Multidisciplinary investigation into stock structure of small pelagic fishes in southern Africa

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Multidisciplinary investigation into stock structure of small pelagic fishes in southern Africa

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Declaration

I know the meaning of Plagiarism and declare that all the work presented in this thesis is my own, except where it is properly acknowledged in the text. This thesis has not been submitted in whole or in part for a degree at another university.

S.L. Hampton

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Multidisciplinary investigations into stock structure of small pelagic fishes in southern Africa

Three abundant small pelagic fish species co-occur in the Benguela upwelling ecosystem off southern Africa: sardine (*Sardinops sagax*), anchovy (*Engraulis encrasicolus*) and round herring (*Etrumeus whiteheadi*). It is hypothesised that populations of these species have a complex structure that reflects the complexity of their varying habitats. On the basis of the locality and timing of spawning, and morphological, meristic and parasite studies, it has been proposed that there are separate stocks of sardine in South African waters west and east of Cape Agulhas. Consequently, new operational procedures for managing the fishery are based on the premise of two such stocks, termed the west and south coast stocks respectively. In this study, it is hypothesized that the factors causing purported stock differences in sardine should also apply to anchovy and redeye. Three different approaches were used to further test the multi-stock hypothesis for sardine in southern Africa and to provide new information for understanding possible stock differences in anchovy and round herring. The population structure of sardine was investigated through studies on samples taken from the Cape west and south coasts as well as from Namibia and KwaZulu-Natal. Two dimensionless otolith shape indices, otolith elemental signatures, seven microsatellite loci and the mitochondrial DNA marker, ND2, were used to supplement existing information on sardine. Otolith shape, while influenced by fish length and season of capture, distinguished the east coast samples from those taken elsewhere, while the otolith elemental signatures indicated differentiation among sites on a small scale and between samples taken from the west and south coast for certain elements. Both genetic indicators showed high levels of genetic diversity and variation among individuals. There were some genetic differences among sites within a single year, but overall the results suggest that the South African sardine population is well mixed, with weak evidence of genetic patchiness that is not temporally stable. This genetic pattern is evidence of sweepstake recruitment. For anchovy, two spawning locations have been identified in South African waters, but there have been no previous investigations into stock structure. The current study aimed to apply different methods to anchovy samples to determine their usefulness for differentiating population structure,. An analysis of otolith shape revealed that it was primarily influenced by the length of the fish, with no differentiation among sites, whereas an investigation into genetic differentiation using five microsatellite markers revealed high levels of genetic diversity and differentiation among sites. Since there were relatively few samples for a study of this sort, further work is recommended to investigate stock structure in South African anchovy. For round herring, only otolith shape was analysed to test for stock structure. Otolith shape was found to be primarily related to the length of the fish (as in anchovy) and sex, but no significant difference was found between coasts. There were different magnitudes of variability in the results for the three species using different methods. It is likely that the complex, variable environment causes complex patterns of mixing and micro-structuring within all three species, linked to their different niches.

Chapter 1

General Introduction

"No one supposed that all individuals of the same species are cast in the very same mould. These individual differences are highly important for us… "
(Charles Darwin 1859)

Chapter 1: General Introduction

Abstract

The importance of small pelagic fish to the marine ecosystem is described and their economic importance in South Africa and globally. Three main species of small pelagic fish co-occur in the Benguela: anchovy (*Engraulis encrasicolus*), round herring (*Etrumeus whiteheadi*), and sardine (*Sardinops sagax*). These three species are responsible for the largest fishery, by volume, in South Africa. The biology of anchovy, round herring and sardine are discussed in the context of the variable environment in which they live. The importance of understanding stock structure and its application to responsible fisheries management is discussed. Given the multiple stock hypothesis in sardine, the prospect of multiple stocks in two co-occurring small pelagic fish, anchovy and round herring, is discussed, taking into account differences in life history characteristics. Three tools are introduced with which to test the hypothesis of multiple stocks in South Africa. Otolith morphology contributes to other studies on phenotypic variability within sardine and as a first look at differentiation within anchovy and round herring. Otolith elemental chemistry is used as a measure of environmental differences to which sardine are exposed on different coasts within South Africa. Two genetic tools, microsatellite and mitochondrial DNA, are used to test the hypothesis of multiple sardine stocks and the degree of inherent genetic variability, and thus the capacity for resilience to changing environmental conditions within the population. Microsatellite DNA is used in a pilot study to test the hypothesis of multiple stocks in anchovy. This multidisciplinary approach aims to supplement other studies of stock differentiation in sardine and how this should influence fisheries management in South Africa.

Introduction

Small pelagic fish (including sardines, anchovies and herring) are found in abundance across the world where plankton productivity is high as a result of coastal upwelling or freshwater input. Small pelagic fishes can have large populations and are capable of large-scale movement that can result in extensive gene flow. They occur over broad geographical ranges, with a preference for water temperatures between 12 and 26ºC and regions with high plankton productivity, but are capable of surviving in variable environments. Two or more species of small pelagic fish frequently co-occur, adapting to different niches in the environment. For example, when sardine and anchovy occur together, anchovy tend to be found closer inshore than sardine (Checkley *et al*. 2009). The distribution, where they occur together, is often patchy within their range, particularly when the population is small, and groups will contract into refugia (Ward *et al.* 2006, Checkley *et al.* 2009, Izzo *et al.* 2012).

Small pelagic fishes are also an important component of marine ecosystems, occupying the mid-trophic levels, with many other commercially valuable fish, seabirds and marine mammals relying on them as a food source (Cury *et al.* 2000, 2012, Pikitch *et al*. 2012). Pikitch *et al.* (2012) highlight the crucial role that these so-called forage fish play in marine food webs and suggest that the ecosystem services provided by this trophic level could outweigh the direct commercial value of the fisheries for them. This is particularly true in coastal upwelling regions, where top predators are dependent on forage fish. Many of these predators could be vulnerable to extinction. In the Benguela region, seabird species such as the African Penguin, *Spheniscus demersus* (Vulnerable), Cape Cormorant, *Phalacrocorax capensis* (Endangered) and Cape Gannet, *Morus capensis* (Endangered), rely on small pelagic fishes as a major part of their diets, and the collapse of many seabird populations is largely attributed to the decline in small pelagic fishes (Cury *et al.* 2012, Hamann *et al.* 2012).

Maintaining functionality at all levels of the marine ecosystem is particularly important given South Africa's commitment to an ecosystem approach to fisheries management (EAF). This ecosystem approach incorporates fishery, ecosystem, social and economic considerations into management procedures in order to maintain not just fishery and ecosystem health, but also a

functional, economically viable industry that can support those communities that are reliant on the fishery (Hara 2013, Jarre *et al.* 2013).

Small pelagic fishes are well known for large population fluctuations and changes in distribution that occur suddenly as a result of environmental changes (e.g. Alheit *et al.* 2009, Field *et al.* 2009, Izzo *et al.* 2012). These fluctuations are ubiquitous in upwelling regions throughout the world and can span as much as three orders of magnitude (Lecomte *et al.* 2004, Coetzee *et al.* 2008). This is particularly prevalent in regions where sardine and anchovy co-occur. Decadal-scale fluctuations in the abundance of the two species are frequently observed (frequently out of phase with each other), likely driven by oceanographic conditions (Schwartzlose *et al.* 1999, Lecomte *et al.* 2004, Coetzee *et al.* 2008, Field *et al.* 2009). The changes in sardine and anchovy abundance have consequences throughout the ecosystem. If small pelagic fish are depleted within a particular marine ecosystem, they are likely to be replaced by other species, for example the relatively energy-deficient jellyfish in the northern Benguela (Roux *et al.* 2013). This will have consequences throughout the ecosystem, negatively impacting large, commercially-valuable fish species and top predators (Roux *et al.* 2013). Small pelagic fishes are also potentially vulnerable to climate change, and in certain regions a poleward shift in distribution has already started to occur (Checkley *et al.* 2009), which is of particular concern in South Africa and Australia, where there is no suitable habitat for a poleward shift to occur (Kasapidis 2014).

Southern African regional oceanography

South Africa has a relatively wide shelf and smooth coastline (Figure 1.1) that is bordered by two currents: the cool Benguela Current that runs along the west coast to Namibia and the warm Agulhas Current that flows south along the east coast. The Benguela Current is a wind-driven coastal upwelling system bounded by the warm Angolan Current in the north and warm Agulhas Current in the south. There are strong seasonal trends in upwelling intensity in the region (Hutchings *et al.* 2009). In summer, on the west coast, strong upwelling brings cold, nutrient-rich waters towards the surface, resulting in a highly productive system. Associated with this is the offshore movement of filaments of surface water (Ekman drifts) that can result in fish eggs and small larvae being lost offshore (Hutchings *et al.* 2009).

A year-round upwelling cell persists near Lüderitz in Namibia that separates the southern and northern Benguela ecosystems and is a major source of nutrients for central Namibia (Shannon *et al.* 1988, Cole 2002, Hutchings *et al.* 2009). It is an effective barrier to the movement of fish eggs and larvae because the water is too cold for eggs to survive, and strong winds result in water turbulence and offshore transport (Checkley *et al.* 2009, Hutchings *et al.* 2009). The northern Benguela is characterised by upwelling up to the Angola-Benguela frontal zone in the north of Namibia (Shannon *et al.* 1988), from which occasional intrusions of warm water from Angolan surface waters can result in unusually warm waters in northern Namibia, which are characteristic of Benguela Niño events (Shannon *et al.* 1988, Gammelsrød *et al.* 1998).

Summer on the Agulhas Bank is characterised by easterly and westerly winds. The east wind results in subtropical surface waters becoming separated from a cool water ridge by a strong thermocline (Hutchings *et al.* 2002, 2009). The subsurface waters tend to flow east and the bottom current runs west, resulting in clockwise eddies forming and the local retention of fish eggs and nutrients. The summer thermocline breaks down in winter when westerly winds dominate and mixing occurs. The Agulhas Current retroflects on the edge of the continental shelf and flows back towards the central Indian Ocean (Hutchings *et al.* 2002, 2009). A strong jet current transports eggs and larvae that were spawned on the western Agulhas Bank (WAB) to the west coast (Hutchings *et al.* 2002), although some are lost to filaments that extend off the shelf (Penven *et al.* 2001). For the purposes of this study, the South Africa-Namibia border will extend in a straight line seaward at the Orange River. The west and south coast are separated at 20ºE, which is the convention used in fisheries management. The east coast is divided from the south coast at the border of KwaZulu-Natal and the Eastern Cape, although only sardine associated with the sardine run are included in this study (Figure 1.1).

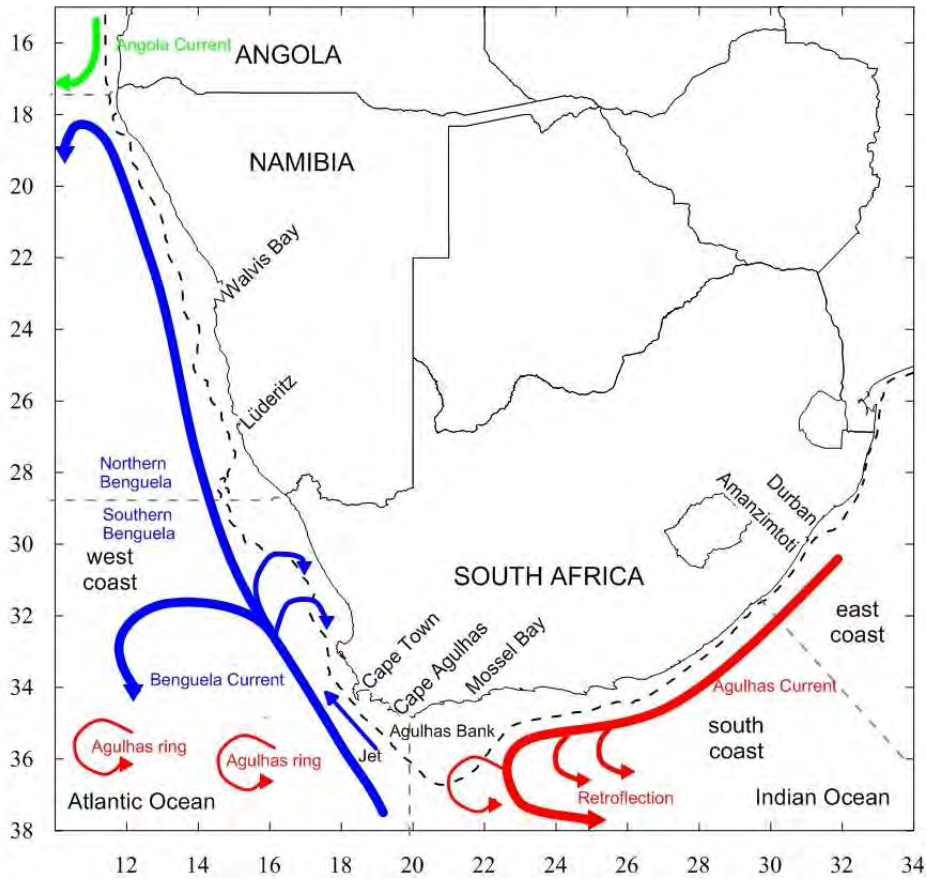


Figure 1.1: A map of southern Africa showing the names of towns and regions that are mentioned in the text. Divisions between Namibia and South Africa's west coast, south coast and east coast are shown with grey dashed lines. The Angolan Current (green), Benguela Current (blue) and Agulhas Current (red) and some of the associated oceanographic patterns are shown. The 200m depth isobath is shown with the black dashed line.

Cape anchovy *Engraulis encrasicolus* (Linneaus 1758), (previously *E. capensis* (Gilchrist 1913))

Engraulis encrasicolus are thought to have arrived in South Africa following a colonisation event from the European population after the last glacial maximum (Kasapidis 2014). As a result of its commercial value and dominance in the Benguela ecosystem during the last five decades, anchovy has been well studied off the South African coast. They are small fish, reaching 15 cm (caudal length - CL), and occur on the west and south coast of South Africa and occasionally off Namibia (Hutchings *et al.* 1998), in the upper ocean layers (0 – 200m) of the continental shelf (Barange *et al.* 1999, Fairweather *et al.* 2006). Anchovy are particulate feeders that favour cool conditions. They typically feed on large zooplankton, including

calanoid copepods and euphausiids (van der Lingen *et al.* 2009). They have a short life span of approximately three years.

Anchovy are sensitive to changes in the environment, resulting in large population fluctuations because recruitment success can differ drastically from year to year (Fairweather *et al.* 2006). Acoustic surveys to estimate spawner stock biomass have been conducted in South Africa each November since 1984 (Barange *et al.* 1999) to correspond with the peak spawning period of anchovy, which occurs between October and February (Huggett *et al.* 1998). Spawning occurs mainly on the Agulhas Bank, sometimes extending past the edge of the shelf (Figure 1.2) (Hutchings *et al.* 1998). Anchovy have also undergone a distributional shift of the bulk of the population towards the east, and it was hypothesised that this was a result of environmental change associated with sea surface temperature and food availability (Roy *et al.* 2007). The possibility of two separate stocks, as hypothesised for sardine, and the disproportionate success of the south coast stock in recent years, could have contributed to the persistence of anchovy on the south coast.

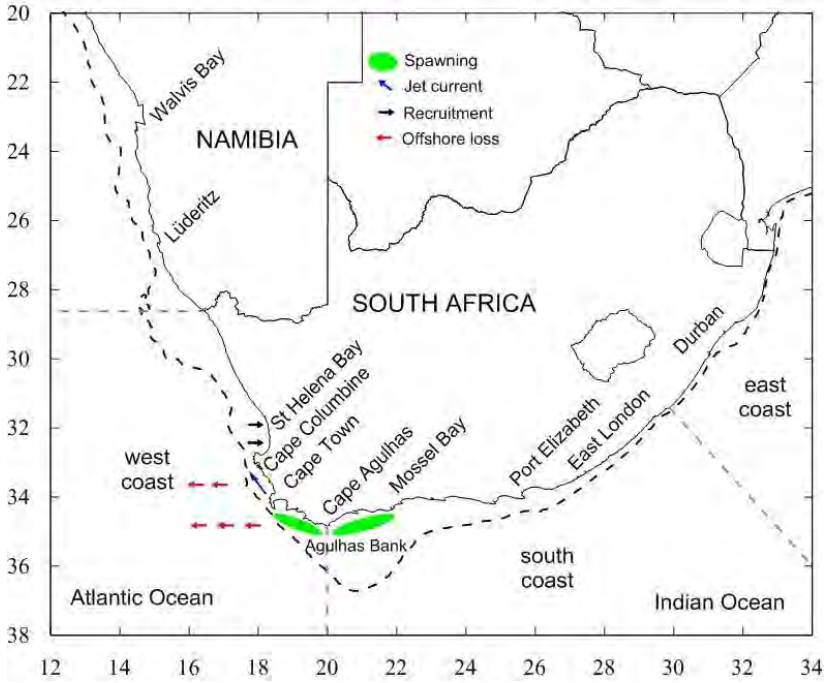


Figure 1.2: A simple schematic representation of approximate location of spawning areas (green ovals), recruitment (black arrows), jet current (blue arrow) and offshore loss (red arrows) of anchovy in South Africa (adapted from Roel and Armstrong 1991).

During the day, anchovy form large schools in the water column. At night, they disperse within the upper ocean layer to feed (van der Lingen *et al.* 2009). This is when spawning occurs. Each female spawns every 11 – 14 days, and has the potential to release between 110 000 and 160 000 eggs per year (Huggett *et al.* 1998). The majority of the eggs are found between Cape Point and Cape Agulhas in water temperatures between 17 – 21°C (Checkley *et al.* 2009) (Figure 1.3) and take, on average, three days to hatch (Korrûbel *et al.* 1998). In the 1990s, Barange *et al.* (1999) showed that, in years when the population was small (1995, 1996, 1997), two stocks of anchovy seemed to exist on either side of the Agulhas Bank. This stock separation was not evident in years when the population was at its maximum (1991, 1992) (Barange *et al.* 1999). A composite map of egg distribution for anchovy (over the period 1984 – 2000), also suggests a break in distribution at the widest point of the Agulhas Bank (Figure 1.3).

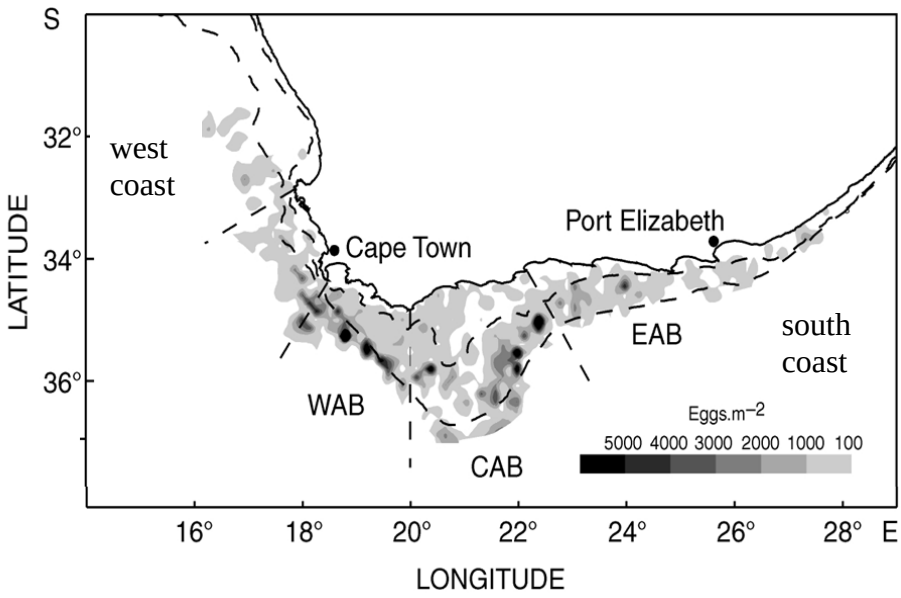


Figure 1.3: Composite map of anchovy egg distribution from 1984 – 2000 during the annual spawner biomass surveys showing the western Agulhas bank (WAB), central Agulhas bank (CAB) and eastern Agulhas bank (EAB) as well as the 20°E line that separates the west coast from the south coast (van der Lingen and Huggett 2003).

After three to four days, the newly-hatched anchovy larvae are able to move on a small scale to find food. However, most dispersal is passive, and if they survive it is because they are transported by a jet current to nursery areas on the west coast, St Helena Bay and north thereof (Hutchings *et al.* 1998). Many eggs and larvae are lost due to Ekman drift between Cape Point and Cape Columbine as a result of the alongshore, south-easterly winds that prevail in summer, or offshore divergences of the jet current along the edge of the shelf (Boyd *et al.* 1992). By the time the larvae reach 9 mm, some of them will have travelled approximately 350 km in the single month since hatching (Huggett *et al.* 1998). Pre-recruits are widespread offshore and tend to move inshore as they grow (van der Lingen and Merkle 1999). The vast majority of anchovy recruits (6 – 9 cm CL) is found inshore on the west coast (Barange *et al.* 1999), with less than 5% inshore on the south coast (Hampton 1992). At one year, or 11 cm, they reach sexual maturity and begin to move towards the south coast to spawn (Figure 1.2).

Round herring, *Etrumeus whiteheadi* (Wongratana 1983)

Round herring adults are widespread off the west coast of South Africa from the Orange River to the Cape Peninsula, and particularly over the outer shelf areas of the central and eastern Agulhas Bank, where temperatures are approximately 17ºC (Coetzee *et al.* 2010), but range between 10°C (at depth) and 20°C (Roel and Armstrong 1991). There is some distributional overlap with east coast round herring, *Etrumeus teres*, north of East London (Coetzee *et al.* 2010). Spawning occurs in deep waters throughout the year, with a late winter peak (Crawford *et al.* 1987, Roel and Armstrong 1991, van der Lingen *et al.* 1998, Hutchings *et al.* 2002), and is most intense on the west coast on the edge of the continental shelf (van der Lingen *et al.* 1998) (Figure 1.4). Eggs and larvae are found mostly in surface waters (upper 50 m), where they drift northwards to the nursery grounds off the west coast (van der Lingen *et al.* 1998, Hutchings *et al.* 2002). Juveniles and young adults are predominantly found in water up to 100 m deep. Reproductive maturity is reached at approximately 13 cm (CL) or two years (Roel and Armstrong 1991), although males tend to mature earlier than females (Geja *et al.* In Press). As they get older, round herring tend to move offshore into deeper water. At two to three years they occur throughout the water column from 0 – 300 m, and are typically found in small schools about 200 m deep towards the edge of the continental shelf. During the day they form small schools close to the ocean floor, and at night they disperse in surface waters to feed. They feed on large zooplankton such as calanoid copepods and euphausiids (Roel and Armstrong 1991), but small anchovy have also been found in their stomachs (S. Hampton, pers. obs.). On the south coast, where the continental shelf is widest, they occur farther offshore (van der Lingen *et al.* 1998).

The fact that they occur far offshore and at great depths makes round herring difficult to catch. Consequently, although still of commercial importance, they are relatively underexploited. The majority of round herring are caught between Cape Columbine and Cape Point (Roel and Armstrong 1991) as adults and used for the production of fishmeal (van der Lingen 2003).

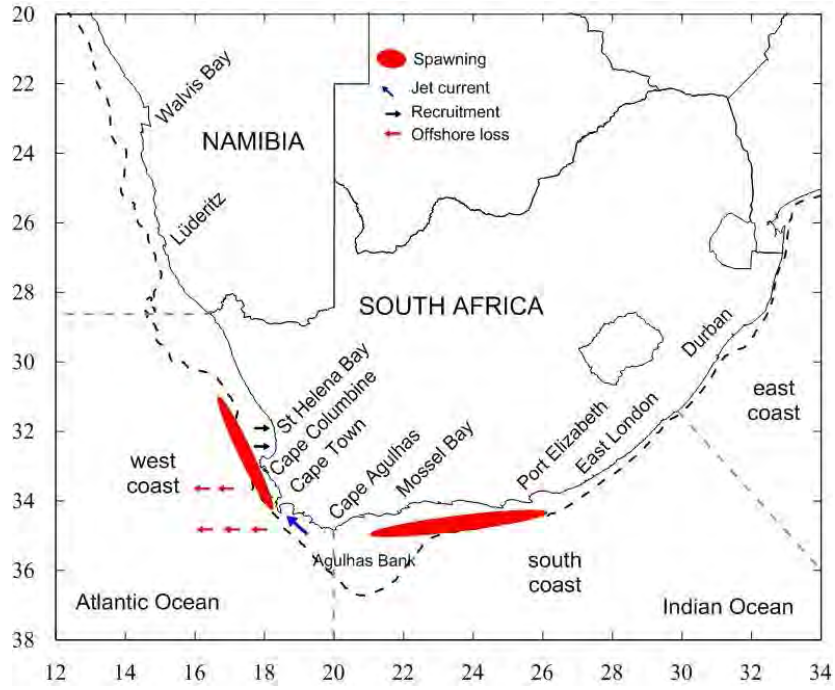


Figure 1.4: A simple schematic representation of the approximate location of spawning areas (red ovals) and recruitment (black arrows), jet current (blue arrow) and offshore loss (red arrows) of round herring in South Africa (adapted from Armstrong 1991).

Sardine *Sardinops sagax* (previously *S. ocellatus*) (Jenys 1842)

Sardinops sagax are thought to have arrived in South Africa from Austalia and New Zealand approximately 200 - 500 thousand years ago (Kasapidis 2014). Sardine is distributed widely over coastal and shelf waters in southern Africa, from southern Angola to KwaZulu-Natal (Figure 1.5) (Beckley and van der Lingen 1999). South African and Namibian sardine stocks are managed separately by the two countries (Boyer *et al*. 2001). The sardine in the northern Benguela are separated from sardine in South Africa by the strong upwelling cell that persists off Lüderitz (Figure 1.1, 25°S) for most of the year (Boyd and Cruickshank 1983, Lett *et al*. 2007). During periods of low abundance, migration between central and northern Namibia is reduced and the northern Benguela sardine spawning area tends to be smaller and occurs farther north (Lluch-Belda *et al.* 1989).

Sardine can live for six to eight years and reach 25 cm (CL). Adults filter feed throughout the day (van der Lingen *et al.* 2009) and their distribution is strongly linked to sea surface temperature (SST), with a preference for water temperatures of about 12 – 24°C. Most sardine reach sexual maturity at two years, or once they have reached at least 16 cm (CL), but

this can vary according to environmental conditions and population density (van der Lingen *et al.* 2006c). Gaggiotti and Vetter (1999) have demonstrated that, for Pacific sardine and European anchovy, the larger generational overlap of sardine is likely to make them more resilient than anchovy to changes in the environment.

Although there has been evidence of spawning all along the South African coast throughout the year (Melo 1994), the bulk of spawning occurs on the Agulhas Bank, away from the dispersive influence of the upwelling system, or on the west coast (Figure 1.5, Roel and Armstrong 1991, Penven *et al.* 2001). Eggs are generally found in water of temperatures between 15 – 21°C (Checkley *et al.* 2009). Hutchings *et al.* (2002) reported an eastward shift in the distribution of the bulk of spawning sardine. Spawning peaks in January with a second peak in September (Huggett *et al.* 1998). Each female spawns repeatedly and can release up to 27 500 eggs per spawning event (Beckley and van der Lingen 1999). The eggs generally hatch within four days of spawning (Shelton 1986). The larvae move passively in a jet current towards the west coast. There is a high risk of being swept into the open ocean as a result of Ekman drift and divergent offshore jet currents (Hutchings *et al.* 2002). Two to three months after spawning there is a peak in pre-recruit numbers off Cape Columbine. There can be some local retention of sardine eggs on the south coast and recruits have been found off Algoa Bay and even farther east (van der Lingen *et al.* 1998, Coetzee *et al.* 2008). In the northern Benguela, sardine spawning peaks twice - once in September-November, and again in February-April (Le Clus 1979), and eggs are found in waters between 15 – 20ºC.

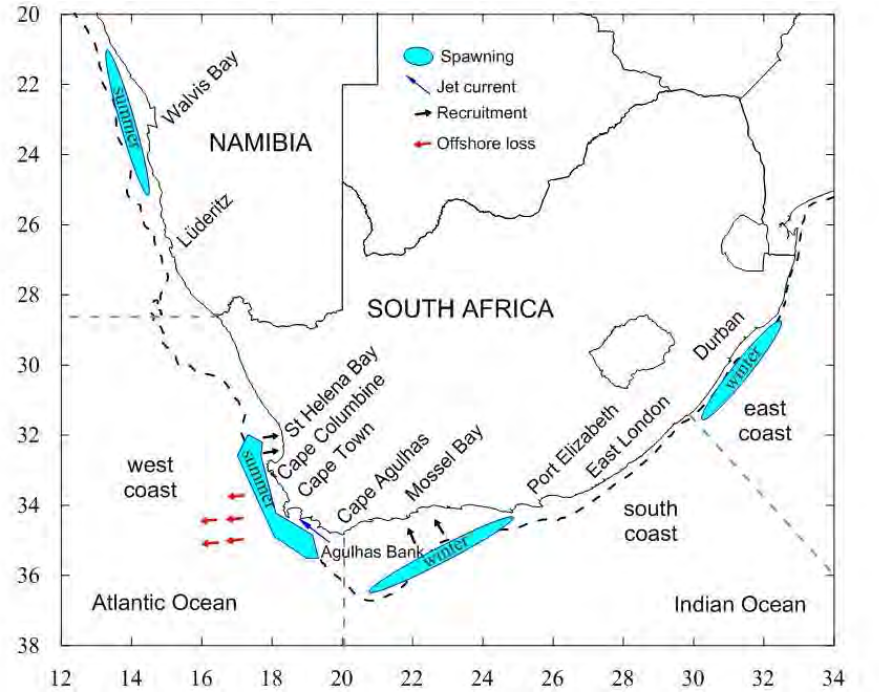


Figure 1.5: A simple schematic representation of the approximate location and season of spawning sardine (blue) and recruitment (black arrows) and offshore loss (red arrows) of sardine eggs and larvae and fast moving jet current (blue arrow) that transports eggs and larvae from the western Agulhas Bank to the west coast (adapted from Roel and Armstrong 1991).

A South African stock division is hypothesized between sardine west and east of Cape Agulhas (Figure 1.3) (Miller *et al.* 2006, Coetzee *et al.* 2008). Acoustic surveys have identified an area on the Agulhas Bank that appears to separate the west coast and western Agulhas Bank sardine from those on the eastern Agulhas Bank. Only in times of high biomass does there appear to be some mixing in this central area of the Agulhas Bank near Cape Agulhas (Coetzee *et al.* 2008) (Figure 1.5). The composite map of sardine egg distributions (Figure 1.6) shows two major spawning grounds separated by the region around Cape Agulhas. Eggs spawned east of this boundary are found in water temperatures of 19°C to 22°C; those spawned to the west occur in water temperatures of 14°C to 17°C (Twatwa *et al.* 2005).

There is the possibility of another distinct sardine stock occurring on the east coast during the austral winter when fish move up the east coast to spawn (Connell 2010). This is known as the sardine run and has been widely studied (van der Lingen *et al.* 2010a). There is a small-scale (up to 700 tons) seasonal beach seine fishery reliant on the sardine run (van der Lingen

et al. 2010a). A tourism industry has also developed around the sardine run and the charismatic predators that follow the sardine from the Agulhas Bank up the coast (van der Lingen *et al.* 2010a). It was hypothesised that the sardine run could be a spawning migration of a genetically distinct subpopulation (Fréon *et al.* 2010).

Between 1970 and 1997 the bulk of the sardine catch in South Africa occurred on the west coast between 32°S and Cape Agulhas (Barange *et al.* 1999), but since 2000 a higher proportion of the total catch has been on the south coast. In 2004, for the first time, the South African sardine fishery did not reach the total allowable catch (TAC) for the year, and in 2005 catches on the south coast exceeded those on the west coast (Coetzee *et al.* 2008). It is unclear whether the shift in distribution was due to environmental forcing, local depletion of the western stock or recurrent successful recruitment of the southern stock as a result of natal homing, or due to a combination of the above. The shift has implications for the fishing industry in South Africa, which has most of its infrastructure on the west coast. This change in relative distribution tends to vary over the years, and in 2009 and 2010 some fish returned to the west coast (Roux *et al.* 2013). Coetzee *et al.* (2008) convincingly showed that much of the increase on the south coast came from west coast recruitment. It is important for fisheries management to know whether the shift is as a result of changes in environmental conditions, or because there are multiple stocks of sardine with the western stock declining.

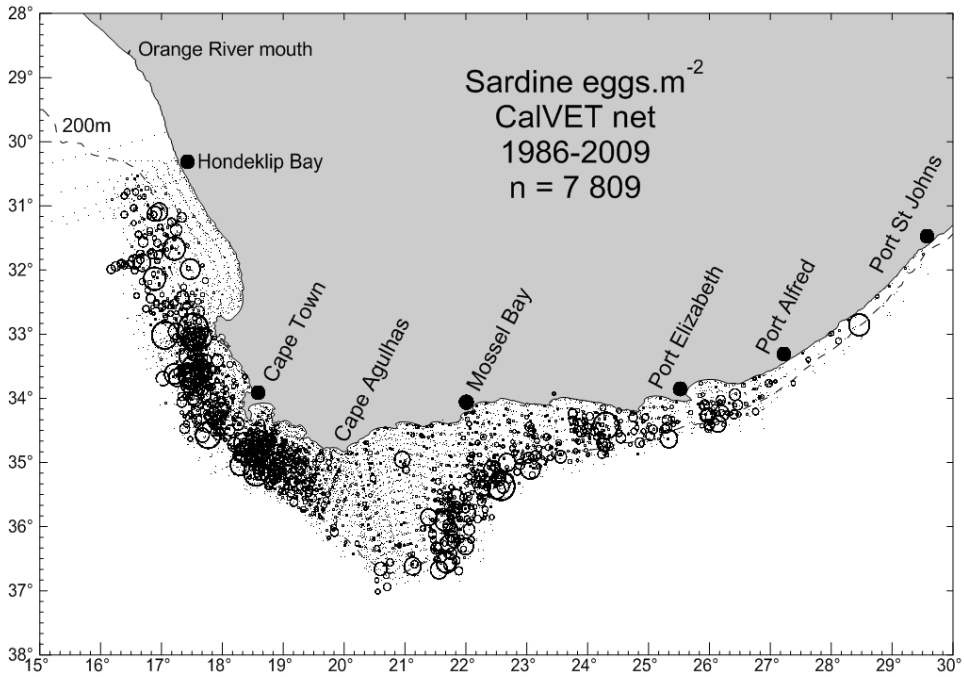


Figure 1.6: Composite figure of sardine egg distribution from 1986 – 2009 during the annual spawner biomass surveys. (Taken from van der Lingen 2011, updated from van der Lingen and Huggett 2003).

Small pelagic fishery

Small pelagic fishes contribute as much as 30% of fish landings worldwide (Lecomte *et al.* 2004, Smith *et al.* 2011a). Up to 90% of this is used for fishmeal production (Pikitch *et al.* 2012). The global value for forage fisheries is estimated at US$16.9 billion, nearly 20% of the total world catch fisheries value (Pikitch *et al.* 2012). The world's largest single-species fishery is that of the Peruvian anchoveta (*Engraulis ringens*) (Checkley *et al.* 2009).

Small pelagic fish stocks of anchovy, round herring and sardine dominate an important commercial fishery in South Africa. Sardine and anchovy account for between 60 and 90% of total fish landings, which average 375 000 tons annually (Figure 1.7) (Fairweather *et al.* 2006).

The anchovy and sardine fisheries are managed together, with a total allowable catch (TAC) allocated for each and an upper limit for sardine bycatch in the anchovy fishery (de Moor *et al.* 2011). The TAC limit is initially set based on adult population size and then adjusted during the year, depending on recruitment strength (DAFF 2012). Most anchovy are caught

as recruits on the west coast, with juvenile sardine caught as bycatch in the anchovy-directed fisheries off the west coast during winter (van der Lingen and Huggett 2003). The anchovy TAC is based on a conservatively estimated adult population size (with the possibility of a mid year adjustment in the final TAC) and incorporates the sardine total allowable bycatch limit.

There is no quota in place for the round herring fishery, although there is an upper precautionary catch limit (of 100 000 tons) that has yet to be reached (van der Lingen 2003). Adult sardine are caught as bycatch in the round herring-directed fishery, so an increase in targeted fishing for round herring will have implications for the sardine population (van der Lingen 2003). There appears to be an increase in round herring biomass in recent years, although this can be attributed as much to an increase in survey effort as to an increased population size (DAFF 2012).The fish are caught using purse-seiners and the majority of anchovy and round herring are processed into fishmeal and fish oil (Hara 2013).

The commercial sardine fishery collapsed in Namibia in the late 1970s and has yet to recover (Le Clus 1979, Roux *et al.* 2013). The lack of small pelagic fishes has resulted in a significant ecosystem change, including a substantial increase in jellyfish (Roux *et al*. 2013). The South African sardine fishery historically has been centered on the west coast. It collapsed in the 1960s, primarily as a result of over exploitation. Catches were low for 30 years, then increased steadily through the 1980s and 1990s until they peaked in 2004, and subsequently declined due to low recruitment (Figure 1.7). Sardine is caught year-round on the west and south coast (Coetzee *et al.* 2008). The sardine TAC is set annually based on adult population size. Sardine is processed for human consumption, pet food, fish oil or fishmeal (DAFF 2012). The majority of the infrastructure for the processing of small pelagic fish has been centered on the west coast of South Africa. The shifting distribution of small pelagic fishes has required fisheries to transport their catches on the south coast to the west coast for processing, negatively impacting the economic value of the fishery (Hara 2013).

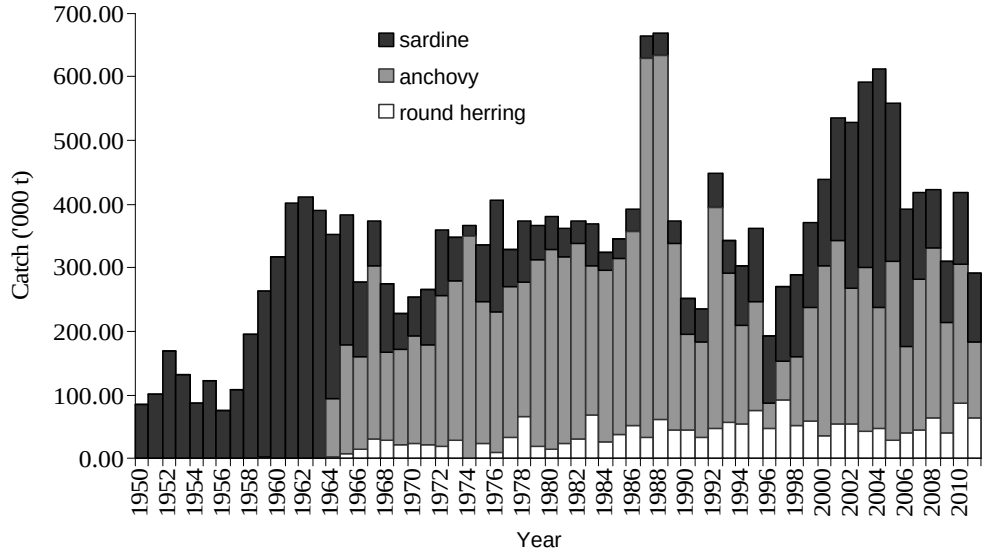


Figure 1.7: South African annual catch data for sardine (dark grey), anchovy (light grey) and round herring (white) from 1950 to 2011 (DAFF 2012).

Investigating stock structure

As a result of the large geographic range of most small pelagic fish species, the management units reflect logistical and political boundaries rather than biology. Biological stocks can be overlooked, not least because the term 'stock' is loosely defined and the definition changes according to the situation. There is debate about the definition of a stock. This debate has been reviewed comprehensively by Cadrin et al. (2005) and hinges on two divergent schools of thought. The ecological paradigm defines a stock as a set of individuals that are found in the same space and time and interact with each other (Secor 1999). Alternatively, the evolutionary paradigm defines a stock as a group in which any member could potentially mate with any other member (Waples and Gaggiotti 2006, Waples *et al.* 2008). Ovenden's (1990) genetic stock concept required that stocks are reproductively isolated units that are genetically distinct, but there is debate about that too (Begg and Waldman 1999, Begg *et al.* 1999, Ward 2000). Begg and Waldman (1999 pg 36) summarise the debate simply by stating that: "Regardless of its precise definition and biological underpinnings, the stock concept really has to do with the interaction between a fish species and its management. Consequently, the purpose of such definitions is to direct management efforts to taxon levels below that of the species." For the purposes of this study, a stock is therefore regarded as "an

intraspecific group of randomly mating individuals with temporal and spatial continuity", as defined by Ihssen *et al.* (1981).

An increased awareness of stock differentiation in small pelagic fish has been permeating the literature. There is a particular emphasis on combining the results of multiple techniques to provide a holistic approach to investigate regional stock structure (Begg and Waldman 1999). For example, Izzo *et al*. (2012) integrated the results of genetic (allozymes and mitochondrial DNA (mtDNA) analysis), morphological, meristic, otolith shape and chemistry analyses, regional catch compositions, fishing seasonality, size at maturity, time of spawning, distribution patterns of larvae and eggs, and larval, adult and juvenile growth patterns to justify the hypothesised complex stock structure of sardine in Australia. Similarly, in the Pacific, Baldwin *et al.* (2012) have added parasite data to previous studies on fish morphometrics, meristics, otolith shape and chemistry, artificial tags and genetics (allozymes, mtDNA, microsatellites and single nucleotide polymorphisms) (references in Baldwin *et al.* 2012) to provide a holistic approach to identifying sardine stock structure. The multidisciplinary nature of both these studies allowed for the identification of phenotypic stocks where reproductive mixing still occurs. The multidisciplinary approach is particularly important in abundant, mobile species such as small pelagic fishes.

In South Africa, a suite of studies has investigated the hypothesis of multiple sardine stocks. A study on vertebral counts showed more vertebrae in the Namibian sardine compared to those found off South Africa's west and south coasts, which did not differ significantly (Wessels *et al.* submitted). In subsequent studies, van der Lingen *et al*. (2010b) found sardine run fish on the east coast to have fewer vertebrae than those of all other regions. Similarly, the spacing between gill rakers was largest for individuals from Namibia, but instead of a continuum, the west coast fish had the smallest gill raker spaces, which then increased in the south and more so in the east coast fish (Idris 2009, Idris *et al*. submitted). Morphometric measurements showed significantly different body shapes for sardine from Namibia and the South African west and south coasts (Wessels 2009, Wessels *et al.* submitted), as well as the east coast (van der Lingen *et al.* 2010b).

Reed *et al.* (2012) investigated the use of parasites as biological tags to examine population structure in South African sardine and found a geographic pattern associated with infection

rates of a tetracotyle metacercariae, presumed to be of the genus *Cardiocephaloides*, suggesting that this parasite can be used as a biological tag for identifying stocks. This work supports the hypothesis that there are two distinct, but mixing stocks in South Africa and that fish spawned in November are possibly the source of mixing between the west and south coasts (Smith *et al.* 2011b).

The use of otolith morphology in South African sardine, anchovy and round herring stock identification

High phenotypic polymorphism in fish populations does not necessarily relate to genetic diversity (Tudela 1999). Given this, it is important to maintain a multidisciplinary approach to stock identification. Phenotypic variability can suggest that individuals are spending a significant proportion of their lives in different environmental conditions and could be locally adapted to those conditions. These adaptations can be important to the success of particular stocks and therefore need to be considered. Increasing the number of variables that are investigated will strengthen any comparisons that are made, and using a holistic approach will provide a clear understanding of the presence or absence of distinct stocks of small pelagic fish species on the South African coast (Begg and Waldman 1999).

To add to the range of investigative tools that have been listed above, this study also investigates the potential use of the outline morphology of otoliths in stock identification studies. Otoliths are relatively easy to extract from fish and are routinely collected for fish ageing studies. The technology involves a microscope and a digital camera to obtain photographs of otoliths. Because otoliths are not destroyed in the process, they can subsequently be used for ageing studies.

Otolith morphology has been used in the identification of stocks in important fisheries such as Irish Sea herring *Clupea harengus* (Burke *et al.* 2008a) and Atlantic cod *Gadus morhua* (Galley *et al.* 2006). Using fish morphology to differentiate stocks has the disadvantage that the size and shape of a fish are subject to short term variability at different reproductive stages or different states of body condition. Such concerns are less marked for internal structures such as otoliths (Cadrin *et al*. 2005). The comparative analysis of their shape can thus be used to identify phenotypic stocks.

The shape of the otolith is laid down early in life and is subsequently inert (Sweeting *et al.* 2004), although the exact cause of individual differences in otolith shape is not fully understood. Otolith shape is influenced by both genetic predisposition to a specific form and environmental factors, including food availability, depth, water temperature and individual variability (sex and age) (Vignon and Morat 2010). Since studies of otolith morphology are a cost-effective way to investigate stock structure, the method has been used in this study as a starting point to investigate the geographic stock structure in anchovy and round herring, as well as to contribute to the suite of studies investigating stock differentiation in sardine.

The use of otolith elemental chemistry in studies of stock differentiation

Although otoliths are predominately calcium carbonate, there are approximately 37 trace metals that can be incorporated into the otolith in small concentrations. The trace elements are incorporated into the otolith as a substitute for calcium, through co-precipitation with calcium or into the interstitial spaces in the crystal structure. The proportion of elements incorporated in the otolith will differ according to their availability in the environment. Otoliths are metabolically inert, so the trace elements that are incorporated into the otolith over time create a record of the environmental conditions to which a fish has been exposed in its life-time. Thus, differences in otolith elemental composition serve as a signal of fish spending significant periods of their lives in different environments. Elemental chemistry has been successfully used to differentiate geographic stocks in Mediterranean horse mackerel (*Trachurus mediterraneus*, Turan 2006), Icelandic cod (*Gadus morhua*, Jónsdóttir *et al.* 2006) and blue whiting (*Micromeistius australis australis*, Arkhipkin *et al.* 2009).

In addition to providing a measure of geographic differentiation, the difference between the elemental composition of the edge of the otolith (representing the most recent environment in which the fish has been) and the core of the otolith (representing the natal environment of the fish) can provide information on the movement of fish from their natal grounds and their spawning patterns (Cook 2011, Guidetti *et al.* 2013). The use of elemental otolith composition will be used to test the hypothesis of multiple stocks in sardine of the southern Benguela.

The use of genetics in studies of stock differentiation

Marine environments comprise extensive habitats that are generally open, with few (if any) barriers to gene flow in large populations (Waples 1998). This has resulted in the perception that there is little genetic differentiation within marine species (Carvalho and Hauser 1995). Such traditional ideas have been challenged recently, with evidence of genetic differentiation on even small geographic scales (Ruzzante 1998, Knutsen *et al.* 2003, 2007, Nielsen *et al.* 2004, Sanz *et al*. 2008). Despite knowledge of genetic make up of stocks being highlighted as a priority for fisheries management in 1988 (Kapuscinski and Philipp 1988), there has been no attempt to incorporate genetic information in the management of the South African pelagic fishery to date.

For exploited marine living resources, it is important to maintain a species' capacity for resilience against the effects of fishing and environmental change (Hauser and Carvalho 2008). Low levels of genetic variation within a population could render it more vulnerable to the spread of infectious disease and less resilient to changes in its natural habitat (Avise 1986), such as those caused by anthropogenic influences and climate change (Frankham 2010). Maintenance of genetic variability therefore provides a buffer to change (Hauser and Carvalho 2008), and intraspecific genetic variability allows for potential resilience to change and is essential for sustainability of fisheries (Sinclair *et al.* 1985, Cury and Anneville 1998, Fréon and Misund 1999, Hauser and Carvalho 2008, Selkoe *et al.* 2008). When genetic bottlenecks occur, rare alleles integral to the survival of the species and to the capacity of species to adapt to future conditions can be lost (Ryman *et al.* 1995, Selkoe *et al.* 2008). However, it is difficult to quantify what level of genetic variability is required to be an effective and sustainable buffer to fishing pressure and environmental change (Hauser and Carvalho 2008).

A variety of genetic markers is available to investigate population structure. Allozymes, mtDNA and nuclear DNA (nDNA) can all be used to identify genetic stocks (Begg and Waldman 1999), each with its advantages and limitations. In order to be useful in stock analysis, the genetic markers need to be sufficiently variable to detect any population differentiation and sufficiently reliable to be repeatable. Hauser and Seeb (2008) highlighted the need for a number of independent loci, which show sufficient levels of variability at each

locus, to be used in order to obtain reliable results. Baldwin *et al.* (2012) pointed out that the interpretation of stock differentiation is complicated by the type of markers and number of loci used in a given analysis; thus, the best way of investigating stock differentiation meaningfully requires the compilation of a number of these methods. It is necessary to also consider life history parameters that influence genetic variability, including distribution and abundance, age, growth, mortality, reproduction, spawning, maturity and recruitment (Begg 2005). A divergence from panmixia in highly fecund and widespread populations could be explained by behavioural attributes, such as natal homing, or a combination of behavioural and environmental factors, such as local retention of eggs (Knutsen *et al*. 2007).

Small pelagic fish populations have traditionally been considered to be homogenous and panmictic (Grant 1985a). There have been mixed results in previous genetic studies on small pelagic fish around the world, suggesting that there is no global pattern to genetic differentiation. Allozyme studies showed no genetic structure in anchovy in South Africa (10 loci, Grant 1985b) or the north-west Mediterranean stocks (29 loci, Tudela *et al.* 1999), but two stocks have been identified in *E. japonicus* of the East China Sea (Yu *et al.* 2002). Further studies of anchovy in the Mediterranean show genetic differentiation between the Mediterranean and the Bay of Biscay sub-populations (24 allozyme loci, Bembo *et al.* 1995), use of mtDNA showed genetic differentiation between sub-populations (Grant 2005b) and, most recently, three genetic groups were identified in the western Mediterranean and eastern Atlantic (28 allozyme loci, Sanz *et al.* 2008). Complex population structure in *E. mordax* off North America was found in two studies, which investigated 39 (Hedgecock *et al.* 1989) and 10 allozyme loci (Hedgecock *et al.* 1994) respectively, but no geographic structure was found using the cytochrome b region of mtDNA (Lecomte *et al.* 2004). In the Pacific, sardine showed no geographic structure in studies using 32 allozyme loci (Hedgecock *et al.* 1989), mtDNA cytochrome b (Lecomte *et al.* 2004), mtDNA control region (García-Rodrígueze *et al.* 2011) or microsatellites (Gutiérrez-Flores 2007). There was no genetic differentiation based on mtDNA cytochrome b in *Sardina pilchardus* from the Adriatic and Ionian Sea (Tinti *et al.* 2002), but there was significant differentiation in the mtDNA control region between *S. pilchardus* from the Mediterranean Sea and the Atlantic Ocean (Atarhouch *et al.* 2006). Regional populations thus fall on a continuum between full panmixia and highly structured populations (Figure 1.8), following either a stepping stone model or isolation by distance (Figure 1.8) (Wright 1943, Kimura and Weiss 1964, Waples and Gaggiotti 2006).

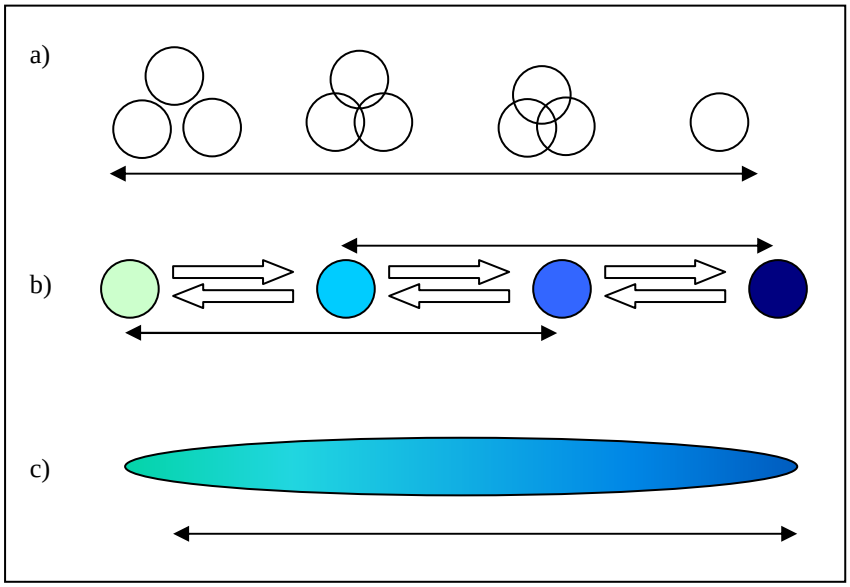


Figure 1.8: A simple graphical representation of: a) the continuum of population differentiation, from complete isolation of populations (represented by unfilled circles) on the left to no differentiation on right (adapted from Waples and Gaggiotti 2006); b) a one dimensional stepping stone model where each circle represents a population and the arrows proportional gene flow (adapted from Kimura and Weiss 1964) and c) the isolation by distance model where geographically proximate individuals in a continuous population are more likely to be genetically similar to each other than to individuals farther away (Wright 1943).

The use of mitochondrial DNA in South African sardine and anchovy stock identification

Mitochondrial DNA is double-stranded circular DNA found in the mitochondria, and is passed from generation to generation through just one parent, usually the female. There is therefore a risk of missing important patterns that are paternally controlled (Waples *et al.* 2008). Different regions of mtDNA have different rates of mutation (Baldwin *et al.* (2012) report mutation rates of 10^{-1} – 10^{-3} per generation for mitochondrial DNA), which are of consequence for stock structure studies. The NADH dehydrogenase subunit 2 (ND2) and control region evolve rapidly and are thus useful in stock identification studies, whereas other regions, such as ribosomal RNA genes, are less variable and are therefore useful for identifying interspecific differences and even deep phylogenetic patterns (Avise 1986, Magoulas 1996).

Bowen and Grant (1997) showed that there was shallow structuring in global sardine and anchovy populations with their study of mtDNA control region sequences. Mitochondrial

DNA investigations of small pelagic fish have suggested that there have been many local depletion and recolonisation events over time, which were caused by climate-related regime shifts (Bowen and Grant 1997), and that current populations are unstable. Despite the capacity of different species to rebound from the occasional population crash, added fishing pressure could lead to local depletions.

It is uncertain whether anchovy found on the south coast, east of Cape Agulhas, form an independent stock from those on the west coast. No population structure was found when Grant (1985b) used 10 protein coding loci to investigate differentiation of anchovy along the southern African coast. The genetic differentiation followed a stepping stone model, but as few as 13 migrants per generation could account for the extensive variability found (Grant 1985b). No stock differentiation was found between the Namibian and South African sardine using mtDNA (Bowen and Grant 1997). However, with the use of highly polymorphic loci, such as those in nDNA, stock differentiation between Namibian and South African sardine (with the Lűderitz upwelling cell acting as a barrier to gene flow), as well as multiple stocks within South Africa, could still be found.

The use of microsatellites in South African sardine and anchovy stock identification

Throughout the genomic DNA, but particularly in non-coding regions, are areas of repetitive short DNA sequences known as microsatellites. Microsatellite repeat motifs range in size from one to five base pairs of DNA (Jarne and Lagoda 1996). These loci are mostly selectively neutral and are highly polymorphic in natural populations, with mutation rates of between 10^{-4} and 10^{-5} mutations per generation (Dewoody and Avise 2000, Baldwin *et al*. 2012). They are also co-dominant and follow Mendelian inheritance models (Jarne and Lagoda 1996). Certain loci are highly variable in nature and have fast mutation rates, and thus are able to show individual identity, relatedness and gene flow, which are useful for high resolution population genetics and stock identification (Hauser and Seeb 2008). Multiple loci need to be screened in order to obtain between four and ten loci with sufficient sensitivity to differentiate stocks (Wirgin and Waldman 2005). Microsatellites are most useful for intraspecific studies, as they can be used to identify events that have occurred in previous generations, including bottlenecks and population expansions that occur on a decadal time scale.

Study aims and thesis outline

Should genetic stock differentiation be found in South African sardine or anchovy, it is important that each stock is appropriately managed, especially if the eastward shifts in sardine and anchovy distribution are a result of a subpopulation decline on the west coast. This could mean that putative west and south coast stocks need to be managed separately to prevent the complete collapse of one stock and the loss of genetic variability associated with it. Thus, each local population needs to be maintained at a level of sufficient capacity to be able to respond independently to environmental change and fishing pressure. If no stock differentiation is found, it can be assumed that there is considerable gene flow between the west and south coast and that the small pelagic fish populations in the southern Benguela are panmictic. This would require further investigation as to why there has been a recent shift of the bulk of the sardine population towards the east, and whether or not this is a result of climate change or environmental variability, as hypothesised for anchovy (Roy *et al.* 2007).

Not only is the South African coastline influenced by two major currents, the Benguela and the Agulhas, it has been divided into as many as nine bioregions (Lombard 2004), or four marine biogeographic provinces (Teske *et al.* 2011). Within each region there is also small-scale variability and complexity of habitat; a review of phylogeographic studies in South Africa found that, while certain species exhibited abrupt genetic breaks corresponding to the biogeographic provinces, other breaks occurred within provinces and other species did not exhibit any genetic differentiation throughout their ranges (Teske *et al.* 2011). The dynamic environment in the Benguela upwelling region leads to major pelagic habitat differences around the coast. This impacts small pelagic fish, which are able to exploit the differences through phenotypic variability, while maintaining the ability to do so through genetic variability and reproductive mixing.

The aim of this study is to use a three-pronged approach to support existing data for the hypothesis of distinct sardine stocks while testing, for the first time, the degree of genetic variability. It is hypothesised that populations of sardine, anchovy and round herring in South African waters are not continuous, but have a complex structure that shows differentiation between multiple stocks, reflecting the complexity of the varying habitats along the South

African coastline. It is expected that there will be complex stock structure that is not necessarily temporally stable (given the variability in oceanographic conditions across seasons and years) and will differ in the three species as a result of their different life history strategies and habitat usage. The work forms part of a multidisciplinary investigation into stock differentiation of sardine in South Africa, builds on previous genetic work on anchovy differentiation and will be the first work done on stock differentiation of round herring in the region. If there are multiple stocks and one stock is being fished at a proportionately higher rate than the other, then that stock is at risk of overfishing if the entire population is managed as a single entity. Thus, a multidisciplinary approach for investigating stock differentiation is important for successful management of small pelagic fishes in the southern Benguela.

Chapter two uses two otolith shape indices, *circularity* and *form factor*, to test the hypothesis that there is a geographic determinant to otolith shape in anchovy, round herring and sardine. One aspect of the study is to determine the usefulness of otolith morphology in the analysis of stock differentiation in small pelagic fishes. Shape indices are compared in anchovy and round herring from the west and south coast, and in sardine from Namibia and the west, south and east coasts of South Africa.

Chapter three uses the elemental composition of otoliths from sardine around the coast of South Africa to test the hypothesis of multiple stocks of sardine in South Africa. Different elemental compositions of otoliths suggest that individuals spend significant amounts of time in different environments. A comparison between elemental composition at the core of the otolith to the edge of the otolith will test the degree of dispersal of fish from their natal spawning grounds.

Chapter four investigates long term gene flow inferred from mitochondrial DNA variation and fine scale population differentiation based on seven microsatellite DNA markers in sardine. Four alternative hypotheses are tested in chapter four: *(i)* There is no difference within the population, *(ii)* putative stocks are entirely separated with no reproductive mixing between them, *(iii)* there is limited differentiation, with some gene flow in the west-to-south direction, or there is limited differentiation, with some gene flow in the south-to-west direction, or *(iv)* there has been recent segregation but no differentiation, as it has occurred too recently to be evident in this study. The putative stocks are west-, south- and east-

(sardine-run) coast sardine. In addition, stock differentiation is investigated between South African and Namibian stocks.

Chapter five describes a preliminary study into microsatellite diversity of anchovy in South Africa, planned to test the hypothesis about multiple stocks. Gene flow in anchovy is complicated by interactions with the variable environment in which they live.

Chapter six synthesises the results of this thesis and compares them with previous studies on morphological and meristic differentiation in sardine, to establish what differentiation occurs in sardine. A composite hypothesis is presented of sardine life history at decadal to centennial scales. Any stock differentiation analysis needs to take into account the life history parameters of the species and the oceanographic features that could influence the geographic distribution and limitations to mixing, as well as limitations to practical management operations. This chapter also examines whether, given the preliminary work presented, a multidisciplinary study in stock differentiation of round herring and anchovy should be undertaken.

Chapter 2:

Investigating stock structure in small pelagic fishes of southern Africa using otolith morphology

Chapter 2: Investigating stock structure in small pelagic fishes of southern Africa using otolith morphology

Abstract

Metabolically inert structures like otoliths can provide a useful index of stock differentiation in fish. Two dimensionless otolith shape indices, *circularity* and *form factor*, were used to investigate possible stock differentiation in anchovy (*Engraulis encrasicolus*), round herring (*Etrumeus whiteheadi*) and sardine (*Sardinops sagax*) off southern Africa. *Circularity* describes how closely the otolith resembles a circle whilst *form factor* measures the regularity of the circumference of the otolith. General linear modelling was applied to assess the effects of fish length, coast (South African west and south coast for anchovy and round herring, and the South African west, south and east coasts - as well as the Namibian coast - for sardine), sex and season (only in sardine) on these indices. In anchovy, *circularity* and *form factor* were significantly influenced by fish length ($N = 512$, $P < 0.05$) but there was no differentiation between fish from the west and south coast. Round herring otolith shape indices were significantly influenced by both fish length and sex ($N = 563$, $P < 0.01$), with female round herring having longer and more irregularly-shaped otoliths than male round herring. Sardine otolith shape was also significantly influenced by fish length, with shape indices also differing between seasons, and the otoliths of sardine from the east coast were significantly different to those of Namibian, west and south coast sardine. There was no evidence of differentiation between sardine from the west and south coast. Although otolith shape has been useful in significantly differentiating fish stocks elsewhere, they are unlikely to be useful for anchovy and round herring off South Africa, because there is either no spatial differentiation in otolith shape or the signal is obscured by high levels of individual variability and the effects of length (and sex in round herring). In sardine, otolith shape is useful in discriminating east coast sardine from those in other regions.

Introduction

Morphological differences evolve over time scales that are useful for the purposes of fisheries management (Begg and Waldman 1999). Genetic differentiation can occur slowly, particularly in highly mobile and variable populations (Ward *et al.* 1994). Random genetic drift can hide underlying differentiation, despite only a few individuals mixing per generation (Waples 1998). In a species with extensive dispersal capabilities, particularly in the larval stage, there is increased likelihood of mixing. However, it is plausible that individuals, or groups of individuals, can spend the majority of their lives in a particular environment (Campana 1999). Thus phenotypic variability can indicate geographic variability within a species, and local adaptations to particular environments could influence reproductive success.

While morphological markers cannot be used to delineate breeding stocks, they can be used to indicate whether groups of individuals spend most of their lives in different environments. In the Pacific, *Sardinops sagax* from different water temperatures were shown by morphometric analysis to be two stocks, despite no molecular differences (Garcia-Rodriguez *et al.* 2011). These morphometric differences could be important for locally-adapted phenotypic stocks, and population structure is an important management consideration. When using morphological markers, it is necessary to differentiate between traits that are influenced by short-term factors, such as food availability and environmental stresses, and those that are geographically determined. While body shape and condition can reflect temporary changes (Cardinale *et al.* 2004), hard structures such as otoliths are laid down early, grow throughout life and are metabolically inert. Therefore, differences in otolith shape within a species suggest that individuals have spent most of their time in different environments (Campana and Neilson 1985, Campana and Casselman 1993, Turan 2006).

Otoliths are calcium carbonate ($CaCO_3$) structures in the inner ear of all teleost fishes (Tomás *et al*. 2004). Sagittal otoliths are the largest of the three otoliths present in each ear (Ponton 2006). The $CaCO_3$ that forms otoliths generally precipitates as aragonite but occasionally a more translucent vaterite form occurs (Tomás *et al*. 2004). Otolith shape is influenced by both genetic predisposition to a specific form

and environmental factors (such as food availability, water temperature and individual variability), which can cause variability around the specific form (Ponton 2006, Vignon and Morat 2010). Larvae and recruits tend to have a more general, spherical otolith that, through the accretion of minerals during growth, develops into the form characteristic of the species. The causes of individual variability in otolith shape are not fully understood, although some authors have suggested that, in addition to temperature, depth and salinity influence otolith shape (Vignon and Morat 2010).

Otolith shape analysis has been used as a tool to identify species composition of prey items in seabirds (Crawford and Jahncke 1999, Bugoni and Vooren 2004) and marine mammals (Zeppelin *et al.* 2004, Mecenero *et al.* 2006, Ponton 2006). It has been used for fisheries management to discriminate between species and, within species, to delineate stocks (examples of recent studies are shown in Table 2.1). Although broadly clumped as otolith shape studies, the methods used differ: geometric morphometrics, various forms of Fourier analysis and shape indices. Tuset *et al.* (2003) highlighted otolith *form factor* and *circularity* as being useful dimensionless indices sensitive to small differences between individuals from different coasts, with *form factor* being particularly informative.

Table 2.1: Some examples of how otolith shape has been used in stock differentiation studies on clupeids; different methods, including shape indices (SI), elliptical Fourier analysis (EFA) or perimeter weight profiles (PWP) have been used either singly or in combination.

Species	**Common Name**	**Method**	**Reference**
Clupea harengus	Atlantic herring	SI	Burke and King 2008
Engraulis encrasicolus	anchovy	EFA	Kristoffersen and Magoulas 2008
Micromestius australis	southern blue whiting	SI and EFA	Leguá *et al*. 2013
Sardinops sagax	Pacific sardine	PWP	Javor *et al*. 2011
Strangomera bentincki	common sardine	SI and EFA	Curin-Osorio *et al*. 2012

In this study, otolith shape analysis is used to contribute to a multidisciplinary investigation into stock differentiation of sardine (*Sardinops sagax*) in South Africa (van der Lingen 2011). In December 2011, the results of initial investigations of stock structure inspired further research on how the operational management procedure for sardine in South Africa can be adapted to accommodate two mixing stocks; this management procedure was meant to be implemented in 2013 (van der Lingen 2011).

Wessels *et al.* (submitted) were able to distinguish between Namibian and west coast sardine using head shape, and between west and south coast sardine using the position of the pectoral fin. Idris *et al.* (submitted) similarly found the number of gill rakers and the sizes of spaces between gill rakers could be used in distinguishing between South African and Namibian sardine, whereas gill arch length could be used to distinguish between west and south coast sardine.

While extensive research has been undertaken to investigate stock structure in South African sardine, no such effort has been undertaken to investigate geographic structure in anchovy (*Engraulis encrasicolus*) and round herring (*Etrumeus whiteheadi*). There is considerable overlap in the distribution of sardine, anchovy and round herring in southern Africa. The South African sardine and anchovy fisheries are closely linked, as sardine juveniles are caught as bycatch in the anchovy fishery (van der Lingen and Huggett 2003). Anchovy are distributed in the upper ocean layers of the continental shelf of South Africa (Barange *et al.* 1999, Fairweather *et al.* 2006). As with sardine, in times of low population abundance there is a break in distribution of anchovy at the Agulhas Bank (Barange *et al.* 1999). This suggests the possibility of two stocks of anchovy around the South African coastline. Round herring are not as popular with commercial fishers as sardine and anchovy, possibly because adult round herring typically occur in small schools offshore in deep water (approximately 200m) (van der Lingen *et al.* 1998). Round herring are not as well studied as sardine and anchovy, and this will be the first investigation of geographic differentiation in round herring in South Africa.

Small pelagic fishes are an important ecosystem component of the southern Benguela upwelling ecosystem (Cury *et al.* 2000, 2012), and represent the largest fishery, by mass, in South Africa (Fairweather *et al.* 2006, DAFF 2012). This valuable resource therefore needs to be carefully managed for long-term sustainability (Cury *et al.* 2000, Verheye 2000). The three species overlap in distribution but differ in certain life history characteristics (Chapter 1). It is therefore important to make sure that management procedures for each species account for any geographic stocks that could be differentially productive. In this chapter otolith shape indices are developed and analysed, to test the hypothesis of geographic structure in sardine, and as an initial

investigation into stock differentiation of anchovy and round herring between South Africa's west and south coast.

Methods

Sample collection

Anchovy were collected in South Africa from 36 stations during the 2008 Recruit Survey (RS) and 2008 and 2009 Pelagic Spawner Biomass (PSB) surveys conducted by the Department of Agriculture, Forestry and Fisheries (DAFF; see Barange *et al.* 1999). Round herring were collected from 29 sites during the 2008 and 2009 PSB surveys. All samples collected from South African waters were collected using a mid-water trawl net. Sardine were collected from 64 sites in South African and Namibian waters. South African samples were collected during the 2009 and 2012 PSB surveys, in 2009 and 2012 from the annual KwaZulu-Natal sardine run beach-seine catches off Amanzimtoti (see van der Lingen *et al.* 2010a), and in winter 2008 and 2012 during the RS. The Namibian samples were collected in 2009, using a purse-seine net, south of Walvis Bay during a pelagic survey conducted by the Namibian Ministry of Fisheries and Marine Resources.

Details of the samples analysed in this study are given in Table 2.2, and their locations are shown in Figure 2.1. Broken, damaged or vaterite otoliths were excluded from the analyses. Fish length, measured as caudal length (CL, to the nearest 1mm), and sex were recorded for each individual. The left otolith from each individual was removed, cleaned and photographed using a Carl Zeiss Stemi 2000-C microscope linked to a digital camera. Otoliths were positioned in the same orientation on a black background (Figure 2.2) and the microscope was adjusted to maximise resolution of photographs.

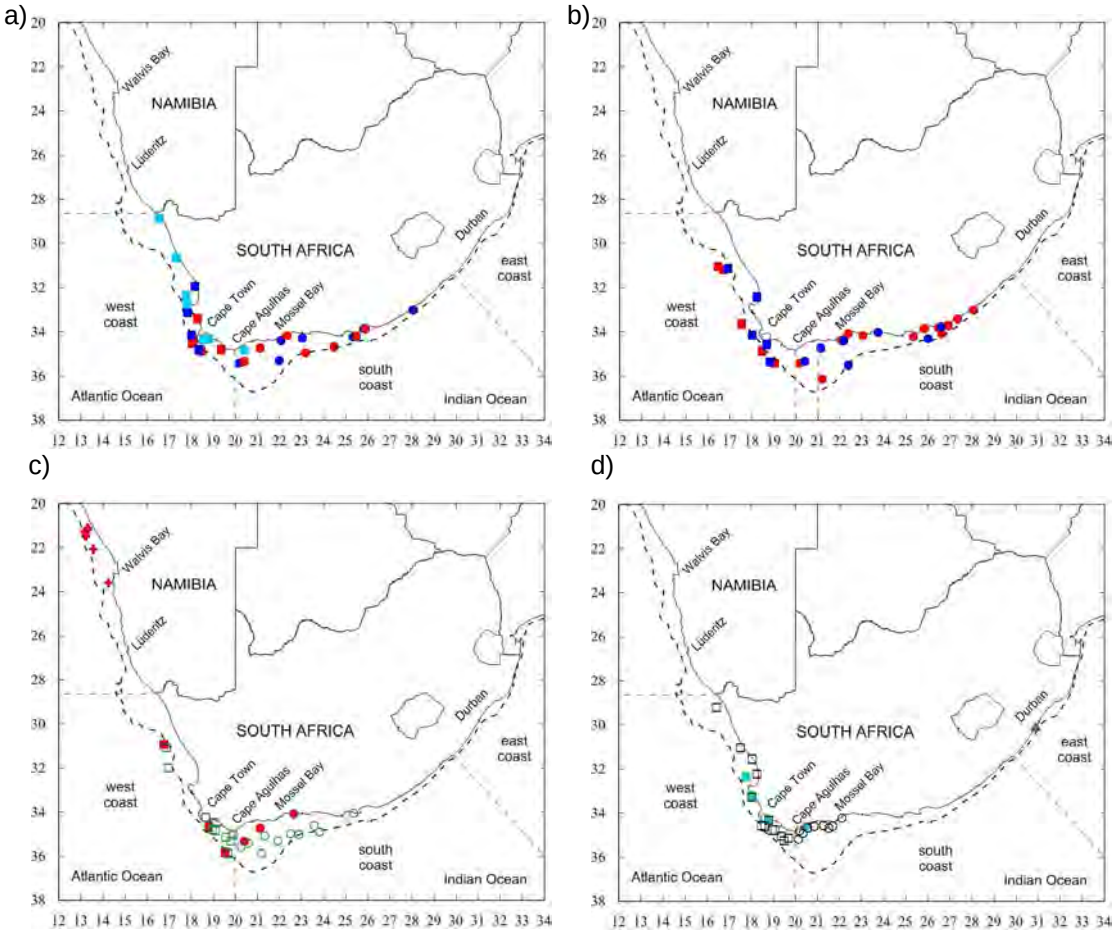


Figure 2.1: Approximate sampling sites for a) anchovy, b) round herring, c) summer sardine and d) winter sardine. Namibian sites are crosses, west coast sites squares, south coast sites circles and east coast sites triangles. Individual surveys are coded turquoise (RS 2008), blue (PSB 2008), red (2009), green outlines (PSB 2012), black outlines (RS 2012) and filled green (east coast sardine run 2009, 2012). The 200m depth contour is shown with the black dashed line.

Table 2.2: Samples of small pelagic fishes used for otolith analysis; the number of trawl sites per coast and number of male (M), female (F) and immature individuals (I) are given, as well as the number of samples per coast and total number of samples for each species. The coast codes are Namibia (NAM), west coast (WC), south coast (SC) and east coast sardine run (EC).

	Code	N sites	M	F	I	Coast total	Total
Anchovy	WC	16	94	80	260	434	980
	SC	20	148	189	209	546	
Round herring	WC	10	148	129	28	305	817
	SC	19	124	162	226	512	
Sardine	NAM	8	72	57	0	129	1987
	WC	31	309	255	497	1061	
	SC	23	225	313	191	729	
	EC	2	29	39	0	68	

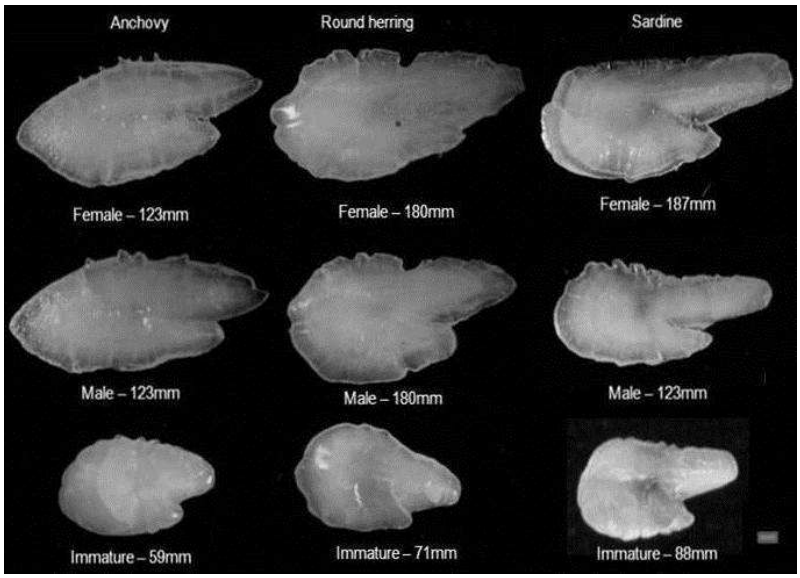


Figure 2.2: Examples of mature female, mature male and immature otolith shapes for anchovy, round herring and sardine. The size of each fish from which the otolith was extracted is shown in mm. The grey block in the bottom right corner represents the approximate actual size of a mature sardine otolith (5mm).

Outline analysis

Area and perimeter were both calculated from outline analysis in tpsDIG v.2.16, which uses an automatic outlining function to differentiate between the white outline of the otolith and the dark background on which it was photographed, according to a

user defined threshold (Rohlf 2010), with manual correction. Shape indices were calculated as described in Tuset *et al.* (2003) and Stransky and Macellan (2005).

$$Circularity = \frac{Perimeter^2}{Area}$$

Circularity is a relative measurement to describe how closely the otolith shape resembles a circle, where a minimum value of 4π (12.566) represents a perfect circle and larger values represent increasingly elongated or oval otoliths.

$$Form\ \ factor = \frac{4\pi * \text{Area}}{Perimeter^2}$$

Form factor measures the regularity of the circumference, where 1 is a perfect circle and less than 1 represents an increasingly irregular outline.

Statistical analysis

Paired t tests were used on a subset of individuals to test for differences in *circularity* and *form factor* between left and right otoliths. A general linear model (GLM) was used to test whether fish length, coast, fish sex and season (only applicable for sardine), significantly influenced otolith shape indices. Otolith *circularity* and *form factor* were analysed separately as response variables. Coast, sex, and season were included as independent categorical explanatory variables and log-transformed fish length as a covariate in the fully crossed model. Each GLM was done for known-sex individuals and immature individuals separately. All non-significant interaction terms were excluded, in a stepwise fashion, from the final model and confirmed by using the lowest Akaike information criterion (AIC) score. Where necessary, log transformations were performed to normalise data after examination of residuals. Normality of residuals and homoscedasticity were examined visually from residual analysis. The significance level was set at $P < 0.05$ for all statistical tests. All statistical tests for shape indices were performed in R v. 3.0.2 (R Core Team 2013). A *post hoc* multiple comparisons of means test was performed using the R package multcomp (Hothorn *et al.* 2008). A model dataset, standardised for covariates, was used to create graphics of least squares means. R code is shown in Appendix 2.

Results

Anchovy were the smallest of the three species. When all anchovy samples were included, they had similar mean CLs to immature sardine (Table 2.3). Round herring and sardine had comparable lengths, although sardine had a larger range of sizes. Immature round herring tended to be small. They were predominately less than 145mm in length and from the south coast, although the smallest individuals, measuring between 70 and 80mm, were from the west coast (Figure 2.3).

Table 2.3: Samples sizes, mean caudal length (sd), minimum and maximum values are shown for all samples, as well as separately for mature (male (M) and female (F)) and immature (I) anchovy, round herring and sardine.

Sex	N	Mean (sd) mm	min - max
		Caudal Length	
Anchovy			
ALL	853	103.7 (18.1)	49 - 137
M + F	512	110.6 (10.2)	90 - 137
I	341	83.1 (16.7)	49 - 122
Round herring			
ALL	817	136.3 (28.1)	70 - 200
M + F	563	146.8 (24.8)	87 - 200
I	254	113.0 (19.8)	70 - 153
Sardine			
ALL	1987	136.2 (34.9)	59 - 218
M + F	1299	153.7 (28.0)	95 - 218
I	688	103.3 (19.2)	59 - 157

Circularity

There were no significant interactions between covariates in any of the models. Otolith *circularity* of immature fish increased with fish length more rapidly than it did in the larger, mature fish (Figure 2.3). For all three species, length was the only significant contributor to the models of immature fish (results not shown). In both mature and immature individuals of all three species, otoliths tended to become less circular (i.e. increased in *circularity*) with an increase in fish CL (Figure 2.3, Table 2.4), but the relationship was most noticeable in sardine and least apparent in mature anchovy (Figure 2.3).

Table 2.4: Samples sizes, mean *circularity* (sd), minimum and maximum values are shown for all samples, as well as separately for mature (male (M) and female (F)) and immature (I) anchovy, round herring and sardine.

Sex	N	Mean (sd)	min - max
		Circularity	
Anchovy			
ALL	853	22.95 (1.79)	17.79 - 33.47
M + F	512	23.29 (1.71)	17.32 - 33.47
I	341	22.05 (1.58)	15.55 – 27.60
Round herring			
ALL	817	21.20 (1.20)	17.48 - 25.56
M + F	563	21.51 (1.11)	19.04 - 25.56
I	254	20.51 (1.08)	17.48 - 23.31
Sardine			
ALL	1987	25.84 (2.96)	18.13 - 39.41
M + F	1299	27.04 (2.57)	21.71 - 39.41
I	688	23.57 (2.21)	18.13 - 32.23

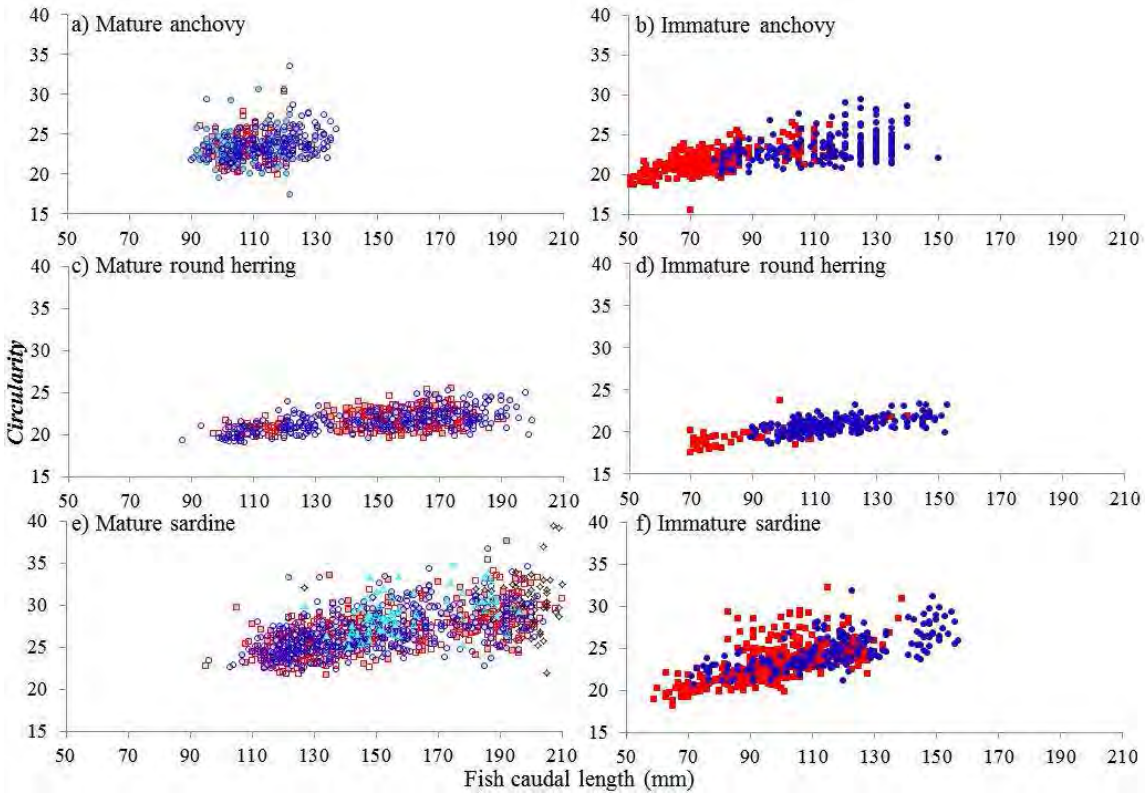


Figure 2.3: *Circularity* in relation to length for a) mature anchovy, b) immature anchovy, c) mature round herring, d) immature round herring, e) mature sardine and f) immature sardine from Namibia (black diamonds), west coast (red squares), south coast (blue circles) and east coast (turquoise triangles). Immature individuals are shown by filled symbols, males are represented by symbols filled with grey and females are represented by open symbols.

In anchovy, fish length was the only significant contributor to the GLM that also included coast and sex as covariates (Table 2.5). There was a large degree of unexplained variability in the model (adjusted $R^2 = 0.06$). Anchovy otolith *circularity* was positively related to the length of the anchovy (Figure 2.4a), with otoliths tending to become slightly less circular with increasing fish length. There was no difference in otolith *circularity* among individuals from the west and south coasts (Table 2.5, Figure 2.4b). This remained true when only immature individuals were used in the analysis; there was no significant difference between samples from the west and south coasts ($F = 566.48$, $df_1 = 1$, $df_2 = 355$, $P = 0.06$). Otolith shape was significantly related to the length of the fish ($P < 0.05$) and the model was better explained ($R^2 = 0.37$).

Table 2.5: Results of anchovy GLM for response variable *circularity* and explanatory variables fish length, coast and sex (male and females only); degrees of freedom (df), residual deviance (Res. Dev), change in deviance (Δ Dev), F and P values are shown. The R^2 for the model is also shown. Significance is indicated in bold.

	df	Res. Dev	Δ Dev	F - value	*p-value*
Null	511	1494.9			
+ log(length)	**1**	**1414.1**	**80.83**	**29.09**	**< 0.001**
+ coast	1	1412.5	1.53	0.55	0.458
+ sex	1	1408.7	3.82	1.37	0.242
Adj R^2	6%				

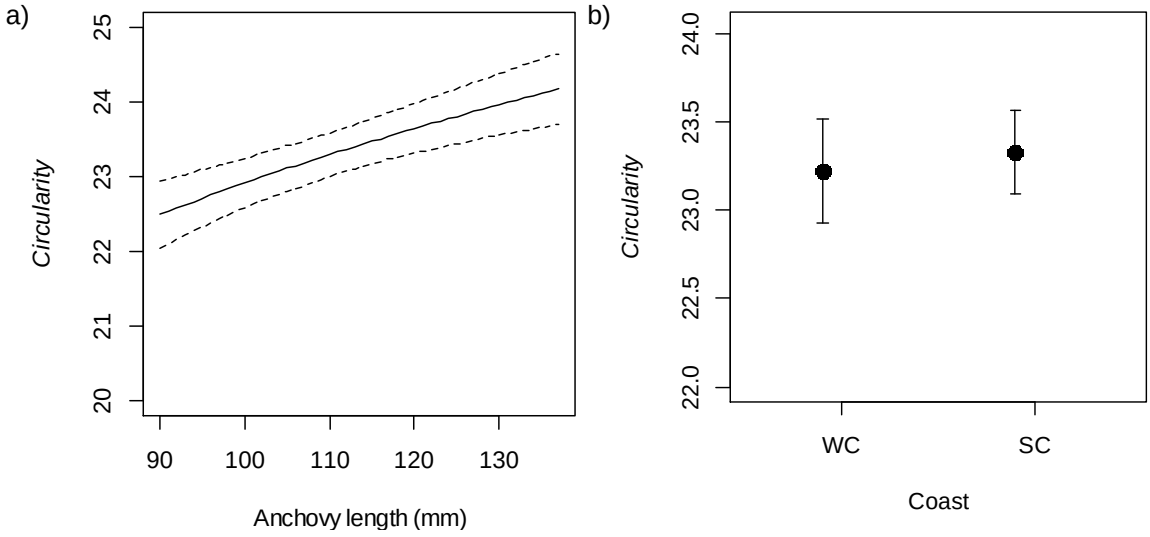


Figure 2.4: Model-predicted otolith *circularity* of anchovy standardised for a) west coast females between 90 and 137mm CL, 95% confidence intervals are shown with dashed lines and b) females of 108mm CL from the west (WC) and south (SC) coasts; bars indicate 95% confidence intervals.

In the round herring GLM analysis, fish length and sex both explained significant amounts of variability in the *circularity* estimates ($P < 0.05$, Table 2.6). There was no difference between coasts ($P = 0.18$, Table 2.6). Round herring females had larger otolith *circularity* values (and were therefore less round) than round herring males (Tukey contrast $z = 2.639$, $P < 0.05$), despite overlap in the 95% confidence intervals (Figure 2.5). Similarly, there was no significant difference in otolith *circularity* between the west and south coast immature round herrings ($F = 0.701$, $df_1 = 1$, $df_2 = 251$, $P = 0.403$).

Table 2.6: Results of round herring GLM for response variable *circularity* and explanatory variables fish length, coast and sex (male and female only); degrees of freedom (df), Residual deviance (Res. Dev), change in deviance (Δ Dev), F and P values are shown. The R^2 for the model is also shown. Significance is indicated in bold.

	df	Res. Dev	Δ Dev	F - value	*p-value*
Null	562	**695.17**			
+ log(length)	**1**	**479.49**	**215.77**	**255.39**	**<0.001**
+ coast	1	477.97	1.53	1.81	0.18
+ sex	**1**	**472.09**	**5.88**	**6.96**	**0.009**
Adj R^2	32%				

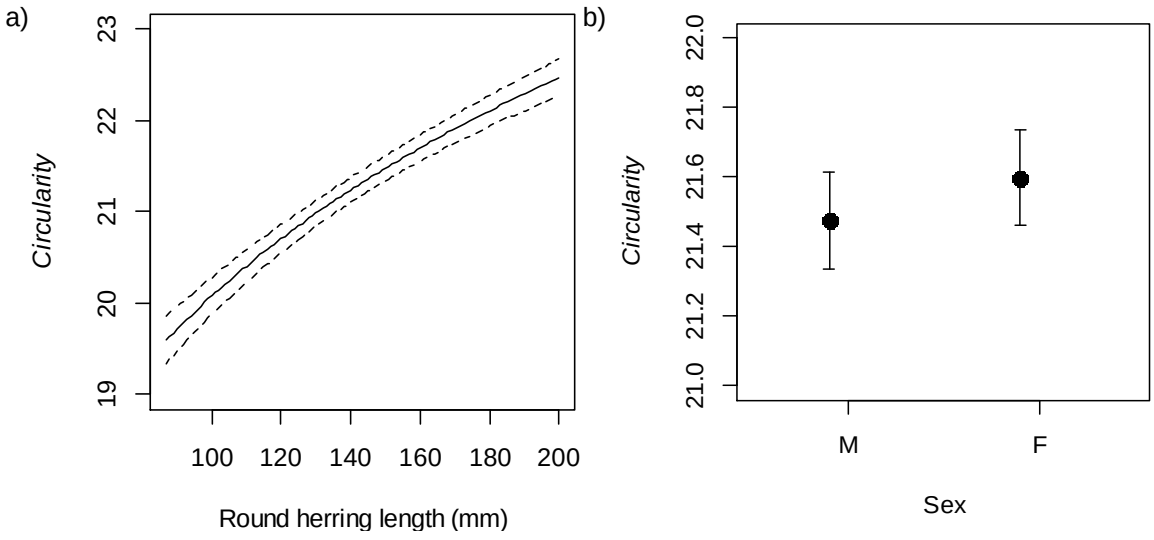


Figure 2.5: Model-predicted otolith *circularity* for round herring standardised for a) west coast females between 87 and 200mm CL, dashed lines indicate 95% confidence intervals and b) west coast males (M) and females (F), standardised to a length of 150mm; bars indicate 95% confidence intervals.

Sardine length, coast and the season in which the fish were caught contributed significantly to the model in which mature sardine otoliths from all four coasts were analysed (Table 2.7, Figure 2.6). The model explained 42% of the observed variance (adjusted R^2 = 0.42). Fish length significantly influenced the shape of the otolith (Figure 2.6a). A multiple comparisons of means test showed that otolith *circularity* from east coast samples was significantly different to otolith *circularity* of samples from Namibia and the west and south coasts (Table 2.8), but there were no differences between the Namibian, west or south coasts. East coast otoliths were less circular than those sampled in the rest of southern Africa (Figure 2.6b). Samples that were collected in summer had significantly higher *circularity* values than those collected in winter (Tukey contrasts z = -5.44, P < 0.05) and were therefore less round than winter-collected otoliths (Figure 2.6c).

Immature sardine were only available from the west and south coasts, and there was no difference in otolith *circularity* between samples from these two coasts (P = 0.163), although the season in which the fish were caught did contribute significantly to the model (F = 14.86, df_1 = 1, df_2 = 684, P < 0.05, R^2 = 0.49). As with the mature sardine, samples collected in summer were less circular than those collected in winter.

Table 2.7: Results of sardine GLM for response variable *circularity* and explanatory variables fish length, coast, sex (male and female only), and season of capture; degrees of freedom (df), Residual deviance (Res. Dev), change in deviance (Δ Dev), F and P values are shown. The R^2 for the model is also shown. Significance is indicated in bold.

	df	Res. Dev	Δ Dev	F - value	*p-value*
Null	1298	**8590.1**			
+ log(length)	**1**	**5281.4**	**3308.7**	**864.42**	**<0.001**
+ coast	**3**	**5060.0**	**221.4**	**19.28**	**<0.001**
+ season	**1**	**4950.5**	**109.5**	**28.60**	**<0.001**
+ sex	1	4945.3	5.2	1.35	0.245
Adj R^2	42%				

Table 2.8: P values of pairwise Tukey contrasts for multiple comparisons of least square means of otolith *circularity* for sardine from Namibia (NAM), west coast (WC), south coast (SC) and east coast (EC). Significance is indicated in bold.

	Nam	WC	SC	EC
Nam				
WC	0.214			
SC	0.537	0.766		
EC	**<0.001**	**<0.001**	**<0.001**	

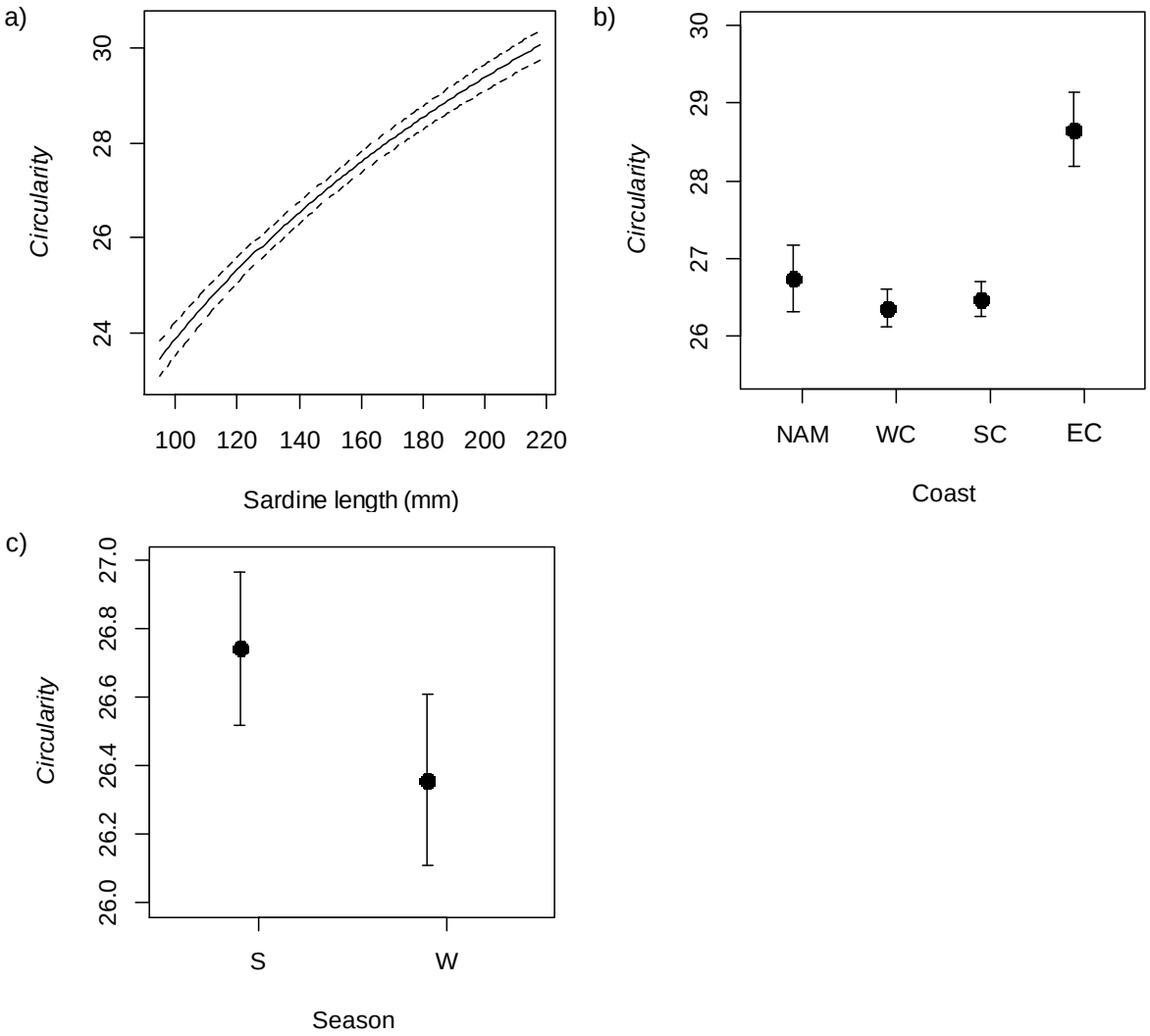


Figure 2.6: Model-predicted otolith *circularity* for sardine standardised for a) winter-caught west coast females from 95 to 218mm CL, dashed lines indicate 95% confidence intervals; b) winter-caught west coast females from Namibia (NAM), west coast (WC), south coast (SC) and KwaZulu Natal and c) 149mm CL west coast females caught in summer (S) and winter (W); bars indicate 95% confidence intervals.

Form factor

Form factor is smaller in mature than immature individuals of all three species (Table 2.9). There is a trend of decreasing *form factor* with an increase in length in mature individuals of all three species and in immature round herring and sardine (Figure 2.7), with otoliths becoming increasingly irregular as the fish grow. Sardine had the lowest and round herring the highest mean otolith *form factor* (Table 2.9) of the three species.

Table 2.9: Samples sizes, mean *form factor* (sd), minimum and maximum values are shown for all samples, as well as separately for mature (male (M) and female (F)) and immature (I) anchovy, round herring and sardine.

Sex	**N**	**Mean (sd)**	**min - max**
		Form factor	
Anchovy			
ALL	853	0.551 (0.041)	0.375 - 0.678
M + F	512	0.542 (0.037)	0.375 - 0.646
I	341	0.572 (0.041)	0.455 - 0.808
Round herring			
ALL	817	0.595 (0.034)	0.492 - 0.719
M + F	563	0.586 (0.030)	0.492 - 0.660
I	254	0.615 (0.033)	0.539 - 0.719
Sardine			
ALL	1987	0.496 (0.056)	0.319 - 0.693
M + F	1299	0.469 (0.043)	0.319 - 0.579
I	688	0.538 (0.049)	0.390 - 0.693

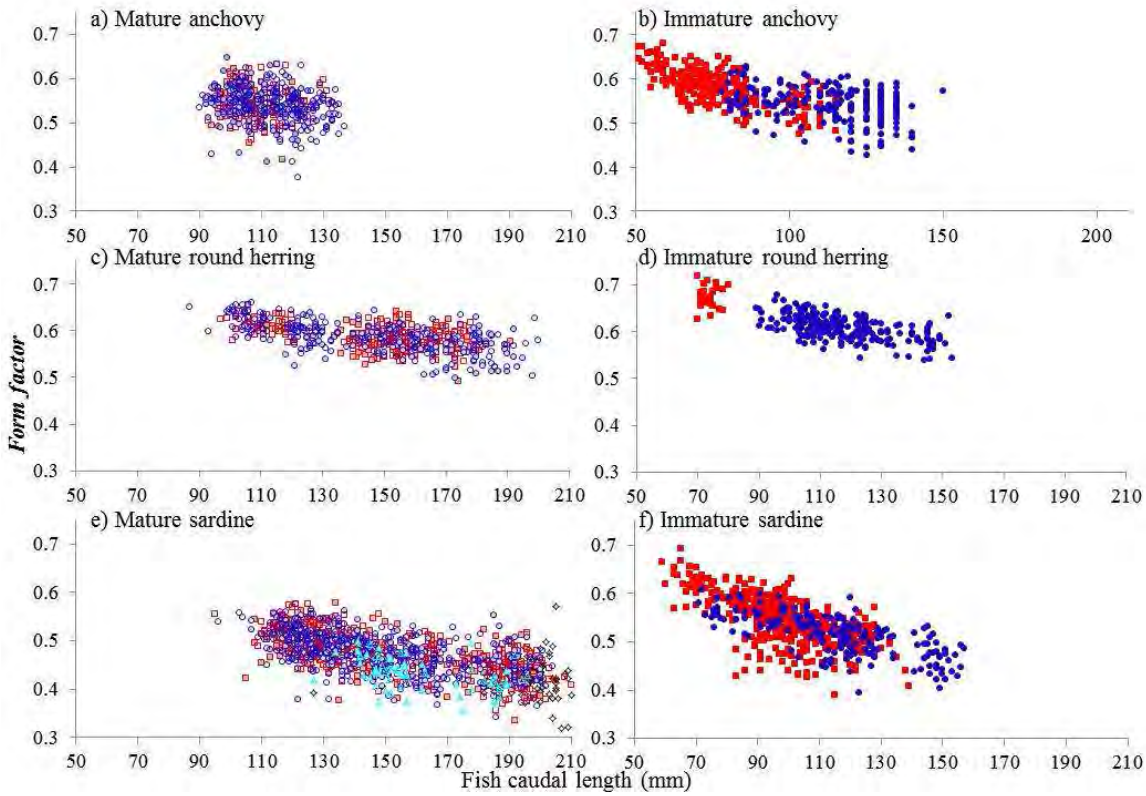


Figure 2.7: *Form factor* versus length relationship for a) immature anchovy, b) mature anchovy, c) immature round herring, d) mature round herring, e) immature sardine and f) mature sardine from Namibia (black diamonds), west coast (red squares), south coast (blue circles) and KwaZulu-Natal (turquoise triangles). Immature individuals are shown by filled in symbols, male symbols are filled with grey and female symbols are open.

In the anchovy GLM, adjusted R^2 values were low (adj. $R^2 = 0.05$) and fish length was the only significant contributor to the model ($P < 0.05$, Table 2.10). Larger anchovy were more likely to have rough-edged otoliths than smaller individuals (Figure 2.8). Similarly, otolith *form factor* also decreased significantly with an increase in fish length in immature anchovy ($F = 208.297$, $df = 356$, $P < 0.001$) but west and south coast otolith *form factor* values did not differ ($F = 3.067$, $df_1 = 1$, $df_2 = 354$, $P = 0.08$, $R^2 = 0.37$).

Table 2.10: Results of anchovy GLM for response variable *form factor* and explanatory variables fish length, coast, and sex (male and female only); degrees of freedom (df), residual deviance (Res. Dev.), change in deviance (Δ Dev), F and P values are shown. The R^2 for the model is also shown. Significance is indicated in bold.

	df	Res. Dev	Δ Dev	F - value	*p-value*
Null	510	**0.709**			
+ log(length)	**1**	**0.034**	**0.675**	**25.596**	**<0.001**
+ coast	1	0.001	0.674	0.644	0.423
+ sex	1	0.001	0.673	0.621	0.431
Adj R^2	5%				

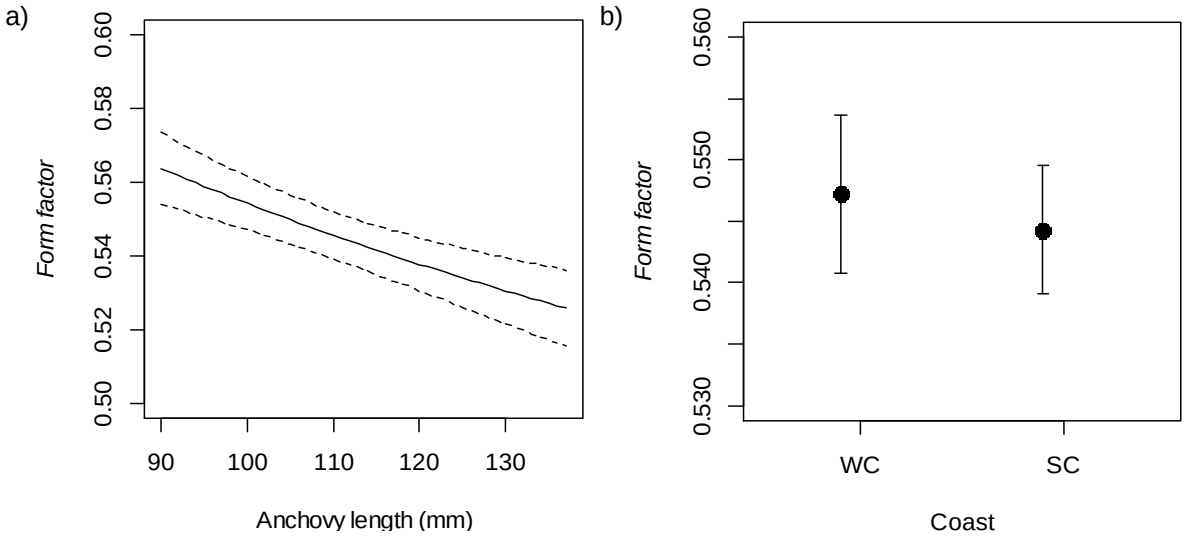


Figure 2.8: Model-predicted means otolith *form factor* for anchovy standardised for a) west coast females between 90 and 137mm CL; 95% confidence intervals are shown with dashed lines and b) females of 108mm CL from the west (WC) and south (SC) coasts; bars indicate 95% confidence intervals.

For the round herring GLM analysis that included only mature individuals, fish length and sex both contributed significantly to the model ($P < 0.05$, Table 2.11), which explained 33% of the variance found in round herring otolith *form factor*. Females tended to have lower otolith *form factor* values and, thus, rougher otolith edges than males (Figure 2.9). There was no difference between west and south coast otolith *form factor* ($P = 0.31$). Immature round herring *form factor* was significantly impacted by fish length ($F = 363.45$, $df = 252$, $P < 0.001$) but was not different between west coast and south coast samples ($F = 3.324$, $df_1 = 1$, $df_2 = 251$, $P = 0.07$).

Table 2.11: Results of round herring GLM for response variable *form factor* and explanatory variables fish length, coast, and sex (male and female only); degrees of freedom (df), residual deviance (Res. Dev), change in deviance (Δ Dev), F and P values are shown. The R^2 for the model is also shown. Significance is indicated in bold.

	df	Res. Dev	Δ Dev	F - value	*p-value*
Null	562	**0.496**			
+ log(length)	**1**	**0.336**	**0.160**	**271.04**	**<0.001**
+ coast	1	0.335	0.001	1.03	0.311
+ sex	**1**	**0.331**	**0.004**	**7.37**	**0.007**
Adj R^2	33%				

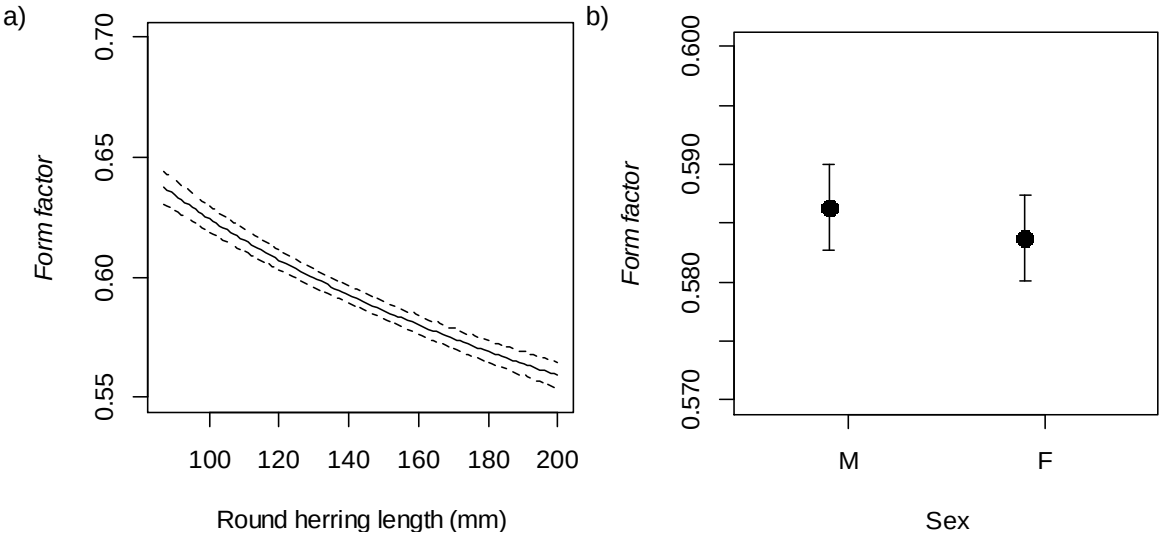


Figure 2.9: Least squares mean otolith *form factor* for round herring standardised for a) west coast females from 87 to 200mm CL; dashed lines indicate 95% confidence intervals and b) west coast males (M) and females (F), standardised to a length of 150mm; bars indicate 95% confidence intervals.

Sardine length, coast and catch season contributed significantly to the model that included mature sardine from all four coasts; the model explained 44% of the variance (adjusted R^2 = 0.44, Table 2.12). Length significantly influenced the shape of the sardine otoliths. A multiple comparisons of means test showed that otolith *form factor* from east coast samples was significantly different to otolith *form factor* of samples from Namibia and the west and south coasts (Table 2.13), but there were no differences among the Namibian, west or south coasts (Figure 2.10). Otoliths collected from the east coast had more irregular outlines than otoliths in the rest of southern Africa. Samples that were collected in summer had a significantly more

irregular perimeter than those collected in winter (Figure 2.10. Tukey contrasts z = 5.77, P < 0.05).

Immature sardine were only available from the west and south coast. As with mature sardine, the length of the fish explained the majority of the variance (F = 753.22, df = 686, P < 0.001). There was no difference in otolith *form factor* between these two coasts (F = 1.70, P = 0.192), although the season in which the fish were caught did contribute significantly to the model (F = 10.29, df_1 = 1, df_2 = 684, P < 0.05, R^2 = 0.52). As with the mature sardine, samples collected in summer had a more irregular outline than those caught in winter.

Table 2.12: Results of sardine GLM for response variable *form factor* and explanatory variables fish length, coast, sex (male and female only), and season of capture; degrees of freedom (df), Residual deviance (Res. Dev), change in deviance (Δ Dev), F and P values are shown. The R^2 for the model is also shown. Significance is indicated in bold.

	df	Res. Dev	Δ Dev	F - value	*p-value*
Null	1298	**2.390**			
+ log(length)	**1**	**1.431**	**0.959**	**926.78**	**<0.001**
+ coast	**3**	**1.372**	**0.059**	**18.85**	**<0.001**
+ sex	**1**	**1.338**	**0.034**	**32.73**	**<0.001**
+ season	1	1.338	0.001	0.64	0.426
Adj R^2	44%				

Table 2.13: Pairwise Tukey contrasts for multiple comparisons of means of otolith *form factor* for sardine from Namibia (NAM), west coast (WC), south coast (SC) and east coast (EC). Significance is indicated in bold.

	Nam	WC	SC	EC
Nam				
WC	0.473			
SC	0.883	0.639		
EC	**<0.001**	**<0.001**	**<0.001**	

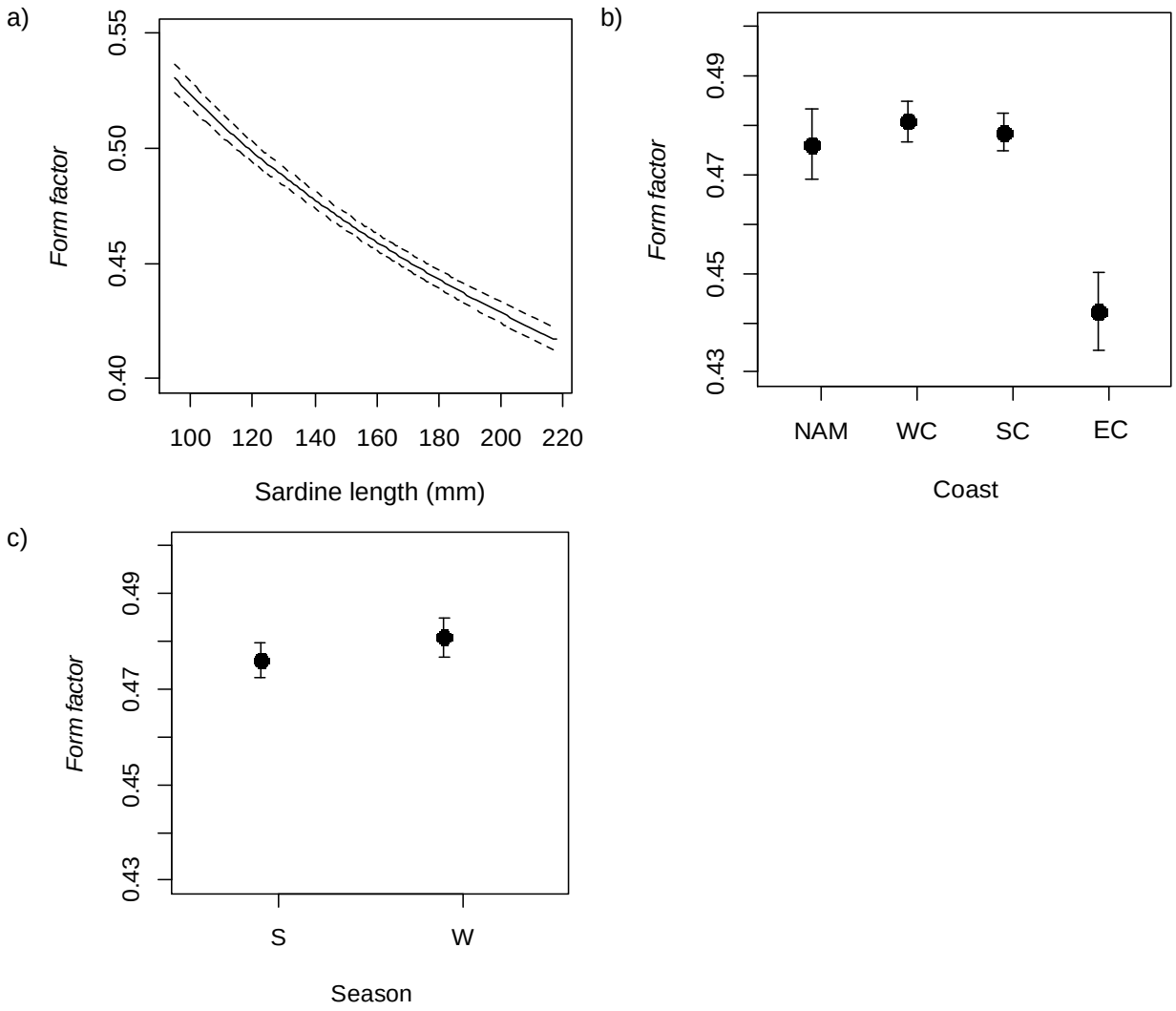


Figure 2.10: Model-predicted mean otolith *form factor* for sardine standardised for a) winter-caught west coast females from 95 to 218mm CL, dashed lines indicate 95% confidence intervals; b) winter-caught west coast females from Namibian (NAM), west (WC), south (SC) and KwaZulu-Natal and c) 149mm CL west coast females caught in winter (W) and summer (S); bars indicate 95% confidence intervals.

Discussion

Previous studies have shown that morphological analysis can be useful in delineating stocks of sardine in the southern Benguela; van der Lingen *et al*. (2010b) and Wessels *et al*. (submitted) have used morphological analysis to distinguish between sardine from Namibia, west coast, south coast and east coast. However, when investigating stock structure, it is important to take a multidisciplinary approach in order to control for short-term differences in phenotypic traits, and identify stocks that have spent

extended periods in different environments (Campana 1999) in the absence of genetic differentiation (Tudela 1999).

In this study, the otolith shape indices, *circularity* and *form factor*, were statistically related to the length of the fish in all three species. Otolith *circularity* values tended to increase with an increase in fish length in round herring and sardine and, to a lesser extent, in anchovy (Figure 2.3). Thus, the otoliths of longer fish tended to be more elongated than those of smaller individuals, which were more circular. Conversely, otolith *form factor* tended to decrease with an increase in fish length. This was most marked in immature anchovy and sardine but the relationship was also evident in mature anchovy, round herring and sardine. Longer fish tended to have increasingly irregular and elongated otoliths. Otoliths from smaller fish tended to be round and to have a smoother edge (Figure 2.11). This confirms the suggestion by Capoccioni *et al.* (2011) that otoliths from small fish tend to be more consistently smooth and round; outline shape changes as fishes grow and the rate of change is relatively faster in smaller, immature fish than mature fish (Hüssy 2008).

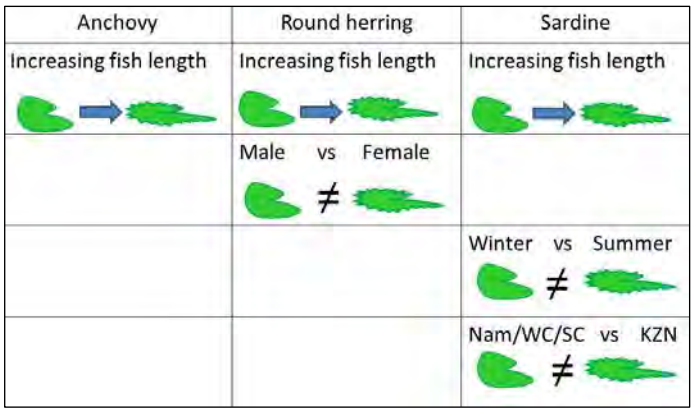


Figure 2.11: Summary of GLM results for otolith shape indices showing which variables contributed significantly to the GLM and how the variables impact on hypothetical otolith shape. The blue arrows show how otolith shape changes with an increase in length of anchovy, round herring and sardine. The slashed equal signs indicate where significant differences occur in otolith shape between sexes (in round herring), seasonality and coast (in sardine).

Fish length was the only variable to contribute to the shape of anchovy otoliths. However, neither the *circularity* nor the *form factor* models were well defined and a large amount of variance remains unexplained. The models performed better for

immature anchovy, wherein both *circularity* and *form factor* were mostly defined by the length of fish. The two shape indices are unlikely to be useful in determining stock structure in anchovy because, in mature fish, the model is ill-defined, and in immature anchovy, only the length of the fish contributes to the shape indices. A large amount of individual variability of otolith shape is evident in anchovy.

Round herring otolith shape differed in male and female fish. Female fish otoliths tended to be elongated with irregular edges. This difference could be a result of differential resource utilisation between the sexes, where females prioritise gonad growth over somatic growth. It is possible that, in resource limited conditions, otolith shape deviates from the smooth, round shape characteristic of small fish (Figure 2.10). It is presumed that male and female round herring would be experiencing similar water temperatures, salinity and bathymetry and thus those environmental indicators are unlikely to be the cause of variability in this case. Male and female round herring in South Africa mature at different sizes and ages; males mature when smaller and younger than females (Geja *et al.* in press). Differences in age at maturity could therefore also account for the sex-related differences in otolith shape.

It is unclear why a sex difference is not seen in this study in all three species. This could be investigated further by comparing condition factors of male and female small pelagic fishes. Agüero and Rodriguez (2004) studied body shape using landmark morphometrics and found sexual dimorphism in sardine in the Pacific. This was not the case in sardine from South Africa (Wessels 2009). Although there was a difference between male and female round herring otolith shape indices, length was the most important explanation of otolith shape.

Sardine from the east coast had a significantly different otolith shape to sardine from Namibia and the west and south coast of South Africa. Since the east coast is less nutrient-rich than the Agulhas Bank and west coast (Hutchings *et al*. 2010), it is possible that this has impacted on otolith shape, resulting in deviation from the smooth, round shape. Environmental conditions vary between coasts, and therefore the reason for differences in otolith shape between the east coast and the other coasts cannot be isolated. Resource availability, water temperature and salinity vary between seasons too, which could explain the seasonal variability in otolith shape. The shape is

probably a result of conditions experienced in the previous season; thus, sardine caught in winter will have experienced a nutrient-rich upwelling season prior to being caught, thus resulting in a smoother, rounder otolith (Hutchings *et al.* 2009). This could be investigated further by comparing otolith shape and condition factors between seasons.

It might be useful to compare the shapes of the annuli (the growth rings laid down annually or seasonally) of the otoliths, in order to better control for age and to allow for an understanding of how otolith shape changes over the lifetime of the fish. This has been done successfully in Atlantic herring (*Clupea harengus*) to differentiate between resident and migrant stocks in the Irish and Celtic Seas (Burke *et al.* 2008b). An investigation of otolith annuli would also help to determine whether the seasonal differences found in sardine otolith shape are as a result of the conditions experienced in the season prior to capture. This is, however, a more time-consuming and labour-intensive method than otolith shape analysis, and is more error-prone because the annuli need to be outlined manually (Dorval *et al.* 2013).

Burke *et al*. (2008a) stated that the factors that determine the shape of otoliths are not well understood. It can be that individuals have different-sized otoliths as a result of past growth rate (Ponton 2006). The current study finds that fish length is the most likely predictor of otolith shape in these three species in southern Africa. Sex was also a confounding variable in the case of round herring, where otolith shape differed significantly between males and females. Simoneau *et al.* (2000) found age, year class and sex to influence the shape of otoliths. The premise behind using otolith shape as an effective stock identification tool is that the shape is laid down early in life and therefore will not change. The evidence presented here shows, however, that larger individuals have longer, thinner otoliths than smaller individuals. The results suggest that, within species, shape differences could be based on previous growth rate (Reznick *et al.*1989) or biological processes (Zelditch *et al.* 2004), but the primary cause of otolith shape remains uncertain.

Alternative means of analysing shape data, such as geomorphometrics or Fourier shape analysis, might give different results to those presented here. However, since otolith shape is statistically related to fish length in the samples available, length will

be a confounding factor, no matter which technique is used. Although Fourier analysis has been successfully used to differentiate between stocks (e.g. Stransky and MacLellan 2005, Hamer *et al.* 2012, Sadighzadeh *et al.* 2012), Cadrin and Friedland (1999) suggest that the cause of significant differences in Fourier analysis and geometric landmark studies are difficult to interpret. Shape indices provide intuitive measures of shape variability, but they are strongly related to length in these three species.

Otolith shape can be successfully used to differentiate between KwaZulu-Natal sardine and those from Namibia and the west and south coast of South Africa. It is not effective at differentiating between small pelagic fishes from the west and south coast, either because there is no difference, or because otolith shape is not an effective tool, given the confounding influences of fish length and sex (in the case of round herring). This further supports the need to use a multidisciplinary approach in any investigation of stock differentiation.

Chapter 3

Spatial and temporal variability in elemental signatures of sardine otoliths in South Africa

"Otoliths… while they are clearly more important to the fish that to the fish biologist, that point is easily forgotten." (Campana 1999, page 263)

Chapter 3*:* Spatial and temporal variability in elemental signatures of sardine otoliths in South Africa

Abstract

Otolith elemental signatures can be used to identify when groups of individuals are spending a significant amount of time in different environments. As part of a multidisciplinary study into the stock structure of sardine in the southern Benguela, elemental signatures were measured from 34 otoliths from juvenile sardine caught in winter 2008 and from 52 otoliths from juvenile sardine caught in 2009 using ICP-MS. The elemental signatures from fish caught in 2009 were measured at both the edge (to represent conditions prior to capture) and core (to represent conditions at time of spawning in 2008) of the otolith. PCAs were used to visualise the relationships of individuals to each other, in terms of their otolith chemistry, in two-dimensional space. MANOVAs were used to investigate differences among sites for samples collected in 2008, and among sites for core and edge measurements of samples collected in 2009. Significant differences among sites were found in each of the three MANOVAs, but the between-site differences varied among the elements. Magnesium concentration tended to decrease from the west to the south coast, whereas barium concentrations tended to increase. There was evidence of some dispersal from natal areas, but two sites at the extreme of sampling did not differentiate between natal measurements of the sardine and measurements from the one year old sardine in the PCA. Otolith microchemistry provides evidence of small scale differentiation in sardine that is not necessarily temporally stable. The southern Benguela is a dynamic and variable environment and this is reflected in the inter-site differences of elemental signatures of juvenile sardine.

Introduction

In the inner ear of fish, there are three acellular, calcium carbonate ($CaCO_3$) structures known as otoliths (Tomás *et al*. 2004). Otolith shape analysis on the largest of these structures has been shown to be useful in discriminating sardine from the putative eastern stock of the east coast of South Africa from those from the rest of South Africa (Chapter 2). However, otoliths show no clear differences in shape between sardine from the putative western and southern stocks (Chapter 2). Otolith shape reflects the entire life history of the fish, but short term differences also need to be investigated. The elemental composition of otoliths is a useful tool for identifying geographic stock differentiation in fish that spend significant amounts of time in different environments, but are not necessarily reproductively isolated. It can also be analysed to provide insight into population structure, and to assess fish movement, including the dispersal of juvenile fish from their natal origin (Thresher 1999, Cook 2011).

Otoliths grow through the addition of material following a circadian rhythm that differs according to season, physiology of the fish and environmental conditions (Jolivet *et al.* 2008). Although the majority (90-99%) of the otolith's non-collagenous organic matrix is $CaCO_3$, there are approximately 37 minor and trace elements that can be incorporated into the otolith during its growth, either as a substitute for calcium and/or a co-precipitate (e.g. magnesium, lithium, barium, strontium), or into the interstitial spaces of the crystal structure (e.g. sodium, chlorine, zinc, potassium) (Campana 1999, Thresher 1999, Tomás *et al.* 2004). In marine fishes, elements are primarily incorporated into the otolith from the intestine (Cook 2011). When concentrations of calcium are low in the marine environment, other elements, particularly strontium and barium, are substituted into the structure of the otolith. Because these and other elements are incorporated into the otolith in concentrations that closely reflect the characteristics of the ambient water (Cook 2011), otolith elemental signatures will reflect ambient elemental concentrations through ontogeny. The concentration of the elements incorporated into the otolith is also influenced by water temperature, salinity, pH and the concentration of dissolved oxygen in the water

(Campana 1999) and possibly influenced by certain types of parasites (Heagney *et al*. 2013).

Otoliths continue to grow throughout the life time of the fish, and thus provide a life time record of the environmental conditions experienced by an individual fish. The elemental signature of an otolith can therefore serve as a natural tag for stock differentiation studies (Campana 1999), should the population under study be distributed across environments that have sufficient spatial variation in water chemistry for this to be reflected in their otoliths.

Because the otolith elemental signature is a record of the environmental conditions that a fish has encountered, it can provide an indication of whether groups of fish spend significant amounts of time in waters of different chemical composition. Otolith microchemistry can also be used to investigate the extent of movement of fish. The elemental composition at the core of the otolith formed after fertilisation of the egg represents the conditions of the spawning site (Phillis *et al.* 2011). The edge of the otolith represents the conditions of the area in which the fish has spent time prior to being caught (the length of time represented will depend on the growth rate of the fish). A comparison of the composition of the core and the edge can therefore provide information about how far from the spawning site the fish has travelled. By examining the difference between core and edge otolith signatures, one can determine the degree of population connectivity, and test hypotheses of natal homing and group fidelity.

Analysis of otolith elemental composition has been widely used in population structure studies (Campana 2005). It has been successfully used, for example, to differentiate between what was regarded as a single stock of Icelandic cod, *Gadus morhua*, in cold northern waters and warmer, more saline southern waters, and was also found to differentiate between cod found deeper than 125m and those found in shallower waters influenced by fresh water run off (Jónsdóttir *et al*. 2006). Otolith chemistry was also used to discern fine scale population structure in the pelagic southern blue whiting (*Micromesistius australis australis*) (Arkhipkin *et al.* 2009).

Turan (2006) was able to differentiate between Mediterranean horse mackerel, *Trachurus mediterraneus*, from the Black Sea and Aegean Sea using sodium, potassium, magnesium and barium concentrations in their otoliths, but found that fish from the eastern Mediterranean Sea and Marmara Sea (Turkey) were indistinguishable. Otolith microchemistry was used to distinguish among five groups of anchovy (*Engraulis encrasicolus*) in the Atlantic in one year, but the analyses failed to demonstrate differentiation the following year, suggesting that the differentiation is not temporally stable (Guidetti *et al.* 2013). Despite some negative results, otolith elemental composition appears to be a reliable differentiator of marine fish stocks. Gillanders *et al*. (2001) reported that more than 90% of the published articles they found that used otolith microchemistry were successful in differentiating between geographically separated groups of fish.

As far as the author is aware, the elemental composition of otoliths has not been used previously in stock differentiation studies of marine fish in South Africa. In order for otolith microchemistry to be useful in stock differentiation studies, there needs to be spatial variability in elemental composition among fish from different sites. In this chapter, data on the otolith microchemistry of juvenile sardine collected around the South African coast will be described and used to test the hypothesis of multiple stocks in South Africa. The data will also be used to identify nursery grounds and assess the degree of mixing in the juvenile life stage.

Methods

Sample collection

Juvenile sardine were collected from inshore waters around South Africa from four mid water trawl samples taken during the 2008 pelagic recruit survey (RS) and five from samples taken during the 2009 RS conducted by the Department of Agriculture, Forestry and Fisheries (DAFF; see Barange *et al.* 1999). A tenth sample was collected from Warner Beach in KwaZulu-Natal in March 2009 using a beach seine net (Figure 3.1, Table 3.1).

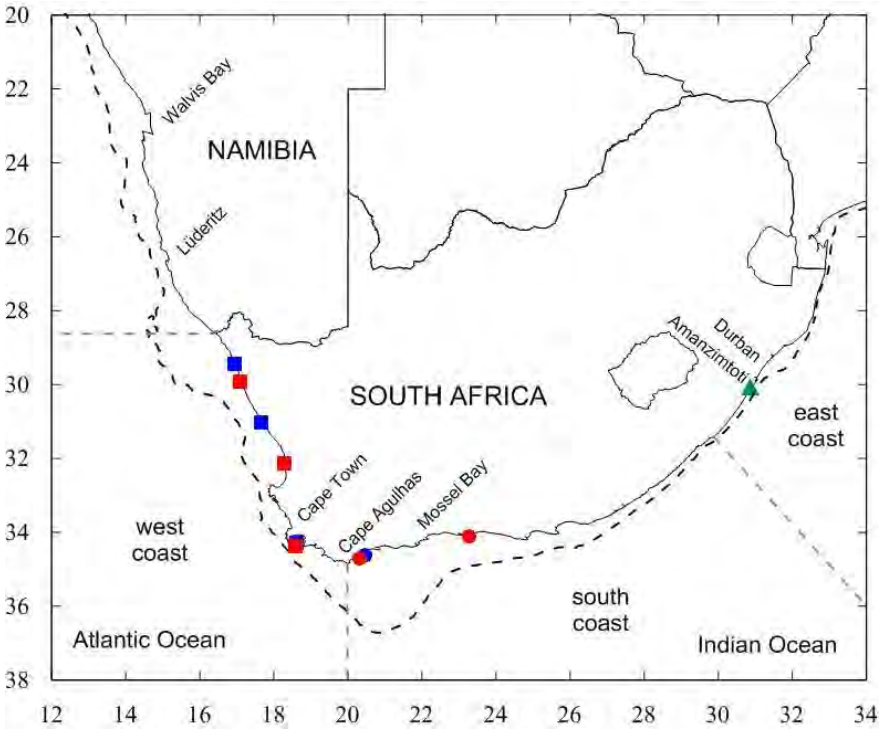


Figure 3.1: Approximate sampling site localities for juvenile sardine used in microchemistry analyses. West coast sites are shown as squares, south coast sites are shown as circles in red (2009) or blue (2008) and the east coast site is shown as a green triangle. The 200m depth contour is shown with the black dashed line.

Table 3.1: Summary of samples of juvenile sardine collected in 2008 and 2009 for use in microchemistry analyses. The summary includes DAFF trawl codes (where applicable), site code (summary of year, coast, sample number), number of individuals collected per trawl (N), average length of fish and standard deviation (CL mm) and the date of sample collection (Date). Sites are labelled in order from west to east within each region. The elemental composition of samples collected in 2009 was taken at both the edge and core of the otolith, and is differentiated with an "E" or "C" in the site code. The sample size for each position is shown.

Trawl code	Site code	N	TL (sd) mm	Date
	2008			
0501A	8EWC1	10	79.23 (3.4)	23-May-08
1301A	8EWC2	5	60.9 (5.1)	29-May-08
3803A	8EWC3	9	80.6 (9.0)	07-June-08
5001A	8ESC1	10	112.8 (8.9)	14-June-08
	2009	**N**		
	edge/core	**edge/core**		
0601A	9EWC1/9CWC1	9/9	69.8 (3.4)	18-May-09
1801A	9EWC2/9CWC2	9/8	109.2 (2.7)	21-May09
3503A	9EWC3/9CWC3	7/7	107.4 (8.8)	29-May-09
5003A	9ESC1/9CSC1	8/8	103.2 (4.8)	04-June-09
6401A	9ESC2/9CSC2	9/9	124.0 (8.7)	09-June-09
Warner Beach	9EEC/9CEC	10/9	143.2 (10.2)	02-March-09

Samples were frozen shortly after capture. In the laboratory, fish were thawed and their total lengths (TL, to the nearest 1mm) recorded. Sampling was restricted to individuals less than 136mm, except for those from Warner Beach, where the maximum limit was 155mm. This size limit aimed to restrict samples to juvenile fish. In each case, both otoliths were removed under a fume hood with ceramic forceps to minimise contamination, dried, and stored in plastic Eppendorf tubes for later analysis.

Otolith microchemistry analysis

The samples were sent to the CHRONOS laboratory in Brest, France, where elemental analysis was conducted on sectioned otoliths (see Labonne *et al*. 2009). Trace elements were analysed using inductively-coupled plasma mass-spectroscopy (ICP-MS). A 60µm spot was analysed on the edge of the otoliths collected in 2008 and 2009, and this was taken to represent the average conditions in which fish were

found in a short period prior to capture. To test the difference between elemental composition immediately prior to capture and that of the natal region of the fish, the 2009 samples were also analysed at a 60µm spot close to the core of the otolith (Figure 3.2). The position of this core measurement differed according to the length of the fish; in fish >110mm (TL) the centre of the otolith was analysed, and in those <110mm (TL) the spot was just off-centre. The core measurement was done to represent conditions in 2008 and the off-centre adjustment in small fish was to ensure elemental conditions for the same period of time were represented, given an approximately linear growth rate of the juvenile fish.

The laser conditions for the analyses were at 5Hz and 5 Joules.cm^{-1}, and a gas blank was run between each otolith sample to monitor for instrument drift. Measurements were attempted for twenty elements but only lithium (^{7}Li), boron (^{11}B), magnesium (^{25}Mg), zinc (^{66}Zn), rubidium (^{85}Rb), strontium (^{88}Sr), barium (^{138}Ba), tin (^{118}Sn) and uranium (^{238}U) were above the detection limits, and these were not uniformly detectable across all sites and in both years. When fewer than 50% of the readings for a particular element were missing for a particular site, the average of the site was used in place of the missing value.

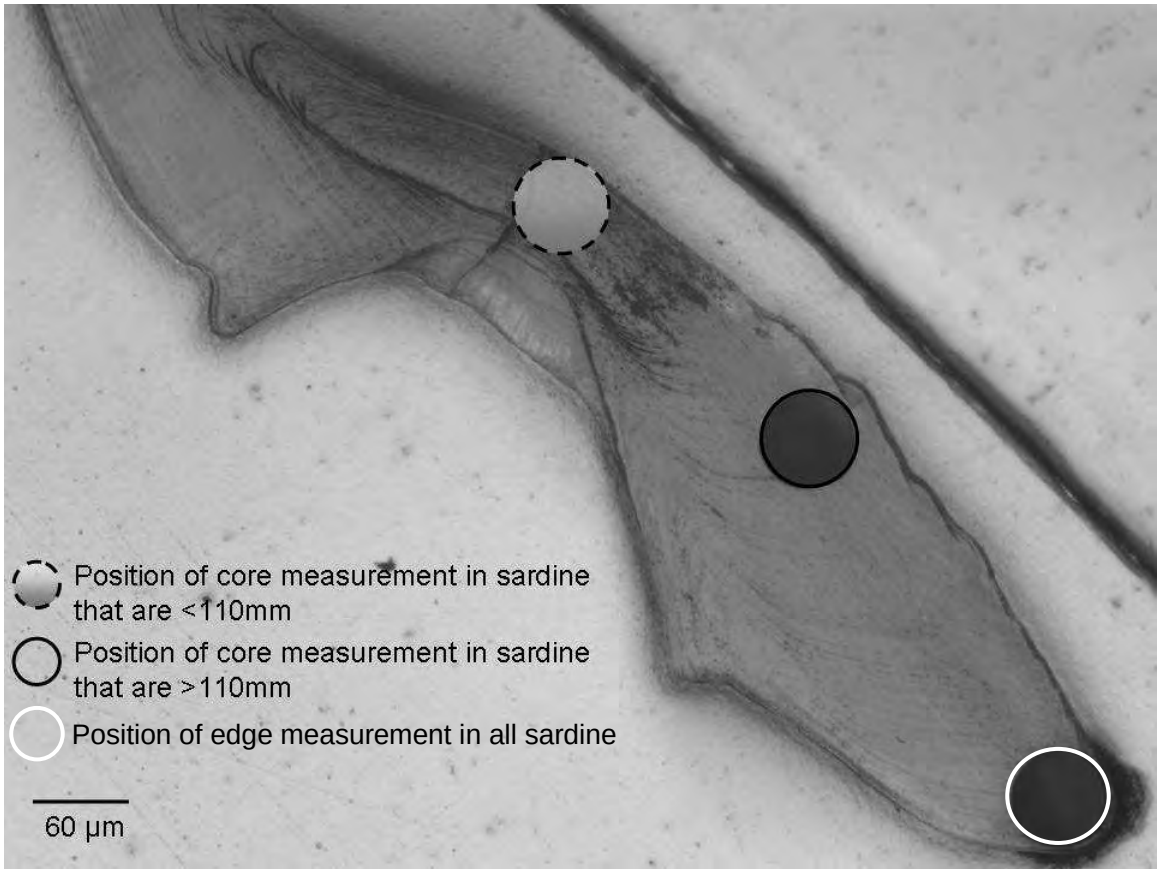


Figure 3.2: Sectioned sardine otolith showing the two 60µm spots at which elemental analysis was conducted in individuals >110mm (solid black outline for core measurement and white outline for edge measurement) and a third hypothetical spot (dashed black outline) at which the core measurement would be taken if this sardine had been <110mm. This otolith was from a 155mm (TL) individual collected off Warner Beach, KwaZulu-Natal in 2009.

Statistical analysis

All measurements were taken in parts per million and standardised by the total of the element for that year. All analyses were conducted on the $\log(x + 1)$ of the standardised values. Similarities in the elemental compositions of the otoliths for each of the three data sets (2008 edge, 2009 edge and 2009 core) were analysed with principal component analysis (PCA), the advantages of which are explained by Agüera and Brophy (2011). The PCA simplifies the analysis, while not excluding variables, by using unconstrained ordination to organise samples on principle component axes, which rank variance in descending order. Issues of colinearity are avoided because components are orthogonal to each other.

Differences in mean concentrations of the elements in the otoliths in each year were tested using MANOVA, with sampling site and length of fish as predictor variables. To determine temporal stability, given by the extent of differentiation between the microchemistry at the point of natal origin and prior to capture, the otolith edge and core measurements of the 2009 samples also were compared using MANOVA.

The Pillai trace test statistic from the MANOVAs was used to test significance; it is robust to violations of homogeneity of covariance and returns an approximate F value (Quinn and Keough 2002). Univariate ANOVAs and *post hoc* Tukey tests were used as procedures to investigate pairwise site differences of individual elements. The chemical elements were ordered by decreasing F value in the final MANOVA (Quinn and Keough 2002). Significance is set at $P < 0.05$ for all tests. Homogeneity of variances and normal distributions of residuals were tested visually with QQ plots, histograms and scatter plots of the residuals. Outliers were excluded once identified by Mahalanobi's Distance tests. All the above statistical analyses were done in R. R code is shown in Appendix 3.1.

Results

The concentrations of various elements in otoliths of juvenile sardine by year, site and location (edge or core) are shown in Figure 3.3. Elements were not at detectable limits in all sites (Table 3.2) and some were measured at much higher concentrations than others. For instance, magnesium and strontium were at higher concentrations than the rest of the elements (Figure 3.3). Only four elements were common to both 2008 and 2009 (edge and core measurements). Sampling site and length of fish were predictor variables in the MANOVA but length of fish was not significant in any tests and was thus excluded from further analyses.

Table 3.2: Summary of elements detected at levels sufficient for analyses in 2008 and 2009 edge of otolith readings and 2009 core of otolith readings. A plus sign indicates that the element was detected at all sites within that year.

Element	2008 Edge	2009 Edge	2009 Core
B	+	+	+
Mg	+	+	+
Zn		+	+
Sn	+		+
Sr	+	+	+
Rb			+
Ba	+	+	+
Li	+		

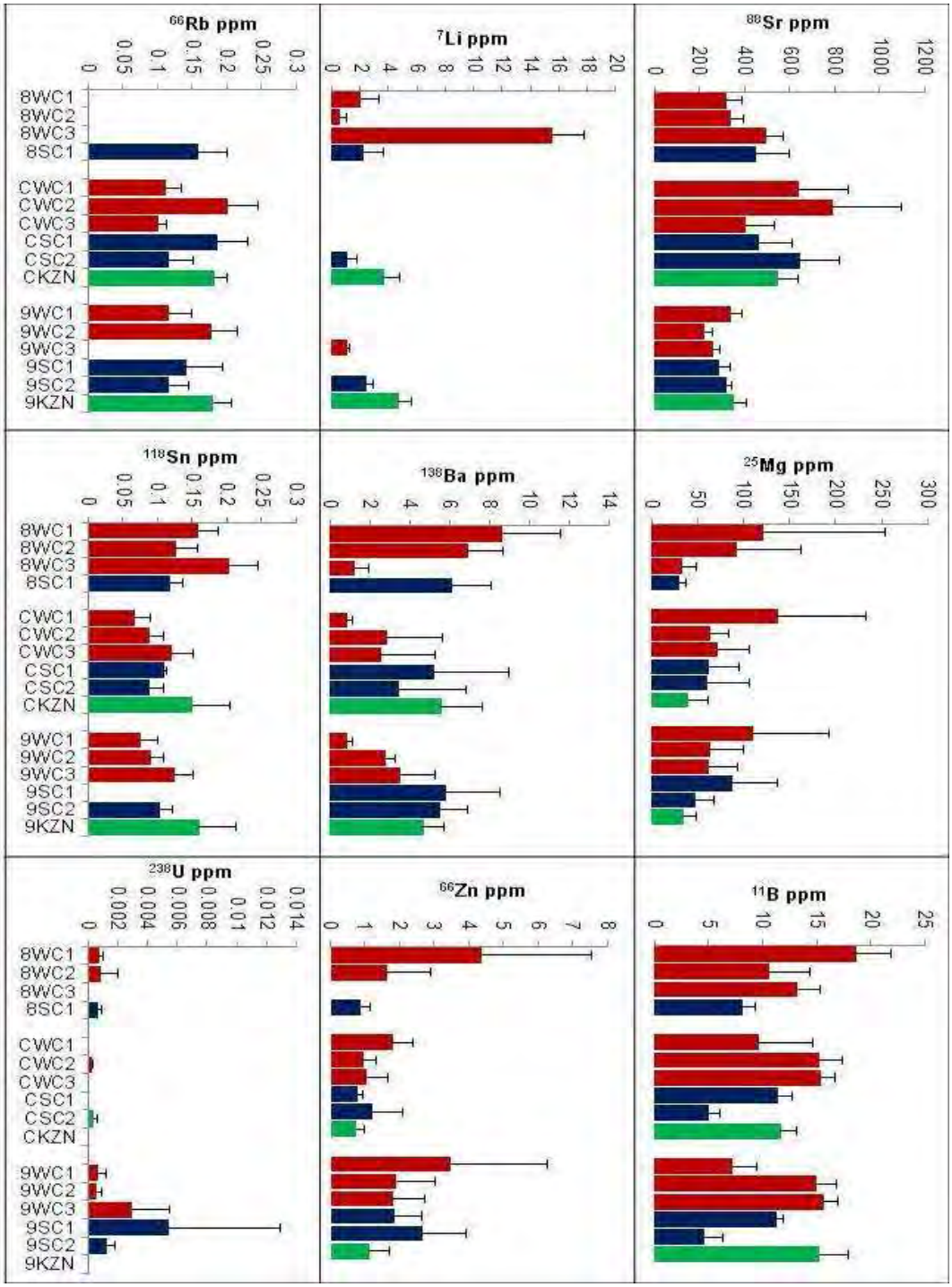


Figure 3.3: Mean (standard deviation) concentrations (ppm) of nine elements that were above detection level, although not always in all sites. The first four bars show edge concentrations from four sites in 2008 (N = 10, 5, 9, 10) followed by core concentrations from six 2009 sites (N = 9, 8, 7, 8, 9, 9) and edge measurements from six 2009 (N = 9, 9, 7, 8, 9, 10) sites. West coast samples are in red, south coast in blue and east coast in green. Note the different scales of the y axes.

2008 Otolith Edge data

The first four principal components explained 95% of the variance and the first two 78% of the variance. Lithium and barium contributed the most to principal component one although in opposite directions Boron and tin contributed most to the second principal component in the same direction. (Figure 3.4). The sampling site 8SC1 separated from the west coast sites along the first axis, and 8WC3 separates along the second axis. One of the five samples from site 8WC2 groups with those from 8WC1 (Figure 3.4). There is some differentiation between west and south coast sites evident from the second principal component.

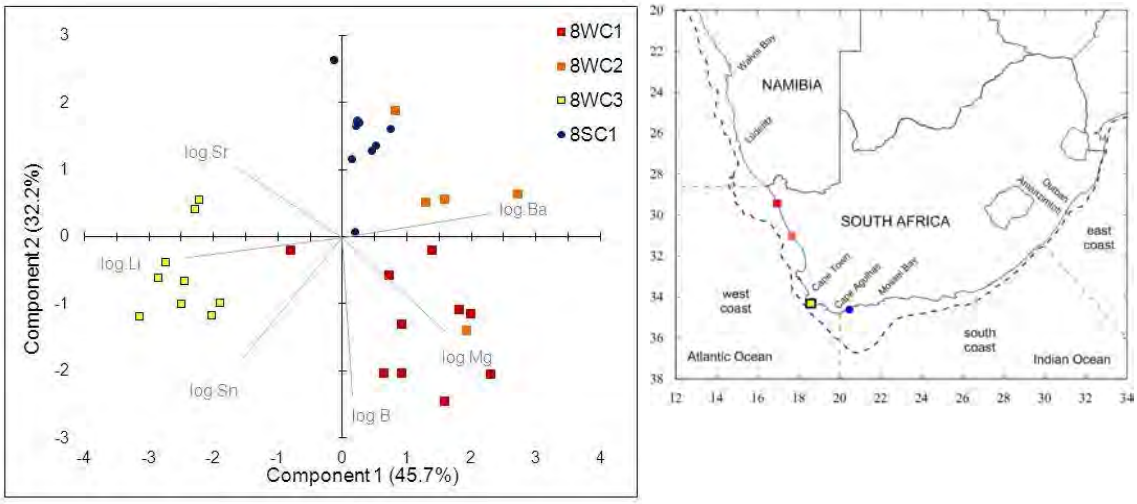


Figure 3.4: a) Component 1 and 2 of the PCA of chemical elements Li, B, Mg, Sr, Ba and Sn for four sites from 2008 otolith edge samples from the west coast (8WC, red, orange and yellow squares) and south coast (8SC, navy circles). Grey lines represent the direction of the component loadings for each log transformed element. A map with sampling sites is shown alongside.

The MANOVA was significant (Pillai's test = 2.089, F = 10.318, df_1 = 18, df_2 = 81, P< 0.05) and sites varied significantly in concentrations of all the elements from the 2008 otoliths. Lithium and barium contributed most to the MANOVA (Table 3.3). Mean lithium concentrations were significantly different among all but one pair of sites: 8WC1 and 8SC1 (Figure 3.5). The individual elements do not provide much clarity on which sites differ because elements showed different patterns of differentiation (Figure 3.5). However, concentrations of Mg, Ba and B appear to decline and Sr increase from the west to south coast. The site 8WC3 differs from the other sites in Li and Ba concentrations (Figure 3.5) and was the only 2008 edge site to

have insufficient information of Zn and U concentrations to be included in the analyses (Figure 3.3).

Table 3.3: MANOVA results for 2008 edge data, degrees of freedom (df), sums of squares (SS), F and P values are shown for each of the elements.

Element		**df**	**SS**	**F**	**P**
^{7}Li	Site	3	8.175	66.626	P < 0.05
	Residuals	30	1.227		
^{138}Ba	Site	3	4.121	62.166	P < 0.05
	Residuals	30	0.663		
^{11}B	Site	3	0.674	38.317	P < 0.05
	Residuals	30	0.176		
^{118}Sn	Site	3	0.286	14.612	P < 0.05
	Residuals	30	0.196		
^{88}Sr	Site	3	0.216	8.679	0.0003
	Residuals	30	0.249		
^{25}Mg	Site	3	1.346	5.227	0.005
	Residuals	30	2.575		

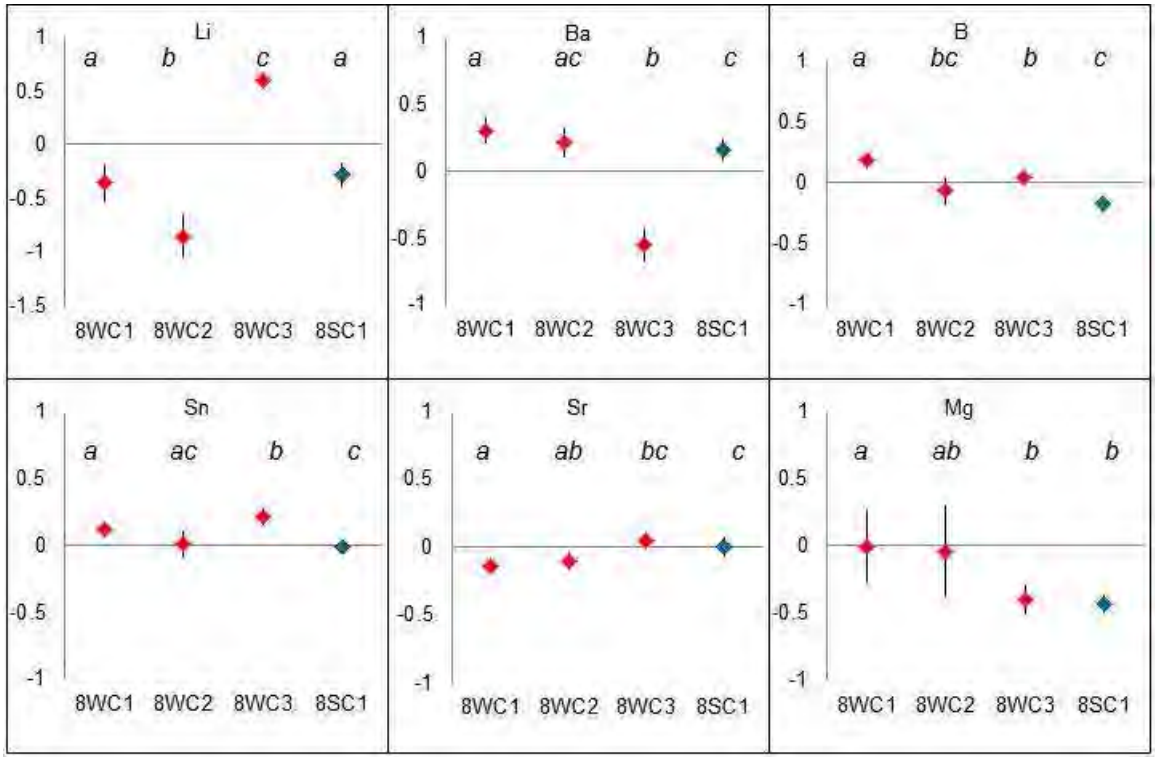
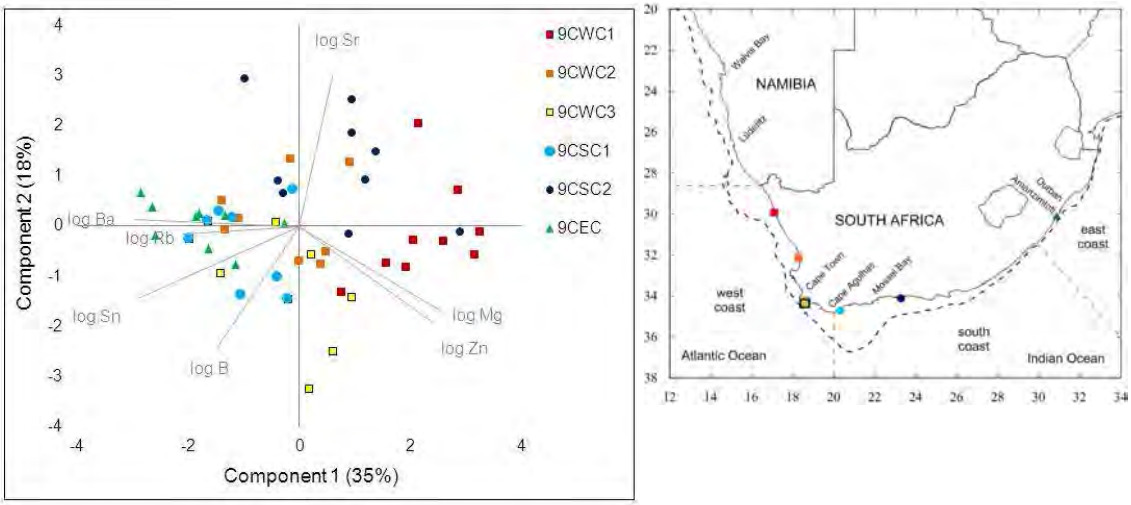


Figure 3.5: Log of standardised mean and confidence intervals of each element for sardine otoliths in each sampling site included in the 2008 MANOVA. *Post hoc* pairwise comparisons between sites for each element are represented with letters. When sites share a letter, there is no difference between them. Sites marked with an asterisk (*) could not be linked unambiguously to only one group. Sites are ordered from west to east coast.

2009 Otolith Core data

There were seven elements available for analyses from the core of the otoliths collected in 2009. The first four components of the PCA explained 82% of the variability in the data, and the first two components 54%. The first component loadings were relatively evenly spread across the elements, although strontium was not represented in the first component, but contributed most to the second component in which rubidium and barium were not represented (Figure 3.6). The sites 9CWC1 and 9CEC separated along the first axis, and 9CWC2 along the second axis.

a)



b)

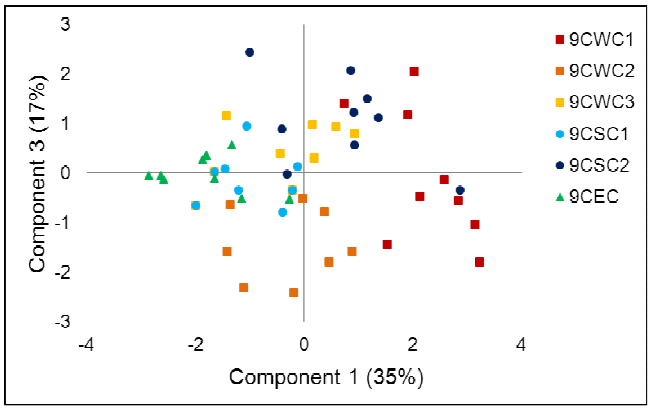


Figure 3.6: Components a) 1 and 2 and b) 1 and 3 of the PCA of chemical elements Li, Mg, Sr, Ba, Rb, Sn and Zn for six sites from 2009 otolith core samples from the west coast (9CWC, red, orange and yellow squares), south coast (CSC, turquoise and navy circles) and east coast (green 9CEC, triangle). Grey lines represent the direction of the component loadings for each log transformed element. A map with sampling sites is shown alongside.

As with the 2008 edge measurements, the MANOVA was significant (Pillai's test = 2.365, F = 5.131, df_1 = 35, df_2 = 200, P< 0.05) and sites were significantly different for all elements. Boron contributed most to the MANOVA, although all elements contributed significantly (Table 3.4). There is a decline in magnesium from the west to the south coast (which was also seen in the 2008 edge) and an increase in barium from the west to the south coast (Figure 3.7).

Table 3.4: MANOVA results for 2009 core measurements, degrees of freedom (df), sum of squares (SS), F and P values are shown for each of the elements

Element		**df**	**SS**	**F**	**P**
^{11}B	Site	5	1.459	63.620	P < 0.05
	Residuals	42	0.193		
^{85}Rb	Site	5	0.707	17.246	P < 0.05
	Residuals	42	0.344		
^{118}Sn	Site	5	0.585	8.237	P < 0.05
	Residuals	42	0.597		
^{138}Ba	Site	5	3.364	4.927	0.001
	Residuals	42	5.735		
^{88}Sr	Site	5	0.443	4.232	0.003
	Residuals	42	0.879		
^{25}Mg	Site	5	1.028	3.760	0.007
	Residuals	42	2.297		
^{66}Zn	Site	5	0.751	3.357	0.012
	Residuals	42	1.879		

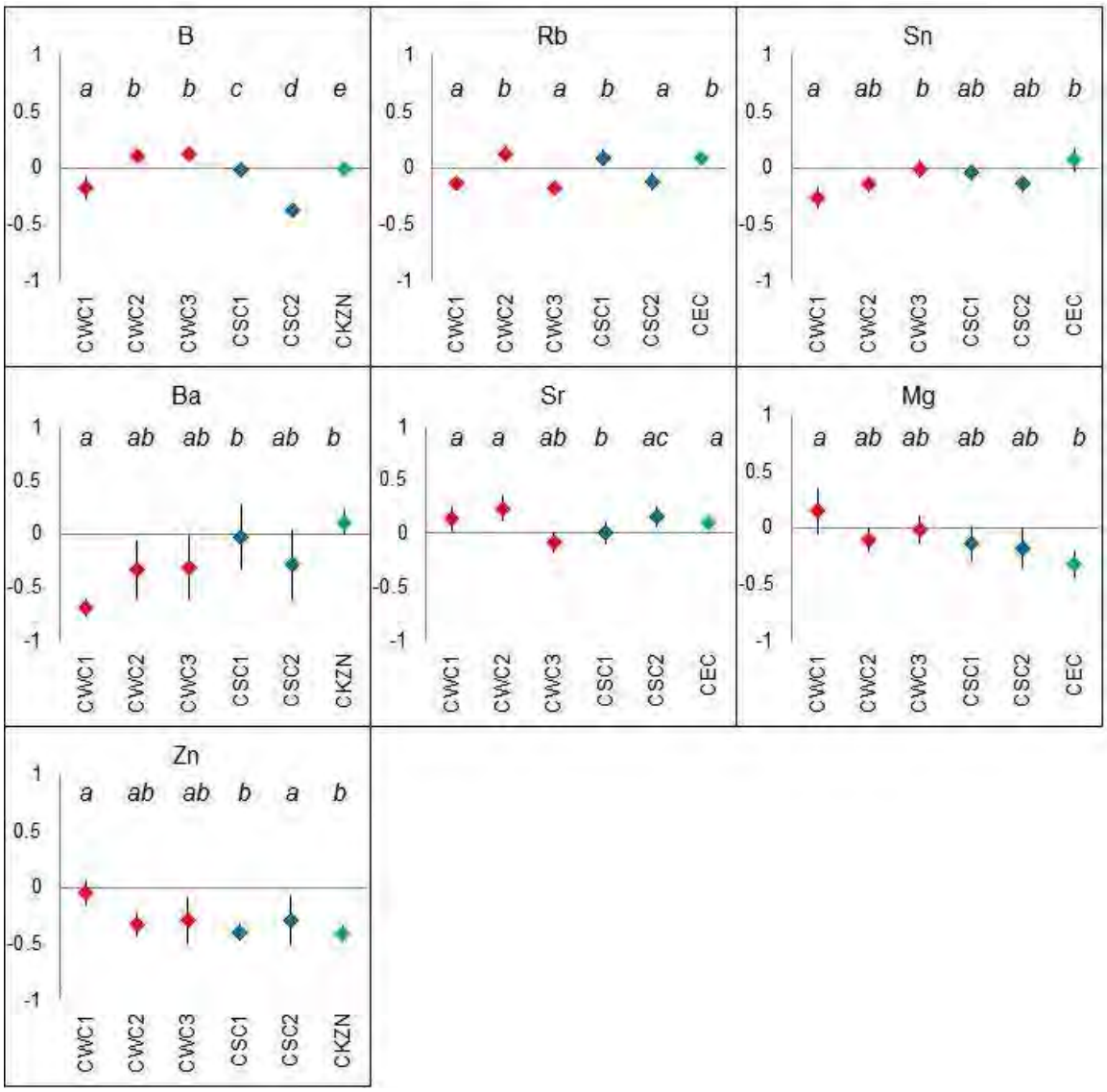


Figure 3.7: Log standardised mean and standard deviations for each sampling site of each element included in the 2009 core MANOVA. *Post hoc* pairwise comparisons between sites for each element are represented with letters. When sites share a letter, there is no difference between them. Sites marked with an asterisk (*) could not be linked unambiguously to only one group. Sites are ordered from west to east coast.

2009 Otolith Edge data

The first four principal components explained 93% of the variance and the first two 64% of the variance (Figure 3.8). The first principal component loadings were relatively evenly spread across four of the elements; magnesium and zinc contributed the most to principal component one (both positively), and barium and boron contributed to the first component in a negative direction. Strontium contributed most

to the second principal component. The sampling site 9EWC1 separates along the first axis and 9EEC and 9SC2 separate along the second axis (Figure 3.8).

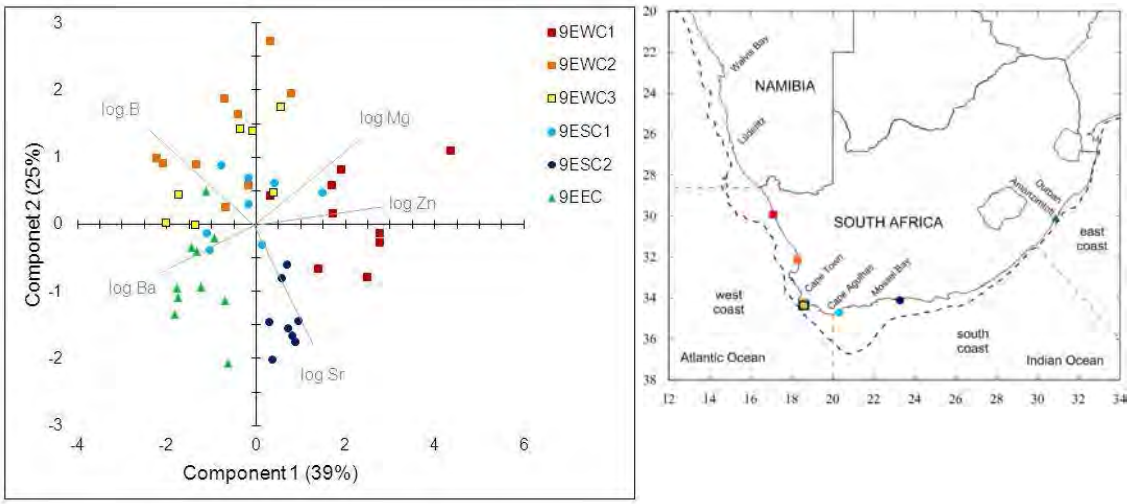


Figure 3.8: Axis 1 and 2 of the PCA of chemical elements Li, Mg, Sr, Ba and Zn for six sites from 2009 otolith edge samples from the west coast (9EWC, red, orange and yellow squares) and south coast (9ESC, turquoise and navy circles) and east coast (9EEC, green triangle). Grey lines represent the direction of the component loadings for each log transformed element. A map with sampling sites is shown alongside.

The MANOVA was significant (Pillai's test = 2.517, F = 9.326, df_1 = 25, df_2 = 230, P< 0.05) and sites varied significantly in all of the elements from the 2009 otolith edges. Boron and barium contributed most to the MANOVA (Table 3.5). Once again, individual elements did not show the same patterns for how sites differed from each other, although Mg concentrations seem to decline from west to south while Ba concentrations appear to increase (Figure 3.3).

Table 3.5: MANOVA results for 2009 edge data, degrees of freedom (df), sums of squares (SS), F statistic and P values are shown for each of the elements

Element		**df**	**SS**	**F**	**P**
^{11}B	Site	5	2.188	59.341	P < 0.05
	Residuals	46	0.339		
^{138}Ba	Site	5	4.197	37.593	P < 0.05
	Residuals	46	1.027		
^{88}Sr	Site	5	0.284	17.435	P < 0.05
	Residuals	46	0.150		
^{25}Mg	Site	5	1.235	4.820	0.001
	Residuals	46	2.357		
^{66}Zn	Site	5	1.193	4.657	0.002
	Residuals	46	2.357		

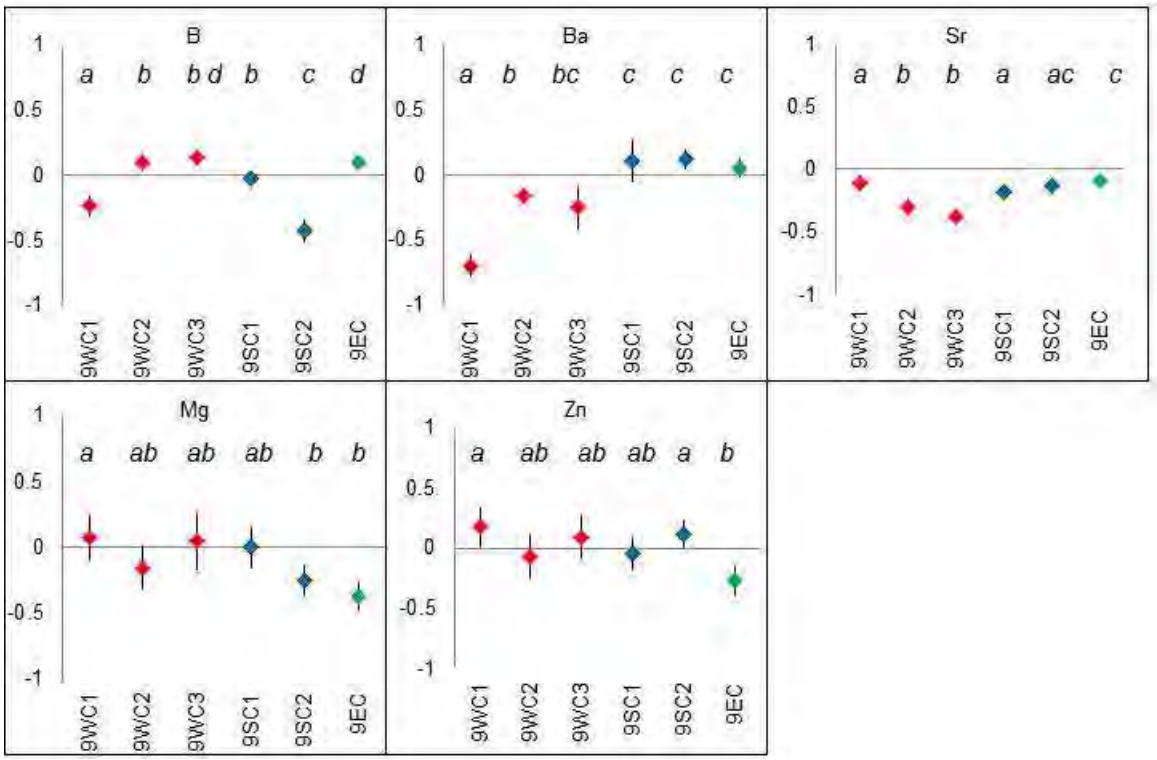


Figure 3.9: Log standardised mean and standard deviations for each sampling site of each element included in the 2009 MANOVA. *Post hoc* pairwise comparisons between sites for each element are represented with letters. When sites share a letter, there is no difference between them. Sites marked with an asterisk (*) could not be linked unambiguously to only one group. Sites are ordered from west to east coast.

Comparing 2009 core and edge samples

The elements B, Mg, Ba, Zn and Sr were common to both core and edge measurements from the otoliths sampled in 2009. The PCA incorporating these elements and all 2009 samples explained 94% of the variance in the first four components and 64% in the first two components. The elements zinc and magnesium contributed most to the first principal component. The second principle component was explained predominately by boron and barium. The edge and core measurements from WC1 clustered together, as did the EC measurements.

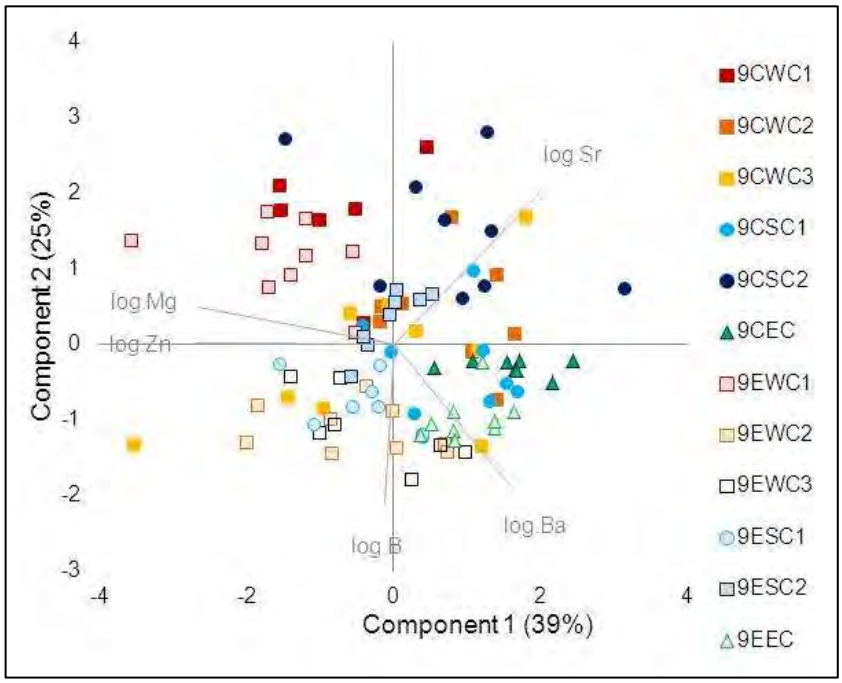


Figure 3.10: PCA component analysis of elements B, Mg, Ba, Zn and Sr in otoliths of all individuals sampled in 2009 from the core measurements (solid colour) and edge measurements (light shaded shapes) from the west coast (red, orange and yellow squares), south coast (turquoise and navy circles) and east coast (green triangle). Grey lines represent the direction of the component loadings for each log transformed element.

In the MANOVA comparing otolith elemental chemistry from all 2009 otoliths using sites and location on otolith as grouping variables, the Pillai test statistic was significant for site and edge/core. Similarly, all five univariate tests were significant for sites ($P < 0.05$) but there was no significant difference between the edge and core measurements of B and Mg (Table 3.6).

Table 3.6: MANOVA results for five elements from otoliths collected in 2009 core and edge measurements. The Pillai statistic, degrees of freedom (df), approximate F statistic (F) and P value are shown for site and edge or core of the otolith. Univariate ANOVA results including degrees of freedom (df), sum of squares (SS), F and P values are shown for each of the four elements common to all samples.

MANOVA		**df**	**Pillai**	**F**	**P**
	Site	5	51.56	8.94	P < 0.05
	Edge/Core	1	0.55	23.37	P < 0.05
	Residuals	99			
ANOVA		**df**	**SS**	**F**	**P**
^{138}Ba	Site	5	7.326	17.546	P < 0.05
	Core/Edge	1	0.500	5.989	P = 0.016
	Residuals	99	8.267		
^{66}Zn	Site	5	1.746	6.358	P < 0.05
	Core/Edge	1	2.258	41.123	P < 0.05
	Residuals	99	5.436		
^{11}B	Site	5	3.684	85.418	P < 0.05
	Core/Edge	1	0.001	0.057	P = 0.812
	Residuals	99	0.854		
^{25}Mg	Site	5	2.606	7.514	P < 0.05
	Core/Edge	1	0.004	0.054	P = 0.816
	Residuals	99	6.866		
^{88}Sr	Site	5	0.734	4.991	P < 0.05
	Core/Edge	1	2.255	76.135	P < 0.05
	Residuals	99	2.932		

Discussion

The South African coastline is subject to constant flux and both temporal and spatial variability. This manifests in small scale variability embedded in large regional differences. This environmental instability means that species that have extensive distributions in coastal waters, such as small pelagic fish, experience a range of environmental conditions across their habitat, which could necessitate local adaptation to certain environmental conditions. The elemental signatures of otoliths provide a permanent record of the environmental conditions an individual fish has experienced. By comparing groups of individuals in different geographical regions, it can be seen

whether the groups of fish have experienced different environmental conditions (Campana 1999).

In this study, the elemental chemistry results showed evidence of small scale differences among sites (particularly in samples collected in 2008), and some large regional patterns of differentiation between the west and south coasts. In particular, certain elements appear to increase (Ba, Sn) or decrease (Mg) from west to south coast. Multivariate statistics that incorporate all elements were more useful at showing differences between sites than individual elements, which showed conflicting patterns of differentiation (Campana 2005). Boron was the element that contributed most to the MANOVA results from the 2009 edge and core measurements, and the third most important (after lithium and barium) contributing to the 2008 MANOVA results, the pairwise comparisons of which showed the most conclusive differentiation among sites. Thus, certain elements seem to consistently influence the MANOVA results more than other elements.

The elemental chemistry of otoliths is determined not only by the ambient environmental conditions. Fish growth, stress and reproductive state, as well as the water temperature and salinity, can all influence the elemental composition of otoliths (Milton and Chenery 1998). Further, Castro (2007) found that there was less discriminatory power in large sardine (*Sardina pilchardus*) along the Atlantic Coast of the Iberian Peninsula, which they hypothesised was a result of the increased mobility in larger fish. With this in mind, life history stage was controlled for in this study by using juvenile fish. No relationship was found between any of the elements and length of fish.

Brophy *et al.* (2004) and Correia *et al.* (2011) found that there are usually higher concentrations of magnesium at the core of the otolith, suggesting that there is an embryonic effect on the magnesium: calcium ratio. In contrast, Tanner *et al.* (2012) found that barium: calcium ratios were higher at the core of hake (*Merluccius merluccius*) otoliths compared to the edge of the otolith. The ontogenetic effects of the incorporation of elements into the otolith is likely to vary among different species (Chang and Geffen 2013). In this study, zinc and strontium were found to be

significantly different between the core and edge. Walther *et al*. (2010) found that strontium and barium differed according to life stage of damselfish (*Acanthochromis polycanthus*). The elemental uptake in otoliths differs among species, and is not fully understood in all species and thus trends need to be taken on a species by species basis (Chang and Geffen 2013). Strontium concentrations have been shown to be correlated with salinity of the water; in more freshwater environments, strontium tends to be found in lower concentrations within the otoliths (Campana 1999). In the present study, the strontium and zinc concentrations were significantly higher in the core of the otolith compared to the edge of the otolith, suggesting either a change in conditions between spawning sites and conditions experienced by the fish a year later, or a physiological difference in the uptake of Sr as the fish grow. There were some sites (WC1 and EC) for which the edge and core measurements of 2009 otoliths grouped together in the PCA, suggesting that the fish have stayed in similar environmental conditions for the past year. Both of the sites were at the geographic extreme of the samples collected. It is possible that the gradient in change of environmental conditions from west to south coast is most noticeable towards the edge of the species distribution.

The pattern found in this study, of small scale differences in one year, but not the next, is similar to what was found in European anchovy, in the Ligurian Sea, where it was suggested that elemental differentiation occurred in some cohorts but not others (Guidetti *et al.* 2013). D'Avignon and Rose (2013) suggest that, where signatures differ among years, a long term study is required to determine temporal stability and, thus, whether the elemental signatures can be used as natural tags of stock differentiation.

Inter-annual variability in elemental composition could represent differences in environmental conditions experienced by different cohorts at the time of spawning, as hypothesised for reef fish (Cook 2011). It is unlikely that, in a dynamic environment such as the southern Benguela, elemental condition will be stable over long periods of time. The temporal instability and small scale variability found in the element signatures of this study suggest that local retention of sardine in areas in some years,

but not others, could be responsible for the elemental signatures found in South African sardine.

Chapter 4

*Mitochondrial and microsatellite loci indicate high diversity with low geographic structure in southern African sardine (***Sardinops sagax***)*

"…and everything revolves around the humble sardine."
David Attenborough in the BBC's Nature's Great Events

Chapter 4: Mitochondrial and microsatellite loci indicate high diversity with low geographic structure in southern African sardine (*Sardinops sagax*)

Abstract

The South African operational management procedure for sardine (*Sardinops sagax*) is currently being updated to incorporate the hypothesised existence of two discrete stocks, namely putative "western" and "southern" stocks. Evidence for the existence of phenotypically-distinct stocks has been collected, but whether the putative stocks are genetically distinct has yet to be determined. In this study mitochondrial ND2 and seven microsatellite DNA loci were used to test the hypothesis of there being more than one southern African sardine stock, by investigating geographic structuring at the genetic level. Mitochondrial diversity from 33 individuals collected in 2007 and 53 collected in 2009 showed high levels of haplotype diversity (h = 0923) and a star-like haplotype network with few shared alleles. No west-south geographic structuring was found, but sample sizes were small considering the level of genetic heterogeneity. All seven microsatellite loci showed high levels of individual variability in the 34, 85 and 176 individuals collected in 2007, 2008 and 2009, respectively. An Analysis of Molecular Variance showed that the majority of the genetic variance was within individuals and sites. In the 2008 sample, weak differentiation between sites near Algoa Bay and those on the rest of the coast were observed, but this was not apparent in subsequent years and there was no west-south genetic differentiation. These results do not indicate the occurrence of genotypically-differentiated stocks, but support a sweepstake hypothesis, where a small number of adult individuals can be disproportionately responsible for successful recruitment in subsequent years. This results in a pattern of small patches of genetically differentiated groups, which are not necessarily temporally stable, in an otherwise genetically well-mixed population.

Introduction

Traditionally, it was assumed that the pelagic zone was effectively without barriers to fish dispersal (Waples 1998, Hauser and Carvalho 2008). However, Pawson and Jennings (1996) have found that random mating throughout the geographic range (i.e. panmixia) is rare in marine fish, and subsequent studies have found distinct geographic population structure in marine animals. These include the Australian bottlenose dolphin (*Tursiops aduncus*, Ansmann *et al.* 2012), the North Atlantic redfish (*Sebastes mentella*, Cadrin *et al.* 2010), the Russian chum salmon (*Oncorhynchus keta*, Afanas'ev *et al*. 2011) and even a broadcast spawning Antarctic limpet (*Nacella concinna*, Hoffman *et al.* 2012). Subtle environmental limitations to species distribution exist, despite the lack of the obvious physical barriers inherent in freshwater and terrestrial environments. Persistent oceanographic features, such as temperature-, depth- or salinity- gradients or powerful currents, have the potential to limit gene flow (Hauser and Carvalho 2008), with larvae and eggs being particularly sensitive to changes in conditions and oceanographic features (Hauser and Ward 1998).

The southern African coastal waters are influenced by two major oceanographic features: the Benguela Current on the west coast and the Agulhas Current on the east coast (Hutchings *et al.* 2002). The Benguela Current is a typical eastern boundary upwelling system in that strong offshore winds result in coastal upwelling and high productivity (Fréon *et al.* 2009). The Agulhas Current flows from Mozambique towards the Agulhas Bank, in the south, where it retroflects, losing velocity and warmth (Roberts *et al.* 2010) (Figure 1.1). Various smaller oceanographic features in the region could be of importance, particularly for passive egg and larval dispersal of small pelagic species. For example, the presence of a jet current in the region has been demonstrated to passively transport egg and larval stages from the Agulhas Bank towards the west coast, where some are lost offshore due to Ekman drift (Hutchings *et al.* 2009, Figure 1.3).

Sardinops sagax in southern Africa occurs in coastal waters from southern Angola to the south coast of South Africa, and extends onto the east coast during the annual migration known as the sardine run (Kreiner *et al.* 2001, van der Lingen *et al.* 2006b, 2010a). In South Africa, there appears to be a consistent break in distribution of sardine on the central Agulhas Bank, particularly during periods of low overall biomass (Coetzee *et al.* 2008). This led to the hypothesis of separate western and southern stocks or "Functionally Distinct Adult Assemblages" (FDAAs; Gaughan *et al.* 2002), and the initiation of a multidisciplinary investigation into multiple stocks in South Africa.

It is important to reliably define stocks, and to use these stocks in formulating management policy (Waldman 2005). Hauser and Ward (1998) state that it is unlikely that locally depleted stocks will be replenished by migration from other geographic stocks. Therefore each stock needs to be managed sustainably. Genetic resilience is also an important buffer for species to be able to respond to environmental change (Hillborn *et al.* 2003).

Small pelagic fish tend to have large populations and the capacity to disperse across large distances through active swimming as adults, and by passive movement as eggs and larvae (Hauser and Carvalho 2008). These factors have contributed to the perception that pelagic fish species were unlikely to have any geographically defined population structuring (Hauser and Ward 1998). Despite this, there have been investigations into stock divisions of *S. sagax* in various eastern boundary systems around the world. Stock structure in the Pacific *S. sagax* has been repeatedly investigated (e.g. Hedgecock *et al.* 1989, Lecomte *et al.* 2004), and a recent study (Garcia-Rodriguez *et al.* 2011) found three morphotypes on the Baja-California Peninsula with weak phylogeographic structure among the three. Previous studies could have failed to detect genetic structure in *S. sagax* because the genetic markers used were inappropriate for the time scales at which differentiation had occurred (e.g. cytochrome b in Lecomte *et al.* 2004). Similarly, stock division has been suggested between *Sardina pilchardus* in the Mediterranean and Atlantic (Atarhouch *et al.* 2006), but not between the Adriatic and Ionian Sea (Tinti *et al.* 2002). The lack of an

overall trend in the stock integrity of sardine supports the need for regional studies of stock structure.

The use of molecular markers to increase our understanding of genetic, temporal and spatial differentiation in marine fish is well established (Hauser and Ward 1998, Hauser and Carvalho 2008). Increasingly sensitive markers (such as microsatellite markers) are making it possible to discern even limited population structure that could be of biological importance and that is up to date enough to be used for stock management purposes (e.g. Tzeng *et al.* 2009, Zarraonaindia *et al.* 2009). Microsatellites are generally selectively neutral, co-dominant, short tandem repeat units that are scattered throughout the genome (Jarne and Lagoda 1996). Variation in the number of repeat units results in alleles with varying sizes, which are, therefore, distinguishable from each other (DeWoody and Avise 2000). The mutation rates of microsatellites vary from 10^{-4} to 10^{-5} mutations per generation (Baldwin *et al.* 2012).

In a review of the use of microsatellites and their applications in fish genetics, Chistiakov *et al.* (2006) highlighted their benefits in revealing population structure over a limited geographic range. Even evidence of shallow genetic divergence is useful in a fisheries context and, if consistent over time, it is a reliable signal of population differentiation (Hauser and Ward 1998). Microsatellites are useful in detecting population dynamics, such as effective population size, recent population bottlenecks, population size fluctuations and, in some instances, the direction of gene flow (Chistiakov *et al.* 2006, Hauser and Ward 1998).

Previous molecular studies used allozymes and mitochondrial DNA markers, such as the cytochrome b gene and the control region, to determine the relationship between southern African *S. sagax* and populations in the rest of the world, as well as to investigate regional population structure (Grant 1985a, Whitehead and Wongratana 1986, Grant and Leslie 1996, Bowen and Grant 1997, Grant and Bowen 1998). Grant and Bowen (1998) found that sardine populations from Australia, South Africa, Japan, Chile and California had most recently shared a common ancestor approximately 200 000 years ago. There is also evidence of shared haplotypes between South African and Australian populations (Grant and Bowen 1998). A star-like haplotype network,

with a few prevalent haplotypes from which many rare, single-step mutation haplotypes expanded, has been reported repeatedly in sardine (Bowen and Grant 1997, Grant and Bowen 1998). These authors suggested three factors that might have been responsible for the shallow genetic structure having occurred on different time scales: sweepstakes recruitment occurring between generations; repeated population crashes and recolonization events occurring at decadal scales; and regional extinctions and recolonizations over thousands of years (Grant and Bowen 1998).

This chapter revisits the population genetic structure of the southern African sardine using a novel mitochondrial DNA marker (NADH dehydrogenase subunit 2 - ND2) and seven microsatellite markers. On one hand, the ND2 gene is less variable than the mtDNA control region, thereby limiting the variability that might hide any geographic patterns that could exist among sardine in southern Africa. On the other hand, it is also more variable than cytochrome b in a number of South African marine fish species investigated to date (P. Bloomer, University of Pretoria, *pers. comm*.), potentially allowing the detection of subtle genetic differences among the different geographic regions of southern Africa. Samples collected yearly from 2007 to 2009 were used to investigate whether there was temporally stable genetic differentiation between sardine from the putative western and southern stocks. Extended sampling in 2009 was used to investigate whether the persistent upwelling cell near Lüderitz provides a barrier to genetic mixing between South African and Namibian sardine stocks, as it does with deepwater hake (*Merluccius paradoxus)*, geelbek (*Atractoscion aequidens*) and leervis (*Lichia amia*), another commercially important species (Henriques *et al.* 2012, 2014; von der Heyden *et al.* 2007). The extended sampling of 2009 also allowed the investigation of whether individuals from the sardine run, off the east coast, form part of a genetically distinct group.

Methods

Sample collection

Sardine were collected in three consecutive years (2007 to 2009) on board the *RV Africana,* in pelagic trawls during the annual Pelagic Spawner Biomass (PSB) surveys of the Department of Agriculture, Forestry and Fisheries (DAFF). These surveys took place from the end of October until the beginning of December each year. Samples from the sardine run were collected off the coast of Amanzimtoti in KwaZulu-Natal by Marc Hendricks of DAFF. Namibian sardine were collected by the Namibian Ministry of Fisheries and Marine Resources in 2009 (N. Morhoff, Namibian Ministry of Fisheries and Marine Resources, *pers. comm.*). Additional sardine were collected in the Lüderitz lagoon using gill nets (J-P Roux, Namibian Ministry of Fisheries and Marine Resources). In all instances, whole fish were collected, frozen and dissected at a later stage to determine sex. A summary of the samples is available in Table 4.1, with the corresponding geographic locations shown in Figure 4.1. Samples were limited to adult fish >17cm (CL) that were reproductively mature, based on gonad developmental phase (van der Lingen *et al.* 2006b). Geographically proximal sites were grouped together for analyses.

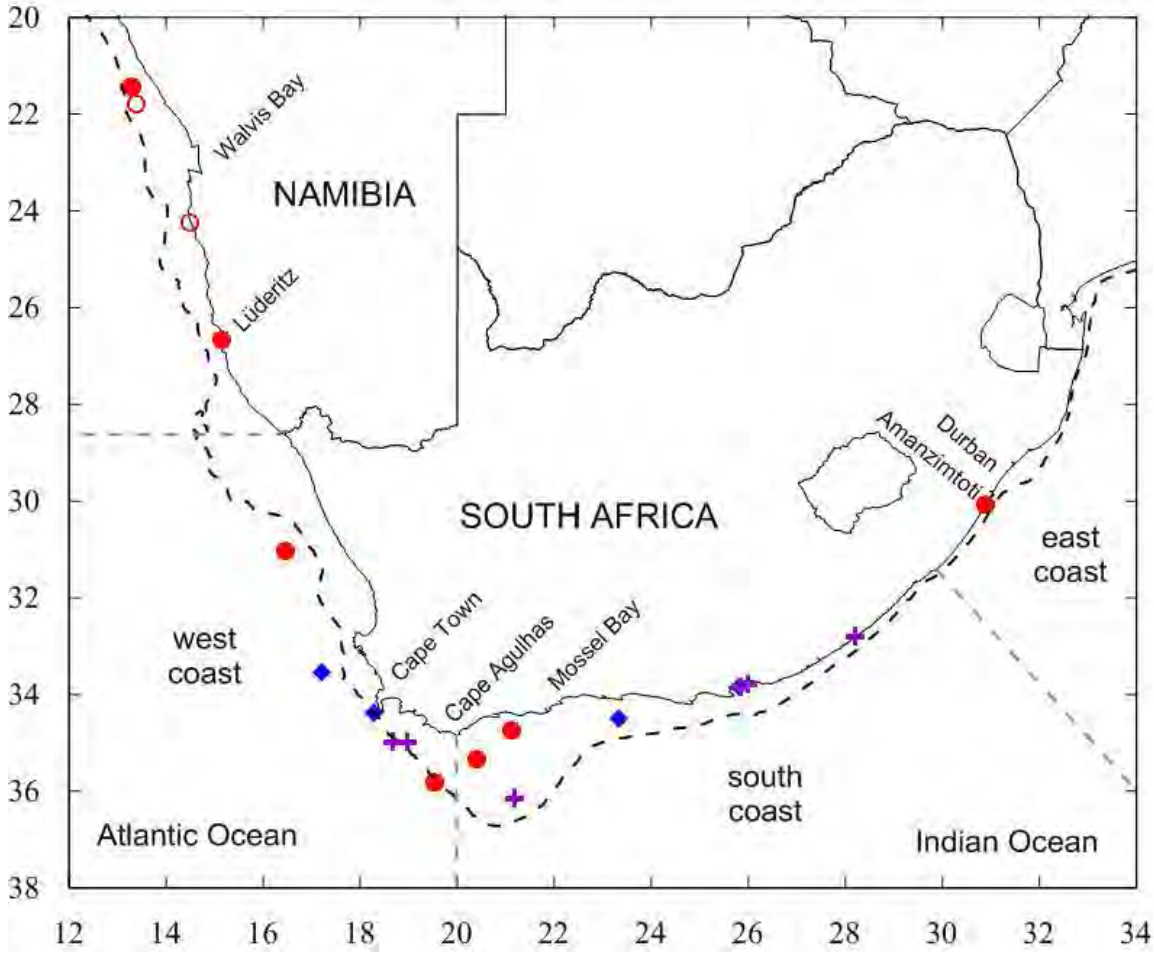


Figure 4.1: Sampling localities for sardine in 2007 (blue diamonds), 2008 (purple crosses, microsatellites only) and 2009 (red circles, unfilled circles represent microsatellite analysis only, whereas filled circles represent mtDNA and microsatellite analysis). Sites are labelled in ascending order from west to east in Table 4.1. Divisions between Namibia and South Africa's west coast, south coast and east coast are shown with grey dashed lines (regional divisions are defined by South Africa's DAFF). The 200m depth contour is shown with the black dashed line.

Table 4.1: Sardine samples collected during 2007, 2008 and 2009. The summary includes DAFF trawl codes (where applicable), site code (summary of year, coast, sample number), number of individuals collected per trawl used in mtDNA analysis (N mt) and microsatellite analysis (N SSR), longitude, latitude, and the date of sample collection. Sites are labelled in ascending order from west to east within each region. *Bracketed samples for these trawls were combined for all analyses.

Site	Site code	N mt	N SSR	Latitude	Longitude	Date of collection
	2007	**33**	**38**			
1209A	7WC1	7	6	33º31.99' S	17º13.05' E	02-Nov-07
1701A	7WC2	6	10	34º23.63' S	18º17.20' E	04-Nov-07
4405A	7SC1	7	16	34º29.68' S	23º19.45' E	28-Nov-07
*5303A	7SC2	9	6	33º49.85' S	25º49.56' E	02-Dec-07
*5901A	7SC3	4		33º29.76' S	27º12.38' E	03-Dec-07
	2008		**85**			
2409A	8WC1		16	34º 58.84' S	18º 41.09' E	22-Oct-08
2507A	8WC2		16	34º 57.87' S	18º 57.47' E	23-Oct-08
3521A	8SC1		12	36º 09.50' S	21º 10.94' E	01-Nov-08
5403A	8SC2		14	33º 49.76' S	25º 49.99' E	18-Nov-08
5501A	8SC3		11	33º 47.28' S	26º 00.10' E	18-Nov-08
6202A	8SC4		16	31º 42.40' S	29º 30.15' E	22-Nov-08
	2009	**53**	**176**			
*Nam	9NAM1	0	34	21º 26.00' S	13º 18.00' E	10-Oct-09
*Nam 1	9NAM1	9		24º 14.00' S	14º 29.00' E	22-Feb-09
*Nam 13	9NAM1	0		21º 46.82' S	13º 22.32' E	10-Nov-09
0209A	9WC1	6	28	31º 01.25' S	16º 27.40' E	12-Oct-09
2615A	9WC2	6	30	35º 49.50' S	19º 33.04' E	30-Oct-09
2909A	9SC1	11	27	35º 20.70' S	20º 24.60' E	01-Nov-09
3207A	9SC2	7	34	34º 43.60' S	21º 06.70' E	05-Nov-09
Sardine Run	9EC	8	43	30º 04.29' S	30º 58.98' E	03-Sep-09

DNA extraction and amplification

Up to 25mg of muscle tissue was removed from the left lateral side of individual fish. Total genomic DNA was extracted with the GenElute^{TM} Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich), following the standard protocol for tissue preparation

as recommended by the manufacturer. Despite being a mammal extraction kit, this was found to yield the best results in laboratory trials of DNA extractions in sardine (*pers. obs.*). Following extraction, DNA was visualised on a 1% agarose gel (Roche Diagnostics) using GelRed™ acid stain (Biotium).

MtDNA PCR and sequencing

Species-specific ND2 primers were designed from a sequence of *Sardina pilchardus* (GenBank accession number AP009233) using Primer Design v.4.20. (SarND2 F - 5' ACT CTG GGT GCT TCC ACT AC 3', and SarND2 R - 5'CCT GCT AGG AGC TTT GAA GG 3'). PCR reactions were made up to a total volume of 25µl, which included 1x buffer, 2.5mM $MgCl_2$ and 0.2mM of each dNTP (Promega), 10pmol forward and reverse primer, 0.5U of SuperTherm® DNA polymerase (Southern Cross Biotechnology) and approximately 100ng of DNA. Sabax water® was used instead of DNA for negative controls. The PCR reaction was performed on a GeneAmp® PCR System 9700 (Applied Biosystems) as follows: an initial denaturation step for 3 minutes at 94°C, 35 cycles of denaturation at 94°C for 45 seconds, primer annealing at 54°C for 1 minute, elongation at 72°C for 1 minute, and an extended final elongation at 72°C for 20 minutes. Polymerase chain reaction fragment amplification was confirmed by electrophoresis through 1% agarose gels. Successful PCR amplified fragments were precipitated using 0.08 volumes 3M NaAc, 3 volumes sequencing grade EtOH and 0.4 volumes Sabax® water. The precipitated DNA was pelleted in a microcentrifuge at 13 000 rpm for 30 minutes and then washed with 70% EtOH. DNA pellets were air-dried and eluted in 15-20 µl Sabax® water. Precipitation success was evaluated by electrophoresis through 1% agarose gels.

Fragments were sequenced in both the forward and reverse direction. Cycle sequencing was performed using the BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 3.0 (Applied Biosystems) and a GeneAmp® PCR System 9700 (Applied Biosystems). Each 10µl cycle sequencing reaction contained approximately 160ng purified PCR product as template, 3.2pmol primer, and 2µl of the BigDye reaction mix. Cycle sequencing reactions were precipitated as described above. The

sequences were screened in an ABI 3130 capillary automated DNA sequencer (Applied Biosystems). Forward and reverse sequences were manually checked, edited and aligned using BioEdit v.7.0.9.0, with ClustalW (Hall 1999). Attempts to use mtDNA control Region and cytochrome b for comparison were met with limited success, and further optimisation was beyond the scope of this project.

Microsatellite genotyping

All the primer sets used in this study were developed for the conspecific Pacific sardine, *S. sagax* (Pereyra *et al.* 2004). The details of the primers, including the primer sequence, repeat motif and GenBank accession numbers from the Pereyra *et al.* (2004) study are summarised in Table 4.2, together with the name of the fluorescent dye used to label forward primers in this study. Microsatellite loci were PCR amplified using the Quantitect multiplex kit (Qiagen®) according to the manufacturer's protocol.

Table 4.2: Summary of primer information including primer names, the forward (F) and reverse (R) primer sequence, repeat motif and GenBank Accession number (AN) as published in Pereyra et al. (2004). The name of the fluorescent dye (Dye) used with each forward primer is also included. The numbers in superscript indicate the multiplex combinations.

Name	**Primer Sequence 5' - 3'**	**Repeat Motif**	**AN**	**Dye**
Sar1A11[1,2]	F: GAG CTG GAA ATC TGG TGA TAT TTA G R: CCT GTT CAC AAG TTA GAG CAT TC	(GATA)2GCTA(GATA) 5GCTA(GATA)8	AY636115	6 – FAM
Sar1-H11[2]	F: TTC CCG GTC TAA ATG TCC AG R: GGC ACG TTA CGT TTC AGA CT	(TG)11TA(TG)6	AY636122	NED
SarBH04F[1]	F: CTC TCG GTG CTT GGA GAG GAA R: GGA GGA GGG GAG AAA AGA TG	(TG)18	AY636119	VIC
SarBH04[2]	F: CGA GTT TGT CCC ACA CCT GGA G R: CTC CAA GCA CCG AGA GCA TC	(GT)9	AY636119	VIC
SarC05[1]	F: GAA CGC AGA CAT AAA AGG GTC R: GGG TAT GTG GTG ATT ATC GTT C	(TC)5TT(TC)4	AY636116	NED
SarB-A08[1]	F: GTG ATA CTC TCT GCC TTG GA R: GCA CTT GTC CTA GTA AAT AGC	(CA)26	AY636115	NED
SarB-D06[1,2]	F: CGG CTA TTC TTA GAC TAG GTG R: CCC CAT CAG CAA TGA ATA AG	(TG)18	AY636123	PET

Primers were diluted according to manufacturer's specifications and combined into one of two primer mixes with 2.5ng/µl of each primer used in two multiplexes (Table 4.2). When individual loci did not amplify they were redone using a different combination of primers or were amplified individually using the Qiagen® multiplex kit and then co-loaded. PCR cycles included an initial step at 95ºC for 15 minutes, 30 cycles of denaturing at 94ºC for 30 seconds, primer annealing at 60ºC for 90 seconds and elongation at 72ºC for 30 seconds, with a final extension step of 10 minutes at 72ºC. All PCR products were visualised with GelRed™ Acid stain (Biotium) on a 2% agarose gel in order to confirm successful amplification before fragment analysis. Successfully amplified PCR product was combined with the LIZ™ 500 size standard (Applied Biosystems) for allele size determination on an ABI 3100 automated DNA sequencer (Applied Biosystems) using GeneScan software.

Allele sizes were scored using GeneMarker v.1.8 software (SoftGenetics LLC, 2008) and checked individually to ensure consistent scoring. Scoring was done twice, immediately after a GeneScan run and then again when all individuals had been

processed to check for consistency. Where sizes could not be clearly determined, the individual was genotyped again for that locus. Subsets of individuals were genotyped repeatedly to determine genotyping error.

MtDNA data analysis

Summary statistics were calculated using DnaSP v.5 (Librado and Rozas 2009). For each sampling site the number of variable sites (S), haplotype diversity (*h*) and nucleotide diversity (π) were determined. Haplotype diversity is explained by frequency of haplotypes, whereas nucleotide diversity takes into account both frequency of haplotypes and sequence divergence of haplotypes (Bowen and Grant 1997). Genetic diversity, theta (θ), is calculated in two ways: either the average number of variable nucleotide positions between pairs of sequences, as with π, or the number of variable positions in the sequence alignment. Deviations from neutrality were tested using Tajima's D statistic (Tajima 1989), which compares these two measurements of θ (Rand 1996). A negative D statistic would indicate population or geographic expansions or contractions, or possibly selection acting on the gene (Tajima 1989). The selective neutrality of a random sample, under the infinite sites model, was tested with Fu's F statistic (over 10 000 simulated samples), which is thought to be a more sensitive indicator of population expansion than Tajima's D. Here, a negative value suggests a recent population expansion, while a positive value represents a deficiency of alleles that characterises a population bottleneck (Fu 1997).

Gene genealogies were constructed for samples collected during 2007 and 2009 in TCS v.1.2.1 using statistical parsimony following Templeton *et al.* (1992) and Clement *et al.* (2000). Hierarchical population structure was further explored in Arlequin v.3.11 (Excoffier *et al*. 2005) using an Analysis of Molecular Variance (AMOVA) over 10 000 permutation to calculate the proportion of variance explained within and among populations. Groupings for this analysis were defined by the region in which the samples were collected: South Africa's west coast and south coast in 2007; and Namibia, and South African west coast, south coast and east coast for 2009. Pairwise F_{ST} values were calculated over 10 000 permutations between sampling

localities and population regions in Arlequin v.3.11 (Excoffier *et al*. 2005) using the genetic distance matrix to calculate the average number of pairwise differences between populations over 10 000 permutations.

A mismatch distribution was performed using DnaSP v.5 (Librado and Rozas 2009) in order to determine whether population size was stable over time, or expanding. The shape of the mismatch distribution provided an indication of the historical changes in population size. The degree of raggedness, measured by the Harpendings raggedness index (Harpending 1994) in Arlequin v.3.11 (Excoffier *et al*. 2005) indicates the population growth trend. A ragged curve (higher value of the raggedness index) is characteristic of a stable population whereas a smooth curve indicates population growth. The level of significance for all analyses was set as 0.05.

Microsatellite data analysis

Individuals missing information for more than two loci were excluded from all analyses. Null alleles, scoring error as a result of stutter and large allele drop out were calculated in Micro-Checker v.2.2.3 (van Oosterhout *et al.* 2004) for all samples combined and each sampling site within each year. LOSITAN was used to test whether loci were selectively neutral, as it calculates the probability of the loci being under selection based on expected heterozygosity and F_{ST}. All analyses were run using 5000 simulations with simulations at neutral F_{ST} (Beaumont and Nichols 1996, Antao *et al*. 2008).

Linkage disequilibrium tests were performed to identify non-random association of alleles at different loci (Excoffier and Heckel 2006), and were conducted using the Black and Krafsur (1985) method in Genetix v.4.05.2 (Belkhir *et al.* 2004). Deviations from Hardy-Weinberg Equilibrium (HWE) were tested in Arlequin v.3.11 (Excoffier *et al*. 2005), using an algorithm modified from Guo and Thomson (1992), and ran for 1 million MCMC steps with 100 000 dememorisation steps.

Given the small sample size available, it was important to determine whether the given sample size and number of loci would be sufficient to detect population differentiation. The power of the loci to detect population differentiation was calculated in POWSIM v.4.1(Ryman and Palm 2006), based on observed allele frequencies calculated in Genepop'007 (Raymond and Rousset 1995, Rousset 2008). Simulations were run for different F_{ST} values, for two populations with a sample size of 32 and 53 (west and south coast sample size respectively in 2008), for 1000 iterations. Simulated F_{ST} values were obtained using multiple combinations of effective population size (Ne) and number of generations since drift (t), based on the equation of $F_{ST} = 1-(1-½N_e)^t$. A total of three different F_{ST} values were estimated using the combined data set: F_{ST} = 0.002 (Ne = 2000 and t=10); F_{ST} = 0.01 (Ne = 2000, t = 50) and F_{ST} = 0.02 (Ne = 200 and t= 10). An F_{ST} of 0.02 is considered the threshold of differentiation between populations of marine fish (Waples 1998). Significance of results was assessed using a Chi^2 test.

Summary statistics such as gene diversity, number or alleles, and F_{IS} were calculated for each locus at each site in FSTAT v.2.9.3.2. (Goudet 2001), allelic richness and private allele richness were calculated in HP-RARE (Kalinowski 2005). The number of alleles, the mean number of alleles, observed and expected heterozygosity, and overall F statistics (F_{ST}, F_{IS} and F_{IT}) were calculated for all loci over all samples, as well as within each of the three years. The 95% confidence intervals of the F statistics were calculated using 1000 bootstraps in Genetix v.4.05.2 (Belkhir *et al.* 2004). The three F statistics measure different levels of inbreeding: F_{IS} measured individual inbreeding relative to the subpopulation and associated decrease in heterozygosity associated with inbreeding; F_{IT} demonstrated inbreeding within individuals, but relative to the total population; and F_{ST} is a measure of inbreeding within subpopulations relative to the total population. Wright's F_{ST} described genetic differentiation among populations using allele frequencies. It assumes an Infinite Alleles Model (IAM) (Kimura and Weiss 1964) and as mentioned above, F_{ST} of 0.02 is considered to show population differentiation in marine populations.

In order to understand if genetic differentiation varied not only geographically, but also temporally (among the sampled years), genotypic and genic differentiation, based

on allelic frequencies, were calculated using the Fisher's Exact Probability test in Genepop'007 (Rousset 2008, Raymond and Rousset 1995). However, given the smaller sample sizes and potential for null alleles and deviation from Hardy Weinberg Equilibrium (HWE), population differentiation among sites was investigated using pairwise $F_{ST,}$ while not assuming HWE, calculated in FSTAT v.2.9.3.2, with statistical significance obtained after 1000 permutations and adjusted for multiple comparisons (Goudet 2001). These results were compared to those calculated in FreeNA (Chapuis and Estoup 2007) which takes null alleles into account, using the ENA correction described by Chapuis and Estoup (2007), and bootstrapping 1000 times over the loci. Null alleles can homogenise differentiation and therefore need to be taken into consideration when determining population structure. Pairwise F_{ST} was first calculated among years, and then with samples grouped within each coast across the three years. Finally sites were compared within 2008 and 2009.

Within each year, AMOVA (Excoffier *et al.* 2005) was performed using Arlequin v.3.11 with 10 000 permutations in order to test the hypothesis of hierarchical population structure by calculating the variance components at different levels of groupings. The sample sites were grouped according to region and then adjusted to maximise F_{CT} (among group) values. In 2007 and 2008, samples were divided between the west coast (WC) and south coast (SC). In 2009, groups defined were: Namibia (NAM), west coast (WC), south coast (SC) or east coast (EC) as summarised in Table 4.1. In addition, spatial autocorrelation for each of the years was investigated using GeneAlEx v.6.0. In an isolation by distance model, it is expected that genetic difference increases with an increase in geographic distance (a positive correlation). The analyses used pairwise matrices of geographic and genetic distance for all loci among individuals from all sampling locations. The significance of the correlation coefficient, r, was determined by the 95% confidence intervals from 1000 bootstrap resampling events. In a plot of r against distance, the distance at which r crosses the x axis (i.e. changes from positive to negative) is an indication of the point at which there is no longer spatial genetic structure. The calculation of r is influenced by the distance class sizes, therefore the analysis was run for multiple distance classes to test for sensitivity of the distance classes used (Peakall and Smouse 2006). Finally, custom distance classes were used to ensure that all distance classes included data.

To visualise population differentiation among sites, Factorial Correspondence Analysis (FCA) was performed using Genetix v.4.03 (Belkhir et al. 1996-2004). This software compares individuals in a pairwise fashion and separated on a three-dimensional grid. This was done first with no prior information about species and then including sampling locality as prior information. In species that are likely to have shallow genetic structure, using prior information on sampling locality as an additional factor helps resolve underlying structure. This was done for each year separately using sampling region as a prior factor and for all years together using the year as a prior factor.

Finally, the coalescent-approach implemented in Structure v.2.3.3 (Pritchard *et al.* 2000) was used to detect structured population genetic variation among individuals. An initial run of 1 to 10 clusters (K) was run with 20 iterations and 100 000 MCMC repeats after a burnin of 100 000. The admixture model with correlated allele frequencies was used with no prior geographic information initially. Structure uses MCMC clustering to proportionally divide individuals into 'K' populations in a way that maximises HWE (Pritchard *et al.* 2000). The most probable number of clusters was determined using a combination of the value and standard deviation of log probability of K and Delta K (ΔK), the rate of change in the log probability between successive values of K as described by Evanno *et al*. (2005). The K-value with the greatest change in ΔK represents the most likely number of populations. The cut off point for individual assignment into a particular group was 80% as determined from the average of three runs.

Results

MtDNA results

In the 33 individuals from 2007, there were 27 haplotypes with a mean (s.d.) haplotype diversity of h = 0.9830 (0.0002) and mean (s.d.) nucleotide diversity of π = 0.0056 (0.0005). The Tajima's D statistic (D = -2.2916) showed statistically

significant deviation from neutrality ($P < 0.01$), as did Fu's F statistic ($Fs = -21.8102$, $P < 0.01$). In the 53 individuals from 2009, there was a total of 41 haplotypes and overall haplotype diversity of $h = 0.929$ (s.d. = 0.030) and nucleotide diversity of $\pi = 0.0068$ (s.d. = 0.0008). Tajima's D statistic for neutrality ($D = -2.6134$) was significant ($P < 0.001$). Overall there were 58 variable sites and 64 mutations. There were 52 and 48 variable sites in 2007 and 2009, respectively. When the two years are combined there were 52 haplotypes with a mean (s.d.) haplotype diversity $h = 0.923$ (0.024) and mean (s.d.) nucleotide diversity $\pi = 0.0058$ (0.0005). Tajima's D statistic for the two years combined ($D = -2.6641$) was significant ($P < 0.001$).

There are high levels of haplotype diversity within all sampling localities. At seven of the 12 sampling localities there were no shared haplotypes. The lowest diversity was found at a 2007 south coast site, 7SC1 (h=0.857). However, nucleotide diversity was low across all sites, ranging from 0.003 in east coast samples to 0.009 in 9SC1 of 2009 (Table 4.3). No clear geographic pattern in diversity values was observed (Table 4.3).

Table 4.3: Molecular diversity indices for all 2007 and 2009 sardine ND2 samples per sampling locality, including: number of samples (N), number of polymorphic sites (S), nucleotide diversity (π, s.d.), number of haplotypes (H) and haplotype diversity (h, s.d.).

Region	**N**	**S**	**π (%)**	**s.d.**	**H**	***h***	**s.d.**
2007							
7WC1	7	14	0.572	0.00144	6	0.952	0.096
7WC2	6	14	0.560	0.00134	5	0.933	0.122
7SC1	7	13	0.612	0.00108	5	0.857	0.019
7SC2	9	18	0.500	0.00085	9	1.000	0.052
7SC3	4	12	0.724	0.00154	4	1.000	0.031
2009							
9NAM1	9	21	0.605	0.00074	9	1.000	0.003
9LUD1	6	16	0.502	0.00084	9	1.000	0.063
9WC1	6	23	0.793	0.00137	7	1.000	0.076
9WC2	6	13	0.469	0.00097	7	1.000	0.076
9SC1	11	14	0.881	0.00150	10	1.000	0.045
9SC2	7	12	0.412	0.00091	6	0.952	0.096
9EC	8	9	0.292	0.00068	6	0.929	0.007

The central haplotype in the TCS minimum spanning haplotype network for samples collected during 2007 (Figure 4.2a) is shared by two individuals, from which many unique haplotypes are removed by up to nine mutational steps, giving rise to a star-like network. The 2009 minimum spanning network (Figure 4.2b) has a central haplotype shared by nine individuals, four of which are from a single sampling locality on the Agulhas Bank. There are many unique haplotypes spreading from the ancestral haplotype in a star-like fashion. The ancestral haplotype is represented in five of the seven sampling sites.

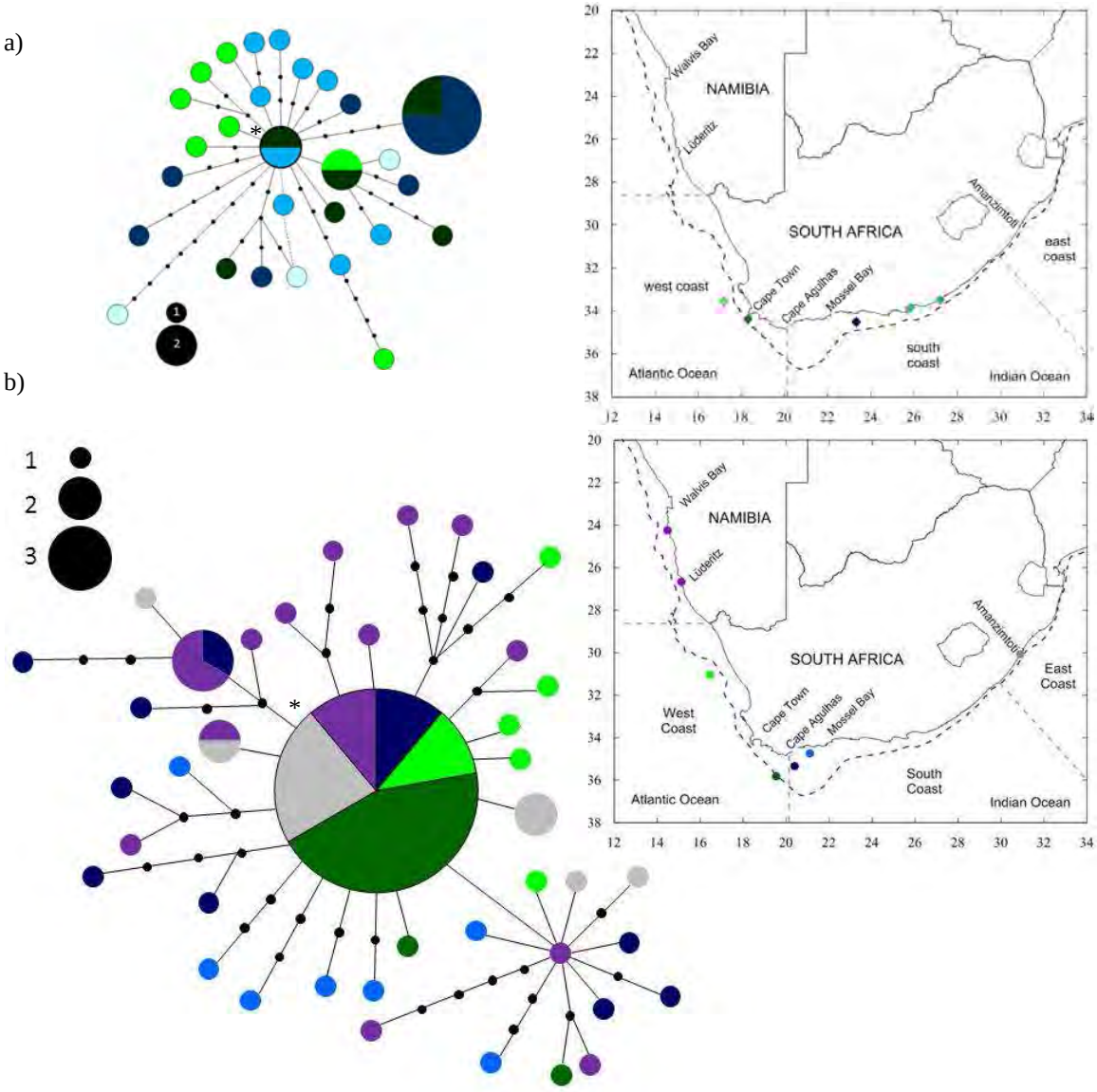


Figure 4.2: Sardine ND2 minimum spanning network for a) 33 individuals, defining 27 haplotypes for samples collected in 2007 and b) 53 individuals, defining 41 haplotypes, for samples collected during 2009. The size of the circle representing each haplotype is proportional to the number of individuals that share that specific haplotype, according to the scale provided. Black circles represent a single, unsampled or extinct haplotype. The colours used represent the sampling localities as shown in the insert. The dotted line in the 2007 network represents an alternative link to the haplotype. The asterisk indicates the central haplotype.

There were no significant differences in pairwise F_{ST} estimates (Table 4.4) across the two years. When the west coast samples were combined and compared to the south coast samples, there was no significant difference (F_{ST} = -0.0199, P = 0.9279). There were no significant differences between any of the sampling sites in 2009. However, when 9WC1 and 9WC2 were combined to form a west coast group and 9SC1 and 9SC2 were combined in a south coast group, there were significant differences between Namibian samples and the west coast (F_{ST} = 0.0168, P = 0.027).

Table 4.4: Sardine ND2 pairwise F_{ST} values between sampling sites for 2007 (a) and 2009 (b) are indicated below the diagonal and P values above the diagonal. The sample size is in the grey block on the diagonal.

a)

2007	7WC1	7WC2A	7SC1	7SC2	7SC3
7WC1	7	0.999	0.188	0.999	0.999
7WC2	-0.0246	6	0.562	0.999	0.999
7SC1	0.071	0.002	7	0.063	0.236
7SC2	0.000	-0.019	0.068	9	0.999
7SC3	0.000	0.000	0.081	0.000	4

b)

2009	9NAM1	9WC1	9WC2	9SC1	9SC2	9EC1
9NAM1	15	0.5496	0.3514	0.2072	0.9550	0.7928
9WC1	-0.0023	6	0.6937	0.4685	0.9910	0.2973
9WC2	0.0037	-0.0044	6	0.3784	0.7298	0.0541
9SC1	0.0128	-0.0040	0.0061	11	0.3243	0.2072
9SC2	-0.0280	0.0000	0.0038	0.0102	7	0.4955
9EC	-0.0150	0.0141	0.0426	0.0254	0.0209	8

*Significance value at P < 0.05

Regions defined for the AMOVA analyses were adjusted to minimise the within-site variability, in order to explain regional variance. In 2007 the west coast sites 7WC1 and 7WC2 were grouped together, and south coast sites 7SC1, 7SC2 and 7SC3 were grouped together. The majority of the variance was among individuals, regardless of the groupings that were evaluated. Similarly, in 2009 the majority of the variance was among individuals. The highest level of regional variance was found when Namibian samples were grouped separately to South African samples, but not significantly so (Table 4.5).

Table 4.5: ND2 AMOVA results for sardine obtained during 2007 and 2009. Groups were defined by their region: Namibia (2009 only), west coast, south coast, and east coast (2009 only) of South Africa. Degrees of freedom (d.f), sum of squares (SS), percentage of variation (%), P values (P) and fixation indices (F) are shown.

Source of Variation	d.f.	SS	%	F	P
2007					
Among regions	1	0.480	0	F_{CT}: -0.01142	0.898
Among sites within regions	3	1.702	2.63	F_{SC}: 0.02596	0.065
Among individuals within sites	28	13.57	98.52	F_{ST}: 0.01484	0.086
2009					
Among countries	1	0.738	2.63	F_{CT}: 0.026	0.196
Among sites within countries	5	2.226	0	F_{SC}: -0.004	0.497
Among individuals within sites	50	22.571	97.78	F_{ST}: 0.022	0.267

In the 2007 mismatch analysis (Figure 4.3a), the mean (s.d.) Harpending's raggedness index (Harpending 1994) was 0.108 (0.106), which was not significant (P = 0.736) Similarly, in the 2009 samples (Figure 4.3b), the mean (s.d.) Harpending's raggedness 0.104 (0.08) index was not significant (P = 0.225) and, therefore, the model of an expansion could not be rejected. The poor fit between observed and expected distribution could be a function of sample size. This is particularly likely in the 2007 analysis, where there were only 33 samples.

a)

b)

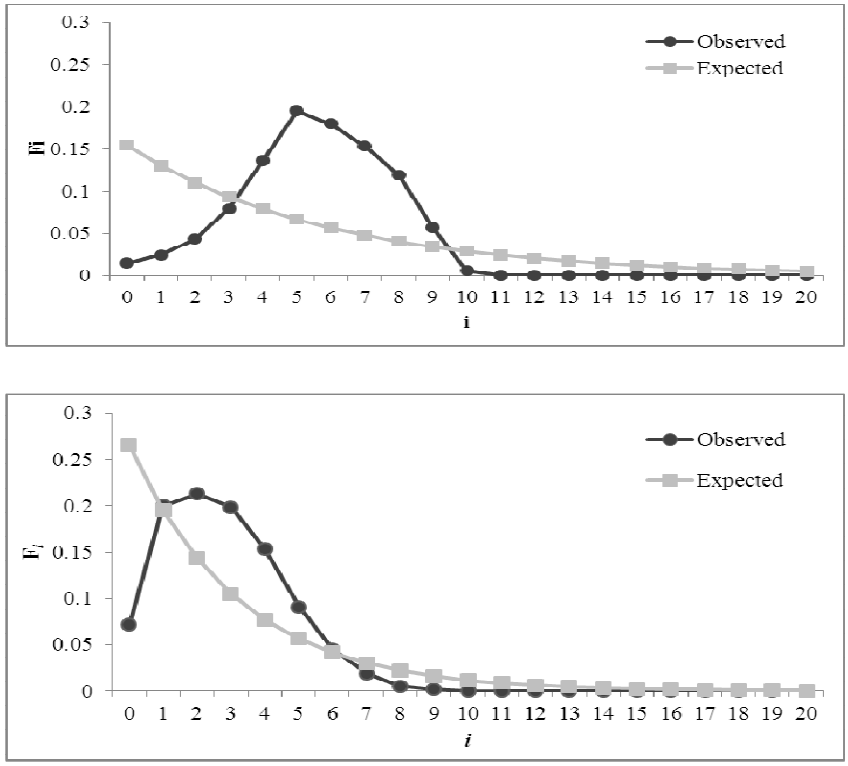


Figure 4.3: The x axis (i) is the number of pairwise differences and y axis the frequency of a particular *i* value (Fi) of expected under constant population size (grey squares) and observed (black circles) pairwise equilibrium distribution for a) 2007 and b) 2009.

Microsatellite results

Amplification success following PCR varied greatly among the different loci. The highest amplification success (98%) was observed for SarD06 across the three years, and the lowest amplification success was for SarBH04F (61% of the 463 samples analysed). The re-genotyped loci showed evidence of genotyping errors at a rate of approximately 10%. The percentage of missing information in the data set that was used in all analyses is shown in Appendix 4.1. The Micro-Checker v.2.2.3 (van Oosterhout *et al.* 2004) results detected no evidence of stutter or large allele dropout. However, there was evidence of null alleles in all but one of the loci (Sar1-H11), although this varied among sites and years (Appendix 4.3). In all but five incidents, the null allele frequency was less than 25%.

LOSITAN did not detect any of the loci to be outside the expected neutral range (Appendix 4.3) and none of the loci were found to be significantly linked (Appendix

4.4), therefore all loci were retained in further analyses. There were significant deviations from HWE; the only locus to be consistently in HWE was Sar1-H11, although it was monomorphic in 7SC2 (Appendix 4.5). The small sample size and high levels of missing data in 2007 mean that all conclusions drawn from this year in particular should be considered preliminary and exploratory.

The analysis of power in POWSIM determined that there was 43% chance of determining differentiation between two populations with these seven loci at F_{ST} of 0.002, using the number of samples available in 2008 and 100% chance of determining differentiation at F_{ST} of 0.02 and 0.01.

Gene diversity was high (He>0.8) in all loci from all sites, but F_{IS} varied greatly among loci within and between sites: F_{IS} = 0.04 (Sar1-H11, for 9WC1) and F_{IS} = 0.61 (SarBH04F, for 9WC1) (Appendix 4.6). Summary statistics for each site are shown in Table 4.6a and per locus in Table 4.6b. The mean number of alleles per locus ranged from 5.43 in 2007 and 6.71 in 2008, to 22.71 in 2009. The site 7SC2 was an outlier in terms of allelic richness, but this was due to a lack of amplification of the SarH11 loci at that site. Allelic richness was highest in 2009, when samples sizes are larger. There were fewer private alleles in the Namibian samples than in 9WC1. When all three years were combined in a single sample, the mean number of alleles for all loci was 34.14 and H_E and H_O were 0.91 and 0.69, respectively. The mean F_{IS} across all three years was 0.248 (variance = 0.0026), and mean F_{ST} (0.007) suggested low levels of differentiation overall.

Table 4.6: Results of sardine microsatellite analyses of seven loci for a) individual sampling site over three years and b) all samples for each locus. Total number of samples (N), average allelic richness over all loci (AR), private alleles (Pvt A), mean number of alleles (MNA), expected (H_E) and observed (H_O) heterozygosity for each sampling site over the three years are summarised. Per locus F_{IS}, number of alleles (NA), allelic richness (AR), MNA, H_E and H_O are shown in b.

	Region	F_{IS}	AR	Pvt A	N	MNA	H_E	H_O
a)	**2007**							
	7WC1	0.318	3.96	0.27	16	5.43	0.724	0.593
	7WC2	0.368	4.51	0.28	6	9.00	0.823	0.562
	7SC1	0.254	4.73	0.30	6	12.86	0.864	0.672
	7SC2	0.446	12.96	0.38	10	7.00	0.838	0.528
	2008							
	8WC1	0.197	4.66	0.31	16	12.71	0.851	0.711
	8WC2	0.312	4.89	0.37	16	14.29	0.876	0.631
	8SC1	0.276	4.68	0.32	12	10.00	0.850	0.657
	8SC2	0.237	4.43	0.27	13	10.43	0.820	0.672
	8SC3	0.356	3.63	0.12	12	6.71	0.658	0.459
	8SC4	0.124	4.76	0.38	16	13.71	0.861	0.782
	2009							
	9NAM	0.247	4.98	0.35	34	18.86	0.900	0.693
	9WC1	0.196	4.97	0.64	28	19.43	0.897	0.738
	9WC2	0.219	4.95	0.40	30	19.14	0.894	0.715
	9SC1	0.252	5.00	0.50	27	19.71	0.901	0.690
	9SC2	0.255	4.93	0.46	34	19.00	0.897	0.682
	9EC	0.188	5.12	0.57	43	22.71	0.915	0.755
b)	**Locus**	**Fis**	**AR**		**NA**	**MNA**	**H_E**	**H_O**
	Sar1A11	0.295	12.363		19	0.61	0.87	0.62
	Sar1-H11	0.018	15.736		31	0.54	0.80	0.78
	SarBH04F	0.421	22.289		36	0.78	0.95	0.55
	SarBH04	0.193	23.664		42	0.79	0.96	0.77
	SarC05	0.175	20.491		32	0.73	0.95	0.79
	SarB-A08	0.386	23.336		40	0.58	0.95	0.59
	SarD06	0.182	16.008		34	0.79	0.90	0.74

Overall F_{ST}, F_{IS} and F_{IT} for the different year groups are shown in Table 4.7. The positive F_{IS} and F_{IT} for all three years indicate a consistent homozygosity excess, as values close to 0 are expected under conditions of random mating. F_{ST} measures difference in allele frequencies between groups and is thus a measure of population differentiation. F_{ST} values were small, particularly in 2007 and 2009. However, F_{ST} for 2008 was larger than the mean F_{ST} for marine fish (Waples 1998). F statistics were significant in all of the years.

Table 4.7: Overall yearly F_{ST}, FIS and FIT and variance for 2007, 2008 and 2009 sardine microsatellite loci are shown, significance was set at 0.05 and all values were significant.

	2007	**2008**	**2009**
F_{IS}	0.314 (0.169 – 0.459)	0.233 (0.111 – 0.343)	0.226 (0.141 – 0.309)
F_{IT}	0.316 (0.169 – 0.466)	0.258 (0.140 – 0.356)	0.226 (0.139 – 0.309)
F_{ST}	0.004 (-0.0002 - 0.0004)	0.033 (0.005 – 0.066)	-0.0004 (-0.00002 – 0.00005)

Although there were significant genic and genotypic differences among years (Table 4.8a), the three years were not significantly different from each other when compared in FSTAT while not assuming HWE, and F_{ST} values were only weakly positive once corrected for null alleles (Table 4.8b).

Table 4.8: a) P values for genic differentiation among years (below diagonal) and genotypic differentiation (above diagonal) both using Fisher's Exact Method. b) F_{ST} values corrected for null alleles using ENA correction (above the diagonal) and P value of pairwise differentiation calculated not assuming HWE and adjusted for multiple comparisons (significance was set at 0.017; below the diagonal). Statistical significant results are indicated by an asterix. The samples size is shown in each of the grey blocks on the diagonal.

a)	**2007**	**2008**	**2009**
2007	38	0.003*	0.009*
2008	0	85	0
2009	0.003*	0	196
b)	**2007**	**2008**	**2009**
2007	38	0.0065	0.0033
2008	0.033	85	0.0067
2009	0.1	0.217	196

However, comparison of all sampling sites, independently of the year, revealed statistically significant pairwise differences between the 2008 south coast and 2009 west and south coast samples (Table 4.9).

Table 4.9: F_{ST} values corrected for null alleles using ENA correction (above the diagonal) and P value of pairwise differentiation calculated not assuming HWE and adjusted for multiple comparisons (significance was set at 0.0018, below the diagonal). Sample size is shown in the grey blocks on the diagonal.

	7WC	**7SC**	**8WC**	**8SC**	**9NAM**	**9WC**	**9SC**	**9EC**
7WC	16	-0.0052	-0.0031	-0.0066	-0.0108	-0.0047	-0.0049	-0.0051
7SC	0.4125	22	-0.0014	0.0070	-0.0073	0.0005	0.0012	-0.0024
8WC	0.2625	0.0143	32	0.0005	-0.0052	-0.0035	-0.0013	-0.0041
8SC	0.4964	0.0107	0.0786	53	-0.0001	0.0041	0.0031	0.0029
9NAM	0.7143	0.6554	0.7286	0.0571	34	-0.0037	-0.0033	-0.0070
9WC	0.6625	0.0232	0.5929	**0.0018**	0.1143	58	-0.0015	-0.0035
9SC	0.4571	0.0143	0.4643	**0.0018**	0.0839	0.4482	61	-0.0028
9EC	0.5214	0.1554	0.9054	0.0393	0.8929	0.4821	0.2893	43

Furthermore, FST comparisons within 2008 and 2009 revealed that combined 8SC2 and 8SC3 were significantly different to combined west coast samples and 8SC1. However, once corrected for null alleles, F_{ST} was not positive for those pairs of sites. No significant differentiation was observed for the 2009 samples, independently of the method used (Table 4.10).

Table 4.10: P values for pairwise comparisons of sardine microsatellite F_{ST} calculated without assuming HWE (below the diagonal) and F_{ST} using ENA correction over 1000 bootstraps (Chapuis and Estoup 2007) (above the diagonal) are given for a) 2008 and b) 2009. The sample size is shown in the grey block on the diagonal. Significance, adjusted for multiple comparisons, is indicated in bold.

a)

2008	8WC1/2	8SC1	8SC2/3	8SC4
8WC1/2	32	-0.0019	0.0023	-0.0023
8SC1	0.142	12	0.0009	0.0018
8SC2/3	**0.008**	**0.008**	25	-0.0017
8SC4	0.067	0.042	0.058	16

b)

2009	9NAM	9WC1	9WC2	9SC1	9SC2	9EC
9NAM	34	-0.0022	-0.0067	0.0006	-0.0039	-0.0018
9WC1	0.070	28	-0.0050	-0.0040	-0.0047	-0.0068
9WC2	0.443	0.503	30	-0.0078	-0.0066	-0.0035
9SC1	0.143	0.373	0.690	27	-0.0028	-0.0060
9SC2	0.047	0.140	0.117	0.160	34	-0.0068
9EC	0.890	0.187	0.800	0.450	0.067	43

AMOVA analyses revealed that the majority of variance was explained by individual differences in all three years and among individuals within regions (Table 4.11). Only in 2008 was there a significant contribution of sites within regions, which is most likely explained by the south coast sites 8SC2/3 being significantly different to 8SC1.

Table 4.11: Summary of AMOVA based on seven sardine microsatellite loci with degrees of freedom (df.), percentage variation, fixation index (F) and its P value shown for each source of variation in each year. Significance is shown in bold. Regions are divided into Namibia (2009 only), west, south and east coast (2009 only) of South Africa.

Source of variation	**df**	**SS**	**%**	**F**	**P**
2007					
Among regions	1	2.75	1.55	F_{CT}: 0.016	0.365±0.017
Among sites within regions	2	3.70	0.00	F_{SC}: 0.000	0.860±0.012
Among individuals within regions	34	77.66	28.29	F_{IS}: 0.283	<**0.001**
Among individuals within sites	38	48.50	71.66	F_{IT}: 0.283	<**0.001**
Total	75	132.61			
2008					
Among regions	1	6.19	0.27	F_{CT}: 0.002	0.515±0.018
Among sites within regions	2	8.55	4.64	F_{SC}: 0.047	<**0.001**
Among individuals within regions	81	140.06	11.20	F_{IS}: 0.118	<**0.001**
Among individuals within sites	85	116.00	83.89	F_{IT}: 0.161	<**0.001**
Total	169	270.80			
2009					
Among regions	3	7.09	0.00	F_{CT}: 0.000	0.739±0.012
Among sites within coasts	2	5.39	0.11	F_{SC}: 0.001	0.319±0.017
Among individuals within coasts	190	485.25	17.78	F_{IS}: 0.178	<**0.001**
Among individuals within sites	196	349.50	82.28	F_{IT}: 0.177	<**0.001**
Total	391	847.23			

Spatial autocorrelation analysis of the 2007 data showed no non-random genetic differentiation for geographically separated individuals (Figure 4.4a). The r coefficient was only significant (P = 0.004) for individuals sampled from the same location. In 2008 there was significant positive correlation (P < 0.05) for individuals up to a distance of 500km (Figure 4.4b), after which there was significant negative correlation of genetic distance with the larger geographic differences. The point at which the coefficient r crossed the x-axis (and genetic differentiation was non-random) was at approximately 500km. In 2009, there was significant positive correlation (P = 0.001) for individuals within the first distance class (200km) (Figure 4.4c). There was significant negative correlation (P < 0.05) with the 300km distance class and again from 600km to 1600km, after which there was significant positive correlation (Figure 4.4c).

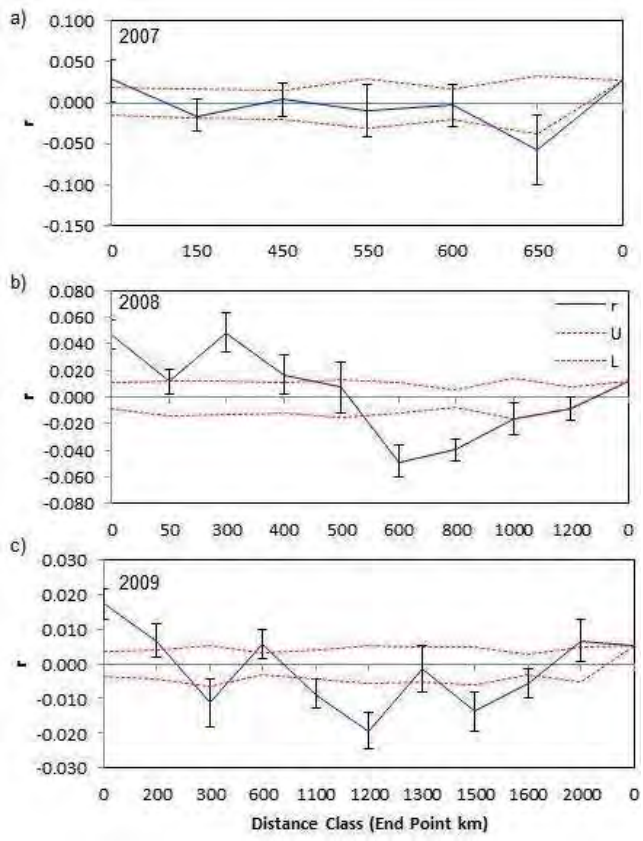


Figure 4.4: Spatial autocorrelation (r, blue) for customised distance classes (km) for a) 2007, b) 2008 and c) 2009 sardine microsatellite data. Error bars represent 95% confidence intervals by bootstrapping over pairs of individuals and the upper (U) and lower (L) 95% confidence intervals are shown in dashed red.

The FCA analyses without prior information on sampling sites explained only a small proportion of the variance in the first three axes, and the samples largely clustered together, but for a few outliers (Figure 4.5a). When the sampling year was included as prior information, 100% of the variance was explained in the first two axes and there was a separation between years despite some overlap (Figure 4.5b).

a) b)

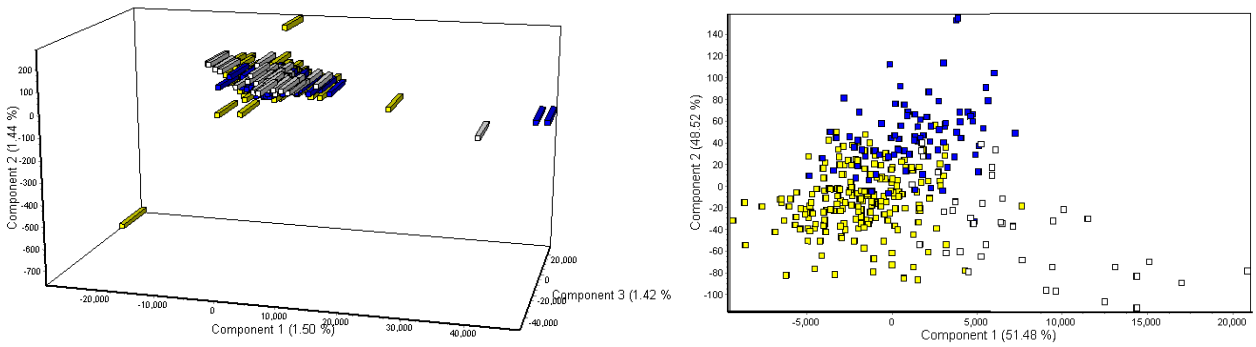


Figure 4.5: FCA based on variation at seven microsatellite loci for sardine between years, with 2009 in yellow, 2008 in blue and 2007 in white with a) no prior and b) years used as prior information. Each block represents an individual sample.

Again, for within-year comparisons, the FCA was first run with no prior information and did not explain the majority of the variance (16.24% in 2007, 9.89% in 2008 and 5.2% in 2009), thus coastal divisions were included as prior information (Figure 4.6). When sampling information was included, the 2007 samples were separated into west coast and south coast, with 100% of variation explained by the first factor (Figure 4.6b). The same was observed for the 2008 and 2009 samples (Figure 4.6c and d). However, even with prior clustering defined, the 2009 west and south coasts remain largely separate, but some overlap was observed between the Namibian and east coast samples (Figure 4.6f).

The FCA from the microsatellite loci showed a degree of structuring among coasts in all three years, although only when prior information was included in the analyses. In 2009, when the samples came from a wider geographic range, there was the same separation between west coast and south coast samples, but there was an overlap between Namibian samples and east coast samples. These results suggest that, on a broad geographic scale, there could be some weak separation between the regions.

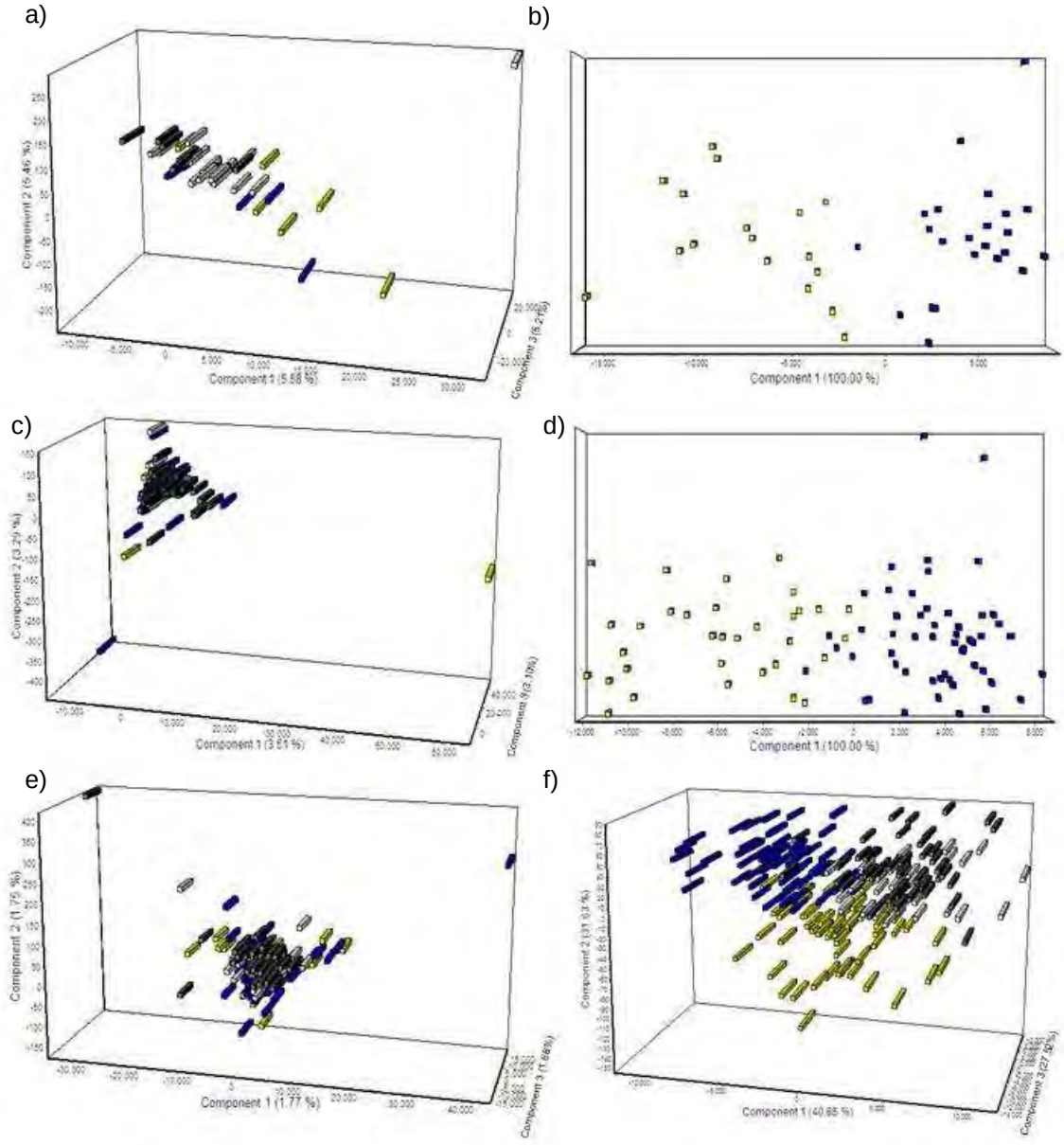


Figure 4.6 FCA based on seven microsatellite loci for sardine a) no prior information was included and b) regional divisions of 2007, WC is yellow and SC is blue. c) No prior information was included and d) 2008 regional division; WC is yellow and SC is blue. e) No prior information included in 2009 and f) individuals are divided into coasts with WC in yellow, SC in blue, NAM in white and KZN in grey. The figure displays each individual sample, represented as a bar in 3D or a square in 2D space.

Results for the program Structure suggested that there were two clusters across the three years, with mixing between the two clusters (Figure 4.7). Individuals from the south coast in 2008 have a higher probability of belonging to cluster 2 (grey) than individuals from 2007 and 2009 do (Figure 4.7).

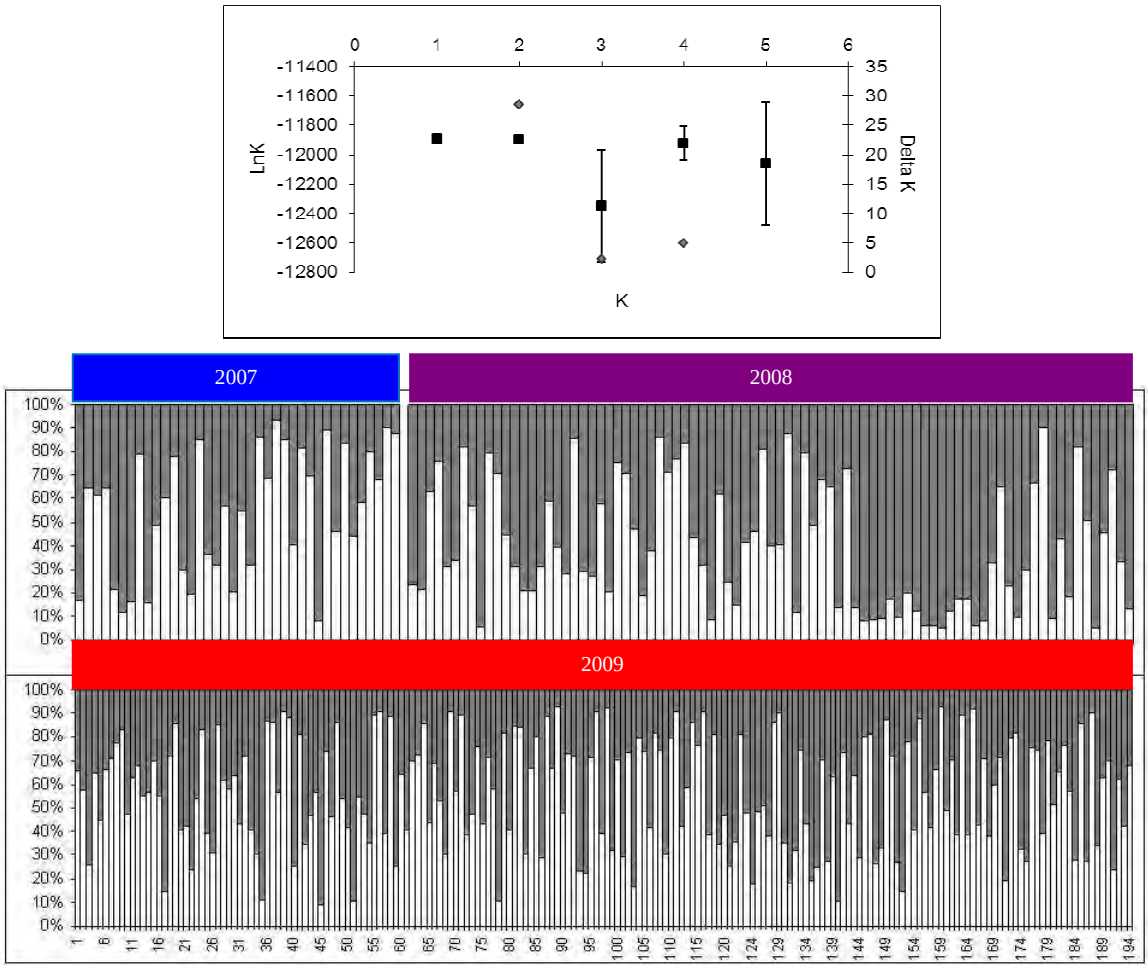


Figure 4.7: Simulation summary from Structure analysis of all sardine across the three years showing a) the likelihood (LnK and s.d., black squares) for each value of K and ΔK (grey diamonds) with no prior information about sites or year and b) an average of three Structure runs for K=2. Each individual is proportionally assigned to one of two clusters (K) and is represented by a single vertical bar. The sampling sites are ordered from west to east within each year, 2007 and 2008 are separated by a blank bar and 2009 is below. The coloured bars above each panel correspond to the year, 2007 in blue, 2008 in purple and 2009 in red.

These findings were further corroborated by the independent analyses for each sampling year. Analyses for 2007 suggest K = 1 as the most likely number of clusters (Figure 4.8a); LnK is least negative, low Δ*K* suggests a lack of discrimination between K values and there is little deviation around K = 1 (Figure 4.8).

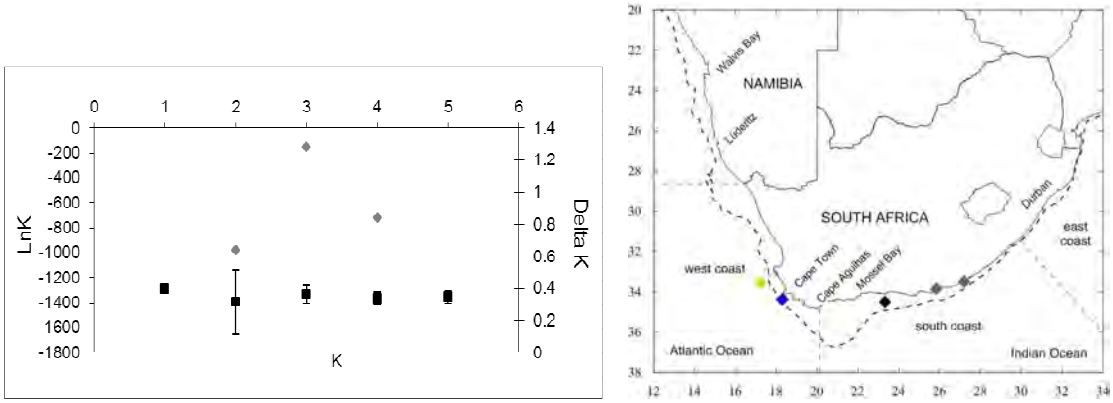
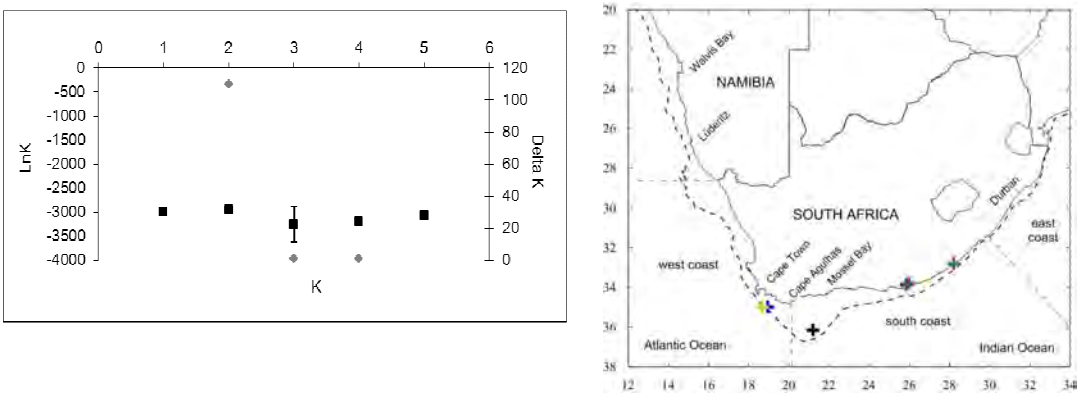


Figure 4.8: Simulation summary from Structure analysis of the 2007 sardine showing the likelihood (LnK and s.d., black squares) for each value of K and ΔK (grey diamonds) with no prior information about sites.

In 2008, the largest value for LnK and *ad hoc* statistic Δ*K* was at K = 2 (Figure 4.9a). The two sample sites near Algoa Bay, 8SC2 and 8SC3 (grey and pink respectively), were mainly assigned to the white cluster and three individuals in 8SC4 were clustered into the white cluster, while individuals from the other sites were mixed between the grey and white cluster (Figure 4.9).

a)



b)

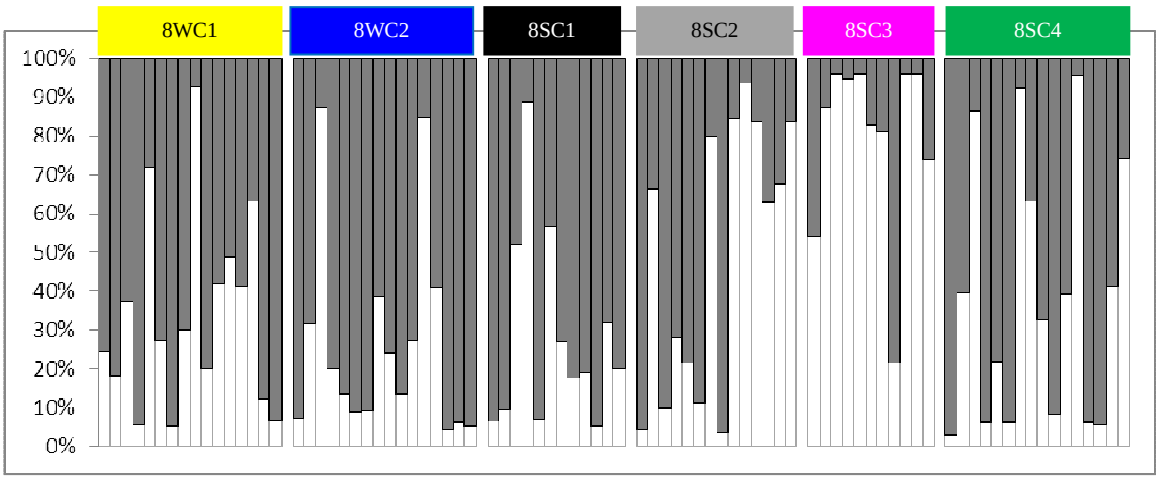
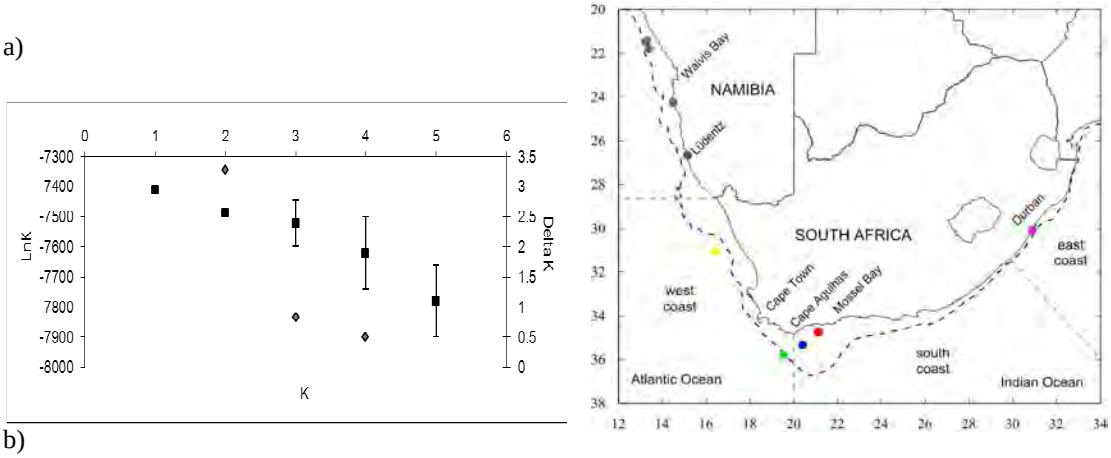


Figure 4.9: Simulation summary from Structure analysis of the 2008 sardine showing a) the likelihood or (LnK and s.d., black squares) for each value of K and ΔK (grey diamonds) ad hoc statistic Delta K. b) Average of three Structure runs for K=2 showing individuals assigned proportionally to cluster (K) 1 and 2. Each individual is represented by a single vertical bar. The sampling sites are in order from west to east and each is separated by an empty column. The coloured bar above each section corresponds to the coloured of the site on the inserted map, 8WC1 in yellow, 8WC2 in blue, 8SC1 in black, 8SC2 in grey, 8SC3 in pink and 8SC4 in green.

Structure results for 2009 did not allow clear differentiation between K = 1 and K = 2, but LnK is largest for K = 1 (Figure 4.9a). For K = 2, few individuals were assigned with more than 80% to either cluster (Figure 4.10b).

a)



b)

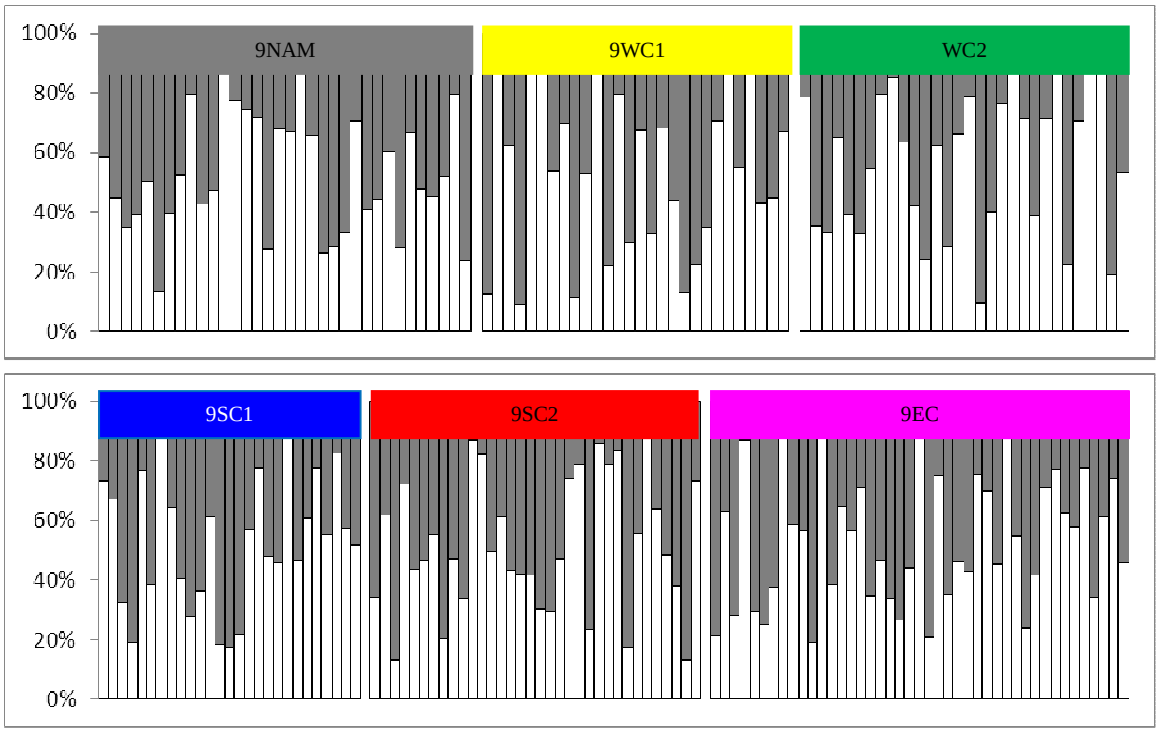


Figure 4.10: Simulation summary from Structure analysis of the 2009 samples showing a) the likelihood or LnK (s.d, black square) for each value of K and ΔK (grey diamond) ad hoc statistic Delta K. b) Example of Structure output of K = 2 showing individuals assigned proportionally to clusters (K = 2). Each individual is represented by a single vertical bar. The sampling sites are in order from west to east and each is separated by an empty column. The coloured bar above each section corresponds to the colour of the site on the inserted map. All Namibian samples are grouped together.

Discussion

Otolith shape was used to differentiate between sardine found on the east coast and the rest of South Africa and Namibia (Chapter 2), and otolith microchemistry was used to identify small scale differences among sites in South Africa (Chapter 3). Although these differences indicate that sardines could be spending significant periods of their life in different environments, it does not necessarily mean that there is genetic isolation between regions. This chapter used ND2 mitochondrial DNA and seven microsatellite markers to investigate whether there was genetic differentiation in sardine around southern Africa. The findings revealed a large amount of individual variability and no significant difference among coasts in either mitochondrial DNA or microsatellite loci. However, there is a suggestion of west-south differentiation and microsatellite loci provided evidence of differentiation between years, and among sites on the south coast in 2008.

There was a high degree of haplotype variability and allelic diversity across all sampling locations in all three years, as expected for a small pelagic species such as sardine. Many of the haplotypes were found in only one sample, although the chosen marker is one of the slower evolving regions of the mtDNA. An excess of low frequency haplotypes and statistically significant deviation from neutrality were confirmed, both with Tajima's D and Fu's Fs statistics (Tajima 1989, Atarhouch *et al.* 2006). Negative values of Tajima's D and Fu's Fs are likely to indicate demographic expansion (Tajima 1989), possibly from the small founding population that colonised South Africa from Australia and New Zealand 200 – 500kya (Kasapidis 2014). Previously, it was found that, for the southern African sardine populations, the nucleotide diversity (π) and haplotype diversity (h) using the mtDNA control region were $h = 0.004$ (n = 15) and $\pi = 1$ respectively (Bowen and Grant 1997). The same study also reported that the nucleotide diversity (π) and haplotype diversity (h), using the mtDNA cytochrome b gene, was $\pi = 0.02$ (n = 15) and $h = 0.62$, respectively (Bowen and Grant 1997, Grant and Leslie 1996). The high haplotype diversity shown in the current study mirrors their findings and can be explained by frequent fluctuations in population numbers (Bowen and Grant 1997).

New haplotypes can emerge in response to population expansions after a bottleneck event (Avise *et al.* 1984). Lecomte *et al.* (2004) explained the high haplotype diversity (between 0.83 and 0.92) they found in the cytochrome b region of Californian sardine by suggesting that, during unfavourable conditions, sardine retreat to small pockets or basins (MacCall 1990, Bakun 2010). The population expands again when conditions improve. Although the time scales for fisheries survey data are much shorter than what is recorded in genetic processes, they suggest a similar life history strategy for the South African sardine (Coetzee *et al.* 2008, de Moor *et al.* 2011).

The mitochondrial DNA AMOVA results revealed that for both 2007 and 2009 most variability is at the level of the individual. When the 2009 samples were separated into Namibian and South African groups, the highest among-sites variance (2.63%) was detected. However, this was still much less than the within-sites variation. This suggests that there could be differentiation between the Namibian and South African sardine, but this is not discernible statistically, given the current sample size.

The microsatellite loci that were used in this study were developed in conspecific sardine from the Pacific (Pereyra *et al.* 2004) and, in that study, high expected heterozygosity (between 0.794 and 0.959) was reported, which is similar to what was found in this study. There was also a similarity in the large number of alleles that were found in both studies. The high levels of observed heterozygosity and mean number of alleles are characteristic of species with large populations and the high mutation rates associated with microsatellite loci (Hauser and Ward 1998).

Overall F_{IS} calculated from the seven microsatellite loci suggests that there is an excess of homozygotes, which could have been influenced by the presence of null alleles. The null alleles observed in this study were most likely caused by missing information at the individual sample level that could be a result of degraded DNA or problems with the PCR. Although null alleles have the potential to homogenise results, previous studies have found that up to 25% of null alleles is acceptable

(Chapuis and Estoup 2007) and the POWSIM analysis showed that there was sufficient power in the data collected for this study to calculate F_{ST}.

Large allelic diversity decreases the capacity of F_{ST} analyses to detect genetic structure (Nauta and Weissing 1996). F_{ST} of marine fish are known to be low; in a review of marine fish genetic studies, Ward *et al.* (1994) showed an average F_{ST} of 0.062 using allozymes, while a median F_{ST} of 0.02 was reported by Waples (1998), again, using allozymes. In the current study, the overall F_{ST} (0.007) indicates limited genetic differentiation but with variation across years. However, F_{ST} in 2008 (0.037) was in the range of what would be expected when there is population differentiation. Given that sardines have large populations, are highly mobile and live in a highly variable and unpredictable environment, it is expected that they will have low F_{ST} values and high levels of individual variability.

Results from the program Structure provide some evidence of geographic differentiation among sites in 2008. In the 2007 Structure analysis, there is most likely a single population. There are no significant pairwise F_{ST} values in 2007 and the AMOVA results suggest that the most important level of variation is that of the individual rather than any regional grouping; this was confirmed by spatial autocorrelation analysis. Similarly, in 2009 there were no significant differences in pairwise F_{ST} values between sampling sites. The 2009 samples cover the broadest geographic range and have the largest sample sizes within each site and are therefore likely to be the most robust results of the three years. The AMOVA results reiterate that the majority of the variance found is at the level of the individual. However, there was evidence of non-random genetic structure in the spatial autocorrelation analysis, although this was limited to individuals that were within 200km of each other, which includes WC2, SC1 and SC2. The Structure results do not differentiate well between one or two clusters. However, the majority of individuals are associated with a single cluster with a high degree of mixing. There is some differentiation in the FCA plot with the west coast and south coast separating from Namibia. This suggests that there could be weak differentiation that is geographically defined but that is not significant enough to affect the pairwise F_{ST} values or the Structure output with current sample sizes. Again, the instability of the environment in which sardine in South Africa live

as well as the large population numbers and mobility will affect the potential to identify significant geographic differences and result in a large amount of individual variability.

The only evidence of genetic differentiation among sites was found in 2008. There were pairwise differences among sites, and the AMOVA identified that there were significant differences among sites within regions. Spatial autocorrelation analysis showed that there was non-random genetic structure among individuals within 500km of each other. Similarly, Structure results identified two clusters, and individuals at two of the south coast sites were more likely to be assigned to the second cluster than individuals on the west coast were (although there was a substantial amount of mixing between the two clusters across all sites). Such a differentiation pattern, where divergence is observed in a pair of sampling sites but not for the entire region, could be linked to sweepstakes events or microgeographic structuring relating to individual breeding success, rather than geographical structuring. Thus, the weak genetic signal at certain sites in 2008 could indicate that a small number of adult fish are disproportionately successful at breeding, and are therefore responsible for a larger proportion of the genetic stock in subsequent years (Hedgecock 1994).

In a similar study on *Sardinops sagax* in Mexico using the mtDNA control region, haplotype diversity was also high (h = 0.9999) (García-Rodríguez *et al.* 2011). In that study, the authors described microgeographic population structure as “chaotic patchiness” (Hedgecock 1994), in which certain individuals are more reproductively successful than others. This was also reported for the Californian anchovy (*Engraulis mordax*, Hedgecock *et al.* 1994). The importance of big, old, fat, fecund, female fish (BOFFFFs) to overall recruitment success has been recorded in multiple species, including clupeids (Hixon *et al.* 2013). Fecundity has been related to body weight in sardine (Ganias *et al.* 2014) and could contribute to chaotic genetic patchiness or a sweepstake recruitment pattern.

In this study, the lack of west-east geographic structure and limited differentiation indicated by the pairwise comparisons in 2008 suggest possible microgeographic structuring in southern African *Sardinops sagax* that is not temporally stable. Sardine

have large populations and are mobile, which will cause any possible geographic patterns of genetic differentiation to be diluted and difficult to detect. Previous studies on southern African sardine showed a similar pattern of a large number of low frequency haplotypes (Bowen and Grant 1997).

A good way to test for the occurrence of sweepstake events would be to use the eggs of sardine from geographically distinct areas, sampled periodically. Eggs are less mobile, and particularly early stage eggs are more likely to have been spawned nearby. If the sweepstakes hypothesis is correct for sardine, one would expect the eggs sampled in the same area to be closely related. This signal would not necessarily be temporally stable. Alternatively, the clumps of genetically similar individuals within sample sites can be as a result of school fidelity (Russell *et al.* 2004). This would need to be tested with tagging studies.

In both mitochondrial DNA and microsatellite markers, the majority of variation was explained by individual differences. In highly variable populations with high levels of allelic and haplotype diversity, such as these, it could be that the relatively short time since colonization of the area has only allowed for shallow divergence (Grant and Bowen 1998) within the area, and ancestral polymorphism is still evident in the population. This, combined with a large effective population size, can result in high levels of genetic mixing. Over the three years, there is no evidence of biogeographic structuring, and only in 2008 is there evidence of small scale geographic structure on the south coast.

These results suggest the possibility of multiple populations with a large degree of genetic mixing between them. Results from modelling studies by De Moor and Butterworth (2013) suggest a high degree of mixing between the putative western and southern stocks, with over 60% of western recruits moving into the southern stock in some years. The geographic differentiation is not consistent over time. The high degree of genetic variability suggests that the population will be less sensitive to environmental change because there is a reservoir of genetic resources (Waples 1998). However, if there was genetic patchiness in 2008, it would be worth further investigation to see if these were isolated sweepstake events or if there is something

particular to that area that prevents mixing with the greater population. Unfortunately, this area was not sampled in 2007 and 2009 and, therefore, there is no way of knowing if this area is consistently different as a result of local oceanographic conditions, or if it was a once-off sweepstake event.

Management strategies need to protect both the genetic diversity and the phenotypic adaptations to local environmental variations (Hauser and Carvalho 2008). Any investigation into stock differentiation should use a multifaceted approach. The South African sardine fisheries operational management procedure is being updated, and it acknowledges the possibility of more than one stock off the South African coast (van der Lingen 2011). The possibility of school fidelity or microgeographic structuring on the south coast in the South African sardine population should be taken into account, particularly if it is as a result of an identifiable portion of the population being disproportionately successful (for instance, BOFFFFs) or spatially and temporally stable, and this is worth further investigation.

Chapter 5

*Preliminary investigations using microsatellite loci hint at geographic breaks in gene flow for anchovy (***Engraulis encrasicolus***) off South Africa*

"Anchovies are fish that seem to be very smart; many of them are business men and nerds."

http://en.spongepedia.org/index.php?title=Anchovy

Chapter 5: Preliminary investigation of genetic differentiation using microsatellite loci hints a break in gene flow between sites for anchovy off the coast of South Africa

Abstract

Anchovy (*Engraulis encrasicolus*) is a commercially important fish species in South Africa. It has been suggested that, when there is a population decrease, the distribution of anchovy becomes divided on either side of the Agulhas Bank. In order to investigate if this distribution change corresponds to a break in gene flow, a total of 52 anchovy was collected from four sites during the 2009 Pelagic Spawner Biomass survey, to test for geographic genetic differentiation. Of the six microsatellites initially used, one was excluded due to missing data. Observed and expected heterozygosity were 0.45 and 0.77 respectively. This, together with a positive F_{IS} (0.41), suggests an excess of homozygotes and null alleles. Pairwise F_{ST} results were not conclusive, possibly due to the small sample size, but suggest some differences among sites, although not between coasts. AMOVA results suggest that the greatest variability was due to individuals and, among individuals, within coasts. Factorial Correspondence Analysis and Structure outputs suggest a level of between-site differentiation that does not follow a stepping-stone model. Although limited, the present results indicate the presence of genetic differences among anchovy along the South African coastline, which could be linked to school fidelity or a sweepstake recruitment effect.

Introduction

The purse seine fishery mainly targets sardine and anchovy in South Africa (Barange *et al.* 1999). The anchovy fishery targets recruits that are less than a year old, which are used to produce fishmeal (Huggett *et al.* 2003). As such, the majority of anchovy are caught as juveniles off the west coast in winter (DAFF 2012). When the sardine fishery declined in the early 1960s, anchovy replaced it as the dominant fishery until the mid 1990s. This was followed by an increase in sardine abundance in the mid-2000s (DAFF 2012). In the early 2000s, both sardine and anchovy populations peaked in abundance, bringing in substantial yields for the pelagic fishery: population biomass was estimated to be approximately 7 million tons for anchovy in 2001 and 4 million tons for sardine in 2002. However, both stocks have subsequently declined, and anchovy recruitment in 2011 was the lowest it has been in 14 years (DAFF 2012).

Anchovy are small, pelagic fish, reaching 15 cm total length (TL). They occur along the entire South African and Namibian coasts (Hutchings *et al.* 2002, Barange *et al.* 1999, Fairweather *et al.* 2006). Anchovy, like sardine, experience large population fluctuations and changes in annual recruitment success (Fairweather *et al.* 2006). The species is known to aggregate on the west coast as juveniles, migrating southwards, past Cape Point, before they reach one year, with spawning taking place on the Agulhas Bank (Huggett *et al.* 2003). Each female spawns every 11 to 14 days and has the potential to release between 110 000 and 160 000 eggs per year (Huggett *et al.* 1998). They spawn at night while dispersed within the near-surface layers, predominantly on the western Agulhas Bank, between October and February, peaking in November (Hutchings *et al.* 2002). The eggs take, on average, three days to hatch (Korrûbel *et al.* 1998) and are primarily found between Cape Point and Cape Agulhas. They are carried towards the west coast in a strong jet current (Nelson and Hutchings 1987). There was an abrupt, persistent shift in relative biomass distribution in 1996, from over 50% biomass west of Cape Agulhas to over 50% biomass east of Cape Agulhas (Roy *et al.* 2007).

In the 1990s, Barange *et al.* (1999) showed that, in years when the population was small (1995, 1996, 1997), two stocks of anchovy seemed to exist on either side of the Agulhas Bank. This stock separation was not evident in years when the population was at its maximum (1991, 1992) (Barange *et al.* 1999). It is uncertain whether anchovy found on the south coast, east of Cape Agulhas, form an independent stock from those on the west coast. During periods of increased biomass, anchovy expand their geographic range (Barange *et al.* 1999) and spread out over a larger area to feed, unlike sardine. This is likely to be a result of differences in feeding strategies. Sardine are primarily filter feeders and, therefore, are not negatively affected by increased densities, whereas anchovy are particulate feeders and a minimum distance between individuals is required to ensure feeding is not impeded (Barange *et al.* 1999).

In recent years, sardine have been the subject of extensive research into stock structure (e.g. Twatwa *et al.* 2005, Coetzee *et al.* 2008, van der Lingen 2011). However, little research has been conducted on anchovy stock structure, despite the fact that anchovy could be more likely to show stock structure because of its particular life history features: short life spans (approximately three years), short spawning periods and recruitment closely correlated to a single cohort's spawner biomass (in contrast to sardine recruitment, which results from multiple year classes) (Barange *et al.* 1999). Grant (1985b) investigated the stock structure of anchovy in southern Africa using allozymes, and found low levels of genetic differentiation but no associated phylogeographical patterns. More recently, and employing microsatellite markers, studies on *E. encrasicolus* in the northern Atlantic showed genetic differentiation between the Mediterranean and the Bay of Biscay (Bembo *et al.* 1995), and between the western Mediterranean and eastern Atlantic (Sanz *et al.* 2008). These findings highlight the advantages of employing faster-evolving markers, such as microsatellites, as they allow the identification of genetic structure that was previously not evident using mitochondrial DNA (Hauser and Seeb 2008), and over a finer geographic scale (Chistiakov *et al.* 2006). As such, this chapter represents a preliminary investigation into geographic genetic differentiation of anchovy in South Africa, in order to understand if the observed population division has a corresponding genetic break and, consequently, whether there is cause to further investigate a potential west coast - south coast divide, such as the one hypothesised for sardine.

Methods

Sample collection

Samples were collected in 2009 by pelagic trawls during the annual Department of Agriculture, Forestry and Fisheries (DAFF) Pelagic Spawner Biomass (PSB) survey onboard the *RV Africana*. The survey took place from the end of October until the beginning of December. The samples were collected from four sites: one on the west coast (WC1), and three on the south coast (SC1, SC2 and SC3) (Table 5.1, Figure 5.1). Whole fish samples were immediately frozen in a -20ºC freezer on board the *RV Africana* and then moved to a -70ºC freezer at the University of Cape Town for further processing. Samples were limited to reproductively mature fish (based on gonad stage), greater than 10cm caudal length (CL).

Table 5.1: Anchovy samples collected during 2009. The summary includes DAFF trawl codes (where applicable), site code (summary of coast, sample number), number of individuals collected per trawl used in microsatellite analysis (N), longitude, latitude, and the date of sample collection. Sites are labelled in ascending order from west to east within each region.

Site	Site code	N	Longitude	Latitude	Date of collection
	2009	**52**			
19-11A	WC1	12	34º51.76' S	18º27.98' E	23-Oct-2009
29-09A	SC1	15	35º20.70' S	20º24.60' E	01-Nov-2009
37-01A	SC2	12	34º10.46' S	22º20.28' E	09-Nov-2009
44-09A	SC3	13	34º41.39' S	24º28.86' E	13-Nov-2009

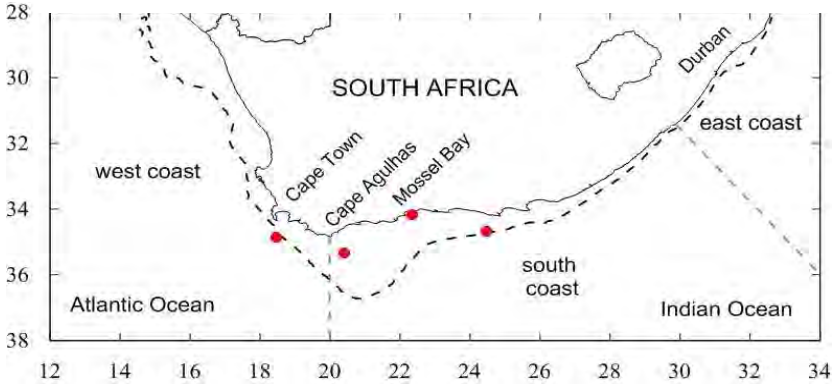


Figure 5.1: Sampling localities for anchovy in 2009. Sites from west to east are WC1, SC1, SC2 and SC3. The divisions between the west coast and south coast are shown with the grey dashed lines (regional divisions are defined by the South African Department of Agriculture, Forestry and Fisheries). The 200m depth contour is shown with the black dashed line.

Microsatellite genotyping

Total genomic DNA was extracted using the GenElute^{TM} Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich) using the manufacturer's standard protocol for mammalian tissue preparation. Following extraction, DNA was visualised on a 1% agarose gel (Roche Diagnostics) with GelRed^{TM} acid stain (Biotium) (see Chapter 4). The quality and quantity of DNA varied greatly between individuals.

All but one of the primer pairs used in this study were developed for Japanese anchovy, *Engraulis japonicus* (Chiu *et al.* 2002, Chiu *et al.* 2008 (unpublished)); An25_6 was developed for European anchovy, *E. encrasicolus* (Zampicinini and Magoulas, unpublished). The details of the primers, including the primer sequence, repeat motif, GenBank accession numbers and name of fluorescent dye used in this study are summarised in Table 5.2. Microsatellite loci were amplified by a Polymerase Chain Reaction (PCR) using the Quantitect multiplex kit (Qiagen®) according to the manufacturer's protocol. Primers were combined into one of two multiplex mixes (Table 5.2) using the same protocol for amplification as outlined in Chapter 4. In cases when individual loci did not amplify they were redone using a different combination of primers or were run alone using the Qiagen® multiplex kit

and then loaded together. Allele sizes were scored using GeneMarker v.1.8 software (SoftGenetics LLC, 2008). Individuals missing information for more than two loci were excluded from all analyses. Micro-checker v2.2.3 (van Oosterhout *et al.* 2004) was used to assess the occurrence of amplification errors, such as large allele dropout rates, null alleles and stuttering.

Table 5.2: Summary of anchovy primer information including primer names, the forward (F) and reverse (R) primer sequence, repeat motif and GenBank accession number (AN). The name of the fluorescent dye (DYE) used with each primer is also included. The letters in superscript indicate the multiplex combinations.

Name	Primer Sequence 5' - 3'	Repeat Motif	AN	DYE	Reference
An25_6[1]	F: GCT GCA CAT CAT GTC TCC TC R: CTG CCA CCA CAC AGT AAG TC	(CA)14	AJ609587	6-FAM	Zampicinini and Magoulas (Unpublished)
EJ43[1]	F: CCT CTG ACT GGC TGG TGA TG R: GCC GAT CCA GTT CCT GTT CC	(CAG)9(CCG)3(CAG)2	EU984142	VIC	Chiu *et al*. 2008 (Unpublished)
EJ2[1]	F: AGC AAG GGA GCA AAC AAT C R: TGC AAT TTG ACA GAA ACC ACA	(CT)43	AF344655	PET	Chiu *et al*. 2002
*EJ34[2]	F: TGA GCA GCT GGT CAG GTA GT R: ATG GCA CCA CCT AGT GTT CG	(TATC)9	EU984137	6-FAM	Chiu *et al*. 2002
EJ9[2]	F: GCC TTA CCC CTT TAG CCA TT R: GCC CTC CGA GTC GAC ATA GT	(TC)39	AF344656	VIC	Chiu *et al*. 2002
EJ27.1[2]	F: GAC TGT GAA GGA ACG CTG GT R: AAT AGG ATT AGT CAT CAC AGG G	(GA)36	AF344657	NED	Chiu *et al*. 2002

*EJ34 was later excluded from analyses due to missing data.

Microsatellite data analysis

The microsatellite data analyses used in Chapter 4 for sardine microsatellites were largely repeated in the analysis of anchovy. A brief summary is outlined here, but details of each analysis can be found in Chapter 4.

Individuals missing data from more than two loci were excluded from all analyses. Null alleles, scoring error and large allele drop out were calculated in Micro-Checker v.2.2.3 (van Oosterhout *et al.* 2004). The frequency of null alleles was calculated in FreeNA (Chapuis and Estoup 2007). LOSITAN was used to test whether loci were selectively neutral, running the simulation with a neutral F_{ST} for 5 000 runs (Beaumont and Nichols 1996, Antao *et al*. 2008). Linkage disequilibrium was tested

using Genetix v.4.03 (Belkhir *et al.* 1996-2004). Deviations from Hardy-Weinberg Equilibrium (HWE) were tested in Arlequin v.3.11 (Excoffier *et al*. 2005) using an algorithm modified from Guo and Thomson (1992), with 1 million Markov Chain Monte Carlo (MCMC) steps and 100 000 dememorisation steps. POWSIM v.4.1 (Ryman and Palm 2006) was used to test the power of the data set to detect population differentiation for three F_{ST} values (0.0025, 0.025 and 0.049) for two populations (N = 15 and 12 individuals) over 1000 simulation runs.

The mean number of alleles, allelic richness, observed and expected heterozygosity were calculated for all loci. Overall F_{ST}, F_{IS} and F_{IT} were also calculated in Genetix v.4.05.2 (Belkhir *et al.* 1996 – 2004). Population differentiation among sampling locations was measured with pairwise F_{ST} calculated in FSTAT, while not assuming HWE, and statistical significance was assessed after 1000 permutations (Goudet 2001). The obtained result was subsequently compared to pairwise F_{ST} calculated to account for null alleles in FreeNA (Chapuis and Estoup 2007). In order to test if there is a significant differentiation between west and south coasts, a hierarchical analyses of molecular variance (AMOVA) was performed using Arlequin v.3.11 (Excoffier *et al.* 2005), with statistical significance assessed after 10 000 permutations. The relationship between genetic and geographic distance was investigated using spatial autocorrelation analysis in GeneAlEx v.6.0 (Peakall and Smouse 2006).

Factorial Correspondence Analysis (FCA) in Genetix v.4.03 (Belkhir *et al.* 1996-2004) was used to visualise individual and among site differentiation. Structure v2.3.3 (Pritchard *et al.* 2000) was used to detect structured population genetic variation among individuals. The analysis was the same as that used in sardine and details are described in Chapter 4. The most likely number of clusters (K) was estimated, and individuals were then proportionately assigned to clusters based on the average of three runs.

Results

Amplification varied among loci, and missing data was a problem in all but one of the loci (EJ27.1). The percentage of missing information in the data set that was used in all analyses is shown in Appendix 5.1. Micro-Checker did not find any evidence of stutter or large allele drop out, but there was a suggestion of null alleles, as evidenced by an excess of homozygotes; this varied among loci within sites (Appendix 5.2). The frequency of null alleles per site for each locus is shown in Appendix 5.3. All loci were selectively neutral (Appendix 5.4).

When tested for linkage equilibrium between loci, An25_6 and EJ43 were significantly linked in some, but not all, sites. However, as neither of these was linked with any of the other loci nor with any consistent pattern, they were still included in the analysis (Appendix 5.5). Although there were deviations from HWE, it was not consistent across all sites for all but two of the loci (EJ2 and EJ9, Appendix 5.2). However, EJ34 was missing information in 66% of the individuals and was therefore excluded from further analysis. POWSIM calculated that there was 77.9% chance of detecting differentiation at an F_{ST} of 0.0245, and 99% probability of detecting differentiation of F_{ST} 0.049 or higher.

Gene diversity was high in all sites (Table 5.3a) and in all but one locus (EJ43, Table 5.3b). Expected and observed heterozygosity were moderately high (H_E = 0.77 and H_O = 0.45). The H_E was consistently higher than H_O for all loci and F_{IS} was positive for all loci (Table 5.4). The mean of F_{IS} was 0.415 (99% C.I.: 0.39 – 0.45) and of F_{IT} was 0.429 (99% C.I.: 0.41 – 0.45), with both metrics indicating a consistent homozygosity excess. Overall F_{ST} was 0.024, varying between -0.003 – 0.046, and was statistically significant ($P < 0.05$).

Table5.3: The F_{IS} per site, total number of samples (N), allelic richness (AR), mean number of alleles per locus (MNA), expected (HE) and observed (HO) heterozygosity for a) each sampling site and b) each locus used in the anchovy microsatellite analyses are shown.

a)	**Site code**	**F_{IS}**	**N**	**AR**	**MNA**	**H_E**	**H_O**
	WC1	0.257	12	3.79	9.0	0.74	0.59
	SC1	0.438	15	3.36	7.6	0.68	0.42
	SC2	0.481	12	3.29	7.0	0.66	0.37
	SC3	0.453	13	3.80	8.0	0.79	0.46
b)	**Locus**	**F_{IS}**	**NA**	**AR**	**MNA**	**H_E**	**H_O**
	An25_6	0.422	21	6.22	8.5	0.88	0.52
	EJ43	0.420	6	2.02	2.5	0.20	0.10
	EJ2	0.422	20	7.94	10.0	0.94	0.54
	EJ9	0.428	21	7.67	9.5	0.90	0.55
	EJ27.1	0.436	24	7.87	9.0	0.95	0.58

Pairwise F_{ST} values between the different locations are shown in Table 5.5. There were no significant differences between pairs of sites when calculated in FSTAT and not assuming HWE. There are also no significant differences when the homogenising effect of null alleles is taken into account. There was no significant difference between the west and south coasts (F_{ST} = 0.012, P=0.25).

Table 5.5: Pairwise comparisons based on five anchovy microsatellites calculated while not assuming HWE in FSTAT (below the diagonal) with significance corrected for multiple comparisons (P < 0.008) and F_{ST} calculated to incorporate the effect of null alleles (above the diagonal).

	WC1	SC1	SC2	SC3
WC1	12	-0.008	-0.004	-0.012
SC1	0.208	15	-0.007	-0.017
SC2	0.175	0.125	12	-0.003
SC3	0.092	0.183	0.125	13

Similarly, the AMOVA results did not detect significant differentiation between the south and west coasts, as the higher percentage variation was observed among individuals within groups (51.89%, p<0.05) and not between groups (0%, p>0.05) (Table 5.6).

Table 5.6: Summary of anchovy microsatellite AMOVA with degrees of freedom (d.f.), sum of squared deviation (SS), percentage variation, fixation index (F) and its P value are shown for each source of variation.

Source of variation	d.f.	SS	% variation	F	P Value
Between west and south coast	1	0.09	-2.22	F_{CT} : 0.002	0.742
Among sites within west and south coast	2	0.34	0.89	F_{SC}: 0.009	0.384
Among individuals within west/south coast	48	7.15	51.89	F_{IS}: 0.512	0.003
Within individuals	52	2.50	49.44	F_{IT}: 0.506	0.000

Spatial autocorrelation analysis was run multiple times with variable distance size classes. In all cases, the correlation coefficient, r, was only significant when geographic distance was less than 200km (Figure 5.2). Since the sampling locations were all more than 200km apart, this only occurs when individuals were sampled from the same location (Figure 5.2).

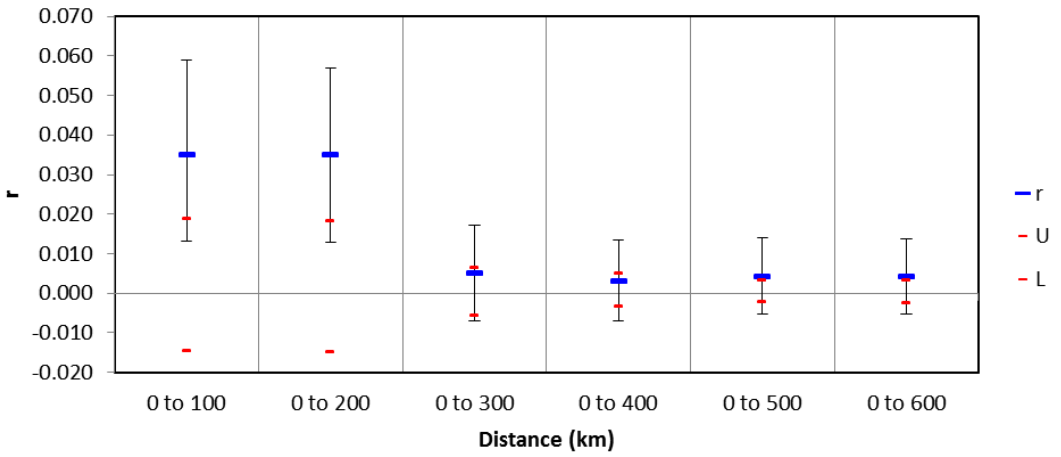


Figure 5.2: Spatial autocorrelation (r) of anchovy microsatellite data when the first distance class was increased in increments of 100km. Error bars represent 95% confidence intervals by bootstrapping over pairs of individuals and the upper (U) and lower (L) 95% confidence intervals are shown in red.

When the FCA was run with no sample site prior, the first three axes only explained 14.15% of the data and, except for two individuals, there was extensive overlap (Figure 5.3a). When the sampling region was included as a prior factor, the first three factors explained 100% of the variance and each of the sample sites was distinct from

the others in three-dimensional space, with limited overlap between SC1 (blue) and SC3 (grey) (Figure 5.3).

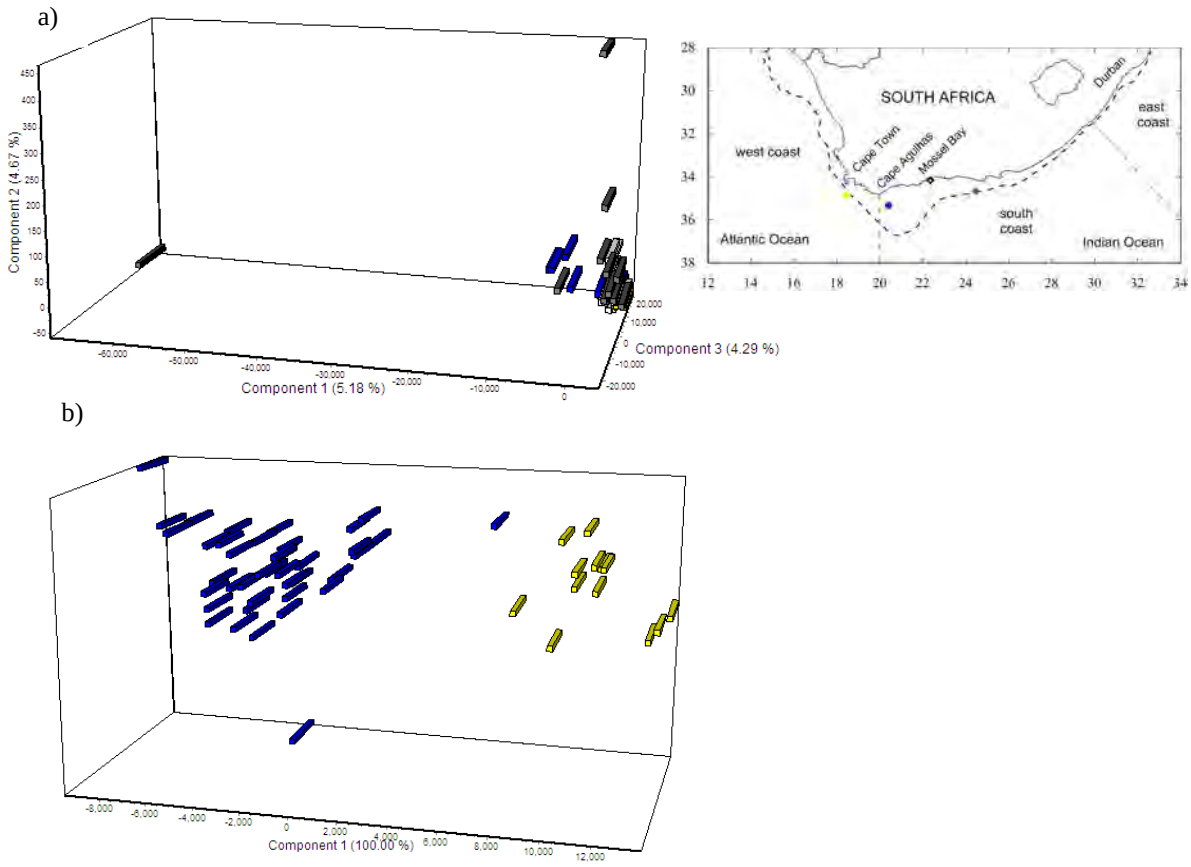


Figure 5.3: Factorial correspondence analysis based on variation at five anchovy microsatellite loci for four sites along the South African coast; each bar represents an individual sample. a) FCA as calculated with no prior information included, and b) with coasts (WC in yellow, SC in blue) as prior information. The map alongside shows each study site in the colour that corresponds to the FCA analysis: WC1 in yellow, SC1 in blue, SC2 in white and SC3 in grey.

The Structure simulations were run with the four sampling sites with no prior information. This analysis detected two possible groups (K), as estimated from the highest mean value with lowest standard deviation of log K (LnK) and *ad hoc* Delta K (ΔK). The majority of individuals assign to the grey cluster but individuals from SC1 and, particularly inshore site SC2, assign to the white cluster (Figure 5.4).

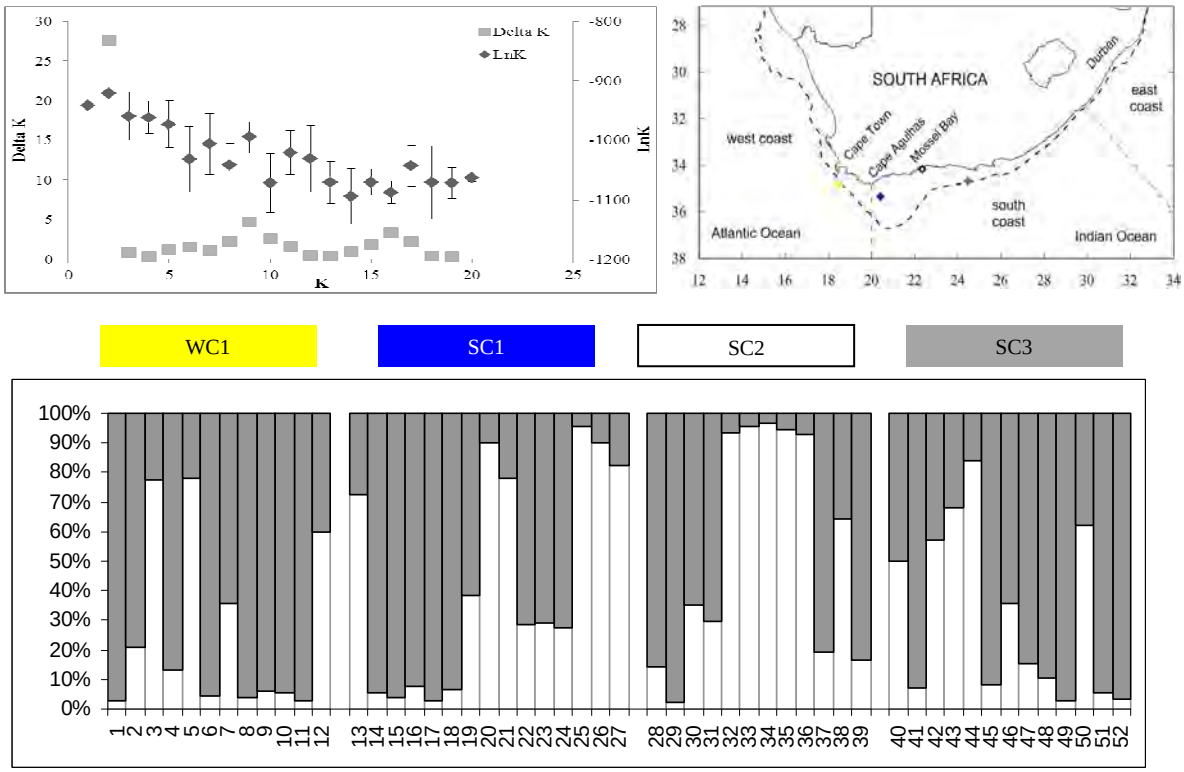


Figure 5.4: Simulation summary from Structure analysis of the five anchovy microsatellites showing; a) LnK with standard deviation and Δ*K* without site information in the simulation n b) The average assignment for three Structure runs at K=2 is shown in the simulation with no prior. Each individual is proportionally assigned to one of two clusters (arbitrarily white or grey) and is represented by a single vertical bar. The sampling sites are ordered from west to east, each site is separated from the next with a blank bar. The colour of the rectangle above the graph indicates the position of the site on the inserted map. The sample sites are shown on the map insert.

Discussion

Although Grant (1985b) reported a panmictic population of anchovy off the southern African coastline, there have since been numerous advances in genetic techniques. Other studies have shown geographic differentiation of anchovy in the Mediterranean (*Engraulis encrasicolus*, Spanakis 1989, Bembo 1996, Magoulas *et al*. 1996, Grant 2005, Magoulas *et al*. 2006, Sanz *et al.* 2008, Silva *et al*. 2014), North America (*E. mordax*, Hedgecock *et al.* 1989 and 1994) and Japan (*E. japonicus*, Funamoto and Aoki 2002, Yu *et al.* 2002). The current study investigated population structure within

South African anchovy (*E. encrasicolus*) using microsatellite loci and identified a possible break in gene flow between west and south coasts, particularly between an inshore site on the south coast and the remaining offshore sites (evident from FCA and Structure results).

The majority of primers that were used in this study were developed for Japanese anchovy (Chiu *et al.* 2002) to investigate potential genetic differentiation between anchovy spawning in the Pacific Ocean and those in the Taiwan Strait (Yu *et al.* 2002). That study revealed high levels of genetic variability and between 25.5 and 32.3 alleles per locus (Yu *et al.* 2002). In the current study, the mean number of alleles per sampling site was much lower (7 – 9), but so were the sample sizes. As such, it is likely that, if more samples had been obtained, the number of alleles would have increased. High levels of microsatellite diversity have been reported in *E. encrasicolus* microsatellite studies, which those authors attributed to the anchovy's ability to disperse quickly *en masse* to optimal habitats (Silva *et al.* 2014).

There was a high level of homozygosity found in the present study, which is likely a result of the null alleles present in the data set. Overall F_{IS} results suggest an excess of homozygotes, which could have been influenced by the presence of null alleles and missing information from failed PCRs. The possibility of null alleles can be higher because the microsatellite markers weren't developed in this species, and thus the PCR amplification could fail to detect certain alleles. However, null alleles previously have been reported as a common feature of *E. encrasicolus* microsatellite analyses (Zarraoindia *et al.* 2009, Silva *et al.* 2014), and An25_6 was developed in *E. encrasicolus* and still has a high F_{IS} value.

The reported F_{ST} was within the range reported for marine fish (Ward *et al.* 1994, Waples 1998), and similar to that found for European sprat (*Sprattus sprattus*), a small pelagic fish that occurs in the North Baltic Sea and in which geographic structure was found (Limborg *et al.* 2009). AMOVA results showed variation among individuals within the west and south coasts, as well as at the level of individuals, but not between sites. Similarly, spatial autocorrelation analysis showed no spatial structuring among sites. F_{ST} calculated in FreeNA showed some differentiation among

sites. However, the results were ambiguous, possibly a result of the small sample size, although POWSIM did calculate that there was 77.9% chance of detecting population differentiation at an F_{ST} of 0.02.

The Structure output confirmed that there was more than one genetic cluster among the samples, but with mixing occurring among sites. There was some evidence of differentiation between the west and south coasts from Structure results. All four samples showed some degree of mixing. However, the inshore sample off Mossel Bay showed a greater proportion of a cluster that was not well represented in any of the other samples.

Borell *et al.* (2011) found that, in a study of breeding success in an anchovy aquaculture facility, there was a disproportionate success of a relatively small number of individuals contributing to the next generation. This has inbreeding consequences in the relatively small gene pool of an aquaculture facility, but in the wild can contribute to genetic differentiation between schools, as certain individuals dominate others. This genetic chaotic patchiness is commonly found in small pelagic fishes (Lecomte *et al*. 2004) and there is some evidence of this in sardine in South Africa (Chapter 4). A similar "sweepstake recruitment" (or Whalund effect) is possible for South African anchovy, and can result in genetic differentiation between sites that are geographically close. This would be best tested by using larvae or eggs to analyse genetic differentiation of natal sites.

If statistical differentiation were found among sites, it could suggest a level of school fidelity in anchovy. Previous studies have provided evidence for school fidelity among fish, including anchovy, and associated morphological and behavioural differences within schools (Tsukamoto and Tsukamoto 1999, Somarakis and Nikolioudakis 2010, Ospina-Alvarez *et al.* 2012). School fidelity is an important anti-predator behaviour (Zheng *et al.* 2005) and is aided by oceanographic currents (Ospina-Alvarez *et al.* 2012). Alternatively, genetic differentiation can reflect recruitment events and, thus, different age-classes (Zarraonaindia *et al*. 2009). This could only be determined with larger sample sizes from more sites and, given the temporal instability found in sardine (Chapter 4), multiple years.

This was an initial investigation into genetic differentiation of anchovy in South Africa, and the first time that microsatellite markers have been used on anchovy in the country. However, the numbers of individuals and numbers of markers were limited, and the results should thus be treated with caution. There was some indication of geographic differentiation that should be investigated further. A further 14 loci have been developed in the Japanese anchovy (Lin *et al.* 2011) and in the conspecific European anchovy (six loci in Landi *et al.* 2005 and 11 in Pakaki *et al*. 2009), which could be used for further analyses. Some initial attempts to use Ee2, Ee10, and Ee16 (Landi *et al.* 2005), as well as EJ41.1 (Chiu *et al.* 2002), were met with limited success and further optimisation was beyond the scope of this project.

In December 2011 at a local workshop to review operational management procedures for small pelagic fishes (van der Lingen 2011), it was recognised that there are likely to be two mixing sardine stocks in South Africa. This hypothesis is based on a suite of studies investigating stock differentiation in sardine. No such broad scale investigation has been undertaken for anchovy, but the present study suggests further work is required, including a multidisciplinary investigation into stock differentiation. Recently, single nucleotide polymorphisms (SNPs) have been successful, in conjunction with mitochondrial and microsatellite data, to elucidate stock structure in the Atlantic and Mediterranean (Zarraonaindia *et al*. 2012, Silva *et al.* 2014) and to identify population expansions in the North Sea (Petitgas *et al*. 2012), They should be an important additional tool in further genetic analysis of anchovy in South Africa.

Chapter 6 Synthesis:

Variable patterns of stock structure in a dynamic environment

"… there are a number of valid approaches to characterize or to define a stock. The question remains: Is this of any help for fisheries managers? The answer is typically scientific: yes and no."
(Hammer and Zimmerman, 2005 pg 632)

Chapter 6: Synthesis: Variable patterns of stock structure in a dynamic environment

The small pelagic fishery is the largest (by mass) in South Africa and contributes significantly to the economy. However, the 2012 anchovy landings were 40% lower than those reported in 2010. Between 2010 and 2011, domestic demand for canned sardine increased, in contrast to the decline in international demand (FAO 2006 - 2012). Natural fluctuations in the population (van der Lingen *et al.* 2006a, Checkley *et al.* 2009), together with fluctuations in demand, highlight the importance of sustainable management and the need to understand causes of variability. South Africa's Department of Agriculture, Forestry and Fisheries (DAFF) identifies protection of indigenous genetic resources as a strategic goal (DAFF 2011). In order to achieve this goal, we need to understand the resource. In the case of small pelagic fish, this requires an understanding of the extent of phenotypic and genetic variability in the population, and whether or not that variability amounts to more than one stock. A multidisciplinary approach to identifying stocks is required in order to identify phenotypic stocks (Begg and Waldman 1999).

In order to investigate stock differentiation in small pelagic fish of the southern Benguela, the first challenge is to define a population. In this work, the definition provided by Ihssen *et al.* (1981) is used, in which a stock is an intraspecific group of randomly mating individuals with temporal and spatial continuity. However, in a continuum from a metapopulation, where mating is completely random and evenly distributed (panmictic), to fully isolated subpopulations, one needs to determine the point at which subpopulations or stocks are to be considered separate. Waples and Gaggiotti (2006) explore this conundrum in depth. They conclude that the criteria used to define a subpopulation depend on the objective of the study. In this study the objective of determining the existence, or not, of separate stocks is for management purposes, to account for different rates of fishing pressure and productivity between stocks so that the harvest of one area does not negatively impact the ability to harvest another area (Kasapidis 2014).

The current study tested hypotheses of stock division in small pelagic fish (sardine (*Sardinops sagax*), anchovy (*Engraulis encrasicolus*) and round herring (*Etrumeus whiteheadi*)) between biogeographic provinces to the west or east of Cape Agulhas, with an emphasis on sardine.

Stock differentiation of sardine

As discussed in Chapter 1, Coetzee *et al.* (2008) have noted a shift in the relative abundance of sardine on the south coast of South Africa, from west of Cape Agulhas to east of Cape Agulhas. This shift impacted negatively on the economic viability of the region's fishing industry - the majority of processing infrastructure was concentrated on the west coast. Most fish caught to the east of Cape Agulhas (referred to as the south coast) have to be transported to the west coast for processing. Increased landings on the south coast therefore substantially increased transportation costs, increasing the industry's overall production cost. Coetzee *et al.* (2008) proposed three possible causes for the shift:

a) the west coast component of the stock constitutes a functionally distinct unit and has been overfished;

b) a shift has occurred in response to a change in environmental conditions [Roy *et al.* (2007) (anchovy) and van der Lingen *et al.* 2011 (sardine) previously found a shift in distribution of sardine and anchovy to correspond to changing ocean temperatures];

c) the south coast sardine, which are largely retained as eggs in that region, have had proportionately higher recruitment success than those on the west coast.

There is also the possibility of a third stock, off the east coast of South Africa, which travels up the east coast to KwaZulu Natal (KZN) around July every year, in what is known locally as the sardine run. While it does not constitute a major proportion of the industry, it does have a seasonal fishery associated with it. It also supports a large tourist industry, built mainly around the charismatic predators associated with the run (van der Lingen *et al.* 2010a). The economic value of the sardine run is predominately from the tourism it generates, rather than from fish catches.

The possibility of distinct stocks of sardine has been further examined in a number of studies. Off Cape Agulhas, a persistent break in spawner biomass has been found at both low and medium biomass, with overlaps only occurring at high biomass levels. Similarly, based on egg distributions, there are distinct spawning grounds to the west and east of Cape Agulhas (Coetzee *et al.* 2008). A multidisciplinary approach (Figure 6.1) has been implemented to determine whether there is more than one stock. Hydrodynamic models (Miller *et al.* 2006) and composite maps of sardine eggs (DAFF 2012) support the hypothesis of multiple sardine stocks. Morphometric and meristic studies support the hypothesis of multiple stocks in some measures [vertebral counts between sardine-run fish and other South African fish, gill arch length, gill raker spacing (Idris *et al.* submitted) and body shape (Wessels *et al.* submitted)] but not others [(gill raker number) (Idris *et al.* submitted)]. Parasites of the genus *Cardiocephaloides*, found in the humour of fish eyes, and *Eimeria sardinae*, found in fish testes, have been used as biological tags, and suggest geographic variability of infection rates between the west, south and east coasts of South Africa (van der Lingen *et al.* 2014). Spatial differences in relative weight indices have also been recorded (Ndjaula *et al.* 2013). The International Review Panel of the International Stock Assessment Workshop, meeting in Cape Town in December 2011, concluded that, together, these results were sufficient to suggest the hypothesis of two stocks with some mixing is most likely (the east coast stock was not discussed at this workshop) (Smith *et al.* 2011b). Subsequently, the two stock hypothesis has been incorporated into assessment models (de Moor and Butterworth 2013).

In this study, otolith shape analysis revealed some distinction between sardine from the east coast and sardine caught on the west and south coasts (Chapter 2). The shape of sardine otoliths in samples from the east coast was statistically distinct from that of sardine collected elsewhere in South Africa and Namibia, in terms of smoothness of the otolith circumference (*form factor*) (less smooth) and overall roundness (*circularity*) (less round). Sardine otolith shape was also influenced by the environmental conditions that the fish experienced in the previous season. Otoliths that were collected in summer were significantly different to the smoother and rounder otoliths that were collected in winter in all regions. This indicates suboptimal environmental conditions could result in deviation from a smooth, round otolith shape. The finding of no difference between sardine taken from the west and south coasts supports the results of a study on vertebral counts (Wessels 2009, Wessels *et al.* submitted) but is

in contrast to what has been found in previous morphometric and meristic work, where differentiation between west and south coast sardine was evident in morphometric body measurements and gill raker spacings (Figure 6.1, Idris 2009, Idris *et al.*submitted). Both otolith shape and the number of vertebrae are features that are laid down early in life, and are possibly less sensitive to small differences in environmental conditions.

Otolith elemental signatures (Chapter 3) showed small scale differences among sites, but there was no temporal stability to those patterns. There were trends from the west to south coast of increasing (strontium) and decreasing (magnesium and barium) concentrations of elements. There was also evidence of dispersal from the natal region (or change in environmental conditions within the natal region), as evidenced by a comparison of elemental signatures between the edge and core of otoliths, which respectively represent conditions approximately one year after spawning and at the time of spawning. This reflects the dynamic nature of the South African coastal environment, and provides evidence of micro-structuring in populations, which was investigated using genetic analyses in Chapter 4.

Genetic analyses showed high levels of polymorphism in both the mitochondrial ND2 marker and seven microsatellite markers. There was minimal genetic differentiation and little evidence for large scale geographic differentiation between sardine from the west, south and east coasts or from Namibia (which was included as a control). There was evidence of microgeographic structuring. Within certain sites, individuals were more likely to be related to each other than individuals were in other sites, despite the geographic proximity of the sites. This supports a sweepstake hypothesis, in which a small number of adults are disproportionately responsible for successful recruitment, resulting in genetic patchiness (Hedgecock *et al*. 1994).

In order for genetic structure to be maintained into adulthood, there must be some school fidelity to maintain the integrity of the genetic patch within the school. The formation and persistence of genetic patchiness is facilitated by eddies and oceanographic filaments that retain eggs and larvae in a region (Ospina-Alvareza *et al.* 2012). Schooling is an effective

predator avoidance behaviour (Zheng *et al.* 2005, Ospina-Alvareza *et al.* 2012), and is an important characteristic of the small pelagic fishes studied here. The oceanographic features of the south coast are particularly conducive to local retention of eggs and larvae, and clockwise eddies on the Agulhas Bank (Hutchings *et al.* 2002, Hutchings *et al.* 2009) will encourage school fidelity at the juvenile stage. Not all sites were genetically distinct from others, and this mixing could be facilitated by a seasonal change in oceanographic features. For example, in winter, the thermocline that drives eddy formation breaks down, allowing for increased mixing compared to summer (Hutchings *et al.* 2002, Hutchings *et al.* 2009).

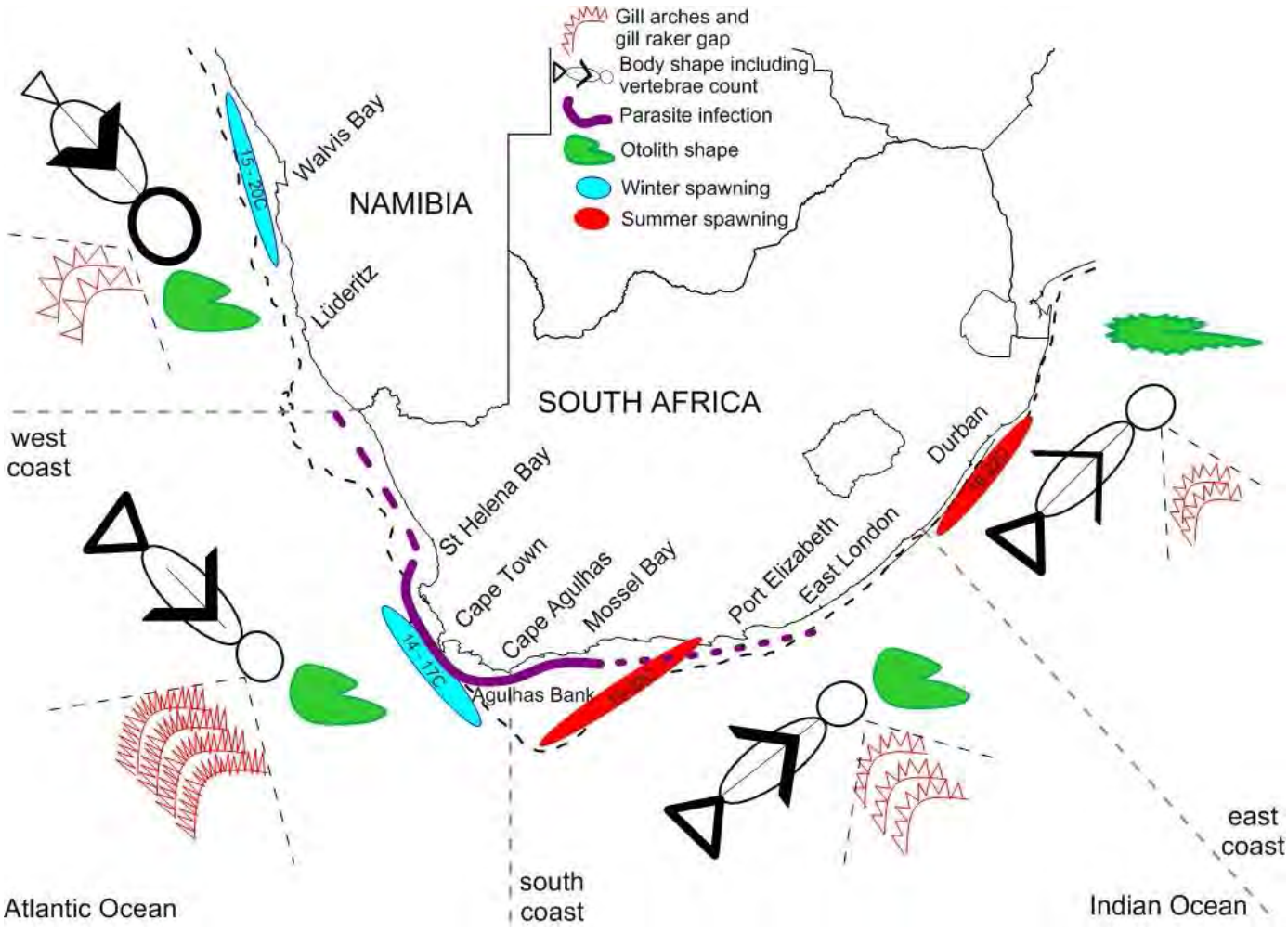


Figure 6.1: A simplified schematic showing some of the differences between sardines in Namibia and South Africa's west, south and east coast as compiled by van der Lingen (2011) with the findings from the current study on differences in otolith morphology and microchemistry included. Differences between the number and length of gill arches as well as the size of the gill raker gap are shown in red. Spawning localities are shown with blue (winter spawning) and red (summer spawning) ellipses and the prevalence of parasite infection is shown in

purple. Where infection rates are high the line is solid and where infection rates decrease, the line is dashed. Morphological differences between fish such as the relative size of head, tail, length, number of vertebrae and body thickness are summarised in black. The thickness of the arrow represents a relative index of the number of vertebra. Otolith morphology derived from the current study is shown in green.

Comparing stock differentiation between three species of small pelagic fish in the southern Benguela

By occupying different niches in the pelagic environment, the distributions of sardine, anchovy and round herring in the southern Benguela can overlap substantially. The different life history strategies can influence the likelihood of anchovy and round herring forming more than one stock in the southern Benguela, as hypothesised for sardine, or displaying school fidelity, as inferred from the microsatellite genetic results for sardine (Chapter 4). To date, research investigating the multiple stock hypothesis has focused on sardine. Little work has been done on the possibility of multiple stocks in anchovy and round herring. This is despite numerous international examples of stock differentiation in anchovy species. For example, studies have identified two or more stocks in *E. japonicus* in East China (Yu *et al.* 2002), and three groups of *E. encrasicolus* in the Mediterranean (Bembo *et al.* 1995).

Stock differentiation in anchovy and round herring has not been investigated using multiple markers, as has been done for sardine. In this study, otolith morphology results showed distinct differences in patterns of differentiation between the species (Chapter 2). Anchovy otolith shape was mostly influenced by the length of the fish, although there was a large amount of individual variability. The shape of round herring otoliths was mainly related to length and gender of the fish. The shape of sardine otoliths, in contrast, did not depend on fish length and gender. Such differences in a single index can reflect different life history strategies between the three species. Further investigation of other traits is, however, required to test this hypothesis (Galarza *et al.* 2009). At present, the cause of variability in otolith shape is not well understood, making it difficult to identify the causal mechanism for the differences in otolith shape patterns among the three species.

It cannot be assumed that when one species in an area displays a particular pattern of stock differentiation, other species in the same area, even if closely related, will show similar patterns (Gaggiotti and Vetter 1999, Ward 2000). Gaggiotti and Vetter (1999) found substantial differences in the genetic variability of sardine (*Sardinops sagax*) and anchovy (*Engraulis mordax*) in the California Current, which resulted in substantial differences in the capacity of these two species to respond to environmental changes. They found that sardine had decreased genetic variability compared to anchovy, and hypothesised that they are predetermined to have lower levels of genetic variability than anchovy, despite favourable environmental conditions. In contrast, in the southern Benguela all three small pelagic fish species studied showed high levels of individual otolith shape variability (Chapter 2), and both sardine and anchovy had high levels of heterogeneity (Chapters 4 and 5 respectively). This is likely to be the result of the dynamic environment of the southern Benguela.

Sardine and anchovy overlap in distribution around the world and often undergo large population fluctuations or regime shifts (Coetzee *et al.* 2008, Checkley *et al.* 2009). Kasapidis (2014) concluded that sardine and anchovy followed different phylogeographical pathways to colonise South African waters; anchovy from European populations to the north and sardine from Australia and New Zealand. Grant and Bowen (1998) suggest that sardine and anchovy are likely to have shallow genetic architecture, in part due to sweepstake recruitment, which is evident in the genetic results shown in the current study (Chapters 4 and 5). Anchovy have shallow genetic architecture from relatively recent colonisation events from Europe, in response to climate driven extinctions (Grant and Bowen 2006, Grant *et al.* 2010). In contrast, sardine are more closely related to Australian sardine than to northern populations (Grant and Bowen 1998, Kasapidis 2014). In order for strong differentiation signals to be observed, the populations would need to have been well established in the region and to have had time to diverge within the population. Neither sardine nor anchovy have been in the region long enough to have lost the signal of their ancestral polymorphism (Kasapidis 2014). On a more regional scale, sardine and anchovy respond differently to population fluctuations. For the same size population, anchovy occupy a larger area than sardine, as a result of different feeding strategies (Barange *et al.* 1999), which influences the degree of mixing between the species.

The marine biogeography of South Africa is complex, as it is influenced by water masses in both the Atlantic and Indian Oceans and two boundary currents: the Benguela and Agulhas Currents. Four phylogeographic provinces have so far been identified, based primarily on coastal species. (Figure 6.2, Teske *et al.* 2011). Despite these distinct environmental regions, not all species that occur in more than one province show genetic differentiation between provinces (e.g. lantern fish, *Lampanyctodes hectoris* (Florence *et al.* 2010), squid, *Loligo reynaudii* (Shaw *et al.* 2010)), and some species have different phylogeographic breaks (e.g. brown mussel, *Perna perna* (Nicastro *et al.* 2008)). Cold water upwelling, freshwater discharge, regional currents and province-specific adaptations all influence stock differentiation in various ways, depending on species distribution, spawning localities and timing, dispersal capabilities, mobility and population dynamics (Teske *et al.* 2011). Furthermore, morphological and genetic variability on a regional scale provide ecological and evolutionary advantages within that region (Grant *et al.* 2010), which could mean that individuals adapted to a particular area are less likely to recruit successfully to other areas (Hauser and Carvalho 2008). Thus, should there be more than one stock of sardine in the southern Benguela, individuals from the south coast would not necessarily recruit successfully to the west coast to supplement a declining population there.

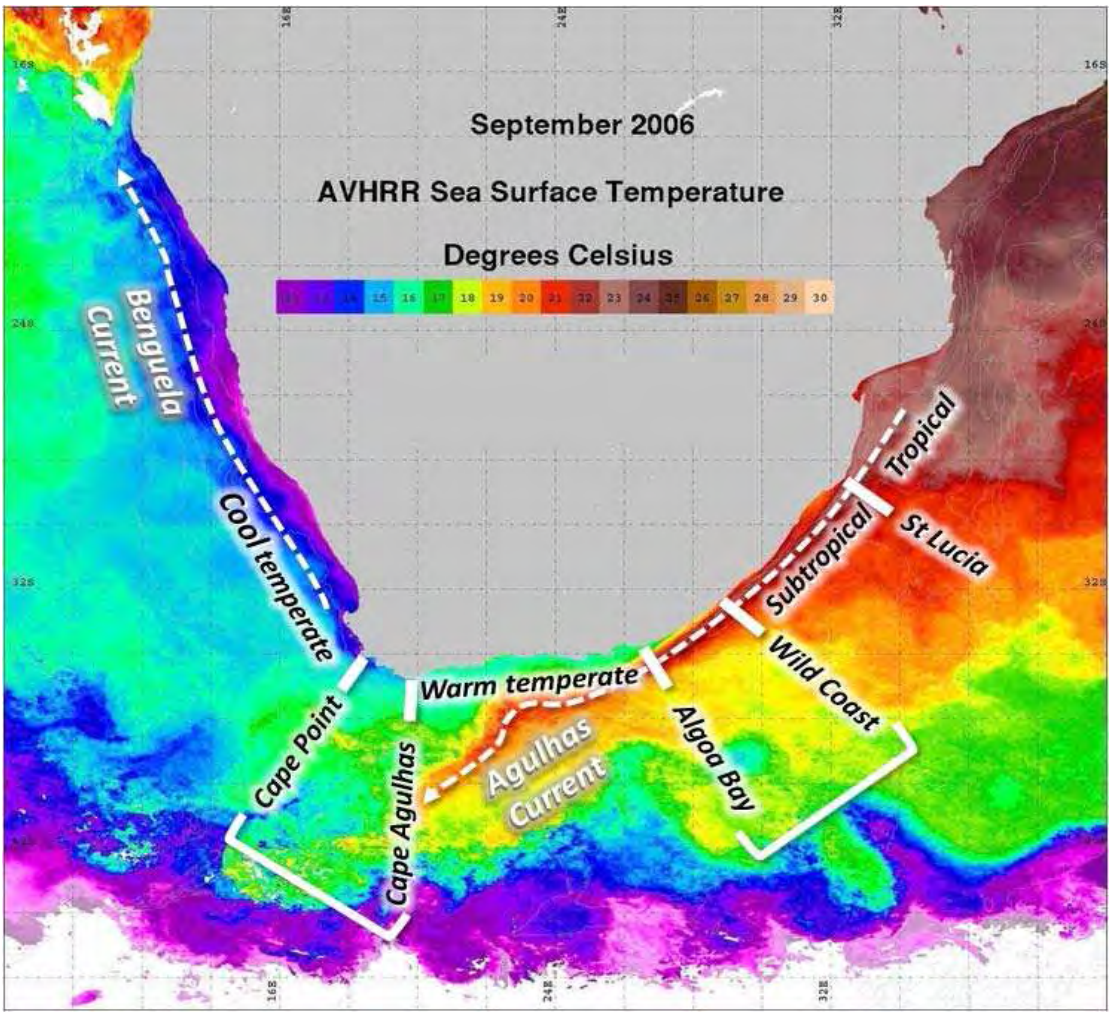


Figure 6.2: Southern Africa regional oceanography and biogeographic provinces (cool temperate, warm temperate, subtropical and tropical) and two transition zones between Cape Point and Cape Agulhas and Algoa Bay and Wild Coast (Teske *et al.* 2011). Source: SST image, Marine Research Institute, University of Cape Town

The formation and maintenance of schools and stocks are influenced by oceanographic filaments (Galarza *et al.* 2009). In the southern Benguela and Agulhas Current systems, eddies can cause or prevent mixing of some schools, but not others. This is a possible explanation for the patchy genetic differentiation found in sardine and anchovy. Combining genetic differentiation with models of oceanographic features, such as was done by Stenevik *et al.* (2008) in work on the hake species *Merluccius capensis* and *M. paradoxus* in the Benguela, could further explain spatial patterns of genetic differentiation in these two species

(White *et al.* 2010). Schooling fidelity and associated behavioural and morphological changes, as previously identified in European anchovy (Ospina-Alvareza *et al.* 2012), are possible further mechanisms for the development of stock structure.

Suggestions for further work

To optimise effort in an investigation of stock structure in small pelagic fish, Baldwin *et al.* (2012) suggested collecting 50 individuals inshore and 50 individuals offshore for each site, at night, during the peak spawning period. The extremes of species distribution should be included in this sampling. Baldwin *et al.* (2012) also advise that sampling between spawning events will help elucidate patterns of migration between spawning grounds. In reality, sampling is often opportunistic and occurs alongside work with different objectives, in order to save on expensive ships' time. What is important, and can be controlled, is that all measures be taken on the same individuals to reduce any inherent variability. These measures should include traits that reflect short term variability and evolutionary history. Short term variability measures can vary between traits that are laid down in early life stages (vertebral counts, gill rakers and blood groups) or reflect conditions during the life time of the fish (parasites as a biotag, otolith morphology, size at age, otolith microchemistry, mark-recapture – although this last technique has had limited success in small pelagic fish because of the high levels of mortality experienced).

Evolutionary history should be investigated using molecular markers. However, the decadal-scale population fluctuations that characterise small pelagic fish could mask some of the historic demographic changes that one would normally be able to discern with molecular markers (Grant and Utter 1984). An holistic and comprehensive sampling strategy was used effectively during horse mackerel stock identification research (HOMSIR) (Abaunza *et al.* 2008). Some of the difficulties in incorporating genetic data into fisheries management have been outlined by Waples *et al.* (2008). The challenge is particularly acute when results are inconclusive but rule out panmixia (as in this study). The lack of common terminology and effective communication among geneticists, managers and fisheries biologists has hampered progress in this field. Furthermore, the power of analyses can be low, evidenced by the fact

that gene flow of 1% can cause genetic homogeneity (Ward 2000). Cornuet *et al.* (1999) have found that, given thirty to fifty individuals from ten populations using ten loci, individuals can be correctly assigned 100% of the time given an F_{ST} of approximately 0.1, but the mean F_{ST} of marine fish is 0.062 (Waples 1998). Larger samples, more sample sites or more loci will increase the statistical power of analyses. However, catching individuals that are reproductively mature from sufficient sites is expensive and difficult. Given this limitation, an increase in the number of loci and the application of new technologies (such as single next generation sequencing, nucleotide polymorphisms, SNPs), or the use of non-neutral coding regions, will strengthen results. Temporal sampling will also decrease artefacts of sampling strategy (Waples 1998).

Conclusion

On the basis of the evidence from multiple sources and what has been presented in this study (including otolith morphology, otolith microchemisty and microsatellites) and international review, it is recommended that the management of sardine in South African waters be founded on the two stock hypothesis. Models incorporating the two stock hypothesis suggest that, in November 2011, there were approximately 298 thousand tons of sardine on the west coast and 1 million tons on the south coast (de Moor and Butterworth 2013). The disparity in the size and productivity of the two stocks would mean that, if managed under a single stock hypothesis, the smaller stock would be over-utilised (Smith 2011a).

The existence of multiple spawning stocks in anchovy (van der Lingen and Huggett 2003), together with the preliminary microsatellite results from the current study (notwithstanding the small sample size), suggest that further work in stock differentiation of anchovy should also be undertaken. Investigations into stock structure of anchovy and round herring should be multidisciplinary, and include morphological, meristic, genetic and parasitic studies (Baldwin *et al.* 2012). Morphological or meristic studies would provide a useful starting point, since they are relatively inexpensive.

The studies here on otolith morphology, elemental chemistry and genetic markers provide evidence of microstructure within the South African sardine population, and do not support the hypothesis that the population is panmictic. The genetic studies on anchovy suggest that there is complex population structure at the level of the sampling sites (a proxy for schools), although both genetic and otolith studies showed high levels of individual variability and no coastal differentiation. Taken together, the results suggest that, while there is little biogeographic structure in the South African sardine and anchovy populations, there is geographic variability in both species. This variability is temporally unstable in sardine and is likely to be in anchovy. There is no evidence from differences in otolith shape of there being structure in the round herring population, but further investigations into population structure in this species are required, using genetic or morphological markers other than otoliths. It is clear that the complex nature of the environment in the southern Benguela translates into complex patterns of mixing and micro-structuring, which can break down seasonally.

> *"Hence these fishes epitomize the uncertainty that unravels fishery management plans." (Lecomte et al. 2004 pg 2170)*

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Glossary and List of Acronyms

AMOVA: Analysis of Molecular Variance

Annuli: Concentric rings on the otolith (or any structure) that can be interpreted in terms of discrete units of time.

Benguela: South Atlantic current flowing northward along the south western coast of southern Africa.

BOFFFFs: Big, old, fat, fecund, female, fish

Caudal length (CL): The measurement taken from nose to tail, or the anteroposterior axis.

Circularity: $Circularity = \frac{Perimeter^2}{Area}$

DAFF: National Department of Agriculture, Forestry and Fisheries (previously known as Marine and Coastal Management).

Decadal: A ten year time period.

Eastern boundary systems: The eastern boundary of currents (on the western coast of continents) characterised by a wide, relatively shallow coastal shelf.

Ekman Drift: Movement of the surface layer of the water due to wind, in the Southern Hemisphere, movement is 90 degrees to the left of the direction of the wind.

FCA: Factorial Correspondence Analysis

FDAA: Functionally distinct adult assemblages (Gaughan et al. 2012)

Form factor: $Form \quad factor = \frac{4\pi * \text{Area}}{Perimeter^2}$

GLM: General linear model

HWE: Hardy Weinberg Equilibrium

MCMC: Monte Carlo Markov Chain

Microsatellite DNA: Short tandem repeat sequences between 2 and 6 bases of DNA

Mitochondrial DNA (mtDNA): Predominately maternally inherited DNA that is only found in the mitochondria.

ND2: Mitochondrial DNA marker, NADH dehydrogenase subunit 2)

Otolith: Structure in the inner ear. They are sensitive to gravity and acceleration and aid hearing.

Panmictic: Population structure where this is no limitations on any individual mating with any other individual in the system.

PCA: Principal Component Analysis

PCR: Polymerase chain reaction.

Pelagic: The open ocean.

Polymorphism: Different forms of members in the same population.

PSB: Pelagic spawner biomass (annual research cruise held in November/December to determine the spawner population of small pelagic fishes.)

Regime shift: 'A regime shift is when there is a rapid change from a quantifiable state and a change in the functioning of the ecosystem that is temporally stable and geographically extensive

RS: Recruit survey (annual research cruise held in November/December to determine the recruit population of small pelagic fishes.)

Sd: standard deviation

Seine: A large fishing net that hangs vertically in the water column with floats at the surface and weights at the bottom.

SNPs: Single nucleotide polymorphisms

SST: Sea surface temperature

Stock: Subpopulations of a fish species that mate randomly in a given geographical area with temporal and spatial continuity (Ihssen *et al.* 1981).

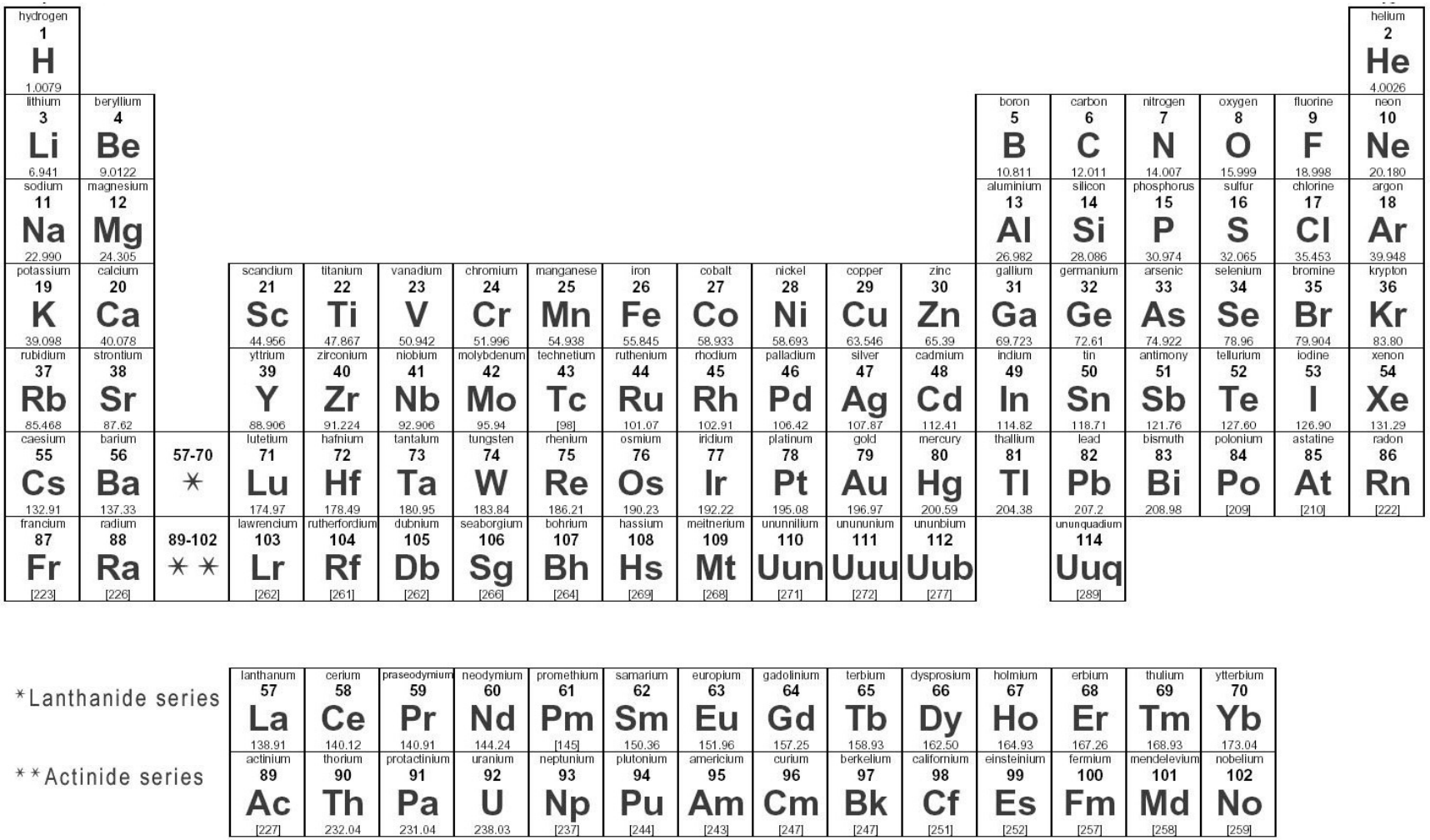
Sweepstakes recruitment: A relatively small number of individuals are disproportionately responsible for recruitment.

TAC: Total Allowable catch

π : nucleotide diversity

h: haplotype diversity

Periodic Table of the elements:



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Appendix 2: R Code used in Chapter 2

Example of R Code

```
#Adapted from code by R. Sherley (ADU, UCT) and H. Winkler (Ma-Re, UCT).
library(MASS)
library("gplots")
library("multcomp")

dat <- read.table("datafile.csv",header=T, sep=",")
dat$y <- dat$ff

names(dat)
summary(dat)

par(mfrow=c(1,1),mex=0.9, cex=0.8)
hist(dat$y, ylab="Frequency", xlab="Form factor",main="",col="gray", breaks=50)

# some boxplots
windows(width=8,height=4)
par(mfrow=c(1,2))
boxplot(y ~ coast, data = dat, col="grey",notch=F,ylab=expression(italic("Form
factor")),xlab="Coast")
boxplot(y ~ sex, data = dat, col="grey",notch=F,ylab=expression(italic("Form
factor")),xlab="Sex")

# GLM analysis # first fit a basic LM with all variables of interest
fit1 <- glm(y ~ coast+sex+log(length), data=dat)
fit2 <- glm(y ~ coast+sex+(length), data=dat)
AIC(fit1,fit2)

drop1(fit1, test="F")
anova(fit1, test="F")
step(fit1)
```

```
drop1(fit2, test="F")
anova(fit2, test="F")
step(fit2)

final_fit <- glm(y ~ log(length)+coast, data=dat)

# model stats results
summary(final_fit)
anova(final_fit,test="F")

# Estimate total variation explained
R2 <- 1-final_fit$deviance/final_fit$null.deviance
R2

# Standard Model Validation # Residuals
res <- residuals(final_fit)

# fitted values
yfit <- fitted.values(final_fit)

par(mfrow=c(1,3),mex=0.85, cex=0.9,mar=c(4,4,1,1))
plot(yfit,res,xlab="Predicted values",ylab="Residuals")
abline(0,0,lty=3)
qqnorm(res,main="")
qqline(res)
hist(res,main= "",xlab="Residuals")

# Make Prediction Datasets - set up for prediction datasets
coast <- c("WC","SC")
sex <- c("M","F")
median.Length <-  median(dat$length)
Length.range <- c(round(min(dat$length)):round(max(dat$length)))
```

```
length.classes <- length(Length.range)
nL = length.classes
#Create prediction data for Coast
pred.dat <- data.frame(coast,sex=rep("F",2),length=rep(median.Length,2))
pred.dat

#  predict Coast
pC <- (predict(final_fit,pred.dat,type="link",se=T))

# predict mean given the "log" link function
mC <- (pC$fit)

#predict lower and upper 95% CI
uC <-  (pC$fit+1.96*pC$se.fit)
lC <-  (pC$fit-1.96*pC$se.fit)

# predict sex
pred.S <- data.frame(coast=rep("WC",2),sex=c("F","M"),length=rep(median.Length,2))
pS <- (predict(final_fit,pred.S,type="link",se=T))
mS <- (pS$fit)

#predict lower and upper 95% CI
uS <-  (pS$fit+1.96*pS$se.fit)
lS <-  (pS$fit-1.96*pS$se.fit)

# predict Length
predS.dat <-
data.frame(coast=rep("WC",2),season=c("S","W"),sex=rep("F",2),length=rep(median.Length
,2))
pS <- (predict(final_fit,predS.dat,type="link",se=T))
mS <- (pS$fit)

#predict lower and upper 95% CI
```

```
uS <-  (pS$fit+1.96*pS$se.fit)
lS <-  (pS$fit-1.96*pS$se.fit)

# predict length
length.classes
predL.dat <- data.frame(coast="WC", sex=rep("F",nL),length=Length.range)
predL.dat

# Predict Length West
pL <- predict(final_fit,predL.dat,type="link",se=T)

# predict mean given the "log" link function
mL<- (pL$fit)
#predict lower and upper 95% CI
uL <-  (pL$fit+1.96*pL$se.fit)
lL <-  (pL$fit-1.96*pL$se.fit)

# Make result plots
maxyC = max(uC*1.02)
maxyS = max(uS*1.02)

par(mfrow=c(1,3),mex=1, cex=1,mar=c(4,4,2,1))

# Length effect plot
plot(Length.range,mL, type="l", ylim=c(0.55,0.7), xlab="Round herring length (mm)" , ylab
= expression(italic("Form factor")))
lines(Length.range,uL,lty=2)
lines(Length.range,lL,lty=2)

# 1)Coast plot
plotCI(x=(c(1:2)-0.1),mC[1:2],uiw=(uC[1:2]-mC[1:2]),liw=(mC[1:2]-
lC[1:2]),ylim=c(0.57,0.6), xaxt="n",pch=16, cex=1.5,xlim=c(0.5,2.5),
xlab="Coast",ylab=expression(italic("Form factor")),gap=0,type="p")
```

```
axis(side=1, at=1:2, labels=coast, cex=0.7)

# 2)Sex plot
plotCI(x=(c(1:2)-0.1),mC[1:2],uiw=(uS-mS),liw=(mS-lS), xaxt="n",pch=16,
cex=1.5,ylim=c(0.57,0.6),xlim=c(0.5,2.5), xlab="Sex",ylab=expression(italic("Form
factor")),gap=0,type="p")
axis(side=1, at=1:2, labels=sex, cex=0.7)

# 3)Sex plot
maxyL =max(uL)
minyL = min(lL)

# Post-hoc test
summary(glht(final_fit, linfct = mcp(sex = "Tukey")))
```

Appendix 3: R Code used in Chapter 3

Example of R Code

```
#Adapted from code from various sources.

setwd("F:/microchem")
edge2008 = read.csv("2008test.csv", header=T) #T=true, take headers as headers

summary(edge2008) #check
names(edge2008) #check
attach(edge2008)

#Viewing the data
#e.g.
plot(Site, logLi, xlab= "Site", ylab="Li")

#subset the data to include elements present in all samples
data <- cbind(logLi, logB, logMg, logSr, logBa, logSn)
#data in order of descending F values
datanew <- cbind(logLi, logBa, logB, logSn, logSr, logMg)

#add column names
colnames(data) <- c("log.Li", "log.B", "log.Mg", "log.Sr", "log.Ba", "log.Sn")
colnames(datanew) <- c("log.Li", "log.Ba", "log.B", "log.Sn", "log.Sr", "log.Mg")

#pairwise scatterplots to check for correlations
pairs(data)

#Principal components Analysis
arc.pcal <- princomp(data, scores=TRUE, cor=TRUE)
summary(arc.pcal)
plot(arc.pcal)
```

```
biplot(arc.pcal)
arc.pcal$scores
arc.pcal$loadings

require(FactoMineR)
result <- PCA(data)

#means model predicting colour and data shape PCA with sites
arc.pcal <- princomp(data, scores=TRUE, cor=TRUE)
summary(arc.pcal)
plot(arc.pcal)

clus = kmeans(arc.pcal$scores[,1:2], centers=5)
key = data.frame(Site=edge2008$Site,
                 shape=as.numeric(edge2008$Site),
                 color=clus$cluster)
plot(arc.pcal$scores[,1:2],
    col=clus$cluster,
    pch=14+as.numeric(edge2008$Site))

#MANOVA Site
MANOVAS <- manova(datanew ~ Site)
summary(MANOVAS, test="Pillai")
summary(MANOVAS, test="Wilks")
summary(MANOVAS, test="Hotelling-Lawley")
summary.aov(MANOVAS)

#testing assumptions - identify multivariate outliers by plotting the ordered squared robust Mahalanobis distances of observations against the empirical distributional function of the MD
#testing assumptions
require(mvoutlier)
```

```
#testing univariate assumptions
#e.g.
par(mfrow=c(3,2))
qqnorm(logLi, ylab="Li")
qqline(logLi)

#testing homogeneity of variance
#e.g.:
bartlett.test(logLi~Site)
#e.g.
fligner.test(logLi~Site)

#testing multivariate normality
require(mvnormtest)

#graphically testing normality QQ plot
par(mfrow=c(1.1))
x <- as.matrix(data)
center <- colMeans(x)
n <- nrow(x)
p <- ncol(x)
cov <- cov(x)
d <- mahalanobis(x,center,cov)
qqplot(qchisq(ppoints(n), df=p), d, main="QQ Plot assessing Multivariate Normality", ylab = "Mahalanobis D2")
abline(a=0, b=1)
```

Appendix 4: Supplementary material from Chapter 4

Appendix 4.1: Percentage of missing data for each of the seven sardine microsatellite loci in each site in 2007, 2008 and 2009.

	Overall	2007	7WC1	7WC2	7SC1	7SC2
Sar1A11	6.85		0	0	0	0
Sar1-H11	11.53		12.5	33.33	33.33	60
SarBH04F	19.31		12.5	0	0	0
SarBH04	2.18		6.25	50	0	0
SarC05	7.79		0	0	0	0
SarB-A08	8.10		25	16.67	50	0
SarD06	0.31		0	0	0	0
2008	8WC1	8WC2	8SC1	8SC2	8SC3	8SC4
Sar1A11	6.25	18.75	16.67	0	0	0
Sar1-H11	12.5	0	0.00	0	0	0
SarBH04F	6.25	31.25	41.67	0	0	0
SarBH04	0	0	0.00	7.14	9.09	0
SarC05	6.25	6.25	16.67	21.43	36.36	6.25
SarB-A08	0	0	8.33	42.86	36.36	6.25
SarD06	0	0	0.00	0	0	0
2009	9WC1	9WC2	9SC1	9SC2	9NAM	9KZN
Sar1A11	3.57	0.00	3.70	14.71	0.00	20.93
Sar1-H11	10.71	30.00	0.00	2.94	17.65	9.30
SarBH04F	14.29	30.00	3.70	2.94	47.06	41.86
SarBH04	0.00	0.00	0.00	0.00	0.00	2.33
SarC05	10.71	0.00	3.70	14.71	5.88	4.65
SarB-A08	7.14	0.00	3.70	2.94	2.94	2.33
SarD06	0.00	0.00	0.00	2.94	0.00	0.00

Appendix 4.2: Expected and observed heterozygosity for each sardine microsatellite locus in each site in each year as determined by Micro-Checker. Null alleles are indicated in bold. No evidence of large allele dropout or stutter was found.

2007	7WC		7SC	
	He	Ho	He	Ho
Sar1A11	4.437	7	**3.318**	**12**
Sar1-H11	2.958	2	3.607	3
SarBH04F	**1.464**	**7**	**1.545**	**8**
SarBH04	**1.416**	**7**	**1.772**	**7**
SarC05	**1.375**	**6**	1.977	4
SarB-A08	**1.409**	**7**	**2.263**	**13**
SarD06	**2.343**	**5**	**2.263**	**13**

2008	8WC1		8WC2		8SC1		8SC2		8SC3		8SC4	
	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho
Sar1A11	4.066	3	**1.499**	**7**	**2**	**6**	**5.035**	**8**	7.545	9	4.75	5
Sar1-H11	4.25	3	2.906	3	2.916	0	3.428	4	4.772	4	3.156	2
SarBH04F	**1.3**	**8**	**1.09**	**3**	**0.928**	**4**	**1.428**	**4**	1.681	4	**1.531**	**6**
SarBH04	1.218	3	**1.218**	**6**	1.041	2	1.192	2	**1.35**	**4**	1.187	2
SarC05	1.233	3	**1.433**	**5**	1.049	1	1.09	3	**1.714**	**4**	1.266	0
SarB-A08	**1.375**	**8**	**1.812**	**9**	**1.227**	**6**	**1.437**	**5**	**1.142**	**5**	**1.366**	**6**
SarD06	2.156	3	3.031	5	**2.083**	**5**	**3.107**	**3**	6.227	6	2.187	3

2009	9WC1		9WC2		9SC1		9SC2		9NAM		9KZN	
	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho	He	Ho
Sar1A11	**3.203**	**7**	3.816	6	2.576	5	**3.344**	**12**	**4.588**	**13**	**4.455**	**14**
Sar1-H11	5.179	4	4.833	7	5.29	6	6.545	8	5.785	6	6.807	9
SarBH04F	**1.583**	**15**	**1.357**	**9**	**2.557**	**18**	**2.106**	**14**	**1.361**	**9**	**1.34**	**6**
SarBH04	1.857	3	**1.816**	**7**	**1.87**	**6**	**2.455**	**8**	**1.838**	**6**	**2.047**	**8**
SarC05	**1.82**	**6**	**2.316**	**6**	1.634	3	**2.362**	**8**	**2.093**	**9**	**2.097**	**5**
SarB-A08	**1.403**	**5**	**2.049**	**11**	**1.634**	**13**	**1.833**	**11**	**1.803**	**8**	**2.166**	**14**
SarD06	3.767	7	**3.349**	**7**	**2.777**	**6**	**4.348**	**10**	**3.647**	**12**	**3.534**	**8**

Appendix 4.3: Estimate of the frequency of null alleles for sardine microsatellite loci for each site in each year. Estimates were calculated using EM algorithm in FreeNA (Dempster *et al.* 1977). Where the frequency of null alleles is 25% or more, it is highlighted in bold.

	Overall	2007	7WC1	7WC2	7SC1	7SC2
Sar1A11	0.135		0.058	0.051	0.153	**0.340**
Sar1-H11	0.001		0.000	0.000	0.000	0.000
SarBH04F	0.204		0.132	0.199	0.092	**0.286**
SarBH04	0.094		0.223	0.205	0.107	0.200
SarC05	0.083		0.088	0.140	0.040	0.000
SarB-A08	0.187		0.001	**0.287**	**0.318**	0.200
SarD06	0.084		0.000	0.120	0.053	0.000
2008	8WC1	8WC2	8SC1	8SC2	8SC3	8SC4
Sar1A11	0.000	0.226	0.220	0.133	0.141	0.000
Sar1-H11	0.000	0.000	0.000	0.000	0.000	0.000
SarBH04F	0.233	0.074	0.245	0.094	0.134	0.156
SarBH04	0.055	0.157	0.036	0.015	0.131	0.005
SarC05	0.057	0.131	0.000	0.095	0.166	0.000
SarB-A08	0.219	0.238	0.229	0.242	**0.300**	0.168
SarD06	0.031	0.036	0.134	0.015	0.000	0.000
2009	9WC1	9WC2	9SC1	9SC2	9NAM	9KZN
Sar1A11	0.080	0.037	0.059	0.159	0.129	0.146
Sar1-H11	0.000	0.027	0.009	0.018	0.000	0.013
SarBH04F	**0.290**	0.188	**0.311**	0.185	0.220	0.100
SarBH04	0.012	0.091	0.068	0.081	0.061	0.075
SarC05	0.084	0.060	0.029	0.101	0.115	0.036
SarB-A08	0.066	0.154	0.226	0.144	0.094	0.145
SarD06	0.075	0.035	0.043	0.093	0.125	0.055

Appendix 4.4: LOSITAN tests for neutrality for the seven sardine microsatellite loci. Heterozygosity, F_{ST} and P value of sample F_{ST} larger than simulated F_{ST} from 50 000 simulations are shown for each locus. The overall F_{ST} was 0.004.

	Heterozygosity	F_{ST}	P (stimulated Fst < sample Fst)
Sar1A11	0.861	0.022	0.950
Sar1-H11	0.786	0.000	0.392
SarBH04F	0.956	0.010	0.838
SarBH04	0.955	0.003	0.441
SarC05	0.951	0.002	0.332
SarB-A08	0.952	0.007	0.681
SarD06	0.901	0.007	0.637

Appendix 4.5: Matrix of correspondent coefficients (below diagonal) and P values (above diagonal) between pairs of sardine microsatellite loci, to test for linkage disequilibrium using the Black and Krafsur calculation in Genetix v.4.05.2.

	Sar1A11	Sar1-H11	SarBH04F	SarBH04	SarC05	SarB-A08	SarD06
Sar1A11		0.0001	0.0001	0.0001	0.0001	0.0001	0.0001
Sar1-H11	0.040		0.0001	0.0001	0.0001	0.0001	0.0001
SarBH04F	0.052	0.043		0.0001	0.0001	0.0001	0.0001
SarBH04	0.038	0.036	0.042		0.0001	0.0001	0.0001
SarC05	0.040	0.046	0.043	0.040		0.0001	0.0001
SarB-A08	0.041	0.037	0.043	0.038	0.043		0.0001
SarD06	0.038	0.046	0.038	0.033	0.043	0.035	

Appendix 4.6: Observed and expected heterozygosity and P values, showing deviation from Hardy Weinberg Equilibrium (in bold), are shown for each site and each of the seven sardine microsatellite loci for 2007, 2008 and 2009.

	7WC1			7WC2		
	H_O	H_E	P	H_O	H_E	P
Sar1A11	0.50	0.70	0.43	0.60	0.80	0.17
Sar1-H11	0.75	0.64	1.00	0.88	0.85	0.96
SarBH04F	0.50	0.82	0.31	**0.50**	**0.92**	**0.00**
SarBH04	**0.40**	**0.89**	**0.01**	**0.43**	**0.86**	**0.01**
SarC05	**0.67**	**0.94**	**0.02**	**0.60**	**0.92**	**0.00**
SarB-A08	0.50	0.83	0.33	**0.33**	**0.92**	**0.00**
SarD06	0.83	0.94	0.39	**0.60**	**0.83**	**0.03**

	8SC2			8SC3		
	H_O	H_E	P	H_O	H_E	P
Sar1A11	**0.56**	**0.88**	**0.00**	**0.17**	**0.83**	**0.00**
Sar1-H11	0.79	0.77	0.89	**mono**		
SarBH04F	**0.75**	**0.96**	**0.00**	**0.33**	**0.91**	**0.00**
SarBH04	**0.75**	**0.95**	**0.00**	**0.50**	**0.92**	**0.00**
SarC05	0.81	0.93	0.11	**0.83**	**0.95**	**0.03**
SarB-A08	**0.23**	**0.85**	**0.00**	**0.50**	**0.92**	**0.00**
SarD06	0.81	0.92	0.12	0.83	0.94	0.39

2008	**8WC1**			**8WC2**			**8SC1**		
	H_O	H_E	P	H_O	H_E	P	H_O	H_E	P
Sar1A11	0.80	0.75	0.65	**0.46**	**0.92**	**0.00**	**0.40**	**0.84**	**0.00**
Sar1-H11	0.79	0.72	0.18	0.81	0.84	0.36	1.00	0.79	0.18
SarBH04F	**0.47**	**0.94**	**0.00**	**0.73**	**0.94**	**0.03**	**0.43**	**0.93**	**0.00**
SarBH04	0.81	0.95	0.08	**0.63**	**0.95**	**0.00**	0.83	0.95	0.16
SarC05	0.80	0.95	0.10	**0.67**	**0.94**	**0.00**	0.90	0.94	0.60
SarB-A08	**0.50**	**0.94**	**0.00**	**0.44**	**0.92**	**0.00**	**0.45**	**0.93**	**0.00**
SarD06	0.81	0.89	0.11	**0.69**	**0.84**	**0.01**	**0.58**	**0.86**	**0.02**

2008	**8SC2**			**8SC3**			**8SC4**		
	H_O	H_E	P	H_O	H_E	P	H_O	H_E	P
Sar1A11	**0.43**	**0.66**	**0.01**	0.18	0.33	0.10	0.69	0.73	0.22
Sar1-H11	0.71	0.78	0.37	0.64	0.59	0.67	0.88	0.83	0.60
SarBH04F	0.71	0.93	0.07	**0.64**	**0.89**	**0.01**	**0.63**	**0.93**	**0.00**
SarBH04	0.85	0.94	0.31	**0.60**	**0.91**	**0.00**	0.88	0.96	0.33
SarC05	**0.73**	**0.94**	**0.01**	**0.43**	**0.81**	**0.00**	1.00	0.95	0.78
SarB-A08	**0.38**	**0.88**	**0.00**	**0.29**	**0.90**	**0.00**	**0.60**	**0.94**	**0.00**
SarD06	0.79	0.81	0.49	0.45	0.45	0.40	0.81	0.89	0.07

2009	9NAM			9WC1			9WC2		
	Ho	He	P	Ho	He	P	Ho	He	P
Sar1A11	**0.62**	**0.88**	**0.00**	0.74	0.90	0.10	**0.80**	**0.89**	**0.01**
Sar1-H11	0.79	0.81	0.23	0.84	0.81	0.57	0.67	0.79	0.09
SarBH04F	**0.50**	**0.95**	**0.00**	**0.38**	**0.95**	**0.00**	**0.57**	**0.96**	**0.00**
SarBH04	0.82	0.96	0.05	0.89	0.95	0.18	**0.77**	**0.96**	**0.01**
SarC05	**0.72**	**0.95**	**0.00**	**0.76**	**0.95**	**0.00**	0.80	0.94	0.06
SarB-A08	**0.76**	**0.96**	**0.00**	**0.81**	**0.96**	**0.00**	**0.63**	**0.95**	**0.00**
SarD06	**0.65**	**0.91**	**0.00**	0.75	0.88	0.11	**0.77**	**0.90**	**0.00**

2009	9SC1			9SC2			9KZN		
	H_O	H_E	P	H_O	H_E	P	H_O	H_E	P
Sar1A11	0.81	0.92	0.29	**0.59**	**0.90**	**0.00**	**0.59**	**0.88**	**0.00**
Sar1-H11	0.78	0.82	0.22	0.76	0.81	0.13	0.77	0.84	0.13
SarBH04F	**0.31**	**0.92**	**0.00**	**0.58**	**0.95**	**0.00**	**0.76**	**0.97**	**0.00**
SarBH04	**0.78**	**0.95**	**0.00**	**0.76**	**0.94**	**0.01**	**0.81**	**0.96**	**0.00**
SarC05	0.88	0.96	0.05	**0.72**	**0.93**	**0.00**	0.88	0.96	0.21
SarB-A08	**0.50**	**0.96**	**0.00**	**0.67**	**0.96**	**0.00**	**0.67**	**0.96**	**0.00**
SarD06	**0.78**	**0.91**	**0.01**	**0.70**	**0.88**	**0.00**	0.81	0.93	0.07

Appendix 4.6: F_{STAT} gene diversity (G), number of alleles (N), allelic richness (AR) (based on a minimum sample size of 18 diploid individuals in 2009, 8 in 2008) and F_{IS} for each of the sardine microsatellite loci at each site in 2007, 2008 and 2009. Allelic richness could not be calculated for 2007 Sar1-H11 because it was monomorphic at 7SC2.

2007	7WC1			7WC2			7SC1			7SC2		
	G	**N**	**F_{IS}**	**G**	**N**	**F_{IS}**	**G**	**N**	**F_{IS}**	**G**	**N**	**F_{IS}**
Sar1A11	0.72	4	0.30	0.81	7	0.26	0.89	11	0.37	0.90	5	0.82
Sar1-H11	0.63	4	-0.20	0.85	9	-0.03	0.77	11	-0.02	NA	NA	NA
SarBH04F	0.88	4	0.43	0.94	10	0.47	0.97	18	0.22	0.97	6	0.66
SarBH04	0.95	6	0.58	0.89	7	0.52	0.96	15	0.22	0.97	7	0.48
SarC05	0.97	9	0.31	0.94	11	0.36	0.94	14	0.13	0.97	9	0.14
SarB-A08	1.00	3	0.50	0.96	10	0.65	0.87	9	0.74	0.97	7	0.48
SarD06	0.95	8	0.12	0.84	9	0.29	0.92	12	0.12	0.95	8	0.12

2008	8WC1				8WC2				8SC1			
	G	**N**	**AR**	**F_{IS}**	**G**	**N**	**AR**	**F_{IS}**	**G**	**N**	**AR**	**F_{IS}**
Sar1A11	0.75	6	5.17	-0.06	0.94	11	8.48	0.51	0.87	7	6.08	0.54
Sar1-H11	0.72	7	4.95	-0.09	0.85	14	8.07	0.04	0.78	7	5.57	-0.28
SarBH04F	0.96	15	9.98	0.52	0.96	14	10.16	0.24	0.98	8	8.00	0.56
SarBH04	0.96	17	10.53	0.15	0.97	18	10.65	0.35	0.96	15	10.52	0.13
SarC05	0.96	17	10.36	0.16	0.95	15	9.60	0.30	0.94	12	9.80	0.05
SarB-A08	0.96	15	9.81	0.48	0.93	15	9.27	0.53	0.96	12	9.31	0.52
SarD06	0.90	12	8.05	0.09	0.84	13	7.68	0.18	0.88	9	7.05	0.33

	8SC2				**8SC3**				**8SC4**			
	G	**N**	**AR**	**F_{IS}**	**G**	**N**	**AR**	**F_{IS}**	**G**	**N**	**AR**	**F_{IS}**
Sar1A11	0.67	8	5.54	0.36	0.34	3	2.76	0.46	0.73	8	5.74	0.05
Sar1-H11	0.79	10	6.43	0.09	0.59	6	4.91	-0.08	0.83	11	7.13	-0.06
SarBH04F	0.94	13	9.18	0.24	0.90	8	7.03	0.29	0.94	16	9.66	0.34
SarBH04	0.95	15	10.09	0.11	0.93	10	8.32	0.35	0.96	19	10.79	0.09
SarC05	0.96	13	9.92	0.24	0.85	7	7.00	0.49	0.95	16	10.16	-0.06
SarB-A08	0.91	8	7.48	0.59	0.95	8	8.00	0.70	0.95	13	9.47	0.37
SarD06	0.81	8	6.03	0.03	0.46	4	3.26	0.00	0.89	13	8.36	0.09
2009	**9NAM**				**9WC1**				**9WC2**			
	G	**N**	**AR**	**F_{IS}**	**G**	**N**	**AR**	**F_{IS}**	**G**	**N**	**AR**	**F_{IS}**
Sar1A11	0.88	11	9.88	0.30	0.90	13	11.68	0.18	0.89	13	11.05	0.10
Sar1-H11	0.81	16	12.97	0.03	0.81	18	14.21	0.04	0.79	16	14.53	0.16
SarBH04F	0.96	18	18.00	0.48	0.97	20	18.26	0.61	0.97	21	19.70	0.41
SarBH04	0.96	29	20.62	0.14	0.95	25	19.48	0.06	0.96	24	19.39	0.20
SarC05	0.95	20	16.84	0.25	0.95	20	17.74	0.20	0.94	19	16.23	0.15
SarB-A08	0.96	24	19.35	0.21	0.97	26	21.50	0.17	0.95	22	17.66	0.34
SarD06	0.91	14	11.79	0.29	0.88	14	11.87	0.15	0.91	19	14.23	0.15
	9SC1				**9SC2**				**9KZN**			
	G	**N**	**AR**	**F_{IS}**	**G**	**N**	**AR**	**F_{IS}**	**G**	**N**	**AR**	**F_{IS}**
Sar1A11	0.92	14	13.03	0.12	0.91	13	12.19	0.35	0.89	12	10.46	0.34
Sar1-H11	0.82	18	14.02	0.05	0.82	15	12.66	0.07	0.84	20	14.59	0.08
SarBH04F	0.93	18	15.04	0.67	0.96	23	17.99	0.40	0.97	23	20.30	0.22
SarBH04	0.95	24	19.02	0.18	0.94	23	17.53	0.19	0.97	30	20.70	0.16
SarC05	0.96	23	19.31	0.08	0.94	18	15.27	0.23	0.96	25	19.44	0.09
SarB-A08	0.97	23	19.31	0.48	0.96	25	19.54	0.31	0.96	29	20.40	0.31
SarD06	0.92	18	14.51	0.15	0.88	16	12.11	0.21	0.93	20	15.33	0.13

*Appendix 4: Mitochondrial and microsatellite loci indicate high diversity with low geographic structure in southern African sardine (*Sardinops sagax*)*

Appendix 5: Supplementary material for Chapter 5

Appendix 5.1: Percentage of missing data for each anchovy microsatellite loci over all, and in each site.

	Overall	WC1	SC1	SC2	SC3
AN25_6	19	8	7	17	46
EJ43	37	8	47	25	62
EJ2	29	50	27	17	23
EJ9	25	0	40	17	38
EJ27.1	2	0	0	8	0

Appendix 5.2: Expected and observed heterozygosity for each anchovy microsatellite loci for each site as determined by Micro-Checker. Null alleles are indicated in bold. No evidence of large allele dropout or stutter were found.

	WC1		SC1		SC2		SC3	
	He	Ho	He	Ho	He	Ho	He	Ho
AN25_6	0.750	1	**2.500**	**8**	**2.000**	**5**	**2.000**	**5**
EJ43	10.166	10	13.133	13	10.045	10	**7.923**	**13**
EJ2	**1.208**	**4**	1.388	3	**1.500**	**8**	**0.875**	**3**
EJ9	1.363	3	**1.857**	**7**	**2.549**	**6**	**0.928**	**3**
EJ27.1	**1.227**	**5**	**1.562**	**4**	1.555	3	0.800	2

Appendix 5.3: Frequency of null alleles for each anchovy microsatellite loci at each sampling site.

	WC1	SC1	SC2	SC3
AN25_6	0.0004	0.2791	0.1685	0.1447
EJ43	0.0000	0.0000	0.0000	0.3081
EJ2	0.1273	0.0983	0.3519	0.1392
EJ9	0.0953	0.1923	0.1850	0.1590
EJ27.1	0.1812	0.1593	0.1065	0.1333

Appendix 5.4: LOSITAN tests for neutrality in loci. Heterozygosity, Fst and P value of sample Fst larger than simulated Fst from 50 000 simulations are shown for each loci. The overall data Fst was 0.026.

	Het	F_{ST}	P (simulated F_{ST} < sample F_{ST})
An25_6	0.873	0.013	0.222
EJ43	0.203	0.033	0.636
EJ2	0.956	0.038	0.685
EJ9	0.957	0.076	0.968
EJ27.1	0.954	0.057	0.900

Appendix 5.5: Matrix of correspondent coefficients (below diagonal) and P values (above diagonal) between pairs of loci to test the against linkage disequilibrium using Black and Krafsur calculation in Genetix v.4.05.2.

	AN25_6	EJ43	EJ2	EJ9	EJ27.1
AN25_6		**0.970**	0.0001	0.0001	0.0001
EJ43	**57.96**		0.0001	0.0001	0.0001
EJ2	0.182	0.108		0.0001	0.0001
EJ9	0.172	0.167	0.144		0.0001
EJ27.1	0.253	0.201	0.144	0.196	

Appendix 5.6: Site summary for five anchovy microsatellite loci in 2009 are provided with the observed and expected heterozygosity and P values showing deviation from Hardy Weinberg Equilibrium (bold).

	WC1			SC1		
	H_E	H_O	P	H_E	H_O	P
AN25_6	0.955	0.833	0.288	**0.810**	**0.273**	**0.000**
EJ43	0.159	0.167	1.000	0.129	0.133	1.000
EJ2	**0.938**	**0.667**	**0.004**	**0.895**	**0.667**	**0.041**
EJ9	**0.918**	**0.727**	**0.042**	**0.899**	**0.500**	**0.000**
EJ27.1	**0.931**	**0.545**	**0.001**	**0.858**	**0.500**	**0.011**
	SC2			SC3		
	H_E	H_O	P	H_E	H_O	P
AN25_6	**0.842**	**0.500**	**0.008**	**0.842**	**0.500**	**0.003**
EJ43	0.091	0.091	1.000	**0.406**	**0.000**	**0.000**
EJ2	**0.895**	**0.260**	**0.000**	**0.950**	**0.625**	**0.007**
EJ9	**0.784**	**0.400**	**0.001**	**0.934**	**0.571**	**0.014**
EJ27.1	0.876	0.667	0.075	0.933	0.600	0.062