Population connectivity of sardines (*Sardinops sagax*) of the KZN sardine run using meristic, morphological and genetic data

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Abstract

The Sardine run occurs annually when large schools of sardine (*Sardinops sagax*) move from the Agulhas Bank towards KwaZulu-Natal, and has significant ecological and anthropogenic importance. Recent investigation has highlighted the nature and mechanisms resulting in the sardine run, however, critical questions about why the sardine run occurs remain unanswered. Therefore, the aim of this project was to elucidate the population diversity, connectivity and structure of sardines undertaking the sardine run. Sardines were sampled at four sites along the South African coast, and their morphology assessed using meristic data, multivariate, and geometric morphometrics. Nine exon-primed, intron-crossing (EPIC) DNA markers and the mitochondrially encoded cytochrome oxidase I (mtCOI) region of DNA were used for population and phylogeographic genetic analyses. Morphological analyses revealed significant differences between head size and shape of sardine run stock compared with other regions, and supports the delineation of a Western Cape and Agulhas Bank stock. Phylogeographic analysis using cytochrome oxidase I data, supported the idea that the *Sardinops* genus is monotypic despite current taxonomy. Genetic analyses using EPIC data confirmed low levels of segregation between sardines from the sardine run and the Western Cape stock. However, larvae spawned in KwaZulu-Natal demonstrated moderate levels of isolation from the Western Cape stock. The results reveal that there is successful recruitment of KwaZulu-Natal juveniles to the adult stock undertaking the sardine run, but not to the Western Cape population. This suggests that although sardines from the West Coast and Agulhas Bank may partake in the sardine run, only a certain subpopulation of the Agulhas Bank stock spawn successfully in KwaZulu-Natal. These results support the hypothesis that the sardine run represents a subpopulation spawning migration of *Sardinops sagax* in South Africa.

Key words: *Sardinops sagax*, KZN sardine run, morphology, genetic connectivity, stock structure

Preface

The work described in this dissertation was carried out at the School of Life Sciences at the University of KwaZulu-Natal, Westville. Field work was conducted at various sites along the KwaZulu-Natal, Eastern Cape and Western Cape coastline, under the supervision of Drs Angus H.H. MacDonald and Sean O'Donoghue. This dissertation represents original work by the author and has not otherwise been submitted in any form for any degree or diploma to any tertiary institute. Where use has been made of the work of others, it is duly acknowledged in the text.

I certify that the above statement is correct:

Signed: _____

Brent Chiazzari; April 2014

As the candidate's supervisor, I agree with the above, and have approved this dissertation for submission:

Signed: _____

Dr Angus H.H. MacDonald, Supervisor; April 2014

Signed: _____

Dr Sean O'Donoghue, Supervisor; April 2014

Declaration

Plagiarism

I, Brent Chiazzari, declare that:

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Publications

Detail of contribution to publications that form part and/or include research presented in this thesis.

Publication 1 (in preparation):

B. Chiazzari, S. O'Donoghue and A.H.H. MacDonald. The population connectivity of the KZN Sardines (*Sardinops sagax*) using meristic, morphological and genetic data.

Author contributions:

Brent Chiazzari, Angus H.H. MacDonald and Sean O'Donoghue conceived the paper. Brent Chiazzari collected and analysed the data, Angus H.H. MacDonald provided financial support for the study, and both Angus H.H. MacDonald and Sean O'Donoghue contributed comments on the manuscript.

Publication 2 (in preparation):

B. Chiazzari, S. O'Donoghue and A.H.H. MacDonald. The optimisation and use of EPIC primers in stock identification of sardine (*Sardinops sagax*) in South Africa; a cheap and reliable alternative to sequence data analyses.

Author contributions:

Brent Chiazzari and Angus H.H. MacDonald conceived the paper. Brent Chiazzari collected and analysed the data, Angus H.H. MacDonald provided financial support for the study, and both Angus H.H. MacDonald and Sean O'Donoghue contributed comments on the manuscript.

Other research outputs

Poster presentation:

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Signed: 

Brent Chiazzari; April 2014

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Chapter one:

General introduction

1.1. The sardine run: an overview

The ecological phenomenon known as the 'Sardine run' occurs annually when large schools of sardines (*Sardinops sagax)* move from the Agulhas Bank towards KwaZulu-Natal (KZN) (van der Lingen, Coetzee*, et al.* 2010). This is a well-known event along the KZN and Eastern Cape coast, primarily because of its value as an ecologically important phenomenon, natural resource and more recently, in the ecotourism industry.

Freon*, et al.* (2010) defines the sardine run as: "the visible effects of the coastal, alongshore movement during early austral winter of a small and variable fraction of the South African population of sardines (*Sardinops sagax*) from the eastern Agulhas bank to the KwaZulu-Natal (KZN) coast, as far as Durban and the north coast of KZN". The sardine run typically includes the movement of sardines and their predators into the neritic waters along the South African east coast from the Agulhas bank, and congregates further to the east near Waterfall Bluff in late May (Natoli*, et al.* 2008, O'Donoghue, Drapeau, Dudley*, et al.* 2010, O'Donoghue, Whittington*, et al.* 2010). The large shoals of sardines continue up the coast, and if suitable conditions prevail, they will move into KZN, and have been known to move as far up the coast as the Tugela Bank (Natoli*, et al.* 2008, Freon*, et al.* 2010, O'Donoghue, Drapeau, Dudley*, et al.* 2010, O'Donoghue, Drapeau and Peddemors 2010, O'Donoghue, Whittington*, et al.* 2010).

The South African sardine, *Sardinops sagax* (Jenyns, 1842), is a member of the family Clupeidae. Females are oviparous and spawn pelagically on the continental shelf and in large open mouth bays, and their larvae are pelagic. Individuals have a life span of about two to three years but can attain five years. *Sardinops sagax* can attain a length of just under 30 centimetres and a weight of just under 300 grams (Matarese*, et al.* 1989). Although this epipelagic shoaling species is considered a non-selective filter feeder, juveniles mostly feed on zooplankton and adults on phytoplankton (Whitehead*, et al.* 1988, van der Lingen 2002). Worldwide distribution of *Sardinops sagax* is restricted to the Indo-Pacific and Atlantic oceans

and concentrated populations are found in areas of ocean with strong upwelling present, favouring cool water in the range of 10°C to 20°C (Whitehead*, et al.* 1988).

Congregation of predatory species and their winter migrations corresponds with the sardine's migration up the east coast. Shoals of sardines usually overlap with the presence of other small shoaling clupeids (Coetzee 1996, Coetzee*, et al.* 2010). The influx of the sardine biomass causes the run to be an important perennial source of energy for the oligotrophic waters off the east coast of South Africa (Coetzee*, et al.* 2010). The sardine run also supports a seasonal beach seine-net fishery in KZN, and is also a focus of international ecotourism along the Eastern Cape and KZN coasts. Myeza*, et al.* (2010) estimated the socioeconomic value of the sardine run and its related activity to R 34 - 54 million (US$ 3.23 – 5.14 million, R10 to US$ 1 accessed on 07/04/2014) annually, which benefits local coastal communities (Myeza*, et al.* 2010).

The poorly understood features of the sardine run prompted a collaborative effort by researchers which recently shed light on the ecology of this unpredictable event, and progress has been made into the mechanisms that trigger the sardine run. Investigations include the biology and ecology of sardines and their predators during the sardine run, physio-chemical aspects of the sardine run, and the socio-economic impacts of the sardine run. Nevertheless, the question of why the sardine run occurs remains unanswered. A recent review of sardine run related literature, conducted by Freon*, et al.* (2010), provided a number of possible hypotheses for further investigation into the reasons for and mechanisms that drive the sardine run.

The aim of the introductory chapter of this dissertation is to provide an overview of the ecology of sardines and a summary of the current understanding of the sardine run up the east coast of southern Africa, report on hypotheses concerning the nature of the KZN sardine run, and test hypotheses proposed in contemporary literature. Finally, a genetic and morphological study on the sardine run is presented, and it will be argued that such research can elucidate the reasons for the sardine run, and will aid in the planning and management of the commercial fishing and ecotourism industry and most importantly the ecology of *Sardinops sagax* in South Africa.

1.2. Ecological phenomenon: The sardine run and it's fishery

The sardine run corresponds with the onset of austral winter, usually beginning in late May, when large shoals of sardines move up the east coast from the Agulhas Bank, usually in association with water between 15 and 18°C (van der Lingen, Hendricks*, et al.* 2010). Sardines from the Western coast south of Luderitz (separated by sardines to the north by a strong upwelling cell in the vicinity) and sardines of the Agulhas Bank extend their range further east to the Agulhas bank and up the Eastern coast of South Africa (Figure 1). Sardines are most frequently observed between Port St Johns and Waterfall Bluff area and are forced close to the coastline by the narrowing continental shelf, and bounded by the unfavourable warmer Agulhas current offshore. Inshore counter currents (Roberts*, et al.* 2010) and the cooling of inshore waters along the eastern coast (Heydorn*, et al.* 1978) have some positive effect on magnitude of the event, but is not the key cause for it (Freon*, et al.* 2010). Thus the effective winter range of Southern African sardines can extend from the Western Cape to KZN (Figure 1).

Connell (2010) interpreted the annual bi-modal peak in sardine presence along the KZN South Coast as two separate movements (north and then south) and that the southward second movement of fish is likely to occur at depth due to the warming of surface layers of the ocean during spring. Thus, in late winter sardines are thought to move offshore at depth and most are thought to return to the Agulhas bank by early summer (Connell 1996; 2010). Sardine eggs are observed in surface plankton trawls in southern KZN from the onset of the sardine run in late May until December, indicating the presence of some sardines into early summer (Connell 1996; 2010). Sardines have also occasionally been caught by KZN ski-boat fishermen on Yusuri Jig and in the gut contents of predatory teleosts into late spring. Nevertheless, the exact estimates on the number of sardines that return to the Agulhas Bank are not known.

The migration of sardines encourages a host of ecological interactions between species. Predation on sardines is the most evident interaction. Cetacean species such as the long-beaked common dolphin (*Delphinus* capensis) and seasonally migrating Indo-Pacific bottlenose dolphin (*Tursiops aduncus*) congregate along the Eastern coast and target the shoaling sardines as the sardines move into KZN (Peddemors 1993, Peddemors*, et al.* 1997, Peddemors 1999, O'Donoghue, Whittington*, et al.* 2010). The abundance of elasmobranch species, like the

copper shark (*Carcharhinus brachyurus*), spinner shark (*C. brevipinna*), and dusky shark (*C. obscurus*) increases significantly in KZN between June and July (Dudley*, et al.* 2010). Teleosts, like the king mackerel (*Scomberomorus commerson*), garrick (*Lichia amia*), giant kob (*Argyrosomus japonicas*), geelbek (*Atractoscion aequidens*), kingfish (*Caranx* sp.), Elf (*Pomatomus saltatrix*) and yellowfin tuna (*Thunnus albacares*) also prey upon the small fish (Fennessy*, et al.* 2010). Many of these species spawning migrations also coincide with the sardine run, possibly to increase the amount of nutrition along their migration route along the KZN and Eastern Cape coasts. Broekhuysen*, et al.* (1961) and Crawford et al. (1983) described the Cape gannet (*Morus capensis*) as non-breeding winter visitor to KZN which exhibit a close association to the sardine run. More recently, spatio-temporal associations between these avian predators and the sardine run have been investigated (O'Donoghue 2009, O'Donoghue, Drapeau, Dudley*, et al.* 2010, O'Donoghue, Drapeau and Peddemors 2010, O'Donoghue, Whittington*, et al.* 2010). These examples of the relationship of different species to the sardine run highlight its interspecific ecological importance.

There are many ecologically important interactions during the sardine run. Nevertheless, the most important aspect of the sardine run, ecologically, is the delivery of atypical amounts of nutrients, in the form of lipid and protein rich sardine biomass, to the oligotrophic East Coast waters (Hutchings*, et al.* 2010). Whilst acknowledging the high level of inter-annual variability of sardine abundance during the sardine run, a mean estimation of 960 tons of organic nitrogen is supplied from the Agulhas bank to KZN waters by migrating sardines. The sardine run can be equivalent to between 24 % and 76 % of the total annual nitrogen input; depending on the strength of the run, and the total nitrogen input from other sources (Hutchings*, et al.* 2010).

The Eastern Cape and KZN sardine fishery is sporadic, seasonal and substantially smaller than the Benguela sardine fishery. In 2010, the catch of sardines by purse seine netters in the southern Benguela was 126 386 tons. Conversely, the total catch by beach seine netters in KZN in 2010 was less than 50 tons. The largest ever annual catch by beach seine netters was just less than 700 tons, in 1993 (van der Lingen, Coetzee*, et al.* 2010). Indeed, from a fishery perspective, the sardine run has considerably less economic value than the fishery in the Western Cape. However, the benefit to local beach seine fishermen and their communities, along with the ecotourism generated, is locally significant (Myeza*, et al.* 2010).

Most recently, a number of papers published in the *African Journal of Marine Science* have dealt with patterns of distribution of sardines and their predators in relation to environmental conditions (Dudley*, et al.* 2010, Fennessy*, et al.* 2010, O'Donoghue, Drapeau, Dudley*, et al.* 2010, O'Donoghue, Drapeau and Peddemors 2010), biological characteristics of the KZN sardine (van der Lingen, Hendricks*, et al.* 2010), long term evidence of spawning of sardines in KZN (Connell 1996; 2010), biomass of sardine and it's ecological impact during the sardine run (Coetzee*, et al.* 2010, Hutchings*, et al.* 2010), socioeconomic impacts to local KZN and Eastern Cape communities (Dicken 2010, Myeza*, et al.* 2010), and environmental factors such as ocean currents and sea surface temperature (SST) (Roberts*, et al.* 2010). This and previous research has culminated in the development of a paper by Freon*, et al.* (2010) citing hypotheses about the proximate and ultimate factors relating to the cause of the sardine run.

In recent years, important insights about the nature and ecology of the sardine run have been revealed. The final publication in a special series of papers by Freon*, et al.* (2010) describes current hypotheses about the cause of the sardine run. Where sufficient data exists, some of these hypotheses were tested. However, for the sake of brevity, only those that were supported by current literature will be discussed.

1.3. Proximate and ultimate factors: current hypotheses about the sardine run

1.3.1. Ultimate factors

Freon*, et al.* (2010) listed a number of hypotheses that may contribute to the ultimate cause (reasons) of the sardine run. In total, seven ultimate hypotheses were presented and tested, but only two were supported. The first of the supported hypotheses was proposed by Baird (1971), who proposed that the sardine run formed part of a subpopulation spawning migration. The second ultimate hypothesis supported was that sardines undertook their migration due to relic behavioural responses and possibly a bet-hedging strategy (Olofsson*, et al.* 2009, Rees*, et al.* 2010, Ripa*, et al.* 2010) during the last glacial maximum (Coetzee, Van der Lingen*, et al.* 2008). Both of these hypotheses were tested by Freon*, et al.* (2010) via the same tests, except one which was used to test the relic hypothesis. The tests used to support the two hypotheses are listed and discussed below.

Baird's (1971) and Coetzee, *et al.'s* (2010) hypotheses state that sardines of the KZN sardine run are in fact undergoing a migration. Freon*, et al.* (2010) lists Dingle's (1996) conditions for a movement to be deemed a migration. Dingle's (1996) migration definition requires that sardines would need to display direct, predictable and continuous motion, swimming outside of their usual home range. Indeed, these fish undergo a journey of hundreds of kilometres clear of their home range (see Figure 1), in a direct manner and towards a predictable area i.e. KZN. Another requirement for the sardine run movement to be deemed migration is that the fish should ignore other resources along the migration route that would usually not be ignored (Dingle 1996). Sardines leave the plankton rich waters of the Agulhas Bank to swim into the nutrient poor, warm oligotrophic waters of KZN (Hutchings*, et al.* 2010). The poor conditioning factor of sardines partaking in the sardine run exemplify the fact that these individuals do not move into KZN to feed (van der Lingen, Coetzee*, et al.* 2010).

Dingle (1996) stated that a migratory movement requires specific departure and arrival behaviour. Although these aspects of the sardine run remain unclear, Freon*, et al.* (2010) report that shoals of sardines on the eastern Agulhas bank leave for KZN in May. Sardine shoals usually arrive in the Northern Eastern Cape near Port St. Johns by June, and if conditions are suitable, they may move into KZN periodically, in accordance with the Waterfall Bluff gateway hypothesis described by Roberts*, et al.* (2010), from June to September (Freon et al. 2010). The use of proxies such as predator occurrence (such as Cape gannets and Common dolphin) has been used to determine the presence of sardine shoals, and thus the beginning of the sardine run (O'Donoghue, Drapeau and Peddemors 2010). In KZN, plankton trawls for sardine eggs, are used to detect spawning sardines. Indeed, sardine eggs are commonly found in plankton trawls until December (Connell 2010).

The last condition stipulated by Dingle (1996), is that the migrant sardines should show physiological storage of energy in the form of fats and lipids. Long term research between 1953 to 2004 (van der Lingen*, et al.* 2006, van der Lingen*, et al.* 2007, van der Lingen, Hendricks*, et al.* 2010) showed that sardines from the southern cape and Agulhas bank (Figure 1) illustrate a higher mean year round conditioning factor than those found on the western

coast (Table 1). Individuals from the sardine run displayed the lowest conditioning factor (van der Lingen*, et al.* 2006) of all sampled areas.

Sardines in southern Africa have been separated into two distinct sub stocks based on anatomical and life history trait differences. Miller (2006) confirmed variability in spawning and retention of sardine eggs and larvae into the Western Cape area and Agulhas Bank (Coetzee, Merkle*, et al.* 2008, Coetzee, Van der Lingen*, et al.* 2008) and the abundance and distribution of two separate groupings of sardines, separated by a transition zone between the west coast and the Agulhas Bank (Figure 1). Based on this, de Moor*, et al.* (2008), de Moor*, et al.* (2009) and Van Der Lingen*, et al.* (2009) described the delineation of two putative sardine stocks in the Southern Benguela, which included differences in physical characteristics; i.e. the western and eastern stocks. The poorer conditioned western stock do not readily interact with eastern stock sardines or move east of Cape Agulhas, apart for some larval transport (Miller*, et al.* 2006). Thus Freon*, et al.* (2010) deduced that sardines partaking in the sardine run likely come from sardines on the Agulhas Bank. Freon*, et al.* (2010) concluded that KZN sardines lost their lipid reserves as they travelled northwards into KZN. Thus, from the above information, the sardine run generally conformed to Dingle (1996) definition of a migration.

Baird (1971) suggested that the sardine run formed part of a subpopulation spawning migration; Freon*, et al.* (2010) questioned whether sardines from the sardine run were phenotypically and genetically different (see Freon' test 3 and 4) from the rest of the SA population. Phenotypic data can be useful for indirect subpopulation separation and show post larval variation between varied environments (Freon*, et al.* 2010), as illustrated in Table 1 (van der Lingen, Coetzee*, et al.* 2010). Most recently, van der Lingen, Hendricks*, et al.* (2010) described the biological characteristics of sardines caught off KZN and compared them with the eastern and western stock, and concluded that the KZN stock represented a distinct stock or possibly a functionally discrete adult assemblage (FDAA). A FDAA is defined as a population of adults that are physically or reproductively isolated from another. These conclusions were drawn from significant differences in conditioning factor, vertebral count and body shape of fish from the Agulhas Bank (eastern stock, Figure 1) and KwaZulu-Natal (van der Lingen, Coetzee*, et al.* 2010, van der Lingen, Hendricks*, et al.* 2010).

Definitive evidence of stock structure can be determined by the use of various modern genetic techniques. Nevertheless, this powerful tool has yet to be applied specifically to the sardine run. Hampton (pers. comm.) has undertaken a study to test the two stock hypothesis (Figure 1) by van Van Der Lingen*, et al.* (2009), de Moor*, et al.* (2008) and de Moor*, et al.* (2009) of the southern Benguela sardines. The lack of molecular data relating to the sardine run has left a key shortage in the collective knowledge of the sardine run. The use of these techniques is discussed further on.

Another method of identifying stock structure, using parasites as biological tags, has been proposed by Reed*, et al.* (2012). In their assessment of possible parasitic biological tags, it was illustrated that some members of the digenean tetracotyle metacercariae (a subclass of parasitic flatworms) have potential as biological tags. The study employing biological tags (Reed, UCT; van der Lingen, DAFF; Froeschke, Stellenbosch University) and the use of modern molecular techniques (within this study) are underway, and should produce a description of the stock structure sardine run complimentary to this study.

Freon*, et al.* (2010) tested another requirement for the spawning migration hypothesis to be fully supported, and this was that migrating sardines would have to spawn in KZN. Connell's (2010) twenty-one year time-series of ichthyoplankton data confirms that sardines do spawn during the sardine run. Occurrences of sardine eggs in plankton trawls begin with the start of the run in June, and continue before slowly declining to almost no observations in December (although sardine eggs have been collected as early as March and late as January), when most sardines are thought to have returned to the Agulhas bank (Freon*, et al.* 2010, van der Lingen, Coetzee*, et al.* 2010). The sixth requirement highlighted by Freon*, et al.* (2010), is that a nursery area should exist in KZN waters. Indeed, juveniles (van der Byl 1978, Beckley*, et al.* 1994, Beckley*, et al.* 2003) and larvae (Connell 1996; 2010; 2012) have been documented in KZN. More recently, Freon*, et al.* (2010) described how Miller*, et al.* (2006) observed juvenile sardines during a survey of the waters between Port Elizabeth and Port St. Johns. Miller*, et al.* (2006) thus reasoned that these sardines were possibly spawned in KZN and travelled south with the Agulhas current. Miller*, et al.* (2006) added that long term data by Connell (2010) suggested a causative link between the winter presence of sardines in KZN and the presence of

eggs in the water column off KZN. Thus KZN is likely a nursery ground for the sardines spawned during the sardine run.

It is not yet known whether KZN spawned juveniles are successfully recruited to the adult stock (Freon*, et al.* 2010). Nevertheless, the number of juveniles recruited to the returning stock of sardines must be significant, especially if the KZN sardine run represents a genetically distinct subpopulation spawning migration or relic behaviour (Freon*, et al.* 2010). The use of modern molecular techniques can be used to compare relatedness of sardine larvae and juveniles sampled in KZN and along the Agulhas bank (Freon*, et al.* 2010). Other techniques such as otolith biochemistry can also aid in determining the origin of larvae and juveniles and biannual sampling of otoliths can allow for one to determine the regions that sardines occupy during the year.

Freon, *et al.'s* (2010) final test of Baird's (1971) hypotheses was whether all spawned sardines, in all year classes, spawn solely in KZN. Allopatric spawning in *Sardinops sagax* is possible; where sardine run individuals only spawn in KZN, and those of the western and eastern stock off the Agulhas bank only spawn on the Agulhas bank (Freon*, et al.* 2010). However, Freon*, et al.* (2010) established that due to the lengthy and bimodal spawning characteristics of the sardines, the possibility of larger fecund females spawning in KZN in June and again later in the year upon returning to the Agulhas Bank was probable. The use of fine scale population genetic techniques, investigating whether allopatric or sympatric spawning populations exist, remains the most feasible method of shedding light on the subject.

Freon *et al.*'s (2010) final test, which only applies to the relic behaviour hypothesis, relates to past environmental conditions that may have influenced distribution and reproductive strategies, including bet hedging (Coetzee*, et al.* 2010). Conditions during the last glacial maximum approximately 18 000 years ago, included lower SST and sea levels (Freon*, et al.* 2010). These past environments are thought to have encouraged a larger suitable range for *Sardinops sagax*. It is likely that the west and east coast sardines were separated by the limits of its suitable habitat (see Figure 1) and that two large areas of the continental shelf off Mozambique were suitable for sardines. This area had increased fluvial input and which may

have been favourable to sardines as a nursery ground and thus could be a relic of a spawning migration of sardines during the last glacial maximum (Freon*, et al.* 2010). Nevertheless, without evidence of the presence of sardines off Mozambique in the fossil record, any assumptions remain conjecture (Freon*, et al.* 2010).

1.3.2. Proximate factors

In the case of the sardine run, proximate factors are those that enable or trigger the sardine run. Freon*, et al.* (2010) tested and ultimately supported two of the five proximate factors involved in the sardine run. The first hypothesis to explain the triggering of the sardine run is the natal homing and imprinting hypothesis, defined by Carr (1967), where adults will return to their place of spawning and Stabell (1984) who added that natal homing was brought on by external signals which are imprinted at the egg or larval stage (Freon*, et al.* 2010). Certainly, this hypothesis could explain why such a small percentage of sardines migrate annually during the sardine run and that the amount of sardines partaking in the run shows no relationship to population size off the Agulhas bank and the Western Cape coast (Freon*, et al.* 2010). Vertebral counts may also yield important information about the above hypothesis. Sardines spawned in warmer waters, such as off the KZN coast during the sardine run have less vertebrae than those spawned off the Western Cape (Freon*, et al.* 2010). This may help to identify where fish may have been spawned. However, the use of vertebral counts as a proxy for spawning locality has a significant caveat in that sea surface temperature (SST) is variable along the Southern African coastline throughout the year (Lutjeharms*, et al.* 2001).

The second proximate hypothesis is environmental forcing of sardines up the east coast via the Waterfall Bluff gateway, first observed by Armstrong*, et al.* (1991) and then confirmed by Roberts*, et al.* (2010). Here, sardines are hypothesised to be forced by currents of cooler water, bounded by unfavourable warm Agulhas current water, and the Natal pulse. Nevertheless, this hypothesis cannot be tested by means of the objectives set out below, thus for the sake of brevity the above mentioned and explanation will suffice.

The findings described in the sardine run special edition were constrained by available ocean current data. However, the need to establish objectives to fully test the hypotheses listed above, is critical. Indeed, the need for targeted research has been reiterated by Freon*, et al.* (2010). One of the tools described as suitable to test all but the Waterfall bluff gateway hypothesis, is the use of modern molecular genetics (Freon*, et al.* 2010). The work proposed, and ultimately conducted in this MSc dissertation, is based primarily on modern molecular techniques briefly outlined in the following paragraphs, with a more in depth description given in chapter 2 (Methodology).

The need to establish population ranges and parameters such as identity, variance, relatedness, demographics, diversity, degrees of inbreeding, and migration is important to understanding the sardine run. Practically, this information could allow fisheries and conservation managers to determine populations under potential threat, and those that may be exploited to an optimum. Multi population fisheries may be implemented if necessary, thus generating a more realistic fisheries model. This minimises the effect of selection differentials on specific populations (Law 2007).

1.4. Motivation and rationale for this study

1.4.1. Population genetic and morphological techniques in population delineation

Taxonomy is important to fishery scientists for the delineation of fished resources, and aids in developing rational conservation strategies (Agüero*, et al.* 2004). The taxonomy of the sardine globally, including other clupeid species has been contentious (Agüero*, et al.* 2004), and this has been the case for *Sardinops sagax* in Southern Africa. Five global sub-species of *Sardinops sagax* were identified worldwide by Grant*, et al.* (1998); including the sub species of *S. sagax* from southern Africa, designated the sub species name: *S. ocellatus*. Nevertheless, studies focused on stock structure and systematics have subsequently shown the genus to be monotypic (Beckley*, et al.* 1999).

The sardine is referred to as *Sardinops sagax*, although it is considered a different sub-species to the South American *S. sagax sagax*. In Southern Africa, *S. sagax* occurs year round on the Agulhas bank between Port Elizabeth in the east and into Namibia to Luderitz to the north

west. Genetic and morphological analysis can be especially valuable in testing the above hypotheses, where broad scale structuring among sardines found on the east and south or west coast during the sardine run may be determined. Finer scale connectivity and population structure amongst sardines within the sardine run may also be determined via genetic analysis. Genetic structuring of the sardines will allow one to test the current hypotheses outlined above, thought to be the cause of the sardine run (Freon*, et al.* 2010). Genetic techniques using microsatellites, exon-primed intron-crossed primers, single nucleotide polymorphisms and many other methods are common now to many fished stocks such as salmon (Ryynanen*, et al.* 2006), Big eye tuna (Martínez*, et al.* 2006) and a host of other species, including Clupeidae (Touriya*, et al.* 2003, Pereyra*, et al.* 2004, Atarhouch*, et al.* 2006, Gonzalez*, et al.* 2007, Keski¨n*, et al.* 2012).

Genetic analyses can be used for testing the hypotheses proposed by contemporary literature, but are also crucial to determining the demography of the sardine stocks which are fished commercially on both the west and east coasts of Southern Africa. This may ultimately lead to the conservation of genetically delineated stocks. This is especially important in ensuring species and stock resilience to anthropogenic pressure and thus the sustainability of the sardine run as a fished stock and an economically important ecological phenomenon primarily through the preservation of genetic diversity.

Based on Miller, *et al.*'s (2006) data, Van Der Lingen*, et al.* (2009) concluded that the Southern Benguela stock illustrates strong evidence as two separate stocks, with a transition zone separating the stock west of Cape Agulhas and east of Mossel Bay (Figure 1). Miller*, et al.* (2006) concluded that apart from some larval exchange within the transition zone, effectively two distinct stocks exist with limited larval and egg exchange. Hampton (*pers. comm.)* found some shallow genetic structuring, using various microsatellite markers.

Sardines found west of Cape Agulhas exhibited different phenotypic characteristics to those found east of the transition zone (see Figure 1). Generally, sardines found east of the transition zone had poorer conditioning, lower fat content and lower reproductive parameters, smaller

relative body size, different otolith shape, body shape and larger head size (Wessels 2009). West coast sardines also fed on smaller copepods and were found in cooler waters, based on data from multiple years (see Table 1; Van der Lingen *et al.* (2009)). Most notable however, is the separation of the breeding stock and nursery grounds of each stock (Miller*, et al.* 2006). Sardines of both stocks illustrate natal homing with separate nursery areas to one another. Although Miller*, et al.* (2006) found some degree of egg and larval transport, mostly from the western stock to the east; the two stocks seem to be on an evolutionarily divergent path.

Although all sardines along the southern African coast have overlapping ranges, new data describing shallow population structuring between the western and eastern Benguela sardine stock have been identified (Shannon and van der Lingen, Pers. Comm.). This may be explained by the fact that it is possible that southern Benguela sardines may form functionally discrete adult assemblages (FDAA's) (van der Lingen, Hendricks*, et al.* 2010). The delineation of these stocks may be caused by factors such as diet, conditioning factor (Van Der Lingen*, et al.* 2009), habit, breeding and dispersal ranges (Coetzee, Van der Lingen*, et al.* 2008, de Moor*, et al.* 2008, de Moor*, et al.* 2009). Limited larval and juvenile exchange in the area between Cape Agulhas and Mossel Bay may be a manifestation of two FDAA (Figure1; Miller*, et al.* (2006)).Thus, molecular techniques employed during this study, must be able to identify fine scale genetic structuring as well as broader scale sub-species and species level phylogeographic relationships that may exist. This requires the use of molecular markers that evolve at different rates, to discern evolutionary relationships at different taxonomic resolutions.

Broad-scale markers, such as Cytochrome-b, D-loop and COI, have been used successfully on clupeids to elucidate phylogenetic relationships within and between species (Ivanova*, et al.* 2007). Recently, universal primer cocktails designed to amplify a 650 base pair region of the cytochrome oxidase I (COI) region have been developed at the Canadian centre for DNA barcoding and has been successfully used to barcode marine fishes. The barcoding of sardines has already been conducted on sardine larvae and adults in KZN and abroad (Ward*, et al.* 2005, Ward*, et al.* 2009, Zemlak*, et al.* 2009, Cawthorn*, et al.* 2011, Steinke*, et al.* 2011, Keskİn*, et al.* 2013).

The nature of the sardine run, as discussed above, may show some amount of genetic structuring. This necessitates the use of a spectrum of very fine, to moderate scale markers such as the microsatellite markers described by Pereyra*, et al.* (2004) and others such as the exon-primed, intron-crossing (EPIC) primers described by Touriya*, et al.* (2003) used on the clupeid *Sardina pilchardus*, and other non-model teleost fish (Hassan*, et al.* 2002, Li*, et al.* 2010). These primers can be utilised in many different ways by modern molecular biology.

The mode of analysis depends on many factors, including the type of data required which best fits the aims of the study, available laboratory equipment and the allocated budget required to process representative numbers of individuals per population. Recently, modern molecular biology has shifted towards the use of DNA sequencing as the means through which molecular analyses are carried out (Sunnucks*, et al.* 2000). Nevertheless, this method can be expensive and time consuming (Sunnucks*, et al.* 2000).The development of fast and relatively cheap yet effective protocols, capable of detecting accurate levels of sequence polymorphism are available and have been used successfully in population genetic studies (Sunnucks*, et al.* 2000). This includes length polymorphism gel electrophoresis, employed in this study, where many individuals may be analysed over multiple marker sets in a short period of time (details illustrated in chapter 2; methodology).

1.4.2. Conclusions

Considering the above, the aim of this project is to investigate the heterogeneity of the South African sardine stock (*Sardinops sagax*) using length polymorphism gel electrophoresis and sequencing of EPIC PCR amplicons. Population structure will also be investigated using meristic and morphological characters, using multivariate and geometric techniques. Samples collected off KZN sites, Port St. Johns, Port Elizabeth and sites in the Western Cape will be used (see methodology). Findings from the genetic analyses will be used to determine whether sardines caught in KZN return to their (possible) natal spawning grounds, and whether sardines

partaking in the KZN sardine run form part of a sub-stock of sardine in South Africa, thus testing Freon*, et al.* (2010) hypotheses.

If it is found that KZN sardines are a sub-stock, then it would have important consequences for the management of this event, particularly from an ecosystem approach to managing the beach-seine and the purse-seine fishery in South Africa. The threat of potential near shore ocean warming, and effects on local upwelling cells, due to the strengthening of the Agulhas Current as a consequence of climate change, may also have negative effects on the sardine run (Rouault*, et al.* 2010). Climate mediated changes in physical and ecological parameters has been observed in changes in the distribution and behaviour of a large number of species globally (see Brander (2010) for examples), including the south and eastward shift in sardine distribution in South Africa (Coetzee, Van der Lingen*, et al.* 2008). Given the importance of the annual sardine run movement, as outlined above, it is critical that further research is undertaken to provide both continuity in research effort and to create the knowledge necessary for the successful marine management of this event.

Information collected in this study will allow stock managers to plan conservation strategies based on morphological and genetic diversity, connectivity, and breeding patterns within the KZN sardine. Ultimately, understanding genetic relationships and breeding patterns of the migrating fish stocks will allow for the conservation of genetic diversity of the fish, and the preservation of the KZN sardine run. This will not only benefit the sardine, but also allow the multitude of taxa such as marine birds, teleosts, elasmobranchs, and cetacea that take advantage of the sardine run.

1.4.3. Arrangement of dissertation

This dissertation consists of five chapters; an introductory chapter (chapter 1), Materials and Methods (chapter 2), two chapters describing the morphometric (using meristic, multivariate and geometric morphology) and genetic (using mitochondrial and exon-primed, intron-crossing markers) population structure of *Sardinops sagax* between KZN and the Eastern Agulhas Bank and Western Cape stock (chapter 3 and chapter 4), and a concluding chapter (chapter 5). The materials and methods in chapter 3 and chapter 4 are presented together in chapter 2 to avoid repetition.

Chapter two:

Materials and methods

2.1 Study sites and sample collection

To assess the population connectivity of sardines along the South African coast and thus allow for comparison of the hypothesised KZN sardine stock, sampling was conducted west of Cape Agulhas, on the Agulhas bank, and in KZN between June 2011 and September 2013. The sites mentioned represented sampling sites within hypothesized populations (Figure 1). A total of 1039 sardines were collected (including 34 larvae samples collected in KZN) from five sites along the South African coast; namely Cape Town, Mossel Bay, Port Elizabeth, Port St. Johns, and the KZN South coast from Port Edward to Park Rynie (see Table 1).

Table 1: *Sardinops sagax* collected in South Africa from July 2011 to 2013, including sample numbers for morphometric, meristic and genetic analyses.

Sample type	Date collected	Location and Coordinates	Capture method	Morphometric analysis	Meristic analysis	mtDNA	EPIC
Adult	01/07/2011	KZN (30°27'S 29°42'E)	BSN	None	204	11	48
Adult	01/07/2012	KZN, (30°56'S 30°18'E)	BSN	None	210	10	48
Adult	01/11/2012	KZN, (30°18'S 30°44'E)	RR	None	7	None	None
Larvae	30/09/2012	KZN (30°18'S 30°44'E)	PN	N/A	N/A	5	22
Larvae	13/07/2012	KZN (30°18'S 30°44'E)	PN	N/A	N/A	None	10
Larvae	22/11/2012	KZN (30°18'S 30°44'E)	PN	N/A	N/A	8	4
Larvae	09/12/2012	KZN (30°18'S 30°44'E)	PN	N/A	N/A	None	None
Adult	04/02/2013	WP, CT (33°04'S 17°50'E)	PSN	122	200	13	48
Adult	09/02/2013	EC, PE (33°44'S 25°58'E)	PSN	200	201	6	None
Adult	04/04/2013	WP, MB (34°35'S 22°27'E)	PSN	200	200	None	None
Adult	08/07/2013	KZN (31°02'S 29°34'E)	RR	17	17	None	None
			Total:	522	1039	40	378

WP=Western Province, EC= Eastern Cape, KZN=KwaZulu-Natal, CT=Cape Town, PE=Port Elizabeth, MB=Mossel Bay,. BSN=Beach seine net, RR=Rod and reel, PN=Plankton net, PSN=Purse seine net. N/A=Not applicable.

Sardines were caught using purse seine nets with a 28mm mesh size aboard commercial purse seining vessels in Cape Town, Algoa Bay (Port Elizabeth) and Mossel Bay (Table 1) for research by the department of Fisheries and Forestry (DAFF). Sardines were collected from beach seine netters operating along the Eastern Cape's wild coast and KZN coast in winter. Sardines were netted in favourable surf conditions along sandy beaches where shoals of sardines came close enough to the shoreline (generally less than 300m from the shore) to be caught in beach-seine nets with a wing mesh size of at least 14mm and at least 13mm for the cod end, with a total net length of less than 100m. Sardines were also caught via Yusuri jig (Kingfisher Sabiki luminous live bait jig, No. 4 hook) on rod and reel in less than 50m depth of water, from a ski-boat in KZN and the wild coast (Table 1). Sardines were frozen at -20°C after capture for approximately two months before morphometric analysis.

Sardine eggs were collected via surface plankton trawls off Park Rynie (KZN) in the months after the sardine run, as part of an on-going ichthyoplankton survey started in 1987 and aimed at understanding the spawning patterns of pelagic fish off the KZN coastline (Connell 1996; 2010; 2012). A two-meter long cone shaped plankton net with an aperture of 1360cm^2 and a mesh size of 300µm was trawled along the water surface at approximately 2km/h for 10 minutes. The plankton trawls were conducted in 40 to 50 meters of water depth. Sardine eggs were sorted from the trawl samples based on external characteristics, hatched overnight, and incubated for five days, so as to aid identification, by Dr Allan Connell. Larvae were stored in 95 % ethanol at room temperature.

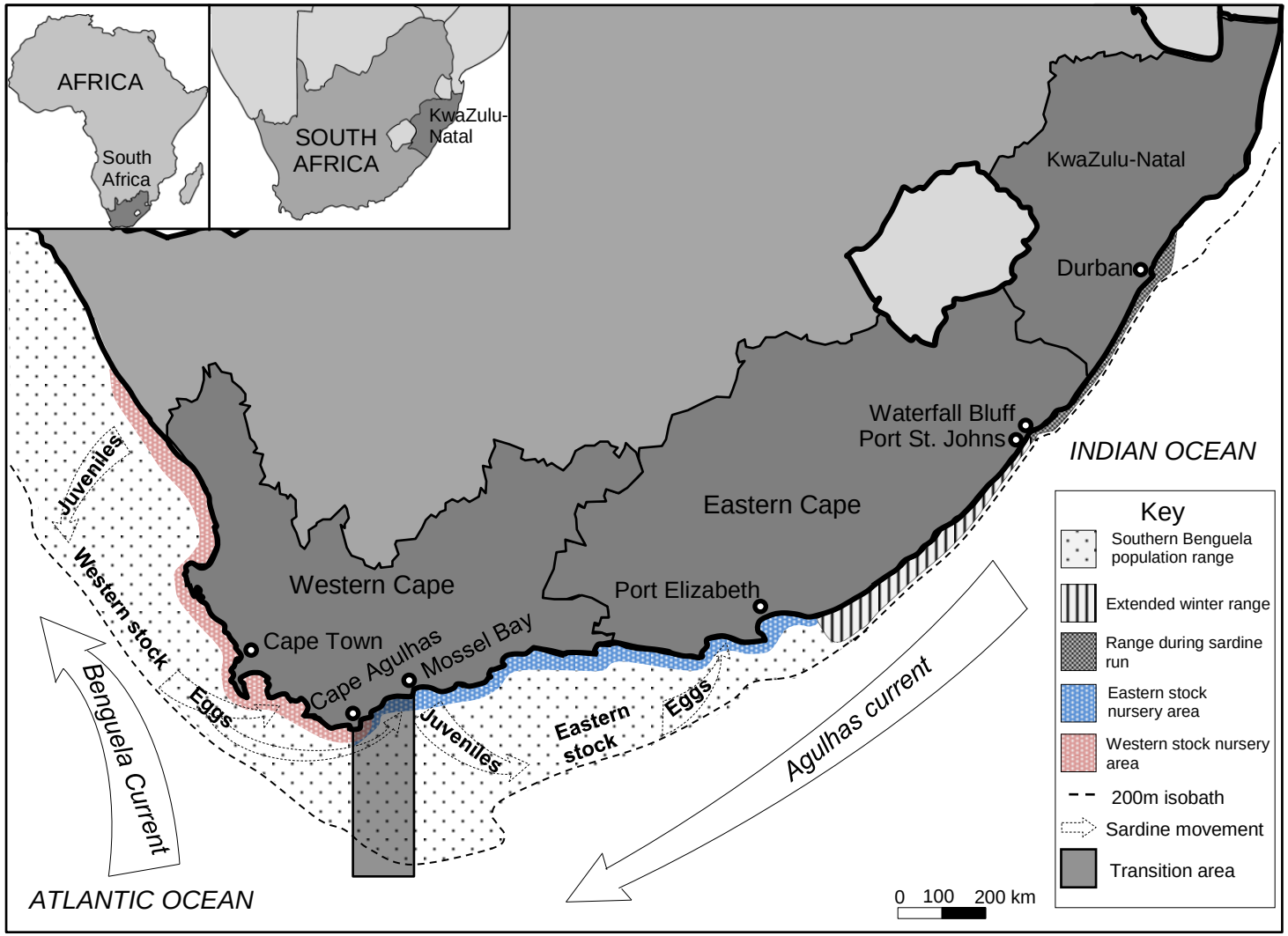

Figure 1: Map of *Sardinops sagax* occurrence and putative stock delineation in Southern Africa, including the winter and summer ranges and the spatial extent of the sardine run.

2.2 Meristic data collection and analysis

Wet body mass, caudal length, sex ratio, and number of vertebrae can be useful indicators of stock identity. These morphological traits are often sensitive to environmental factors such as stress, food type and availability, and water temperature; and can cause differences in these measurements between populations. Hence, these parameters have successfully been used on sardine stocks in South Africa for some time (van der Lingen*, et al.* 2005, Miller*, et al.* 2006, van der Lingen*, et al.* 2007, Coetzee, Van der Lingen*, et al.* 2008, Wessels*, et al.* 2010). Wet body mass and caudal lengths were recorded for all individuals and subsequently frozen at -20°C for two months; this was to standardize the amount of time all sardines were exposed to freezing and thus its effect on fish morphology (Wessels*, et al.* 2010). Vertebrae in the Clupeidae vary in number due to environmental temperature during development. Thus, vertebrae number between individuals of different populations is an important proxy for the origin stock identity of sardine populations in Southern African waters. The difference in population variation in vertebral count is due to the temperature gradients along the coast. Sardines spawned in colder water generally have more vertebrae, and less in warmer water. After photographs were taken for geometric analysis (see: 2.3.2. Multivariate and Geometric analysis), sardines were dissected dorso-ventrally to count vertebrae and determine sex.

A correlation analysis between caudal length and wet body mass of fish was conducted. A non-parametric Spearman's rho correlation was computed after the assumptions for parametric analysis, via a one sample Kolmogorov-Smirnov test, were not met. R^2 values were also calculated for each population.

To test the possibility of sexual dimorphism (and thus the necessity to test population differences according to sex), a non-parametric Kruskal-Wallis ANOVA was conducted for wet body mass, caudal length, and vertebrae count within each population; according to sex with data cases split according to site so within site sexual dimorphism could be measured. This, after the assumptions of homeoscedacity (Levene's test) and normality (1 sample Kolmogorov-Smirnov test) were not met. This meant both sexes could be analysed together, between sites.

A non-parametric Kruskal-Wallis ANOVA was conducted for wet body mass, caudal length, and vertebrae count within each year class of the KZN population so any differences in sardines sampled between years during the sardine run could be measured. There were no significant differences observed between years. This, after the assumptions of homeoscedacity (Levene's test) and normality (1 sample Kolmogorov-Smirnov test) were not met. This meant that all year classes could be analysed together. Vertebrae and sex ratio frequency distributions were also calculated for each population. All statistical tests were undertaken with a 95 % confidence interval using IBM SPSS version 21.0 (IBM Corp. 2012).

2.3. Morphological data collection and analysis

2.3.1. Material collection and preparation

Sardines were stored frozen at -20°C for two months, before being photographed. Sardines were carefully placed in polystyrene boxes, in single layers, laid straight and belly-up to ensure minimal deformation of body shape, resulting from vertebral columns of fish freezing at varied angles or tearing of the soft abdomen flesh. Sardines were slowly air thawed to room temperature before photographs were taken. Sardines were placed individually on a white plastic surface alongside a set square with millimetre marking. Individuals were placed upon a line drawn across the white plastic, with a marking to place the tip of the nose of the fish. This ensured consistent placement of the fish within the camera's focal range. Individuals were manipulated into a natural position with their mouths closed, vertebral column aligned; and flared dorsal, caudal, anal, pelvic and pectoral fins. The camera was placed on a stand 35 cm from the fish and so that the focal point was the centre of the fish. Photographs were taken without flash, and in good lighting conditions. Careful attention was given to keeping all components of the photographic apparatus in the identical position between photograph sessions, thus keeping a standardised photographed area. Digital photographs of the left side of each individual were taken with a Sony DSC-WX7 (resolution: 28.3 pixels.cm^{-1}).

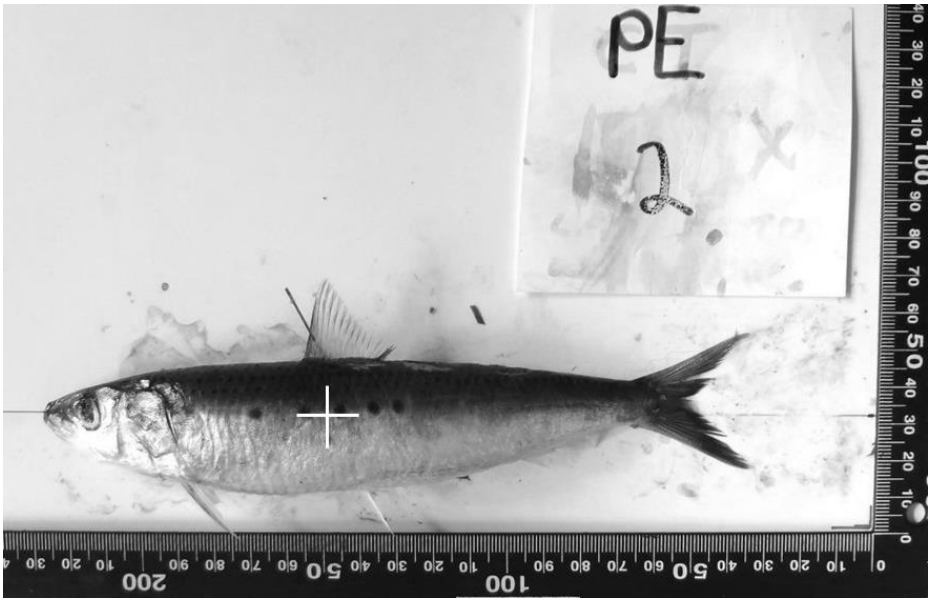

Plate 1: Typical photographed area (cropped to show only scale bar and sample) of *Sardinops sagax* for landmark placement before morphometric analysis. The white cross represents the consistent focal area.

2.3.2. *Multivariate and Geometric analysis*

Morphometric analyses are a powerful set of tools in evaluating stock identity, discrimination, and delineation (Cadrin 2000). Although recently superseded by modern genetic techniques, this technique is important in delineating populations that can only be observed in an individual's phenotype. Thus, in combination with genetic techniques, geometric morphometrics offer a powerful tool to determine population boundaries according to phenotype and genotype (Cadrin 2000).

Eleven digitised landmarks were recorded upon the sardines photographed, with 21 truss measurements (Figure 2), using tpsDig2 software (Adams*, et al.* 2004). Easily recognisable and reproducible points on the sardine were chosen to minimise error associated with placing landmarks, and in accordance with previous studies for continuity and possible future comparison (Silva 2003, Agüero*, et al.* 2004, van der Lingen, Hendricks*, et al.* 2010, Wessels*, et al.* 2010).

Table 2: Description of landmark positions placed on *Sardinops sagax* samples for morphometric analyses.

Land mark coordinate	Landmark description
1	Tip of the snout
2	Top front corner of operculum, below occipital ridge
3	Front insertion point-dorsal fin
4	Back insertion point-dorsal fin
5	Upper insertion point-caudal fin
6	Lower insertion point-caudal fin
7	Front insertion point-anal fin
8	Front insertion point-pelvic fin
9	Front insertion point-pectoral fin
10	Jaw hinge, aligned with operculum
11	Point where last backbone meets caudal fin bones

Ten landmarks (1 - 10, see Figure 2) were used to produce 21 box trusses, for subsequent multivariate analyses, by calculating relative distances between landmarks using PAST version 3.01 (Hammer*, et al.* 2012). A Principal Coordinates Analysis (PCA) was run and outlying samples from the 95 % confidence limits of the scatter plot were removed from further analysis. Seven females were removed due to abnormally distended stomachs. Truss variables were then corrected for by size, using the Burnaby method, after being log transformed (Burnaby 1966, Rohlf*, et al.* 1987). Truss variable size was corrected by projecting truss variables on the subspace orthogonal to the space covered by the size vector in PAST (Hammer*, et al.* 2012). Subsequently, confidence (95 % interval) limits indicated individual size vectors were not significantly different from other individuals, suggesting a common size transformation had standardised all samples.

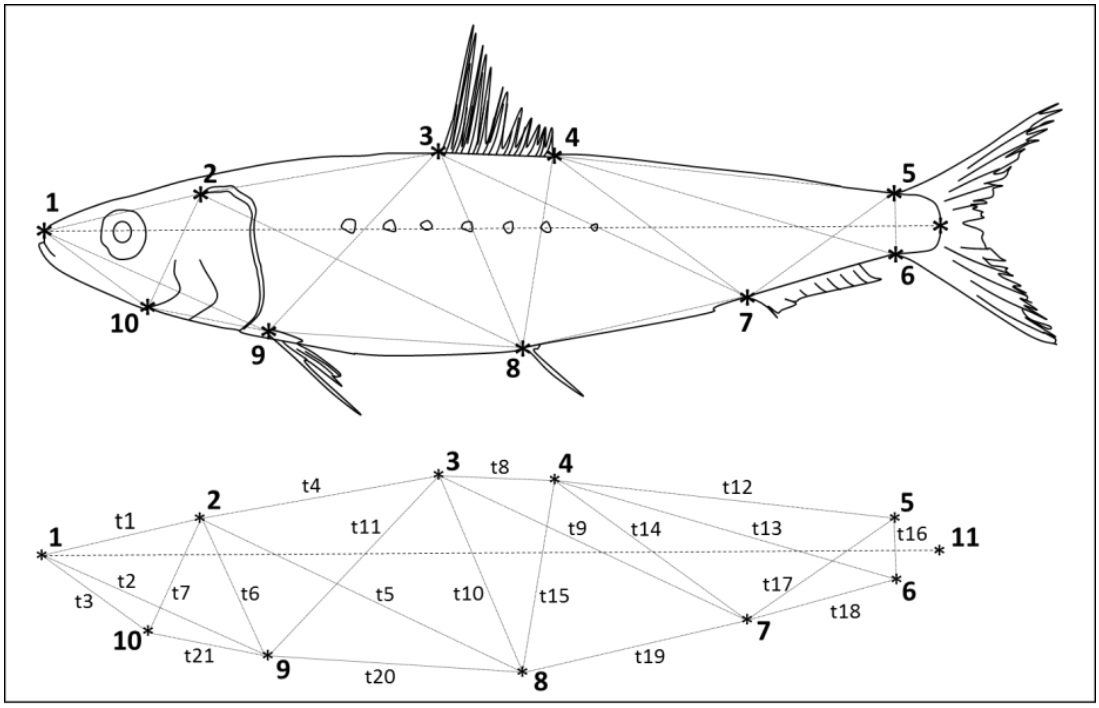

Figure 2: Placement of eleven morphometric landmarks and the truss network (t1 - t21) used in multivariate and geometric analysis, for *Sardinops sagax* caught at eight sites along the South African coastline. Dotted line (landmark 1 - 11) indicates the caudal length measurement.

A Principal Component Analysis (PCA) was conducted for the size-corrected truss variables, and principal coordinates calculated using SPSS (IBM Corp. 2012). Group centroids for individuals of each site, and 95 % asymptotic confidence limits of the scores on the first two principal components, were computed for each sample. Discrimination functions between truss measurements according to site were calculated using a Discriminant Function Analysis (DFA). DFA was used to calculate the contribution of each truss measurement to site separation and individual difference to hypothetical mean measurements (McGarigal 2000). Relative discriminatory power of each truss measurement was also calculated for each truss measurement.

Geometric analyses were conducted using ten landmarks (1 - 10), in PAST (Hammer*, et al.* 2012). A generalised orthogonal Procrustes 2D analysis was completed for each sardine, through superimposition, to determine size corrections for all individuals using PAST (Hammer*,*

et al. 2012). Sample configurations were centred, scaled, and rotated to minimise the sum of squares distance between homologous landmarks for all individuals allowing for the removal of the effect of distance from the shapes. Mean shapes (consensus configurations) were calculated for sites including relative warps. The Procrustes distances were then analysed by hierarchical clustering with 100000 bootstraps (Stevens 2012). Differences among group means were tested using the Mahalanobis distance using single linkage, and the consistency of the groups was evaluated by computing the misclassification rates of new individuals (Stevens 2012).

2.4 Genetic material, primer and data collection, and analysis

2.4.1. Primer selection

Exon-primed, Intron-crossing (EPIC) primers are an invaluable tool to molecular ecologists and taxonomists alike, due to their ability to deliver both inter- and intra-specific level of genetic information relatively cheaply (Li*, et al.* 2010). The homology of EPIC-amplified sequences can also be determined by comparison of their exon or intron (Li*, et al.* 2010). Nevertheless, there have been few markers developed for non-model teleost fishes, thus little work has been conducted using them (Hassan*, et al.* 2002, Touriya*, et al.* 2003, Ryynanen*, et al.* 2006, Chenuil*, et al.* 2010, Li*, et al.* 2010). Nevertheless, ten EPIC primers previously shown to work on teleost fishes were chosen for this study (Table 2). The first aim was to identify EPIC markers that were amplifiable and showed polymorphisms within the sardine DNA. The markers that worked and showed polymorphisms within or between populations were then used for a population level assessment of genetic diversity amongst the Southern African Sardine.

Primers that amplify the mitochondrially encoded cytochrome oxidase I gene (mtCOI), have recently been designed at the Canadian Centre for DNA Barcoding (CCDB), and are the primary markers used in the global effort to genetically barcode the world's teleost ichthyofauna (Ivanova*, et al.* 2007). This marker has been used successfully on Clupeiformes, including *Sardinops sagax* locally and abroad (Ward*, et al.* 2005, Ivanova*, et al.* 2007, Zemlak*, et al.* 2009, Cawthorn*, et al.* 2011, Keskİn*, et al.* 2013).

Table 3: Mitochondrial DNA and (mtDNA) exon-primed intron-crossing (EPIC), primers used on *Sardinops sagax*.

	Gene	Marker abbreviation	Primer name	Sequence set (5'-3')	Ta	Size of focal intron	Reference
mtDNA primer cocktail	Cytochrome Oxidase sub unit 1	COI	FishF2-t1	TGTAAAACGACGGCCAGTCGACTAATCATAAAGATATCGGCAC	46°C	652	(Ivanova, Zemlak et al. 2007)
			FishR2-t1	CAGGAAACAGCTATGACACTTCAGGGTGACCGAAGAATCAGAA			
M13 sequencing primers	-	-	M13F (-21)	TGTAAAACGACGGCCAGT	N/A	N/A	(Messing 1983)
			M13R (-27)	CAGGAAACAGCTATGAC			
EPIC primers	Opsin	Ops	OPS-1-F	GCTCATGGGCCTGCAGACCACAA	52°C	1022	(Touriya, Rami et al. 2003)
			OPS-1-R	CCTGCTCAACCTGGCCATGGC			
	Actin	Act	Act-2-F	GCATAACCCTCGTAGATGGGCAC	58°C		(Touriya, Rami et al. 2003)
			Act-2-R	ATCTGGCACCACACCTTCTACAA			
	Chymo-trypsin B	ChymB	ChymB-6-F	GCATGAGGGCTGTGACTCGGG	54°C	382	(Touriya, Rami et al. 2003)
			ChymB-6-R	ATCGTGTCCGAGGCTGACTGCAA			
	Myosin light chain 3	MLc	MLc-3-F	AGTAATGACGTCGCAGATGTTCT	54°C	674	(Touriya, Rami et al. 2003)
			MLc-3-R	CGACAGGTTCACTCTCGAGGAG			
	Calmodulin	Cam-3	Cam-3-F	TGACGGAGCTCTGCAGCACTGAC	54°C	491	(Touriya, Rami et al. 2003)
			Cam-3-R	GTGAGGAGGAGCTCCGTGAGGC			
	Glyceraldehyde-3-phosphate dehydrogenase	GPd	GPd-2-F	GCCATCAATGACCCCTTCATCG	46°C	303	(Hassan, Lemaire et al. 2002)
			GPd-3-R	TTGACCTCACCCTTGAAGCGGCCG			
	Aldolase B, intron 4	AldoB-4	Aldo-5-F	GCCAGATATGCCAGCATCTGCC	54°C	134	(Hassan, Lemaire et al. 2002)
			Aldo-3.1-R	GGGTTCCATCAGGCAGGATCTCTGGC			
	Gonadotropin-releasing hormone 3	GnRH	GnRH-2-F	AGAAGTGTGGGAGAGCTAGAGGC	Failed	-	(Hassan, Lemaire et al. 2002)
			GnRH-2-R	AGAGACACCACTTCTCCTGTACCC			
	Alpha tropomyosin	TR-1	Tr-1-F	AGGGAACAGAGGATGAGCTGGAC	52°C	841	(Hassan, Lemaire et al. 2002)
			Tr-1-R	TCTCAGCTTCCTCCAGCTTGGTG			
	Aldolase B	AldoB	AldoB1-1-F	GCTCCAGGAAAGGGAATCCTGGC	54°C	262	(Hassan, Lemaire et al. 2002)
			AldoB1-2-R	CTCGTGGAAGAAGATGATCCCGCC			

2.4.2. Material collection and preparation

After morphological and meristic data was collected, fish were rinsed in deionised water and scraped clean of scales behind the dorsal fin where a $1cm^2$ section of muscle tissue was removed. Tissue samples were then individually stored in 80 % ethanol at room temperature. DNA was extracted from adult and whole larvae specimens using a standard phenol-chloroform-isoamyl alcohol (PCI) DNA extraction protocol, according to Barker et al (1998). Tissue was first patted dry and rinsed in distilled water to remove any residual ethanol that can inhibit the enzymatic activity of Proteinase-k. The dried muscle tissue was then added to a 1.5ml Eppendorf tube with 400ml of extraction buffer (0.01M Tris, 0.005M NaCl, 250µM Sodium Laural Sulphate (SDS); pH8.3) and 50µl of proteinase-k. Sample tissue was homogenised using an inoculation loop and incubated on a rocking tray at 57°C overnight.

A 25:24:1 phenol:choloroform:isoamyl (PCI) mix was added to the DNA solution and inverted for five minutes before being spun down at 14 000RPM. The supernatant was then pipetted off and an equal amount of PCI was added to the supernatant and gently inverted for five minutes and spun down a second time. If an interphase layer was still present, a third PCI wash was undertaken. A 24:1 Chloroform:Isoamyl (CI) mixture was then added in equal volume to the supernatant and inverted for two minutes before being centrifuged at 14 000 rpm for one minute. The top aqueous phase was then removed and 2 - 2.5 volume of 100 % molecular grade, ice cold ethanol was added before being placed in a freezer at -20°C overnight. Samples were then spun down for 20 minutes at 14 000RPM. Ethanol was then poured off and the pellets were washed with 800µl of 75 % ethanol and spun down for 20 minutes at 14 000rpm. The ethanol was poured off and the DNA pellets were dried in a vacuum desiccator for twenty minutes. DNA pellets were then dissolved in 100µl of molecular grade water.

DNA was checked for quality and quantity using a Nanodrop 3 000 spectrophotometer and subsequently run on a 1 % agarose gel, using 1 µl of loading dye and 5 µl of PCR product per sample. DNA quality varied greatly between individuals (1 – 1 000 $ng.\mu g^{-1}$ DNA per sample), with varying levels of phenolic and protein contamination. DNA was stored at -20°C before being used in PCR.

2.4.3. EPIC encoded data collection and analysis

Specimen DNA was amplified via Polymerase Chain Reaction (PCR) using several EPIC primers designed to amplify various genes (see table 1) (Messing 1983, Ivanova*, et al.* 2007). PCR reactions for all markers contained 12.5 µl EconoTaq PLUS GREEN 2X Master Mix (Lucigen). The PCR thermal cycle was [94 °C for 3 min], 34x [(94 °C for 30 s) (annealing temperature for 45 s) (72 °C for45 s)] and [72 °C for 10 min], [4 °C for ∞]. PCR product for each sample was checked for quality and quantity using a Nanodrop 3 000 spectrophotometer. PCR amplicons were run on a 3 % agarose gel for 5 hours at 100 volts, adapted from Touriya*, et al.* (2003). O'GeneRuler Plus DNA Ladder, Ready-to-Use, 100 - 3 000 bp (Thermoscientific) and a reference sample was run with every gel. Gels were viewed on a Biorad Molecular Imager, Gel Doc™ XR+. A representative sample was sequenced for each marker using an ABI 3730 capillary sequencer at Inqaba Biotechnical Industries (Pty) Ltd, Hatfield, South Africa.

Gel images were scored using Image lab 4.1 (Bio-Rad Laboratories Inc.). Gel warping and distortion of fragment distance after electrophoresis was accounted for and standardised using various programme tools in Image lab, to attain the most accurate account of the data. Five percent of samples were reamplified and scored to test for repeatability. Samples were scored according to their predicted focal allele band size ranges. Scored allele lengths were then categorized into between 5bp and 20bp groupings and assigned a numerical number, representing different alleles according to GenAlex data requirements (Peakall 2012).

Data were then exported to GenAlex 6.4.1 for analysis. Analysis of molecular variance (AMOVA) was performed on the data, with samples grouped by site and region, relative to the total population. Phi_{PT} (Φ_{PT}) was calculated for within and among the sites and Phi_{RT} (Φ_{RT}) between two regions (in essence a Phi_{PT} value calculated between grouped sites), compared to the total genetic variance (Peakall 2012). A mantel test was performed between genetic and geographic distances with 10 000 permutations, using GenAlex (Peakall 2012). Allele frequencies, heterozygosity, deviations from Hardy-Weinberg equilibrium, Nei's genetic distance, and a PCA of the genetic distance matrix were calculated in GenAlex. Finally, rates of migration between sites were calculated using Migrate 3.2 (Beerli 2008). Default settings for a

Bayesian analysis, with a burn-in of 1 000 and 500 000 iterations (5 000 000 parameter values) with adaptive heating on were run for the data, based on an island model of evolution. GenAlEx was used to calculate immigration between regional groups (those used to calculate Φ_{RT}) using default settings.

Patterns in population structure were assessed using Bayesian clustering analysis in the program Structure 2.3.4 (Pritchard*, et al.* 2000). The analysis was run using five MCMC simulations per run, with 1 000 000 iterations and 500 000 burn in, to test possible population clusters between one and ten (K). Structure harvester (Earl 2012) was used to determine the most likely combination of homogenous clusters (Evanno*, et al.* 2005).

A standard random representative sample (from KZN, 2012) had the first allele of each gene sequenced for reference with an ABI 3730 capillary sequencer at Inqaba Biotechnical Industries (Pty) Ltd, Hatfield, South Africa. Sequence electropherograms were edited using BioEdit version 7.0.9 (Hall 1999). Ambiguities in certain loci were compared assessed and compared to their respective electropherograms, and changed according to the signal peaks. The final sequences were trimmed to a length where definitive bp signals were obtained. The NCBI data base did not have any sequences of the genes tested, thus a BLAST search did not return any similar sequences (Altschul 1990).

2.4.4. mtCOI data collection and analysis

Specimen DNA was amplified via Polymerase Chain Reaction (PCR) using primers designed to amplify a 652 base pair region of the mtCOI gene (see table 1) (Messing 1983, Ivanova*, et al.* 2007). The PCR reactions for both markers contained 21 µl H2O,4 µl 10x buffer, 1.8 µl $MgCl_2$ (25 mM), 0.84 µl forward and reverse 3/550 primer (10 µM), 0.15 µl dNTP mix (10 mM), 1 µl BSA (10 mM) and 0.2 µl Supertherm Taq polymerase5 $u.\mu l^{-1}$ (Lucigen). The PCR thermal cycle was [95 °C for5 min], 30x [(94 °C for 30 s) (55 °C for 45 s for the 3/550 primer and 58 °C for 45 s for the mtDNA primer) (72 °C for45 s)] and [72 °C for 10 min], [4 °C for ∞]. PCR product for each sample was checked for quality and quantity using a Nanodrop 3 000 spectrophotometer

and subsequently run on a 1 % agarose gel, using 1 µl of loading dye and 5 µl of PCR product per sample. Samples were viewed on a Biorad Molecular Imager, Gel Doc™ XR+. Samples were then sequenced using an ABI 3730 capillary sequencer at Inqaba Biotechnical Industries (Pty) Ltd, Hatfield, South Africa.

BioEdit version 7.0.9 (Hall 1999) was used to align and edit the mtDNA sequence electropherograms. A Clustal W multiple-alignment algorithm was used to align sequences, which were subsequently checked and realigned by eye. Ambiguities in certain loci were then checked for by choosing the strongest signal on the chromatogram for each locus. The final alignments were trimmed to a length of 652 bp. A sequence search for highly similar sequences was conducted on a consensus sequence of all sequences, using BLAST (Altschul 1990). Sequences of *Sardinops sagax* and *Sardinops melanostictus* from various localities around the world were retrieved from the NCBI nucleotide collection and added to the sequence set for further analysis (see Table 4). Only sequences retrieved from BLAST of 652bp or more were included to preserve information in data when trimming sequences for analysis. All sample sequences were uploaded onto GenBank with accompanying information (submission # 1705716).

Table 4: COI sequence information of samples included for phylogenetic analyses, from the NCBI Genbank website.

Species	number of sequences	Location (Co-ordinates)	Collection date	Accession number	Reference
Sardinops sagax	2	Durban, KZN, South Africa (29.945 S 31.00 E)	30-Jun-05	JF494411 JF4944112	(Steinke, *et al.* 2011)
	2	Agulhas Bank, Mossel Bay, South Africa (34.873 S 21.618 E)	26-Apr-08	JF494409 JF494410	(Steinke, *et al.* 2011)
	5	British Columbia, Canada (48.93 N 125.34 W)	15-Oct-05	FJ165120 FJ165121 FJ165123 FJ165126 FJ165128	(Steinke, *et al.* 2009)
Sardinops melanostictus	3	Yokosuka, Japan (35.00 N 139.50 E)	07-Jul-05	FJ952841 FJ952842 FJ952843	(Zhang, *et al.* 2011)

DnaSP was used to construct haplotypes from the sequenced genotypes (Scheet*, et al.* 2006, Librado*, et al.* 2009). Gaps were ignored and all variable sites were used. MrModelTest 2.0 (Nylander 2004) and Modeltest 3.7 (Posada*, et al.* 2005) were used to search for the best fit model of evolution that fitted both genetic marker datasets. The HKY model of Hasegawa*, et al.* (1985) was chosen to construct the neighbour joining (NJ) and maximum likelihood (ML) trees in PAUP 4.1b (Swofford 2002) as well as the Bayesian tree searches, using MrBayes 3.1.2 (Huelsenbeck*, et al.* 2005). The mtDNA Bayesian trees were rooted with a *Pomacentrus baenchschi* (accession number:JF435106) and *Sardina pilchardus* (accession number: KC501229) sequence, retrieved from GenBank after a BLAST search of the NCBI nucleotide collection (Altschul 1990). Sequences of *Sardinops sagax* and *Sardinops melanostictus* of various localities from Genbank were also included (Table 4). The NJ and ML trees were bootstrapped for 1 000 iterations. The Bayesian trees constructed with haplotypes were created using 4 Markov chains of 1 000 000 generations each, sampled every 10 generations. The first 10 000 trees were discarded as burn in, with the rest of the genealogies used to construct a 50 % majority-rule consensus tree. A haplotype network was constructed using the median joining method (Bandelt*, et al.* 1999) for the COI sequence data, with gaps coded as missing, and run at a 95 % confidence interval. The number of specimens assigned to haplotypes in DNAsp 5.0 was also supplied for each branch on the minimum spanning tree network (Bandelt*, et al.* 1999).

Data were then exported to GenAlex 6.4.1 for analysis, and was coded as haploid. Analysis of molecular variance (AMOVA) was performed on the data, with samples grouped by region (RSA, Canada and *S. melanostictus*). Phi_{PT} was calculated for within and among the sites and compared to the total (Peakall 2012). A mantel test was performed between genetic and geographic distances with 10 000 permutations, using GenAlex (Peakall 2012). Nei's genetic distance and rates of immigration were also calculated using GenAlex. Finally a PCA for the genetic distance matrix was conducted.

Chapter three:

Morphological characteristics of the sardine (*Sardinops sagax*) in South Africa

3.1. Introduction

The South African sardine (*Sardinops sagax*) occurs throughout the year along the western coast of South Africa and Namibia, from Luderitz to the Agulhas bank as far as Port Elizabeth. Seasonal winter migration during the winter "sardine run" extends the range of *S. sagax* approximately 700km northward into the sub-tropical waters of KZN (van der Lingen, Hendricks*, et al.* 2010). The large biogeographic range of the South African *S. sagax* spans two oceans and a range of physical and ecological conditions and ecosystems. The diverse ecosystem parameters over this large spatial scale certainly plays a role in shaping the biology, ecology and thus the population structure of *S. sagax* in South Africa (de Moor*, et al.* 2009) as it has globally (Parrish*, et al.* 1989, Agüero*, et al.* 2004). Understanding population structure is important to developing accurate modelling of fish stocks and thus the effective management of any fishery (Agüero*, et al.* 2004).

Ecological differences between biogeographic areas shape the phenotypic and behavioural characteristics of individuals (Cadrin 2000). Differences in mean ocean temperature, salinity, food type and availability, and other factors are important factors affecting *S. sagax* along the western, south east and eastern coastlines of South Africa. Thus, phenotypic traits may be useful in testing population structuring between biogeographically separated *S. sagax;* and has been investigated previously (de Moor*, et al.* 2009, Wessels 2009, van der Lingen, Hendricks*, et al.* 2010).

Thus far, stock structure of *S. sagax* in South Africa has been explored using meristic, morphometric, and ecological data such as FDAAs and spawning habitats and other life history

traits (De Goede*, et al.* 2005, de Moor*, et al.* 2009, Van Der Lingen*, et al.* 2009, Wessels 2009, van der Lingen, Hendricks*, et al.* 2010) and has been successful in delineating stock structure. The aim of these studies has focused mostly on the commercially important *S. sagax* west of Cape Agulhas and the eastern stock on the Agulhas bank. Nevertheless, van der Lingen, Hendricks*, et al.* (2010) has focused specifically on meristic characteristics of *S. sagax* of the KZN sardine run, and Wessels (2009) study included KZN sardine in a morphometric study of the South African sardine.

Freon*, et al.* (2010) suggested the use of similar methods to determine whether sardines undertaking the migration up into KZN are morphologically disparate to the Western and Eastern Cape stock. An investigation of this nature would have important implications for the seasonal KZN beach seine fishery and to its management, for the development of a multi-stock model for the South African sardine (Freon*, et al.* 2010).

Indeed, some investigation has been conducted on the KZN sardines. Sardines caught in KZN have been shown to exhibit a lower mean conditioning factor, different mean vertebrae frequencies and are smaller (caudal length) than sardines on the Agulhas Bank and on the Western Cape coast (van der Lingen, Hendricks*, et al.* 2010). Differences in morphology between western and south-eastern stocks (including KZN samples) have also been observed using multivariate morphometrics. Nevertheless, a morphometric study focusing specifically on KZN sardines has yet to be completed. These previous studies have suggested some form of structuring between KZN and southern Benguela sardines.

Therefore KZN sardines are expected to show morphological differences from the Western Cape stock, and possibly between biogeographic regions. To test this hypothesis, sardines were collected at four sites along the South African coast, including samples from the KZN sardine run and subjected to a range of morphometric and meristic analyses to determine whether phenotypic differences existed between sardines from the KZN component of the sardine run and the Western Cape or Agulhas bank.

3.2 Materials and methods

Refer to chapter 2, 2.2. Meristic data collection and analysis and 2.3. Morphological data collection and analysis.

3.3. Results

A total of 1040 sardines were collected for meristic analysis between the four sites along the South African coast, including a three year temporal scale for KZN (Table 2). Morphometric analysis included 540 sardines, with 500 individuals excluded due to being frozen for longer than three months, which influenced the shape of sardines (Wessels*, et al.* 2010), and a further 86 outliers mostly due to distended stomachs and those bent or with torn flesh during the freezing period (95 % confidence interval) were removed.

3.3.1 Meristic results

No sexual dimorphism was detected, after a non-parametric Kruskal-Wallis test showed no significant sex based groupings (Kruskal-Wallis ANOVA: $H_{1, 150} = 22.04$, $p = 0.779$). Sex ratios for all sites were not significantly different from an expected 1:1 sex ratio (Table 5), except KZN for the years 2011, 2012 and the combined KZN grouped samples (2011 - 2013). The KZN sample for 2013 showed the largest male skewed sex ratio, although still not significantly so ($\chi^2 > 0.05$, Table 5). Nevertheless, the low significance was due to the small sample size (n = 17), thus limiting the confidence in this result. Cape Town was the only site with an equal sex ratio. All other sites showed positively male skewed sex ratios. Sex ratio was different between KwaZulu-Natal and Port Elizabeth (Tukey HSD: $p < 0.001$) but not among the other sites (homogeneity subset test: $p < 0.080$).

Table 5: Sex ratio for the four *Sardinops sagax* populations and results of a Chi² test for deviation from an expected 1:1 sex ratio. A combined KZN result is also presented. Significant values are indicated in bold.

Site	Sex ratio (percent male)	*N*	*df*	Chi²	Asymptotic sig.
Cape Town	50	200	1	0.000	1.000
Mossel Bay	56	200	1	2.880	0.090
Port Elizabeth	44	201	1	2.632	0.105
KZN 2011	61	204	1	9.490	**0.002**
KZN 2012	60	217	1	9.332	**0.002**
KZN 2013	71	17	1	2.882	0.090
KZN (2011-2013)	61	438	1	21.041	**0.000**

Vertebral counts between the three years for KZN were very similar according to a Kruskal-Wallis test ($H_{3, 49} = 0.03$, $p = 0.943$), and were thus combined for comparison with the other three sites. Vertebral counts between Cape Town and the other three sites were significantly different from each other (Tukey's HSD: $p < 0.001$), however there were no differences between the other sites (Tukey's HSD homogenous subset: $p = 0.354$). Figure 3 illustrates the frequency distributions of vertebrae between the four sites. Cape Town consisted mostly of individuals with 51 vertebrae whilst the other sites exhibited a median vertebrae number of 49, accounting for more than 60 % of each population (Figure 3). Cape Town had a mean vertebrae count of n = 49.695, Mossel Bay n = 49.005, Port Elizabeth n = 49.08, and KZN n = 49.000. No sardines were recorded with 51 vertebrae for all years of KZN and for Mossel Bay (Figure 3). A graded decrease in individuals with 49 vertebrae and an increase of individuals with 48 and 50 vertebrae was observed between Mossel Bay, Port Elizabeth and KZN.

Overall wet body mass and caudal length was significantly higher for Cape Town, Mossel Bay and Port Elizabeth, than KZN (Tukey's HSD: $p < 0.001$; Figure 4). There was no difference between the former three sites according to a Tukey's HSD *post hoc* test. A decrease in standard deviation was observed among all sites from Cape Town to KZN for both wet body mass and caudal length (Figure 4).

A non-parametric Spearman's rank correlation between wet body mass and caudal length was conducted after the assumptions of normality were were not met using a 1 sample Kolmogorov-Smirnov test; and showed a strong positive correlation ($R^2 = 0.956$) for all data (Figure 5). Individual correlation coefficients were also calculated for each site. A decrease in the correlation coefficient was observed from Cape Town ($R^2 = 0.970$), to Mossel Bay

(R^2=0.856), Port Elizabeth (R^2=0.802) and finally KZN (across all years) illustrated the lowest correlation coefficient (R^2=0.773).

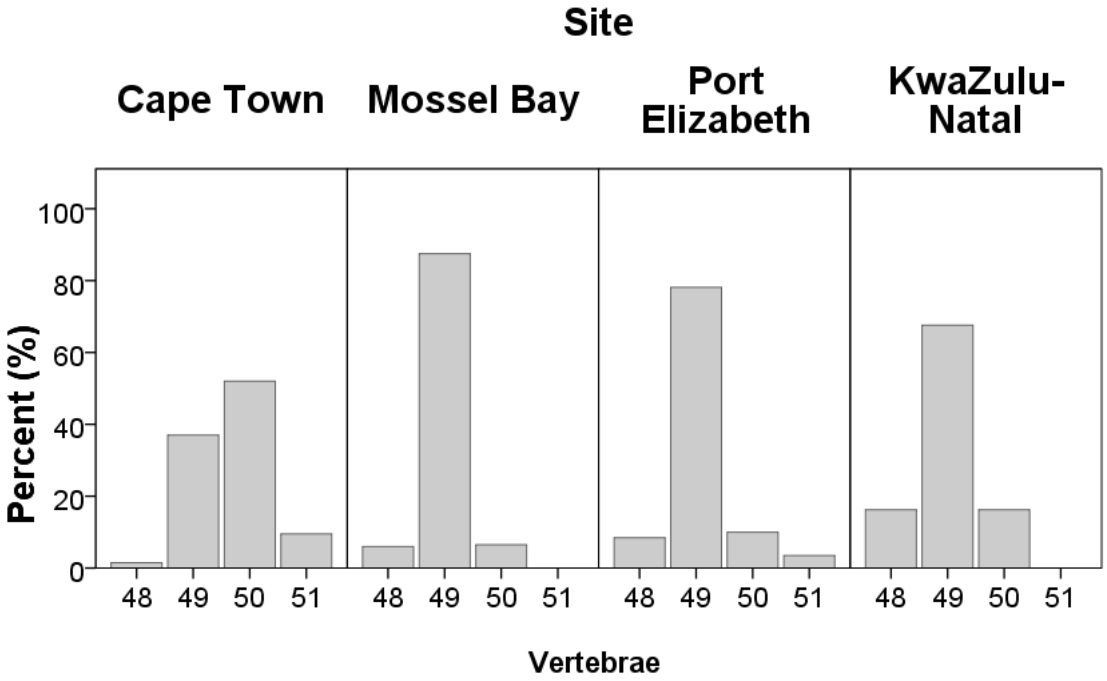

Figure 3: Frequency distributions (percentage) of each vertebrae count at four sites along the South African coastline.

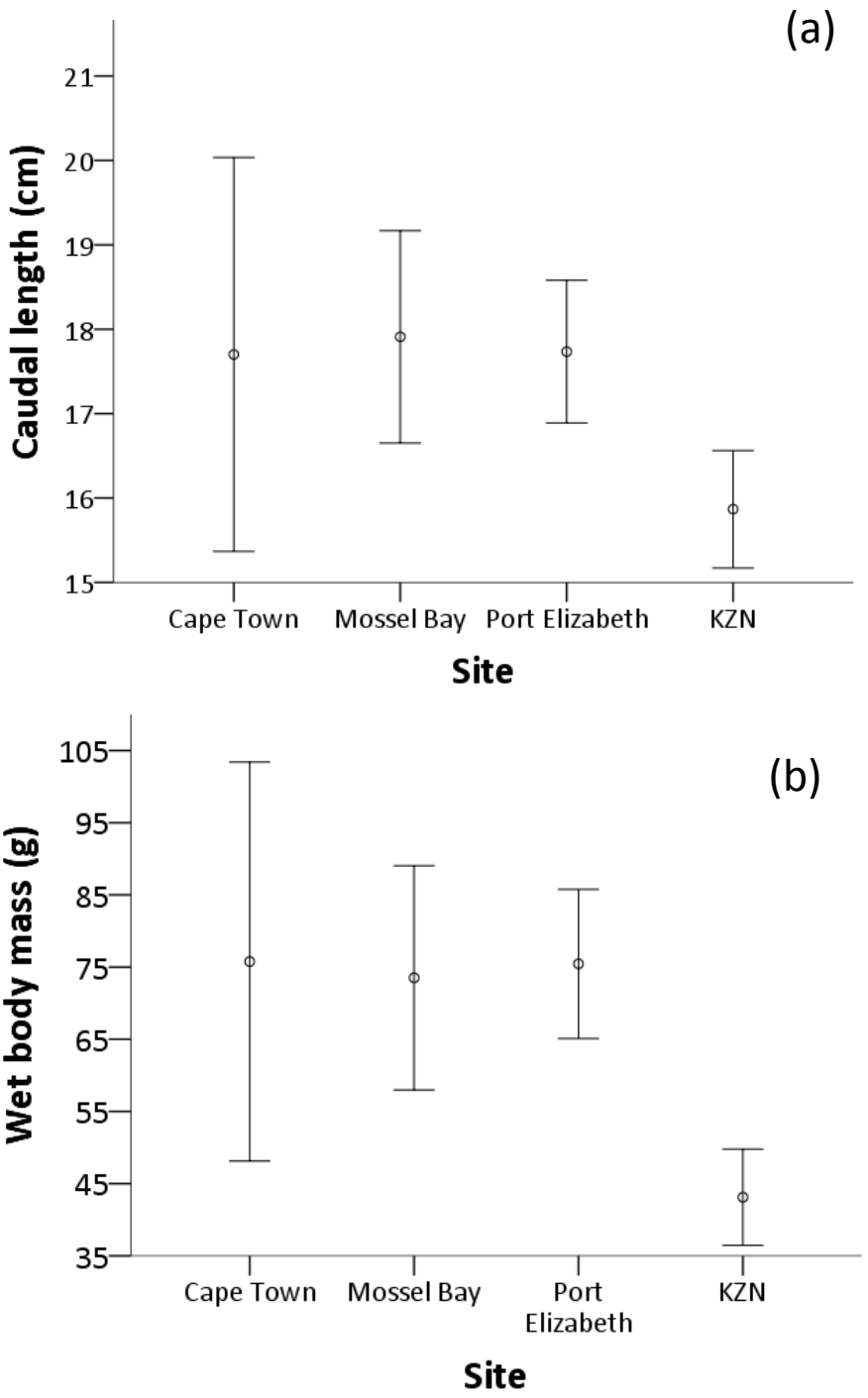

Figure 4: (a) Mean wet body mass and (b) mean caudal length of *Sardinops sagax* at four sites along the South African coastline, with standard deviation.

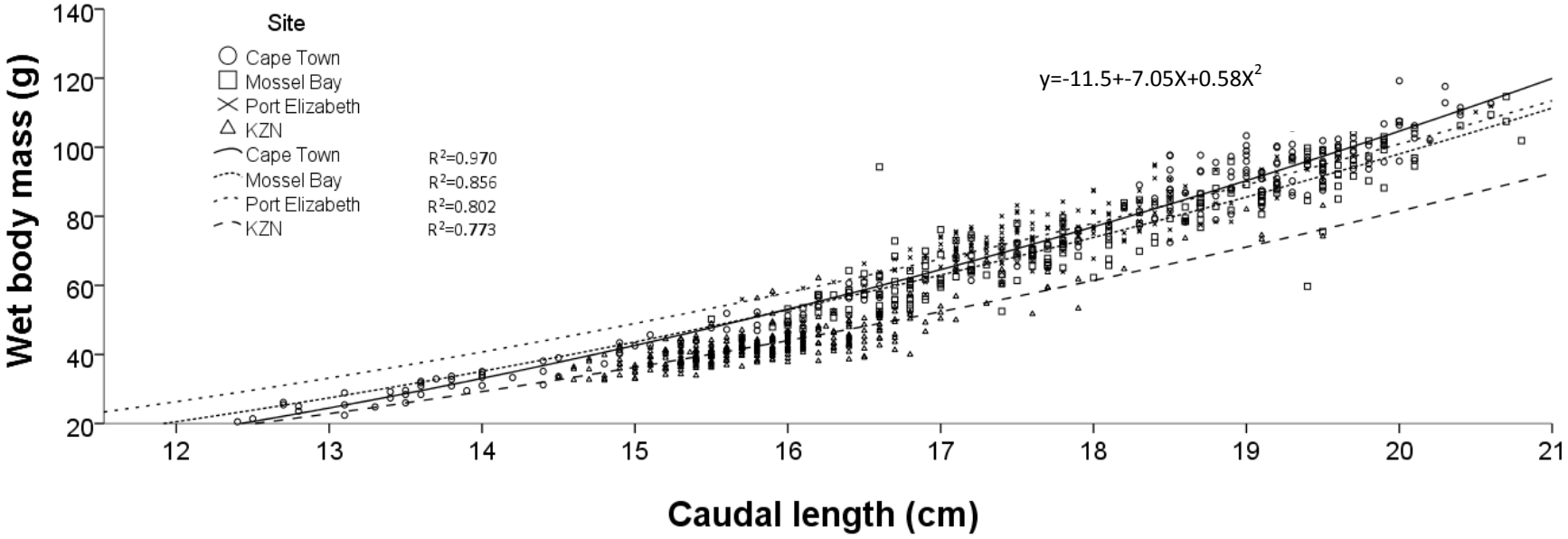

Figure 5: Mass at length scatter plot of *Sardinops sagax* at four sites along the South African coastline during the months of January to November between 2011 and 2013, with fitted power curves representing mean weight at length of fish.

3.3.2. Morphometric results

All morphometric distances (truss measurements) differed between samples, ($F_{5.201}$ = 16.372 – 130.241, $p < 0.05$) except t19 ($F_{3, 1.571}$ = 16.7108, p =0.372), according to a one way ANOVA (table 8). Truss variables that did not differ between sites were the distance between the snout and the point where the jaw hinge meets the operculum and the body area between the front and back insertion points on the dorsal fin (t8), and the ventral fin and front insertion point on the anal fin (t19). All other distances were significantly different between sites tested.

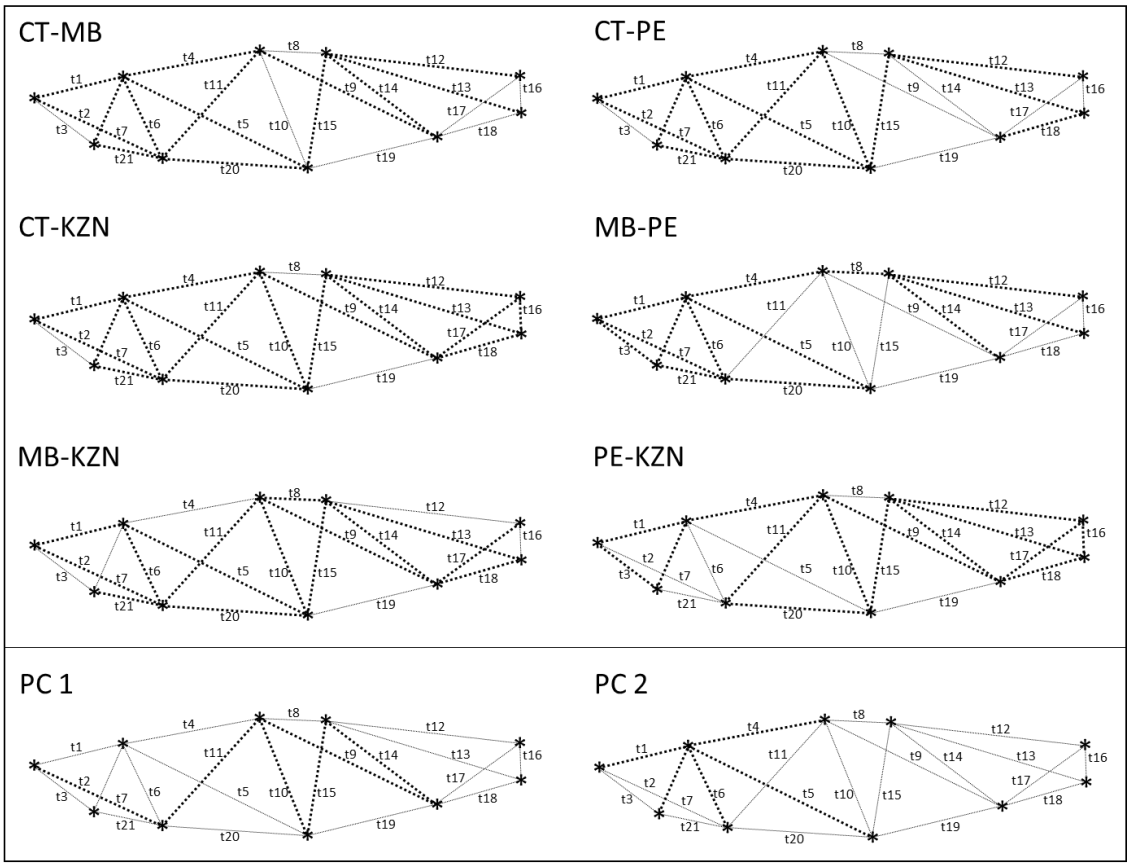

Figure 6: Tukey's HSD *post hoc* test of size corrected truss measurements for *Sardinops sagax* at four sites along the South African coastline. Significant differences in truss length are represented as bold dotted lines. PC1 and PC2 show truss measurements that showed the greatest contribution to variance among samples (bold dotted lines).

These differences indicated an increase or decrease in height and length of sardines between sites. Nevertheless, the largest differences in variability between sites in relation to within site variability (F statistic, table 8) were the distances within the area of the head (t1, t2, t3, t6 and t7), and the lowest were the vertical and horizontal distances behind the operculum (pre dorsal: t4, t5 and t11) and the horizontal and perimeter distances behind the front dorsal insertion and the front anal insertion (post dorsal: t8, t9, t10, t12 and t13).

Table 6: One way ANOVA illustrating significance (*) of size corrected truss measurements for *Sardinops sagax* at four sites along the South African coastline; including PCA eigenvectors with eigenvalues and cumulative variance for the first four principal components (PCs) of a Principal Coordinates Analysis.

One way ANOVA			PCA eigenvectors	
Truss measurement	Sig.	F	PC1	PC2
t1	*	112.622	-0.094	-0.184
t2	*	102.780	-0.140	0.053
t3	*	73.105	-0.064	0.102
t4	*	31.472	0.061	0.222
t5	*	46.112	0.071	0.220
t6	*	94.275	-0.043	0.185
t7	*	130.241	-0.070	-0.117
t8	*	24.427	0.019	0.134
t9	*	30.592	0.124	-0.024
t10	*	39.983	0.114	-0.036
t11	*	42.655	0.109	0.042
t12	*	41.975	0.089	-0.137
t13	*	43.630	0.102	-0.138
t14	*	25.879	0.114	-0.113
t15	*	34.421	0.109	0.000
t16	*	28.925	0.065	-0.052
t17	*	41.671	0.050	-0.059
t18	*	46.476	0.027	-0.038
t19	0.372	16.711	0.077	0.038
t20	*	40.810	0.097	0.067
t21	*	79.277	-0.093	-0.047
Eigenvalue			0.0212	0.0026
Cumulative variation (%)			71.457	80.261

The first two principal components (PC) of the PCA for the size corrected truss measurements accounted for 80.26 % of the total variance explained and was thus a good approximation of the data (Figure 4). The remaining PCs accounted for approximately 10 % of the variance each.

PC3 accounted for 4.75 %, PC4: 3.27 %, PC5: 2.66 and PC6 2.20 %. All eigenvectors were between negative one and one, indicating the effect of size had been removed from the data (Table 6). The greatest contributor to variance in PC 1 was t2 followed by t9, t10 and t11, and t14 and t15 (Table 6). Truss variables associated with the second landmark (top front corner of operculum, below occipital ridge; Table 2) showed the greatest contribution to variance in PC 2 (Table 6). Truss measurements t3 and t16-t21, all lying on the ventral side of the fish perimeter from the tail to the snout, showed the least contribution to the variance between both PCs.

The PCA plot showed substantial overlap of the 95 % confidence intervals (Figure 7). Nevertheless, some differentiation was observed between sites. The KZN samples were mostly negative according to PC1, with Mossel Bay approximately neutral and the Port Elizabeth and Cape Town samples had positive loadings on PC1 (Figure 7). KwaZulu-Natal, Port Elizabeth, and Mossel Bay had a negative loading on PC2, and Cape Town showed a positive loading (Figure 7).

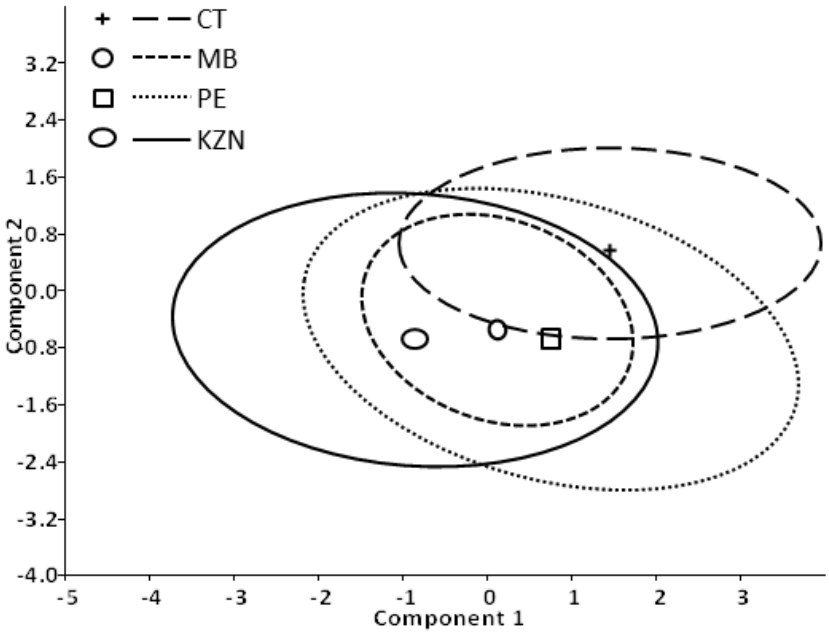

Figure 7: Principal Coordinates Analysis of size corrected truss measurements for PC1 and PC2, with 95 % confidence interval ellipses, for *Sardinops sagax* at four sites along the South African coastline.

Seventeen truss measurements were included in the DFA, with t18-t21 removed subsequent to failing a tolerance test. Overall discrimination between sites was significant according to a DFA (Wilk's-Lambda = 0.088, F = 25.63, $p < 0.001$), thus validating the groupings (Table 7). The canonical analysis identified three discriminant functions although only the first two were significant (chi^2 = 1064.5, 572.9 and 148.5; df = 51, 32 and 15; p = 0.001, 0.001 and 0.09). DF1, DF2 and DF3 had eigenvalues of 2.037, 1.609 and 0.399. DF1, DF2 and DF3 accounted for 50.4 %, 39.8 %. and 9.9 % of discriminant capacity. The highest loadings for DF1 were three truss measurements found in the head of the fish and three in the body; and the tip of the snout to the front insertion of the dorsal fin, and three measurements in the body, for DF2 (Figure 8). DF1 composed of negative values for truss variables that lay along the perimeter of samples from the snout to the tail (t1, t4-t6) and the vertical trusses on the body behind the operculum to the back insertion of the dorsal fin to the ventral fin (t8, t10-t12). DF2 had negative trusses, which were trusses radiating from the top operculum insertion point (t1, t4-t6), and three diagonal trusses across the body (t5, t14, t15).

Table 7: Summary results for the discriminant function analysis of size corrected truss measurements for function 1 and function 2, for *Sardinops sagax* at four sites along the South African coastline. Truss measurements t18 - t21 were excluded after failing a tolerance test.

	Standard coefficient for canonical variables			Discriminant function analysis		
truss measurement	DF1	DF2	DF3	Wilk's Lambda	*F*	*P*
t1	-2.643	-1.151	-1.857	0.593	102.780	0.000
t2	1.779	0.176	1.469	0.672	73.105	0.000
t3	1.948	-0.146	-0.273	0.827	31.472	0.000
t4	-0.011	-1.948	0.065	0.765	46.112	0.000
t5	-0.736	-0.267	0.280	0.614	94.275	0.000
t6	-0.303	-0.462	-0.175	0.535	130.241	0.000
t7	0.423	0.729	-0.077	0.860	24.427	0.000
t8	-0.697	-0.958	-0.581	0.831	30.592	0.000
t9	0.027	1.553	1.020	0.790	39.983	0.000
t10	-1.904	1.377	0.190	0.779	42.655	0.000
t11	-0.508	0.131	-2.320	0.781	41.975	0.000
t12	-0.570	0.263	0.833	0.775	43.630	0.000
t13	1.524	1.009	0.551	0.853	25.879	0.000
t14	0.570	-0.825	0.290	0.813	34.421	0.000
t15	-0.022	-0.043	-0.569	0.838	28.925	0.000
t16	0.298	0.048	1.082	0.783	41.671	0.000
t17	1.232	0.654	1.062	0.763	46.476	0.000

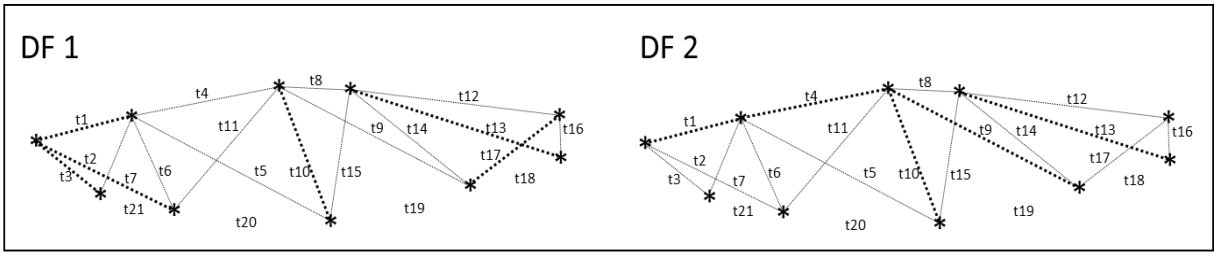

Figure 8: Truss measurements that loaded highest in the discriminant function analysis for each of the two significant discriminant functions (bold dotted lines), for *Sardinops sagax* at four sites along the South African coastline. Truss measurements t18-t20 were excluded after failing a tolerance test and are thus not depicted.

The 95 % ellipses showed substantial overlap between sites (Figure 9). The Mossel Bay and KZN centroids grouped relatively close to Port Elizabeth, more than Cape Town. Cape Town illustrated mostly negative loadings according to DF1, with the other three sites loaded positively (Figure 9). DF2 discriminated sites evenly, from negative (KZN), to Port Elizabeth and Cape Town, to Mossel Bay which was positive (Figure 9). In total, 89.2 % of individuals were correctly reclassified into their original groups, using Mahalanobis distance (Table 8). Cape Town had only 4.6 % of individuals reclassified to different groups (Mossel Bay and Port Elizabeth, Table 8). Mossel Bay had 20.2 % of individuals reclassified as Cape Town, Port Elizabeth and KZN. Port Elizabeth had the least of its individuals reclassified into other groups, with 4.1 % of individuals reclassified to Cape Town and Mossel Bay. KZN had 5.9 % of individuals reclassified, all to Mossel Bay (Figure 9).

Table 8: Reallocation of individuals in new groups, according to discriminant functions, for *Sardinops sagax* at four sites along the South African coastline.

Site	Predicted group membership				Total (%)
	Cape Town (%)	Mossel Bay (%)	Port Elizabeth (%)	KZN (%)	
Cape Town	95.4	1.9	2.8	0	100
Mossel Bay	3.3	84.2	10.4	2.2	100
Port Elizabeth	1.4	6.2	92.5	0	100
KZN	0	5.9	0	94.1	100

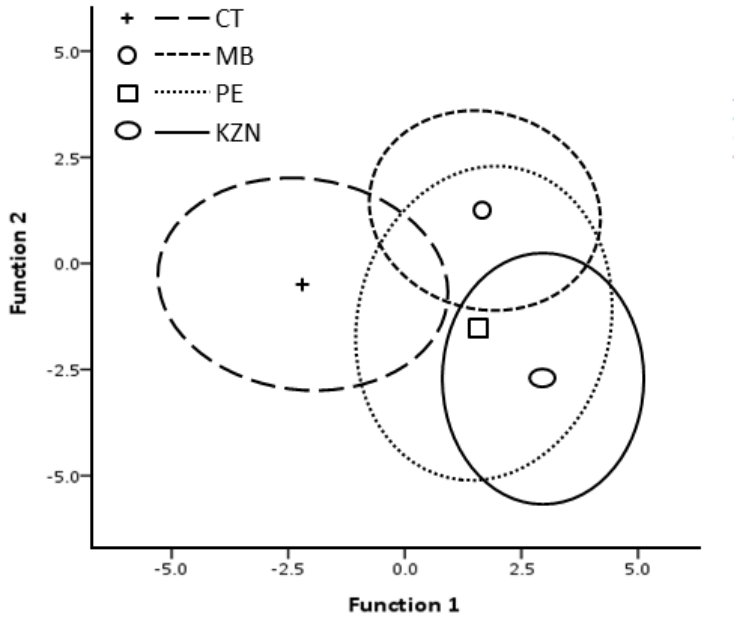

Figure 9: Discriminant function analysis of size corrected truss measurements for function 1 and function 2, with 95 % confidence interval ellipses, for *Sardinops sagax* at four sites along the South African coastline. Truss measurements t18 - t20 were excluded after failing a tolerance test.

Cluster analysis of sardine shape supported the clustering of the mean shapes of Cape Town, Mossel Bay and Port Elizabeth from KZN with 100 % confidence, and the separation of Cape Town from Mossel Bay and Port Elizabeth with 94 % confidence. Finer scale structuring separated Mossel Bay and Port Elizabeth with 75 % confidence. Figure 10 illustrated the mean fish shape for each site.

The difference in geometric shape was mainly focused on the head shape attributed, mostly to the increase of operculum size and the distribution of landmark 2 (Top front corner of operculum, below occipital ridge). Body size also explained variation between sites (Figure 11). KZN and Port Elizabeth illustrated a reduction in head size (both on the vertical and horizontal axes). However, this was less pronounced in the KZN samples due to the overall reduction in their absolute body shape (Figure 11). These observations were supported by the multivariate distances (Figure 6). Mossel Bay samples also showed a reduction in head size, relative to body size, however to a lesser degree than the latter two. Cape Town showed a slight increase in head shape from the mean shape (Figure 11). Port Elizabeth showed a large increase in dorso-

ventral depth and length, demonstrating the largest increase in dorsal height and overall body length, followed by Cape Town, Mossel Bay, and KZN (Figure 11). KZN and Port Elizabeth showed an increase in posterior shape size between the back dorsal fin and front anal fin insertion point to both caudal fin insertion points, whereas Cape Town and Mossel Bay showed a decrease in the same measurements, but an increase in the area between the ventral fin and front anal fin insertion points.

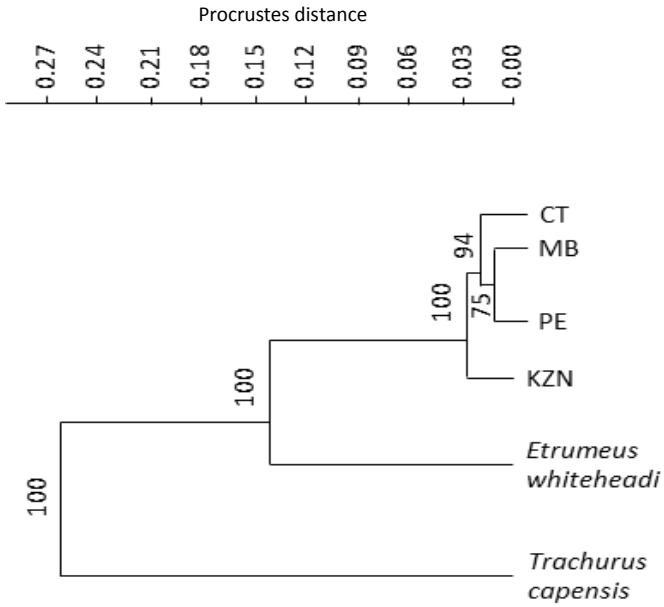

Figure 10: Cluster analysis of mean Procrustes distances of each of the four sites and two outgroups, *Trachurus capensis* and *Etrumeus whiteheadi*, for *Sardinops sagax* at four sites along the South African coastline. Numbers represent percentage confidence via 100 000 bootstrapping.

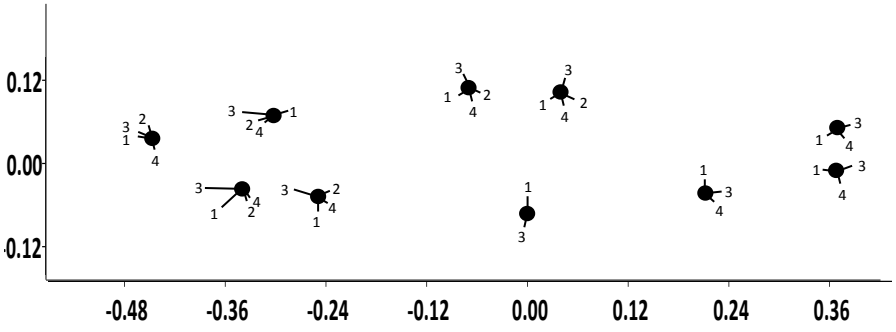

Figure 11: Reallocation of individuals in new groups, according to discriminant functions, for *Sardinops sagax* at four sites along the South African coastline. Black dots represent consensus shape, and lines represent relative warp from the mean shape. Cape Town = 1, Mossel Bay = 2, Port Elizabeth = 3, KZN = 4.

3.4. Discussion

Analysis of morphometric and meristic data ascribed no sexual dimorphism to the South African sardines. However, the presence of heavily egg laden females needs to be considered, and which was not covered by the analyses, apart from the removal of seven females from the Cape Town sample with severely distended stomach cavities. Sex ratios were not different from results previously reported (Akkers*, et al.* 1996; 1998, van der Lingen*, et al.* 2006, van der Lingen*, et al.* 2007, de Moor*, et al.* 2009, van der Lingen, Hendricks*, et al.* 2010, Wessels*, et al.* 2010).

The change in vertebrae frequency along the coastline from the Western Cape to KZN was significant. The mean and modal numbers of vertebrae concurred with Wessels (2009). These differences in vertebrae are attributed to differences in environment, especially temperature during the larval stage of life when the number of vertebrae are set early on in the development cycle (Begg*, et al.* 1999, Florence*, et al.* 2002). Sardines spawned in warm waters tend to develop fewer vertebrae than those spawned in cooler water (Hulme 1995). The differences in vertebrae are consistent with the sardine's range across different biogeographical breaks described by Teske*, et al.* (2011). Sardines on the Agulhas bank had less vertebrae and less variability in vertebral count than sardines caught in the Western Cape west of Cape Agulhas. This suggests that the sardines between the two regions were spawned there. However, vertebral count can be very loosely correlated to environmental conditions and often intra-population variance of vertebral counts can be large (Florence*, et al.* 2002). Added to this, the spatial variability of larvae and adults in sardine distribution can mislead the interpretation of results (Wessels 2009). Thus, the use of vertebral counts for population discrimination and possible larval nursery of a fish, should account for these factors (Begg*, et al.* 1999, Wessels 2009).

The reduction in the standard deviation of both mass and length of individuals from Cape Town to KZN may have been due to the fact that the shoals of sardines sampled in Cape Town had greater differences in size class compared to the shoals sampled in the other regions. The weight to length relationship of sardines showed a decrease among the regions from Cape

Town to KZN. The reduction in weight at length observed in the KZN population have been ascribed to the energetic costs that it takes sardines to swim from the Agulhas Bank, up into KZN during the sardine run (Freon*, et al.* 2010, van der Lingen, Hendricks*, et al.* 2010). The greater deviation (r^2 value) from the weight at length of individuals in KZN may also have to do with the energetic costs with the migration up the east coast, where fish may experience varied food availability and workload. These observations were similar to those of recent sardine runs, described by van der Lingen, Hendricks*, et al.* (2010). Differences in environmental conditions between shoals of sardine or among years of sardine run may also contribute to the lowered conformity of sardines to the weight at length ratio.

According to the multivariate methods, the PCA determined the most important measurements to the distance morphology of the fish as the size of the body and head (from snout to back of operculum). The results of the DFA confirmed those measurements as significant to the variability in the fish morphology. The Western Cape samples grouped separately from the rest of the regions due mostly to larger head size and the depth of body. The reclassification of most (91.5 %) individuals to their original region indicated the statistical significance of the classification. Relative size of the shape provided some discrimination between populations; however population overlap was still significant. The geometric shape analysis supported the multivariate results with the Cape samples showing an increase in the size of the head, and the KZN, Mossel Bay and Port Elizabeth samples showing decreases in head size.

Silva (2003) and Wessels (2009) concluded that differences in head size and shape were discriminating factors to the delineation of stock structure in European *Sardinina pilchardus* and South African *Sardinops sagax*. In the latter case, Wessels*, et al.* (2010) found a similar reduction in head size in the south east samples, the greatest differences however were found between Cape samples and sardines sampled in Namibia. Cluster analysis supports these findings with all sample regions separating out with high confidence. Freon*, et al.* (2010) hypothesis (test 3) that sardines from the run phenotypically distinct from the rest of the population was thus accepted, as multivariate and geometric morphometrics showed differentiation among sites, including the KZN sardine run individuals. Although direct cause-

effect between meristic characters should not be drawn, association between vertebrae and length-body mass relationships between sites was observed.

It must be noted that it would be beneficial to be able to compare sardines from further north, such as from Namibia such as in the study by Wessels (2009) to gain a better resolution and for relative data comparisons. The weak sardine run of the past years, during this study have not allowed for an inter-shoal comparison of sardine morphology. KZN experienced poor sardine run events in 2012 and 2013. Greater resolution would increase the strength of the tests employed in this study. Unequal sample sizes, namely the small sample size of the KZN population is also a factor to account for in comparing the results (Cadrin 2000) even though few sardines lay outside of the 95 % confidence interval.

The observed morphological differences between populations are most probably influenced heavily by environmental factors including temperature, food type and availability and others (Cadrin 2000). The heterogeneous environment that the sardines occupy in South Africa is probably the reason for the observed morphological structuring (Heydorn*, et al.* 1978) which seemed to follow Wessels (2009) and van der Lingen, Hendricks*, et al.* (2010) findings. Nevertheless, one cannot reason that these observed differences are representative of population structuring. Only the addition of genetic analyses can test connectivity and structure of these regions (Palumbi 1994, Cadrin 2000, Hellberg*, et al.* 2002). Only then can morphology can be compared with the genetic structure of populations to determine whether phenotype is an appropriate estimator of population affinity. The genetic structuring in the South African sardine is discussed in the next chapter.

Chapter four:

Genetic population structure of the KZN sardine (*Sardinops sagax*) using mitochondrial and exon-primed, intron-crossing (EPIC) markers

4.1. Introduction

Phylogeny is important to fishery scientists for the delineation of fished resources at a genus, species, and population resolution and aids in developing rational conservation strategies (Palumbi 1994, Agüero*, et al.* 2004, Palsbøll*, et al.* 2007). Population structuring of small pelagic teleosts such as *S. sagax* is influenced by an interaction of a multitude of variables which effect natural selection, genetic drift, mutation and gene flow (Palumbi 1994, Hellberg*, et al.* 2002). These effects can be measured via the allele frequencies of genes between individuals, and thus determine population structure (Palumbi 1994, Hellberg*, et al.* 2002, Hansen 2003). Contrary to this, morphological analyses are under strong selective pressure and may not reflect an individual's phylogeny (Palumbi 1994, Hellberg*, et al.* 2002, Zinetti*, et al.* 2013). Thus, the use of genetic analyses is important in determining population structure of fish stocks (Excoffier*, et al.* 1992, Carvalho*, et al.* 1995, Punt*, et al.* 1997, Begg*, et al.* 1999, Palsbøll*, et al.* 2007, Hauser*, et al.* 2008).

Pelagic teleosts such as *S. sagax* have large range sizes, short generation turnover, dispersal ability, large population size, and mass breeding and spawning, and these life history traits and others such as balancing selective pressures keep populations homogenous (Grant 1985, Palumbi 1992; 1994, Grant*, et al.* 1998, Hellberg*, et al.* 2002, Gonzalez*, et al.* 2007). Conversely, factors contributing to genetic structuring include sexual and other selection pressures, larval retention, spawning migrations, local adaptations, and geographic and biological barriers (Palumbi 1994, Martínez*, et al.* 2006, Gonzalez*, et al.* 2007). Historical factors such as previous climate and sea level fluctuation, such as that of the last glacial maximum 18000 years ago, may also influence genetic structure (Palumbi 1994, Martínez*, et al.* 2006).

In Southern Africa, *S. sagax* occurs across two heterogeneous ocean current systems and numerous biogeographic regions (Teske, et al. 2011). The sardine run, a seasonal migration of *S. sagax* up the east coast of South Africa, is a feature which has also been hypothesised to occur due to some form of genetic structure. Freon*, et al.* (2010) determined that there were three hypotheses relating to the sardine run that are amenable to genetic testing. These hypotheses include subpopulation spawning migration (Baird 1971), natal homing and imprinting (Carr 1967, Stabell 1984), and relic behaviour (Wyatt*, et al.* 1991). These hypotheses and the testing of larger scale population structure of the Southern African sardines are complimentary, and can be tested simultaneously.

Nevertheless, thus far, studies have investigated the population structure of *S. sagax* across these regions based on body morphology and meristic characters (de Moor*, et al.* 2009, Van Der Lingen*, et al.* 2009, van der Lingen, Hendricks*, et al.* 2010). As mentioned in chapter three, an investigation of this nature would have important implications for the seasonal KZN beach seine fishery and to its management, for the development of a multi-stock model for the South African sardine, and answer key questions relating to the nature of the sardine run (Freon*, et al.* 2010). Such a study would also corroborate or contradict the use of morphology in determining population structure in *S. sagax*.

Incorporation of both mtDNA and nuclear DNA data to population studies offers a thorough investigation of genomic variation between individuals and a robust genetic technique (Carvalho*, et al.* 1995, Hauser*, et al.* 1998, Ward 2000, Ward*, et al.* 2009). Broad-scale markers, such as mitochondrially-encoded cytochrome oxidase 1 (mtCOI), have been used successfully on clupeids to elucidate phylogenetic relationships within and between species (Ward*, et al.* 2005, Ivanova*, et al.* 2007) and can be used to validate morphological species identification. Recently, universal primer cocktails designed to amplify a 650 base pair region of the mtCOI region, have been developed at the Canadian centre for DNA barcoding and has been successfully used to barcode marine fishes. The barcoding of sardines has already been conducted on sardine larvae and adults in KZN and abroad (Ward*, et al.* 2005, Ward*, et al.* 2009, Zemlak*, et al.* 2009, Cawthorn*, et al.* 2011, Steinke*, et al.* 2011, Keskİn*, et al.* 2013).

The nature of the sardine run, as discussed above, may show shallow but significant structuring due to large effective populations and balancing selection. This necessitates the use of a spectrum of very fine, to moderate scale markers. One such solution is to use exon-primed, intron-crossing (EPIC) primers described by Touriya*, et al.* (2003) used on the clupeid *Sardina pilchardus*, and other non-model teleost fish (Hassan*, et al.* 2002, Li*, et al.* 2010). These primers have the advantage of being applied to a wide array of organisms, and it is possible to analyse the exon or intron component of a sequence separately to determine homology (Li*, et al.* 2010). The exon and intron regions are often under different selective pressures, thus it is possible to analyse genetic material at separate scales of phylogeny (Li*, et al.* 2010). The use of genetic analyses, specifically EPIC markers and mtCOI data are thus appropriate for testing population structure and in testing Freon*, et al.* (2010) hypotheses about the sardine run.

The aim of this study is to determine both fine scale and broad scale structure of the South African *Sardinops sagax*. A mediated interaction between life strategies and environmental factors in *S. sagax* are predicted to show some form of structuring, however at similar rates to other small pelagic teleosts. Low but significant genetic differentiation is expected between the Cape and KZN samples and similarly, individuals of the sardine run are expected to show little seasonal temporal differentiation over separate years if they are to be recognised as a separate subpopulation. To test these hypotheses, samples from Cape Town and KZN, including juveniles spawned in KZN were analysed using mtCOI data and nine EPIC markers, and investigated using standard population genetic analyses.

4.2. Materials and methods

Refer to chapter 2, 2.2. Meristic data collection and analysis and 2.3. Morphological data collection and analysis.

4.3. Results

4.3.1. Population genetic analyses, using EPIC primers

Ten EPIC markers were tested for the presence of polymorphisms, and only the Gonadotropin-releasing hormone 3 (GnRH) marker failed to amplify with *S. sagax* genomic DNA. The rest were amplifiable across all populations and possessed detectable polymorphisms. The representative samples sequenced ranged from 262bp - 1022bp.

Genetic diversity indices for each marker studied were number of individuals = 30.750 – 41.000, number of alleles = 2.750 – 6.500, number of effective alleles = 1.653 – 4.515, Shannon's information index = 0.172 – 0.274, expected heterozygosity 0.389 – 0.776, and observed heterozygosity 0.241 – 0.911 (Table 10). Amongst the markers, MLc showed the most genetic diversity with the highest number of alleles, effective alleles, Shannon's diversity index and effective heterozygotes. Act had the largest number of individuals and observed heterozygotes. AldoB-1 showed the least genetic diversity with the lowest number of alleles, effective alleles, Shannon's diversity index, observed and effective heterozygotes. Tr-1 had the lowest number of individuals (Table 10).

Genetic diversity indices among all the markers, according to site were number of individuals = 31.111 – 42.556, number of alleles = 4.000 – 4.778, number of effective alleles = 2.686 – 3.178, Shannon's information index = 1.084 – 1.224, expected heterozygosity 0.598 – 0657, and observed heterozygosity 0.513 – 0.657 (Table 10). Genetic diversity per site was highest at KZN, with the highest number of individuals, effective alleles, Shannon's diversity index and effective heterozygotes and observed heterozygotes. Cape Town had the largest number of alleles. KZN larvae had the lowest number of individuals, alleles, effective alleles, Shannon's diversity index, and effective heterozygotes. Cape Town had the lowest number of observed heterozygotes (Table 10).

Table 9: Reference sequences for each of the nine EPIC markers used for *Sardinops sagax* at four sites along the South African coastline. A random individual from the KZN 2012 population was used.

Locus	Size (bp)	Reference sequence (5' - 3')
Ops	1022	GAGTTCATTCTCACTCCATGCTGTAAGCACGCCCATTTCATGACTAGTGTTCCTTCAGGTTACGTTGCGGGGGGGGAATTTGCAGTCTGATCGAAGGTCCTGGGTTGCAGATGCAAAGGCTCCTTATTGGAAAGGATTATGAACCCCGGTCTT TTTCTGGGAAATAAGTCCTGACAGGCCGGACTGCACCCCGACGCCTCATTTTTTGGTTTTCCCCTATTTTTGGGCATGATGCTTATTTTTGTTTTTCCTATTGGGAACTTTTTCCGGTAAATGGACCCTTCTACTGGCAATGTGTTGAGCTGGA TGGTAGCCTTTTTTGTTTCATACTGTTTTTTGGTTGGGGTTTCTTACAGTCGATTTTGCTATACGCCTATCAATGCGCCAAAGATGGTGACCCATCACACACAGACAATTTGTTTAGGAATGGATGCCACTGGTTCTCTATAAACATAATATTTGT TATGTAAGCTATAGGCTACATGCTTTTTGTTATGATACAAAAAAAGACAAATGCTTCTGAAATAGACTACCCTTTTTATCATTTTTTTTCAGAAAAAAAACCCCACACCCGCTATGAGCTGCCTGACAATTTGCATTTTTTCCAATGGACATTTT TTTCCAAAACACCTATATCCACCCATATAATTGTATTGGCTGTAAATAACCAGCGACAACAATCCCAACCAAATGTCCCAGTGGAATTCAAACAATTTTCCCACATCCTGCTCTGTAATTATAGCCCCGCTAGAGAAAACTTCTACTCCTGGAT CAACACATCAAAAAAAAGCACCCTGTCCTCCAAATCTCTGCACAGCCTTGGTTAACGTTTCCTGAAATAGACTCTGGCCCGGAAAAACAAAACAGAAAATCGACCCCAAGTCCCCAAGAAAGCCAAACATCCAGAACTGTCACCGGACCCG GGACTGTCAGCGTACTGACCCTTCATTTTACACAGGGTCAGCCGGGGATTCCTCTATTCCTGTGCCATAGGCAGCCTAATGCCCACTGTCCCTTGGGATCAG
Act	382	GGGCACCTTTGATCCTACTCTCCGTGACTCCCACCGTCACCCCTGGCTGGGGGGATCCCCCAGGCCAGATTTAAACAAGCTCCCCCAGCTCCACCAAGGCCCCACTTCCCTCTCTCTTTCTCTCCCTCCCCTGTCTCTCTCTCTGAATCTCTCTC TTTCTCCCTCTCCCTCTCTCTGTCTTTCTCCCTCGCCCTCTCCCCCAGTCTCCCTCTTTCTCTCTCTCCTTCCTCTCTCTCTTGTGCTCTCTGGCCTGCGTGTTTGTGCAGAGACGGCTCGCTCTCGCACGTCCGAGGTTGGCTCTGGTGCTCTGGG GACAGCTGGTAAGAGCCAACCTGGTAAGAGGCACGGAGAGAGGGGCCCCTGTGTTGCAGTCAGCCTCGGA
ChymB	382	GGGCACCTTTGATCCTACTCTCCGTGACTCCCACCGTCACCCCTGGCTGGGGGGATCCCCCAGGCCAGATTTAAACAAGCTCCCCCAGCTCCACCAAGGCCCCACTTCCCTCTCTCTTTCTCTCCCTCCCCTGTCTCTCTCTCTGAATCTCTCTC TTTCTCCCTCTCCCTCTCTCTGTCTTTCTCCCTCGCCCTCTCCCCCAGTCTCCCTCTTTCTCTCTCTCCTTCCTCTCTCTCTTGTGCTCTCTGGCCTGCGTGTTTGTGCAGAGACGGCTCGCTCTCGCACGTCCGAGGTTGGCTCTGGTGCTCTGGG GACAGCTGGTAAGAGCCAACCTGGTAAGAGGCACGGAGAGAGGGGCCCCTGTGTTGCAGTCAGCCTCGGA
MLc	674	GTCGTCCATCTGGGGGGAAGCGGCCCACAGGTTGGTCATCTGAGGAGGAGGAGGGGGAGGAAGAGGAATAGCAGTTAGATAGATAGATATATACTTTATTGTCCCCATAGGGAAATTTGTCTTGGACTTCTCAAAGTATATGACCCGGT ACAAAAACATATAAAACAACCACCTCAGATACAACATGACATCCATATCCTCATGATAAATCCAAAATACATTTGACAGAAATATTAATTACAGCAATCTGTGTATGATGCTTCACATGTACATAAAACAAATATTAGTGCTGTTTTACTTTGT TGATTAGGACAAAGCTGGCTTGTCACACAACATATCATACCCTATTCATCTGAAATACACTACTTTGTTGGCTCTGAAGCCTACATCATCATTATCGTGTATGTGAAGTGGTTTTGGATTTTTAAAAAGTGCAGACATTTCCTGTGTATAACCC ACAGGACAGTGTTTTATGCAAGTTAAAAGAAACAAACCCATATTAATACCCTATTAACTGCCTCATAGCTGAAAAAATTTTAAGTCAATGTATACGCCATGGCTAATAGTTAGGAAATTACGGTATTTCTATGTCTTACAATCATGAACATTA TCATCAACGTAATCATGAACTTAATTCATGAACACTCACCTCCTCGAGAGGAAACCCCTGTCGA
Cam-3	491	CATAGAGTAGGATGTGTATGAGAGAGAAGCTACAGCATGTGTGTGTGTATGTGTGTGTCGGCGTGTGTGTCATGCAAATGATCTTTCACCACATTTTGAATGTCTTGGATAGATCTACTCTGCCAAAGACGCTGAACGCTAACAAAATGAT GGCCCAACCACTGAAGGTCAAGAATGATGAGGGACTGCACATTCCCTTTCATAGACTGCTCCCATTTACTTAACCAGAGCAATCTAAAGACAGCCTGACTCATAATCTTATATGCACAAATGTGCCAACTCTCAGAGATAGGCCTATAATCA CATTCTAACATGGCTAACACCATGCAATCACATCAGTAGGAAGGCCATTATCTTGGCTATCCAAGTGATGGGAAAAAAAAACCTTATCTGGTCTTGTCAATGTCCATGACCTCTCACCACATAGATATGCCCACACACACCCACACCAAAGT CTCACCTTGTCAAACACTCTGAAGGCCTCACGGAGC
GPd	303	TCCGGCACTGTCTCTACTCTTTAGTGAATATTAAGAGATGACAACTGATTTTAAACACAAAATGATCAATTATGAACAAGTAACATTTGTGTGTTGTTGATAGAAAGACAAAACACTTTGATAATTTCATACTGTTAATTATTAACTGTAAACA
AldoB-4	135	TCTTCTGGCTGACAGGCTTACATGTTCAAGTATGACTCCACCCACGGCCGCTTCAAGGGTGAGGTCAAACCCCGCTCCTCGGGGGAGATGTCAAAGCCTCATACATCTACCTCCTGCCCGGGCGCTTCAAGGTAGAGGGAAAAGTCAAA
TR-1	848	TGTTACTCGATCACTTTTACTCCGTTACTCAGTAGTTTTATGTGATGCATGGTGCAAGTTGGTGACTGTTTCTTGCTCCTTTCAGAATGGTCTCGTGCCCATTGTGGAGCCAGAGATCCTGCCTGATGGAACCCA
AldoB-1	262	GCAATGCTCTTGCCATGGTTTAGGAGCAACAGTCGTGCCTCTTGAAGGGATCAGCGGCATAGTCTCAGGGTGTTTACAATAAAGTGTTTGACACACACACAACTTACTTGTTAGGGTCTGATTCTTTATGGTTATGGTTATGGTTATTGAAT TTGGCAGACGCTTTTGTCCAAAGTTTAAAAGTGTATGTATACTCCTCATACACAAACTCTACTAAATAAGTGTTATTCTTATAAAGGGATGTCTGTGCTGTCAACACAAAAAAAAAAGAATGAGACCAGCATCGTTATGATTTAGATGACTTCC TCGTCAAGTTATTTTGTTGTTGTTATGATGTCATATAATGATGATAAAAAGCAGGTCACGATAGCAACCATATTCACCCCACATTTTTTATGGTTCAATTATCTGGTCTTGTTTCATGGTGCTGTGCAACGAAGAGAACATCACCCGGTGTTGT GAAAAAGATATTAAAAAAGTAGTATCCGGCTCGGAGGTGCCTCTTTTATAAAAACCACCCTGTGTAGCAGGAGGGGTGTGACTCTGCTGATGTAGCACCACTCTCTATTCAGAGTGGAAGGGAGATCTCACTTTGCTCCGATTCTGGATCT TGGGGTGCTCTCCTCGCATAGAACACACCTAACGCAGAGAAACAAAATATCTTAAAATCTTCTTATCTCTTCTTTTGTTATAAAATTTTGTTGTGGCGTGTGTATGCCCAATACCGAAGATAAAATAATCGAACACAGTATGATGTGGAGCG AGGTACGATACTTCACCCCCCTCCCCCCCTTTTTTTTTTAAAAAAAATAATTTCTCCACACCGCTCTACAAACTTTTTTTTGTCC
		GCTACGGCATCAACAGCTATTGTGCCTCTTGGGTGTTGGCCATGACCTGATTGCATTTGTGTTGTCCTTGCTTATTTGTGTTGAAATGCGTCGGTCAACTTTTTTATTTATTATTTTTATTTCCTAGGTACCATGGGCAACCGTCTTCAGAAGAT CAACGTGGAGAACACCGAGGAGAACCGCCGCTACTTCCGTGACCTCCTCTTCTCCGTTGACCAATCCATCAACCAGTGTGTGGGCGGGATCATCTTCTTCCACGAGA

Table 10: Genetic diversity indices for *Sardinops sagax* at four sites along the South African coastline, based on frequencies of nine EPIC markers. Standard errors are presented in parentheses. *N* = number of samples, N_h = number of haplotypes, N_a = number of alleles, N_e =number of effective alleles; *I* = Shannon's information index, H_e = expected heterozygosity, H_o = observed heterozygosity.

Locus	*N*	N_a	N_e	*I*	H_o	H_e
MLc	38.500 (3.476)	6.500 (0.289)	4.515 (0.273)	1.637 (0.057)	0.836 (0.038)	0.776 (0.015)
ChymB	32.750 (3.881)	3.750 (0.250)	2.526 (0.154)	1.057 (0.037)	0.392 (0.014)	0.599 (0.027)
aldoB-1	39.500 (2.754)	2.750 (0.250)	1.653 (0.092)	0.658 (0.047)	0.241 (0.103)	0.389 (0.036)
Ops-1	36.250 (4.589)	3.000 (0.000)	2.313 (0.068)	0.928 (0.018)	0.605 (0.106)	0.566 (0.013)
Tr-1	30.750 (5.513)	6.500 (0.646)	4.069 (0.604)	1.536 (0.125)	0.401 (0.083)	0.737 (0.038)
AldoB-4	40.750 (3.400)	4.250 (0.479)	2.796 (0.404)	1.110 (0.157)	0.596 (0.148)	0.619 (0.054)
Cam-3	41.000 (2.345)	5.500 (0.289)	3.051 (0.248)	1.275 (0.065)	0.563 (0.108)	0.666 (0.027)
Act	42.750 (2.657)	4.500 (0.500)	3.469 (0.309)	1.324 (0.108)	0.911 (0.025)	0.704 (0.029)
GPd	35.750 (1.250)	3.000 (0.000)	2.392 (0.224)	0.944 (0.079)	0.649 (0.066)	0.568 (0.049)
Population	*N*	N_a	N_e	*I*	H_o	H_e
KZN 2011	35.889 (1.822)	4.444 (0.475)	2.983 (0.239)	1.169 (0.105)	0.586 (0.086)	0.635 (0.036)
KZN 2012	42.556 (1.215)	4.444 (0.530)	3.178 (0.303)	1.224 (0.110)	0.637 (0.088)	0.657 (0.037)
KZN Larvae 2012	31.111 (2.220)	4.000 (0.441)	2.686 (0.282)	1.084 (0.094)	0.573 (0.079)	0.598 (0.038)
CT	40.667 (2.483)	4.778 (0.641)	3.058 (0.459)	1.175 (0.145)	0.513 (0.096)	0.610 (0.057)

Allele frequencies were significantly different from those expected under Hardy-Weinberg equilibrium. All populations showed a significant heterozygote excess (Table 10). The genetic markers showed a significant heterozygote excess, except for ChymB, aldoB-1, and Tr-1, which had a heterozygote deficit (Table 10). Expected heterozygosity, a measure of gene diversity, was highest for MLc, and lowest for aldoB-1, among the genetic markers (Table 10). An AMOVA indicated 89 % intra-population variance which was significant (Φ_{PT} = 0.105, p < 0.001), and 11 % inter-population variance (Φ_{PR} = 0.111, p < 0.001; Table 11). Genetic difference between the two regions was negligible and not significant (Φ_{PR} = -0.003, P=0.736; Table 11). Total genetic difference among all populations was negligible and not significant (Φ_{PT} = 0.091, p = 0.010; Table 11 and Table 12). AMOVA revealed the two most genetically different sites to be KZN 2012 and the KZN larvae collected in the same year (Φ_{PT} = 0.127, p = 0.001; Table 11). The most similar were KZN 2011 and KZN 2012 (Φ_{PT} = 0.073, p = 0.001; Table 11).

AMOVA according to each EPIC marker was also calculated (Table 12). Tr-1 had the lowest Φ_{RT} value which was significant, while Mlc had the highest (Table 12). Ops-1 showed the lowest

Φ_{PR} value, while AldoB-5 had the highest (Table 12). ChymB had the lowest Φ_{PT} value although not significant, and AldoB-5 had the highest (Table 12). A Mantel test revealed no measurable isolation by distance ($p < 0.001$, $r^2 = 0.1039$), even after outliers were removed. Levels of migration among the four populations differed among sites and years (Table 11) with mean Nm values ranging from 20.6 – 311.1. Migration rate between the two tested regions was 2.355 (Table 11). A PCA of the EPIC data failed to resolve any observable groupings (Figure 12).

Table 11: Summary of geographic distances, Φ_{PT} values and their level of significance, and Nei genetic distance (NeiP) among four populations and two regions, for *S. sagax* at two sites and over two years along the South African coastline, based on nine EPIC markers. Probabilities for Φ_{PT} values and confidence intervals (2.5–97.5 %) for Nm are represented in parentheses. Significant Nm values are shown in bold.

Population 1	Population 2	Spatial and temporal separation	Φ_{PT}	NeiP	Nm (Migrate)	
					Pop 1 to pop 2	Pop 2 to pop 1
KZN 2011	KZN 2012	0km, 1 year	0.073 (P=0.001)	0.127	111.9 (54.0-106.7)	**122.8 (106.6-192.0)**
KZN 2011	KZN larvae 2012	0km, 1 year	0.091 (P=0.001)	0.108	57.9 (712.0-1000.0)	203.8 (0-72.6)
KZN 2011	CT	1400km, 16 months	0.092 (P=0.001)	0.108	**37.7 (12.6-62.0)**	160.4 (0-38.0)
KZN 2012	KZN larvae 2012	0km, N/A	0.127 (P=0.001)	0.189	**42.6 (18.6-66.0)**	**254.4 (182.0-379.3)**
KZN 2012	CT	1400km, 4 months	0.091 (P=0.001)	0.121	311.1 (4.0-44.0)	51.9 (7.3-51.3)
KZN larvae 2012	CT	1400km, N/A	0.090 (P=0.001)	0.180	64.2 (0-38.0)	**20.6 (2.6-38.6)**
Region 1	Region 2	Geographic distance (km)	Φ_{RT}		Nm (Genalex)	
All KZN	CT	1400km, N/A	-0.003 (P=0.736)		2.355	

Table 12: $\Phi_{PT/RT}$ values for the nine EPIC markers, for *S. sagax* at two sites and over two years along the South African coastline.

	MLc	Chymb	aldob1	Tr1	Ops1	AldoB5	Cam-3	Act	GPd	Total
Φ_{RT}	0.099 (0.010)	-0.060 (1.000)	-0.028 (0.950)	-0.099 (1.000)	0.090 (0.010)	0.026 (0.020)	0.022 (0.100)	-0.065 (1.000)	0.009 (0.210)	-0.003 (0.740)
Φ_{PR}	0.055 (0.010)	0.069 (0.020)	0.056 (0.030)	0.159 (0.010)	0.041 (0.040)	0.174 (0.010)	0.035 (0.020)	0.135 (0.010)	0.097 (0.010)	0.094 (0.010)
Φ_{PT}	0.149 (0.010)	0.013 (0.120)	0.029 (0.070)	0.076 (0.010)	0.127 (0.010)	0.195 (0.010)	0.056 (0.010)	0.076 (0.010)	0.105 (0.010)	0.091 (0.010)

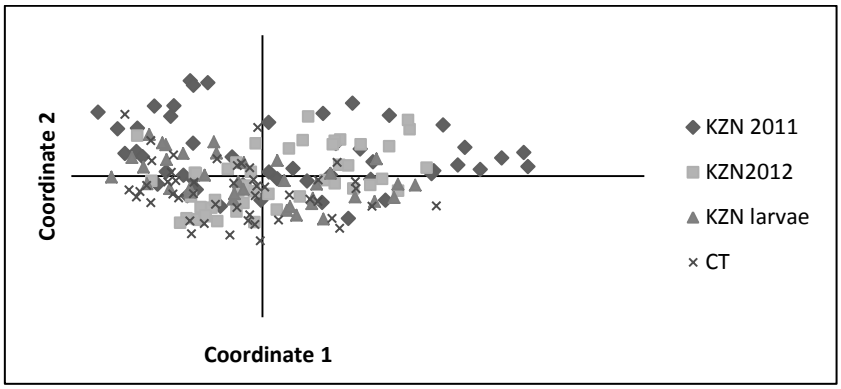

Figure 12: Principal coordinates analysis depicting the first two principal components of genetic distance among four populations, for *S. sagax* at two sites and over two years along the South African coastline, based on nine EPIC markers.

Bayesian analysis of population structure revealed the most likely number of population clusters for the sardines sampled were three (mean LnP(K) = -3214.780, Delta K = 155.644; Figure 13). KZN 2011 and the KZN larvae of 2012 were most likely to have originated from two separate clusters, mostly red and approximately 20 % blue (Figure 14). KZN 2012 adults and the Cape Town samples were composed approximately equally of the same green and blue population (Figure 14).

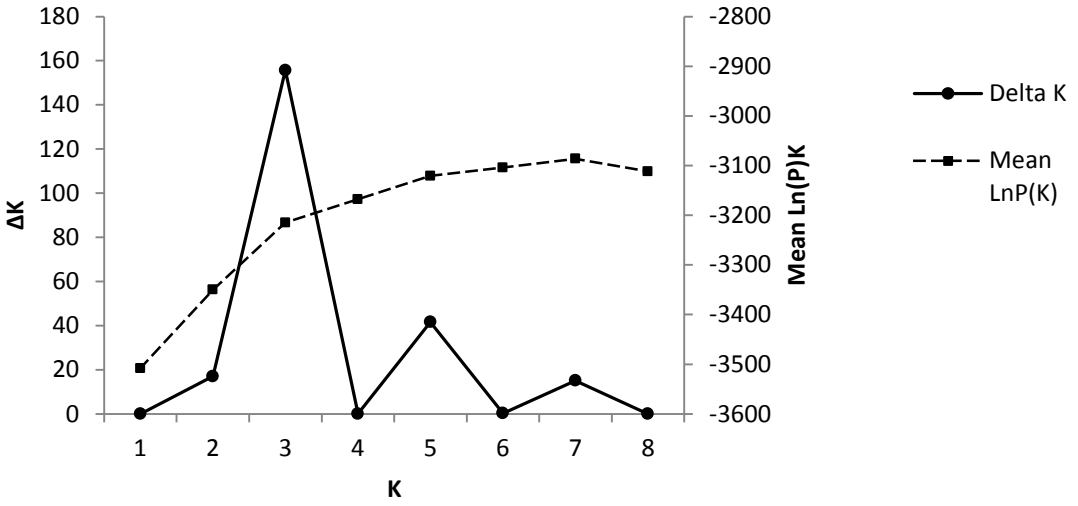

Figure 13: Delta (Δ) K (circles, solid line) versus corresponding values for the mean likelihood (squares, dashed line), for *S. sagax* at two sites and over two years along the South African coastline, based on nine EPIC markers (as derived from Structure harvester) (Earl 2012).

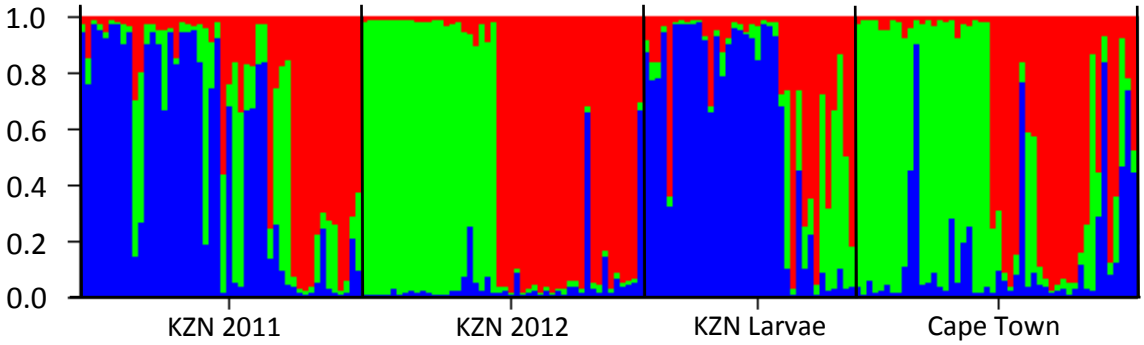

Figure 14: Bayesian analysis of population structure with three clusters (shown in red, green and blue), for *S. sagax* at two sites and over two years along the South African coastline, based on nine EPIC markers.

4.3.2. Phylogenetic analyses, using mtCOI primers

Between 665pb and 680bp of the mtCOI gene was amplified for 53 *S. sagax* individuals, collected at Cape Town, Port Elizabeth, KZN sardine run for 2011, 2012 and juveniles collected in KZN in the months following the sardine run in 2012 (Table 1). A further two *S. sagax* individuals from the Agulhas Bank near Mossel Bay and two individuals sampled near Durban, were included from two previous studies (Steinke*, et al.* 2009, Steinke*, et al.* 2011), retrieved

from Genbank (Table 4). Three sequences of another species, *S. melanostictus* were also included. Results of the reconstructed mitochondrial COI data yielded 41 haplotypes with posterior probabilities all over 0.9. The trimmed, aligned sequence set was 652 bp in length with a G+C content of 49 %. There were 51 variable sites and 61 mutations. The mitochondrial COI gene generated a haplotype diversity of 0.916 (SD = 0.030) and an average number of nucleotide difference between sequences of 5.941. The most common haplotype was haplotype two which constituted 19 individuals from all the sites sampled. The next most common haplotype was haplotype 10, constituted of two individuals from KZN 2012 and one of KZN 2011. Haplotype three constituted one KZN 2011 and a KZN 2012 individual. Haplotype 19 consisted of two larvae. The three *S. melanostictus* samples were assigned to two haplotypes. All other samples were allocated as individual haplotypes.

Grant*, et al.* (1998) concluded that the *Sardinops* genus is monotypic. Based on this, all *Sardinops sp.* can then theoretically be analysed via population analyses. AMOVA revealed 52 % of genetic difference between South African populations was due to inter-population variation. The Φ_{PT} values between the KZN larvae and PE, Mossel Bay and Cape Town were lowest and were not significant (Table 13). The highest pairwise Φ_{PT} values were for KZN 2012, compared to all the other sites, which was significant (Table 13). Mossel Bay also showed significantly large pairwise Φ_{PT} values compared with KZN 2011 and Cape Town (Table 13). The Nei's pairwise genetic distance (NeiP) were all lower than 0.03. Rates of migration were all between 1.51 and 3.22.

AMOVA revealed 74 % of genetic difference between the three global regions was due to inter-regional genetic variation. Genetic difference among the three regions tested was high, with all Φ_{RT} values above 0.750 (Table 14). Genetic distance (NeiP) ranged 0.015 – 0.021. A Mantel test revealed a positive correlation of isolation by distance ($p < 0.001$, $r^2 = 0.6383$). A PCA resolved strong groupings, with all tested populations separating (Figure 15).

Table 13: Summary of geographic distances, Φ_{PT} values and their level of significance among six South African populations based on the mtCOI marker.

Population 1	Population 2	Spatial and temporal separation	Φ_{PT}	NeiP	Nm (GenAlex)
KZN 2011	KZN 2012	0km, 1 year	0.124 (P=0.001)	0.001	3.516
KZN 2011	KZN larvae	0km, 1 year	0.195 (P=0.001)	0.001	2.055
KZN 2011	Port Elizabeth	540km, 16 months	0.196 (P=0.001)	0.001	2.048
KZN 2011	Mossel Bay	890km, 16 months	0.345 (P=0.001)	0.002	0.950
KZN 2011	CapeTown	1400km, 16 months	0.165 (P=0.001)	0.002	2.519
KZN 2012	KZN larvae	0km, N/A	0.238 (P=0.001)	0.002	1.599
KZN 2012	Port Elizabeth	540km, 16 months	0.216 (P=0.001)	0.002	1.804
KZN 2012	Mossel Bay	890km, 16 months	0.340 (P=0.001)	0.003	0.970
KZN 2012	CapeTown	1400km, 16 months	0.202 (P=0.001)	0.003	1.974
KZN larvae	Port Elizabeth	540km, 16 months	0.054 (P=0.105)	0.002	8.632
KZN larvae	Mossel Bay	890km, 16 months	0.055 (P=0.281)	0.001	8.537
KZN larvae	CapeTown	1400km, 16 months	0.011 (P=0.253)	0.001	44.757
Port Elizabeth	Mossel Bay	350km, 0 months	0.010 (P=0.430)	0.001	48.643
Port Elizabeth	CapeTown	970km, 2 months	0.000 (P=0.364)	0.015	-
Mossel Bay	CapeTown	620km, 2 months	0.000 (P=0.001)	0.001	-
Region 1	Region 2	Geographic distance (km)	Φ_{RT}		Nm
All KZN	CT	1400km, N/A	-0.003 (P=0.736)		2.355

Table 14: Summary of geographic distances, Φ_{RT} values and their level of significance, and Nei genetic distance (NeiP) among three regions, based on the mtCOI marker. Probabilities for Φ_{RT} values and confidence intervals (2.5 - 97.5 %) for Nm are represented in parentheses.

Region 1	Region 2	Spatial distance	Φ_{RT}	NeiP	Nm (GenAlex)
RSA (*S. sagax*)	Canada (*S. sagax*)	17300 km	0.752 (P=0.001)	0.015	0.083
RSA (*S. sagax*)	Japan (*S. melanostictus*)	14500 km	0.750 (P=0.001)	0.018	0.083
Canada (*S. sagax*)	Japan (*S. melanostictus*)	6700 km	0.881 (P=0.014)	0.021	0.034

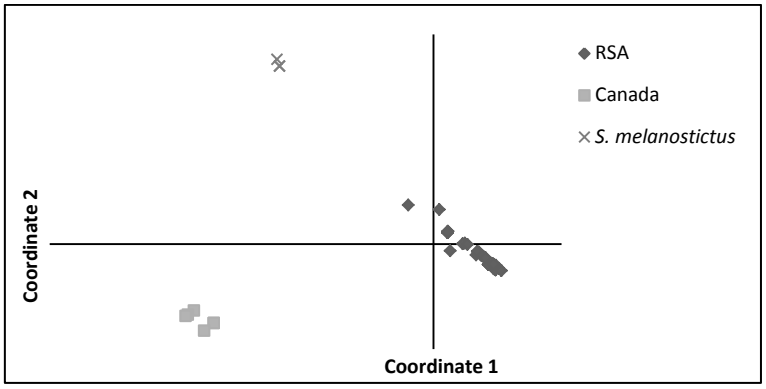

Figure 15: Principal coordinates analysis depicting the first two principal components genetic distance among three regions, for *S. sagax* and *S. melanostictus*, based on the mtCOI marker.

The 50 % majority-rule consensus Bayesian phylogram, based on the mtCOI data, illustrated the phylogeny among 38 *S. sagax* haplotype and, two *S. melanostictus* haplotypes, rooted with a *Pomacentrus baenschi* and *Sardina pilchardus* individual (Figure 16 A). The Japanese *S. melanostictus*, Canadian *S. sagax* showed strong nodal support, correlating with accepted species boundaries (Figure 16 A). Two clades (clade A and B) of South African *S. sagax* demonstrated strong nodal support, (Figure 16 A). Clade A representing hap 23, 24, 29 and 31 comprised of two Cape Town and Port Elizabeth samples each (Figure 16 A), and the clade B representing hap 7, 10, 11, 14 and 15 comprised of seven KZN individuals from 2011, 2012 and juveniles from 2012 (Figure 16). The polytomy of 44 *S. sagax* individuals and 23 haplotypes included individuals of all the sites and showed poor nodal support (Figure 16 A). A minimum spanning haplotype network of the mtCOI data suggested three homogenous clades, namely South African *S. sagax*, Canadian *S. sagax*, and *S. melanostictus* (Figure 16 B). These clades represented species level boundaries with weak levels of reticulation. A large genetic distance was observed between *S. melanostictus* and *S. sagax* from Canada (Fig. 15, 16b), and where a hypothesised common ancestor was estimated by the network analysis, than those two and the South African *S. sagax* clade (Fig. 16a, b).

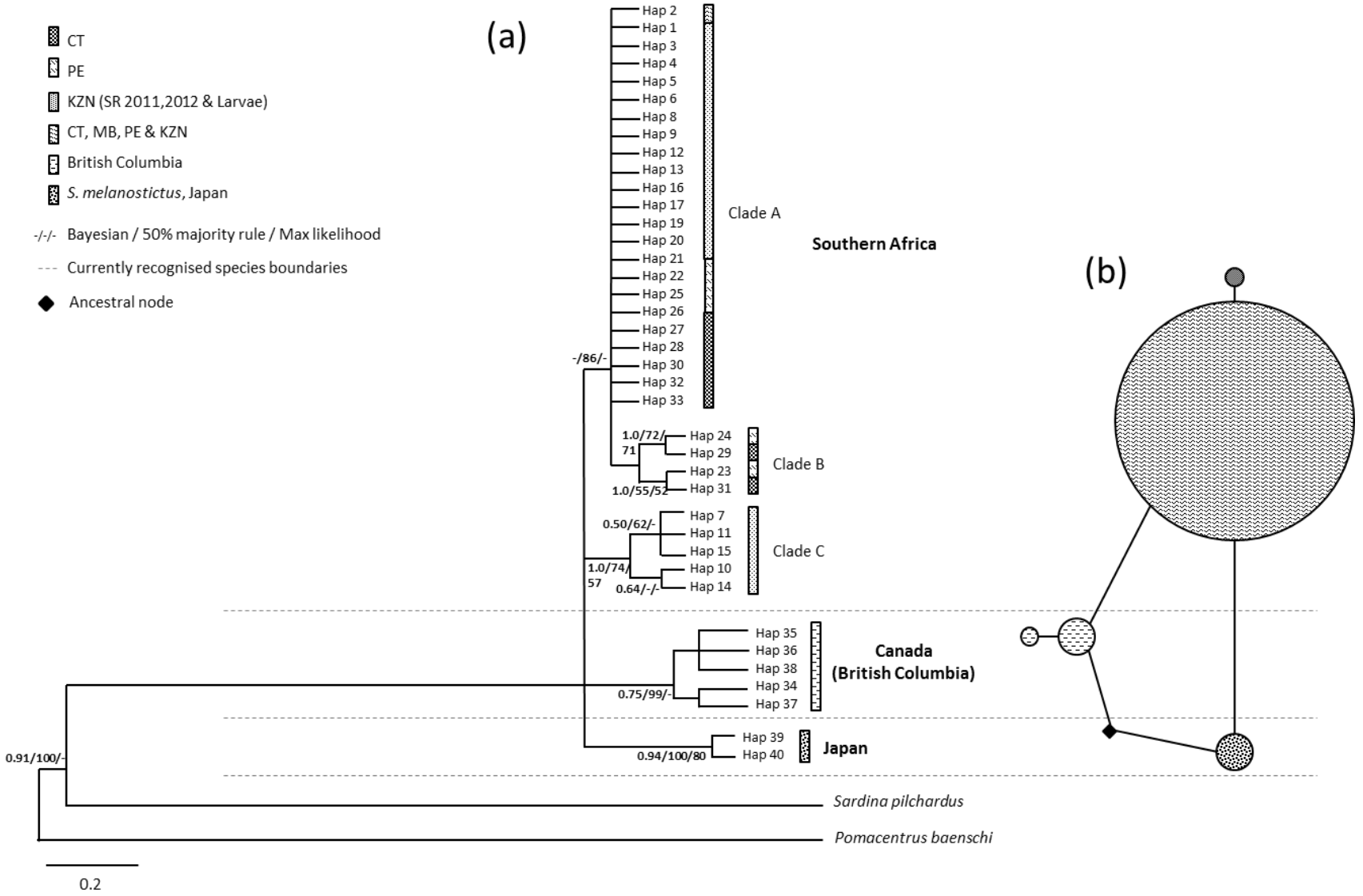

Figure 16: (a) Rooted (*Pomacentrus baenschi* and *Sardina pilchardus*) phylogram indicating evolutionary species level relationships and distribution for the mitochondrially encoded COI sequence data for *Sardinops sagax* in Southern Africa, with additional sequences from British Columbia and *Sardinops melanostictus* sequences from Japan; (b) a minimum spanning tree network of the COI sequence data using the median joining method, showing reticulation and haplotype distribution and number of haplotypes (relative size of circles).

4.4. Discussion

4.4.1 Phylogenetics of Sardinops sagax in South African waters using mtCOI genetic data

Phylogenetic analysis of *S. sagax* and *S. melanostictus* between South Africa, Canada and Japan has yielded distinct clades for each, and has shown genetic distance between each of approximately 1 %, which according to the phylogram is recent, when compared to the distance observed relative to the outgroups (Figure 16A). This suggests that *S. Sagax* between Southern Africa and Canada represent separate sub-species. This is corroborated by the large genetic differences, (Φ_{RT} > 0.25 are considered very great genetic differentiation), at sub-species resolution (Rice 1989), and extremely low estimated rates of migration among to three regions. The observations are indicative of populations that are no longer interbreeding, and are thus reproductively isolated (Palumbi 1994). Indeed, the three regions are positioned in separate ocean basins (south east Atlantic, western north Pacific, and the eastern north Pacific) over thousands of kilometres. Thus, even larval dispersal among regions is highly improbable, as the chance of random larval transport is further minimised through the diffusion effect and physiological traits of larvae (Palumbi 1994, Hellberg*, et al.* 2002). The strong genetic structuring among these groups are based on shallow evolutionary divergences (based on the genetic distance on the phylogram, Figure 16 A).

As these regions are so genetically disparate, and reproductively isolated the genus *Sardinops*, proposed by Parrish*, et al.* (1989) could in some respects be considered separate species or subspecies under the biological-isolation species concept, and the genetic species concept (Claridge*, et al.* 1997). The use of mtCOI data to delineate species is extensive and accepted (Frézal*, et al.* 2008). Nevertheless Grant*, et al.* (1985), Parrish*, et al.* (1989), and Grant*, et al.* (1998) argued that although genetic differences were significant, the genetic distances and relatively recent radiation (approximately 500 000 years before present) observed did not warrant further taxonomic delineation.

Nevertheless, data generated from the mtCOI data for the three regional populations suggests genetic difference and distance among regions is approximately equal. Thus logically, *S.*

melanostictus should either be considered monotypic and assigned as *S. sagax*, or the *S. sagax* between RSA and Canada should be considered separate sub-species or species. The lack of data from other populations, known as "shadow" populations since they were not sampled, such as the Peruvian (*Sardinops sagax sagax*) and Australian (*Sardinops neopilchardus*) sardines may confound these results.

Phylogenetic analysis of *S. sagax* indicates well supported structuring of two clades (Clade B and C) within a polytomy (Clade A) of the rest of the South African samples (Figure 16 A). These clades displayed relatively low genetic distance from the rest of the South African samples. These groups indicate genetic structuring between a fragment of the Cape and Agulhas Bank (PE) samples, and some KZN samples from a relatively homogenous South African population. The branch lengths indicate that these clades show shallow genetic distances representing moderate genetic differences within species boundaries. These clades occur within the spatial ranges of the greater South African population, and thus represent possible sympatric population differentiation (clade A and B; Figure 16 A, B). Pairwise Φ_{PT} values showed the smallest genetic differences to be between the KZN larvae and the Western Cape (Cape Town) and Agulhas Bank samples (Mossel Bay and Port Elizabeth). This followed the EPIC data results, and is discussed in detail in chapter 4.5.2 and chapter 5.

Freon*, et al.* (2010) predicted that sardines from the Western Cape and KZN would show genetic population structuring as there was evidence of population structure between the western coast and Agulhas Bank sardines based on morphology and meristic characters, and life history traits (de Moor*, et al.* 2009, Van Der Lingen*, et al.* 2009). It is likely that the observed structuring is due to recent divergence in sardine populations, where a component of Clade B and C have an intra-population breeding preference. The grouping of Cape Town and Port Elizabeth samples together in clade B and among the polytomy (Clade A) concurs with Van Der Lingen, *et al.*'s (2009) and de Moor, *et al.*'s (2009) two stock hypothesis for sardines along the Western Cape coastline and Agulhas Bank. Van Der Lingen*, et al.* (2009) and de Moor*, et al.* (2009) hypothesised that the southern Benguela sardines share a gene pool with the "spillover" of juvenile migrants into each of the populations, allowing for moderate gene flow to persist between the areas each side of Cape Agulhas (Figure 1). This appears to

correlate with the mtCOI data, although this relationship may also represent remnants of ancient population structure or some other life history traits or ecological factors (Palumbi 1994, Hellberg*, et al.* 2002).

To test Baird's (1971) hypothesis (H_U1) Freon*, et al.* (2010) listed the following questions that may be answered using genetic techniques:

- (Test 4) Are sardines from the sardine run genetically distinct from the rest of the population?
- (Test 7) Is there successful recruitment arising from individuals spawning in KZN waters?
- (Test 15) Are Sardine run sardines mostly spawned in KZN?

Relating to test four, KZN 2012 sardines appear to be genetically different with relatively low migration rates between the populations tested, according to mtCOI data and the phylogram (clade C). Nevertheless, the rest of the individuals caught in KZN showed groupings with all the other populations. KZN 2011 also demonstrated a lower difference with the other populations. Thus, the data shows that some of the sardines undertaking the sardine run are genetically distinct, and show structuring from the Cape Town, Mossel Bay and Port Elizabeth samples. However, if the sardine run were a true spawning migration of a certain sub population, one would expect all members of the KZN sub population to show structuring from the rest (Cape Town, Mossel Bay, and Port Elizabeth). Added to this, only one of the individuals belonging to clade C was a larva (out of a total of 13; Figure 16 A). Thus, the bulk of the sardines from clade A, were spawning in KZN waters, not just the genetically structured individuals from clade C (Figure 16 A).

Relating to test seven, the mtCOI data suggests successful recruitment based on the genetic similarity of larvae to adults, across the populations tested, based on all samples collected during the sardine run. Rates of migration among the KZN Larvae and all the other sites, especially Port Elizabeth, Mossel Bay and Cape Town support the hypothesis that sardines spawned in KZN are recruited to the adult breeding populations in the Western Cape and on

the Agulhas Bank. Test eight was not able to be answered as the study had only analysed larvae from a single year class (2012).

Freon*, et al.* (2010) natal homing hypothesis (H_p1), based on previous work by Carr (1967) and Stabell (1984), requires that sardine run sardines are mostly spawned in KZN (Test 15). The mtCOI data suggested that most sardines belong to low structured interbreeding population, where most of the larvae grouped with all other populations (Clade A, Figure 16 A). Most larvae showed little genetic difference and moderate migration rates between the Cape populations. Thus, most sardines undertaking the sardine run may have not been spawned in KZN.

It must be noted that mtCOI is primarily a species genetic barcoding marker, used to delineate species level genetic differences (Ward*, et al.* 2005). Thus, although its use in population level studies is common, it may fail to resolve some fine scale structuring, which should be taken into account. The sample sizes used are adequate for phylogenetic techniques used in this study, however, more samples would aid resolution for population level techniques, thus caution must be exercised when interpreting this data.

4.4.2 Population structure of KZN Sardinops sagax using EPIC genetic data

The nine EPIC markers used, showed similar allele diversities as observed in the original publications that they were described (Hassan*, et al.* 2002, Touriya*, et al.* 2003), and had sufficient polymorphism and gene diversity (H_e) for population analysis (Table 10). Alleles were found within similar size ranges. The increased diversity of these markers allow for increased power of inference of the marker set (Hedgecock*, et al.* 1989). The relative ease of PCR amplification illustrated the universality of these primers for use among non-model teleosts, and confirmed that these primers are suitable for fine scale population genetic analyses.

The excess of heterozygosity observed among most markers and all populations is indicative of outbreeding among populations (Palumbi 1994, Hellberg*, et al.* 2002). The increased levels of intra-population genetic difference over inter-population difference, and overall moderately low Φ_{PT} values indicate low to moderate levels of population genetic structure is also an indication of outbreeding (Weir*, et al.* 1984). *Sardinops sagax*, like most clupeid species occupy large ranges, and have large effective population sizes (Parrish*, et al.* 1989), and most likely explains the heterozygote excess (Palumbi 1994, Hellberg*, et al.* 2002). Heterozygote excess may also be due to balancing selection due to heterosis; an improved fitness of hybrid due to outbreeding (Nei*, et al.* 1974, Birchler*, et al.* 2010). Evolution favouring heterozygosity within a gene or set of genes is often due to changes in allelic fitness over spatial and temporal scales in a species environment (Hubby*, et al.* 1966, Nei*, et al.* 1974). The heterogeneous marine environment along the South African coastline (Lutjeharms*, et al.* 2001, Lutjeharms 2006) coincides with three biogeographic breaks, namely the sub-tropical, warm temperate, and cool temperate according to Teske*, et al.* (2011). These environmental factors may play a role in causing balancing selection, to increase gene fitness to cope with these varied and changing selection pressures.

Sardine run individuals between 2011 and 2012 showed the least genetic differentiation, and almost equally high migration rates between populations. They share shallow genetic structuring and the same lineages of sardines undertaking the sardine run appear to mate and spawn in KZN annually. The rates of migration and genetic differentiation supported test four; that sardines of the sardine run are genetically distinct from the rest of the population.

Sardines of the sardine run and larvae caught in KZN in the months following the run showed the most genetic difference (considered moderate). In contrast, KZN larvae showed the greatest genetic similarity to the Western Cape population. Although the Phi_{PT} results show low structure between Western Cape and larval samples, there is relatively low recruitment of the KZN larvae to the Adult Western Cape population (Figure 14).

Disparity in the relationships between genetically different populations may be due in part to the timing of the sampling of the populations in this study. If genetically distinct shoals occur during the run, then this could explain the moderate genetic difference between adults and larvae of the 2012 sardine run. Overall, genetic differences in the population's tests showed little structure (Figure 12).

Rates of migration among the KZN Larvae and Cape Town support the hypothesis that sardines spawned in KZN are recruited to the adult breeding populations in the Western Cape and Agulhas Bank for the COI gene (Test 7). Test eight could not be directly answered as the study had only analysed larvae from a single year class (2012). Nevertheless, the fact that Cape Town samples and KZN larvae showed low levels of structure and moderate levels of migration between themselves, suggests sardine individuals do not only spawn in KZN, but breed and spawn on the Agulhas bank and on the western coast too. This is supported by the sardine's bimodal spawning regime (de Moor*, et al.* 2009). It is likely that the breeding and spawning of these individuals twice a year would have the effect of lowering any structuring that a separate spawning event such as the sardine run would have on genetic isolation.

It seems then, that *Sardinops sagax* in South Africa exhibits genetic structuring not unlike other clupeids globally (Whitehead*, et al.* 1986, Parrish*, et al.* 1989, Tinti*, et al.* 2002, Croft*, et al.* 2003, Silva 2003, Gonzalez*, et al.* 2007, Baibai*, et al.* 2012). The increased gene diversity (H_e) of fishes in KZN, compared to Cape Town, may be suggestive of the heavy fishing pressures of the western coast sardines, the target of the South African industrial pilchard fishery. Selective pressure on fished stocks has a biological effect of not only reducing genetic diversity, but also includes the selective targeting of certain phenotypic characters (Law 2000). Fishing induced phenotypic selection, such as a minimum size of individuals in a targeted fishery, often results in a selection pressure for smaller fish at sexual maturity, as reproduction at a smaller size is favoured (Law 2000; 2007). Western Cape sardines, however, are larger than KZN sardines.

Although the census population size of the sardines undertaking the sardine run is much smaller than the west coast, they exhibit higher gene diversity. This observation can be

indicative of heavily fished stocks (Carvalho*, et al.* 1995, Turner*, et al.* 2002, Hauser*, et al.* 2008). Alternately, the observed structure may be descriptive of KZN being a zone of admixture for all sardine populations. The low levels of structure and moderate levels of migration between larvae and the cape populations (supported by the Cape Town, Mossel Bay, and Port Elizabeth mtCOI data) is suggestive of admixture. Nevertheless, the lower expected heterozygosity shown by KZN larvae over KZN adults and Cape samples may not be indicative of admixture, where recombination of new gene combinations and the sharing of alleles between populations is expected (Durand*, et al.* 2009). The slightly lower heterozygosity indices among the Cape Town population follow the Wahlund effect, (where decreases in heterozygotes often represent a structured sub-population). Evolutionarily, this phenomenon would be beneficial in that it would serve to increase the transfer of genetic material between the Western Cape and Agulhas Bank population (Nielsen*, et al.* 2003, Durand*, et al.* 2009). Evolutionarily then, this type of strategy would be a stable strategy. These observations were mirrored by the COI data, where the KZN larvae illustrated low genetic difference from samples from the Western Cape (Cape Town) and Agulhas Bank (Cape Town, Mossel Bay and Port Elizabeth).

According to the EPIC nuclear DNA data (Freon*, et al.* (2010) test 4), sardines of the sardine run did show structuring from the other populations, however this did not accord with the KZN larvae, which illustrated less genetic differentiation to the Cape populations than KZN 2012. The rates of migration of sardines spawned in KZN, to the Western Cape and KZN adult populations suggest there was successful recruitment due to individuals spawned in KZN (Freon*, et al.* (2010) test 7). Nevertheless, migration among the Cape and KZN larvae samples was reduced, compared with KZN 2011 sardine run adults. The Bayesian analysis of population structure, confirmed the findings that KZN larvae showed a population demographic similar to the KZN 2011 sardine run. Test eight (Freon*, et al.* 2010) was not answered as the study had only analysed larvae from a single year class and only in KZN, and comparisons of larval structure from the Cape are also needed (2012). The EPIC nuclear data suggested that most sardines belong to a finely structured interbreeding population, where most of the larvae grouped with all other populations, and most closely to the Western Cape stock. Thus, most sardines undertaking the sardine run may have not been spawned in KZN according to Freon*, et al.* (2010) test 15.

Chapter five:

Conclusions

The Sardine run is an important ecological and economic phenomenon (van der Lingen, Coetzee, et al. 2010). Little is known about why and how sardines undergo this migration up the east coast of South Africa. The hypotheses about the nature of the sardine run can allow for insights into these questions. Information collected in this study could allow scientists to plan management strategies based on morphological and genetic diversity, connectivity, and breeding patterns within the KZN sardines. Ultimately, understanding genetic relationships and breeding patterns of the migrating fish stocks will allow for the conservation of genetic diversity of the fish, and the preservation of the KZN sardine run. In this study, these questions have been tested and the structure of the KZN sardines based on genetic and morphological techniques has been investigated.

The global genetic structure of *Sardinops sagax* suggests that the South African stock has diverged enough to be considered a separate subspecies to the Canadian *S. sagax*. These findings reflect previous allozymes and mtDNA genetic analysis by Grant*, et al.* (1998). However, it is argued that shallow divergence of these species does not validate a separate species grouping. Nevertheless, it can be argued that the genetic divergence between *S. melanostictus* is similar to that of the two populations of *S. sagax* between Canada and South Africa. Thus, based on the above, *S. sagax* between Canada and South Africa could be delineated into two separate subspecies. Nevertheless the benefits of reclassifying individuals may not warrant the effort. Further study into defining the species *Sardinops* worldwide would ultimately allow for a final solution to the above dilemma in taxonomy.

Genetic and morphological analyses both described differences in population structure between regions. Although there seemed to be larger population differences observed according to morphology, genetic structuring was considered to be moderately low but significant, especially considering the nature of clupeid life history and ecology. Nevertheless, morphology and genetic analyses seemed to show a positive association of difference among

the populations tested in this study. Mitochondrial (mtCOI) and nuclear (EPIC) markers conferred the observed genetic structuring.

Tests for the ultimate and proximate factors of the sardine run (Freon*, et al.* 2010) were tested and are summarised below (Table 15). Test 3, the only test not related to genetics was supported by the morphology of the sardines. KZN sardines are genetically different from the rest of the population, with an overall smaller head and body size. This formed part of the questions that were used to test whether sardines undertaking the sardine run form part of a subpopulation spawning migration (H_{U1}). Test 3, 4 and 7 were investigated in this study, and all could not be rejected, thus supporting the notion that sardines undergoing the sardine run are a sub population spawning migration. Test 4 and 7 were also used to test whether sardines undergo the sardine run because they exhibit some form of relic behaviour (H_{U7}), which was also supported. The hypothesis that sardines undergo natal homing and imprinting (H_{P1}) was rejected with the rejection of test 15, where the data showed that sardine run sardines did not only spawn in KZN. Genetic analysis has shown that most sardines belong to a finely structured interbreeding population, where most of the larvae grouped with other populations, not only the sardine run adults. Test eight could not be assessed using the scope of the analyses in this study. Thus, all the tests for H_{U1} and H_{U7} except test eight were supported.

Table 15: Conclusions of tests based on morphometric and genetic data in this study, used to test the ultimate and proximate hypotheses relating to the sardine run, including caveats to the analyses and proposed further analysis. Strength and reliability of the tests were based on the statistical analyses undertaken and their results.

Test	Question	Result	Strength of result and reliability	Analyses used in test	Caveats	Further analysis
3	Are sardines from the run phenotypically distinct from the rest of the population?	Yes, there are significant morphological differences between the sardine run fish compared with other regions. Sardine run individuals more phenotypically related to Port Elizabeth and Mossel Bay samples than the Cape Town individuals.	++ ***	Meristic, multivariate and geometric morphometric analyses.	Small KZN morphometric size.	Larger sample sizes, including more west coast and KZN samples.
4	Are sardines from the sardine run genetically distinct from the rest of the population?	Yes, KZN adults illustrate some isolation from Cape samples. However, KZN larvae illustrate significant genetic affiliation to Cape Town, Mossel Bay and Port Elizabeth adults.	+ *	mtCOI and EPIC marker phylogenetic and population analyses	EPIC data analyses compared sardine run sardines to Cape samples only.	More temporal samples for KZN and addition of Agulhas bank samples for EPIC data analysis.
7	Is there successful recruitment arising from individuals spawning in KZN waters?	Yes. Migration rates and structure between larvae and all populations suggest this.	+ **	mtCOI and EPIC marker phylogenetic and population analyses.	-	Collection and genetic analysis of more larvae samples from all regions is needed. Multiple temporal samples during and after the sardine run should be taken.
8	Do all individuals of the sardine run subpopulation only spawn in KZN?	Inconclusive. Structuring suggests not.	0 *	Comparisons with Cape larvae needed for test, thus reducing the null effects of a shadow population.	N/A	"
15	Are Sardine run sardines mostly spawned in KZN?	Yes, according to EPIC data. Sardines of the sardine run from 2011 belong to a structured interbreeding population, where most of the larvae grouped with the KZN larvae population. This was, however, not true for the KZN 2012 sardine run sardines, which showed migration and genetic affiliation to the Cape Town population. mtCOI data however may have been too broad scale in scope to be able to delineate population level structure, thus failing to show genetic structure.	+ ***	mtCOI and EPIC marker phylogenetic and population analyses.	These results represent the sardine population over two years only. Within sardine run differences in shoals may yield more accurate results.	"

Strength of Result: - negative; - - strongly negative; 0 inconclusive; + positive; ++ strongly positive. Strength and reliability of test: * low; ** medium; *** high.

Although the results of genetic analysis supported the ultimate hypotheses, the observed structure may have also been descriptive of KZN being a zone of admixture for the sardine populations of the Western Cape and Agulhas Bank. To test this hypothesis however, a more comprehensive study of population structure, by including larvae samples from all regions and better spatial sampling coverage of the sardine run is needed. These tests would serve to answer the final question (test 8) that remains to be *answered* and including the notion of admixture of populations in KZN.

Having said this, the current study has raised important points for consideration by fisheries managers. Most importantly, the sardine run is genetically diverse and distinct from the rest of the stock, at the population level. Thus, fisheries managers should take this into account when developing fishery models of *S. sagax* in South Africa. This study has also raised the importance of utilising many samples from the sardine run as possible. The usage of the nine EPIC markers was considered successful.

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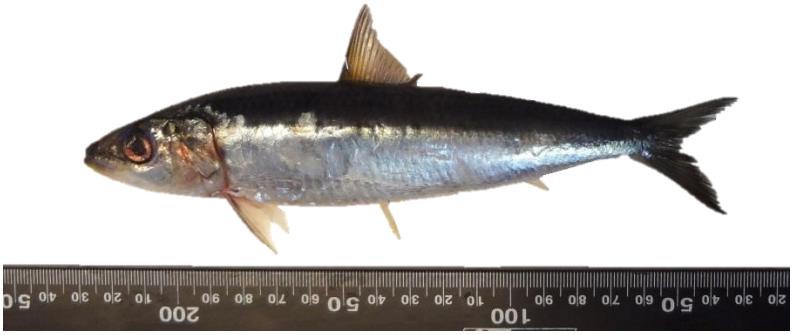
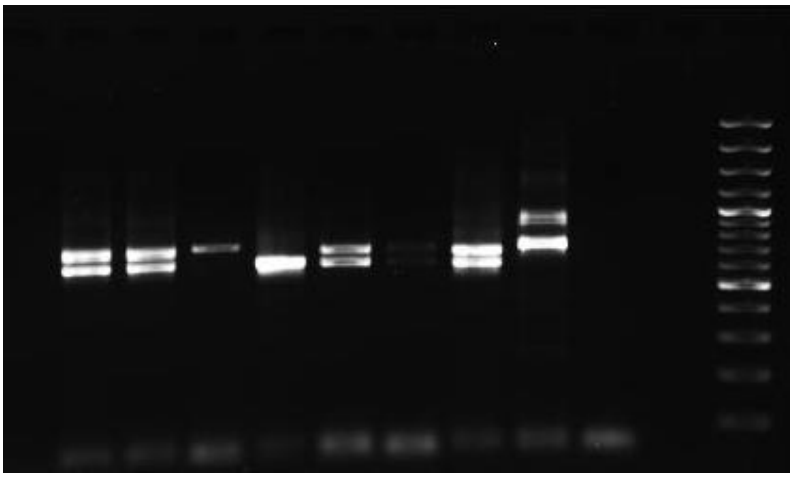
Appendix 1

Typical photograph used in the morphometric analysis of *Sardinops sagax* in this study.



Appendix 2

A 3 % agarose gel electropherogram run for 5 hours at 100 volts. The gel electrophoresis depicts polymorphisms used to genotype sardines for each of the nine EPIC markers in this study.



Appendix 3

Hardy Weinberg Equilibrium test for sardines, including the significances of heterozygosity for each EPIC marker between the sites.

Population	Locus	DF	Chi^2	Probability	Significance
KZN 2011	MLc	21	36.168	0.021	*
	Chymb	6	13.296	0.039	*
	aldob1	3	6.158	0.104	Ns
	Tr1	15	70.348	0.000	***
	Ops1	3	22.427	0.000	***
	AldoB5	6	17.149	0.009	**
	Cam-3	10	31.241	0.001	***
	Act	10	37.106	0.000	***
	GPd	3	23.802	0.000	***
KZN 2012	MLc	15	49.384	0.000	***
	Chymb	6	51.069	0.000	***
	aldob1	1	37.349	0.000	***
	Tr1	21	61.331	0.000	***
	Ops1	3	13.358	0.004	**
	AldoB5	10	12.350	0.262	ns
	Cam-3	10	35.134	0.000	***
	Act	10	30.071	0.001	***
	GPd	3	8.374	0.039	*
KZN Larvae	MLc	15	30.853	0.009	**
	Chymb	6	10.026	0.124	ns
	aldob1	3	4.112	0.250	ns
	Tr1	10	28.189	0.002	**
	Ops1	3	4.301	0.231	ns
	AldoB5	3	5.383	0.146	ns
	Cam-3	15	27.508	0.025	*
	Act	3	27.590	0.000	***
	GPd	3	9.029	0.029	*
CT	MLc	21	55.947	0.000	***
	Chymb	3	12.600	0.006	**
	aldob1	3	33.757	0.000	***
	Tr1	28	118.110	0.000	***
	Ops1	3	5.996	0.112	ns
	AldoB5	10	21.555	0.018	*
	Cam-3	15	50.704	0.000	***
	Act	10	15.909	0.102	ns
	GPd	3	1.359	0.715	ns

Appendix 4

Haplotype list with individual assignment to haplotypes in DNAsp, for the mtCOI gene.

Haplotype number	Number of individuals representing haplotype	Individual identification
1	3	B1, B9, C13
2	19	B2, C18, F1, F4, H3, H4, H6, H7,PE3,PE8, CT1, CT2, CT8, CT9, CT11, JF494407.1, JF494410.1, JF494409.1, JF494412.1
3	1	B3
4	1	B4
5	2	B5, C12
6	1	B6
7	1	B7
8	1	B8
9	1	B11
10	3	C2, C3, C6
11	1	C4
12	1	C14
13	1	C15
14	1	C19
15	1	F2
16	1	F3
17	1	F6
18	1	H5
19	2	H8, H10
20	1	H9
21	1	PE1
22	1	PE6
23	1	PE7
24	1	PE11
25	1	CT3
26	1	CT4
27	1	CT5
28	1	CT6
29	1	CT7
30	1	CT10
31	1	CT12
32	1	CT13
33	1	JF494411.1
34	2	FJ165126.1, FJ165128.1
35	1	FJ165121.1
36	1	FJ165125.1
37	1	FJ165123.1
38	1	FJ165120.1
39	2	JF952843.1, JF952841.1
40	1	JF952842.1