anthropogenic freshwater inputs. Average rainfall is 465 mm/month, and average riverine input is 135 m³ s⁻¹. The Manapouri Hydroelectric Power Station introduces an additional 350 m³ s⁻¹ of freshwater into the head of the fiord (Bowman and Dietrich 1995). The large freshwater inputs result in a
surface low salinity layer (LSL) deepest at the head of the fiord. Mixing of the LSL with the denser saltwater layer below induces a weak gravitational estuarine circulation pattern where the surface LSL flows seaward. Water from the saline layer below is entrained and replaced by a slow injection of seawater that moves back up the fiord (Stanton and Pickard 1981). Maximum tidal range is 1.4 m (Lamare 1998), and sea temperatures at 10 m depth typically range from 12-14°C annually (Wing, pers. obs.).

Within Doubtful Sound, there is a horizontal zonation of habitat along the main axis of the fiord (Fig. 2.2). The mouth is characterized as an open coastal advective environment influenced by strong westerly and southwesterly storms, tidal mixing, and coastally trapped waves. A diverse macroalgal assemblage dominates this area. Up to 50% of the outer fiord habitat is made up of turf and kelp dominated substratum (Wing and Vasques 2001). Conversely, the inner fiord is more sheltered from winds and coastal waves and has a high percentage of bare rock-coraline pavement habitat. This environmental gradient from the outer fiord to the inner fiord has been shown to influence photosynthetic and morphological parameters of the macroalgae Ecklonia radiata, and it likely influences abundance and growth of other organisms. The inner fiord and outer fiord habitat types represent different compilations of biotic and abiotic factors that can affect growth and abundance of organisms (e.g. Anthony and Hoegh-Guldberg 2003; Defeo and Martinez 2003; Haase 2003).

The diverse brachiopod fauna of New Zealand is composed of twelve species in nine genera. The articulate brachiopod Terebratella sanguinea is one of the most common. This species is abundant along the rock walls of the fiords and has been found in the Marlborough Sounds, around Stewart Island, and in the Auckland Islands (Foster 1974). This is the first report focusing on the life history characteristics of T. sanguinea. In this study the density and size of T. sanguinea throughout Doubtful Sound and growth at an outer and an inner fiord site within Doubtful Sound were measured to determine how these animals respond to the habitat gradient within the fiord. In addition, the reproductive cycle of T. sanguinea at an inner fiord site was characterized to confirm the reproductive cycle of this brachiopod.
Materials and Methods

Density and size estimates

The distribution and abundance of the populations of *Terebratella sanguinea* in Doubtful Sound were evaluated at ten sites distributed along two transects. One transect was located along the axis of Doubtful Sound and one along the axis of Thompson/Bradshaw Sound, and five sites were located along each transect (Fig. 2.1a). A stratified random quadrat design was used to count brachiopod abundance at each of the ten sites. A diver counted individual *T. sanguinea* present within randomly placed 0.25 m\(^2\) quadrats (Witman and Cooper 1983). The number of animals within 15 quadrats at each of three depths (10 m, 15 m, and 20 m) was counted at each site. Surveys were conducted during January through December of 2000. Data could not be transformed to meet normality assumptions, so a Kruskal-Wallis analysis was used to test for differences among sites.

SCUBA divers randomly removed 25 brachiopods from the rock walls at the ten sites within Doubtful Sound (Fig. 2.1a). The length of each animal, defined as the distance between the pedicel opening and the shell margin, was measured to the nearest 0.5 mm using Vernier callipers. An ANOVA was performed to determine whether brachiopods from different sites within the two arms of Doubtful Sound differed in size. A linear regression determined whether a correlation existed between distance separating a site from the open coast and mean animal length for that site.
Fig. 2.1. (a) Sampling sites for *T. sanguinea* natural history study in Doubtful Sound. Sites used for density and size sampling are indicated in red (sites 1-5, Doubtful Sound; sites 6-10, Thompson Sound). Growth data were collected at sites indicated in yellow, and reproduction data were from site “B” marked in yellow.
Fig. 2.1(cont’d). (b) Longitudinal sections from the main arm of Doubtful Sound (Doubtful Sound) showing the location of sills and deep basins (from Stanton and Pickard 1981); (c) Color-coded freshwater profile of the main arm of Doubtful Sound illustrating the change in freshwater over depth and distance from the head of the fiord. (Data from S. Wing).
Fig. 2.2. Underwater photographs from Doubtful Sound illustrating the habitat differences between the outer fiord (a) and inner fiord (b) environments. Both photos were taken at 20 m. (Photos M. Lamare and H. Nollens.)
Growth estimates
During January 2000, 30 animals were marked at each of two sites in Doubtful Sound, an outer fiord site and an inner fiord site (Fig. 2.1a). For each animal, a plastic number was attached around the pedicel using a cable tie. After marking each animal, length (from pedicel to shell margin) was measured to the nearest 0.5 mm using vernier callipers. Brachiopods were collected in April 2001 and measured (to the nearest 0.5 mm) in the lab. Final length was plotted against initial length, and Manzer and Taylor regression equations were calculated for each site (Poore 1972). Regressions were tested for significance of slope and homogeneity of slopes (McShane and Naylor 1995; Zar 1999) to determine whether location within a fiord influenced growth. Growth rates were calculated using the general von Bertalanffy growth curve:

\[ L_t = L_\infty (1 - e^{-K(t-t_0)}) \]

The von Bertalanffy growth parameters, the relative growth coefficient (K) and the asymptotic length (\(L_\infty\)), were derived from the regression equation for each site.

Reproductive cycle
The periodicity of reproduction was determined by monitoring the state of development of female gonadal tissues throughout a single year. SCUBA divers collected 20 - 30 adult individuals (20 - 37 mm) every 4 - 6 weeks from January to December 2000; this ensured the presence of at least ten females in each collection. All collections were from the rock walls of the inner fiord site (site B) used in the growth rate analysis (Fig. 2.1a). Animals were returned to the laboratory where length and width of each individual were measured to the nearest 0.5 mm using Vernier callipers and animals were fixed in Bouin’s solution. For histological preparation, brachiopods were opened, and gonads were removed from both valves and rinsed in 70% ethanol. Gonad tissues were processed using standard dehydration and embedding procedures (Humason 1981). Samples were sectioned (7 \(\mu\)m thick), mounted, and stained with haematoxylin/eosin (to differentiate nuclei from cytoplasmic structures). Histological slides were used to sex animals, and female gonads were assigned to one of four gametogenic stages based on the description of the development of genital lamella and associated oocytes in the brachiopod Terebratulina retusa (James et al. 1991b) and the gonad staging of the brachiopod Glottidia pyrimidata (Paine 1963). Oocyte size-
frequency distributions were determined by measuring the diameter (nearest 0.1 μm) of the first 50 oocytes encountered sectioned through the nucleolus. Oocytes from ten female brachiopods from each collection were measured on a computer running NIH image software and linked to a microscope mounted with a video camera.

Results

Density and size estimates

Densities between the ten sites differed significantly (Kruskal-Wallis test statistic = 329.656, df = 9, p < 0.001). In the main arm of Doubtful Sound, brachiopod densities were highest at Site 2 (the sheltered point behind the mouth of the fiord; a maximum of 192 brachiopods/m²) and declined toward the head of the fiord (Sites 3-5; a minimum of 0 brachiopods/m²) (Fig. 2.3). In contrast, densities of brachiopods in Thompson’s Sound were more consistent across the sites (Sites 6-10; mean 14 brachiopods/m²) with one exception – at Site 7 (the sheltered site behind the mouth of the fiord) mean density was < 1 brachiopod/m² (Fig. 2.3).

Brachiopod lengths were significantly different when sites were compared (F = 7.602, df = 9, p < 0.001). Distance separating a site from the outer coast explained 56% of the variation in mean *T. sanguinea* lengths from the ten sites (p = 0.0124) (Fig. 2.4).
Fig. 2.3. Mean density values for *T. sanguinea* from ten sites located along the two arms of Doubtful Sound. Site numbers correspond to those in Fig. 2.1 with site 1 being the outermost site in Doubtful Sound and site 6 being the outermost site in Thompson’s Sound. Error bars represent standard deviation. Statistical analysis was performed using the Kruskal Wallis function in Systat 10 (SPSS, Inc.). The solid line represents mean length (mm) of 25 animals collected at each site.

Fig. 2.4. Relationship between the distance separating each site from the outer coast and the mean length (pedicel opening to shell margin) of *T. sanguinea* samples from ten sites along the two arms of Doubtful Sound.
Growth estimates

Of the 30 animals tagged at each site, 24 were recovered from the site near the mouth of the fiord and 25 from the site along the main reach. This is a small sample size, and therefore data should be interpreted with caution. Analysis of this dataset suggests animals from Site A (near the mouth of the main arm of Doubtful Sound) displayed higher growth rates when compared to their counterparts from Site B (the central reach of the main arm) (Site A: \( K = 0.4575 \); Site B: \( K = 0.1266 \)), but they reached a smaller final size (Site A: \( L_\infty = 28.15 \); Site B: \( L_\infty = 30.88 \)) (Figs. 2.5 and 2.6). The animals from the outer site had a higher growth rate at small sizes, though the regression line crosses the zero growth line indicating larger sized brachiopods from the outer site had stopped growing (Fig. 2.5 (a)). At the inner site, growth was more constant over all lengths (Fig. 2.5 (b)). Growth curves suggest that animals from the site near the mouth reach a growth plateau sooner and at a smaller maximum size than animals from the inner site (Fig. 2.6). Our analysis suggested the growth curves of the populations were not significantly different (\( t = 1.352, df = 37, p > 0.05 \)), however a power analysis suggested our sample size would have to be an order of magnitude larger (243 animals per site, which was logistically unfeasible) to detect differences of such a small magnitude (power = 0.297).

Reproductive cycle

*Terebratella sanguinea* was found to be gonochoristic, and the sex ratio within the study population was 1:1 (80 females of 151 animals). Except the June samples, which showed no active oogenesis, animals examined for histological analysis had developing ovaries; the smallest animal examined was 23 mm. *T. sanguinea* was found to have an annual reproductive cycle with spawning occurring in the austral autumn (May) (Fig. 2.7). Synchrony in oogenesis and spawning was observed.

Oocytes showed a characteristic pattern of maturation. Oocytes were large (mean size 69.4 \( \mu \)m) and nucleated when the study began in January and continued to grow through May (mean size 97.6 \( \mu \)m) (Table 2.1; Fig. 2.7). Growing oocytes in samples taken during June were either difficult to differentiate or absent, or, in some cases, necrotic oocytes of multiple stages were present within the same ovary; animals were
assumed to have recently spawned during this period. Because the June ovaries did not contain distinguishable growing oocytes, no measurements were made for this sampling period. Oocyte diameters increased progressively from their smallest in July (mean size 5.2 μm) until the end of the study (Table 2.1; Fig. 2.7).

Development of the ovaries was divided into four broad stages based on the size and development of the oocytes. Stage 1 was defined by the presence of clusters of oocytes with no visible nucleus (resting or undeveloped gonad) (Fig. 2.8), Stage 2 by the development of a nucleus in the oocytes (developing gonad) (Fig. 2.9), Stage 3 by the presence of mature oocytes with larger oocytes detaching from the genital 'amella (mature gonad) (Fig. 2.10), and Stage 4 by the presence of necrotic tissue/oocytes (post-spawning or spent gonad) (Figs. 2.11 and 2.12). Staging of the ovaries illustrated complete synchrony (data not shown). However, in a small proportion of the July (post-spawning) samples, both early developing oocytes and necrotic oocytes were visible.
Fig. 2.5. Manzer Taylor plots of *T. sanguinea* growth from (a) outer and (b) inner fiord site in Doubtful Sound. The dashed line indicates the 1:1 line of zero growth.
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Fig. 2.6. Von Bertalanffy growth models for T. sanguinea from outer fiord (dotted line) and inner fiord (solid line) sites in Doubtful Sound.

Fig. 2.7. Mean diameter of T. sanguinea oocytes as measured from animals collected every 4-6 weeks throughout 2000. All animals were collected from an inner fiord site (Site B) in Doubtful Sound.
Table 2.1. Frequency (%) of oocyte diameters (µm) for each collection of *T. sanguinea* at an inner fiord site in Doubtful Sound. Each sampling period, 50 oocytes from 10 animals were measured.

<table>
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<th>Month</th>
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<th>10 - 20</th>
<th>20 - 30</th>
<th>30 - 40</th>
<th>40 - 50</th>
<th>50 - 60</th>
<th>60 - 70</th>
<th>70 - 80</th>
<th>80 - 90</th>
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<th>130 - 140</th>
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<td>6.6</td>
<td>10.2</td>
<td>22</td>
<td>37.8</td>
<td>18.4</td>
<td>3.4</td>
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<tr>
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<td>0</td>
<td>1</td>
<td>2.4</td>
<td>2.2</td>
<td>7.8</td>
<td>12</td>
<td>32</td>
<td>29.8</td>
<td>12</td>
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<td>0</td>
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<tr>
<td>May</td>
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<td>0.2</td>
<td>0.4</td>
<td>1.6</td>
<td>5.2</td>
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<td>31.2</td>
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<td>July</td>
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<tr>
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<td>12</td>
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<td>0</td>
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<tr>
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<td>32.4</td>
<td>34.6</td>
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<tr>
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Fig. 2.8. Stage 1, "resting", ovary in which oocytes (large arrows) are small and densely packed. Eosinophilic spherules involved in phagocytosis are indicated by the hollow arrow. Animals with Stage 1 ovaries were found in July and August, 2000.

Fig. 2.9. Early stage 2, "developing", ovary. The genital lamella extends as vitellogenic oocytes increase in size. Note the clear nucleus in the deeply stained oocytes. Animals with Stage 2 ovaries were encountered in January, March, September, October, and December, 2000.
Fig. 2.10. Stage 3 ovary shows mature genital lamella. Late stage vitellogenic oocytes have separated from the genital lamella and float freely while they complete their development prior to spawning. Stage 3 ovaries were encountered in May, 2000.

Fig. 2.11. Stage 4, “post-spawning”, ovary showing karyolysis and necrosis of unspawned oocytes and that will be phagocytosed. The next generation of oocytes will develop beneath the necrotic oocytes. Stage 4 ovaries were encountered in June, 2000.
Fig. 2.12. Partially spawned ovary illustrating basophilic, necrotic oocytes (large arrow) and eosinophilic spherules involved in phagocytosis (hollow arrow).
Discussion

The density survey indicated that *T. sanguinea* in Fiordland has an uneven distribution. The patchy distribution of *T. sanguinea* was similar to *Calloria inconspicua* in low tide areas in New Zealand harbours that are packed closely on one boulder and absent on adjacent boulders (Rudwick 1962). Our highest mean density (192 animals/m²) was similar to the average density of a brachiopod assemblage in a British Columbia fiord (190 animals/m²) (Tunnicliffe and Wilson 1988) as well as the density of *Terebratulina septentrionalis* from two habitats in the Gulf of Maine (120 animals/m² and 230 animals/m²). However, the densities in the current study were markedly lower than the maxima in the Canadian fiords of 945 animals/m² (Tunnicliffe and Wilson 1988) and 800 animals/m² (Thayer 1977). The maximum density measured was higher than that of the brachiopod *Magellania fragilis* on the Antarctic shelf (26.15 animals/m²) (Brey et al. 1995). Densities reflect a number of factors including the size of the animals and the productivity of the environment and it is not unexpected that densities should differ between such widely separated areas. The largest animal measured in the current study was 43 mm and the maximum annual growth rate was 8 mm. This is comparable to the brachiopod *Terebratalia transversa* from British Colombia that reached a maximum size of 30 mm and had a maximum growth of 7.8 mm per year (Thayer 1977).

The size and shape of brachiopods appears to be affected by differences in environmental factors. In the present study animals from the outer fiord were significantly smaller than animals located at the sites within the fiord. Sites near the entrance of the fiord are dominated by macroalgal assemblages and are exposed to an open coastal environment influenced by storms, tidal mixing, and coastally trapped waves. Inner sites are more sheltered from winds and coastal waves and bottom coverage is nearly 100% bare rock - coralline pavement (Wing and Vasques 2001). Previous studies have documented that some brachiopods will not occur where wave action is strong (McCammon 1973), and others have reported differences in morphology between brachiopods in rough water and calm water. *Calloria inconspicua* from around New Zealand illustrated phenotypic stunting and a more gibbous form in the animals exposed to rough water (Rickwood 1977). Similarly, *T.*
sanguinea from around New Zealand’s South Island exhibited shape differences based on geographic location (Rickwood 1977). Observational data indicated that the New Zealand brachiopods *C. inconspicua, Notosaria nigricans, Pumilus antiqua*us, *T. sanguinea*, and *Neothyris lenticularis* are intolerant of sedimentation and wave-action (Rudwick 1962). Data from the present study suggested *T. sanguinea* is sensitive to differences in environmental conditions and that this sensitivity is expressed phenotypically. Furthermore, competition for settling space has been determined to play an important role in structuring brachiopod populations (Rudwick 1962; Noble *et al.* 1976; Collins 1991). In the New Zealand fiords, the outer fiord has less bare rock available for settling than does the inner fiord. Therefore, differences between outer and inner fiord environments may also relate to competitive interactions during settlement.

Similar to density, size, and shape, growth estimates also reflect the environment in which animals live. In the current study, growth rates did not differ significantly between animals from an inner fiord site and outer fiord site. Brey *et al.* (1995) calculated a growth rate for the Antarctic brachiopod *Magellania fragilis* that was substantially lower than those in the current study (\( K = 0.020 \), Antarctic shell versus \( K = 0.4575 \), outer fiord site and \( K = 0.1266 \), inner fiord site). Peck *et al.* (1997) calculated growth rates for another Antarctic brachiopod, *Liothyrella uva*, from two sites. As with *M. fragilis, L. uva* had low growth rates (\( K = 0.0297 \) and \( K = 0.0451 \)) (Peck *et al.* 1997). However, a third Antarctic brachiopod, *Neorhynchia strebeli*, had a growth rate similar to that of *T. sanguinea* in Fiordland (\( K = 0.228 \)) (Barnes and Peck 1997). The temperate, inarticulate *Lingula unguis* on the intertidal flats of Korea had a markedly higher growth rate (\( K = 0.88 \)) than those calculated for the New Zealand fiords or the Antarctic (Park *et al.* 2000).

The difference between the growth rates at the two sites in the present study was of a smaller scale than the interspecific differences. Although not statistically different, the growth difference between the sites may be of biological significance. The growth models suggested that, if the age of first reproduction remains constant between sites, it would take more than twice as long for animals to reach 20 mm at the inner site as compared to the outer site (20 mm representing an approximate size at maturity based
on reproductive measures) and for those animals to contribute to the gamete pool. Furthermore, the attainment of a size refuge is the only strategy by which articulate brachiopods counter fatal overgrowth or disturbance (Doherty 1979). The different growth rates in Doubtful Sound translate to animals from the outer site reaching this size refuge faster than those at the inner site.

Nearly all brachiopods are gonochoristic (James et al. 1991a; Williams et al. 1997), and sex ratios of 1:1 have been found for several brachiopod species (Curry 1982; Williams et al. 1997). Data from the current study are consistent with the observational data of Tortell (1981) indicating that T. sanguinea fits this pattern. Additionally, T. sanguinea were found to have mature oocytes of similar size to other articulate (hinged shell) brachiopods, suggesting that, like other living articulate for which data are available (Valentine and Jablonski 1983), T. sanguinea has nonplanktrophic larvae. Although adults vary in size, oocyte diameter appears consistent across articulate brachiopod species. The mean diameter of mature oocytes was 97.11 μm in T. sanguinea, and comparably sized Terebratulina retusa and Terebratalia transversa both have late mature oocytes of a mean diameter of 130 μm (James et al. 1991a; Stricker and Folsom 1997). The smaller Pumilus antiquatus (3 mm length) from New Zealand harbours has mean ova diameters of 150-200 μm (Rickwood 1968). This suggests that indeed T. sanguinea, like other articulate brachiopods, have nonplanktrophic larvae. Furthermore, the absence of maturing larvae within the mantle space indicated that this articulate brachiopod has lecithotrophic, not brooded, larvae.

Because articulate brachiopods produce non-feeding larvae, no selective pressure to spawn exists during periods of high larval-food availability. Indeed, articulate brachiopods reproduce throughout the year. Pumilus antiquatus from New Zealand harbours has an annual breeding cycle with a reproductive peak in the austral spring (September to November) (Rickwood 1968). Similarly, the deep brachiopod Frieleia halli illustrates synchronized spawning in spring (January to April) (Rokop 1977). The Antarctic brachiopod, Liothyrella univa, showed a decrease in large oocytes between October and November (austral spring) suggesting spawning in this period (Meidlinger et al. 1998), but large interannual differences have been noted, and a second study indicated spawning occurred in January (austral summer) (Peck and Robinson 1994).
In New Zealand, *Liothyrella neozelanica* spawned by late February (austral summer) (Tortell 1981; Chuang 1994). *Argyrotheca codata* and *A. cistellula* from the Mediterranean have continuous breeding activity while *A. cuneata* breeds in autumn (September and November) (Grobe and Luter 1999). *Terebratulina retusa* in Scotland was found to have a single synchronized spawning event in late November to the end of January (winter) in one population and repeatedly throughout spring and summer with a peak in late autumn in a second population where food availability was greater (James *et al.* 1991b). A third population of *T. retusa* was highly synchronized in reproductive cycle; spawning in late spring and late autumn (Curry 1982). The northern hemisphere brachiopod *Terebratalia transversa* illustrates peak spawning in winter (Stricker and Folsom 1997) as do the southern hemisphere brachiopods *Notosaria nigricans* and *Calloria inconspicua* (Williams and Rowell 1965) (Percival 1944; Rickwood 1968; Tortell 1981), similar to *T. sanguinea* in this study.

Organisms with feeding larvae must spawn when larval-food is available in order to have recruits in the next generation. Phytoplankton blooms in Doubtful Sound occur in spring (Goebel 2001). The endemic sea urchin, *Evechinus chloroticus*, has planktrophic larvae, and spawning occurs in the spring (Lamare 1998). The lack of planktrophic among the articulate brachiopod larvae has decoupled food availability and spawning. Spawning in autumn means *T. sanguinea* spawns when chlorophyll *a* (a measure of phytoplankton productivity) is low (Goebel 2001; Peake *et al.* 2001); in addition, freshwater input is low, salinity is high, and the water column is not highly stratified (Goebel 2001; Peake *et al.* 2001). Because features of the low salinity surface layer are linked to the estuarine circulation within the fiord, lower freshwater input (and the related high salinity and reduction of water column stratification) would decrease the strength of this circulation. Therefore, spawning in the autumn may affect passive dispersal by larvae mediated by water movement in the fiords; estuarine circulation might be less of an influence for *T. sanguinea* than for organisms that spawn when freshwater input is higher.
Conclusions
Size and density of the brachiopod *T. sanguinea* are influenced significantly by the environmental gradient within a fiord, however more data are necessary to determine if growth rates may also be subject to this influence. Animals located at the coastal influenced outer sites were significantly smaller and less dense than those at the more sheltered inner fiord sites. Growth rates between inner and outer sites did not differ significantly, though further study with larger sample sizes might illustrate a stronger pattern than the suggestive trend found in the current study. A reproductive survey illustrated that *T. sanguinea* in Doubtful Sound are gonochoristic and spawn synchronously in the austral autumn.

Acknowledgements
We would like to thank all of the people who made this work possible, especially G. Hopkins, whose help with tagging, collecting, and counting animals was invaluable. P. Meredith was irreplaceable as a field coordinator. Thanks also to M. Clarke, H. Nollens, M. Lamare, P. Brewin, and S. McDonald for their help in counting and “capturing” brachiopods, and to H. Smith and H. Nollens for their assistance with oocyte measurements. And a most sincere note of appreciation to G. Stokes who prepared the histological slides. E. Greiner, H. Nollens, and R. Thompson, were of great help in editing the manuscript. This work was supported by a Marsden Grant obtained by P. Mladenov, M. Roy, and S. Wing as well as PADI AWARE and AMNH Lerner Gray grants in aid of research received by D. Ostrow.
Chapter 2: Natural history of Terebratella sanguinea

Literature Cited


Chapter 2: Natural history of Terebratella sanguinea


Population genetics of *Terebratella sanguinea* in the New Zealand fiords: structure and potential hydrographic influences

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Abstract

Studies have suggested that estuaries, lagoons, enclosed embayments, and fiords may offer opportunities for local subdivision in marine species. Currently the New Zealand fiords are characterised by gravitational estuarine circulation that may influence planktonic dispersal and ensuing population genetic patterns in two ways: 1) fiords may have unidirectional gene flow (into the fiords only) and act as sinks for the Tasman Sea, or 2) gene flow between the Tasman Sea and the fiords may be restricted in both directions. To investigate the influence of hydrodynamic features of the New Zealand fiords on planktonic dispersal, genetic structure of an articulate brachiopod with short-duration planktonic larvae (*Terebratella sanguinea*) was assessed across Fiordland, New Zealand, and compared to hydrodynamic variables that approximated estuarine circulation. Ten sites separated by 16-171 km (322 individuals) were included in a preliminary allozyme analysis, and 20 sites separated by 4-255 km (358 individuals) were used for the amplified fragment length polymorphism (AFLP) analysis. Genetic differentiation was low (allozymes: $\theta_{ST} = 0.0089$; AFLP’s: $\theta_{ST} = 0.0193$), however a slight genetic divergence between Long Sound and the other Fiordland sites was detected with AFLP’s. Isolation by distance was only detected when the Long Sound site was compared to other populations; because the marine environment in the fiords has only recently been colonized, these populations may not have reached drift/migration equilibrium, which can influence detection of isolation by distance. The low levels of genetic differentiation between sites suggest the fiords are not highly isolated from each other or the open ocean. However, hydrodynamic features of Long Sound may inhibit planktonic dispersal.

Introduction

Genetic connectivity among populations of organisms with pelagic larvae is difficult to predict. Barriers to dispersal in the marine environment are perceived to be weak and ephemeral, so marine species that produce pelagic larvae are expected to maintain genetic connectivity among geographically distant populations (Palumbi 1992). However, a growing body of evidence suggests that genetic differentiation can occur

The New Zealand fiords provide an interesting environment in which to explore the influence of cryptic barriers on gene flow among localized populations. The 14 deep-water fiords along the southwest coast of New Zealand’s South Island are a relatively young marine system (8,000 to 12,000 years (Smith 2001)). The marine habitat was created at the end of the Pleistocene when the glaciers that carved the fiords receded, and the fiords were inundated with seawater (Smith 2001). Features of the glacially-carved, deep-water fiords may affect the water movement in the marine environment of Fiordland, which, in turn, would influence the movement of passively dispersing planktonic organisms.

Currently, a gravitational estuarine circulation pattern characterizes the water movement in these fiords (though this may not have been the case during the entirety of the fiords’ history). Large amounts of rainfall (up to 7 m per annum) in addition to glacier melt and river drainage produce a high freshwater runoff into the fiords. This produces a low-salinity surface layer (LSL) typically 5-10 m thick that is separated from the saline layer (SL) below it by a prominent halocline (Stanton and Pickard 1981). Mixing of the LSL with the denser SL below induces a weak gravitational estuarine circulation pattern where the barotropically driven surface layer flows seaward, entraining water from the saline layer below. Outgoing saline water is replaced by a slow injection of seawater that moves back up the fiord below the LSL (Stanton and Pickard 1981). The two-layer estuarine circulation pattern is of variable strength among and within fiords due to physical and hydrographic characteristics (Gibbs et al. 2000), most notably the amount of freshwater and the thickness of the LSL.
Because the outgoing water has very low salinity, it is generally assumed that osmotic stress would be too great for larvae of stenohaline species to survive within the LSL. Therefore, the estuarine circulation pattern is thought to retain pelagic larvae of passively dispersing stenohaline species within the fiords (Lamare 1998). Additionally, each fiord has a characteristic terminal sill or moraine deposited by the glacier that formed each of the fiords (Stanton and Pickard 1981). Most of these sills are deep (50 – 100 m below the water surface) leaving some 45 – 90 m for the seawater to flow over the sill uninhibited. However shallow sills (for example, 25 m in Long Sound) can restrict water moving over the top of the sill and may affect the estuarine circulation pattern (Gibbs et al. 2000). The age of the fiord also is related to the depth of the terminal sill since fiords with deeper sills were flooded before those with more shallow sills (see Pickrill et al. 1992). Therefore the age of the marine habitat in different fiords may vary by thousands of years (Smith 2001). Furthermore, each fiord has a unique signature of internal features such as total fiord length, internal sills, narrow constrictions, and overall fiord shape (such as the dog-legged shape of Bligh Sound versus the straight path of Caswell Sound, see Fig. 3.1). Identifying whether genetic differentiation is correlated to features such as sill depth and freshwater input across the fiords provides insight into the factors that influence larval exchange.

The brachiopod *Terebratella sanguinea* occurs at depths accessible to SCUBA divers throughout most of Fiordland. Brachiopods have a sessile adult phase during which organisms are permanently attached to a hard substrate via a muscular pedicel (Richardson 1981). Therefore, the only dispersal occurs through the passive dispersal of the planktonic larvae. Few accurate accounts of brachiopod reproductive cycles exist, and spawning has rarely been witnessed (see Long and Stricker 1991; James et al. 1992; Williams et al. 1997 for reviews). Consequently, the reproductive cycle of *T. sanguinea* is not well understood (but see Chapter 2), and the duration of the planktonic larval period is not known. It is assumed that the free-swimming stage lasts for a few days, similar to that of other non-brooding articulate brachiopods (*e.g. Hemithiris psittacea*: 5.3 days (Reed 1987), *Terebratulina retusa*: 1.75 - 2 days (James et al. 1992), and *Calloria inconspicua*: 1-1.25 days (Percival 1944)). Because *T. sanguinea* larvae are likely to be in the plankton for a relatively short amount of time, dispersal is probably limited to less than 100 km. If this limited dispersal restricts gene flow
among populations, this species should demonstrate genetic structuring of populations at the scale of tens of kilometres.

Previous studies have found that the New Zealand fiords exert a range of effects on larval dispersal of benthic invertebrates. A number of studies have targeted the sea urchin *Evechinus chloroticus*, which produces passive larvae that spend approximately 1 - 2 months in the plankton. Whereas Mladenov et al. (1997) used allozymes to illustrate that a population of located inside the fiord system was genetically differentiated from coastal populations, a comparison of allozyme loci for *E. chloroticus* in several of the fiords revealed genetic homogeneity among populations (Wing, unpublished data). A microsatellite analysis throughout Fiordland illustrated that *E. chloroticus* from inner fiord and outer fiord sites differed significantly (Perrin 2002). For the sea star *Coscinasterias muricata* (planktonic larval duration of 1 – 2 months), allozyme analysis (Skold *et al.* 2003) and a mitochondrial D-loop analysis (Perrin 2002) found that geographic distance alone could not explain the genetic divergence between populations and suggested that fiord hydrography might increase the effects of genetic drift. Fiord populations of the black coral (*Antipathes fiordensis*), which produces short lived planula larvae, displayed genetic differentiation between sites within fiords, but genetic structuring among fiords was not evident using allozyme data (Miller 1997a). The snake star (*Astrobrachion constrictum*), presumed to have short-lived planktonic larvae, showed no genetic structuring either within a fiord or among fiords based on mitochondrial RFLP data or allozyme data (Steel 1999). An overall pattern of genetic structuring of fiord invertebrates is not evident from this body of research; only *E. chloroticus* illustrates genetic patterning that has been correlated with the physical environment. Furthermore, no overall pattern is apparent based on life history. The current study compliments the previous studies by exploring the genetic differentiation within and between populations of an articulate brachiopod species within this complex system using a novel genetic technique (AFLP).

Genetic studies involving brachiopods have focused primarily on the location of the phylum within the phylogeny of protostomes versus deuterostomes (Cohen *et al.* 1998a and b; Stechmann and Schlegel 1999; Noguchi *et al.* 2000; Saito *et al.* 2000; Helfenbein *et al.* 2001). Other evolutionary questions such as the interaction between
genetic variability and the mass extinction of brachiopods (Ayala et al. 1975), genetic variation and speciation across ocean basins (Cohen et al. 1991; Endo et al. 2001), and measures of genetic variation of a brachiopod species (Hammond and Poine: 1984; Balakirev and Manchenko 1985) have received limited attention. However, the current study is the first comprehensive account of brachiopod population structure and larval dispersal using the indirect measure of population genetic structure.

In this study we examine the population genetic structure of *T. sanguinea* from several sites across Fiordland using both allozyme and AFLP markers. Allozyme surveys remain an efficient way of indirectly estimating gene flow because they are less expensive and require less specialized laboratory equipment than other genetic methods. Furthermore, the statistical estimations of genetic connectivity have been scrutinized and refined since the introduction of the allozyme technique in the 1960's (Neigel 1997). AFLP markers are dominant markers that allow the examination of thousands of polymorphisms across the genome (Kai et al. 2002). Accordingly, they are appropriate markers for looking at rapidly evolving systems (Albertson et al. 1999) as well as patterns of genetic variation in evolutionarily young systems (Porter et al. 2001). Furthermore, the AFLP technique requires no previous information about the DNA sequence of an organism, making these markers particularly useful for taxa for which genetic markers have not been developed previously (Mueller and Wolfenberger 1999). We compare the genetic structure to traditional population genetic models and to geographic and hydrological patterns across the Fiordland landscape in order to create a better understanding of what factors are likely to influence genetic structure in Fiordland. The isolation by distance model was selected as an example of a traditional model that might explain the genetic relationships in Fiordland. If hydrodynamic features are not interrupting gene flow, then organisms with limited dispersal capability (such as *T. sanguinea*) should demonstrate increased levels of genetic differentiation over increased geographic distances. However, if features such as hydrodynamics are influencing gene flow, then a strict correlation between genetic and geographic distance would be less visible and interactions between variables including water circulation and genetic differentiation would exist. Sill depth, fiord length, and freshwater input were selected as variables that might influence historical colonization of Fiordland or the
continuing patterns of passive dispersal of planktonic larvae. Relationships between these variables and genetic differentiation of *T. sanguinea* were explored.

**Materials and Methods**

**Sample collection**

*Terebratella sanguinea* samples were collected from a total of 23 sites in Fiordland between December 1998 and October 2002. We collected only adult brachiopods (length >20 mm), however animals of different sizes over 20 mm were collected. Because brachiopods grow relatively slowly, take several years to reach sexual maturity, and grow more slowly after reaching sexual maturity (Rudwick 1962; Rickwood 1977; Doherty 1979), collections in any given year likely contained animals of many ages. Sampling different sites at different times was not likely to have influenced genetic structure because each collection did not reflect a given settling event but rather the entire accumulation of adults in an area. Additional samples from Stewart Island (Paterson’s Inlet and Port Pegasus), two ‘non-fiord’ sites presumed to be representative of an unrestricted open ocean population, were collected in April of 2000 (Table 3.1, Fig. 3.1). Further sampling of non-fiord populations in the Marlborough Sounds and along the West Coast of the South Island was attempted, but divers could not find *T. sanguinea* at these sites. Collections were made by SCUBA at shallow subtidal sites ranging from 5 to 20 m depth. Divers randomly selected brachiopods 20-40 mm in length from the rock wall at each site. The length of each animal was measured from the pedicel opening to the outer edge of the dorsal valve. Lophophore tissue was removed from each animal and frozen in liquid nitrogen in the field.

Samples were maintained at -80°C until used for genetic analysis. All tissue samples are housed at the University of Otago, Dunedin, New Zealand. In total, 322 individuals of *T. sanguinea* from 10 sites were analysed for allozymes and 358 individuals of *T. sanguinea* from 20 sites were analysed for AFLP’s.
Table 3.1. Study sites for *Terebratella sanguinea* allozyme and AFLP analysis. Sites are numbered according to decreasing latitude (numbers correspond to the map in Fig. 3.1). The letter codes following the number refer to the fiord (first three letters) and the specific position of the site within the fiord (I = inside fiord, O = near outer coast; sites within Doubtful and Thompson are given individual codes because of the multiple sampling sites in these fiords, and sites are listed with the outermost first and the innermost last). For sites at which both allozyme and AFLP data were collected, the first of the two values in a column corresponds to allozyme data and the second value to AFLP data. Samples were collected over multiple years; collection date refers to the year in which samples were collected.

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Latitude/Longitude</th>
<th>N</th>
<th>Collection</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-BLI O</td>
<td>Bligh Sound</td>
<td>44°48'S/167°32'E</td>
<td>20</td>
<td>1999</td>
<td>AFLP</td>
</tr>
<tr>
<td>1-BLI I</td>
<td>Bligh Sound</td>
<td>44°52'S/167°31'E</td>
<td>42/17</td>
<td>1998/1999</td>
<td>Allozymes/ AFLP</td>
</tr>
<tr>
<td>2-GEO O</td>
<td>George Sound</td>
<td>44°52'S/162°20'E</td>
<td>23/20</td>
<td>1998/1999</td>
<td>Allozymes/ AFLP</td>
</tr>
<tr>
<td>2-GEO I</td>
<td>George Sound</td>
<td>44°58'S/167°24'E</td>
<td>24/21</td>
<td>1998/1999</td>
<td>Allozymes/ AFLP</td>
</tr>
<tr>
<td>3-CAS I</td>
<td>Caswell Sound</td>
<td>45°02'S/167°18'E</td>
<td>19</td>
<td>1999</td>
<td>AFLP</td>
</tr>
<tr>
<td>4-CHR I</td>
<td>Charles Sound</td>
<td>45°02'S/167°18'E</td>
<td>22</td>
<td>1998</td>
<td>AFLP</td>
</tr>
<tr>
<td>5-NAN I</td>
<td>Nancy Sound</td>
<td>45°10'S/167°06'E</td>
<td>20</td>
<td>1999</td>
<td>AFLP</td>
</tr>
<tr>
<td>6-THM RP</td>
<td>Thompson Sound</td>
<td>45°17'S/167°01'E</td>
<td>11</td>
<td>2000</td>
<td>AFLP</td>
</tr>
<tr>
<td>6-THM BD</td>
<td>Thompson Sound</td>
<td>45°17'S/167°05'E</td>
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<td>1998</td>
<td>Allozymes</td>
</tr>
<tr>
<td>6-THM CM</td>
<td>Thompson Sound</td>
<td>45°18'S/167°09'E</td>
<td>13</td>
<td>2000</td>
<td>AFLP</td>
</tr>
<tr>
<td>7-DFL CC</td>
<td>Doubtful Sound</td>
<td>45°17'S/165°54'E</td>
<td>15</td>
<td>2000</td>
<td>AFLP</td>
</tr>
<tr>
<td>7-DFL FA</td>
<td>Doubtful Sound</td>
<td>45°19'S/165°58'E</td>
<td>15</td>
<td>2000</td>
<td>AFLP</td>
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<tr>
<td>7-DFL CA</td>
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<td>17</td>
<td>1999</td>
<td>AFLP</td>
</tr>
<tr>
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<td>AFLP</td>
</tr>
<tr>
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<td>Allozymes/ AFLP</td>
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<td>1998</td>
<td>AFLP</td>
</tr>
<tr>
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<td>20</td>
<td>1999</td>
<td>AFLP</td>
</tr>
<tr>
<td>10-WTJ O</td>
<td>Wet Jacket Sound</td>
<td>45°40'S/166°44'E</td>
<td>20</td>
<td>1999</td>
<td>AFLP</td>
</tr>
<tr>
<td>10-WTJ I</td>
<td>Wet Jacket Sound</td>
<td>45°38'S/166°51'E</td>
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<td>1999</td>
<td>AFLP</td>
</tr>
<tr>
<td>11-DSK O</td>
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<td>45°47'S/166°34'E</td>
<td>29</td>
<td>1998</td>
<td>Allozymes</td>
</tr>
<tr>
<td>11-DSK I</td>
<td>Dusky Sound</td>
<td>45°44'S/166°54'E</td>
<td>22</td>
<td>1998</td>
<td>Allozymes</td>
</tr>
<tr>
<td>12-LNG I</td>
<td>Long Sound</td>
<td>45°59'S/165°50'E</td>
<td>18</td>
<td>2002</td>
<td>AFLP</td>
</tr>
<tr>
<td>13-STW PT</td>
<td>Stewart Island</td>
<td>46°56'S/168°08'E</td>
<td>16</td>
<td>2000</td>
<td>Allozymes</td>
</tr>
<tr>
<td>13-STW-PG</td>
<td>Stewart Island</td>
<td>47°12'S/167°41'E</td>
<td>38</td>
<td>2000</td>
<td>Allozymes</td>
</tr>
</tbody>
</table>
Fig. 3.1. *Terebratella sanguinea* sampling sites across Fiordland and Stewart Island. Site locations and sample sizes are provided in Table 3.1. Solid grey circles indicate allozyme data, hollow circles indicate AFLP data, and solid black circles indicate both allozyme and AFLP data.
Allozyme Electrophoresis

Tissue was homogenized with an equal volume of buffer (2 ml Tris-HCl (pH 8.0), 0.1 ml mercaptoethanol, 10 g sucrose, and 25 mg NADP per 100 ml H₂O – adjusted to pH 7.2 with HCl). Electrophoresis was performed on cellulose acetate plates (Titan III Helena Laboratories, Beaumont, Texas). A preliminary survey of 42 enzymes detected four enzyme loci, coding for four different enzyme systems that were polymorphic and consistently resolvable (see Appendix IV). These enzymes were used for the analysis and were as follows (abbreviation and enzyme commission number in parentheses): hexokinase (HK, E.C. 2.7.1.1), mannose-6-phosphate isomerase (MPI, E.C. 5.3.1.8), 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44), and phosphogluccose isomerase (PGI, E.C. 5.3.1.9).

Tris-glycine (pH 8.5) was used as the running buffer for Hk, Mpi, and Pgi. Tris-malate (pH 7.8) was used as the running buffer for 6Pgd (see Richardson et al. (1986) for buffer recipes). Enzyme stains were modified from Hebert and Beaton (1989).
Numerous side-by-side comparisons of electro-morphs were made to confirm relative electrophoretic mobilities; alleles were assigned letter codes with ‘A’ representing the fastest allele (travelling the farthest during an electrophoretic run) at a locus.

AFLP Assay

Genomic DNA was extracted using the commercially available DNAzol Genomic DNA Isolation Reagent (Molecular Research Center, Inc., Cincinnati, OH) (see Appendix III). Dried DNA was re-suspended in 100μl to 200μl deionized, sterile H₂O. Presence of high quality, high molecular weight DNA was verified by running samples on a 2.0% agarose gel alongside a molecular weight ladder. Only samples yielding predominantly high molecular weight DNA were included in the study.

The AFLP assay was carried out essentially following Vos and Kuiper’s (1997) Procedure II for small genomes; modifications to this protocol are detailed here. Total genomic DNA was digested with 5 units Eco RI (Roche Diagnostics) and 5 units of Tru 9I (Roche Diagnostics - Tru 9I is an isoschizimer of Mse I) in a total reaction volume of
40μl. Digestions were performed in the reaction buffer supplied with the Tru 9I. Reagents were overlayed with mineral oil, and DNA was digested at 37°C for 3 hours in a PCR thermal cycler. Subsequently, 10μl of a ligation mixture containing 5pmol Eco RI adaptor, 50pmol Tru 9I adaptor, and 1 unit of T4 DNA Ligase was added. The ligation reactions took place at room temperature (approximately 13-20°C) overnight (approximately 12 hours). Immediately after ligation, 2μl of the digested/ligated DNA was used as PCR template (Fig. 3.2).

AFLP fingerprints were made using two rounds of PCR with the ligated adaptors serving as targets for primer annealing. Selective nucleotides added to the 3’ end of the primers act to selectively reduce the number of restriction fragments amplified. The first round of PCR (pre-amplification) was carried out using a primer pair based on the sequences of the Eco RI and Tru 9I adaptors with a single selective nucleotide added to the 3’ end (Fig. 3.2). Two additional nucleotides (therefore 3 selective nucleotides in total) were added to both primers for the second round of PCR (selective amplification). Initially, the primers for the selective amplification consisted of a Tru 9I based primer with three selective nucleotides and an Eco RI based primer with two selective nucleotides. These primers were selected according to the recommendations of Vos and Kuiper (1997) for animals with small genomes. However, banding produced with these primers was too dense to score reliably, so an additional selective nucleotide was added to the Eco RI based primer. In this study, AFLP bands were produced using two primer pair combinations: 1) Tru 9I: 5’-GATGAGCTGTAGTAACAG-3’ plus Eco RI: GACTGCGTACCAATTCACT, and 2) Tru 9I: 5’-GATGAGCTGTAGTAACACTG-3’ plus Eco RI: GACTGCGTACCAATTCAATA (underlined sequences correspond to pre-amplification primers; selective amplification primers include the additional two nucleotides).

Adaptors and primers from the Gibco BRL AFLP Small Genome Primer kit were used, and additional adaptors and primers were obtained from Gibco BRL. Pre-amplification reactions (20μl) contained 25 ng of each primer, 0.2 mM dNTP’s, 1X PCR buffer (ABGene) and 0.5 units of Taq. Cycling conditions for the pre-amplification were: 30 sec at 94°C; 60 sec at 56°C, and 60 sec at 72°C for 30 cycles. Pre-amplification products were verified on a 2% agarose gels and then diluted 10-fold with sterile, MilliQ H2O. The diluted pre-amplification product was stored at -20°C and used as a
template for selective PCR. Selective amplification conditions were as described above with the exception that 5 ng of $^{33}$P-labelled Eco RI primer and 30 ng of the unlabelled Tru 9I primer were used. Selective amplification cycling conditions were identical to those in Vos and Kuiper (1997). Reaction products were separated on 5% denaturing polyacrylamide gels (Long Ranger gel solution, FMC) using a sequencing electrophoresis apparatus and run for 1.75 h at 60W in 0.5X TBE. For alignment purposes, 3-5 standard fingerprint samples were run on each gel in addition to replicate samples within each gel. Gels were visualised using autoradiography (Biomax film, Kodak) with exposure times ranging from 24-120 hours depending on the strength of the radioactive signal. To test reproducibility, duplicate reactions (at each step of the process: extraction, restriction/ligation, pre-amplification, and selective amplification) were run for random samples.

![Restriction/Ligation Diagram](image)

**Fig. 3.2.** Schematic of the template DNA preparation preceding the radioactive amplification for amplified fragment length polymorphism analysis (Mse I, often used in AFLP analysis, is an isochizimer of Tru 9I used in this study).
The presence or absence of AFLP markers was scored by eye from the autoradiographs and coded as a binary character (presence or absence). For each lane, the relative intensity of bands was considered in addition to the intensity of a given band across lanes; bands of similar intensity (within a lane and across lanes) were scored. Negative controls were run at the extraction, restriction/ligation, and PCR stages; some bands were present in controls, but these rarely matched “real” bands in either size or intensity. Bands present in control lanes were disregarded unless they matched “real” bands in size and intensity and were present in all of the animals run with the control (R. Poulter, pers com). For all analyses, data from the two primer combinations were used to create a single raw data matrix. In order to avoid bias introduced into the estimation of population genetic parameters through the use of dominant markers (Isabel et al. 1995), bands with a frequency less than \((3/N)\) (where \(N\) = the total number of samples) were pruned from the analysis (Lynch and Milligan 1994). A final data set comprising 182 polymorphic bands across 358 animals was used for analysis.

Data Analyses

Genetic diversity
For co-dominant allozyme data, allele frequencies were calculated using FSTAT (Goudet 1995). The GENEPOP program (version 3.1d) (Raymond and Rousset 1995) was used to test for linkage disequilibrium and to carry out exact tests for deviations from Hardy-Weinberg equilibrium.

For the dominant AFLP data, levels of within-population genetic diversity were characterized by calculating Nei’s (1978) unbiased expected heterozygosity \((H)\) and the proportion of polymorphic presumptive loci \((P)\). Estimates of \(H\) were calculated from allele-frequencies (obtained from the Lynch and Milligan (1994) estimator). Because AFLPs are dominant markers and heterozygotes cannot be distinguished directly, Lynch and Milligan’s Taylor expansion was used to estimate allele frequencies and determine indirect levels of heterozygosity. This approach assumes: 1) populations are in Hardy-Weinberg equilibrium, and 2) AFLP’s produce two alleles per locus. Percent
polymorphic presumptive loci estimates were calculated as the proportion of presumptive loci at which the most common allele had an estimated frequency of less than 0.95. Both $H$ and $P$ were estimated using Tools For Population Genetic Analysis (TFPGA) (Miller 1997b). Sample size was regressed against $H$ and $P$ to determine whether the number of animals sampled at each site was related to $H$ and $P$ measurements (see Busch et al. 2000).

Because larger sample sizes would increase the probability of detecting rare alleles, the total and rare alleles (for allozymes) or fragments (for AFLP’s) found in each population was tallied. Alleles or fragments found only in a single population and those found in only 2 or 3 populations were tallied. For allozymes, the average diploid sample size (each animal contributed two alleles at each locus) over all loci per population was regressed against the number of alleles per population found in 1 population, alleles found in 2 or 3 populations, and total number of alleles per population. For AFLP’s, the number of animal per site was regressed against fragments found in 1 population, fragments found in 2 or 3 populations, and total number of alleles per population (SigmaPlot 5.0, S SPSS Science, Chicago, Illinois) to determine whether the number of animals sampled at each site was related to these measurements.

**Population structure**
Total genetic variation was partitioned among groups (among fiords for Fiordland populations or Stewart Island for the two Stewart Island populations), among sites, and among individuals within sites by carrying out a hierarchical analysis of molecular variance (AMOVA) on Euclidean pairwise distances ($\Phi_{ST}$) using GenAlEx (Peakall and Smouse 2001). Distance measures for the AMOVA analysis were based on the genetic distance measure used by Huff et al. (1993). The AMOVA calculation of GenAlEx followed that of Excoffier et al. (1992), Peakall et al. (1995), and Michalakis and Excoffier (1996). Departure of the estimated among-population variation ($\Phi_{ST}$) from random expectations was evaluated using a permutation test consisting of 999 replicates.
Chapter 3: **Terebratella sanguinea** in the New Zealand fiords

Estimates of among population genetic variation were also characterized by calculating Weir and Cockerham's (1984) $\theta$ (an unbiased estimator of Wright's (1978) $F_{ST}$ values). $\theta$ estimates the reduction in heterozygosity from Hardy-Weinberg equilibrium that results from population subdivision. $\theta$ values range from zero, indicating no genetic differences between populations, and one, indicating fixed allelic differences between populations. This indirect measure of gene flow assumes an infinite number of equal-sized populations are exchanging migrants at a constant rate, selection and mutation are not occurring, gene flow is not affected by distance separating populations, and the populations have reached equilibrium between migration and drift. Because natural populations do not meet all of these assumptions, $F_{ST}$ and related estimators provide a convenient abstraction that isolates the opposing effects of gene flow and genetic drift, allowing the measure of relative population divergence (Neigel 2002).

For allozyme data, $\theta$ (Wright 1978; Weir and Cockerham 1984) was calculated using FSTAT (Goudet 1995) to examine the genetic structuring of populations. $\theta$ was calculated for each locus as well as across loci; calculations across loci were compared for all pairs of populations. Levels of significance were calculated for $\theta$ estimations for each locus according to Waples (1987). For pairwise comparisons of $\theta$, the probability that a $\theta$ value was greater than zero was tested using 4500 permutations. For cases in which multiple independent tests were performed, a sequential Bonferroni adjustment (Rice 1989) was used to modify significance levels to account for experiment-wide error. For AFLP data, $\theta$ was calculated using TFPGA and used to test for differences in allele frequencies among sites ($\theta_{ST}$) as well as between fiords ($\theta_{FR}$). Confidence limits (95%) of $\theta$ estimates were obtained from a bootstrap procedure in which 10,000 new data sets were constructed by re-sampling with replacement over presumptive loci. Confidence limits of $\theta$ that did not overlap 0 (no evidence of differentiation) were interpreted as evidence for significant differences of allele frequencies between populations. For each of the two primer combinations, $\theta$ was calculated separately due to the large size of the data set when the bootstrap procedure was invoked; the two $\theta$ estimates then were averaged. Additionally, $N_{m0}$ (theoretical number of migrants per generation when subpopulations are at genetic equilibrium) was estimated (according to $N_{m0} = 0.25(1 - \theta)/\theta$) for both allozyme and AFLP data. $N_{m0}$ is an estimate of the combined influence of the effective population size ($N_e$) and the migration rate ($m$) and,
therefore, can be used to obtain estimates for these parameters. An average effective population size (using census data (see Chapter 2) and assuming an average density over a 25m depth and along the perimeter of Doubtful Sound (fiord considered a population)) was estimated and a migration rate extrapolated using $N_{mg}$. The time required for a population to reach drift-migration equilibrium was calculated as $1/[2m + 1/(2N)]$ (Crow and Aoki 1984).

In order to visually demonstrate the level of relatedness of populations and identify those that may be genetically distinct, relationships between sampling sites were visualized by non-metric multidimensional scaling (MDS) using the program PRIMER v5 (Clarke and Gorley 2001). MDS ordinations were created using a pairwise matrix of genetic distances; two different genetic distance measures were used to create MDS plots. The first of these was the distance measure of Nei (1978). Nei’s distance measure is proportional to evolutionary time when drift and mutation are both taken into account (Weir 1996). The second of these was the coancestry distance of Reynolds (1983), appropriate for short-term evolution when mutation can be neglected (Reynolds et al. 1983). The coancestry distance measures the genetic distance between populations that evolve under the effects of genetic drift and gene flow and is calculated as a simple transformation of $\theta$. Because the marine environment in Fiordland is a young system, the Reynolds coancestry distance was judged to be an appropriate estimator; however, Nei’s genetic distance is most often reported in the literature, so this estimator was calculated in order to make comparisons between this study and others. MDS is a method of data reduction and structure detection that reproduces the relative position of a set of points in a reduced space given, not the points themselves, but only a matrix with interpoint distances (McArdle 2001). Using MDS analysis, a very large, multi-variate data set can be distilled to a two-dimensional image in which groupings of data points become more readily apparent. Two-dimensional MDS ordinations are presented with a ‘stress’ value that measures how well the multi-dimensional data have been fitted into two dimensions. Stress values between 0 and 0.10 indicate that the relative distance between samples has been well preserved; 0.10-0.20 indicates poorer representation, but the plots are adequate for interpretation (Connell et al. 1998). The ordination technique adopted in the non-metric MDS is complex but it makes few model assumptions about the form of the data.
or the inter-relationship of the samples. If stress values are low, the MDS ordination is probably a more useful representation than a cluster analysis (Clarke and Warwick 1994). We used ten restarts of the algorithm to determine stress values of MDS ordinations.

Isolation by distance using allozymes and AFLP's

If the fiords do not present a discrete barrier to gene flow but dispersal is limited (e.g. by larval duration), isolation by distance might influence the process of genetic differentiation between fiord populations. In order to test for a correlation between genetic and geographic distance among populations, for both marker sets, θ estimates were obtained for each pair of sites using the coancestry distance (Reynolds et al. 1983) and Nei's unbiased genetic distance (Nei 1978). Bivariate plots of genetic versus geographic (along fiord and open coastline distances measured on nautical charts and reported in km) distance were constructed for each genetic distance measure, and the significance of the correlation between genetic and geographic distance was evaluated using the Mantel test (Mantel 1967; Sokal and Rohlf 1995) procedure in TFPGA. A normalized Z-test was performed in which the observed value after 999 permutations should be significantly larger than expected by chance in order for the null hypothesis, no association between the pair of matrices, to be rejected. The general framework of Hutchinson and Templeton (1999) was used to interpret the bivariate plots and infer the relative importance of drift and migration in this system. Assuming a stepping stone model (Kimura and Weiss 1964) and populations at drift/migration equilibrium, simple diffusion would produce a positive correlation between genetic and geographic distance (gene flow more common over short distances). However, if either gene flow or genetic drift is overly influential, no correlation is predicted. In order to differentiate these two scenarios, the scatter among genetic distances can be examined. Minimal scatter of genetic distances will occur if gene flow is the overriding force and gene flow is acting as homogenizing factor (creating near-equal genetic distances) regardless of geographic distance. In contrast, wide scatter will occur when genetic drift is more influential and populations are differentiating randomly.
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Congruence between genetic markers

For each type of marker (allozyme and AFLP) pairwise estimates of Nei’s (1978) genetic distances were compared using a Mantel test in TFPGA. Locus-independent processes such as drift and migration are the primary drivers of evolution. If a group of populations experienced different evolutionary histories involving drift and migration, then they would differ for their level of diversity in a similar manner for the two markers. A lack of correlation between the two distance matrices would indicate that ‘marker-specific factors’ are sufficiently different between populations to generate different levels of genetic diversity.

Historical and hydrographic tests using allozymes and AFLP’s

Age of fiord habitat vs. genetic diversity and genetic differentiation

At the end of the last ice age, fiords changed from oligotrophic lakes to extensions of the Tasman Sea as the outer sills were overtopped by rising seawater. As a result, the depth of the terminal sill of a fiord can be used as a proxy for when the marine habitat became available for colonization (Smith 2001). It is generally assumed that older populations contain higher levels of genetic diversity through the persistent accumulation of alleles (via mutation and migration) than do younger populations, and empirical tests suggest this is the case (Bottin et al. 2005; Haag et al. 2005). To test for a correlation between genetic diversity and the estimated age of a fiord marine habitat, linear regression analyses were performed using SigmaPlot 5.00 (SPSS Science, Chicago, Illinois). Because outer sites are located on the sill or near the entrance to a fiord (and therefore may have been accessible to marine organisms before a fiord was flooded), only sites within the fiords were used for this analysis. Expected heterozygosities for both allozyme and AFLP data were used as an estimate for genetic diversity. Sill depth was calculated using fine scale bathymetric data from Land Information New Zealand (LINZ) (Smith 2001) (Table 3.2). In addition to testing whether the age of individual fiords correlates to levels of genetic diversity, it is also possible to test whether colonization of sites correlates with the predicted age of the site (sites closer to the outer coast should have been accessible for colonization before inner
sites with roughly 3000 years difference between flooding of outer versus inner sites (Smith 2001)). To test for a correlation between genetic diversity and the location of a site relative to the outer coast, linear regression analyses were performed using SigmaPlot 5.00 (SPSS Science, Chicago, Illinois). As with the previous analysis, estimated heterozygosities were used as estimates for genetic diversity. Distance from the outer coast was measured on nautical charts and reported in kilometres (Table 3.2).

Table 3.2. Entrance sill depth, fiord age (estimated from entrance sill depth), distance from the open ocean, and amount of fresh water (measured as Effective Freshwater Depth) for individual sites.

<table>
<thead>
<tr>
<th>Fiord</th>
<th>Entrance Sill Depth (m)</th>
<th>Estimated Age (yr BP)</th>
<th>Distance from the outer coast (outer site/inner site) (km)</th>
<th>Effective Freshwater Depth (outer site/inner site)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bligh Sound</td>
<td>77</td>
<td>14,000</td>
<td>7.5/17</td>
<td>1.25/1.15</td>
</tr>
<tr>
<td>George Sound</td>
<td>45</td>
<td>11,000</td>
<td>5/17</td>
<td>1.13/1.10</td>
</tr>
<tr>
<td>Caswell Sound</td>
<td>66</td>
<td>13,000</td>
<td>19.1</td>
<td>1.93</td>
</tr>
<tr>
<td>Charles Sound</td>
<td>83</td>
<td>14,500</td>
<td>7.3</td>
<td>1.39</td>
</tr>
<tr>
<td>Nancy Sound</td>
<td>77</td>
<td>14,000</td>
<td>11</td>
<td>1.15</td>
</tr>
<tr>
<td>Thompson Sound</td>
<td>115</td>
<td>18,000</td>
<td>11.5/18/32</td>
<td>NA</td>
</tr>
<tr>
<td>Doubtful Sound</td>
<td>101</td>
<td>16,000</td>
<td>5/13.5/18.5/22.5</td>
<td>1.53/1.97/2.29/2.28</td>
</tr>
<tr>
<td>Dagg Sound</td>
<td>42</td>
<td>10,500</td>
<td>8</td>
<td>3.98</td>
</tr>
<tr>
<td>Breaksea Sound</td>
<td>93</td>
<td>15,500</td>
<td>8/27</td>
<td>1.44/1.36</td>
</tr>
<tr>
<td>Wet Jacket Sound</td>
<td>93</td>
<td>15,500</td>
<td>2/12</td>
<td>1.90/1.87</td>
</tr>
<tr>
<td>Dusky Sound</td>
<td>65</td>
<td>12,500</td>
<td>9/35</td>
<td>2.01/2.12</td>
</tr>
<tr>
<td>Long Sound</td>
<td>25</td>
<td>9,000</td>
<td>12/37</td>
<td>4.41</td>
</tr>
</tbody>
</table>
On the other hand, according to population genetic theory, populations in the older fiords would be the most likely to show high levels of genetic divergence due to longer periods of isolation and ensuing drift over time, assuming populations are in drift migration equilibrium and that populations are indeed isolated (e.g. Giles and Goudet 1997). The probability that sills affect the flow of seawater in most of the fiords is small, however, since most of the sills are deep (Table 3.2). To test for a correlation between genetic divergence and sill depth, linear regression analyses were performed using SigmaPlot. Because $\theta_{ST}$ values are calculated as pairwise comparisons, MDS coordinates were used to represent the genetic divergence of each population. Coordinates on the MDS axis showing the most variation among populations were used as an estimator of genetic divergence (coordinates on the second MDS axis showed similar patterns and therefore are not presented).

**Estuarine circulation vs. genetic differentiation**

Because brachiopods with planktonic larvae disperse passively within the water column, hydrology of their environment plays a key role in dispersal. Within the fiords, estuarine circulation dominates overall water movements (Gibbs 2001). The amount of freshwater and the thickness of the LSL relative to the catchment area, in combination with the entrance sill topology (e.g. the sill depth), will affect the strength of the estuarine circulation within a fiord. In those fiords where the entrance sill is shallower, water flow may be constricted. However, since most of the sills in Fiordland are deep, the amount of freshwater and the thickness of the LSL can be used as a proxy for the strength of estuarine circulation. Effective freshwater depth (EFD) is described as the integrated thickness of the LSL at each site over time (Gibbs et al. 2000); this measurement was used as a proxy for the strength of estuarine circulation (Perrin 2002). EFD measurements were accomplished using conductivity, temperature, and depth surveys (CTD) across Fiordland in both 1998 and 1999 (a mean value was calculated). To test for a potential relationship between estuarine circulation and larval dispersal, linear regression analysis was performed using SigmaPlot. As with the previously described analysis of fiord age, coordinates from the MDS axis showing the most variation among populations were used as an estimator of genetic divergence.
Results

Four allozyme systems were consistently polymorphic. In total, five alleles were identified at the \( H_k \) locus, nine at the \( Mpi \) locus, four at the \( 6Pgdl \) locus, and six at the \( Pgi \) locus. A total of 182 scoreable, polymorphic AFLP presumptive loci were generated for \( T. \ sanguinea \) using two primer combinations. Only 2 of the 296 bands generated (0.68\%) among the 16 individuals analysed in duplicate were not reproducible; both of these bands were in a single individual, and in all other cases duplications from multiple extractions and amplifications produced identical banding (Fig. 3.3). This very low error rate indicated the consistency of the AFLP procedure.

Genetic diversity

The most common alleles at each allozyme locus were present in all of the fiord and non-fiord populations at similar frequencies, though less common alleles were not represented in all of the populations (Table 3.3). Some of these ‘uncommon’ alleles were represented in only a few populations. The slowest electromorph at \( Mpi \) and an intermediate electromorph at the \( 6Pgdl \) locus were found only at 2-GEO I. An intermediate electromorph at the \( Pgi \) locus was found only at 1-BLI I. In all cases, these alleles were found only in a single heterozygous individual. Overall, the presence of allozyme alleles did not suggest a geographic correlation. In addition, there was no correlation between sample size and the number of private or rare alleles \( (r^2 = 0.0362, p = 0.6827; r^2 = 0.5935, p = 0.8818 \) respectively). However, there was a significant, positive correlation between sample size and number of alleles found at a site \( (r^2 = 0.9985, p = 0.0013) \). All possible combinations of alleles at different loci were found to be independent \( (p ≥ 0.231 \) for all comparisons) when tested for linkage disequilibrium.
Fig. 3.3. A portion of an amplified fragment length polymorphism gel for *T. sanguinea*. Lanes A and B are from two separate extractions from the same animal while lane C is the same animal again but the entire process has been replicated from the extraction used in lane A. Lanes 1 and 2 represent two extractions from the same animal, and lanes 3 and 4 are replicates from the extraction used in lane 1 (lane 2 is darker than the others because slightly more sample was loaded in this lane). Note the consistency of banding among replicates from an animal. This contrasts with the unique banding patterns between individuals.
Table 3.3. *Terebratella sanguinea* allele frequencies for 10 localities from Fiordland and Stewart Island, New Zealand. Allele codes indicate relative mobility with ‘A’ indicating the fastest allele (greatest electrophoretic mobility) at a locus. Numbers of animals from a population genotyped for each locus are given. Private alleles are indicated by bold type.

<table>
<thead>
<tr>
<th>Locus</th>
<th>Bligh</th>
<th>George O</th>
<th>George I</th>
<th>Thompson</th>
<th>Bradshaw</th>
<th>Dagg</th>
<th>Dusky</th>
<th>Giriles Is.</th>
<th>Paterson’s</th>
<th>Pegasus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hk</td>
<td>n = 42</td>
<td>23</td>
<td>24</td>
<td>40</td>
<td>44</td>
<td>44</td>
<td>29</td>
<td>22</td>
<td>16</td>
<td>38</td>
</tr>
<tr>
<td>A</td>
<td>0.000</td>
<td>0.000</td>
<td>0.021</td>
<td>0.000</td>
<td>0.091</td>
<td>0.000</td>
<td>0.034</td>
<td>0.000</td>
<td>0.000</td>
<td>0.053</td>
</tr>
<tr>
<td>B</td>
<td>0.190</td>
<td>0.217</td>
<td>0.292</td>
<td>0.300</td>
<td>0.148</td>
<td>0.148</td>
<td>0.155</td>
<td>0.205</td>
<td>0.156</td>
<td>0.105</td>
</tr>
<tr>
<td>C</td>
<td>0.536</td>
<td>0.609</td>
<td>0.479</td>
<td>0.450</td>
<td>0.568</td>
<td>0.414</td>
<td>0.523</td>
<td>0.469</td>
<td>0.313</td>
<td>0.211</td>
</tr>
<tr>
<td>D</td>
<td>0.167</td>
<td>0.152</td>
<td>0.188</td>
<td>0.237</td>
<td>0.239</td>
<td>0.261</td>
<td>0.345</td>
<td>0.227</td>
<td>0.063</td>
<td>0.013</td>
</tr>
<tr>
<td>E</td>
<td>0.107</td>
<td>0.022</td>
<td>0.021</td>
<td>0.013</td>
<td>0.034</td>
<td>0.023</td>
<td>0.052</td>
<td>0.045</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Mpi</td>
<td>n = 49</td>
<td>27</td>
<td>28</td>
<td>44</td>
<td>43</td>
<td>49</td>
<td>28</td>
<td>28</td>
<td>11</td>
<td>40</td>
</tr>
<tr>
<td>A</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.034</td>
<td>0.000</td>
<td>0.020</td>
<td>0.000</td>
<td>0.018</td>
<td>0.000</td>
<td>0.013</td>
</tr>
<tr>
<td>B</td>
<td>0.020</td>
<td>0.019</td>
<td>0.018</td>
<td>0.045</td>
<td>0.000</td>
<td>0.071</td>
<td>0.071</td>
<td>0.054</td>
<td>0.091</td>
<td>0.025</td>
</tr>
<tr>
<td>C</td>
<td>0.184</td>
<td>0.241</td>
<td>0.107</td>
<td>0.239</td>
<td>0.081</td>
<td>0.204</td>
<td>0.179</td>
<td>0.179</td>
<td>0.182</td>
<td>0.087</td>
</tr>
<tr>
<td>D</td>
<td>0.214</td>
<td>0.204</td>
<td>0.214</td>
<td>0.205</td>
<td>0.174</td>
<td>0.296</td>
<td>0.089</td>
<td>0.214</td>
<td>0.136</td>
<td>0.300</td>
</tr>
<tr>
<td>E</td>
<td>0.429</td>
<td>0.296</td>
<td>0.304</td>
<td>0.273</td>
<td>0.442</td>
<td>0.235</td>
<td>0.232</td>
<td>0.411</td>
<td>0.455</td>
<td>0.300</td>
</tr>
<tr>
<td>F</td>
<td>0.102</td>
<td>0.222</td>
<td>0.304</td>
<td>0.136</td>
<td>0.233</td>
<td>0.133</td>
<td>0.304</td>
<td>0.089</td>
<td>0.091</td>
<td>0.138</td>
</tr>
<tr>
<td>G</td>
<td>0.031</td>
<td>0.000</td>
<td>0.000</td>
<td>0.068</td>
<td>0.058</td>
<td>0.031</td>
<td>0.071</td>
<td>0.036</td>
<td>0.045</td>
<td>0.087</td>
</tr>
<tr>
<td>H</td>
<td>0.020</td>
<td>0.019</td>
<td>0.036</td>
<td>0.000</td>
<td>0.012</td>
<td>0.010</td>
<td>0.054</td>
<td>0.000</td>
<td>0.000</td>
<td>0.050</td>
</tr>
<tr>
<td>I</td>
<td>0.000</td>
<td>0.000</td>
<td>0.018</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>6Pgld</td>
<td>n = 41</td>
<td>26</td>
<td>32</td>
<td>33</td>
<td>25</td>
<td>46</td>
<td>27</td>
<td>24</td>
<td>15</td>
<td>41</td>
</tr>
<tr>
<td>A</td>
<td>0.085</td>
<td>0.000</td>
<td>0.016</td>
<td>0.015</td>
<td>0.000</td>
<td>0.043</td>
<td>0.000</td>
<td>0.063</td>
<td>0.000</td>
<td>0.024</td>
</tr>
<tr>
<td>B</td>
<td>0.890</td>
<td>0.942</td>
<td>0.891</td>
<td>0.955</td>
<td>1.000</td>
<td>0.935</td>
<td>0.881</td>
<td>0.938</td>
<td>0.900</td>
<td>0.963</td>
</tr>
<tr>
<td>C</td>
<td>0.000</td>
<td>0.000</td>
<td>0.016</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>D</td>
<td>0.024</td>
<td>0.058</td>
<td>0.073</td>
<td>0.030</td>
<td>0.000</td>
<td>0.022</td>
<td>0.019</td>
<td>0.000</td>
<td>0.100</td>
<td>0.012</td>
</tr>
<tr>
<td>Pgl</td>
<td>n = 52</td>
<td>29</td>
<td>29</td>
<td>43</td>
<td>45</td>
<td>52</td>
<td>32</td>
<td>26</td>
<td>47</td>
<td>20</td>
</tr>
<tr>
<td>A</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.012</td>
<td>0.000</td>
<td>0.010</td>
<td>0.000</td>
<td>0.018</td>
<td>0.011</td>
<td>0.000</td>
</tr>
<tr>
<td>B</td>
<td>0.038</td>
<td>0.052</td>
<td>0.034</td>
<td>0.035</td>
<td>0.022</td>
<td>0.038</td>
<td>0.094</td>
<td>0.036</td>
<td>0.084</td>
<td>0.050</td>
</tr>
<tr>
<td>C</td>
<td>0.673</td>
<td>0.759</td>
<td>0.828</td>
<td>0.744</td>
<td>0.711</td>
<td>0.760</td>
<td>0.766</td>
<td>0.661</td>
<td>0.628</td>
<td>0.725</td>
</tr>
<tr>
<td>D</td>
<td>0.010</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>E</td>
<td>0.280</td>
<td>0.190</td>
<td>0.138</td>
<td>0.209</td>
<td>0.267</td>
<td>0.183</td>
<td>0.141</td>
<td>0.268</td>
<td>0.255</td>
<td>0.200</td>
</tr>
<tr>
<td>F</td>
<td>0.019</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.010</td>
<td>0.000</td>
<td>0.016</td>
<td>0.043</td>
<td>0.025</td>
</tr>
</tbody>
</table>
A pattern of genetic diversity, measured as the presence of rare AFLP fragments, was not consistent with geography. The number of fragments found at only one site ranged from 0 (1-BLI I, 2-GEO I, 5-NAN I, 6-THM CM, 7-DFL CC, 7-DFL FA, 9-BRK O, 9-BRK I, 10-WTJ O) to 3 (1-BLI O, 3-CAS I, 4-CHR I) (Table 3.4; see Fig. 3.1 for site locations). The number of fragments found at only 2 or 3 sites ranged from 1 (2-GEO O, 3-CAS I, 6-THM RP, 9-BRK O) to 9 (7-DFL FA) (Table 3.4; see Fig. 3.: for site locations). In addition, there was no correlation between sample size and the number of private or rare fragments \( r^2 = 0.0693, p = 0.2622; r^2 = 0.0092, p = 0.6872 \) respectively. However, there was a significant, positive correlation between sample size and number of fragments (presumptive loci) found at a site \( r^2 = 0.2991, p = 0.0126 \).

**Heterozygosity**

At two of the inner fiord sampling sites, heterozygosity levels differed significantly from Hardy-Weinberg expectations for an individual allozyme locus. Site 1-BLI I exhibited a significant heterozygote excess at the \( Hk \) locus at the corrected table-wide significance level \( p < 0.01 \); 2-GEO I exhibited a significant heterozygote deficit (after sequential Bonferroni correction) at both the \( Hk \) and \( Pgi \) loci (for each locus \( p < 0.01 \)) (Table 3.5). A significant heterozygote deficiency over all four loci was detected at the sites in George Sound and the sites in the Thompson arm of Doubtful Sound \( p < 0.05 \).

Nei’s (1978) unbiased expected heterozygosity \( (H) \) estimations for AFLP data ranged from 0.0717 at 9-BRK O to 0.1322 at 12-LNG I; the proportion of polymorphic presumptive loci \( (P) \) ranged from a minimum of 21.98% (6-THM RP) to a maximum of 45.25% at 12-LNG I (Table 3.4). Neither \( H \) nor \( P \) was significantly correlated with the number of animals sampled in a population \( H: r^2 = 0.00874, p = 0.7034; P: r^2 = 0.0763, p = 0.1333 \); the Long Sound site was removed before calculating the regressions because it was judged to be an outlier for both \( H \) and \( P \).
Table 3.4. Location, sample size, Nei’s (1978) unbiased expected heterozygosity ($H$), the proportion of polymorphic presumptive loci ($P$), the number of fragments found only at one sampling site, the number of fragments found in two or three sampling sites, and the total number of fragments found at each of the sampling sites.

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>n</th>
<th>Expected Heterozygosity</th>
<th>% polymorphic</th>
<th>Fragments in 1 pop</th>
<th>Fragments in 2 or 3 pops</th>
<th>Total Fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-BLI O</td>
<td>Bligh Sound Bligh George</td>
<td>20</td>
<td>0.0877</td>
<td>34.07</td>
<td>3</td>
<td>4</td>
<td>92</td>
</tr>
<tr>
<td>1-BLI I</td>
<td>Bligh Sound George</td>
<td>17</td>
<td>0.0868</td>
<td>28.57</td>
<td>0</td>
<td>4</td>
<td>96</td>
</tr>
<tr>
<td>2-GEO O</td>
<td>Sound George</td>
<td>20</td>
<td>0.0766</td>
<td>30.77</td>
<td>1</td>
<td>1</td>
<td>81</td>
</tr>
<tr>
<td>2-GEO I</td>
<td>Sound Caswell Sound Charles</td>
<td>21</td>
<td>0.0899</td>
<td>26.37</td>
<td>0</td>
<td>4</td>
<td>106</td>
</tr>
<tr>
<td>3-CAS I</td>
<td>Sound</td>
<td>19</td>
<td>0.0751</td>
<td>26.92</td>
<td>3</td>
<td>1</td>
<td>84</td>
</tr>
<tr>
<td>4-CHR I</td>
<td>Sound Nancy</td>
<td>22</td>
<td>0.0865</td>
<td>25.82</td>
<td>3</td>
<td>5</td>
<td>100</td>
</tr>
<tr>
<td>5-NAN I</td>
<td>Sound Thompson</td>
<td>20</td>
<td>0.0948</td>
<td>42.86</td>
<td>0</td>
<td>8</td>
<td>110</td>
</tr>
<tr>
<td>6-THM TM</td>
<td>Sound Thompson</td>
<td>16</td>
<td>0.0793</td>
<td>26.92</td>
<td>2</td>
<td>6</td>
<td>84</td>
</tr>
<tr>
<td>6-THM RP</td>
<td>Sound Thompson</td>
<td>11</td>
<td>0.0801</td>
<td>21.98</td>
<td>1</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>6-THM CM</td>
<td>Sound Doubtful Doubtful</td>
<td>13</td>
<td>0.0762</td>
<td>23.63</td>
<td>0</td>
<td>2</td>
<td>73</td>
</tr>
<tr>
<td>7-DFL CC</td>
<td>Doubtful</td>
<td>15</td>
<td>0.0779</td>
<td>24.18</td>
<td>0</td>
<td>2</td>
<td>76</td>
</tr>
<tr>
<td>7-DFL FA</td>
<td>Doubtful</td>
<td>15</td>
<td>0.0890</td>
<td>27.47</td>
<td>0</td>
<td>9</td>
<td>85</td>
</tr>
<tr>
<td>7-DFL CA</td>
<td>Doubtful</td>
<td>17</td>
<td>0.0799</td>
<td>28.02</td>
<td>1</td>
<td>3</td>
<td>82</td>
</tr>
<tr>
<td>7-DFL OZ</td>
<td>Doubtful Dagg</td>
<td>15</td>
<td>0.0861</td>
<td>28.57</td>
<td>1</td>
<td>3</td>
<td>80</td>
</tr>
<tr>
<td>8-DAG I</td>
<td>Breaksea</td>
<td>17</td>
<td>0.0852</td>
<td>27.47</td>
<td>1</td>
<td>3</td>
<td>76</td>
</tr>
<tr>
<td>9-BRK O</td>
<td>Breaksea</td>
<td>20</td>
<td>0.0717</td>
<td>27.47</td>
<td>0</td>
<td>1</td>
<td>79</td>
</tr>
<tr>
<td>9-BRK I</td>
<td>Breaksea</td>
<td>20</td>
<td>0.0883</td>
<td>31.87</td>
<td>0</td>
<td>2</td>
<td>89</td>
</tr>
<tr>
<td>10-WTJ O</td>
<td>Wet Jacket</td>
<td>20</td>
<td>0.0777</td>
<td>30.22</td>
<td>0</td>
<td>5</td>
<td>80</td>
</tr>
<tr>
<td>10-WTJ I</td>
<td>Wet Jacket Long</td>
<td>22</td>
<td>0.0740</td>
<td>21.43</td>
<td>2</td>
<td>3</td>
<td>86</td>
</tr>
<tr>
<td>12-LNG I</td>
<td>Sound</td>
<td>18</td>
<td>0.1322</td>
<td>47.25</td>
<td>2</td>
<td>8</td>
<td>110</td>
</tr>
</tbody>
</table>
Table 3.5. *Terebratella sanguinea* genetic variability in the ten populations where allozyme loci were sampled. For each of the allozyme loci, the number of direct count heterozygotes and the number of heterozygotes expected under Hardy-Weinberg equilibrium are shown. 6PGD was monomorphic for the individuals at the Bradshaw site, therefore an estimation of expected levels of heterozygosity could not be calculated for this site/locus combination. For overall probabilities and standard errors, a score test was used instead of a probability test. This test was a one-tailed test for which the alternate hypothesis was heterozygote deficiency. Values in bold print are significantly different from zero at the table-wide significance level. Site numbers as in Table 3.1.

<table>
<thead>
<tr>
<th>Population</th>
<th>Hk</th>
<th>Mpi</th>
<th>6PGD</th>
<th>Pgi</th>
<th>Overall</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Obs</td>
<td>Exp</td>
<td>p</td>
<td>SE</td>
<td>Obs</td>
</tr>
<tr>
<td>1-BLI 1</td>
<td>0.6905</td>
<td>0.6452</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.6531</td>
</tr>
<tr>
<td>2-GEO O</td>
<td>0.4783</td>
<td>0.5710</td>
<td>0.2672</td>
<td>0.0000</td>
<td>0.6296</td>
</tr>
<tr>
<td>2-GEO I</td>
<td>0.4167</td>
<td>0.6561</td>
<td>0.0008</td>
<td>0.0004</td>
<td>0.7857</td>
</tr>
<tr>
<td>6-THM TM</td>
<td>0.6500</td>
<td>0.6592</td>
<td>0.0884</td>
<td>0.0000</td>
<td>0.7273</td>
</tr>
<tr>
<td>6-THM BD</td>
<td>0.5682</td>
<td>0.6808</td>
<td>0.0161</td>
<td>0.0018</td>
<td>0.6512</td>
</tr>
<tr>
<td>8-DAG 1</td>
<td>0.5909</td>
<td>0.5933</td>
<td>1.0000</td>
<td>0.0000</td>
<td>0.7143</td>
</tr>
<tr>
<td>11-DSK O</td>
<td>0.6552</td>
<td>0.6939</td>
<td>0.5898</td>
<td>0.0091</td>
<td>0.8929</td>
</tr>
<tr>
<td>11-DSK 1</td>
<td>0.5455</td>
<td>0.6459</td>
<td>0.0462</td>
<td>0.0000</td>
<td>0.7857</td>
</tr>
<tr>
<td>13-STW PT</td>
<td>0.6250</td>
<td>0.6754</td>
<td>0.6807</td>
<td>0.0000</td>
<td>0.6364</td>
</tr>
<tr>
<td>13-STW PG</td>
<td>0.5263</td>
<td>0.5667</td>
<td>0.1907</td>
<td>0.0104</td>
<td>0.7000</td>
</tr>
</tbody>
</table>
Chapter 3: Terebratella sanguinea in the New Zealand fiords

Population structure

Results from the AMOVA indicated that genetic differentiation at all of the hierarchical levels did not differ significantly from 0 (among fiords ($\Phi_{ST} < 0.001, p = 0.529$), among sites within fiords ($\Phi_{ST} < 0.001, p = 0.890$), and among individuals within sites ($\Phi_{ST} < 0.001, p = 0.990$)) at allozyme loci. Although there was no significant differentiation among individuals within sites, one hundred percent of the variation was found among individuals within sites. Genetic differentiation among sites, measured as $\theta$, calculated for each of the four allozyme loci did not differ significantly from zero at any of the loci ($p \geq 0.44$; data not shown). When $\theta$ values were averaged over all four loci, estimates for data from all 10 sites were not significantly different from zero ($\theta_{ST} = 0.0089, p = 0.98$), however, pairwise comparisons between sampling sites uncovered significant differences between some of the sites (Table 3.6). The most consistent differences for the pairwise comparisons were between 11-DSK O and the other sites; six of the nine comparisons including 11-DSK O produced $\theta$ values significantly different from 0, and one of the largest $\theta$ values obtained was between the two sites located within Dusky Sound (11-DSK O and 11-DSK I). Additionally, both 1-BLI I and 2-GEO I (the two sites out of Hardy-Weinberg equilibrium) each were significantly different in four of nine pairwise comparisons.

Analysis of AFLP data using $F$-statistics and AMOVA suggested significant but subtle genetic differentiation at all hierarchical levels, and the largest amount of genetic differentiation occurred within sites. Ninety-eight percent of the genetic variation was observed within sites ($p = 0.001$) whereas only 1% occurred among fiords ($p = 0.001$) and 1% among sites within fiords ($p = 0.002$). The $\theta$ estimate for among site differentiation ($\theta_{ST}$) was also higher than the among fiord estimate ($\theta_{ST}$) ($\theta_{ST} = 0.0193$, 95% CI = 0.0108 to 0.0301; $\theta_{ST} = 0.0130$, 95% CI = 0.0087 to 0.0220). Confidence limits of $\theta$ did not overlap 0 indicating that genetic differentiation among sites and among fiords was statistically significant. However, the low values generated by each method imply that differentiation is slight. Point estimates of $\Phi$-statistics were within the confidence intervals obtained for the corresponding $\theta$-values suggesting that the analysis of estimated allele frequencies and raw marker phenotypes yielded similar information.
Table 3.6. Pairwise comparisons (between ten sampling locations) of $\theta_{ST}$ calculated over four allozyme loci. Significance of $\theta_{ST}$ was tested using 4500 permutations and a Bonferroni correction applied (* $p < 0.05$, ** $p < 0.01$). Site numbers as in Table 3.1.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-BLI I</td>
<td>0.0026</td>
<td>0.0167*</td>
<td>0.0086</td>
<td>0.0070</td>
<td>0.0104*</td>
<td>0.0331*</td>
<td>-0.0122</td>
<td>-0.0091</td>
<td>0.0078*</td>
<td>-0.0074</td>
</tr>
<tr>
<td>2-GEO O</td>
<td></td>
<td>-0.0067</td>
<td>-0.0025</td>
<td>0.0099</td>
<td>-0.0036</td>
<td>0.0132</td>
<td>-0.0019</td>
<td>-0.0018</td>
<td>0.0247</td>
<td>0.0188**</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>0.0027*</td>
<td>0.0109</td>
<td>0.0116</td>
<td>0.0069*</td>
<td>0.0123**</td>
<td>0.0050</td>
<td>0.0188**</td>
<td>0.0040</td>
</tr>
<tr>
<td>6-THM TM</td>
<td></td>
<td></td>
<td></td>
<td>0.0154</td>
<td>0.0020</td>
<td>0.0100**</td>
<td>-0.0024</td>
<td>-0.0040</td>
<td>0.0087</td>
<td>0.0037</td>
</tr>
<tr>
<td>6-THM BD</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0196*</td>
<td>0.0161</td>
<td>-0.0011</td>
<td>-0.0043</td>
<td>0.0057</td>
<td>0.0319**</td>
</tr>
<tr>
<td>8-DAG I</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0164*</td>
<td>0.0012</td>
<td>0.0017</td>
<td>0.0090</td>
<td></td>
</tr>
<tr>
<td>11-DSK O</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0227**</td>
<td>0.0057</td>
<td></td>
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</tr>
<tr>
<td>11-DSK I</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.0170</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13-STW PT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.0002</td>
<td></td>
</tr>
<tr>
<td>13-STW PG</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.0090</td>
</tr>
</tbody>
</table>
AMOVA analyses were re-calculated after removing the Long Sound site. Without the Long Sound site, 99% of genetic variation occurred within sites (p = 0.001) and 1% among sites within fiords (p = 0.001); none of the variation occurred between fiords. The θ estimates showed a similar pattern (θ_ST = 0.0105, 95% CI = 0.0050 to 0.0167; θ_TT = 0.0029, 95% CI = -0.0011 to 0.0064). However, less differentiation was observed among sites (θ_ST values were lower) when the Long Sound site was removed than in the previous analysis.

For both the allozyme and the AFLP data, the estimates of gene flow were >> 1, the theoretical limit at which substantial genetic divergence between populations is prevented (Wright 1931) (overall loci and populations for each marker allozymes: N_m = 30.6; AFLP: N_m = 12.7). Further N_m estimates based on pairwise comparisons varied by several orders of magnitude and were also >> 1 (allozymes: 7<N_m<2499; AFLP: 65<N_m<2499). If hydrodynamics limit dispersal and therefore the fiord is considered a population, an estimate of the time required to near equilibrium > 1 million years (using allozyme N_m estimates: 1,075,641; using AFLP N_m estimates: 2,562,434).

The MDS ordination of allozyme data suggested a lack of population structuring. No distinct separations were visible along either the vertical or horizontal axis of the MDS plot (Fig. 3.4). The MDS ordinations of AFLP data suggested differentiation between the Long Sound site and the rest of the sites sampled, and much lower levels of differentiation among the “non-Long” sites (Fig. 3.5 (a)). When the Long Sound site was removed from the ordination analysis, no distinct clusters of other sites emerged, however site 6-THM RP separated from the other sites (Fig. 3.5 (b)). For both allozyme and AFLP data, both of the distance measures illustrated the same pattern; therefore, only the ordinations from the coancestry distance measure are shown.
Fig. 3.4. Multi-dimensional scaling ordination calculated from allozyme data (4 loci) of *Terebratella sanguinea*. Site numbers as in Table 3.1. Ordinations were created in PRIMER v5 (Clarke and Gorley 2001).
Fig. 3.5 (a). *Terebratella sanguinea* multi-dimensional scaling (MDS) ordination for AFLP data (182 loci) calculated from the coancestry distance matrix (Reynolds et al. 1983). (b) The same ordination as described in (a) but with the Long Sound site removed. For both A and B, site numbers are as in Table 3.1. Ordinations were created using PRIMER v5 (Clarke and Gorley 2001).
Isolation by distance using allozymes and AFLP’s

Use of the Mantel test revealed non-significant relationships between genetic distance measures and geographic distances for allozyme data (Coancestry: $r = -0.0988$, $p = 0.6760$; Nei: $r = 0.0952$, $p = 0.6200$) (Fig. 3.6(a)). The overall Mantel test for the AFLP data, however, showed a significant correlation (Coancestry: $r = 0.5604$; Nei: $r = 0.6516$) between pairwise matrices of the two genetic markers and geographical distances (Coancestry: $p = 0.0240$; Nei $p = 0.0010$) (Fig. 3.6(b)). When pairwise comparisons including the Long Sound site were removed from the analysis, the correlation was no longer significant (Coancestry: $r = 0.0690$, $p = 0.2660$, Nei: $r^2 = 0.0696$, $p = 0.2580$) (Fig. 3.6(b)).

Congruence between markers

For those sites where both allozyme and AFLP data were available, genetic distances from the two markers were compared using a Mantel test; there was no significant relationship between genetic distances produced by the two markers ($r = -0.2605$, $p = 0.3020$).

Historical and hydrographic tests using allozymes and AFLP’s

Age of fiord habitat vs. genetic diversity and genetic differentiation

Regression analysis of sill depth versus estimated heterozygosity suggested no trend in genetic diversity based on allozyme data ($r^2 = 0.31$, $p = 0.1966$) (Fig. 3.7(a)), however a significant negative correlation was detected with the complete AFLP data set ($r^2 = 0.42$, $p = 0.0314$) (Fig. 3.8(a)). However, the assumption of constant variance was violated in the analysis of the complete AFLP data set; transforming the data could not rectify this. Regression analysis is robust to violations (Zar 1999), but these results should be interpreted with caution. When the Long Sound site was removed from the analysis, the correlation between sill depth (a proxy for fiord age) and heterozygosity (a measure of genetic diversity) was no longer significant ($r^2 = 0.10$, $p = 0.3696$) (Fig. 3.8(a)).
3.9(a)). All three analyses had lower power than the 0.8000 power desired for a regression analysis.

No significant correlation between sill depth and genetic differentiation was detected (allozyme data: \( r^2 = 0.05, p = 0.6356 \); AFLP data: \( r^2 = 0.11, p = 0.3379 \) (Figs. 3.7(b) and 3.8(b)). When the Long Sound site was removed from the data set, the relationship between sill depth and genetic differentiation did not change \( (r^2 = 0.11, p = 0.3835) \) (Fig. 3.9(b)). All three analyses had lower power than the 0.8000 power desired for a regression analysis.

A pattern similar to the sill depth/heterozygosity analysis was observed in the relationship between distance to the open coast and expected heterozygosity. No significant correlation was found with the allozyme data \( (r^2 = 0.14, p = 0.2882) \), but a significant correlation was found with the AFLP data (a positive correlation in this case; \( r^2 = 0.43, p = 0.0426 \) (Figs. 3.7(c) and 3.8(c)). If the Long Sound site was removed, again a significant relationship no longer existed \( (r^2 < 0.0001, p = 0.9681) \) (Fig. 3.9(c)). All three analyses had lower power than the 0.8000 power desired for a regression analysis.

**Estuarine circulation vs. genetic differentiation**

A significant positive correlation was found between the effective freshwater depth and genetic differentiation (measured as MDS scores), but only with the complete AFLP data set \( (r^2 = 0.68, p < 0.0001) \) (Fig. 3.8(d)). With the allozyme data set as well as the AFLP data set minus the Long Sound site, no significant trend was detected \( (r^2 = 0.34, p = 0.2246; r^2 = 0.18, p = 0.1061 \) respectively) (Figs. 3.7(d) and 3.9(d)). The analyses performed with the allozyme data and the AFLP data without the Long Sound site were below the desired power level.
Fig. 3.6. Linear regression of Nei's (1978) genetic distance versus geographic distance (km) for each pair of sites. The $p$-values were obtained from the application of a Mantel matrix correlation analysis (Mantel 1967) using TFGPA (Miller 1997b). (a) Genetic distance values calculated from 4 allozyme loci and (b) Genetic distance values calculated from 182 AFLP loci; (■) comparisons including Long Sound sites; (∆) comparisons including all other fiord sites.
Fig. 3.7. Plot and linear regression of historical and hydrographic data versus allozyme data. The relationship between: (a) sill depth (a proxy for fiord age) and overall heterozygosity (an estimate of genetic diversity). (b) sill depth (a proxy for fiord age) and MDS scores (an estimate of genetic differentiation).
Fig. 3.7(cont’d). Plot and linear regression of historical and hydrographic data versus allozyme data. The relationship between: (c) distance to the outer coast (a proxy for the relative age of the marine habitat) and overall heterozygosity (an estimate of genetic diversity). (d) effective freshwater depth (a proxy for estuarine circulation) and MDS scores (an estimate of genetic differentiation).
Fig. 3.8. Plot and linear regression of historical and hydrographic data versus AFLP data. The relationship between: (a) sill depth (a proxy for fiord age) and expected heterozygosity (an estimate of genetic diversity). (b) sill depth (a proxy for fiord age) and MDS scores (an estimate of genetic differentiation).
Fig. 3.8 (cont’d). Plot and linear regression of historical and hydrographic data versus AFLP data. The relationship between: (c) distance to the outer coast (a proxy for the relative age of the marine habitat) and expected heterozygosity (an estimate of genetic diversity), and (d) effective freshwater depth (a proxy for estuarine circulation) and MDS scores (an estimate of genetic differentiation).
Fig. 3.9. Plot and linear regression of historical and hydrographic data versus AFLP data after the Long Sound site has been removed from the data set. The relationship between: (a) sill depth (a proxy for fiord age) and expected heterozygosity (an estimate of genetic diversity), (b) sill depth (a proxy for fiord age) and MDS scores (an estimate of genetic differentiation).
Fig. 3.9(cont'd). Plot and linear regression of historical and hydrographic data versus AFLP data after the Long Sound site has been removed from the data set. The relationship between: (c) distance to the outer coast (a proxy for the relative age of the marine habitat) and expected heterozygosity (an estimate of genetic diversity). (d) effective freshwater depth (a proxy for estuarine circulation) and MDS scores (an estimate of genetic differentiation).
Discussion

Both allozyme and AFLP data revealed genetic differentiation is very slight between *Terebratella sanguinea* populations across Fiordland. The inclusion of Long Sound in the AFLP analysis illustrated slight but significant genetic differentiation between Long Sound and the other sites in Fiordland. Overall there was no relationship between the genetic data and historic or hydrographic variables; because of the low power of these analyses it is difficult to determine whether historic and hydrographic variables influence genetic structure across Fiordland, however the data suggest historic and hydrographic factors likely contribute to the isolation of Long Sound.

Genetic diversity and population structure

Population structure, as measured by the genetic differentiation among populations, reflects the opposing forces of genetic drift and gene flow as well as the evolutionary forces of selection and mutation. In order to interpret genetic data, some of these forces often are assumed negligible. For example, when $F_{ST}$ is used to estimate gene flow, it has often been assumed that selection and mutation can be ignored (Slatkin and Barton 1989). Furthermore, because $F_{ST}$ is used to estimate the magnitude of gene flow relative to genetic drift, inferences about the strength of gene flow involve assumptions about the strength of genetic drift. The confounding of gene flow and genetic drift is particularly problematic for populations of marine species. In marine populations, drift may be weak because effective population size is large (no barriers to dispersal and high reproductive output) or stronger because of small effective population sizes (reproductive success is limited to a few individuals) (Hedgecock 1994; Neigel 2002). Although there are difficulties associated with using $F_{ST}$ to estimate population genetic structure, $F_{ST}$ remains a useful measure of the average effects of gene flow and provides a means for comparing gene flow among populations.

The overall pattern of $F_{ST}$ estimates calculated from allozymes is reminiscent of the genetic mosaics found in limpets, zoanthids, and sea urchins studied in Australia (Johnson and Black 1982; Johnson and Black 1984; Watts *et al.* 1990; Burnett *et al.* 1994) and sea urchins in California (Edmands *et al.* 1996), where genetic differences
over short geographic distances in some cases exceeded differences over larger
distances. In the current study, differentiation between the two sites within Dusky
Sound (11-DSK O and 11-DSK I: 28 km separates sites, $\theta = 0.0227, p < 0.001$) was
larger than genetic differentiation between sites separated by over 400 km (e.g. 1-BLI I
and 13-STW PG: 406 km separates sites, $\theta = 0.0078, p < 0.05$). This sort of patchy
genetic differentiation may have been more interpretable had the sampling in the
current study included larger scale comparisons, similar to the findings with the
brachiopod *Lingula anatina*. Although initial investigations of *L. anatina* populations
suggested genetic homogeneity over a range of geographic distances (Hammond and
Poiner 1984), further analysis indicated populations across Japan and between Japan
and Australia differed significantly while the geographically proximate populations
within Australia were largely homogenous (Endo *et al.* 2001).

Genetic differentiation at allozyme loci was comparable to allozyme surveys of other
marine organisms with a planktonic larval phase and separated by similar geographic
distances (e.g. *Synalpheus pectinger*, $F_{st} = 0.000 - 0.002$ (Duffy 1993); *Patiriella
calcar*, $F_{st} = 0.000$ (Hunt 1993); *Siphonaria jeanae*, $F_{st} = 0.0019 - 0.0032$ (Johnson and
Black 1984)); the highest levels of differentiation (such as the divergence between 11-
DSK O and 11-DSK I) are lower than values reported for organisms lacking a pelagic
larval phase (e.g. *Synalpheus brooksi*, $F_{st} = 0.064 - 0.214$ (Duffy 1993); *Patiriella
exigua*, $F_{st} = 0.462$ (Hunt 1993)), suggesting that the lecithotrophic larvae of *T.
sanguinea* larvae were capable dispersers at this temporal and spatial scale. However,
it is difficult to explain the genetic patchiness of *T. sanguinea* in Fiordland. Other
studies demonstrating genetic patchiness uncovered significant heterozygote
deficiencies (Edmands *et al.* 1996) that were attributed to a temporal Wahlund effect
arising from an accumulation of genetically differentiated cohorts. Within *T.
sanguinea* populations, four of the ten sites exhibited significant heterozygote
deficiencies. These were confined to two of the five fiords sampled at allozyme loci. It
is likely that specific features of George Sound and Doubtful Sound influenced
populations such that heterozygote deficiencies were fiord specific. However, the
random genetic differentiation observed across Fiordland was not limited to these two
fiords, and the highest levels of genetic differentiation were between sites that
conformed to Hardy-Weinberg equilibrium expectations.
Chapter 3: Terebratella sanguinea in the New Zealand fiords

*Terebratella sanguinea* demonstrated similar levels of genetic variation at allozyme loci as other brachiopod species for which allozyme data are available. *Terebratella sanguinea* illustrated high polymorphism (all four resolvable loci were polymorphic) and heterozygosity was consistent with Hardy-Weinberg expectations. This was comparable to the inarticulate brachiopods *Lingula antina* and *L. murphiana*, (Hammond and Poiner 1984) and the articulate brachiopods *Frieleia halli* (Valentine and Ayala 1975), *Coptothryis grayi* (Balakirev and Manchenko 1985), *Terebratulina septentinalis* and *T. retusa* (Cohen et al. 1986; Cohen et al. 1991) that demonstrated high levels of polymorphism and generally conformed to Hardy-Weinberg expectations of heterozygosity. Contrary to the hypothesis that old lineages harbour very high levels of heterozygosity (Soule 1976) and the alternative hypothesis that phylogenetic relics should demonstrate very low levels of variation (Selander et al. 1972), genetic structure among brachiopods is not exceptional despite the exceptionally long evolutionary history of these organisms. This pattern is similar to previous studies of "living fossils" that have found no biochemical evidence to indicate nucleotide sequence evolves differently in very old lineages (Ramshaw et al. 1971; Shimizu 1971; Shimizu and Miura 1971; Hackman and Goldberg 1975; Schopf 1984; Avise 1994).

The AFLP data illustrated the same small-scale genetic patchiness as well as larger scale pattern. MDS ordinations consistently split populations separated by short geographic distances (*e.g.* the 6-THM RP site versus the other 6-THM sites). This small-scale differentiation was similar to that found with the allozymes. Small-scale genetic divergence, though a more patterned divergence, has also been noted among fiord populations of the sea urchin *Evechinus chloroticus*. Analysis of 6 microsatellite markers grouped sea urchin samples within fiords into two categories according to distance from the outer coast (Perrin 2002). Although similar patterning of genetic divergence was not detected in the current study, a small but significant amount of genetic divergence between Long Sound and the other fiords was uncovered. This separation between Long Sound and the other fiords was also visible in the microsatellite data from *E. chloroticus* (Perrin 2002) as well as mitochondrial D-loop data from the sea star *Cocinasterias muricata* (Perrin 2002; Perrin et al. 2004). In contrast, differentiation between Long Sound and the other fiords was not seen in
previous studies of the black coral *Antipathes fiordensis* (Miller 1997a) and the snake star *Astrobrachion constrictum* (Steel 1999). It is important to note, however, that the snake star showed very low levels of genetic diversity across Fiordland (Steel 1999), and that the black coral has a much longer generation time than the other organisms studied and the extended generation time could reduce the opportunity for genetic divergence.

The forces driving marine invertebrate population structure appear to extend beyond larval dispersal potential, and multi-locus, dominant markers have been useful tools for indirectly assessing dispersal. AFLP analysis of populations of the Lusitanian sea star (*Asterina gibbosa*) indicated this direct developer disperses differently in two ocean basins; gene flow within the Atlantic basin was detected but not within the Mediterranean basin (Baus *et al.* 2005). The marine bryozoan *Alcyonidium gelatinosum* broods embryos and releases short-lived, lecithotrophic larvae (Porter *et al.* 2002). High levels of genetic structuring among bryozoan populations were found using RAPD’s, though sampling was at a larger scale than in the current study. The blacklip abalone (*Haliotis rubra*) produces lecithotrophic larvae with a relatively brief pelagic phase of 3-11 days (Huang *et al.* 2000). RAPD analysis revealed increasing genetic differentiation with increasing geographic distance in *H. rubra* (Huang *et al.* 2000), though some of the sampling sites were separated by up to 5 times the greatest distance in the current study. The greenshell mussel, *Perna canaliculus* has larvae that can remain viable for > 4 weeks. Despite the large potential for gene flow in *P. canaliculus*, RAPD analysis indicates significant allele frequency differences coinciding with major hydrodynamic features at 42°S latitude. Multi-locus markers are appropriate for resolving the differentiation between populations of organisms with dispersing larvae, though the spatial scales over which differentiation occurs in organisms with passively dispersing larvae are generally larger than those separating sites within fiords or different fiords.

**Migration and isolation by distance**

Overall calculations of migration indicate substantial gene flow across Fiordland. Estimates of *Nm* calculated from allozyme and from AFLP data are >>1. One effective migrant exchanged between populations per generation is theoretically sufficient to
stop the fixation of different neutral alleles in separate populations (Ridley 1993), which occurs when historically connected populations become completely isolated. It is important to note, however, that this is only the case in large populations at drift/migration equilibrium, and the time required to approach this equilibrium could be far greater than the age of most populations. It is likely that the marine populations in Fiordland have not reached this equilibrium, and gene flow estimates based on non-equilibrium data are likely to produce unrealistic results (Neigel 2002) because drift and migration jointly influence the product $Nm$ (the effective number of migrants per generation); drift is proportional to $1/N$ and $N$ is the effective population size. For many species, dispersal is constrained by distance, and identification of potential equilibrium conditions is possible by identifying patterns of isolation by distance.

The analysis of our data from an isolation-by-distance perspective gave us insight into patterns of genetic exchange throughout Fiordland. From our allozyme data, the scatterplot of genetic versus geographic distances of sites indicated no correlation. This could be interpreted as an island system in an equilibrium state or as a stepping-stone system out of drift/migration equilibrium where drift is more influential than gene flow (Hutchinson and Templeton 1999) (Fig. 3.6(a)). Because of the long distances, short time in the plankton, and the potential for hydrographic interruption of dispersal, the former explanation of continuing long distance exchange is less plausible than the latter. The AFLP data without the Long Sound site illustrated a similar pattern to the allozyme data (Fig. 3.6(b)); the large variation in genetic distances observed between pairs of populations indicates a system where gene flow is minimized and the effect of drift pronounced (Hutchinson and Templeton 1999). However, the complete AFLP data set produced a scatterplot of genetic versus geographic distances indicating a significant, positive correlation. Under a stepping stone model, this relationship indicates the presence of drift/migration equilibrium; gene flow is more common over small spatial scales but is reduced as geographic distance increases (Fig. 3.6(b)). A second hypothesis is that the pattern of isolation by distance represents two populations. High levels of genetic differentiation correspond to comparisons between “northern” sites and sites in Long Sound, whereas low levels of genetic differentiation correspond to comparisons among the northern sites. This pattern is similar to that found among populations of the pocket gopher *Thomomys bottae* presented by Slatkin
(1993). In his plots, there was a slight overall pattern of isolation by distance, however sample locations fell into two distinct groups; Group I had migration rates $> 1$ and Group II had migration rates $< 1$. Slatkin (1993) concluded that Group I is not in equilibrium, but rather has only recently colonized the area, and that the time scale of approach to drift-migration equilibrium is on the order of the local effective population size (Slatkin 1993). In marine populations, local effective population size can be difficult to estimate; as mentioned previously, factors influencing effective population size, such as the geographic range over which migration connects populations and the number of animals successfully reproducing, often are not evident. Based on densities of animals through Doubtful Sound (see Chapter 2), an estimate of effective population size indicated that *T. sanguinea* populations in the fiords would reach drift-migration equilibrium more than 9,000,000 years after colonization. The marine habitat in Fiordland is estimated to be 9,000 – 16,000 years old; therefore, if these calculations are a realistic representation of the time necessary to reach equilibrium, the marine populations in Fiordland have not attained drift-migration equilibrium. Similar to the analysis of *T. bocconei* by Slatkin, the separation between the two groups of *T. sanguinea* sampling locations in Fiordland suggests that indeed the system is not in equilibrium. Fiordland populations of the sea star *C. muricata* also fell into two distinct groups when testing for isolation by distance (Perrin *et al.* 2004). Perrin *et al.* (2004) suggested this split might arise from secondary contact between a northern population and a southern population (*i.e.* a Long Sound population that has differentiated via isolation and ensuing drift) separated by a contact or mixing zone. Under such a scenario, the more isolated southern populations may have smaller effective population sizes, and therefore, may reach an equilibrium state more quickly than the larger northern population.

It has been suggested previously that Fiordland is a geologically young system that has not reached drift-migration equilibrium (Miller 1997a). Disequilibrium with drift more influential than gene flow would be consistent with the original hypothesis of restricted gene flow in Fiordland. If indeed drift is more influential than gene flow, then a mechanism must be found to explain the low levels of differentiation between sites and fiords. Low levels of genetic differentiation could be attributed to historical patterns, such as a common colonizing source population. However, the youngest of the fiords,
Long Sound, showed the highest level of differentiation. This suggests that sufficient
time has elapsed for statistically significant amounts of genetic differentiation to
accumulate. The alternate explanation, that the system is in drift/migration equilibrium
and that gene flow decreases as geographic distance between sites increases, also seems
unlikely when some of the highest levels of genetic differentiation were found between
geographically proximate sites (see the preceding section). The correlation between
genetic and geographic distances is likely an artefact of the high levels of genetic
differentiation between Long Sound and the other sites, and that factors other than
geographic distance have influenced the genetic differentiation of Long Sound (see the
following section ”Effects of historic and hydrographic factors” for possible
mechanisms driving the genetic differentiation of Long Sound).

**Congruence between markers**

Overall estimates of genetic differentiation based on AFLP data were higher than those
based on allozyme data. For most markers, the contribution of mutation to
differentiation has been generally ignored because mutation rates of traditional
markers, such as allozymes, are negligible when compared to migration rates. This
may not be the case with AFLP’s. The dominant nature of AFLP markers is
responsible for a bias in the estimation of differentiation. Simulation and experimental
results have illustrated that $F_{ST}$ is biased upward when using the dominant RAPD
markers to estimate allelic frequencies (Jenczewski *et al.* 1999), and the same bias may
be possible with dominant AFLP markers. Although the lack of correlation between
allozyme and AFLP data suggests factors such as mutation and biased estimation affect
the two markers differently, both data sets lead to similar conclusions: 1) genetic
variation in the Fiordland region is generally low, and 2) the genetic differentiation that
exists is patchy.

**Effects of historic and hydrographic factors**

In this study, the exploration of the role of historic and hydrographic factors is
hampered by the low power of the regression analyses, however the patterns suggest
Long Sound differs substantially from the other sites. As compared to the other fiords,
the Long Sound has an exceptionally shallow entrance sill, a particularly long and
convoluted path along the major fiord axis, and high amounts of freshwater (Fig. 3.10). Therefore it is difficult to determine if the relationships suggested by the correlation analyses might be continuous or if the heavy influence of an outlier is skewing the overall pattern. Because the significant relationship between factors disappears when the Long Sound site is removed from the three correlation analyses, it is likely that these historical and hydrographic factors only have an effect at extreme values. However, since both allozyme and AFLP data indicate similar trends in the relationship between heterozygosity and sill depth and genetic differentiation and EFD, further sampling is necessary to better elucidate these trends. Additional sampling would be of benefit in addressing the lack of power in the regression analyses reported here, particularly the comparison of sill depth and genetic differentiation. The difficulty arises in the lack of sites with values of sill depth and EFD intermediate to those used in the current study.

Only the scatterplots produced from the complete (i.e. including Long Sound) AFLP data set indicated that historic and hydrographic variables were correlated with the genetic data. The negative correlation between heterozygosity and sill depth suggests that heterozygosity is higher in younger fiords with more shallow sills (flooded by the Tasman Sea most recently). We do not have the leverage in this study to disentangle the mechanisms behind a correlation between the shallow sill of Long Sound and genetic differentiation. The original hypothesis that genetic diversity should accumulate in older populations is contradictory to the supposition that the young age of Long Sound is related to increased heterozygosity. However, it is possible that entrance sills could affect population genetic structure in an alternate manner. If the sill is very shallow, the flow of seawater over the sill may be constricted, affecting genetic exchange between populations. Thus populations in fiords with shallow sills may be more isolated and, therefore, more likely to have differentiated over time through genetic drift. Another surprising result was the positive correlation between heterozygosity and distance to the outer coast. The correlation suggests that the youngest sites, farthest from the open ocean, and also those sites least likely to maintain a continued exchange with the open ocean, have the highest levels of genetic diversity. One possible explanation for such a pattern is differences in age between sites may be too small for genetic differences to accumulate; instead the pattern may arise from the
continuing isolation of sites (due to restricted flow, which is also linked to the shallow sill) from the open ocean that may allow genetic diversity to accumulate. However, the correlation between heterozygosity and effective freshwater depth (EFD) was in keeping with our original hypotheses. Sites with higher levels of freshwater (which would indicate a stronger estuarine circulation) illustrated higher levels of genetic differentiation. It is difficult to determine the relative influence of these factors as well as the biological significance of these correlations. Furthermore, additional data are necessary before conclusions can be drawn due to the low power of the correlation analyses.

Fig. 3.10. Map and longitudinal section of Long Sound illustrating the shallow sills and the constrictions along the main axis of the fiord. (from Stanton 1984)
Conclusions

The AFLP markers resolved small but significant genetic differences between the Long Sound site and other sites within Fiordland despite the relatively young age of this system and the short distances (10's to 100's of kilometres) separating sites. This result suggests that elements that differentiate the Long Sound site have contributed to genetic isolation. However these factors only have created genetic isolation at the extremes of the spectrum, as in the case of Long Sound. For example, Long Sound has, by far, the shallowest terminal sill, Long Sound has shallow internal sills and constrictions in addition to being very long (as the name would suggest), and Long Sound has much higher levels of freshwater. Furthermore, because Long Sound was the only fiord to illustrate consistent differences with other sites and Long Sound was an outlier amongst the other fiords for more than one of the factors explored, it is impossible to determine which factor plays the strongest role in creating genetic differentiation. Among the other fiords, continued exchange with the outer coast may continue to genetically homogenize the entire area. The continental shelf in the Fiordland area is narrow, and it is possible that a slow injection of seawater facilitated by the estuarine circulation pattern provides a small but continuous input of larvae into the fiords from a large, offshore population. The similarity of the Stewart Island sites to the fiord sites at four allozyme loci supports this hypothesis. Alternatively, the lack of genetic structure across Fiordland could be the signature of a recent colonization and a current lack of drift-migration equilibrium. Because the fiord system is an evolutionarily young system, a quickly evolving, co-dominant marker (as compared with allozymes that may be evolutionarily constrained), such as a microsatellites, might uncover subtler structuring among fiord sites with intermediate sill heights, fiord lengths, or freshwater inputs. Indeed such a subtle structure might exist if there is a restriction of gene flow due to sills, fiord length, or freshwater input, though the structure may be difficult to detect due to the novelty of this system in evolutionary time frames. Additionally, a co-dominant marker could contribute information about genetic structure at the most highly differentiated site, in Long Sound. Because the Long Sound site was not part of the preliminary allozyme survey, data from a co-dominant marker would be an important addition to the current study. Furthermore, continuing investigation of brachiopod genetic structure would allow a better understanding of the seemingly random genetic differentiation between fiords uncovered by allozymes. Sequence data
might also allow a better understanding of this system. Sequence data lends itself more
easily to the more powerful coalescent models of determining population structure.
Coalescent models are based upon fewer assumptions regarding genetic drift and
migration and, therefore, may be better tools for disentangling population structure in
marine populations.

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Chapter 3: Terebratella sanguinea in the New Zealand fiords


Chapter 3: Terebratella sanguinea in the New Zealand fjords


Genetic population structure in two southern hemisphere brachiopods that differ in ontogeny: assessing the influence of a fiord environment

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Abstract

Dispersal of planktonic larvae in a marine system can be influenced by many factors. As a result, studies of environmental influences on gene flow and population structure can present an appreciable challenge. In order to understand how the fiord environment influences population structure of organisms that disperse via planktonic larvae, we compared population genetic structure of two sympatric brachiopod species that differ in larval development. Genetic analysis using the amplified fragment length polymorphism technique revealed population structure corresponding to the contrasting modes of larval dispersal. Populations of Liothyrella nzelenica, a brachiopod that broods its larvae, were differentiated among sites within and among fiords, whereas populations of Terebratella sanguinea, a brachiopod that does not brood its larvae, were differentiated among but not within fiords. The fiord hydrographic conditions appear to constrain gene flow and create opportunities for local genetic differentiation in organisms capable of long distance dispersal whereas organisms with lower potential for dispersal are influenced more by ontogeny than by basin scale (mesoscale) hydrography.

Introduction

Dispersal in marine organisms has been compared to that in plants - millions of small propagules transported long distances with few discernable barriers to movement (Palumbi 1992). As with plants, directly measuring dispersal amongst the tiny travellers of the marine realm is a daunting task. Therefore, excepting species with very limited dispersal capabilities (e.g. Olson 1985; Davis and Butler 1989), tracking larval movements has been accomplished via indirect estimates based on a variety of sources, including oceanography (Lee et al. 1994; Wing et al. 1995; Limouzy-Paris et
al. 1997; Wing et al. 1998a and b; Wing et al. 2003a and b), species invasions (Carlton and Scanlon 1985; Hicks and Tunnell 1995), and genetics (see Palumbi 1995; Hellberg et al. 2002). Geographical surveys of genetic variation provide an especially useful tool for tracing movements of larvae between populations because measures of genetic differentiation are highly sensitive to migration. Successful migrants should leave a genetic trail of their movement (Hellberg et al. 2002), and indirect measures of population connectivity interpret this trail through population genetic models (Neigel 1997; Waples 1998; Whitlock and McCauley 1998).

In general, a positive correlation exists between time in the plankton and dispersal distance (Shanks et al. 2003). Long duration in the plankton (> 300 hr (Shanks et al. 2003)) often is associated with low genetic structuring whereas a shorter duration in the plankton with greater heterogeneity among populations (e.g. Liu et al. 1991; McMillan et al. 1992; Stickle et al. 1992; Duffy 1993; Hunt 1993; Williams and Benzie 1993; Hellberg 1996; Gold et al. 2001; Goldson et al. 2001). However, a growing body of evidence challenging this paradigm exists (Gaarde and McClanahan 1982; Borsa and Benzie 1996; Ayre and Hughes 2000; Sponer and Roy 2002), and studies suggesting larvae often fail to achieve their dispersal potential (Knowlton and Keller 1986; Todd et al. 1988; Burton and Lee 1994; Ayre and Hughes 2000; Taylor and Hellberg 2003) or recruit back to parental populations (Swearer et al. 1999; Strathmann et al. 2002; Swearer et al. 2002) are mounting. Moreover, the overriding effects that population history can exert on existing genetic population structure may confound the interpretation of genetic data since high dispersal over evolutionary time scales may be consistent with low dispersal over ecological time scales (Waples 1998; Palumbi 2003).

Brachiopods constitute one of the oldest lineages of organisms still in existence today. Common in the fossil record worldwide, brachiopods have survived for some 600 million years with no basic change in form (James et al. 1992). At their peak there were over 3000 genera of brachiopods, and they were an abundant and diverse component of coastal shelf ecosystems (Brusca and Brusca 1990); however, after the Permian extinction and subsequent radiation of mollusks, many brachiopod species became extinct. Today there are about 335 extant species of brachiopods that generally inhabit marginal areas with high current velocity or cold temperatures (Brusca and
Chapter 4: Two brachiopod species with differing life histories

Brusca 1990; Peck 1996). Whereas brachiopods occur in abundance as fossils throughout the world, they are not common in modern seas; the New Zealand fiord system, with eight different brachiopod species, is one of the few areas in which the study of living brachiopod populations is possible.

*Liothyrella neozelanica* and *Terebratella sanguinea* both live in subtidal New Zealand waters with *T. sanguinea* occurring only in the southern portions of this range (the closely related *T. haurakiensis* occurs in the northern part of the range (Richardson and Mineur 1981)). Within the fiords, the two species are sympatric from Doubtful Sound south. *Liothyrella neozelanica* and *T. sanguinea* are density stratified with *L. neozelanica* occurring at deeper depths (*L. neozelanica*: 20 m and deeper; *T. sanguinea*: 5 – 15 m (Ryan and Paulin 1998)). The microhabitats and distributions of the two differ with *L. neozelanica* found in aggregated clusters (Richardson 1981) under overhangs or in caves (Wing, pers. com.) and *T. sanguinea* broadly dispersed across the rock faces (Stewart 1981) (Fig. 4.1). Both species are dioecious (Tortell 1981) with even sex ratios (Tortell 1981; Chuang 1994) (see also Chapter 2) and both produce lecithotrophic larvae (Williams et al. 1997).

Reproductive peaks in *L. neozelanica* are not known, but animals with partially spawned gonads have been reported in the late austral summer and early austral fall (February and March) (Tortell 1981). Mature ova measure 100 to 200 μm (Chuang 1994), and larvae are brooded within the mantle cavity of the female (Chuang 1994). Mature gonads have been reported in animals as small as 20 mm in length (Chuang 1994).

*Terebratella sanguinea* reaches a reproductive peak in the late austral fall/early austral winter (April, May, June) (Tortell 1981) (see also Chapter 2). *Terebratella sanguinea* are probably broadcast spawners with external fertilization and larval development because there is no evidence of brooding (Tortell 1981) (see also Chapter 2). Mature ova of *T. sanguinea* measure approximately 100 μm (Tortell 1981) (see also Chapter 2). As in *L. neozelanica*, animals with mature gonads have been found as small as 20 mm (Tortell 1981).
Fig. 4.1. (a) *Liothyrella neozelanica* are more clumped in their distribution than are *T. sanguinea*. Examples of some of the many *L. neozelanica* visible in the photo are indicated with arrows. (b) *Terebratella sanguinea* is more dispersed across the rock walls in the fiords. The two *T. sanguinea* visible in the photo are marked with arrows. (Photos M. Lamare and H. Nollens)
For both species, larval duration and subsequent dispersal capabilities remain unknown. Larval brooding suggests that *L. neozelanica* should spend a shorter time in the plankton (if they enter the plankton at all). Furthermore, the clumped aggregations of *L. neozelanica* and the more specific habitat suggest that the best strategy for larval survival might be to remain close to a parent. In contrast, external fertilization and development coupled with a more continuous distribution would suggest *T. sanguinea* larvae are in the plankton for a longer time and have a greater ecological tolerance than *L. neozelanica*, facilitating dispersal farther from a natal population.

To better understand the effects of the complex hydrography of the fiords on planktonic movement, we compared the genetic population structure of these two brachiopods that occur sympatrically in the fiords but differ in their putative dispersal capacities. Within the fiords, the complex hydrography (a gravitational estuarine circulation pattern) and recent colonization (8,000 to 12,000 years ago (Smith 2001)) may play a large role in shaping population genetic structure. Historical factors should influence population structure similarly among organisms inhabiting the fiords. However, hydrographic factors should influence populations of organisms with poorly dispersing, brooded larvae differently than those with planktonic larvae. Comparisons between taxa are viewed as an effective way to test hypotheses regarding the effects of microevolutionary forces on population differentiation (Bohonak 1999; Wares *et al.* 2001). The most powerful comparison would be that between sister species, however the brachiopods of the New Zealand fiords are all of different genera.

In general, local adaptation and the stochastic effects of genetic drift counteract the unifying effects of gene flow. Populations of a poorer dispersing species should be less homogenous whereas gene flow will maintain genetic connectivity between populations of an organism with higher dispersal capacity. In previous allozyme studies, *L. neozelanica* demonstrated divergence between two sites within a fiord (Suring 2001) whereas *T. sanguinea* showed little genetic structure over the whole of Fiordland (see Chapter 3). However, both species illustrated low levels of polymorphism at all but one allozyme locus. In the present study, the amplified fragment length polymorphism (AFLP) technique was used to further elucidate the genetic population structure of the two brachiopod species in three fiords. It is
important to note that AFLP analysis assumes populations are in Hardy-Weinberg equilibrium, and previous allozyme analysis illustrated that: 1) *L. neozelanica* was not in equilibrium at two of the three polymorphic allozyme loci, though this may be a result of low levels of polymorphism (Suring 2001) and 2) *T. sanguinea* populations were in equilibrium for most loci and at most sites, though populations in two fiords did not meet Hardy-Weinberg expectations (see Chapter 3). We hypothesized that in the poor disperser, *L. neozelanica*, AFLP data would reveal population genetic structure on a small geographic scale (on the scale of a few meters to a few kilometres, similar to marine invertebrates with crawl-away larvae (e.g. Hellberg 1996). In an organism whose range exceeds its dispersal capacity, the probability of exchanging migrants should decrease as geographic distance separating populations increases. Therefore, we expected genetic differentiation to show a positive correlation with geographic distance separating populations of *L. neozelanica*. We expected that population structure of the pelagically dispersing brachiopod, *T. sanguinea*, would occur on a larger scale (10’s to 100’s of kilometres, similar to other marine invertebrates with similar planktonic durations (McMillan *et al*. 1992; Palumbi 1995)). However, we hypothesized populations in different fiords should be differentiated, even if the geographic distance separating the populations was within the dispersal potential of *T. sanguinea* because fiordic estuarine circulation should interrupt planktonic rafting. Under such a scenario, the interruption of genetic connectivity should be most evident those fiords with strong estuarine circulation and strong retention (*i.e.* Long Sound).

Materials and Methods

Collection of samples

We collected individuals of both *L. neozelanica* and *T. sanguinea* from each of the same four localities representing an inner (within the fiord and near the head) and an outer (near the outer coast) site in Doubtful and Breaksea Sounds (Table 4.1, Fig. 4.2). Sampling both inner and outer sites allowed us to examine whether genetic patterns could be associated with the environmental heterogeneity of the fiords. In a third fiord, *T. sanguinea* could not be located at the outer site (Table 4.1, Fig. 4.2). Therefore, *L.
neozenica individuals were collected from both an inner and outer site in Long Sound, and T. sanguinea individuals were collected only at the inner site. Inner and outer sites ranged from 9 to 25 km apart while distances between fiords (measured as the distance between the mid-points of fiord mouths) ranged from 68 to 175 km. We collected only adult brachiopods (length >20 mm), however animals of different sizes over 20 mm were collected. We sampled all localities between 1998 and 2002. Because brachiopods grow relatively slowly, take several years to reach sexual maturity, and grow more slowly after reaching sexual maturity (Rudwick 1962; Rickwood 1977; Doherty 1979), collections in any given year likely contained animals of many ages. Sampling different sites at different times was not likely to have influenced genetic patterns because each collection did not reflect a given settling event but rather the entire accumulation of adults in an area. For each animal collected, the lophophore was removed and frozen in liquid nitrogen in the field. Samples were subsequently stored at -80°C.

AFLP assay
DNA was extracted using the DNazol Genomic DNA Isolation Reagent. The protocol for this extraction is detailed in Chapter 3. Subsequent AFLP analysis followed the protocol reported in Chapter 3 (see also Appendix II).
Data analyses

Population structure

Estimates of hierarchical F-statistics were characterized by calculating Weir and Cockerham’s (1984) $\theta$. $\theta$ was calculated using Tools for Population Genetic Analysis (TFPGA) (Miller 1997b) and used to test for differences in allele frequencies among sites as well as between fiords. Confidence limits (95%) of $\theta$ estimates were obtained from a bootstrap procedure in which 10,000 new datasets were constructed by re-sampling with replacement over loci. Confidence limits of $\theta$ that did not overlap 0 (no evidence of differentiation) were interpreted as evidence for significant differences of allele frequencies between populations. Total genetic variation was also partitioned among fiords, among sites within fiords, and among individuals within sites by carrying out a hierarchical analysis of molecular variance (AMOVA) on Euclidean pairwise distances ($\Phi_{ST}$) using GenAlEx (Peakall and Smouse 2001). This procedure generated an $F_{ST}$ analog based solely on AFLP marker phenotypes as opposed to the estimated allele frequencies used in the calculation of $\theta$. Distance measures for the AMOVA analysis and permutation tests were as described in Chapter 3. Pairwise comparisons of genetic distance (Nei 1978) measures among all sites were calculated for each species. Statistical significance was calculated as combined probabilities over all loci for each pairwise comparison. Exact tests for pairwise population differentiation (Raymond and Rousset 1995) utilized 1000 dememorization steps, 10 batches, and 1000 permutations per batch. Exact tests of statistical significance and genetic distance calculations were performed in TFPGA (Miller 1997b).

Genetic structure of fiordic samples was visualized by creating UPGMA dendrograms. Genetic distance measures (Nei 1978, Reynolds et al. 1983) were used to construct the dendrograms in Tools for Population Genetic Analysis (TFPGA) (Miller 1997b). In addition, non-metric multidimensional scaling (MDS) ordinations were created using the program PRIMER v5 (Clarke and Gorley 2001) to illustrate any groupings of sites based on the genetic data. Nei’s unbiased genetic distance (Nei 1978) and Reynolds coancestry distance (Reynolds et al. 1983) matrices created in TFPGA were used for the MDS ordinations. Bootstrapping procedures for the UPGMA dendrograms and stress value calculations for the MDS ordinations were as described in Chapter 3.
Table 4.1. Study sites and sample sizes for \textit{Liothyrella neozelanica} and \textit{Terebratella sanguinea} AFLP analysis. Sites are numbered according to decreasing latitude (numbers correspond to the map in Fig. 4.2). The letter codes following the number refer to the fiord (first three letters) and the specific position of the site within the fiord (I = inside fiord, O = near outer coast). Sample sizes are reflective of the number of individuals of each species that could be collected at a given site where species distributions were overlapping. Samples were collected over multiple years; collection date refers to the year in which samples were collected.

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Lat/Long</th>
<th>n (L.n.)</th>
<th>Collection</th>
<th>n (T.s.)</th>
<th>Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-DFL O</td>
<td>Doubtful Sound</td>
<td>45°19'S/166°58'E</td>
<td>16</td>
<td>2002</td>
<td>15</td>
<td>2000</td>
</tr>
<tr>
<td>1-DFL I</td>
<td>Doubtful Sound</td>
<td>45°26'S/167°06'E</td>
<td>24</td>
<td>1999</td>
<td>15</td>
<td>2000</td>
</tr>
<tr>
<td>2-BRK O</td>
<td>Breaksea Sound</td>
<td>45°34'S/166°46'E</td>
<td>17</td>
<td>1998</td>
<td>20</td>
<td>1998</td>
</tr>
<tr>
<td>2-BRK I</td>
<td>Breaksea Sound</td>
<td>45°31'S/166°55'E</td>
<td>21</td>
<td>1999</td>
<td>20</td>
<td>1999</td>
</tr>
<tr>
<td>3-LNG O</td>
<td>Long Sound</td>
<td>46°04'S/166°43'E</td>
<td>19</td>
<td>1998</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3-LNG I</td>
<td>Long Sound</td>
<td>45°59'S/166°50'E</td>
<td>20</td>
<td>1998</td>
<td>18</td>
<td>2002</td>
</tr>
</tbody>
</table>

Fig. 4.2. \textit{Liothyrella neozelanica} and \textit{Terebratella sanguinea} sampling sites across Fiordland. Site locations and sample sizes are provided in Table 4.1. Red circles indicate sites where both species were collected; the yellow circle indicates the site where only \textit{L. neozelanica} was found.
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Genetic diversity

Levels of within-population genetic diversity were characterized by calculating Nei’s (1978) unbiased expected heterozygosity ($H$) and the proportion of polymorphic loci ($P$). Estimates of $H$ were calculated from allele-frequencies obtained from the Lynch and Milligan (1994) estimator. Percent polymorphic loci estimates were calculated as the proportion of loci at which the most common allele had an estimated frequency of less than 0.95. Both $H$ and $P$ were estimated using TFPGA. Sample size was regressed against $H$ and $P$ to determine whether the number of animals sampled at each site was related to $H$ and $P$ measurements (see Busch et al. 2000). In addition, the number of rare fragments found in each population was tallied. Rare fragments were those found in only 1, 2, or 3 populations per species. Because larger sample sizes would increase the probability of detecting rare alleles, sample size was regressed against the number of rare fragments (SigmaPlot 5.0, SPSS Science, Chicago, Illinois) to determine whether the number of animals sampled at each site was related to these measurements.

Isolation by distance

If the fiords do not present a barrier to gene flow, restricted larval dispersal might influence genetic differentiation to produce isolation by distance. In order to test for a correlation between genetic and geographic distance among populations, estimates of $\theta$ were obtained for each pair of sites using Nei’s unbiased genetic distance (Nei 1978). Bivariate plots of genetic versus geographic (along fiord and open coastline distances measured in km) distance were constructed (SigmaPlot 5.0, SPSS Science, Chicago, Illinois), and the significance of the correlation between genetic and geographic distance was evaluated using the Mantel test (Mantel 1967; Sokal and Rohlf 1995) procedure in TFPGA. A normalized $Z$-test was performed in which the observed value after 999 permutations should be significantly larger than expected by chance in order for the null hypothesis, no association between the pair of matrices, to be rejected. We used the general framework of Hutchinson and Templeton (Hutchinson and Templeton 1999) to interpret the bivariate plots and infer the relative importance of drift and migration in this system.
Conditional average frequency

Slatkin’s conditional average frequency (CAF), $\bar{p}(i)$, depends on both the level of gene flow and the geometric arrangement of demes (Slatkin 1981); therefore, this measure is appropriate for obtaining a rough estimate of the levels of gene flow in subdivided populations, and can provide a measure of relative dispersal capability of organisms with different life histories (Waples 1987). Slatkin’s CAF was calculated as the average frequency of each allele over all populations in which it was found. Alleles occurring in the same number of populations (i) were grouped, and an overall average frequency ($\bar{p}$) was calculated for each group of alleles conditioned on i. The value i is made relative to the number of populations ($d$ = demes or populations sampled). This relationship was plotted using SigmaPlot 5.0 (SPSS Science, Chicago, Illinois); consistent and low values of $\bar{p}(i)$ are associated with organisms with high apparent gene flow whereas increasing or consistent and high values of $\bar{p}(i)$ are associated with organisms with medium and low (respectively) apparent gene flow (Waples 1987) (Fig. 4.3).

Fig. 4.3. Conditional average frequencies versus the fraction of all populations in which an allele was found (i/d). Patterns typically found in species with low (red), medium (green), and high (blue) levels of gene flow (after Slatkin 1981, Fig. 1).
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Results

A total of 79 and 182 (L. neozelanica and T. sanguinea respectively) scoreable, polymorphic AFLP presumptive loci were generated using two primer combinations. Of the 119 L. neozelanica bands among the 5 individuals analysed in duplicate, all were reproducible; a sixth individual was run in duplicate but restriction was incomplete in one of the two DNA extractions and the resultant banding was not scored. In T. sanguinea a total of 119 bands were generated among six individuals run in duplicate and all of these were reproduced exactly. This result supports the consistency of the AFLP procedure.

Population structure

Results from the AMOVA, $F$-statistics, and pairwise comparisons indicated that overall, more gene flow occurs among T. sanguinea populations than L. neozelanica populations. In both species, the highest level of variation was observed within sites (L. neozelanica: 86% of the total variation, $p = 0.010$; T. sanguinea: 92% of the total variation, $p = 0.010$). In L. neozelanica, significant variation among sites within fiords was also observed (10% of the total variation, $p = 0.010$). In T. sanguinea, there was no significant variation found at this level. Both species demonstrated significant variation among fiords, however T. sanguinea showed more variation among fiords than L. neozelanica (L. neozelanica: 4% of the total variation, $p = 0.010$; T. sanguinea: 8% of the total variation, $p = 0.010$). $F$-statistic estimates also suggested more genetic variation among sites ($\theta_{ST}$) than among fiords ($\theta_{FT}$) in L. neozelanica ($\theta_{ST} = 0.1198$, 95% CI = 0.0802 to 0.1560; $\theta_{FT} = 0.0401$, 95% CI = 0.0086 to 0.0758). $\theta$-estimates for between site differentiation and between fiord differentiation were of similar magnitude in T. sanguinea ($\theta_{ST} = 0.0471$, 95% CI = 0.0279 to 0.0697; $\theta_{FT} = 0.0428$, 95% CI = 0.0230 to 0.0656). All $F$-statistic estimates produced confidence intervals that did not overlap 0 indicating that genetic differentiation at both hierarchical levels and in both brachiopods was statistically significant. For L. neozelanica, point estimates of $\Phi$-statistics were within the confidence intervals of the corresponding $\theta$-values; for T. sanguinea, however, the $\Phi$-statistics were slightly larger than the upper confidence intervals of the corresponding $\theta$-values. This suggests that the analysis of estimated
allele frequencies and raw marker phenotypes yielded similar but not identical information.

Pairwise comparisons of genetic distance indicated statistically significant differentiation of *L. neozelanica* between sites in Long Sound and those in Doubtful and Breaksea Sounds (Table 4.2). *Terebratella sanguinea* illustrated statistically significant differentiation only between 2-BRK O and 3-LNG 1 (Table 4.3). Overall, levels of genetic differentiation were higher for *L. neozelanica* than for *T. sanguinea*.

Both Nei’s genetic distance and coancestry distance produced similar relationships between sites for the UPGMA and MDS analyses (but with different scales of differentiation as was the case in Chapter 3). Therefore, only the analyses using Nei’s genetic distance are discussed and shown.

Analyses suggested that exchange among sites within a fiord occurs less frequently among *L. neozelanica* than *T. sanguinea*. In both species, however, a break between Long Sound and the other fiords was visible (Fig. 4.4). The UPGMA dendrograms illustrated this overall pattern. The most evident feature on both trees was the prominent division with a high bootstrap support between the Long Sound sites and the other sites sampled (Fig. 4.4). Also important was the manner in which the other sites grouped together; in *L. neozelanica*, sites within a fiord (excepting the Long Sound sites) did not group in the topology (Fig. 4.4 (a)) whereas in *T. sanguinea* the two Breaksea Sound sites formed the shallowest grouping, and the two Doubtful Sound sites branched from this (Fig. 4.4 (b)). It is important to note, however, that the bootstrap values were low on the *L. neozelanica* dendrogram, and that there were many tied topologies with the *T. sanguinea* dendrogram. Therefore, the specific topologies must be interpreted with caution.

MDS ordinations suggested the same pattern. Excepting the two Long Sound sites, sites within a fiord did not fall along the same line in either the horizontal or vertical dimension for *L. neozelanica* (Fig. 4.5 (a)). For *T. sanguinea*, the x-axis separated the sites by fiord (Fig. 4.5 (b)).
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Fig. 4.4. UPGMA dendrogram calculated from AFLP data for *L. neozelanica* (a) and *T. sanguinea* (b). Scale indicates Nei’s (1978) genetic distances. Site numbers as in Table 4.1. Values associated with the nodes refer to the proportion of 1000 bootstrap replicates over loci that resulted in the formation of a cluster similar to the one shown above (bootstrap values of less than 75% are not shown). Dendrograms were created in TFPGA (Miller 1997b).

Fig. 4.5. *L. neozelanica* (a) and *T. sanguinea* (b). Multi-dimensional scaling (MDS) ordinations, calculated from Nei’s (1978) genetic distance matrices for pairs of samples. For both analyses, stress value = 0. Ordinations were created using PRIMER v5 (Clarke and Gorley 2001).
Table 4.2. Pairwise comparisons of *L. neozelanica* genetic distance (Nei 1978) measures among all sites. Statistical significance calculated as the combined probabilities over all loci for each pairwise comparison. Exact tests of significance (Raymond and Rousset 1995) and genetic distance calculations were performed in TFGPA (Miller 1997b).

<table>
<thead>
<tr>
<th></th>
<th>1-DFL O</th>
<th>1-DFL I</th>
<th>2-BRK O</th>
<th>2-BRK I</th>
<th>3-LNG O</th>
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<tr>
<td>1-DFL O</td>
<td>-----</td>
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<td></td>
</tr>
<tr>
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<td></td>
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<td>0.0081</td>
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<td>-----</td>
<td></td>
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</tr>
<tr>
<td>3-LNG O</td>
<td>0.0176</td>
<td>0.0211**</td>
<td>0.0115</td>
<td>0.0176</td>
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<tr>
<td>3-LNG I</td>
<td>0.0237**</td>
<td>0.0219*</td>
<td>0.0127</td>
<td>0.0197*</td>
<td>0.0121</td>
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</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01

Table 4.3. Pairwise comparisons of *T. sanguinea* genetic distance (Nei 1978) measures among all sites. Statistical significance calculated as the combined probabilities over all loci for each pairwise comparison. Exact tests of significance (Raymond and Rousset 1995) and genetic distance calculations were performed in TFGPA (Miller 1997b).

<table>
<thead>
<tr>
<th></th>
<th>1-DFL O</th>
<th>1-DFL I</th>
<th>2-BRK O</th>
<th>2-BRK I</th>
<th>3-LNG I</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-DFL O</td>
<td>-----</td>
<td></td>
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<tr>
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<tr>
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<td>0.0079</td>
<td>0.0095**</td>
<td>0.0088</td>
<td>-----</td>
</tr>
</tbody>
</table>

**p < 0.01

**Genetic diversity**

Nei’s unbiased expected heterozygosity (*H*) was higher among *L. neozelanica* than *T. sanguinea* except in Long Sound (Table 4.4). The pattern in Long Sound was anomalous for both species (Table 4.4). The two sites in Long Sound had the lowest *H* values of the *L. neozelanica* sites (3-LNG O, *H* = 0.0792; 3-LNG I, *H* = 0.0744); in *T. sanguinea*, the Long Sound site had a much higher *H* value than the other sites (3-LNG I, *H* = 0.1322) (Table 4.4). The same was true for the proportion of polymorphic loci (*P*); in *L. neozelanica* the lowest *P* estimates were in Long Sound whereas in *T. sanguinea* the highest *P* estimate was in Long Sound. In *L. neozelanica*, *P* values ranged from a maximum of 39.24% at 1-DFL O to a minimum of 24.05% 3-LNG I
(Table 4.4). P values for T. sanguinea ranged from a maximum of 47.25% 3-LNG I to a minimum of 27.47% at 1-DFL O and 2-BRK O (Table 4.4). In L. neozelanica, H values were higher at the inner sites in Doubtful Sound and Breaksea Sound; in T. sanguinea, P values were consistently higher at the inner sites.

Table 4.4. Location, sample size, expected heterozygosity, and the percent of polymorphic loci for both of the brachiopod species. Expected heterozygosity estimates and percent polymorphic loci calculations were conducted in TFPGA (Miller 1997b).

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Liothyrella neozelanica</th>
<th>Terebratella sanguinea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expected</td>
<td>% polymorphic</td>
<td>Expected</td>
</tr>
<tr>
<td></td>
<td>Heterozygosity</td>
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</tr>
<tr>
<td>n</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>Doubtful Sound</td>
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<td>Doubtful Sound</td>
<td>24</td>
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<tr>
<td>9-BRK O</td>
<td>Breaksea Sound</td>
<td>17</td>
<td>0.0979</td>
</tr>
<tr>
<td>9-BRK I</td>
<td>Breaksea Sound</td>
<td>21</td>
<td>0.1134</td>
</tr>
<tr>
<td>12-LNG O</td>
<td>Long Sound</td>
<td>19</td>
<td>0.0792</td>
</tr>
<tr>
<td>12-LNG I</td>
<td>Long Sound</td>
<td>20</td>
<td>0.0744</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td>17.8</td>
<td>0.0682</td>
</tr>
<tr>
<td><strong>Standard Error</strong></td>
<td>1.8</td>
<td>0.0067</td>
<td>2.05</td>
</tr>
</tbody>
</table>

*Nei's (1978) unbiased expected heterozygosity (H)*

*percent polymorphic loci (P)*

Table 4.5. Location and AFLP fragments (relative to the total number of alleles per species) found at only 1, 2, or 3 sites for each of the brachiopod species.

<table>
<thead>
<tr>
<th>Population</th>
<th>Location</th>
<th>Liothyrella neozelanica</th>
<th>Terebratella sanguinea</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fragments</td>
<td>Fragments</td>
<td>Fragments</td>
</tr>
<tr>
<td></td>
<td>in 1 pop</td>
<td>in 2 pops</td>
<td>in 3 pops</td>
</tr>
<tr>
<td>1-DFL O</td>
<td>Doubtful Sound</td>
<td>0.114</td>
<td>0.101</td>
</tr>
<tr>
<td>1-DFL I</td>
<td>Doubtful Sound</td>
<td>0.114</td>
<td>0.089</td>
</tr>
<tr>
<td>2-BRK O</td>
<td>Breaksea Sound</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>2-BRK I</td>
<td>Breaksea Sound</td>
<td>0.038</td>
<td>0.063</td>
</tr>
<tr>
<td>3-LNG O</td>
<td>Long Sound</td>
<td>0.038</td>
<td>0.038</td>
</tr>
<tr>
<td>3-LNG I</td>
<td>Long Sound</td>
<td>0.025</td>
<td>0.013</td>
</tr>
<tr>
<td><strong>Mean</strong></td>
<td></td>
<td>0.059</td>
<td>0.055</td>
</tr>
<tr>
<td><strong>Standard Error</strong></td>
<td>0.016</td>
<td>0.013</td>
<td>0.010</td>
</tr>
</tbody>
</table>
Neither $H$ nor $P$ was significantly correlated with the number of animals sampled in a population ($L$. neozelanica: $H$: $r^2 = 0.0321$, $p = 0.7339$; $P$: $r^2 = 0.0999$, $p = 0.5417$; $T$. sanguinea: $H$: $r^2 = 0.3482$, $p = 0.2949$; $P$: $r^2 = 0.0676$, $p = 0.6726$).

In general, the two brachiopod species had similar mean frequencies of rare fragments, though the sites with the highest frequencies differed (Table 4.5). In $L$. neozelanica, the number of fragments present in only a single population was highest at the two Doubtful Sound sites (9 at each site) whereas the other four sites had fewer (Table 4.5). This pattern was consistent among fragments that were present in two and three populations with the two Doubtful Sound sites having the highest numbers of fragments. In $T$. sanguinea, nearly twice as many “singletons” (fragments found only in one population) were found in the Long Sound site as compared to the site with the next greatest count (16 in 3-LNG 1 versus 9 at 2-BRK O) (Table 4.5). Fragments present in only two populations showed the same pattern with the Long Sound site having nearly twice as many as any of the other sites where $T$. sanguinea were sampled (Table 4.5). All of the populations had similar numbers of fragments present in three populations (Table 4.5). No relationship between sample size and the number of rare fragments was observed in either species ($L$. neozelanica: fragments in 1 pop: $r^2 = 0.0161$, $p = 0.8385$; fragments in 2 pops: $r^2 = 0.0153$, $p = 0.8153$; fragments in 3 pops: $r^2 = 0.0643$, $p = 0.6279$; $T$. sanguinea: fragments in 1 pop: $r^2 = 0.0188$, $p = 0.8260$; fragments in 2 pops: $r^2 = 0.0913$, $p = 0.6211$; fragments in 3 pops: $r^2 = 0.0446$, $p = 0.7330$).

**Isolation by distance**

The Mantel test revealed a significant relationship between genetic distance estimates and geographic distance for $L$. neozelanica ($r = 0.7760$, $p = 0.0120$) (Fig. 4.6). For $T$. sanguinea, this relationship was not significant ($r = 0.7926$, $p = 0.1260$) (Fig. 4.6).
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Fig. 4.6. Plot and linear regression of Nei’s (1978) genetic distance versus geographic distance for *L. neozelanica* (open circles) and *T. sanguinea* (filled circles). Significance values were calculated using a Mantel test in TFPGA (Miller 1997b).

Fig. 4.7. Slatkin’s (1981) conditional average frequency for *L. neozelanica* (open circles) and *T. sanguinea* (filled circles).
Conditional average frequency

The $\bar{p}(i)$ values were low for both species. The $\bar{p}(i)$ values for *L. neozelanica* resembled those of an organism with moderate dispersal capacity. The $\bar{p}(i)$ values of *T. sanguinea* were consistently lower and resembled the pattern found for moderate to high dispersal organisms (Fig. 4.7).

Discussion

Population structure and larval dispersal

In concordance with our predictions, population structuring was found to correspond to the contrasting modes of larval dispersal. The UPGMA, MDS, and AMOVA analyses, as well as conditional average frequency calculations, suggested *L. neozelanica*, which broods its larvae (Chuang 1994) is a poorer disperser than is *T. sanguinea*, which does not brood its larvae. With *T. sanguinea*, sites within fiords clustered in the UPGMA and MDS analyses, again suggesting exchange among *T. sanguinea* populations, especially those within a fiord. AMOVA results illustrated not only less variation among *T. sanguinea*, but also elucidated the hierarchical levels over which this variation occurred. Among *L. neozelanica*, the majority of variation occurred within a site, but a considerable amount of variation also occurred between sites within fiords. Among *T. sanguinea*, the majority of variation again occurred within sites, however no significant variation occurred between sites within fiords and instead a variation occurred among fiords, indicating genetic exchange between sites within a fiord and interruption of gene flow between fiords. Structuring of populations similar to that in *L. neozelanica* has been reported in other organisms with low dispersal capability. The black coral (*Antipathes fiordensis*) in Fiordland, a taxon with non-feeding, negatively buoyant, short-lived (approximately 10 days) planulae larvae (Miller 1996), displayed genetic differentiation between sites within fiords, but genetic structuring among fiords was not evident (Miller 1997a). This similarity suggests that *L. neozelanica* larvae possess dispersal capabilities similar to the planulae larvae of *A. fiordensis*. Miller (1997a) hypothesized that the pattern she observed in *A. fiordensis* might be the result
of a young system that has yet to reach equilibrium. Although the very long generation
time of the black coral could slow the system from reaching drift-migration
equilibrium, pronounced relationships between gene flow and distance at small but not
large spatial scales have been identified in a number of marine species with limited
dispersal capabilities and shorter generation times (Hellberg 1994; Johnson and Black
1998; Todd et al. 1998).

A system in equilibrium? Interpreting isolation by distance patterns
The shallow structure of the UPGMA dendrograms in both species as well as the low
\( \theta_{ST} \) estimates indicated that the genetic differentiation of Fiordland populations was not
large. The relative youth of this system in evolutionary time frames, especially in light
of the 2 - 4 year maturation period of brachiopods (Rickwood 1977; Doherty 1979),
suggests that not enough time has elapsed for strong genetic structure to develop.
Indeed, probably fewer than 6,000 brachiopod generations have inhabited the fiords,
and rough estimate based on densities of animals through Doubtful Sound (see Chapter
2) suggests that more than 3,000,000 generations would be necessary for \( T. \) sanguinea
populations to reach drift-migration equilibrium. Gene flow estimates based on non-
equilibrium data are likely to produce unrealistic results, convoluting historical
exchange and recent patterns of restricted gene flow. However, the significant
correlation between genetic and geographic distance matrices among \( L. \) neozealanica
samples (geographic distance explained 60% of the pattern of genetic differentiation)
indicated \( L. \) neozealanica populations have reached regional equilibrium between gene
flow and drift (Hutchinson and Templeton 1999). While \( T. \) sanguinea is found along
rock faces across Fiordland, \( L. \) neozealanica occurs in aggregated clusters (Richardson
1981) under overhangs or in caves (Wing, pers. com.). Although population sizes were
not estimated for \( L. \) neozealanica, the more patchy distribution and lower dispersal
capacity of \( L. \) neozealanica should be associated with lower effective population sizes.
Because the time scale of approach to drift-migration equilibrium is on the order of the
local effective population size (Slatkin 1993), \( L. \) neozealanica populations should reach
drift-migration equilibrium more quickly than \( T. \) sanguinea. In addition, detecting
isolation by distance suggested that dispersal in \( L. \) neozealanica followed a stepping-
stone model and that the scale of differentiation matched the scale of sampling. In
contrast, no significant correlation between genetic and geographic distances was found
among \textit{T. sanguinea} samples, suggesting drift-migration equilibrium in the fiords does
not yet exist for this organism at this scale. The slope of the \textit{T. sanguinea} regression is
lower than that of \textit{L. neozelanica}, indicating fewer dimensions in the stepping-stone
model describing dispersal of \textit{L. neozelanica} and lower dispersal ability (Wright 1943;
Vrijenhoek 1997). Because \textit{L. neozelanica} has lower dispersal capacity, the expanse of
deep water across a fiord (with no substrate for settlement) may act as a dispersal
barrier. This would limit dispersal in \textit{L. neozelanica} to a narrow zone along the rock
walls (a 1-dimensional stepping stone model), whereas higher dispersal capacity of \textit{T.
sanguinea} may allow larvae to cross the fiord (a 2-dimensional stepping stone model).
A stepping-stone model within a narrow linear habitat (such as along a fiord wall) can
produce much greater divergence among populations than area models that allow gene
flow to occur in many directions simultaneously (Pogson \textit{et al.} 2001). Further
assessment of cross-fiord versus up/down-fiord genetic connectivity in each brachiopod
species would provide a better understanding of the dispersal model for each species.

The lack of significant isolation by distance in \textit{T. sanguinea} was not surprising.
Isolation by distance was detected in \textit{L. neozelanica} at the scale of our sampling, and
the dispersal capability of \textit{L. neozelanica} is less than that of \textit{T. sanguinea}. This suggests
that the scale of our sampling was not appropriate for detecting a pattern of isolation by
distance for a brachiopod with higher dispersal capacity. Indeed, the scale of sampling
is an important factor in uncovering isolation by distance, and marine species with
limited dispersal capabilities often show different relationships between gene flow and
distance at differing spatial scales (e.g. Hellberg 1994; Johnson and Black 1998; Todd
\textit{et al.} 1998). However, an alternate mechanism behind the lack of correlation between
geographic and genetic distance exists. The influence of fiord hydrography on
passively dispersing planktonic larvae may have disrupted larval movement such that a
pattern of isolation by distance does not exist in the fiords for \textit{T. sanguinea}. The
genetic population structure of \textit{T. sanguinea} was similar to that of the sea star
\textit{Coscinasterias muricata}, an organism with long-lived planktonic larvae (Perrin 2002),
in which a large-scale (around New Zealand) pattern of isolation by distance was
observed, but no pattern between genetic and geographic structure within the fiords was
detected (Perrin 2002; Skold \textit{et al.} 2003). Taken together these data suggest
hydrographic features of the fiords may interrupt gene flow in some organisms with
planktonic larvae, but the fiords are not closed to gene flow. However, unambiguously attributing the pattern in *T. sanguinea* to a lack of equilibrium in an organism with high dispersal or interrupted gene flow due to hydrography is not yet possible. Investigation of *T. sanguinea* population genetic structure in a continuous environment would provide a useful comparison and an understanding of the scale at which dispersal is limited by larval duration in this species.

Colonization or hydrography?
One of the most apparent features of the population structure of both brachiopod species was the genetic differentiation between Long Sound and the other fiords. Both the MDS and UPGMA analyses illustrated a break between Long Sound and the other sites in both brachiopods. This break mirrors that found in the sea urchin *Evechinus chloroticus* and the sea star *C. muricata* (Perrin 2002). Oceanographic surveys of the fiords indicate that Long Sound has the most limited exchange with the open ocean due to sills and constrictions along the major axis of the fiord (S. Wing, pers. comm.). However, the presence of this division in the brooding brachiopod *L. neozelanica*, whose dispersal should not be influenced by hydrography, suggested that the isolation of Long Sound was not due, exclusively, to the hydrography of Long Sound. Estimates of fiord age based on height of the terminal sill separating each fiord from the Tasman Sea suggest that Long Sound is the youngest of the fiords (estimated age: Doubtful Sound 16,000 yrs; Breaksea Sound 15,500 years; Long Sound 9,000 years) (Smith 2001). Both the poor disperser and the better disperser illustrated the genetic break between Long Sound and the other fiords indicating historical factors such as the later colonization of Long Sound may have contributed to the differentiation. However, *L. neozelanica* populations in Long Sound sites had few rare fragments whereas the *T. sanguinea* samples from the inner Long Sound site (the only Long Sound site for *T. sanguinea*) had the highest number of fragments found in only one or two populations. The presence of so many rare fragments at the Long Sound site indicated little exchange between *T. sanguinea* from 3-LNG I and the other sites. If the rare alleles are interpreted as an indicator of isolation within Long Sound, then this indicates that, in addition to historical factors, the hydrography has influenced movement of planktonic larvae.
Terebratella sanguinea illustrated higher levels of percent polymorphic loci at inner fiord sites, suggesting that effective population sizes at inner sites may be larger than those at outer sites. L. neozelanica did not show a similar increase in polymorphic loci at inner sites. Planktonic larvae of T. sanguinea transported by the LSL would be expected to move toward the inner fiord whereas advection near the outer fiord might decrease successful settlement; non-planktonic larvae would not be subject to such forces. These processes affecting larval transport and settlement could lead to discrepancies of larval recruitment that might influence effective population size between inner and outer fiord sites. However, neither L. neozelanica nor T. sanguinea populations demonstrated genetic structure corresponding to environmental variation (outer versus inner) within a fiord. Although L. neozelanica populations within a fiord illustrated genetic differentiation, there was no genetic similarity between similar habitats in different fiords that would suggest an environmental driver. This is in contrast to the sea urchin Evechinus chloroticus that demonstrated genetic differences corresponding to ecotypes (Perrin 2002) as well as to the genetic patterns observed in the eelpout Zoarces viviparus in the Danish Mariager Fjord (Olsen et al. 2002). Therefore, there is no current evidence to support the hypothesis that these two species of brachiopod are influenced by the environmental gradient within the fiords over evolutionary time scales.

Among T. sanguinea, the highest H and P estimates occurred at the Long Sound site. However, excepting the Long Sound sites, estimates of H and P were higher among L. neozelanica populations than T. sanguinea populations. Increased levels of gene flow should slow the loss of alleles due to drift, so heterozygosity should be positively correlated with dispersal and gene flow (Bohonak 1999). Therefore, the relationship between higher heterozygosity estimates and poor dispersal capacity may first appear to be counterintuitive. This pattern of higher genetic diversity in an organism with higher gene flow has been observed in topshells on the Great Barrier Reef (Borsa and Benzie 1996) and marine fish (Fauvelot and Planes 2002). Indeed, heterozygosity is a direct function of effective population size ($N_e$) and mutation rate ($\mu$) according to the model $H = 4 N_e \mu (4 N_e \mu + 1)$. In a number of studies, higher average heterozygosity estimates from AFLP data were associated with larger populations (Travis et al. 1996; Keiper and McConchie 2000; Rivera-Ocasio et al. 2002). Increasing evidence suggests that
recruitment of marine invertebrates with planktonic larvae occurs in a sweepstakes fashion where only a few individuals “win” by producing the majority of recruits (Watts et al. 1990; Hedgecock 1994a and b; Edmands et al. 1996; Johnson and Wernham 1999; Moberg and Burton 2000; Bernardi et al. 2001; Flowers et al. 2002). Under such a scenario, effective population sizes of the planktonic dispersing T. sanguinea may be smaller than those of L. neozelanica. In the case of the lower H values in Long Sound, hydrography likely restricted gene flow; populations in a closed environment may be more likely to be self-seeding (and have larger effective population sizes and resulting higher levels of heterozygosity as well as more rare alleles – see above). Conversely, the lower H values for L. neozelanica in Long Sound illustrated the influence of historical and hydrographic restriction of the stepping-stone migration. Further analysis of the genetic differentiation between larval recruits from multiple years would determine if cohorts differ due to sweepstakes recruitment. Additionally, the analysis of a codominant marker would assist with the interpretation of these results. Because previous allozyme studies have indicated that some brachiopod populations in Fiordland may not be in Hardy-Weinberg equilibrium, the heterozygosity estimates from AFLP data must be interpreted with caution.

Conclusions
As is the case with most marine systems, populations in the fiords have only existed since the glacial retreat 10,000 to 12,000 years ago. Disentangling the genetic signal of thousands of past generations from the more recent signal of exchange among the fiords is difficult. A comparison of population genetic structure of two brachiopods with differing larval durations was used to approach this question. Although the relationship between larval duration and gene flow has been challenged, comparisons between the genetic structure of the brooding brachiopod, L. neozelanica, and the non-brooding T. sanguinea indicated that mode of dispersal corresponded with population genetic structure. In addition, the low dispersal brachiopod species appears to have reached migration-drift equilibrium while the higher dispersal brachiopod species does not. Finally, the comparison of the two brachiopod species suggested that the genetic
break between Long Sound and the other fiords has been a result of both historical and hydrographic features.

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General discussion

Introduction

The effect of the fiord environment on the articulate brachiopod *Terebratella sanguinea* over ecological and evolutionary time scales was investigated. The influence of a habitat gradient on *T. sanguinea* population structure within a single fiord provided insight into the mechanisms that might affect population structure across Fiordland (shorter ecological time scale). Additionally, animals from Fiordland were analysed to determine the impact of hydrographic barriers and geographic distance on population genetic structure (longer evolutionary time scale). Finally, the effects of pelagic larval dispersal were assessed by comparing genetic patterns of *T. sanguinea* to those of the brooding brachiopod *Liothyrella neozelanica*.

Genetic patterns in light of natural history observations in Doubtful Sound

The environmental gradient of Doubtful Sound significantly influenced aspects of *T. sanguinea* population structure. Density and size differed significantly between animals at sites along the main axis of the fiord (Fig. 2.3). The lower density and smaller size of the outer fiord animals suggested that the outer site is a less favourable environment for *T. sanguinea*. This probably is related to the potential for overgrowth by other organisms at the outer sites.

Direct evidence of a genetic component of spatial differences in growth and life history features requires molecular markers that are linked to genes that control adaptive variation in life history traits, and comparisons of traits among populations after environmental effects are controlled. However, if gene flow is reduced between populations, analysis of genetic structure using neutral markers will identify the divergence.
Studies of *T. sanguinea* genetic patterns used presumptive neutral genetic markers. Therefore it was not possible to determine if a genetic component was related to differences in size of *T. sanguinea* at inner and outer fiord sites. However, analyses of neutral marker data for populations of *T. sanguinea* across Doubtful and Thompson Sounds (presented in Chapter 3) did not detect a restriction of gene flow. Genetic differentiation between the 6-THM RP site and other sites was observed (Fig. 3.5), although there was no concordant pattern in the ecological data. It must be noted that it is possible that self-seeding over ecological time-scales, due to frequent short dispersal, and genetic mixing over evolutionary time-scales, due to less frequent long-distance gene flow, are completely compatible. Although genetic and ecological studies provide complementary information about organisms, they address larval dispersal on extremely different temporal scales.

Histology of the female gonads of *T. sanguinea* indicated that, within a population, individuals reproduced synchronously and produced pelagic larvae (Fig. 2.7). Marine organisms with pelagic larvae are generally highly fecund but demonstrate variability in gamete quantity, quality, and competence (Lannan et al. 1980; Lannan 1980a; Lannan 1980b; Muranaka and Lannan 1984; Levitan 1993; Levitan 1996; Marshall et al. 2002; Marshall and Keogh 2003; Huchette et al. 2004; Marshall et al. 2004; Marshall and Evans 2005; Styan et al. 2005; Levitan 2006). Furthermore, reduced fertilization success of externally fertilizing species can occur due to dilution of sperm and low density of spawning adults (Denny and Shibata 1990; Levitan et al. 1992; Babcock et al. 2000). Additionally, pelagic larvae are highly vulnerable while drifting in the plankton, and passive movement to or from suitable settling sites influences successful larval settlement. The aforementioned factors contribute to the patterns of uneven recruitment. In each generation, it is possible for a small minority of individuals to replace the entire population by a sweepstakes-chance matching of reproductive activity with oceanographic conditions (Hedgecock 1994; Flowers et al. 2002). The reproductive success of the minority and failure of the majority creates population variance in offspring number orders of magnitude larger than binomial or Poisson variance (Hedgecock 1994). The reproductive and life history traits of *T. sanguinea* suggest sweepstakes recruitment can occur. Further genetic analysis of individual cohorts would be necessary to definitively conclude whether effective
population sizes are smaller than census population sizes. Additionally, further genetic analysis using a co-dominant marker would provide insight. With dominant markers such as AFLP, it is not possible to explore allele combinations within individuals and populations, and, therefore, it is impossible to uncover the heterozygote deficiencies associated with heterogenous groups of recruits. Preliminary allozyme analysis of T. sanguinea in Fiordland indicated heterozygote deficiencies in populations in two of the five fiords examined, though patterns in the remaining fiords are unknown. During sampling for the current study, animals from a newly settled cohort were collected for an analysis of recruit heterogeneity; however these individuals were not analysed due to constraints in time and funding.

Spawning of T. sanguinea occurred during the austral autumn (May) (Fig. 2.7). During winter months, less freshwater enters the fiords when rivers and glaciers freeze, and the influence of estuarine circulation is less pronounced when the freshwater layer is reduced. In the absence of the consistent influence of a pronounced estuarine circulation, the stochastic effects of storms will play a larger role in the movements of planktonic larvae. Performing similar genetic analysis on an ecologically similar species (with a similar planktonic larval duration) that spawns when freshwater input is higher could test the effect of winter spawning on dispersal.

Population genetics of organisms of the New Zealand fiords compared to organisms from other fiord systems

The New Zealand fiords provide an exceptional opportunity to study genetic partitioning in a marine environment. Unlike other marine environments, fiords represent a physically fragmented marine system. Similar to fiords in Chile and Norway, the New Zealand fiords are characterized by a mean estuarine circulation, a shallow sill separating the fiord from the outer coast, and environmental gradients (salinity, temperature, and productivity). These factors influence the organisms inhabiting the fiords. The articulate brachiopods T. sanguinea and L. neozeланica illustrated low levels of genetic differentiation among fiord populations, though, unlike
ecological patterns, genetic patterns did not correspond to the environmental gradient but rather suggested that Long Sound may be isolated from the other fiords (Fig 3.5, Fig. 3.8, Fig. 4.5). Genetic analysis of T. sanguinea populations suggested that the Long Sound site has differentiated via isolation and ensuing drift and that genetic patterns across Fiordland represent secondary contact between the Long Sound group and the other fiords. Comparisons of the genetic patterns of the two brachiopod species with different larval dispersal capabilities also illustrated that the unique hydrography of Long Sound (Fig. 4.5), and its effects on circulation patterns, is a likely mechanism for the genetic differentiation. Furthermore, the comparison of the two brachiopod species indicated that the effect of the fiord environment on genetic patterns differs between organisms with contrasting ontogenies (Fig 4.6, Fig. 4.7). Although the increased isolation of Long Sound influenced the genetic patterns of L. neozelanica, this brooding brachiopod still showed a stepping stone migration pattern throughout Fiordland (Fig. 4.6). This contrasted with the genetic patterns of T. sanguinea, a brachiopod with planktonic larvae. Terebratella sanguinea showed high levels of exchange and little genetic differentiation both within a fiord and between fiords (Fig. 3.5, Fig. 3.6). At the larger scale, T. sanguinea showed a slight reduction of genetic exchange, especially among Long Sound populations and other populations (Fig. 3.5, Fig. 3.6). These results indicated that indeed the estuarine circulation of fiords influenced gene flow, but that the fiords were not closed.

Different organisms and different markers do not illustrate a consistent response to the New Zealand fiord landscape and environment. Allozyme studies of other benthic invertebrates in the fiords exhibited random genetic subdivision (black coral, Antipathes fiordensis, (Miller 1997); sea star, Coscinasterias muricata, (Skold et al. 2003)). Similar to the current studies, in these previous studies the young age of the fiords and the potential restriction of gene flow resulting from the estuarine circulation pattern have been implicated as potential mechanisms in shaping the population structure in Fiordland. This differs from the genetic patterns among the endemic sea urchin populations. Evechinus chloroticus populations differed between inner and outer fiord sites (Perrin 2002), a difference attributed to the ecological gradients in the fiords.
The genetic differentiation of organisms in the Norwegian fiords also showed conflicting patterns. Several organisms illustrated genetic differentiation between offshore samples and fiord samples (glacier lanternfish, *Benthosema glaciale*, (Suneetha and Salvanes 2001); pearside, *Maurolicus muelleri*, (Suneetha and Naevdal 2001); blue whiting, *Micromesistius poutassou*, (Giaever and Stien 1998); Atlantic cod, *Gadus morhua*, (Jorstad and Naevdal 1989; Dahle 1991; Fevolden and Pogson 1997); euphausiid, *Meganyciphanes norvegica*, (Bucklin et al. 1997)). Genetic differentiation has been attributed to partial isolation of the fiords. A number of mechanisms have been proposed to explain the partial isolation of the Norwegian fiords. Mechanisms include: 1) physical barriers in the form of a shallow entrance sill that would restrict passive drift of organisms, 2) behavioural barriers in the form of active swimming of larvae and adults against currents and active positioning in the water column, and 3) local adaptation to temperature and salinity regimes, and 4) reproductive isolation (Suneetha and Naevdal 2001; Suneetha and Salvanes 2001).

Although a number of organisms demonstrated differentiation of Norwegian fiord populations, the pattern among other organisms is less distinct. Conflicting results have been reported for the Atlantic herring, *Clupea harengus*. Microsatellite and allozyme analysis of population structure illustrated differences between Norwegian fiord stocks and other samples (Turan et al. 1998; Shaw et al. 1999), though mtDNA analysis suggested that the fiord populations were not genetically distinct (Turan et al. 1998; Hauser et al. 2001). Additionally, genetic patterns of resident plankton in the Norwegian fiords contrasted the lack of genetic differentiation of drifting plankton (Bucklin et al. 2000). Finally, haddock (*Melanogrammus aeglefinus*) illustrated an “isolation by distance” pattern among samples collected in the Norwegian fiords and coastal waters without evidence of genetically distinct fiord populations (Giaever and Forthun 1999).

Studies within the Norwegian fiords have focused on organisms capable of behaviourally altering their movements whereas studies in the New Zealand fiords have focused on benthic invertebrates with passive dispersal. Even so, the growing number of studies in both the New Zealand and Norwegian fiords indicates that fiord systems do not affect all organisms uniformly. As this body of information continues to grow,
the mechanisms structuring populations in fiords will become increasingly clear. However current evidence suggests that fiord systems are not closed, but that gene flow can be interrupted for some organisms.

Suggestions for future research
The current study investigated horizontal movement along the fiord axis. For benthic invertebrates, the deep-water expanse of the central fiord may present a barrier to larval dispersal. The potency of this barrier would be more pronounced in organisms with a shorter larval duration, such as *L. neozeulanica*. To investigate such constraints on larval movement, it would be necessary to compare the genetic differentiation of populations along the same rock wall versus those on opposite sides of the fiord. Higher differentiation between populations separated by a geographic distance encompassing deep-water than those separated by an equal distance along a rock wall would indicate that larval dispersal is limited in more than the horizontal dimension along the rock wall. In rivers, genetic differentiation can occur in one dimension, and the fiords may similarly limit movement for some organisms. Samples for this analysis were collected but have not been analysed due to financial and time constraints of the current study.

In this study we examined genetic patterns within the fiords. However, by comparing genetic patterns within and between fiords to populations on the outer coast separated by similar distances, we could further expand our understanding of the fiord environment on larval dispersal. Difficulty arises in locating these samples. The sampling in this study was limited by the known distribution of *T. sanguinea*. *Terebratella sanguinea* has not been collected along the West Coast of New Zealand’s South Island outside of the fiords. Other known populations are located in the protected bays of Stewart Island and the Marlborough Sounds. Adding these populations to the AFLP analysis would provide a representative example of non-fiord populations, however, it would be necessary to find more continuous distributions of non-fiord populations to compare fiord larval movement to open coast larval movement.
Finally, the current study assumed the dispersal capacity of brachiopods producing lecithotrophic larvae is limited. A growing body of evidence has suggested intra-specific (even intra-brood) flexibility of settlement and metamorphosis of larvae (Hadfield and Strathmann 1996; Krug 2001). In order to correctly interpret genetic patterns it is necessary to understand larval life history. During the current study, animals were kept at the Portobello Aquarium, however spawning was not observed. Induction of spawning and observation of larval behaviour would expand our understanding of the genetic patterns presented in this thesis.

Conservation and management implications

Understanding the population structure of some of the marine fauna of Fiordland is an important cornerstone for the developing management plan for the area. The existing management plan for the area regards the whole of Fiordland as a single unit. However, the genetic patterns revealed in this study illustrate that Long Sound is distinct from the other fiords. The unique genetic patterns of Long Sound suggest this area should be managed separately. A proposal for a series of newly defined marine protected areas within Fiordland incorporates this information, designating the whole of Long Sound as a protected area. In protecting all of Long Sound, the genetic diversity harboured within individual populations of organisms that disperse in a stepping stone fashion, similar to *L. neozelanica*, is conserved. Examining the genetic patterns of multiple species across Fiordland has provided the information necessary to understand movement in this physically fragmented system, which benefits management of the system. Conservation of the underwater resources of this World Heritage Area can be successful if the structure of the system and the mechanisms driving this structure are taken into account.
Chapter 5: General discussion

Literature cited


The AFLP technique

The method is a multi-step technique that combines both Restriction Fragment Length Polymorphism (RFLP) and Random Amplified Polymorphic DNA (RAPD) technologies. The generation of AFLP fingerprints involves three steps: 1) restriction of the DNA and ligation of oligonucleotide adapters, 2) amplification of sets of restriction fragments, and 3) gel analysis of amplified fragments. The first step of the process involves the restriction digestion of genomic DNA with a rare cutter (e.g. the six-base recognition restriction enzyme Eco RI) and a frequent cutter (e.g. the four-base recognition restriction enzyme Mse I). A frequent cutter is used to generate small fragments that amplify well and are in the optimal size range for separation on sequencing gels; the rare cutter is used to limit the number of fragments to be amplified since the AFLP technique preferentially amplifies restriction fragments with two different ends (Vos et al. 1995). Double-stranded oligonucleotide adapters then are ligated to the ends of the restriction sites. These adapters subsequently serve as "universal" binding sites for primer annealing during PCR amplification (step two). In this way, restriction fragments can be amplified with "universal" AFLP primers that correspond to the restriction site and adapter sequence with high specificity. However, for most genomes, amplification of all of the restriction fragments would exceed detection capabilities. As a result, the number of fragments amplified by PCR is reduced through the addition of selective bases at the 3’ end of the AFLP primers. This results in the selective amplification of those fragments in which the 3’ primer extensions match the nucleotides in the flanking regions of the restriction site. The number of restriction fragments amplified through PCR can be fine tuned through the selection of the number and identity of the selective bases in the AFLP primers, and the number of fragments simultaneously amplified should be limited to 50-100 to optimise the detection of fragments. The final stage of the AFLP technique involves separating and detecting the amplified fragments on denaturing polyacrylamide gels.

Literature cited

Appendix II: MtDNA

Preliminary studies to identify mtDNA markers for population analysis of *T. sanguinea* and *L. neozealanica*

**Introduction**
Attempts were made to locate a mitochondrial marker that would complement the whole genome AFLP data. A non-coding region should evolve more quickly than coding regions of the mitochondrial genome. For this reason, a non-coding region was sought to uncover the recent population structuring within the New Zealand fjords. However, the mitochondrial genome has not been sequenced for either genus of brachiopod involved in this study, and, therefore, the location of non-coding regions has not been identified. The articulate brachiopod *Terebratella transversa* possesses a 69 nucleotide non-coding region between the Cytochrome Oxidase I and Cytochrome b coding regions (Helfenbein et al. 2001). Efforts were made to amplify this region of the *Terebratella sanguinea* mitochondrial genome using a number of methods. An alternative strategy was to examine coding regions within the mitochondrial genome, which can be amplified with universal primers, for sufficient polymorphism to differentiate population differentiation.

**Non-coding region amplification**
Primers targeting the longest of the *T. transversa* non-coding regions identified by Helfenbein et al. (2001) were designed using the program PRIMERselect (DNASTAR). The primers were as follows: NCR1F, 5’ TTT TTA TGG TTT GAG AGG CCT TTA 3’, and NCR1R, 5’ TGA ACC ACC CGA CCA GGA CT 3’. Amplification reactions (31μl) contained 0.32 mM of each primer, 0.19 mM dNTP mix, 1.6 mM MgCl₂, 1X PCR buffer (ABGene), and 1 unit of *Tag* DNA polymerase (ABGene). After an initial denaturation of 2 min at 95°C in a PTC-100 thermal cycler (MJ Research), 35 cycles were carried out as follows: 30 s at 94°C, 40 sec at 46°C, and 1 min at 72°C, with a final extension of 4 min at 72°C. These conditions were made more stringent when multiple bands were produced, and the annealing temperature was raised to 48°C, and then finally to 50°C. In all cases, two bands of equal intensity were produced, the larger of which was approximately 500 bp and the smaller approximately 300 bp. For
some individuals, a third and fourth band (both <200 bp – the size anticipated) were also produced, but these amplified less strongly than the other two. In addition to altering the annealing temperature, MgCl₂ concentrations and primer concentrations were also altered. The above protocol was used with an annealing temperature of 48°C (this gave the best amplification) and MgCl₂ concentrations of 1.2 mM and 0.8 mM. The same bands were present with these amplification conditions. Finally, the above protocol (48°C annealing temperature) was used with 1.2 mM MgCl₂ and 0.16 mM of each primer. As with the other permutations, the banding did not change with the reduced primer concentrations. Permutations with different MgCl₂ and primer concentrations were conducted with different concentrations of DNA, but in all cases the same suite of bands was produced.

Because amplification conditions could not be optimised to produce a single band, bands were separated on agarose gel (2.0%) and then cut from the gel. Bands cut from the agarose gel were purified according the manufacturer’s instructions using a Qiaquick gel extraction kit (Qiagen). DNA retrieved using this procedure was then re-amplified (1.6 mM MgCl₂, 50°C annealing temperature) and cycle sequenced with the Big-Dye sequencing kit (Applied Biosystems) for automatic sequencing using both the NCR1F and NCR1R primers. Termination fragments were separated and read on an ABI 377 automated sequencer. Clean sequences could not be obtained using this protocol. Alternatively, PCR products were cloned using the following procedure. PCR was carried out using the above protocol (48°C annealing temperature) using Pwo DNA polymerase (Roche Diagnostics) in place of Taq DNA polymerase (Pwo DNA polymerase has an inherent 3’-5’ exonuclease proofreading activity making it 10 times more accurate than Taq DNA polymerase; in addition, Pwo DNA polymerase creates blunt-end fragments that can be directly ligated into a plasmid without polishing whereas Taq DNA polymerase attaches an additional nucleotide at the 3’ end of an amplified fragment). Amplified fragments were purified with High Pure PCR product purification kit (Roche Diagnostics), and PCR products were diluted in 30μl of deionized, sterile water. PCR products (11μl) were blunt-end ligated into Sma I cut/BAP dephosphorylated pUC18 (100μg) (Amersham Pharmacia Biotech) by action of 5 units of T4 DNA ligase (Roche Diagnostics) in a volume of 20μl for 16 hr at 4°C.
Ten µl of the ligation reaction were cloned in 100µl of XL1-Blue competent cells (Cecile Perrin, pers comm.). After overnight growth (37°C) on Luria-Bertani (LB) agar plates containing ampicillin (100mg/ml), 50 mg/ml X-gal, and 40 mg/ml I.P7G, white colonies were picked and transferred to 300µl of ampicillin/LB broth in Eppendorf tubes and incubated at 37°C for 1 hr. Subsequently, clones were used as PCR template using the same conditions as above (with 48°C annealing temperature) but using the M13 universal primers. Products were visualized by agarose gel electrophoresis (2.0%) under UV light and using ethidium bromide. Those clones with a single band visible on the agarose gel were cycle sequenced using the M13R primer and the Big-Dye sequencing kit (Applied Biosystems) for automatic sequencing. Termination fragments were separated and read on an ABI 377 automated sequencer. This protocol did not produce clean sequences suitable for BLAST searching or designing T. sanguinea specific primers for the potential non-coding region identified in T. transversa by Helfenbein et al. (2001).

The original primers designed for the non-coding region (Helfenbein et al. 2001) did not amplify well with the second species of interest, L. neozelanica. Further attempts to locate a non-coding region in L. neozelanica were not undertaken.

Cytochrome Oxidase I amplification
The COI region was amplified using two sets of primers, the universal primers CO1a (5’ CTG ATG GGT CTG CGA ATA AGT – 3’)/CO1f (5’-CCT GCA GGA GGA GGA GAY CC 3’) (Palumbi 1996) and the COI–7 (5’ ACN AAY CAY AAR GAY ATY GGN AC 3’)/COI D (5’ TCN GGR TGN CCR AAN ARY CAR AA 3’) primers (sensu Endo et al. 2001)). Amplification reactions (31µl) contained 0.32 mM of each primer, 0.19 mM dNTP mix, 1.2 mM MgCl₂, 1X PCR buffer (ABGene), and 1 unit of Taq DNA Polymerase (ABGene). PCR conditions were: 2 min at 95°C followed by 35 cycles of 30 s at 94°C, 40 s at 48°C, and 1 min at 72°C with a final extension of 2 min at 72°C (PTC-100 thermal cycler, MJ Research). Amplifications with the CO1a/CO1f primer combination produced a strong band for both T. sanguinea and L. neozelanica when PCR products were run on an agarose gel (2%) (band size was approximately 500
bp in *T. sanguinea* and 600 bp in *L. neozelanica*. The COI-7 and COI D primers also produced a strong band with both species (of approximately 700 bp), but shadow banding at lower molecular weights was visible even when the annealing temperature was raised to 52°C. Additionally, the band obtained with both *T. sanguinea* and *L. neozelanica* was considerably larger (approximately 700 bp) than that reported by Endo (2001) (200 bp). The CO1a/CO1f primer pair was selected for further use. DNA from five *T. sanguinea* and five *L. neozelanica* was amplified using the CO1f primer and cycle sequenced with the Big-Dye sequencing kit (Applied Biosystems) for automatic sequencing. Termination fragments were separated and read on an ABI377 automated sequencer. Sequences were submitted for BLAST searching (Altschul et al. 1997) to verify that the COI region had been amplified. BLAST searches produced matches to COI sequences from other organisms, but not for other brachiopods. Sequences were aligned by eye using the program ESEE, version 3S (Cabot and Beckenbach 1989). One change was found in the *L. neozelanica* samples, and no changes were found in *T. sanguinea*. Further sequencing of COI amplifications was not pursued due to time and financial constraints.

**Cytochrome b amplification**

The Cyt b region was amplified using universal primers modified for echinoderms (CB1 Urchin -5' CCC TCC AAC CTT TCC ATT TGG TGA AA 3', and CB2 Urchin -5' ACC CCC GTT TAC AGG AAA ACT CGA 3' (Palumbi 1996)). Amplification reactions (10μl) contained 0.375 mM of each primer, 0.8 mM dNTP mix, 1.5 mM MgCl₂, 1X PCR buffer (ABGene), and 0.25 units of Taq DNA polymerase. PCR conditions were: 5 min at 95°C followed by 40 cycles of 40 s at 94°C, 30 s at 50°C, and 2 min at 72°C (PTC-100 thermal cycler, MJ Research). This amplified a product of approximately 700-800 bp in *T. sanguinea*, but also faint banding at lower molecular weights. Increasing the annealing temperature to 53°C and then 55°C marginally reduced this background banding. *L. neozelanica* was amplified at 55°C, but the product size for *L. neozelanica* was inconsistent; in some animals a band of 300 bp amplified and in others a band of 800 bp amplified (with a series of lower molecular weight bands amplifying less strongly).
The Cyt b region also was amplified using the universal primers CB1 (5' CCA TCC AAC ATC TCA GCA TGA TGA 3')/CB2 (5' ACT CCT GTT TAT AGT AAG ACT CCC 3') (Simon et al. 1994; Palumbi 1996). These primers are the same CB1 and CB2 primers reported previously but without the modifications for echinoderms (Palumbi 1996). Amplification reactions were identical to those used for the preceding Cyt b primers. PCR conditions were an initial denaturation of 5 min at 95°C followed by 35 cycles of 40 sec at 94°C, 30 s at 49°C, and 30 s at 72°C (PTC-100 thermal cycler, MJ Research). This amplified a product of approximately 600 bp in both T. sanguinea and L. neozelanica (the expected sized fragment). A 600 bp fragment is considered large for Single Stranded Conformation Polymorphism (SSCP), and it is possible that some variation may not be detected with such a large fragment. However, this technique allows screening for variation in a large number of individuals.

SSCP analysis (Orita et al. 1989) was used to look for sequence variation within the Cyt b region for 23 L. neozelanica and 31 T. sanguinea individuals. The labelling reaction (20μl) contained 100 pmol of either primer (CO1a and CB2), 1.5μl of γ33ATP (2500 Ci/mmol, 10mCi/ml), 10 units of T4 Polynucleotide Kinase (T4 PNK, Roche Diagnostics), and 1X T4 PNK buffer (Roche Diagnostics). Amplifications were carried out in a 31μl reaction mixture containing 0.65 mM of each primer, 0.19 mM dNTP mix, 1.2 mM MgCl2, 1X PCR buffer (ABGene), and 1 unit of Taq DNA polymerase (ABGene). PCR cycling conditions were: 2 min at 95°C followed by 40 cycles of 30 s at 94°C, 40 s at 50°C, and 1 min at 72°C with a final extension of 4 min at 72°C (PTC-100 thermal cycler, MJ Research). Twenty μl of denaturing solution (95% Formamide, 20 mM EDTA pH 8.0, 0.05% Bromophenol blue, and 0.05% Xylene cyanol) were added to each PCR reaction. PCR products then were heat denatured for 5 min at 94°C and immediately put on ice. Samples were run on a non-denaturing polyacrylamide gel (6% 37.5:1 bis:acrylamide (Biorad Laboratories), 5% glycerol, and 0.5X TBE) in 0.5X TBE buffer. Gels were run at 4°C for 20 hrs at 10W. Large format sequencing gel rips were used. Haplotypes were visualized using autoradiography (Biomax film, Kodak). Low levels of polymorphism were detected in both brachiopod species; further attempts to use the Cyt b region as a mtDNA marker were not made.
Amplification of the intergenic region between Cytochrome Oxidase I and Cytochrome b

COI and Cyt b universal primers were combined to determine whether the region between these two genes could be amplified. Ten combinations of the primers CO1a, CO1f, COI-7, COI-D, CB1 Urchin, CB2 Urchin, Cytb-F (5' - GGA ATA TTT YTA GCA ATG CAC TA) (A. Chenril, pers. com.), and Cytb-R (5' - CAT TCC GGY TGW ATG TGT GGG GGA) (A. Chenril, pers. com.) were used (the unmodified CB1 and CB2 primers were not included in these permutations because these primers were added to the study after this initial exploration of the region between COI and Cyt b). Of these, only four combinations resulted in a product visible when run on an agarose gel (2%). The combinations CO1f/CB2 Urchin, CO1a/CB2 Urchin, CO1f/CB1 Urchin, and CO1f/CBR successfully amplified in at least two of the five individuals. However, only the combination CO1a/CB2 Urchin amplified in all five individuals, and only the CO1a/CB2 Urchin product showed a single, strong band (approximately 300 bp \textit{L. neozelanica} and 400 bp in \textit{T. sanguinea}) when visualized on the agarose gel. This pair of primers was anomalous, however, as it was composed of two reverse primers. A rearrangement within the mitochondrial genome could allow two reverse primers to work in tandem. This primer combination was selected for further examination, though we recognized that it would be necessary to establish the identity of the fragment before using it for population genetic analysis.

PCR products were sequenced in order to determine the identity of the fragment generated with the two reverse primers. Amplifications were carried out as before. Amplified fragments were purified using a High Pure PCR product purification kit (Roche Diagnostics) and cycle sequenced using Big-Dye cycle sequencing kit (Applied Biosystems) for automatic sequencing. Sequences were generated with either CO1a or CB2 Urchin in order to obtain the complete sequence of the amplified fragment. Termination fragments were separated and read on an ABI 377 automated sequencer. Sequences were submitted for BLAST searching (Altschul \textit{et al.} 1997) to verify that the COI and Cyt b regions had been amplified. BLAST searches produced matches to both COI and Cyt b regions of other organisms, however the matches never exceeded 23 bp of the 330 bp amplified fragment (excepting the primer sites), and other brachiopods were never among the matches found. Based on this information, coupled with the fact
that both primers are reverse primers, the identity of the fragment could not be assigned with certainty. It is possible that a rearrangement in the mtDNA has occurred, but it is also possible that a portion of the nuclear genome was amplified with this primer combination. Because it was impossible to be certain that the amplified fragments were part of the mitochondrial genome, no further attempts were made to use these fragments as mtDNA markers.

Literature cited
DNA extraction protocols

Introduction
Two methods of DNA extraction were used for the AFLP analysis. Initial extractions (used for samples from Dagg Sound, Doubtful Sound (BD site), Dusky Sound, and Stewart Island) were performed using a salting out technique (see below for protocol). DNA yield using this technique was inconsistent, and the AFLP assays from these extractions produced legible and reproducible fingerprints for only a small proportion of the samples. Therefore, excepting the samples from Dagg Sound, samples extracted with the original extraction protocol were eliminated from analysis. A second method was used for the remaining samples. DNA was extracted using the commercially available DNAzol Genomic DNA Isolation Reagent (Molecular Research Center, Inc., Cincinnati, OH). Fifty to 75 mg of lophophore tissue was added to 500 μl of DNAzol and 200-300 μg Proteinase K. Samples were incubated at 37°C for 30 – 120 minutes, until most of the tissue was broken down. DNA yield seemed to improve if samples were not incubated for long periods; therefore, samples were removed from the incubator even if some of the lophophore tissue remained intact after 120 minutes. Samples then were spun for 10 min at 10,000 g, and the resulting supernatant was removed. I added 400 μl of cold 100% EtOH, at which point DNA would become visible immediately. The DNA was retained and the EtOH and DNAzol were removed. DNA was rinsed twice with 800 μl 70% EtOH. Ethanol was removed, and the DNA was air dried overnight.

DNA extraction reagents

2X CTAB
100mM Tris-HCl, pH 8.0
1.4 M NaCl
20mM EDTA
2% Hexadecyltrimethylammonium bromide (CTAB)
0.2% 2-mercaptoethanol
Appendix III: DNA extraction

TNE
10 mM Tris, pH 8.0
0.1 M NaCl
1 mM EDTA

TNES
1 M Tris
5 M NaCl
0.5 M EDTA
20% Sodium dodecyl sulphate (SDS or sodium lauryl sulfate)

CTAB Extractions

CTAB and TNE Extraction
Lophophore tissue was digested by action of 600μl of 2X CTAB buffer, 75μl TNE and
20μl of proteinase K (10 mg/ml) (Sigma). Samples were incubated at 65°C for 1.5 h.
Genomic DNA was extracted from the digest using 250μl of chloroform: isoamyl
alcohol (24:1 v/v 1X). Tubes were agitated and then centrifuged for 2 min at 2,000g.
The supernatant then was transferred to a new tube, and DNA was precipitated by the
addition of 150μl ice cold 100% EtOH and 18μl NaOAc. Tubes were centrifuged at
4°C for 5 minutes at 15,300g. DNA was washed twice with 700μl of cold 70% EtOH
(each wash was followed with a spin cycle of 4°C for 2 minutes at 15,300g). Ethanol
was removed and DNA was dried at 65°C. Dried DNA was re-suspended in 30μl of
deionized sterile water.

Basic CTAB Extraction
Lophophore tissue was homogenized and digested by action of 600μl of 2X CTAB
buffer and 20μl of proteinase K (10 mg/ml) (Sigma). Samples were incubated at 65°C
for 1-24 h. Genomic DNA was extracted from the digest using 600μl of chloroform:
isoamyl alcohol (24:1 v/v 1x). Tubes then were centrifuged for 20 min at 15,300g.
The supernatant then was transferred to a new tube, and DNA was precipitated by the
addition of 600μl ice cold 100% isopropanol. After 1 h, tubes were spun at 4°C for 45
minutes at 15,300g. DNA was washed twice with 1,000μl of cold 70% EtOH. Ethanol
was removed and DNA was dried at 65°C. Dried DNA was re-suspended in 100μl of deionized sterile water.

**Salt Extractions**

**Basic Salt Extraction**

Lophophore tissue was digested by action of 200μl of 2X CTAB buffer and 20μl of proteinase K (10mg/ml) (Sigma) incubated at 65°C for 3 hours. Fifty μl of 6M NaCl were added and tubes were centrifuged at 3,000g for 10 minutes. After spinning, the supernatant was collected in new tubes. To precipitate the DNA, 700 μl of ice cold 100% EtOH was added to the supernatant, and the tubes were spun at 4°C for 20 minutes at 15,300g. Subsequently, DNA was washed twice with 700μl of cold 70% EtOH (each wash was followed with a spin cycle of 4°C for 20 minutes at 15,300g). Ethanol was removed, and DNA was dried at 65°C. Dried DNA was re-suspended in 30μl of deionized sterile water. In addition, this protocol was also used substituting TNES buffer for CTAB.

**Salt Extraction with Chloroform Rinse**

Lophophore tissue was digested by action of 200μl of 2X CTAB buffer and 20μl of proteinase K (10mg/ml) (Sigma) incubated at 65°C for 3 hours. Fifty μl of 5M NaCl were added and tubes were centrifuged at 3,000g for 10 minutes. After spinning, the supernatant was collected in new tubes, and 275μl of chloroform were added. Samples were spun for 10 minutes at 8,000g, and the clear supernatant was transferred to a new tube. To precipitate the DNA, 700 μl of ice cold 100% EtOH was added to the supernatant, and the tubes were spun at 4°C for 20 minutes at 15,300g. Subsequently, DNA was washed twice with 700μl of cold 70% EtOH (each wash was followed with a spin cycle of 4°C for 20 minutes at 15,300g). Ethanol was removed, and DNA was dried at 65°C. Dried DNA was re-suspended in 30μl of deionized sterile water.
Results

In all cases, the DNA yield using the above protocols was inconsistent. In addition, large low molecular weight smears were visible in addition to the DNA when extraction products were run on an agarose gels. Furthermore, success with AFLP protocols using DNA extracted according to the above protocols was inconsistent in that many of the samples did not work. Finally, DNA obtained from the above protocols that produced AFLP fingerprints demonstrated lower levels of reproducibility than those extracted using the DNAzol protocol used in the latter stages of this research. For those animals for which both an initial extraction and a DNAzol extraction were performed, banding between original DNA extractions and subsequent DNAzol extractions illustrated slight to marked differences in pattern. Because it was impossible in almost all cases to re-extract previously extracted samples due to degradation of tissues or lack of remaining tissue, animals used from the initial stages of the project could not be included in the final analysis of AFLP results.
Appendix IV. Enzyme names and running buffers used for a preliminary allozyme survey of *Terebratella sanguinea*. Buffer recipes for Tris-glycine (pH 8.5), Tris-malate (pH 7.8), and Phosphate (pH 7.0) were as in Richardson *et al.* (1986) (see Chapter 3 for full citation).

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<td>HBDH</td>
<td>Tris Glycine</td>
<td>No Staining</td>
</tr>
</tbody>
</table>
GENETIC DIFFERENTIATION OF _Terebratella sanguinea_ IN THE NEW ZEALAND FIORDS: A DISPERsal BARRIER IN THE MARINE ENVIRONMENT?

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Abstract:

The lack of known effective physical barriers in the marine environment in addition to high dispersal capabilities and long dispersal phases of marine organisms have led to the perception that gene flow is high and inter-population genetic differences are low in marine systems. Due to the hydrography of the 14 deep-water fiords on the western coast of New Zealand's South Island, inhabitants of Fiordland's marine environment may be limited in their dispersal; passive transport of larvae out of natal fiords may be limited by a weak estuarine circulation pattern. In order to test whether genetic differentiation among fiord populations is occurring, we used allozyme electrophoresis to determine the genetic structure of _Terebratella sanguinea_ populations in five fiords. Preliminary data from four allozyme loci suggest that, in general, fiordic brachiopod populations are not highly isolated. Eight of ten populations sampled were in Hardy-Weinberg equilibrium, and estimates of gene flow indicated little differentiation at a large scale (overall \(F_{st} = 0.0089\)). However, smaller scale comparisons (between pairs
of sampling sites) suggest that while dispersal may not be limited through the whole of Fiordland, genetic exchange in certain areas may be restricted.

Introduction:

The majority of marine fauna have a planktonic larval phase potentially able to drift for hundreds to thousands of kilometers before settling out of the water column (Palumbi, 1992). Typically, the populations of these species are large and the fecundities very high, numbering in the millions of eggs per adult female (Palumbi, 1992). Speciation of such taxa is poorly understood since these attributes should lead to high gene flow that would slow the process of species formation. However, marine faunal groups are typically speciose with complex patterns of phylogenetic partitioning. A central question in marine evolutionary biology is: what forces promote speciation in the marine environment?

Physical barriers between populations are recognized as a major force in promoting reproductive isolation and genetic divergence that precedes speciation (Mayr, 1942). For this reason, Fiordland, an area along the west coast of New Zealand’s South Island, is particularly interesting. Within Fiordland, the marine environment potentially is subdivided by a series of long, narrow fiords carved out by glaciers and inundated with seawater about 10,000 years ago (Pillans et al., 1992). The marine environment in Fiordland differs from other coastal inlets in both physical terrain and hydrography. At the entrance to each fiord is a high, submarine sill composed of rubble carved from the sides of valleys during periods of glaciation (Grange, 1990). Hydrographically, each fiord is characterized by a surface low salinity layer (LSL), created by the high rainfall and fresh water catchment of the area, which flows in a seaward direction (Stanton and Pickard, 1981). Dissolved minerals including salt from the fiordic seawater are entrained by the LSL and transported out to the open ocean. Salt equilibration between
the open ocean and the fiordic seawater occurs by a net movement of seawater from the open ocean into the fiord, moving between the submarine sill and the LSL. It is assumed that marine plankton within the fiords can gain access to the open ocean only if they, too, become entrained in the LSL, however the osmotic stress of this environment is presumed to inhibit such movement. As a result, populations within the fiords may be isolated from each other, depending on the specific hydrography of the fiords.

If gene flow between populations is limited, then divergence due to natural selection and drift may arise. Genetic data from sea urchins (Evechinus chloroticus) and sea stars (Coscinasterias muricata) collected in Fiordland suggest hydrographic features of the fiords may interrupt large-scale gene flow (Mladenov et al., 1997, Skold et al., in prep, Wing et al., in prep). However, patterns of genetic differentiation in a sessile organism with lecithotrophic larvae, such as the articulate brachiopod Terebratella sanguinea have not been described. In the present study we examine the spatial scale of genetic population structure in the brachiopod T. sanguinea collected in five fiords as well as from two ‘non-fiord’ sites by addressing the following questions: 1) Are animals from the open ocean differentiated from fiord populations? 2) Are animals from different fiords genetically distinct? 3) Are animals from different areas within a fiord genetically distinct?
Amplification of the intergenic region between Cytochrome Oxidase I and Cytochrome b

COI and Cyt b universal primers were combined to determine whether the region between these two genes could be amplified. Ten combinations of the primers CO1a, CO1f, COI-7, COI-D, CB1 Urchin, CB2 Urchin, Cytb-F (5′ – GGA ATA TTT YTA GCA ATG CAC TA) (A. Chenuil, pers. com.), and Cytb-R (5′ – CAT TCC GGY TGW ATG TGT GGG GGA) (A. Chenuil, pers. com.) were used (the unmodified CB1 and CB2 primers were not included in these permutations because these primers were added to the study after this initial exploration of the region between COI and Cyt b). Of these, only four combinations resulted in a product visible when run on an agarose gel (2%). The combinations CO1f/CB2 Urchin, CO1a/CB2 Urchin, CO1f/CB1 Urchin, and CO1f/CBR successfully amplified in at least two of the five individuals. However, only the combination CO1a/CB2 Urchin amplified in all five individuals, and only the CO1a/CB2 Urchin product showed a single, strong band (approximately 300 bp L. neozezanica and 400 bp in T. sanguinea) when visualized on the agarose gel. This pair of primers was anomalous, however, as it was composed of two reverse primers. A rearrangement within the mitochondrial genome could allow two reverse primers to work in tandem. This primer combination was selected for further examination, though we recognized that it would be necessary to establish the identity of the fragment before using it for population genetic analysis.

PCR products were sequenced in order to determine the identity of the fragment generated with the two reverse primers. Amplifications were carried out as before. Amplified fragments were purified using a High Pure PCR product purification kit (Roche Diagnostics) and cycle sequenced using Big-Dye cycle sequencing kit (Applied Biosystems) for automatic sequencing. Sequences were generated with either CO1a or CB2 Urchin in order to obtain the complete sequence of the amplified fragment. Termination fragments were separated and read on an ABI 377 automated sequencer. Sequences were submitted for BLAST searching (Altschul et al., 1997) to verify that the COI and Cyt b regions had been amplified. BLAST searches produced matches to both COI and Cyt b regions of other organisms, however the matches never exceeded 23 bp of the 330 bp amplified fragment (excepting the primer sites), and other brachiopods were never among the matches found. Based on this information, coupled with the fact...
from Bligh Sound and Dagg Sound represent animals collected over both years. Animals from Stewart Island were collected in February 2000. At all sites, brachiopods were collected by divers and dissected upon collection. Lophophore tissue was removed from each animal and frozen in liquid nitrogen in the field. Samples were maintained at -80 °C until used for electrophoresis. Animals from a given sampling location were considered to represent a population, therefore the terms ‘site’ and ‘population’ are used synonymously. All tissue samples are housed at the University of Otago, Dunedin, New Zealand.

Allozyme Electrophoresis:

Tissue was homogenized with an equal volume of buffer (2 ml Tris-HCl (pH 8.0), 0.1 ml mercaptoethanol, 10 g sucrose, and 25 mg NADP per 100 ml H₂O – adjusted to pH 7.2 with HCl). Electrophoresis was performed on cellulose acetate plates (Titan III Helena Laboratories). A preliminary survey of 42 enzymes detected four enzyme loci, coding for four different enzyme systems that were polymorphic and consistently resolvable. These enzymes were used for the analysis and were as follows (abbreviation and enzyme commission number in parentheses): hexokinase (HK, E.C. 2.7.1.1), mannose-6-phosphate isomerase (MPI, E.C. 5.3.1.8), 6-phosphogluconate dehydrogenase (6PGD, E.C. 1.1.1.44), and phosphoglucose isomerase (PGI, E.C. 5.3.1.9).

Tris-glycine (pH 8.5) was used as the running buffer for Hk, Mpi, and Pgi. Tris-malate (pH 7.8) was used as the running buffer for 6Pgd (see Richardson et al. (1986) for buffer recipes). Enzyme stains were modified from Hebert and Beaton (1989). Numerous side-by-side comparisons of electro-morphs were made to confirm relative electrophoretic mobilities; alleles were assigned letter codes with ‘A’ representing the fastest allele (travelling the furthest during an electrophoretic run) at a locus. In total,
five alleles were identified at the \( Hk \) locus, nine at the \( Mpi \) locus, four at the \( 6Pgd \) locus, and six at the \( Pgi \) locus.

**Statistical analysis:**

The GENEPOP program (version 3.1d; Raymond and Rousset, 1995) was used to test for linkage disequilibrium and to carry out exact tests for deviations from Hardy-Weinberg equilibrium. \( F_{st} \) (Wright, 1978; Weir and Cockerham, 1984) and allele frequencies were calculated using FSTAT (Goudet, 1995) to examine the genetic structuring of populations. \( F_{st} \) estimates the reduction in heterozygosity from Hardy-Weinberg equilibrium that results from population subdivision. \( F_{st} \) values range from zero, indicating no genetic differences between populations, and one, indicating fixed allelic differences between populations. \( F_{st} \) was calculated for each locus as well as across loci; calculations across loci were compared for all pairs of populations. Levels of significance were calculated for \( F_{st} \) estimations for each locus according to Waples (1987). For pairwise comparisons of \( F_{st} \), the probability that an \( F_{st} \) value was greater than zero was tested using 4500 permutations. For cases in which multiple independent tests were performed, a sequential Bonferroni adjustment (Rice, 1989) was used to modify significance levels to account for experiment-wide error.

**Results:**

**Allele Frequencies and Linkage Disequilibrium:**

The most common alleles at each locus were present in all of the fiord and non-fiord populations at similar frequencies, though less common alleles were not represented in all of the populations. These ‘uncommon’ alleles included the fastest electromorph at the \( Hk \) locus (allele A was found only at George Inner, Bradshaw, and Dusky), the fastest and two intermediate electromorphs at the \( Mpi \) locus (allele A was found only at
Thompson, Dagg, and Girlies Island, allele G was absent at the two sites in George Sound, and allele H was absent in Thompson, Girlies Island, and Paterson), the fastest and slowest electromorphs at \textit{6Pgd} (allele A was absent from George, Bradshaw, Dusky, and Paterson's, and allele D was absent from Bradshaw and Girlies Island), and the fastest and slowest electromorphs at \textit{Pgi} (allele A was found only at Thompson, Dagg, and Girlies Island, and Bligh, and allele E at Dagg, and Girlies Island). All possible combinations of alleles at different loci were found to be independent (p ≥ 0.231 for all comparisons) when tested for linkage disequilibrium.

\textit{Unique Alleles:}

In addition to uncommon alleles, unique alleles were found at the \textit{Mpi}, \textit{Pgd}, and \textit{Pgi} loci at two of the fiord sampling sites. At each locus, a single allele was found to occur in only one of the ten populations sampled. The slowest electromorph at \textit{Mpi} (allele I) was found only at the George Inner site as was an intermediate electromorph at the \textit{6Pgd} locus (allele C). An intermediate electromorph at the \textit{Pgi} locus was found only at Bligh. In all cases, the unique alleles were found only in a single heterozygous individual.

\textit{Heterozygosity:}

At two of the fiord sampling sites, heterozygosity levels differed significantly from Hardy-Weinberg expectations. Bligh exhibited a significant heterozygote excess at the \textit{Hk} locus at the corrected table-wide significance level (p < 0.01); George Inner exhibited a significant heterozygote deficit (after sequential Bonferroni correction) at both the \textit{Hk} and \textit{Pgi} loci (for each locus p < 0.01) (Table 1). The Bligh and George Inner populations also diverged significantly (after correction) from Hardy-Weinberg expectations when data from all four loci were analyzed together (p < 0.01 in each case).
Table 1. *Terebratella sanguinea* genetic variability in the 10 populations sampled. For each of the allozyme loci, the number of direct count heterozygotes and the number of heterozygotes expected under Hardy-Weinberg equilibrium are shown. $6Pgd$ was monomorphic for the individuals at the Bradshaw site, therefore an estimation of expected levels of heterozygosity could not be calculated for this site/locus combination. Values in bold print are significantly different from zero at the table-wide significance level.

<table>
<thead>
<tr>
<th>Population</th>
<th>Hk</th>
<th></th>
<th></th>
<th></th>
<th>Mpi</th>
<th></th>
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<tr>
<td></td>
<td></td>
<td>Het Obs</td>
<td>Het Exp</td>
<td>p</td>
<td>SE</td>
<td>Obs</td>
<td>Exp</td>
<td>p</td>
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<td>0.6452</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.6531</td>
<td>0.7320</td>
<td>0.1492</td>
<td>0.0112</td>
</tr>
<tr>
<td>George Outer</td>
<td>0.4783</td>
<td>0.5710</td>
<td>0.2672</td>
<td>0.0000</td>
<td>0.6296</td>
<td>0.7771</td>
<td>0.1829</td>
<td>0.0096</td>
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<tr>
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<td>0.0008</td>
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<td>0.6512</td>
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<td>0.7143</td>
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<td>0.5898</td>
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<td>0.8929</td>
<td>0.8156</td>
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<td>Gilies Is.</td>
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<td>0.0000</td>
<td>0.7857</td>
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<tr>
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<td>Pegasus</td>
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<td>0.5667</td>
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<td>0.7000</td>
<td>0.7924</td>
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<table>
<thead>
<tr>
<th>Population</th>
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<th></th>
<th></th>
<th></th>
<th>Pgi</th>
<th></th>
<th></th>
<th></th>
<th>H-W p - all</th>
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<tbody>
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<td></td>
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<td>Exp</td>
<td>p</td>
<td>SE</td>
<td>Obs</td>
<td>Exp</td>
<td>p</td>
<td>SE</td>
<td></td>
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<tr>
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<td>0.4615</td>
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<td>0.0011</td>
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<tr>
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<td>0.0000</td>
<td>0.3103</td>
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<td>0.2452</td>
<td>0.0000</td>
<td>0.3553</td>
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<tr>
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<td>0.0000</td>
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<td>0.0000</td>
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<td>0.0000</td>
<td>0.4651</td>
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<td>0.0648</td>
<td>0.0000</td>
<td>0.0121</td>
</tr>
<tr>
<td>Bradshaw</td>
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<td>0.4750</td>
<td>0.4222</td>
<td>0.4222</td>
<td>0.2500</td>
<td>0.4222</td>
<td>0.7181</td>
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</tr>
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<td>0.4151</td>
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<tr>
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<td>0.0370</td>
<td>na</td>
<td>na</td>
<td>0.4063</td>
<td>0.3914</td>
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<td>0.5000</td>
<td>0.4987</td>
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<tr>
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</tr>
<tr>
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<td>0.0000</td>
<td>0.6170</td>
<td>0.5406</td>
<td>0.0869</td>
<td>0.0069</td>
<td>0.2904</td>
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</tbody>
</table>
\section*{Appendix V: Published allozyme survey}

\textit{F}st:

\textit{F}st values calculated for each of the four allozyme loci did not differ significantly from zero at any of the loci (\(p \geq 0.44\); data not shown). When \(F_{st}\) values were averaged over all four loci, estimates for data from all 10 sites were not significantly different from zero (\(F_{st} = 0.0089, p = 0.98\)), however, pairwise comparisons between sampling sites uncovered significant differences between some of the sites (Table 2). The most consistent differences for the pairwise comparisons were between Dusky and the other sites; six of the nine comparisons including Dusky produced \(F_{st}\) values significantly different from 0, and the one of the largest \(F_{st}\) values obtained was between the two sites located within Dusky Sound (Dusky and Girlies Island). Additionally, both Bligh and George Inner (the two sites out of Hardy-Weinberg equilibrium) each were significantly different in four of nine pairwise comparisons.
Table 2. Pairwise comparisons (between 10 sampling locations) of Weir and Cockerham (1984) estimates of Wright’s $F_{st}$ calculated over four allozyme loci. Significance of $F_{st}$ was tested using 4500 permutations and a Bonferroni correction applied (* $p$<0.05, ** $p$<0.01).

<table>
<thead>
<tr>
<th>Population</th>
<th>Bligh</th>
<th>George O</th>
<th>George I</th>
<th>Thompson</th>
<th>Bradshaw</th>
<th>Dagg</th>
<th>Dusky</th>
<th>Girly</th>
<th>Paterson’s</th>
<th>Pegasus</th>
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<tbody>
<tr>
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<td>0.0026</td>
<td>0.0167**</td>
<td>0.0086</td>
<td>0.0070</td>
<td>0.0104**</td>
<td>0.0331**</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0078*</td>
</tr>
<tr>
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<td>0.0000</td>
<td>0.0099</td>
<td>0.0000</td>
<td>0.0132</td>
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<td>0.0000</td>
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<td>George Inner</td>
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<td>0.0109</td>
<td>0.0106</td>
<td>0.0069**</td>
<td>0.0123**</td>
<td>0.0000</td>
<td>0.0000</td>
<td>0.0050</td>
<td>0.0188**</td>
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<td>0.0100**</td>
<td>0.0000</td>
<td>0.0000</td>
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<td>0.0000</td>
<td>0.0000</td>
<td>0.0087</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Dagg</td>
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<td>0.0012</td>
<td>0.0227**</td>
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<td>0.0090</td>
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</table>
Discussion:

Excepting the Bligh and George Inner populations, the *T. sanguinea* populations examined were in Hardy-Weinberg equilibrium. We are hesitant to speculate what forces might produce the non-equilibrium situations at these two sites. It is possible selection acts upon those loci that are not in equilibrium. It is interesting both populations out of Hardy-Weinberg equilibrium occur at the heads of fiords, areas that receive large amounts of fresh water input and therefore may be osmotically challenging to marine organisms. However, we do not have ecological data to confirm what sort of stressors might exist in these two locations or whether the two locations might differ enough to explain why one population shows an excess of heterozygotes and the other a heterozygote deficit at the same allozyme locus. Without having concordant ecological data, we are hesitant to conclude that allozyme loci are subject to selection in *T. sanguinea*.

The low $F_{st}$ values may indicate continued gene flow between most of the populations we sampled, however, these preliminary results begin to suggest specific areas within Fiordland may be less connected to each other and to the open ocean. The presence of unique alleles at the George Inner and Bligh sites and the significant $F_{st}$ values for the pairwise comparisons including George Inner, Bligh, and Dusky suggest these three areas are more isolated than the other sites. Because these genetic differences do not correlate with obvious geographic patterns, we cannot partition the genetic differentiation at allozyme loci according to a hierarchical population structure. Continuing work with other genetic markers should help us to uncover at what spatial scale genetic population structure in brachiopods occurs.

Allozyme electrophoresis is a useful and cost-effective tool for uncovering genetic differentiation over long time spans (Burton, 1996; Parker *et al.*, 1998). However,
because these markers evolve slowly, they may not provide enough information to uncover the subtle changes between fiord populations isolated for less than 10,000 years. As a result, it is possible that larger amounts of genetic divergence between fiords would be found at more quickly evolving loci (indicating that the fiord environment acts as a barrier, but that not enough time has passed since isolation for slowly evolving markers to reflect interrupted gene flow). It is also possible that the low levels of differentiation are an indication that larvae from a panmictic open ocean population continue to move into the fiords thereby homogenizing fiord populations. However, the true scenario may be neither of these extremes but rather a combination of the two; small pockets (such as the area where the Dusky sampling site is located or the heads of George and Bligh Sounds) maybe be isolated by geographic or hydrographic features of a fiord whereas other areas continue to act as genetic sinks for the open ocean. Increasing allozyme sample sizes and adding genetic data from faster evolving mitochondrial and nuclear markers will provide a more focused view of how the fiords affect planktonic dispersers such as brachiopods.

References:


SKOLD, M., WING, S. R., and MLADENOV, P. V., In Prep., Genetic subdivision of the sea star *Coscinasterias muricata* in the fiords of New Zealand.


