Investigation of the prevalence and characterisation of infection by *Kudoa thyrsites* and *K. paniformis* in South African marine fish species

By

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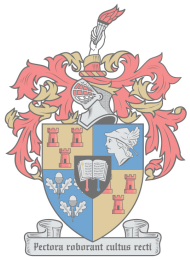

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Declaration

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Summary

Post-mortem myoliquefaction and formation of black pseudocysts in marine fish muscle are associated with infection by *Kudoa thyrsites* and/or *K. paniformis*. These quality defects become apparent only upon filleting, resulting in economic losses. Methods to detect infection include microscopy and polymerase chain reaction (PCR) techniques. These methods are time consuming, expensive, and destructive to the sample. Near-infrared (NIR) spectroscopy is a rapid, non-destructive method, used for identifying nematodes-infected fish. The aims of this study were to investigate the prevalence of *K. thyrsites* and *K. paniformis* in South African (SA) sardine (*Sardinops sagax ocellatus*), kingklip (*Genypterus capensis*) Cape hake (*Merluccius capensis* and *M. paradoxus*), and to use NIR spectroscopy to discriminate between infected and uninfected samples.

A real-time quantitative PCR (qPCR) method was designed for use as qualitative determination of *K. thyrsites*. A total of 536 fish samples (296 SA sardine, 70 kingklip and 170 Cape hake) were analysed with qPCR. The SA sardine samples had *K. thyrsites* prevalence of 92%, Cape hake 79%, and SA kingklip 40%. Results showed that there was no significant difference in *K. thyrsites* prevalence between male and female (SA sardines, kingklip and Cape hake), season (SA sardines) and area of capture (SA sardines). Cape hake samples showed conflicting results for the relationship between *K. thyrsites* prevalence and size. A higher prevalence for smaller (39.63 ± 10.88 cm) than larger sized (49.46 ± 13.94 cm) samples was found in one study, while the other independent study showed no significant relationship to size. A limited number of Cape hake samples were used, resulting in larger sized fish to be poorly represented in this study.

Infection by *K. thyrsites* had no significant effects ($P > 0.05$) on moisture and ash contents of SA sardine. Similarly, the moisture, ash and protein contents of infected Cape hake did not differ from uninfected samples. Moisture, ash and protein contents were therefore not useful in prediction of *K. th*yrsites infection.

No NIR spectral differences between *K. thyrsites* infected and uninfected fish sample were observed when NIR spectroscopy and principal component analysis (PCA) were investigated. In addition, NIR spectroscopy, in combination with soft independent modelling of class analogy (SIMCA) and partial least square discriminant analysis (PLS-DA), was unable to distinguish infected from uninfected samples. Fish samples used in this study were frozen, resulting in water loss, protein degradation and aggregation, thus possibly masking the chemical and textural changes associated with *K. thyrsites* infection. NIR spectroscopy, in combination with SIMCA and PLS-DA, respectively, was therefore not a useful method to predict *K. thyrsites* infection in frozen fish samples.

Further studies are suggested where the relationships between level of infection (number of *K. thyrsites* spores), myoliquefaction, and texture analyses, in combination with NIR spectroscopy, are investigated. However, since SA sardine has a high prevalence of infection, such a study may require more than 500 samples in order to ensure an equal amount of infected and uninfected samples for the development of reliable NIR classification models. For Cape hake, it is suggested to investigate

the use of imaging spectroscopy in an attempt to differentiate between infected and uninfected samples based on optical properties of fish muscle and black pseudocysts.

Opsomming

Die nadoodse afbreking van spierweefsel en vorming van swart pseudoblaas in seevis word geassosieer met besmetting van *Kudoa thyrsites* en/of *K. paniformis*. Hierdie agteruitgang van viskwaliteit word eers waargeneem tydens filering wat bydrae tot ekonomiese verliese. Metodes om besmetting te bepaal behels mikroskopiese en polymerase ketting reaksie (PKR) tegnieke. Hierdie metodes is tydrowend, duur en destruktief tot die produk. Naby-infrarooi (NIR) spektroskopie is ‘n vinnige metode wat gebruik word om vis wat met nematodes besmet is te identifiseer. Die doelstellings van hierdie studie was om die teenwoordigheid van of *K. thyrsites* en/of *K. paniformis* in Suid-Afrikaanse sardien (*Sardinops sagax ocellatus*), stokvis (*Merluccius capensis* en *M. paradoxus*) en koningklip (*Genypterus capensis*) te bepaal, asook om NIR spektroskopie te gebruik om tussen besmette en onbesmette monsters te onderskei.

‘n Reëele kwantitatiewe PKR metode was ontwikkel om kwalitatief die teenwoordigheid van *K. thyrsites* te bepaal. Totaal van 536 visse (296 sardien, 70 koningklip and 170 stokvis) was geanaliseer met diè PKR metode. Suid-Afrikaanse sardien, stokvis en koningklip was besmet met *K. thyrsites*. Meeste (92%) van die sardien monsters was met *K. thyrsites* besmet, terwyl 83% van die stokvis monsters besmet was. Slegs 40% van die koningklip monsters was besmet.

Resultate het geen noemenswaardige verskille in *K. thyrsites* besmetting tussen manlike en vroulike monsters (sardien, stokvis en koningklip), seisoen (sardien) en area van vangs (sardien) gewys nie. Monsters van stokvis het ‘n hoër voorkoms van *K. thyrsites* infeksie in kleiner (39.63 ± 10.88 cm) as groter monsters (49.46 ± 13.94 cm) getoon in die een studie; terwyl resultatke in ‘n onafhanklike studie geen verskil getoon het nie. ‘n Beperkte aantal monsters was gebruik. Gevolglik was groter stokvis swak verteenwoordig in hierdie studie.

Resultate van besmetting met *K. thyrsites* het geen noemenswaardige effek ($P > 0.05$) op vog en as inhoud van sardien getoon nie. Soortgelyk, daar was geen noemenswaardige verskil in proteïen inhoud tussen besmette en onbesmette stokvis nie. Vog, as en proteïen inhoud was dus nie ‘n goeie voorspelling van *K. th*yrsites infeksie nie.

Geen NIR spektrale verskille tussen *K. thyrsites* besmette en onbesmette vis monsters was waargeneem wanneer NIR spektroskopie en hoof komponent analises (PCA) ondersoek was nie. NIR spektroskopie, in kombinasie met sagte onafhanklike modelleerwerk van klas ooreenstemming (SIMCA) en gedeeltelik minste kwadraat ondreskeid analise (PLS-DA), was ook nie in staat om tussen besmette en onbestemme monsters te ondoerskei nie. Vis monsters gebruik in hierdie studie was bevrore, wat gelei het tot vogverlies en denaturasie van proteïne. Hierdie chemiese en struktureële veranderinge het moontlik die chemiese en tekstuur veranderinge wat geassosieer word met *K. thyrsites* besmetting verbloem. NIR spektroskopie, in kombinasie met SIMCA en PLS-DA, was dus nie suksesvol in die voorspelling van *K. thyrsites* infeksie in bevrore vis-monsters nie.

Verdere navorisng word aanbeveel waar die verhouding tussen die vlak van besmetting (aantal *K. thyrsites* spore), nadoodse afbreking in speirweefsel, en die tekstuur van vis, in kombinasie met NIR spektroskopie, ondersoek word. Aangesien daar in sardien ‘n hoë voorkoms van besmetting bestaan, sal verdere navorsing meer as 500 monsters vereis om sodoende te verseker dat ‘n gelyke aantal besmette asook onbesmette monsters bekikbaar is vir die ontwikkeling van betroubare NIR klassifikasie modelle. Betreffende stokvis word navorisng deur die toepassing van beeldspektroskopie voorgestel in ‘n poging om tussen besmette en onbesmette monsters te onderskei, gebaseer op optiese verskille van visspierweefsel en swart pseudoblaas.

Biographical sketch

Mrs. Suné St. Clair Henning is a lecturer and researcher at the Department of Food Science and Technology, Cape Peninsula University of Technology (CPUT), Belleville campus, since January 2008. In 2010 she accepted the role as Departmental ECP co-ordinator in addition to lecturing and supervising BTech students. She successfully supervised 19 BTech projects since 2008, one MTech student and is currently supervising three MSc students. She also co-supervised one MSc student with Stellenbosh University in 2016.

She has been involved in several fish and fish processing related projects since 2010. Many of these projects were Service Learning projects: for example, the optimisation and verification of food safety for a fish drying/fermentation process in collaboration with Environ Choice and ATS (Agri-Foods Technology station, CPUT). Another example is the training of fish-cleaners at Kalk Bay harbour in basic food hygiene and food safety in collaboration with DAFF (Department of Agriculture, Forestry and Fisheries). In 2011, her Service Learning project received the “Most outstanding project” at the CPUT Service Learning Open day showcase. In addition, from her involvement and experience with Service Learning and ECP lecturing, she published an article in a non-accredited journal (*Case studies of Epistemological access in Foundation/Extended Programme studies in South Africa*) discussing the values of Service Learning as a teaching tool in ECP. Mrs. Henning has published in total nine peer-reviewed journal articles since 2006, of which five were during 2008 and 2017 while she worked at CPUT and studied towards her PhD.

Mrs. Henning was the grant holder of a multi-million THRIP project during 2015-2016, in collaboration with Blue-Karoo Trust; which involved the investigation of several aspects (HACCP, Industrial Engineering, and sensory analysis) of the processing and manufacturing of cat fish products.

She has received Thuthuka National Research Funds (NRF) since 2014 for the study to investigate the prevalence of *Kudoa thyrsites* infection in South African marine fish species, such as Cape snoek, sardines, and Cape hake. Mrs. Henning supervised two Master students related to this NRF funded project. Prior to lecturing at CPUT, Mrs. Henning worked as administrative and research assistant at Stellenbosch University, Department of Animal Sciences. She was involved in administration of the USAID Partnership for Food Industry Development in Meat, Seafood and Poultry (PFID-MSP) project. She presented several workshops in Seafood HACCP in Stellenbosch, Lusaka, Zambia and Walvis Bay, Namibia. Mrs. Henning completed her M.Sc. studies in Food Science at the Stellenbosch University in 2004.

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Preface

This dissertation is presented as a compilation of 8 chapters. Each chapter is introduced separately and is written according to the style of the *International Journal of Food Science and* Technology. Part of Chapter 2 was published as a review article in *South African Journal of Science*.

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Lists of definitions, abbreviations, Tables and Figures

List of definitions for terms used in this dissertation:

Allele -	a variant form of a given gene and can arise by mutation and are found at the same place on a chromosome.
Caudal length -	the length of a fish measured from the tip of the snout to the posterior end of the last vertebra of the mid-lateral portion of the hypural plate, excluding the length of the caudal fin.
Juvenile -	the time that fish spend developing from fry (the stage where they are capable of feeding themselves) into reproductively mature adults
Myoliquefaction -	extensive proteolytic breakdown of muscle tissue and structure as a result of proteolytic enzymes released by the marine myxosporean parasites, *Kudoa thyrsites* and *K. paniformis*, after the death of the fish (*post-mortem*). Also referred to as 'jelly flesh', 'pap vis', and 'mushy texture'.
Post-mortem -	after death of the fish.
Prevalence -	the number of fish infected with a particular parasite, divided by the number of fish examined. Typically expressed as a percentage.
Pseudocyst -	an aggregate of *K. thyrsites* or *K. paniformis* spores within the somatic muscle fibre, resembling a cyst but lacking a wall or lining.
TaqMan® assays -	5'-nuclease, linear, target-specific probes which are generally double-labelled with a fluorescent reporter dye and a quencher. Also known a hydrolysis probes and are used in real-time quantitative polymerase chain reaction (qPCR) assays.

List of abbreviations used in this dissertation:

ALA -	alpha-linolenic acid
AMP -	adenosine monophosphate
ANCOVA -	analysis of covariance
ANOVA -	analysis of variance
AOAC -	Association of Official Agricultural Chemists
ATP -	adenosine tri-phosphate
BLAST -	Basic Local Alignment Search Tool
BPNN -	and back-propagation neural networks
CAF -	Central Analytical Facility
CHAID -	Chi-squared automatic interaction detector
CPEG -	Centre for Poverty, Employment and Growth
CPGR -	Centre for Proteomic and Genomic Research
DAFF -	Department of Agriculture, Forestry and Fisheries
DHA -	docosohexaenoic
DMA -	dimethylamine
ELISA	enzyme-linked immunosorbent assay
EPA -	eicosapentaenoic acid
FA -	fatty acid
FAM -	6-carboxyfluorescein
FAO -	Food and Agriculture Organisation of the United Nations
FPH -	fish protein hydrolysates
FN -	false negative
FP -	false positive
FRET -	fluorescence response energy transfer
h -	hours
GLM -	Generalised Linear Models
HIS -	hyperspectral imaging

HSRC	Human Sciences Research Council
Hx -	hypoxanthine
IMP -	inosine monophosphate
Ino -	inosine
kb -	kilobases
KZN -	Kwazulu-Natal
LSU -	large subunit
LW-NIR -	long wave near infrared
MAP -	modified atmosphere packed/packaging
min -	minutes
MIR -	mid-infrared
MORS -	Meullenet-Owens razor shear
MSC -	multiplicative scatter correction
MUFA -	mono-unsaturated fatty acids
NCBI -	National Center for Biotechnology Information
NIR -	near-infrared
NIT -	near-infrared transmittance
NMC -	number of misclassifications
NMRI -	nuclear magnetic resonance imaging
NRQ -	non-fluorescent quencher
NTC -	no template control
PC -	principal component
PCA -	principal components analysis
PCs -	principal components
PCR -	polymerase chain reaction
PLS-DA -	partial least squares discriminant analysis
PLS -	partial least squares
PUFA -	poly-unsaturated fatty acids
rDNA -	ribosomal DNA
RMSECV -	root mean square error for cross-validation
RNA -	ribonucleic acid
SA -	South African
QIM -	quality index method
qPCR -	real-time quantitative polymerase chain reaction
s -	seconds
SASSI -	South African Sustainable Seafood Initiative
SECV -	standard error of cross-validation
SFA -	saturated fatty acids
SIMCA -	soft independent modelling of class analogy
SNP -	single nucleotide polymorphism
SSF -	slice shear force
SSU -	small subunit
SW-NIR -	short wave near infrared
TN -	true negative
TP -	true positive
VIC -	relates to a fluorescent dye, proprietary to Life Technologies
VIS/NIR -	visible/near-infrared
TMA -	trimethylamine
TMAO -	trimethylamine oxide
WBSF -	Warner-Bratzler shear force
3D -	three-dimentioinal

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List of Addendum items

Addendum A Review article published as: Henning, S.S., Hoffman, L.C. & Manley, M. (2013). A review of *Kudoa*-induced myoliquefaction of marine fish species in South Africa and other countries. *South African Journal of Science*, **109** (11/12), Art. #2012-0003, 5 pages.

Addendum B Ethics letter from Department of Agriculture, Fisheries and Forestry (DAFF), for donating SA sardine, Cape hake and SA kingklip samples for the purpose of this study.

Addendum C National Center for Biotechnology Information (NCBI) database showing alignment for *Kudoa thyrsites* (AY542481.1, AY54482.1 and AY941819.1) and *K. paniformis* 18 SSU rDNA partial sequence (AF034640 GenBank accession number) with other *Kudoa* species: *K. inornata* (FJ790311.1); *K. megacapsula* (AB188529.1); *K. dianae* (AF414629.1); *K. paraquadricornis* (FJ792719.1); *K. trifolia*, (AM183300.2); *K. quadricornis* (FJ792721.1 and AY078428.1.1); and *K. thyrsites* The alignment allowed for identification of unique sequences that was used for *K. paniformis* and *K. thyrsites* specific primers, as well as unique SNP sites.

Addendum D Figure 1. Nucleotide BLAST search showing primer, SNP, and probe design for TaqMan® *Kudoa paniformis* and *K. thyrsites* specific assays.

Chapter 1
Introduction

1.1 Introduction

The presence of parasites in marine fish muscle tissue is seen as both a quality defect and a food safety risk (Huss, 1994; Huss, 1995). *Kudoa thyrsites* and *K. paniformis* are microscopically small Myxozoan parasites of marine fish species. These parasites are associated with extensive *post-mortem* proteolytic breakdown (myoliquefaction) and/or formation of unsightly white, brown and black pseudocysts (Morado & Sparks, 1986; Dawson-Coates *et al*., 2003; Samaranayaka *et al*., 2006; Funk *et al*., 2008). Although the infection of fish by *K. thyrsites* and *K. paniformis* does not pose any health risk (Alvarez-Pellitero & Sitja-Bobadilla, 1993), the associated myoliquefaction and/or pseudocysts result in consumer complaints and economic losses (Samaranayaka *et al*., 2006; Dawson-Coates *et al.*, 2003; St-Hilaire, Hill *et al*., 1997; D. Brickles, 2016, Group Quality Assurance Manager, Irvin and Johnson Ltd., Cape Town, South Africa, personal communication).

The infection by *K. thyrsites* and *K. paniformis* and associated fish quality defects have thus been researched extensively (Stehr & Whitaker, 1986; Moran *et al*., 1999; Dawson-Coates *et al*., 2003; Samaranayaka *et al*., 2006; Marshall *et al*., 2016). In some fish species infected with *K. thyrsites* and/or *K. paniformis*, such as Cape hake (*Merluccius capensis*) and Pacific hake (*M. productus*), black pseudocysts were observed (Kabata & Whitaker, 1981; Tsuyuki *et al*., 1982; Morado & Sparks, 1986; Webb, 1993) before *post-mortem* myoliquefaction was evident. In other fish species, such as salmonids (Dawson-Coates *et al*., 2003), South African (SA) sardine (*Sardinops sagax ocellatus*) (Webb, 1990) and Cape snoek (*Thyrsites atun*) (personal observations), there were no formation of black pseudocysts, however, myoliquefaction was still evident several hours *post-mortem*. In fish species not showing black pseudocysts the muscle fibres can become completely infected and plasmodia formation may be evident and/or ruptured without any macroscopic evidence of infection (Morado & Sparks, 1986). In addition, myoliquefaction is typically evident only 38-56 hours (Levsen *et al*., 2008) *post-mortem*, resulting in infected fish reaching the processor and/or consumer.

Several methods to detect the infection of *K. thyrsites* and/or *K. paniformis*, before myoliquefaction is evident, have been investigated (Webb, 1990; Morrissey *et al*., 1995, Cunningham, 2002; Yokoyama & Itoh, 2005; Young & Jones, 2005; Meng & Li-Chan, 2007; Piazzon *et al*., 2012). Methods of detection included visual counting of white and/or black coloured pseudocysts for sorting Pacific hake according to level of infection (Morrissey *et al*., 1995; St-Hilaire, Ribble *et al*., 1997), ultraviolet fluorescence (Webb, 1990), microscopic preparations of fish muscle tissue (St-Hilaire, Ribble *et al*., 1997; Dawson-Coates *et al*., 2003), immunological detection (Chase

et al., 2003), and deoxyribonucleic acid (DNA) based polymerase chain reaction (PCR) technologies (Cunningham, 2002; Meng & Li-Chan, 2007; Grabner *et al*., 2012; Piazzon *et al*., 2012). Identification of *K. thyrsites* and *K. paniformis* was achieved with the use of PCR primers that amplified the 18 SSU rDNA regions (Hervio *et al*., 1997; Meng & Li-Chan, 2007); while quantification of the Myxozoan parasite, *Enteromyxum scophthalmi*, has been demonstrated with the use of a real-time quantitative PCR (qPCR) assay (Piazzon *et al*., 2012). However, these methods are time-consuming, are expensive, and/or result in destruction of the fish sample, and are thus impractical for routine quality control analyses on fishing vessels and/or within fish processing facilities.

In the absence of myoliquefaction (due to the parasitic protease) and visible pseudocysts, infection by *K. thyrsites* and *K. paniformis* is not macroscopically visible (St-Hilaire, Hill *et al*., 1997). Canning infected fish, not showing myoliquefaction at the time, may result in disintegration and unacceptable product quality (Webb, 1990). During the 1990's, SA sardines to be used for canning in South Africa were left to 'soften' (develop myoliquefaction) for several hours before canning. This was done in order to remove heavily infected fish. The drawback of this procedure was that fish with a moderate *Kudoa* infection also deteriorated and became unsuitable for canning. If these moderately infected fish had not been left for myoliquefaction to become apparent, they could still have been processed into suitable quality products. If infected fish can be identified at an early stage *post-mortem*, moderately infected fish may then be processed and canned before myoliquefaction increases to an unacceptable level.

South African (SA) snoek (*T. atun*) (Gilchrist, 1924), Cape hake (*M. capensis*) (Webb, 1993), SA sardine (*S. sagax ocellatus*) (Webb, 1990) and Cape dory (*Zeus capensis*) (Davies & Beyers, 1947) are known to be infected by *K. thyrsites*, with sardines among the most heavily infected fish in SA waters (Webb, 1990). In 1924, Gilchrist reported that 5% of the snoek catch were so heavily infected by *K. thyrsites* as to be of no commercial value. In the case of Cape dory, Davies and Beyers (1947) reported that 25% of the landed catch were myoliquefied to such an extent that the fish could not be filleted. Limited information is available on the prevalence of *Kudoa thyrsites* and *K. paniformis* in SA marine fish species of economic importance, such as SA sardine (Reed *et al*., 2012), Cape hake (Webb, 1993) and SA kingklip (*Genypterus capensis*). South African kingklip, considered as bycatch (Badenhorst & Smale, 1991; WWF-SA, 2011), is a popular eating fish among consumers and is readily available in restaurants (Cawthorn *et al*., 2011). Kingklip is believed to be also infected with *K. thyrsites* due to development of myoliquefaction (C. van der Lingen, 2016, senior researcher, Branch: Fisheries Management, Department of Forestry and Fisheries, Cape Town, South Africa, personal communication).

At present, there are no known economically feasible, fast, and non-destructive method to distinguish between *K. thyrsites* and/or *K. paniformis* infected and uninfected fish in South Africa. The SA fish processing industry is losing money due to the occurrence of myoliquefaction in Cape hake (D. Brickles, 2016, Group Quality Assurance Manager, Irvin and Johnson Ltd., Cape Town, South Africa) and SA sardines (C. van der Lingen, 2016, senior researcher, Branch: Fisheries

Management, Department of Agriculture, Forestry and Fisheries, Cape Town, South Africa, personal communication). The development of an inexpensive, routine, rapid, and non-destructive quality control method for detection of *Kudoa* parasite infection before myoliquefaction is visible, is of great interest to SA fish research and fish processing industries. Heavy infected, together with myoliquefied fish, may potentially be used for alternative processing (fish meal and/or fish oil) and thus improve food security and reduce the loss of animal protein and economic losses.

Due to the time-consuming nature and impracticality of using manual sorting methods to separate parasite infected from uninfected fish, several studies have demonstrated the application of visible/near-infrared (VIS/NIR) spectroscopy (Isaksson *et al*., 2001; Wold *et al*., 2001) and hyperspectral imaging (HSI) (Stormo *et al*., 2004; Sivertsen *et al*., 2011; Sivertsen *et al*., 2012) as effective quality control methods for industrial, non-destructive detection of parasitic nematodes in fish and fish fillets. According to Petursson (in Wold *et al*., 2001) NIR hyperspectral imaging (800-1200 nm) gave better results for deeply embedded nematode parasites compared to absorption spectra in only the visible range of 400-600 nm. Near-infrared (NIR) spectroscopy is a rapid and non-destructive method that has been extensively researched (Weeranantanaphan *et al*., 2011; Cheng *et al*., 2013) for application in the monitoring of fish quality attributes, for example: texture analysis (Isaksson *et al*., 2001), microbial spoilage, (Lin *et al*., 2006; Sone *et al*., 2011), freshness (Isaksson *et al*., 2001; Bøknæs *et al*., 2002), proximate, fatty acid, and other chemical composition (Khodabux *et al*., 2007; Folkestad *et al*., 2008; Malcolm *et al*., 2012), and authenticity of fish species (O'Brien *et al*., 2013).

NIR spectra, acquired with the use of a MicroNIR™ 1700 (VIAVI Solutions, previously JDSU, USA) spectrophotometer, in combination with classification models [(principal component analysis (PCA) and soft independent modelling of class analogy(SIMCA)], was able to discriminate between closely related fish species of winter cod and cod, samlet and salmon trout, and red mullet and mullet (O'Brien *et al*., 2013). The MicroNIR™ 1700 spectrophotometer, is applicable for on-line and off-line measurements (Alcalà *et al*., 2013; Unger *et al*., 2016) due to its light-weight, hand-held, and portable features. However, no research has been documented to date on the use of NIR spectroscopy for detection of the presence of specifically *K. thyrsites* and/or *K. paniformis* or infected fish.

The aim of this study was two-fold, namely to investigate the prevalence of *Kudoa thyrsites* and *K. paniformis* in SA sardine (*S. sagax ocellatus*), Cape hake (*Merluccius capensis* and *M. paradoxus*), and SA kingklip (*Genypterus capensis*), and to discriminate *K. thyrsites* infected from uninfected fish samples with the use of near-infrared (NIR) spectroscopy.

The specific objectives of this study were to:

1 develop a real-time quantitative PCR (qPCR) protocol to be used as a qualitative, routine diagnostic analytical tool for detection of the presence of *K. thyrsites* and *K. paniformis* in fish muscle tissue;

2 investigate the prevalence of *K. thyrsites* and *K. paniformis*, and their relationships with area (west, east, and south coast), season (summer, autumn, winter and spring), sex (male and female), and size of fish in SA sardines, Cape hake, and SA kingklip;

3 investigate the effect of *K. thyrsites* infection on the moisture and ash contents of SA sardine, and the moisture, ash and protein contents of Cape hake; and

4 discriminate between *K. thyrsites* infected and uninfected SA sardine, Cape hake, and SA kingklip samples using NIR spectroscopy.

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DECLARATION

With regards to Chapter 2 (literature review 1, pp. 9-24), part thereof has been published as: Henning, S.S., Hoffman, L.C. & Manley, M. (2013). A review of *Kudoa*-induced myoliquefaction of marine fish species in South Africa and other countries. *South African Journal of Science*, **109** (11/12), Art. #2012-0003, 5 pages.

The nature and scope of the candidate's contribution were as follows:

Nature of contribution	Percentage of contribution
Compiled the manuscript and edited the document in its entirety during the different stages of the publication process.	96%

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Declaration by co-authors:

The undersigned hereby confirm that:

1. The declaration above accurately reflects the nature and percentage of contributions of the candidate and the co-authors to Chapter 2 (pp. 9-24).
2. No other authors contributed to Chapter 2 (pp. 9-24) besides these specified above, and
3. Potential conflicts of interest have been revealed to all interested parties and that the necessary arrangements have been made to use the material in Chapter 2 (pp. 9-24) of this dissertation.

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Chapter 2

Literature review 1

A review of *Kudoa*-induced myoliquefaction of marine fish species in South Africa and other countries*

Abstract

Myoliquefaction of fish musculature results in customer quality complaints and in huge economic losses, especially with regard to Pacific and Cape hake (*Merluccius productus* and *M. capensis*), farm-reared Atlantic salmon (*Salmo salar*), South African (SA) sardine (*Sardinops sagax ocellatus*) and Cape snoek (*Thyrsites atun*). Myoliquefaction, or 'jelly flesh', is caused by proteolytic enzymes released by the marine myxosporean parasites, *Kudoa thyrsites* and *K. paniformis*, after the death of the fish (*post-mortem*). Several methods of detection of infected fish have been investigated, but most of these methods are time-consuming, are expensive and/or result in destruction of the fish, and are impractical for fishing vessels and fish processors. This leaves opportunities for research into detection methods that are rapid and non-destructive, such as spectroscopic techniques. Methods to prevent the development of myoliquefaction focused on inhibiting the *post-mortem* activity of the parasitic proteolytic enzymes. Means such as manipulating *post-mortem* pH, temperature control and the application of enzyme inhibitors have been suggested. While some of these methods of proteolytic inhibition may be applicable to the aquaculture sector, they are impractical for wild-caught fish.

Keywords: Cape snoek, Cape hake, *Kudoa paniformis*, *K. thyrsites*, muscle texture, myxozoan, protease, pseudocysts, South African kingklip, South African sardine

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2.1 Introduction

Post-mortem myoliquefaction of fish muscle, commonly known as 'milky flesh', 'soft flesh' or 'jelly flesh', is a phenomenon associated with parasitic infection by *Kudoa thyrsites* and/or *K. paniformis*. *Kudoa* infection has been researched extensively (Stehr & Whitaker, 1986; Dawson-Coates *et al*., 2003; Marshall *et al*., 2016) as a result of the economic implications for marine and aquaculture industries. Pacific hake (*Merluccius productus*) (Samaranayaka *et al*., 2006) and farm-reared Atlantic salmon (*Salmo salar*) have been studied extensively (St-Hilaire, Hill *et al*., 1997; St-Hilaire *et al*., 1998; Moran *et al*., 1999a, 1999b; Moran, Kent *et al*., 1999; Moran, Margolis *et al*., 1999; Dawson-Coates *et al*., 2003) as these species are of economic importance.

Parasites from the family *Kudoidae* (order Multivalvulida, subclass Myxosporea, and class Myxozoa) result in *post-mortem* myoliquefaction and, in certain fish species, in visible pseudocysts (muscle fibres containing *Kudoa* spores) in the musculature (Moran *et al*., 1999a). The economic implications are of great concern for the salmon aquaculture industry, as infected fish show no external signs of infection. Myoliquefaction becomes apparent only several hours (38 to 56) *post-mortem* (Levsen *et al*., 2008). Currently there is no rapid, non-destructive method to identify and quantify *Kudoa* infection. A visual counting method, by which visible white pseudocysts are counted, has been suggested for Pacific hake. Although this method is time consuming (Morrissey *et al*., 1995), as it requires careful examination, it could be implemented as a means to remove heavily infected fillets with black streaks caused by black pseudocysts. White pseudocysts are only one of the few stages in the *Kudoa* life cycle. While some kudoid species are responsible for *post-mortem* myoliquefaction and pseudocyst formation, others seem to have relatively little effect in host species (Whipps & Kent, 2006).

Many kudoid species show low host specificity; for example, *K. thyrsites* has been recorded from 18 different fish families representing nine fish orders (Lom & Dyková, 2006). Taxonomies of the class Myxosporea (Langdon *et al*., 1992; Moran *et al*., 1999b) and the genus *Kudoa* (Moran *et al*., 1999b) have been elaborated in previous review articles, and are not the focus of this review.

Information about host species-specific kudoids, level of infection and effects on host musculature, in commercially important fish species, is of great economic importance. However, limited information about *Kudoa* infections and the effects thereof on marine fish quality and the economic impact thereof in South Africa is available. This review discusses some of the published global research on *K. thyrsites* and *K. paniformis* in several marine fish species of economic importance, with reference, where applicable, to South African (SA) marine fish species.

2.2 Myoliquefaction in marine fish in South Africa and other countries

Myoliquefaction is a common phenomenon in SA Cape snoek (*Thyrsites atun*). The musculature of infected snoek becomes completely soft and jelly-like (Figure 2.1) and is commonly known as 'pap snoek'. Soft flesh in *T. atun* was described for the first time in Australia and South Africa as early as 1910 and 1924, respectively (Gilchrist, 1924). Myoliquefaction in Cape snoek is caused by the

myxosporean parasite *K. thyrsites*. This parasite was shown to infect up to more than 27 fish species worldwide (Moran *et al*., 1999b), including mahi-mahi (*Coryphaena hippurus*) (Langdon, 1991), Atlantic salmon (*Salmo salar*), Pacific hake (St-Hilaire, Hill *et al*., 1997; Moran *et al*., 1999b; Lom & Dyková, 2006), Cape hake (*Merluccius capensis*) (Webb, 1993) and SA sardine (*Sardinops sagax ocellatus*) (Webb, 1990). Black scraper (*Thamnaconus modestus*, Günther, 1877) from the Sea of Japan (Kasai *et al*., 2016) has recent been documented as a new host species for *K. thyrsites*.

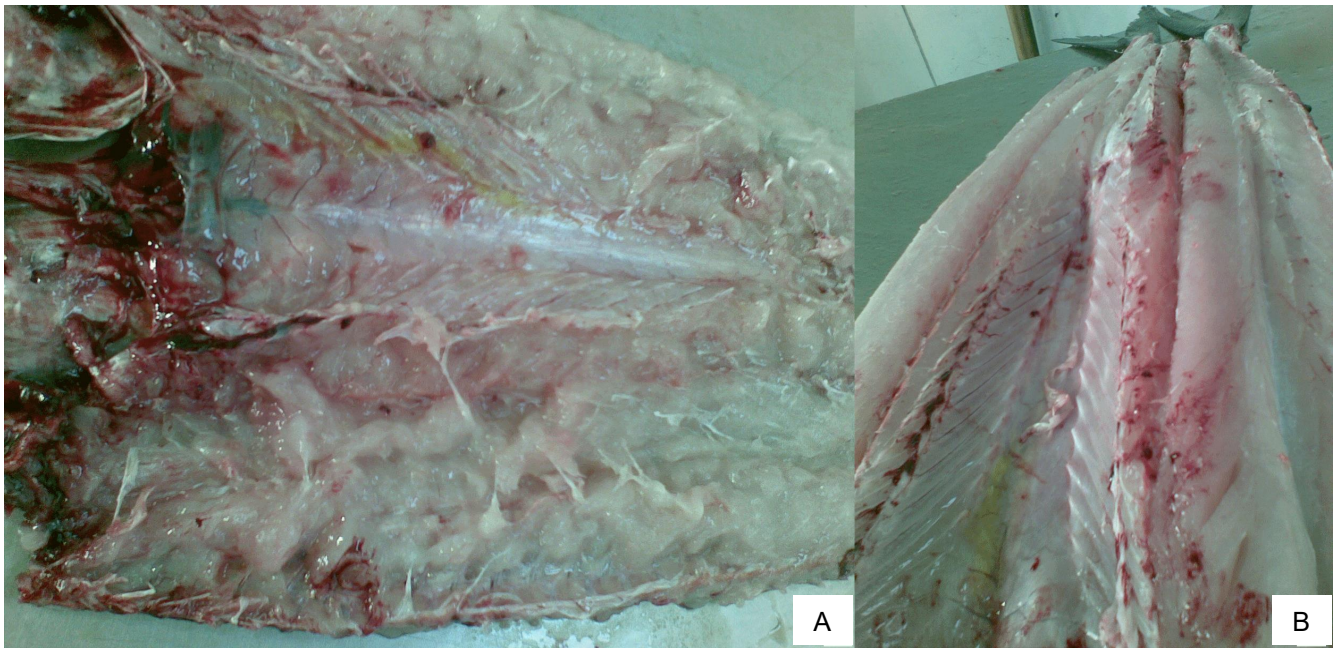

Figure 2.1 Refrigerated (5ºC) flecked Cape snoek (*Thyrsites atun*) musculature at 48 h *post-mortem* (A) with extensive myoliquefaction and (B) without myoliquefaction. Photos were taken with a Canon PowerShot S2 IS camera.

More than 68 species of Kudoid myxozoans (Multivalvulida) have been described worldwide (Anon., 2017; Whipps & Kent, 2006). Three new species, *K. trachuri*, *K. thunni* (Matsukane *et al*., 2011) and *K. ogawai* (Yokoyama *et al*., 2012), have been described in the somatic muscle of fish that are consumed on a daily basis in Japan. Although many *Kudoa* species are associated with *post-mortem* myoliquefaction (Kabata & Whitaker, 1981; Hervio *et al*., 1997; Moran *et al*., 1999a; Yokoyama & Itoh, 2005) not all *Kudoa* species found in fish muscle result in this quality defect.

Martinez de Velasco *et al*. (2007) have reported that some *Kudoa* species may induce an allergic reaction in some people. Concern about food poisoning has also become an important topic. Kawai *et al*. (2012) and Iwashita *et al*. (2013) found that *K. septempunctata* resulted in food poisoning but did not result in myoliquefaction. *Kudoa thyrsites* is not associated with food poisoning (Alvarez-Pellitero & Sitja-Bobadilla, 1993) but results in extensive myoliquefaction and huge economic losses (Webb, 1990; St-Hilaire, Hill *et al*., 1997; St-Hilaire *et al*., 1998; Dawson-Coates *et al*., 2003; Samaranayaka *et al*., 2006).

South African sardine (*Sardinops sagax ocellatus*), also known as pilchards (Grant *et al*., 1998), Cape hake (*Merluccius capensis*) and Cape dory (*Zeus capensis*) are also known to be infected by

K. thyrsites (Table 2.1), with sardines being among the most heavily infected fish species in SA waters (Webb, 1990). The presence of *K. thyrsites*, associated with myoliquefaction in dried fillets exported to Japan, was reported for the first time in 2005 in splendid alfonso (*Beryx splendens*) from South Africa (Yokoyama & Itoh, 2005). In the Pacific Northwest in western North America, the occurrence of *K. thyrsites* is of great economic importance as it has a significant impact on farmed Atlantic salmon and, to a lesser extent, on coho salmon (*Oncorhynchus kisutch*) (Whitaker & Kent, 1991).

Table 2.1 A summary of some reports on marine fish species, from different geological areas, infected by *Kudoa thyrsites*

Geographic region	Fish species	Common/regional name
South Africa[1,2,3,4]	*Sardinops sagax ocellatus*	South African sardine/pilchard
	Thyrsites atun	Cape snoek
	Lepidopus caudatus	Silver scabbardfish (beltfish)
	Merluccius capensis	Cape hake
	Zeus capensis	Cape dory
	Beryx splendens	Splendid alfonso
Southern Australia[3]	*Thyrsites atun*	Barracouta (snoek)
Western Australia[1]	*Coryphaena hippurus*	Common dolphinfish
	S. sagax neopilchardus	Pilchard
	Spratelloides robustus	Blue sprat (round herring)
Canadian Pacific[1,5,6]	*Merluccius productus*	Pacific hake
	Oncorhynchus kisutch	Coho salmon
Washington Pacific[1]	*Salmo salar*	Atlantic salmon
Japanese sea[1]	*Cypsilurus agoo*	
	Engraulis japonicus	Japanese anchovy
Mauritania (Atlantic)[1]	*Zeus faber*	John dory
North Sea[7]	*Scomber scombrus L.*	Atlantic mackerel

[1]Lom & Dyková (2006)
[2]Gilchrist (1924)
[3]Langdon (1991)
[4]Davies & Beyers (1947)
[5]Whitaker & Kent (1991)
[6]Tsuyuki *et al*. (1982)
[7]Moran *et al*. (1999a)

Pacific hake was found to be predominantly infected with *K. paniformis* (Samaranayaka *et al*., 2006; Funk *et al*., 2008), while only a small number of *K. thyrsites* spores were detected (Samaranayaka *et al*., 2006). In a study by Morado and Sparks (1986), during which a total of 178 Pacific hake (*Merluccius productus*) samples were investigated for the presence of both *K. thyrsites* and *K. paniformis*, it was also found that the predominant infection was due to *K. paniformis* (prevalence of 51%). The prevalence of *K. thyrsites* was only 7%, while 17% of samples were infected with both species. Pacific hake infected with both *K. paniformis* and *K. thyrsites* is believed to undergo accelerated myoliquefaction because *K. paniformis* produces both acidic and neutral proteolytic

enzymes, while *K. thyrsites* only produces acidic enzymes (Tsuyuki *et al*., 1982; Stehr & Whitaker, 1986). However, in contrast to the results from studies by Tsuyuki *et al*. (1982) and Stehr and Whitaker (1986), Funk *et al*. (2008) demonstrated that the protease from *K. thyrsites* showed stability and activity over a broad pH range (pH 4.5-8.8). Zhou and Li-Chan (2009) demonstrated that Pacific hake samples that were infected with only *K. thyrsites*, showed higher protease activities, compared to samples infected with *K. paniformis* at similar levels of infection. These authors also noted that fish infected at relatively low levels of *K. thyrsites* still showed significant reduced firmness in cooked fish mince.

2.3 Mode of action of *Kudoa thyrsites* on fish tissue

Kudoa thyrsites releases a cysteine protease in the form of a pro-enzyme from the pre-sporogenic plasmodia (Stehr & Whitaker, 1986; An *et al*., 1994; Funk *et al*., 2008) that support its development by breaking down fish tissue without causing harm to the live fish. Ultra-structural evidence presented by Stehr and Whitaker (1986) suggests that while the fish is alive, neighbouring muscle fibres and myofibrils of the infected muscle fibre that are not in direct contact with *K. thyrsites* plasmodia, are unaffected by the presence of the parasite. However, when these pro-enzymes continue to be released by the parasite after the fish is killed, myoliquefaction of the musculature occurs due to pH-induced activity of the protease (Funk *et al*., 2008). The pro-enzyme form of the protease showed high activity at pH 6.0 but low activity at neutral pH (6.5-7.0). In contrast, the processed protease showed activity over a broad pH (4.5-8.8) range, with a maximum activity at pH 5.5, resulting in *post-mortem* myoliquefaction.

The cytoplasmic cysteine proteases from *K. thyrsites* and *K. paniformis* have been identified and characterised in Pacific hake (Funk *et al*., 2008). Cathepsin L proteases are responsible for myoliquefaction in infected fish and it is clear that this cysteine protease is derived from the parasite and not from a host response to the parasite (Funk *et al*., 2008). Several studies (An *et al*., 1994; Samaranayaka *et al*., 2006; Funk *et al*., 2008) have indicated that the optimum conditions for endogenous enzymes from *K. paniformis* in Pacific hake were at a pH range of 5.25–5.50 and a temperature range of 52–55°C. Cathepsin L protease from *K. thyrsites* showed maximum activity at pH 5.5, the iso-electric point of muscle proteins, and a marked loss of activity at pH 6.5 (Funk *et al*., 2008). Cathepsin L from *K. thyrsites* differs from other cathepsin L proteases in that it contains only four of the six cysteine residues believed to be involved in disulphide bonds. Cathepsin L associated with *K. thyrsites* is translated as a pro-enzyme that is then chemically altered to an active enzyme by removal of the pro-region during limited proteolysis (Funk *et al*., 2008; An *et al*., 1994). This process is regulated by pH, with a decrease in pH resulting in the destabilisation of the pro-region, thus exposing the cleavage site. As the pH in fish muscle drops from 7.0 to 6.5 during early *post-mortem* storage (Chéret *et al*., 2007; Françoise, 2010) myoliquefaction is not evident early *post-mortem*. However, as the muscle pH drops below 6.0, the cysteine protease becomes activated, resulting in muscle breakdown. Reducing the amount of glycogen in Atlantic salmon muscle cells

prior to harvesting might possibly reduce the amount of lactic acid generated in the muscle *post-mortem* and consequently reduce the activity of cathepsin L proteases released from the parasite (Samaranayaka *et al*., 2006).

2.4 Characteristics of *Kudoa thyrsites* infection

Kudoa thyrsites is a multivalvulid myxosporean parasite of marine fish and, although very little is known about the life cycle of this parasite, it is suggested to be complex with more than one host (Tsuyuki *et al*., 1982; Moran *et al*., 1999b; Young, 2002). Based on the life cycle of freshwater myxosporean species, a life cycle for *K. thyrsites* has been hypothesised (Shaw *et al*., 1997; Young, 2002). Parasitic plasmodia (cysts containing many spores) grow within the myocytes. Sporulation of plasmodia within the somatic musculature of the host fish results in many myxospores inducing a chronic inflammatory response but without any apparent harm to the health of the fish. The parasite penetrates the core of muscle fibre and spreads along myocytes without damaging the sarcolemma, resulting in individual muscle fibres being filled with spores without involving the connective tissue (Tsuyuki *et al*., 1982). This aggregate of spores within the somatic muscle fibre is referred to as the pseudocyst (Harrel & Scott, 1985).

The myxospores are released from the pseudocyst upon death of the host fish and are then taken up by an intermediate host (Tsuyuki *et al*., 1982; Harrel & Scott, 1985). Within the intermediate host, a second sporulation results in the development of actinospores. These actinospores are released to infect a new host fish and continue the parasite's life cycle. Whilst the parasite remains within the muscle fibre (where it contains both developing and mature spores), infected fibres appear white (Stehr & Whitaker, 1986). If the sarcolemma is destroyed by the parasite, there is a rapid development of a fibroblast layer around the parasite and the pseudocyst becomes black in appearance. In certain fish host species, such as salmonids, there is no formation of black pseudocysts (Harrel & Scott, 1985) and the muscle fibre can be completely infected and rupture without any macroscopic evidence of infection. Although the stage of *K. thyrsites* that is infective to fish has not yet been identified, the presence of plasmodia in myocytes of Atlantic salmon has been observed after only nine weeks of exposure to contaminated seawater (Moran, Kent *et al*., 1999).

Kudoa thyrsites is different from other members of the myxozoan species in that it infects a wide variety of fish species around the world (Table 2.1). The route of infection is unknown; however, transmission of the parasite does not occur directly among fish (Tsuyuki *et al*., 1982; Moran *et al*., 1999b; Young, 2002). Infection by *K. thyrsites* occurs only in seawater and not in fresh water (Moran, Kent *et al*., 1999). South African Cape snoek and SA sardine appear to be major host reservoir species for *K. thyrsites* (Webb, 1990; Langdon *et al*., 1992) in SA waters. It cannot, however, be assumed that transmission of *K. thyrsites* occurs through ingestion of infected fish by other predatory fish, firstly, because the complete life cycle and route of transmission and infection are unknown. Secondly, the Australian pilchard (*S. sagax neopilchardus*), a planktivorous fish species, is also heavily infected with *K. thyrsites* and is suggested to be a major reservoir host in Southwest

Australian waters. It is thus suggested that infection of planktivorous fish occurs through ingestion of infected invertebrate hosts (Langdon *et al*., 1992).

2.5 Factors affecting the level of *Kudoa thyrsites* infection

The degree of visible myoliquefaction from *K. thyrsites* infection may be influenced by several factors, such as time of evaluation *post-mortem*, the number of *K. thyrsites* spores present in the flesh, temperature during storage of the fish, and the inherent quality of the muscle (Dawson-Coates *et al*., 2003). The latter is affected by factors such as growth and feeding condition of the fish, water temperature, and processing conditions.

From a study that investigated the correlation between *post-mortem* myoliquefaction and intensity of *K. thyrsites* infection in Atlantic salmon (St-Hilaire, Hill *et al*., 1997), it was found that high intensity of infection resulted in severe autolysis of the somatic musculature. An average plasmodium count of 0.3 mm^{-2} or a mean spore count of 4 x 10^5 spores·g^{-1} tissue was suggested to be indicative of severe myoliquefaction (Dawson-Coates *et al*., 2003).

The age and size (St-Hilaire *et al*., 1998; Levsen *et al*., 2008) of fish as factors affecting the degree of *post-mortem* myoliquefaction were demonstrated in 2008 by Levsen *et al*. (2008) with large (> 600 g) Atlantic mackerel having a significantly greater prevalence of myoliquefaction than medium-sized (400 to 600 g) mackerel. Pen-reared sexually mature Atlantic salmon (*S. salar*) was also more likely to be infected with *K. thyrsites* than their sexually immature counterparts (Levsen *et al*., 2008). Similar to the results from a study by Levsen *et al*. (2008), it was found that the prevalence of infection was greater in adult tube-snouts (*Aulorhynchus flavidus*) (up to 100%) than in young tube-snouts (about 50%) (Shaw *et* al., 1997). A local snoek processor (R. Theron, 2012, fish processor, Die Visfabriek, Brackenfell, Cape Town, South Africa, personal communication) indicated that large Cape snoek tended to develop myoliquefaction to a greater extent than smaller fish. Levsen *et al*. (2008) hypothesised that mackerel became infected at a late stage of their lifespan or that the parasite developed relatively slowly. Previous studies (Tsuyuki *et al*., 1982; Moran, Kent *et al*., 1999) have indicated that *K. thyrsites* has a slow plasmodial development in fish and develops into microscopically visible plasmodia in adult fish although a histologically undetectable parasite stage may be present in younger fish. The presence of *K. thyrsites* was identified in young tube-snouts using polymerase chain reaction (PCR) when visual or histological methods were negative (Shaw *et al*., 1997). Early stages of the parasite may be detected by (PCR) (Hervio *et al*., 1997; Moran, Margolis *et al*., 1999; Young & Jones, 2005) and immunohistochemistry (Young & Jones, 2005) techniques.

The prevalence of *Kudoa* infection is also affected by season, with the level of infection tending to be higher during summer than during winter months (Munday *et al*., 1998; St-Hilaire *et al*., 1998; Moran *et al*., 1999a, 1999b), indicating that warmer water temperatures might increase transmission and/or the level of infection. The effect of season on *K. thyrsites* and *K. paniformis* infection levels in

SA marine fish species, such as Cape snoek, Cape hake, Cape dory and SA sardine, is not well documented.

2.6 Detection and identification of *Kudoa thyrsites* infection

Kudoa thyrsites myxospores (Figure 2.2) are the most characteristic stage of infection and are suggested to be the infective stage of invertebrate hosts or vertebrate predatory fish species (Langdon 1991; Langdon *et al*., 1992; Young, 2002; Levsen *et al*., 2008). The myxospores of *K. thyrsites* vary between 12.0 µm (Kabata & Whitaker, 1981) and 16.7 µm (Lom & Dyková, 2006; Whipps & Kent, 2006) in diameter. In the absence of visible pseudocysts, infection with *K. thyrsites* is therefore not macroscopically visible (Morrissey *et al*., 1995; St-Hilaire, Ribble *et al*., 1997).

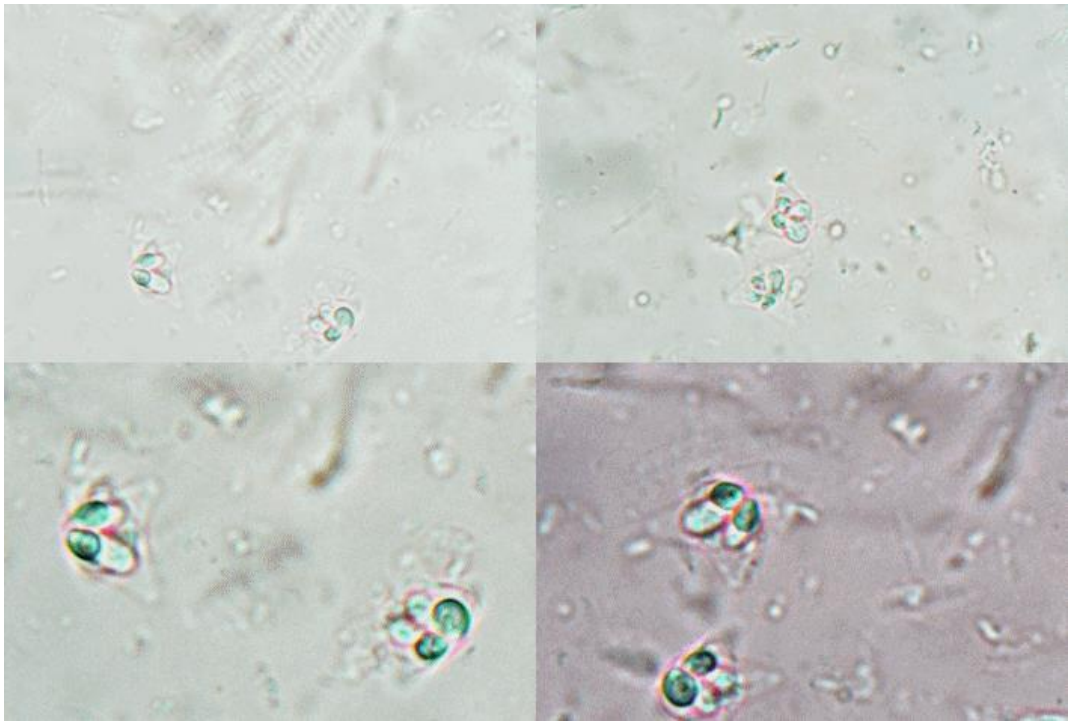

Figure 2.2 Light micrographs (1000X magnification, Olympus CX31, Nikon DS-Fi1 camera, Nikon NisElements Imaging software version 3.22) of *Kudoa thyrsites* spores from infected Cape dory (*Zeus capensis*) muscle.

The appearance of myoliquefaction may be an indication of *Kudoa* infection. However, myoliquefaction may only be apparent several hours (24 to 56) *post-mortem* (personal observations; Levsen *et al*., 2008) resulting in infected fish to be processed. Examination of both muscle and blood samples of Atlantic mackerel (*Scomber scombrus* L.) immediately after catch was unsuccessful in identifying infection by *K. thyrsites* (Levsen *et al*., 2008). A positive correlation between the number of white pseudocysts, protease activity and texture of Pacific hake has been found (Morrissey *et al*., 1995; St-Hilaire, Ribble *et al*., 1997). The counting of visual white pseudocysts as a potential method for sorting Pacific hake according to level of infection has been suggested. The drawback of this method is that it requires careful and time-consuming examination since white pseudocysts are not readily detectable. This method is therefore not feasible for a fish production line.

Detection of the parasite by means of ultraviolet fluorescence has been investigated (Webb, 1990), but this method was unreliable because the parasitic cysts did not glow in dark-coloured muscles or in *post-mortem* flesh. Modern methods, such as amplification of deoxyribonucleic acid (DNA) with the use of PCR technologies, have made important contributions to the screening, diagnosis and taxonomy of parasitic infections in fish species (Cunningham, 2002; Meng & Li-Chan, 2007; Grabner *et al*., 2012). Primers have been designed for amplification of the 18 SSU rDNA region for detection of specific *K. thyrsites*. The primer pair, Kt18S6f and Kt18S1r, amplified a 909-bp region of the *K. thyrsites* 18 SSU rDNA and did not produce a PCR product from fish host DNA (Young & Jones, 2005; Meng & Li-Chan, 2007).

Historically, the identification of infection by *K. thyrsites* was usually done by microscopy, using either wet mounts or histological sections of muscle tissue (Moran *et al*., 1999b). This method resulted in disfiguration of the fish and thus negatively influenced its market value. However, sampling from the *hyohyoideus ventralis* muscle (striated muscle) from under the operculum of Atlantic salmon provided a non-destructive method for assessing fish for the presence or absence of *K. thyrsites* spores (St-Hilaire, Ribble *et al*., 1997). This method required only a small amount of fillet. Counting the number of plasmodia in the *opercular mandibular* muscle of Atlantic salmon did not reflect the number of plasmodia in the fillets and was not a good indicator of fillet quality (namely it did not correlate with the number of pits in the fillet) (Dawson-Coates *et al*., 2003). Because the number of plasmodia and spores varies significantly among individual fish and among tissue samples within a fish, it was recommended that multiple samples be taken from a single fish (Dawson-Coates *et al*., 2003).

Marshall *et al*. (2016) have demonstrated an inverse log relationship (R^2 = 0.998) between false negatives from PCR tests and the number of anatomical areas per fish used for the composite DNA sample. In order to avoid false negatives and to improve detection when using the PCR test method, it is therefore important to use a large sample of tissue from several anatomical areas from a single fish (St-Hilaire, Ribble *et al*., 1997; Marshall *et al*., 2016).

An alternative quantitative method is an antigen-capture enzyme-linked immunosorbent assay (ELISA) (Taylor & Jones, 2005; Jones *et al*., 2012) that utilises uncharacterised soluble parasite antigens to estimate the severity of the infection. This assay is similar in sensitivity to PCR and may be used, with an appropriate sampling plan, as an early diagnostic tool. In comparison with microscopic methods, through which only myxospores (Figure 2.2) are detected, the PCR method is useful for detecting less severe and early stages of infections when no myxospores are present (Grabner *et al*., 2012). Although PCR testing for *K. thyrsites* is specific and more sensitive than wet mount and histological examinations, it provides limited information on parasite locality in specific tissues. Although histological staining provides for localisation of recognisable mature infections, it does not allow characterisation of obscure developmental stages of the parasite. By combining methods of *in-situ* hybridisation and immunohistochemistry, previously undescribed developmental stages of *K. thyrsites* were characterised (Young & Jones, 2005). *In-situ* hybridisation can be used

as a tool to localise *K. thyrsites* in specific tissues and to visualise cryptic stages, whereas immunohistochemistry can be used to detect early developmental stages, but not pre-sporogenic stages or mature spores of *K. thyrsites*.

2.7 Technologies to prevent myoliquefaction

The level of proteolytic activity seems to be linked to the stage of parasitic infection (Tsuyuki *et al*., 1982) as well as to the level of infection (Samaranayaka *et al*., 2006; Zhou & Li-Chan, 2009). Endogenous protease activities in Pacific hake (*M. productus*) have been related to the level of *K. thyrsites* and *K. paniformis* infection (Zhou & Li-Chan, 2009) where the protease activity increased as the number of *Kudoa* spores increased at levels $\geq 10^5$ spores·g^{-1} fish mince. Zhou and Li-Chan (2009) documented that the protease activity was higher in fish infected with *K. thyrsites* than in fish infected with *K. paniformis* at similar levels of infection. However, the level of proteolytic activity was related to the infective stage. Fish infected with mainly young white pseudocysts of *K. thyrsites* had up to seven times higher proteolytic activity at acidic pH values compared to neutral pH. Fish muscle infected with mainly black pseudocysts had a smaller difference in proteolytic activity between acidic and neutral pH values (Tsuyuki *et al*., 1982).

Several studies (Tsuyuki *et al*., 1982; Funk *et al*., 2008; Zhou & Li-Chan, 2009) have investigated the optimum pH and temperature ranges of activity for the protease associated with *K. thyrsites* and *K. paniformis* infections. Funk *et al*. (2008) studied pH-dependent regulation of *K. thyrsites* cysteine protease and documented that the cathepsin L pro-enzyme had maximum activity at pH 6.0, reduced activity at pH 6.5 and no activity at pH 7.0. In contrast to the pro-enzyme, the processed enzyme showed stability and activity over a broad pH range of 4.5 to 8.8.

The maximum activity of the protease from *K. thyrsites* has been shown to be at a pH of 5.5 (Funk *et al*., 2008). Tsuyuki *et al*. (1982) studied the differences in proteolytic activity in Pacific hake infected with *K. thyrsites* and *K. paniformis*. These researchers documented that for hake infected with both *Kudoa* species, or with *K. paniformis* alone, protease activity was evident in the acid pH range with an optimum at pH 3.8 and very little activity at neutral pH at 35ºC. When the temperature was increased to 60ºC, protease activity of infected samples was evident over a broad pH range including both acidic and neutral pH. Pacific hake infected with *K. thyrsites* alone showed little protease activity in the neutral pH range while samples infected with *K. paniformis*, showed strong protease activity at neutral pH at 60ºC. These authors finally documented that infection by *K. thyrsites* resulted in a significant increase in protease activity in the acidic pH range, while *K. paniformis* resulted in an increased protease activity in both the acidic and neutral pH ranges. This suggests that the possibility of pH regulation of the development of myoliquefaction may be mainly related to the pro-enzyme by reducing the fall in *post-mortem* pH. It was suggested that this may be done by reducing the amount of glycogen in fish muscle prior to harvesting (Funk *et al*., 2008). However, this strategy may be a possibility only for farmed fish and not applicable to wild-caught fish.

The effects of chilled and frozen storage on the development of soft texture in cooked Pacific hake infected with *K. thyrsites* and *K. paniformis* have been studied by several researchers (Tsuyuki *et al*., 1982; Zhou & Li-Chan, 2009). These studies showed that short-term freezing (up to three months) and/or chilled storage had little effect on preventing or reducing soft texture development in cooked Pacific hake flesh. Tsuyuki *et al*. (1982) also documented that even with increase chilled storage times (up to 120 hours), the acidic and neutral proteases in Pacific hake infected with *K. paniformis* decreased only slightly, while hake infected with *K. thyrsites* showed little to no reduced protease activity. The effects of prolonged frozen storage (6 to 10 months) on cooked texture of *Kudoa* infected Pacific hake was inconclusive due to the effects of protein denaturation and aggregation, resulting in desiccation and textural hardening of the flesh. Geist and Crawford (1974) demonstrated that frozen storage (-26ºC) for 25 days did not have any effect on cathepsin activity in muscle homogenates of English sole. Freezing did not inhibit cathepsin activity, although, it might reduce the activity. It is well documented that frozen storage of fish does not inhibit enzymatic activities (Mukundan *et al*., 1986; Huss, 1995). In addition, the crystallisation rate of ice (Moore & Molinero, 2011) and freeze-thawing cycles (Huss, 1995; Zhou & Li-Chan, 2009) have significant effects on enzyme activities and textural quality of fish.

Proteolytic enzyme inhibitors may contain specific proteins that compete for the active site of the protease, binding as substrates, therefore inactivating the protease (Otto & Schirmeister, 1997; Song & Markley, 2003). In an attempt to reduce the effects of *post-mortem* soft flesh, Porter *et al*. (1993) investigated the use of potato extract, egg white, and bovine plasma protein as inhibitors of protease enzymes in protein extracts and surimi from Pacific hake (*M. productus*) and arrowtooth flounder (*Atheresthes stomias*). Although these authors did not distinguish between *Kudoa* infected and uninfected fish samples, the extracts from Pacific whiting showed maximum proteolytic activity at a pH range of 5.0 to 5.2 and a temperature of 50ºC, while extracts from arrowtooth flounder showed maximum activity at pH 5.5 and 55ºC. These optima pH ranges are very similar to the maximum activity for the cysteine protease from *K. thyrsites* of pH 5.5, with significant loss of activity at pH 6.5 (Funk *et al*., 2008). Porter *et al*. (1993) concluded that potato extract, egg white, and bovine plasma protein were all effective at inhibiting protease activity responsible for the degradation of Pacific hake texture. Potato extract was, however, more effective than egg white and bovine plasma protein. In the case of the arrowtooth flounder, bovine plasma was the more effective protease inhibitor, although all three inhibitors investigated showed > 85% inhibition. However, for these inhibitors to be effective, the fish needs to be in the form of a minced product. Due to this, the use of protease inhibitors is not practical for the fresh and frozen SA hake market. In addition, due to the relatively high cost of egg white and questionable sustainability of bovine serum, potato extract has the greater potential for use as an inhibitor of protease in minced fish products, such as surimi. However, the use of potato extract as inhibitor of texture deterioration may be applicable to the minced sardine canning products in SA.

A study by Jones *et al*. (2012) investigated the efficacy of dietary nicarbazin, an equimolar complex of 4,4'-dinitrocarbanilide and 2-hydroxy 4,6-dimethylpyrimidine used in poultry feeds for the prevention of coccidiosis (Chapman, 1993), against *K. thyrsites* in seawater-reared Atlantic salmon post-smolts. Dietary nicarbazin was found to significantly reduce the prevalence and severity of *K. thyrsites* in fish. Effects on mortality of fish and of high residues of nicarbazin in skin, liver and muscle warrant further studies. Development of treatment regimes, using medicated diets, is possible for farm-reared (aquaculture) fish, but such strategies are not possible for wild-caught fish. In contrast to direct administration of dietary vaccinations, Jones *et al*. (2016) suggested a management strategy for aquaculture grown Atlantic salmon (*Salmo salar*) where preliminary exposure of fish to *K. thyrsites* resulted in resistance and a significant reduction in the prevalence and severity of infection upon re-exposure. If initial exposure to *K. thyrsites* could be timed in such a way as to reduce infection levels before harvest of the fish, quality defects related to myoliquefaction could be reduced.

2.8 Conclusions

While infection by *K. paniformis* is mainly problematic in fish from the northeast Pacific Ocean, infection of marine fish species by *K. thyrsites* is of global concern due to myoliquefaction caused by enzymatic degradation of the host musculature. In terms of food security, the most important concern with regard to *K. thyrsites* and *K. paniformis* infections is that it results in waste of animal protein and economic losses. While certain strategies to reduce infection and/or prevent *post-mortem* myoliquefaction may be possible for farmed fish, they are impractical for wild-caught fish. At present, the South African fisheries sector does not have any marine fish aquaculture activities, and relies heavily on wild-caught marine fish species. In the case of wild-caught fish, rapid methods of identifying infected fish as soon as possible after harvest or capture at sea will be of benefit to the fishery industry. Up to date, there are no known rapid, inexpensive, and non-destructive methods to distinguish between infected and uninfected fish. If infected fish could be identified at an early stage *post-mortem*, it may be separated from uninfected fish and used for alternative processing activities. In combination with enzyme inhibitors, infected fish may be used for minced products.

2.9 References

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Chapter 3
Literature review 2
Review of fish texture, *Kudoa*-induced myoliquefaction and various analytical detection methods

Abstract

The texture of fish is one of the first sensory attributes to change during *post-mortem* storage. Textural changes are associated with endogenous proteolysis. Exogenous proteolytic enzymes from *Kudoa thyrsites* and/or *K. paniformis* result in extensive muscle deterioration, namely myoliquefaction. Detection and identification of infection by *K. thyrsites* and *K. paniformis* can be done by polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) methods. qPCR is often used as a routine analytical method for fast screening for detection and quantification of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). However, qPCR is not used by the fish industry for detection of *Kudoa* infection, but is more readily used in research applications to qualify (i.e. as diagnostic tool) and quantify the presence of *Kudoa* species. The texture of fish and fishery products is an important quality characteristic and is used as assessment method for freshness. Evaluating the texture of fish by pressing parts of the fish body with the finger are of high importance for fish inspection and forms part of the quality index method (QIM). In addition to sensory texture measurements, many mechanical methods, including Kramer, Warner-Bratzler, puncture, compression, textured profile analysis (TPA), and stress relaxation tests, have been developed for measuring the texture of fish. Some studies have investigated the correlation of near-infrared (NIR) spectroscopy and mechanical tenderness measurement in fish. The application of NIR spectroscopy for quantitative and qualitative analysis of quality attributes of fish and fishery products have been extensively researched. Presently there are no rapid, non-destructive methods available to distinguish between *Kudoa*-infected and uninfected fish. It will be of great value if a rapid, non-destructive method could be developed to detect *K. thyrsites* and/or *K. paniformis* infection before myoliquefaction occurs. This may result in infected fish to be separated from uninfected fish, from which infected fish may then be used for alternative processing such as fish protein hydrolysates (FPH) or fish oil.

Keywords: Autolysis, fish freshness, *Kudoa thyrsites*, near-infrared (NIR) spectroscopy, polymerase chain reaction (PCR), real-time quantitative PCR, texture analysis

3.1 Introduction

The texture of fish is one of the main characteristics used to evaluate the freshness and quality of fish (Huss, 1995). Changes in fish texture is one of the first autolytic, *post-mortem* sensory changes which develops during fish storage. *Post-mortem* textural changes occur due to firstly, enzymatic autolysis. Autolysis occurs typically during the first 24 hours (h) to 6 days, depending on intrinsic and extrinsic factors, such as species of fish, state of feeding of the fish when captured, temperature during storage, method of capture and killing of the fish, to mention only a few. Secondly, textural changes occur due to microbial activity (typically from day 5 *post-mortem*). The texture of fish muscle is softer (more tender) as compared to red meat muscle (veal, beef, pork, and avian muscle) since the muscle fibres of fish, the myotomes, contain muscle cells that are only a single layer deep and are connected to each other through heat-labile connective tissue, myocommata (Ashie & Simpson, 1997). In addition, fish tissue contains higher levels of proteases, for example, fish muscle contains up to 10 times higher levels of cathepsins than mammalian muscle (Karmas, 1978).

Autolysis in fish is due to the activities of proteolytic enzyme systems (proteases responsible for proteolysis), specifically the cathepsins (cysteine peptidases; cathepsins B, L, H, S, F, and K), calpains (cysteine peptidases), calpastatin (calpain inhibitor proteases), and collagenases (Lawrie, 1998; Sentandreu *et al*., 2002). However, the rate and extend of autolysis from endogenous proteases are different for different species of fish. In a study by Geist and Crawford (1974) it was demonstrated that endogenous cathepsin activities differ between different species of sole. Cathepsin from English sole (*Parophrys vetulus*) had a slightly higher pH optimum of 3.2-3.8, compared to 3.0-3.5 for rex sole (*Errex zachirus*) and petrale sole (*Eopsetta jordani*). It was also found that the main enzyme activities for the three species of sole varied significantly.

The rate and extend of autolysis are also affected by infection by intracellular parasites *K. thyrsites* and *K. paniformis* (Funk *et al*., 2008). *Post-mortem* myoliquefaction of marine fish muscle is a result of proteolytic enzymes released by the marine myxosporean parasite of the genus *Kudoa* (Gilchrist, 1924; Dawson-Coates *et al*., 2003; Samaranayaka, *et al*., 2006; Levsen *et al*., 2008). The species *Kudoa thyrsites* is one of the major *Kudoa* parasites responsible for myoliquefaction in economic important fish species (Dawson-Coates *et al*., 2003), including Cape snoek (*Thyrsites atun*) (Gilchrist, 1924) and SA sardine (*Sardinops sagax ocellatus*) (Webb, 1990). Myoliquefaction due to *Kudoa* infection may be apparent before other chemical quality changes, such as fat oxidation and microbiological deterioration, takes place (personal observations in Cape snoek and SA sardines, 2014, 2015, 2016; Levsen *et al*., 2008). Funk *et al*. (2008) identified the proteolytic enzymes from *K. thyrsites* and *K. paniformis*, responsible for myoliquefaction in fish muscle, to be cysteine proteases of the cathepsin L type. These researchers showed that the *K. thyrsites* and *K. paniformis* cathepsin L proteases were not derived from a host response to the parasite. The *K. thyrsites* and *K. paniformis* derived proteases differ in their amino acid sequences over the 23 residue N-terminal. The cathepsin L proteases from *K. thyrsites* is a unique cytoplasmic enzyme and is translated as a pre-proenzyme. It was also found that the cathepsin L from *K. thyrsites* has only

four cysteine residues as compared to six cysteine residues in other cathepsins (An *et al*., 1994; Funk *et al*., 2008).

Endogenous cathepsins are found in cellular lysosomes and are generally referred to as acid or aspartyl proteases since cathepsins exhibit optimum activity at low pH values (Ashie & Simpson, 1997). The optimum pH for maximum activity of the cathepsin L protease from *K. thyrsites* is 5.5, which is also the iso-electric point for most of the main muscle proteins. At the iso-electric point, where the net charge on proteins is zero, denatured proteins are liable to attach by proteolytic enzymes (Lawrie, 1998). The cathepsin L protease from *K. thyrsites* showed noticeable loss of activity at pH 6.5 (An *et al*., 1994; Funk *et al*., 2008) and increased stability at alkaline pH values (pH 8.8).

Strategies to manipulate the *post-mortem* pH of muscle tissue in farm-reared Atlantic salmon (*Salmo salar*) have been suggested by several researchers (Watson, 1995; Funk *et al*., 2008) as a possible means to reduce the activities of cathepsin proteases, however, this strategy is impractical for wild-caught fish. The *post-mortem* decrease in muscle pH is due to anaerobic glycolysis, resulting in the production of lactic acid, and is in part dependent on the glycolytic potential of the muscle cells (Watson, 1995; Lawrie, 1998). If the amount of glycogen in the muscle cells prior to harvesting the fish can be reduced, it may be possible for the amount of anaerobic lactic acid generated to be reduced, thus reducing the fall in muscle pH, and ultimately reducing cathepsin activity. However, fish muscle contains low levels of carbohydrates, ranging between 0.2 to 1.5% (Françoise, 2010) resulting in fish muscle to obtain a relative higher *post-mortem* pH as compared to mammalian muscle. The pH in fish muscle drops from 7.0 to 6.3 during the early *post-mortem* storage (Ofstad *et al*., 1996; Chéret *et al*., 2007; Françoise, 2010) while in bovine muscle the pH falls to 5.7 to 5.4 at 24 h *post-mortem* (Lawrie, 1998).

The texture of fish is an important quality characteristic to consumers as well as to the fish processing industry (Wesson *et al*., 1979; Huss, 1995; Love, 1997). Soft fillets are problematic to the fish industry, for examples, soft sardines cannot be used by the sardine canning industry, and may result in huge economic losses (C. van der Lingen, 2016, senior researcher, Branch: Fisheries Management, Department of Forestry and Fisheries, Cape Town, South Africa, personal communication). In South Africa, myoliquefaction of fish musculature results in customer complaints and economic losses, especially with regard to SA sardine (*Sardinops sagax ocellatus*), Cape snoek (*Thyrsites atun*) and Cape hake (*Merluccius capensis* and *M. paradoxus*) (D. Brickles, 2016, Group Quality Assurance Manager, Irvin and Johnson Ltd., Cape Town, South Africa, personal communication).

This literature briefly reviews the use of polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR) as methods for DNA detection. This is followed by a short discussion about *post-mortem* muscle texture changes in fish and analyses thereof. The use of near infrared (NIR) spectroscopy as a non-invasive, non-destructive, rapid method in routine fish quality control and management, as well as in research applications, is finally considered.

3.2. Molecular detection methods

Many Myxosporea of the genus *Kudoa* infect fish muscle tissue and cause *post-mortem* myoliquefaction (Kabata & Whitaker, 1981; Hervio, *et al*., 1997; Moran *et al*., 1999a; Yokoyama & Itoh, 2005). Examples include: *K. hitolytica* in Atlantic mackerel (*Scomber scombrus*), *K. mirabilis* in ribbonfish (*Trichiurus haumela*), *K. musculoliquefaciens* in swordfish (*Xiphias gladius*), *K. megacapsula* in red barracuda (*Spyraena pinguis*), *K. cruciformum* in Japanese seaperch (*Lateolabrax japonicas*), *K. fundulid* in mummichog (*Fundulus heteroclitus*), and *K. clupeidae* in Atlantic herring (*Clupea harengus*). *Kudoa* species are generally host specific, however, *K. thyrsites* has been reported from more than 27 fish species around the world (Hervio *et al.*, 1997) and are of great concern to the aquaculture industry as it is associated with myoliquefaction in pen-reared Atlantic salmon and coho salmon (Whitaker & Kent, 1991). PCR assays, based on the 18 SSU rDNA sequences, sensitive enough to identify and detect all life stages of *Kudoa* parasites, have been developed (Hervio *et al*., 1997; Kent *et al*., 2001).

3.2.1 Polymerase chain reaction (PCR)

A thermostable polymerase enzyme is responsible for a PCR reaction. A polymerase will synthesise a complementary sequence of DNA bases to any single strand of DNA providing it has a double stranded starting point. Due to the morphological similarities between the spores of *K. thyrsites* and *K. paniformis* (Moran *et al.*, 1999b), it is sometimes difficult to accurately distinguish between these two *Kudoa* species when using microscopic methods (Meng & Li-Chan, 2007). Meng and Li-Chan (2007) developed a PCR assay for the detection of *K. thyrsites* and *K. paniformis*, respectively, in Pacific hake (*Merluccius productus*). These authors designed specific primers that could distinguish *K. thyrsites* and *K. paniformis* DNA from the host fish DNA. In addition, the primers were found to be specific to *K. thyrsites* and *K. paniformis*, respectively, and thus could be used to distinguish between these two *Kudoa* species. The PCR method was found to be more sensitive and specific than the traditional wet-mount microscopic methods for detecting low levels of infection. In PCR assays, DNA analysis requires amplification of the DNA by PCR in order to have enough DNA to give a detectable signal for quantification or qualification (Brunstein, 2013). Marshall *et al*. (2016) also concluded PCR to be a sensitive method for detection of *K. thyrsites* infected Atlantic salmon; however, false negatives should be compensated for by including tissue samples from several different anatomical locations per fish sample.

3.2.2 Real-time quantitative polymerase chain reaction (qPCR)

Real-time quantitative polymerase chain reaction (qPCR) is often used as a routine analysis method for fast screening as it is a very sensitive and reliable method for detection (i.e. qualitative diagnostic tool) and/or quantification of DNA and ribonucleic acid (RNA) (Löfström *et al*., 2015). Detection occurs during the accumulation of the PCR product with each cycle of amplification; allowing for monitoring of the PCR reaction during early and exponential PCR phases where the first significant

increase in the amount of PCR product correlates to the initial amount of target template. This same principle of amplification in PCR is employed in qPCR, but instead of identifying bands on a gel at the end of the reaction, the qPCR process is monitored in "real-time". The PCR reactions are visualised (or detected) with a camera or detector based on detection and quantification of fluorescence emitted from a "reporter" molecule, either fluorescent DNA-binding dyes or target-specific fluorescently labelled primers or probes. Fluorescent labelling can be performed on primers or on probes. Primer-based fluorescence is based on the ability of a fluorophore to change fluorescence properties upon annealing to complementary nucleic acids and subsequently extension. The accumulation of PCR amplicons can then be monitored by labelling one of the primers in the reaction. Although primer-mediated detection is cost effective compared to probe-mediated detection, it is less specific. This is because the fluorescence emissions are changed only upon annealing and extensions, and unspecific amplicons and primers dimers may also give rise to PCR signals. In contrast, probe-based detection technologies are more specific by ensuring that the fluorescence change measured is not caused by false binding of primers or primer dimers (Lind *et al*., 2006; Löfström *et al*., 2015).

There are three major categories of probe-based detection technologies, namely hydrolysis probes, linear hybridisation probes, and conformational probes. Hydrolysis probes are most widely applied. Hydrolysis probes are also known as 5'-nuclease or TaqMan® probes and are linear, target-specific probes that are generally double-labelled with a fluorescent reporter dye and a quencher (McGuigan & Ralston, 2002; Löfström *et al*., 2015). A fluorescence signal is given off when the primers are extended during PCR and the probe is encountered and subsequently hydrolysed, separating the reporting dye from the quencher. Further development of hydrolysis probes has been successfully applied for genotyping and detection of single-nucleotide polymorphism (SNP), allowing for shorter probe types and improved sensitivity and specificity.

Genotyping of SNP is the measurement of genetic variations of single-nucleotide polymorphisms between members of a species where a SNP is a single base pair mutation or difference at a specific position of a chromosome (locus), usually consisting of two alleles (the variant form of a given gene) (Löfström *et al*., 2015). In TaqMan® assays two probes are used in a bi-allelic system, each specific to an allelic variant, with forward and reverse PCR primers designed to amplify a region that includes the SNP polymorphic site (McGuigan & Ralston, 2002). Allele discrimination is achieved by using a fluorescence response energy transfer (FRET) mechanism (fluorophore and quencher) where each probe is labelled with a different reporter fluorophore. If the allele-specific probe is perfectly complementary to the SNP allele, the probes contain a fluorophore at the 5'-end and a quencher at the 3'-end, will bind to the target DNA strand and consequently become degraded by 5'-nuclease activities of the Taq-polymerase as it extends the DNA from the PCR primers. The degradation of the probe during the PCR amplification step results in the separation of the fluorophore from the quencher, thus allowing the fluorophore to generate a detectable signal. If the allele-specific probe is not perfectly complementary, it will have a lower melting temperature and not

bind efficiently, preventing the nuclease from acting on the probe. When the PCR is complete, a laser collects a fluorescence spectrum, and with the use of algorithms within specific software, the contribution of each component dye to the observed spectrum is extracted to produce an allelic discrimination plot (McGuigan & Ralston, 2002).

The labelling of probes for qPCR has been done with various types of fluorophores and quenchers (Löfström *et al*., 2015), where the fluorophores are able to absorb energy from a light source of a certain wavelength and reemit the energy as light at another wavelength. The fluorescent dye VIC, of which its chemical structure is not currently publicly available (Anon., 2017) and is proprietary to Life Technologies (Life Technologies Corporation is a biotech company founded in November 2008 through a merger of Invitrogen Corporation and Applied Bios stems Inc.), has an absorbance of 538 nm and an emission maximum of 554 nm and emits light in the green-yellow part of the visible spectrum (Anon., 2017). The fluorescent dye VIC is typically used to fluorescently label oligonucleotides at the ‘5-end for use as probes in qPCR applications. The purpose of quencher molecules is to quench the fluorescence emitted by the fluorophore when excited by the cycler’s light source. While the fluorophore and the quencher is in close proximity of each other, the quencher inhibits any fluorescence signals. Within the many probe based qPCR systems available, TaqMan® probes, within the TaqMan® probe chemistry system, are designed to anneal within a DNA region amplified by a specific set of primers (McGuigan & Ralston, 2002; Löfström *et al*., 2015). In this system, the probe binds to single stranded DNA. As the Taq-polymerase extends the primer and synthesise the nascent strand on a single-stranded template, the 5’ to 3’ exonuclease activity of the Taq-polymerase degrades the probe that has annealed the template. Degradation of the probe releases the fluorophore from the probe and breaks the close proximity to the quencher, thus relieving the quenching effect and allowing fluorescence of the fluorophore. The fluorescence detected is thus directly proportional to the fluorophore released and the amount of DNA template present in the PCR.

The application of qPCR to quantify specific genes or parts of DNA can be done by relative, or absolute quantification (Löfström *et al*., 2015). Relative quantification is mainly used for gene expression studies, where all resultant data is compared to a control, for example to a wild-type or an untreated sample, and expressed as a target/reference ratio. The disadvantage of relative quantification is that it requires normalisation, typically by using housekeeping genes or genes encoding ribosomal RNA, in order to correct for differences in RNA template with regard to quality and quantity. In absolute quantification, the quantification cycle values are compared with a standard curve consisting of different known concentrations of the target organism and used for gene expression studies and for enumeration of a specific micro-organism in a given sample. This method is dependent on the quality of the sample and the standard curve and thus requires similar amplification efficiencies for all samples and standards. Absolute quantification is usually normalised to a unit amount of sample, for example, number of cells, volume, or nucleic acid content. When using the standard curve method for diagnostics within food matrices, the standard curve must be

created in that particular matrix; whereas, when using pure cultures of microorganisms, the interference of the matrix and its inhibitors will be neglected.

In a study by Piazzon *et al*. (2012), a qPCR assay for the detection and quantification of the Myxozoan parasite, *Enteromyxum scophthalmi*, in aquaculture turbot (*Scophthalmus maximus*) was developed. These authors demonstrated good sensitivity and repeatability of the qPCR assay for detection and amplification of *Enteromyxum scophthalmi*. These authors also showed the usefulness of the qPCR assay to monitor infection in fish throughout the entire life cycle of the parasite. It is usually very difficult to impossible to cultivate myxozoan parasites *in vitro*.

3.3. Measurement and analyses of fish freshness and muscle texture

The texture of fish is considered as one of the first quality attributes influencing consumer acceptance and marketability of the final product (Wesson *et al*., 1979). Degradation of fish muscle can result in unwanted softening and/or gaping of the muscle (Love, 1997) rendering the fish unsuitable for further processing. The sensory evaluation of raw fish in markets and at landing sites is typically done by assessing the appearance, texture and odour of the fish (Huss, 1995). The first textural change *post-mortem* is the onset of *rigor mortis* when muscles become hard and inflexible due to the decrease in concentration of adenosine tri-phosphate (ATP), which also acts as a muscle plasticiser.

The two most important ATP-splitting enzymes in live, normal resting muscles are myosin-ATPase and Ca^{2+}-ATPase (Pearson & Young, 1989; Honikel *et al*., 1983). In *post-mortem* muscles, the cells attempt to maintain ATP at physiological levels for as long as possible by minimising ATP-hydrolysis to essential processes (Pearson & Young, 1989). The development of *rigor mortis* does not occur until approximately half of the ATP is depleted. With the decrease in ATP levels, there is not enough energy available for pumping Ca^{2+} back across the membranes and therefore Ca^{2+} slowly leaks into the cytosol. The excess Ca^{2+} ions result in contraction of the muscle fibre bundles and the depletion of ATP leads to the formation of permanent cross-bridges between the action and myosin filaments (actomyosin). These cross-bridges cannot be broken in the absence of ATP, leading thus to constant isometric tension (Pearson & Young, 1989).

The rate of *post-mortem* glycolysis will tend to be higher in muscles that are slow to cool, since higher temperatures are known to speed up the rate of chemical reactions (Pearson & Young, 1989; Lawrie, 1998). The condition of *rigor mortis* usually lasts for a day or longer, after which *rigor* resolves and the muscle becomes relaxed again, but no longer elastic (Huss, 1995). The softening of the muscle during resolution of *rigor* is coincidental with the autolytic changes within the muscle. Among these autolytic changes is the degradation of ATP-related compounds. ATP degrades in a relative predictable and similar manner in most fish to form adenosine diphosphate (ADP), adenosine monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino), and hypoxanthine (Hx). The concentrations of these ATP catabolites during *post-mortem* storage may be used to calculate the “freshness” index or K-value with the following formula:

$$K\% = \frac{[Ino] + [Hx]}{[ATP] + [ADP] + [AMP] + [IMP] + [Ino] + [Hx]} \; x\; 100$$

The higher the K-value, the lower the freshness of the fish, however, some fish species reach a maximum K-value well in advance of the shelf life and therefore is not considered reliable as a freshness index for all marine species. In addition, the degradation of nucleotide catabolites is only coincidental with the perceived changes in freshness and not necessarily related to the cause of freshness deterioration. This is becaus only Hx is considered to have a direct effect on the perceived bitter off-flavour of spoiled fish. The analysis and measurement of the concentration of these catabolites are expensive and time consuming and not suitable for quick decision making at harvest or landing sites.

The quality index method (QIM) is a well-established sensory method for the evaluation of fish freshness and is based on well-defined characteristic changes of quality-related attributes of the raw fish (eyes, skin, gills, and smell) and corresponds to a score system of index points (Hyldig *et al*., 2010). The measurement of chemical indicators, such as nucleotide catabolism or production of amines [such as trimethylamine (TMA)], are used as acceptability indices in guidelines and specifications for the measure of freshness of fish. Trimethylamine (TMA) is an enzymatic breakdown product of trimethylamine oxide (TMAO) from both autolysis and microbiological activity and often used to measure fish spoilage. However, none of the above-mentioned chemical methods are used in the fish industry since they are time consuming and expensive (Nychas & Drosinos, 2010).

Evaluating the texture of fish, as assessment method for freshness, by pressing parts of the fish body with the finger are of high importance for fish inspection and have been incorporated as parameters that comprise the QIM (Hyldig *et al*., 2010; Sánchez-Alonso *et al*., 2010). In addition to sensory texture measurements, many instrumental methods have been developed for measuring the texture of fish. These methods include the Kramer, Warner-Bratzler, puncture, compression, textured profile analysis (TPA), and stress relaxation tests. The analysis of cutting force and compression are the most widely used techniques for measuring the texture of fish (Sigurgisladottir *et al*., 1999).

The Kramer test makes use of the Kramer shear-compression cell that consist of an upper part which holds 10 blades and a lower part (the cell) which holds the fish samples (Sigurgisladottir *et al*., 1999; Hyldig *et al*., 2010; Sánchez-Alonso *et al*., 2010). The blades pass through the fish sample during which complex combinations of compression, extrusion, shear, and friction takes place. Parameters such as maximum force per sample weight, slope and energy of the force-deformation curve are subsequently calculated. The Warner-Bratzler test is connected to an Instron machine, where a blade with two cutting edges penetrates a device with a slot, cutting the fish sample like a guillotine. As the blade cuts through the sample, it is subjected to a combination of tension,

compression, and shearing; from which parameters such as maximum force during the shearing is calculated.

The compression test makes use of a uniaxial compression force, usually between two parallel flat surfaces, that is exerted upon the sample (Sigurgisladottir *et al*., 1999; Hyldig *et al*., 2010; Sánchez-Alonso *et al*., 2010). Parameters such as degree of deformation, and energy can be calculated from the force-deformation curves. The puncture test is also connected to an Instron machine. In this test, a plunger is pushed into the fish sample. The sample is subjected to a combination of compression and shearing in proportion to the area and perimeter of the cross-section of the plunger. The measurement of parameters such as maximum shear stress, stiffness, and strain at failure, depends on the deformation of the sample when the punch force is at its maximum.

The TPA method is an imitative test wherein the sample is compressed twice to mimic the action of the jaw (biting or chewing) (Hyldig *et al*., 2010; Sánchez-Alonso *et al*., 2010). Texture parameters such as hardness, cohesiveness, springiness, adhesiveness, “fracturability” (originally termed brittleness), gumminess, and chewiness are the most used physical definitions of the mechanical texture attributes that can be calculated from the force deformation curves.

The stress relaxation test wherein the fish sample is subjected to a sudden deformation and the force required to hold the deformation constant is measured over time (Hyldig *et al*., 2010; Sánchez-Alonso *et al*., 2010). Parameters such as relaxation times, viscous and elastic moduli, and degree of solidity, can be calculated from the relaxation curves.

In recent years, research has focus on developing rapid, non-destructive, methods (Nychas & Drosinos, 2010; Mathiassen *et al*., 2011; Cheng *et al*. 2013), such as near-infrared (NIR), visible (VIS), combining VIS and NIR (VIS/NIR) spectroscopy, mid-infrared (MIR) spectroscopy, Raman, nuclear magnetic resonance (NMR) spectroscopy, hyperspectral imaging (HSI) and nuclear magnetic resonance imaging (NMRI), to analyse the freshness and quality of fish.

3.4 Application of near-infrared (NIR) spectroscopy for quality control of fish

Methods used for the detection of *Kudoa* infection early *post-mortem* include, PCR and qPCR (Funk *et al*., 2007; Jones *et al*., 2016), microscopic examination of tissue sample preparations (Harrel & Scoctt, 1985; Langdon *et al*., 1992), visual observation of myoliquefaction and/or black pseudocysts (Dawson-Coates *et al*., 2003; Samaranayaka *et al*., 2008) and antigen-capture enzyme-linked immunosorbent assay (ELISA) (Young, 2002; Chase *et al*., 2003; Taylor & Jones, 2005). These methods are time consuming, expensive and destructive to the fish sample (Morrissey *et al*., 1995; St-Hilaire *et al*., 1997). Presently there are no rapid methods that can be used to determine whether fish are infected before myoliquefaction occurs. Near-infrared (NIR) spectroscopy is a rapid, non-destructive, economical, and environmental friendly method extensively researched for the application thereof in fish quality-attribute monitoring (Weeranantanaphan *et al*., 2011).

3.4.1 Basics of near-infrared (NIR) spectroscopy

The wavelength region of NIR spectrum is from 780 to 2500 nm (Osborne, 2011; Cheng *et al*., 2013). This region lies between the visible (380-780 nm) and the mid-infrared (MIR) (2500-15 000 nm) regions (Manley, 2014). The NIR region is usually divided into two sections: short wave near infrared spectrum (SW-NIR) of 780 to 1100 nm, and long wave near infrared spectrum (LW-NIR) of 1100-2526 nm (Osborne, 2011; Cheng *et al*., 2013). NIR spectra results from energy absorption by organic molecules and relates to the reflection, transmission and/or scattering of light in/or through a food material. When an organic molecule absorbs infrared radiation, its chemical bonds vibrate, either by stretching, contracting, or bending. The NIR spectra of food and food products comprise of broad bands arising from overlapping absorptions corresponding mainly to overtones and combinations of vibrational modes involving the X-H chemical bonds, such as C-H, N-H, O-H, and C-O (Osborne, 2011; Cheng *et al*., 2013; Manley, 2014). NIR spectra can thus be used to indicate the chemical and physical changes in food samples in the assessment of food quality (Liu *et al*., 2013). The major source of variation in NIR spectra arise from the solid and highly heterogeneous nature of food (Weeranantanaphan *et al*., 2011).

One of the advantages of NIR is that it allows several constituents to be measured simultaneously. Other advantages (Weeranantanaphan *et al*., 2011; Manley, 2014) of NIR are that i) it can be collected directly from samples without the need for dilution, ii) it is applicable to on-line analysis, thus facilitating control and monitoring at the production line in commercial and/or trading environments, iii) it is cheap, and iv) it is environmental friendly as it does not require the use of chemicals for analysis. On the other hand, a disadvantage is that the initial cost to obtain required NIR equipment and the setting-up standard calibration models may be high. NIR requires calibration, typically with the use of multivariate mathematics (chemometrics), against a reference method for the constituent(s) of interest (Osborne, 2011; Manley, 2014). The NIR analysis of food and food products is therefore based on calibration against a reference method by using a set of samples that represents all the variability in the population from which they are taken. Many review articles (Weeranantanaphan *et al.,* 2011; Liu *et al*., 2013; Cheng *et al*., 2013; Manley, 2014) have extensively discussed quantitative and qualitative applications of NIR in food products. For the development of prediction or calibration models pertain to quantitative analysis, the first step is to acquire a set of calibration samples with known reference values. The reference values are determined by an independent analytical method and should cover the range of variation expected in unknown samples. A regression model, namely mathematical linear relationship, between the NIR spectra and the reference values is then developed. Regression models typically used include principal component regression (PCR), partial least squares (PLS) regression, and multiple linear regression (MLR). For any prediction or calibration model, the predicted values of the calibration set should be compared with the reference values of the same set using the coefficient of determination (R^2), and the standard error of cross-validated (SECV) data (Osborne, 1988). For good calibration equations, high R^2 values and low SECV values, while using the minimum number of model factors (principal

components), is recommended. Shenk and Westerhaus (in Malcolm *et al*., 2012) documented that coefficient of determination (R^2) values more than 0.83 are considered good, while values above 0.90 are suitable for providing quantitative information about unknown samples.

NIR spectra may also be calibrated for qualitative analysis, for example, authenticity testing, where the aim is to classify samples into defined groups or classes (Osborne, 2011; Liu *et al*., 2013). When generating classification models for application of qualitative analysis, a discriminative analysis approach is used where a model is developed from which the mean and standard deviation of each sample type or group can be defined in multidimensional space. The model is then used to test to which group an unknown sample belongs to (Osborne, 2011).

Soft independent modelling of class analogy (SIMCA) and partial least square discriminant analysis (PLS-DA) are examples of classification models. SIMCA is where two classes can overlap (hence "soft" modelling) and an object may belong to both (or neither) class simultaneously (Esbensen, 1994). The idea behind "soft" modelling is that, in chemistry, an object may fit into more than one class simultaneously, for example, a compound may have an ester and an alkene group, and will exhibit spectroscopic characteristics of both functionalities. A "hard" modelling method, where it is assumed that the compound must be either an ester or and alkene, is unrealistic. It is, however, possible to calculate class distances from discriminant analysis that are close to two or more groups. The main steps of SIMCA include: i) PCA, ii) calculation of class distance, iii) definition of modelling power of each variable for each separate class, iv) calculation of the discriminatory power, and v) validation of the SIMCA model. The modelling power varies between 1 (excellent) and 0 (no discrimination). Variables with a modelling power below 0.5 are of little use. The discriminatory power is a measure of how well a variable discriminates between two classes. Validation of the developed SIMCA model is determined by whether unknown samples are classified adequately.

Partial least squares discriminant analysis (PLS-DA) is a multivariate data analysis method based on the partial least squares (PLS) model in which the dependent (Y) variable is linked to the independent (X) variable (Szymańska *et al*., 2012; Brereton & Lloyd, 2014; Grootveld, 2014; Gromski *et al*., 2015). In a two-class model, the Y-variable represents membership of a particular classification (class membership) using the response variable -1 for members in one class, and +1 for members in the second class (Szymańska *et al*., 2012; Brereton & Lloyd, 2014; Gromski *et al*., 2015). Several studies (Ivorra *et al*., 2013; Ottavian *et al*., 2013) have documented the use of NIR spectroscopy and PLS-DA to discriminate between different sets of samples.

3.4.2 Applications of NIR spectroscopy and hyperspectral imaging (HSI)

Several studies on the application of NIR spectroscopy for quantitative (to measure chemical composition in fish) and qualitative analysis (freshness quality parameters) of fish and fishery products, have been reported in many research papers; of which a few are briefly discussed. Some studies demonstrating the use of NIR and/or hyperspectral imaging as technique for the analysis of fish quality were also considered. Hyperspectral imaging (HSI) is a technique that combines optical

spectroscopy and digital imaging to obtain both spectral and spatial information of a food sample (Cheng & Sun, 2014) providing information about organoleptic and chemical properties thereof.

3.4.2.1 Proximate composition

The proximate composition of food and food stuffs, usually expressed as a percentage, entails the crude composition of total protein, fat, moisture, ash, and carbohydrates. Carbohydrates are usually determined by calculating the difference of protein, fat, moisture, ash out of a hundred, since accurate carbohydrate analysis usually involves the use of enzymatic reactions, which are typically costly and time consuming. In general, fish muscle contains very low quantities of carbohydrates, depending of the species of fish, ranging between 0.2 and 1.5% (Françoise, 2010).

In NIR spectra, the absorption bands for moisture are typically between 1440-1470 nm (O-H stretch first overtone) and 1920-1940 nm (combination of O-H stretch and O-H deformation, O-H bending second overtone) (Osborne *et al*., 1993; Liu *et al*., 2013; Manley, 2014), making it possible to predict water content within food products. Similarly, the concentration of protein in food products can be predicted because of the absorption bands of the N-H bonds at 1560-1670 nm and 2080-2220 nm. The absorption of the C-H bonds of fatty acids at 1680-1670 nm and 2300-2350 nm, is typically used to predict the amount of fat; while the amount of carbohydrates can be predicted due to the specific absorbance at 2060-2150 nm for the C-O and O-H bonds.

In a study by Khodabux *et al*. (2007), R^2-values for the prediction values against reference values of the constructed models were reported as 0.98, 0.99, 0.95 and 0.96 for moisture, protein, total fat, and free fat, respectively in skipjack and yellowfin tuna. In a different study by Folkestad *et al*. (2008), the chemical reference method and a computerised tomography (CT) analysis method, respectively, for total fat content in Atlantic salmon were correlated to NIR spectra. These authors reported root mean square error of prediction (RMSEP) of 1.0% fat and a correlation with chemical reference values of $r = 0.94$ ($P < 0.0001$). Predictions for fat content from NIR also correlated well with the CT analysis ($r = 0.95$). Nortvedt *et al*. (1998) used near-infrared transmittance (NIT) spectroscopy, in the range of 850-1048 nm, to predict fat, protein and dry matter in wet homogenised Atlantic halibut (*Hippoglossus hippoglossus*) fillets. Prediction errors, expressed as root mean square error of cross-validation (RMSECV), of 2.7 $g \cdot kg^{-1}$ fat, 5.2 $g \cdot kg^{-1}$ protein, and 4.2 $g \cdot kg^{-1}$ dry matter, using partial least squares regression (PLSR) analysis, were reported.

Prediction models for moisture, protein, glycogen, and fat in oysters (*Crassostrea gigas* and *Saccostrea glomerate*) gave excellent predictions with R^2-values of 0.94 for glycogen and 0.97 for fat (Malcolm *et al*., 2012). The SECV values were 0.14 for fat and 0.52 for moisture; while RPD values (standard error of NIR prediction values) were 2.7 and 5.5. These authors concluded that NIR is suited for application in the oyster farming industry where rapid analysis of many individual oysters is required for selective breeding programs.

Quantitative measurement of moisture and fat distribution, using an online imaging spectroscopy setup, proved to be suitable for industrial application of high-speed assessment of quality parameters

in fish fillets (ElMasry & Wold, 2008). NIR and pixel spectra were converted into distribution maps of moisture and fat by applying PLS regression, creating pixel-wise models for the prediction of moisture (R^2 = 0.94, RMSECV = 2.73%) and fat (R^2 = 0.91, RMSECV = 2.99%) content in fish fillets.

3.4.2.2 Fatty acid concentrations

The high nutritional quality and health benefits of consuming marine fish and fishery products have been well documented over many years (Steffens, 1997; Usydus *et al*., 2011) with most of the focus being on the health benefits of n-3 poly-unsaturated fatty acids (PUFA). Dietary recommendations for the prevention of dietary deficiency symptoms has been made for omega-3 and omega-6 fatty acids, including alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosohexaenoic (DHA), to achieve nutrient adequacy and to prevent and treat certain diseases (Gebauer *et al*., 2006; Balanza-Martinez *et al*., 2011; Grosso *et al.,* 2014; Nestel *et al*., 2015). Knowledge of fatty acid (FA) composition and classes in food products is useful in raw material quality aspects, consumer perception, and shelf life of food products.

The prediction of FA composition with NIR spectroscopy has commonly followed two different approaches, namely i) quantitative models of individual FA constituents, or ii) a model to predict fatty acid classification in classes such as saturated fatty acids (SFA), total mono-unsaturated fatty acids (MUFA), total poly-unsaturated fatty acids (PUFA), and omega-6 fatty acids (Weeranantanaphan *et al*., 2011). In general, accuracies for prediction of fatty acid content in meat and meat products (beef, lamb, and chicken) by NIR spectroscopy have been reported to range from 0.03 to 0.98, with prediction errors between 0.01% and 4.45% (Pla *et al*., 2007; Sierra *et al*., 2008; Riovanto *et al*., 2012; Pullanagari *et al*., 2015). Poor prediction of individual fatty acids is usually due to low concentrations of individual fatty acids (Guy *et al*., 2011), a narrow range of reference values used for calibration, and difficulties arising from the very similar spectral profiles exhibited by fatty acids in general (Realini *et al*., 2004). However, the accuracy of predictions may be improved when fatty acids are combined into classes based on degree of saturation (SFA, MUFA, and PUFA). This is not always the case, as in a study by Riovanto *et al*. (2012), R^2-values for SFA and MUFA were 0.90 and 0.93, respectively, while for PUFA, the R^2-value was 0.62 for chicken meat. However, in a study by Malcolm *et al*. (2012), prediction models for PUFA and long-chain PUFA (C ≥ 20) in oysters (*Crassostrea gigas* and *Saccostrea glomerate*) gave R^2-values of 0.94 and 0.93, respectively.

3.4.2.3 Colour indicators

With regards to the fish flesh colour of Atlantic salmon (*Salmo salar*) and pen-reared, aquaculture-bred trout and salmon fishes (including a number of species of freshwater fish belonging to the genera *Oncorhynchus*, *Salmo* and *Salvelinus*, all of the subfamily Salmoninae of the family Salmonidae), the colour of the flesh is typically used to determine the market value (Sigurgisladottir *et al*., 1997; Alfnes *et al*., 2006) of the fresh or final product (either fresh or processed, for example smoked or canned). The colour parameters of food are typically expressed and/or measured as L*

(lightness), a* (red-green), and b* (yellow-blue) of the colour sphere. In the colour space, L* indicates lightness and a* and b* are the chromaticity coordinates, where a* is the red-green range, and b* the yellow-blue range of the colour spectrum (MINOLTA, 1998). However, instead of correlating NIR spectra with L*, a*, and b*, Folkestad *et al*. (2008) demonstrated coefficients of determination of 0.83 to 0.94 and prediction errors (RMSEP) of 0.4-0.9 $mg \cdot kg^{-1}$ pigment of astaxanthin concentrations, with the use of VIS/NIR in whole and filleted salmon (*Salmo salar* L).

3.4.2.4 Mechanical tenderness and texture analyses

The texture of fresh fish and fishery products significantly influences consumer acceptance and re-purchasing behaviour (Risvik, 1994; Issanchou, 1996). Myoliquefaction of Cape snoek, locally known in South Africa as “pap snoek”, result in consumers hesitating in purchasing that product. Limited research (Isaksson *et al*., 2001) has been done to investigate the correlation of NIR spectra and mechanical tenderness measurement [Warner-Bratzler shear force (WBSF) and slice shear force (SSF)] in fish. Non-destructive texture analysis with the use of visible (VIS) and NIR reflectance spectroscopy of farmed Atlantic salmon (*Salmo salar*) has been investigate by Isaksson *et al*. (2001). These authors correlated VIS/NIR to Kramer shear force measurements and to texture profile analysis (TPA), respectively, of salmon fillets. It was concluded that VIS/NIR does not give very accurate predictive measures of fish texture, with a correlation coefficient of 0.84 and a prediction error (RMSEP) of 0.72 x 10^{-2} $J \cdot g^{-1}$ for 2-hour *post-mortem* Kramer values, and non-significant correlations for TPA. However, when these authors investigated the ability of VIS/NIR to classify texture quality into three broad categories, namely, low, medium, and prime, 79% of all samples were correctly classified.

3.4.2.5 Microbial spoilage of fish (spoilage indicators)

Several review articles (Weeranantanaphan *et al*., 2011; Cheng *et al*., 2013) discuss the use of NIR spectroscopy as a useful and reliable technique for detecting microbial behaviour and spoilage of fish. In a study by Lin *et al*. (2006), using visible-shortwave NIR reflectance spectra (600-1100 nm), a high prediction accuracy (R^2 = 0.94, SEP = 0.38 log colony forming units per gram) was demonstrated for a prediction model to predict total viable counts in intact muscle of Rainbow trout. Tito *et al*. (2012) used NIR spectroscopy to predict microbial spoilage of Atlantic salmon and reported PCA and PLS regression models suitable to discriminate between fresh fillets and fillets stored for 9 days at 4ºC. Hyperspectral imaging spectroscopy in the SW-NIR was used to discriminate between expired vacuum-packed smoked salmon and non-expired samples (Ivorra *et al*., 2013). PLS-DA showed that a red-green-blue colour model had the better R^2 in cross-validation (82.7%) compared to random pixel selection and mean and median colour models.

3.4.2.6 Fresh vs. frozen-thawed fish

Frozen fish has a lower market value than fresh fish, and the substitution of frozen-thawed fish is an important authenticity and adulteration issue (Cheng *et al*., 2013; D. Brickles, 2016, Group Quality Assurance Manager, Irvin and Johnson Ltd., Cape Town, South Africa, personal communication). Structural changes in water molecules can be observed within NIR spectra and the interaction between water molecules and other chemical species can be used for monitoring physiochemical quality traits in fish and fishery products, for example discriminating between frozen and frozen-thawed fish (Weeranantanaphan *et al*., 2011). Reis *et al*. (2017) demonstrated the application of VIS/NIR, in combination with PLS-DA, to distinguish between fresh and frozen-thawed tuna fillets. The PLS-DA, using repeated double cross-validation, showed a 92% probability that a fresh sample was predicted correctly as fresh, and 82% that a frozen-thawed sample was predicted correctly. VIS/NIR spectra was capable of capturing structural changes in the C-H bond from groups such as CH, CH_2 and CH_3, as well as differences in the concentrations of myoglobin, oxymyoglobin, deoxymyoglobin, and metmyoglobin.

Since freezing and thawing of fish muscle result in the disruption of cellular organelles and consequent release of cellular contents into the drip (natural juices) (Lawrie, 1998; Hedges & Nielsen, 2000), chemical differences between fresh and freeze-thawed fish can be measured. Uddin and Okazaki (2004) investigated the potential of NIR to differentiate between fresh and frozen-thawed fish based on physical and chemical differences in the natural drip juices from horse mackerel (*Trachurus japonicas*) fillets and whole fish samples. These authors preformed dry extract spectroscopy by infrared reflection on the natural meat juices (drip), followed by principal component analysis (PCA) and stepwise MLR to develop a classification calibration model for fresh and frozen-thawed fish. This model was able to separate frozen-thawed samples from fresh samples with 100% accuracy.

Species independent discrimination between fresh and frozen-thawed Gilthead sea bream, swordfish and carp was demonstrated with the use of NIR and PLS-DA (Ottavian *et al*., 2013). In a study by Bøknæs *et al*. (2002), using NIR spectroscopy to predict the freshness [frozen and chilled storage period, drip loss, water holding capacity (WHC) and dimethylamine (DMA)] of thawed-chilled modified atmosphere packed (MAP) cod (*Gadus morhua*) fillets, good correlation between NIR spectra and chilled storage period was demonstrated. Bøknæs *et al*. (2002) reported a correlation coefficient of 0.90 between measured and predicted duration (days) of chilled storage (2ºC) period and a root mean square error for cross-validation (RMSECV) of 3.4 days at 2ºC. However, a low correlation coefficient (< 0.1) between NIR spectra and DMA (dimethylamine) content was obtained. Sivertsen *et al*. (2011) also demonstrated that fresh cod (*Gadus morhau*) fillets can be differentiated from frozen-thawed fillets with the use of VIS/NIR. Freshness, expressed as storage days on ice, were determined with NIR spectroscopy with an accuracy of 1.6 days.

3.4.2.7 Authenticity and adulteration of fish and fishery products

Seafood fraud, where seafood species at restaurants and grocery stores are mislabelled, has been a hot topic since 2010. According to Warner *et al.* (in O'Brien *et al*., 2013) the fishing company Oceana from the United States conducted a two-year study (2010-2012), performing DNA testing on more than 1200 fish samples, and found that one third of seafood species were mislabelled. A similar study has been conducted by Cawthorn *et al*. (2015) in South Africa, where 149 fish samples were collected from three provinces (Kwazulu-Natal, Western Cape, and Gauteng). These authors reported that overall 18% of the fish samples were incorrectly described in terms of species, with similar frequencies of misrepresentation rates in restaurants (18%) and retail outlets (19%).

O'Brien *et al*. (2013) investigated the use of NIR spectroscopy to differentiate between different species of fish in an attempt to develop a model to test for the authentication of seafood. These authors used a hand-held MicroNIR 1700 spectrophotometer (wavelength range of 887-1667 nm) to obtain classification models to distinguish between related species of fish. Although a limited number of samples were included, it was demonstrated that SIMCA provided a suitable and reliable analytical tool for the correct assignment of the spectra and subsequent authentication.

3.4.2.8 Parasites in fish

Parasites naturally occur in fish and the presence thereof is very common. Most parasites in fish are of little concern with regard to economic losses or public health (Huss, 1994), however, parasites in fish fillets is a serious quality concern and many species of parasites cause illness in humans. Several species of nematodes (round worms), cestodes (tape worms), and trematodes (flukes) may cause illness in humans if consumed alive. Measures to control parasites in fish are particularly important for fish products that are to be eaten raw. Parasites are killed by cooking, freezing, curing (with high salt concentrations) and drying. All nematodes are killed when heated at temperatures above 55ºC and when frozen to -20ºC for at least 24 hours (Huss, 1994).

The presence of parasites in fish to be eaten raw is unacceptable, however, for the fish industry to avoid customer complaints, the presence of parasites in cooked fish or fish to be cooked, is also unacceptable. The most common methods in the fish industry to ensure parasite-free fish products are manual inspection, trimming of fillets and/or manual removal of parasites from fillets. These methods are very laborious and costly. Several researchers (Wold *et al*., 2001; Heia *et al*., 2007; Sivertsen *et al*., 2012) have thus investigated the use of spectroscopic methods to detect and classify fish as infected or not-infected. Most of the studies done to develop a rapid, non-destructive method for parasitic nematode detection in fish muscle and fillets involves spectral imaging technologies.

In a study by Wold *et al*. (2001), the ability of multispectral imaging and NIR spectroscopy to classify cod fillets as either infected or not-infected with parasitic nematodes, was investigated. These authors demonstrated that multispectral imaging in combination with SIMCA classification (based on a PCA model for multispectral imaging pixels belonging to specific classes) is able to detect nematode parasites at depths of 6 mm into the fish fillet and that this method gives fairly good

classification of infected cod fillets. Petursson (in Wold *et al*., 2001) also documented that NIR in the region of 800 to 1200 nm gives better results for deeply embedded parasites compared to absorption spectra in the visible range of 400 to 600 nm. The optical properties of white and dark fish muscle differed most significantly from that of parasites in the visible spectra range of 400 to 600 nm. Heia *et al*. (2007) demonstrated the ability of imaging spectroscopy to identify parasites in cod (*Gadus morhau*) fillets embedded as deep into the fillet as 0.8 cm, enabling detection of 0.2 to 0.3 cm deeper into the fillet than typically possible by manual inspection. In a similar study Sivertsen *et al*. (2012) demonstrated that hyperspectral imaging (HSI) techniques could be effective for industrial automatic, online, non-destructive detection of parasites in fish and fish fillets. Based on corresponding spectra from hyperspectral images between 448 and 752 nm, a Gaussian maximum likelihood classifier was used to classify pixels as nematodes or not. This was tested at a fish processing plant and detection rates of 70.8% and 60.3% for dark and pale nematodes, respectively, were achieved at a conveyor belt speed of 400 $mm \cdot s^{-1}$.

3.4.2.9 Other chemical components

Salt content plays an important role in the quality and shelf life of fish and fishery products. Lin *et al*. (2003) developed PLS prediction models, from SW-NIR spectra, for the salt content in hot smoked king salmon (*Onorhynchus tshawytscha*) (R^2 = 0.83, SEP = 0.32%) and chum (*O. keta*) salmon (R^2 = 0.82, SEP = 0.25%). In a similar study, Huang *et al*. (2006) successfully used SW-NIR and a back-propagation neural networks method (BPNN) to determine moisture [R^2 = 0.946, root mean square error (RMS) = 2.44] and sodium chloride (R^2 = 0.824, RMS = 0.55) contents in smoked Atlantic salmon (*Salmo salar*).

3.5 Conclusions

Post-mortem myoliquefaction of marine fish species due to the infection of *Kudoa thyrsites* and *K. paniformis* is seen as a texture quality defect. The South African (SA) fishing and fish processing industries find it challenging to control the occurrence of *Kudoa*-associated *post-mortem* myoliquefaction in economic important fish species, such as SA sardine, Cape hake, and SA kingklip. Although PCR and qPCR are often used as routine analytical methods for fast screening for detection and quantification of DNA and RNA, these methods are impractical for use by the fish industry to detect *Kudoa* infection. At the time of writing this dissertation, there were no known technologies (industrial or aquaculture related) to prevent the occurrence of myoliquefaction in wild-caught fish infected with *K. thyrsites* and/or *K. paniformis*. NIR spectroscopy has been shown to be an applicable fast and non-destructive analytical method for measuring several fish quality attributes. If *K. thyrsites* and/or *K. paniformis* infected fish can be identified before processing (filleting and/or canning) commences, the infected fish could be removed before it reaches the processor and/or consumer. This may also create opportunities to channel infected fish toward alternative usage, such as processing into fish meal, fish oil, and/or fish protein hydrolysates (FPH). The prevalence of *K.*

thyrsites and *K. paniformis* in the SA marine fish species: SA sardines (*Sardinops sagax ocellatus*), Cape hake, (*Merluccius capensis* and *M. paradoxus*) and SA kingklip (*Genypterus capensis*) is unknown. In addition, there is presently no rapid, non-destructive method available to the fishing and fish processing industries to distinguish between *Kudoa*-infected and uninfected fish. It will be of great value if a rapid, non-destructive method to detect *Kudoa* parasite infection, before myoliquefaction is visible, could be developed. Thus, the main purposes of this study were to investigate the prevalence of *K. thyrsites* and *K. paniformis* in SA sardine, Cape hake, and SA kingklip musculature tissue with the use of a newly designed qPCR protocol (consisting of two independent assays targeting two heterologous alleles; one for *K. paniformis*, and the other for *K. thyrsites*, respectively, and two heterologous alleles; *K. thyrsites*/*K. inornata*/*K. megacapsula* and *K. paniformis*/*K.* miniauriculata/K. *dianae*, respectively). The purpose of designing the qPCR protocol was to be used as a qualitative, fast and cost-effective diagnostic tool for research purposes to distinguish between *K. thyrsites* and *K. paniformis* infections in marine fish muscle tissue. Secondly, this study investigated the use of near-infrared (NIR) spectroscopy as a rapid, non-destructive method to discriminate between *Kudoa thyrsites* infected and uninfected fish samples.

The lay-out of the rest of this dissertation is as follows: Chapter 4 discusses the development of a qPCR protocol (with the above-mentioned alleles) for the routine, qualitative detection (i.e. presence) of *K. thyrsites* and *K. paniformis* in marine fish muscle tissue. Chapter 5 discusses the study conducted to determine the prevalence of *K. thyrsites* and *K. paniformis* in SA sardine, Cape hake, and SA kingklip. In addition, the prevalence of *K. thyrsites* as influenced by species of Cape hake: *M. capensis* and *M. paradoxus*, season of capture, geological area of capture (sardines only), size (causal length in cm), weight (g), and sex (male and female) were also investigated. Chapter 6 discusses the effects of *K. thyrsites* infection on moisture and ash content of SA sardine and moisture, ash, and crude protein of Cape hake, respectively. Chapter 7 discusses the investigation of NIR as a non-destructive, rapid method for the detection of *Kudoa* infection in SA sardines, Cape hake, and SA kingklip. Chapter 8 gives an overall conclusion and recommendations.

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Chapter 4

Development of a real-time qPCR assay for qualitative detection of *Kudoa thyrsites* and *Kudoa paniformis* in marine fish muscle tissue

Abstract

The myxosporean parasites *Kudoa thyrsites* and *K. paniformis* are known to infect many marine fish species worldwide. These parasites are associated with *post-mortem* myoliquefaction. Several South African (SA) marine fish species are infected with *K. thyrsites*. Cape snoek and SA sardines often show severe myoliquefaction. Up to date, it is unknown whether these fish species are infected with *K. thyrsites* and/or *K. paniformis*, or other *Kudoa* species. Polymerase chain reaction (PCR) results indicated that *post-mortem* myoliquefaction in Cape snoek is mainly due to the presence of *K. thyrsites*. The primers used in the PCR study showed poor alignment to the reference sequence obtained from a nucleotide similarity search. Based on the PCR study on Cape snoek, a real-time quantitative PCR (qPCR) method, that targets the nucleotide sequence within the 18S ribosomal RNA (rRNA) genes of *K. thyrsites* and *K. paniformis*, respectively, was designed for specific and qualitative detection of these two parasites in SA sardine muscle tissue. The majority (88%) of sardine samples were infected with *K. thyrsites*. The presence of *K. paniformis* could not be verified, therefore, no co-infection with *K. thyrsites* was found in the SA sardine samples. No other *Kudoa* species were present in the SA sardine samples. The newly designed single nucleotide polymorphism (SNP) genotyping assays for *K. thyrsites* and *K. paniformis* for qPCR showed sensitivity for fast and routine detection of *K. thyrsites* in fish muscle tissue.

Key words: Cape snoek, *Kudoa thyrsites*, *K. paniformis*, myoliquefaction, qPCR, *Sardinops sagax ocellatus*, single nucleotide polymorphism

4.1 Introduction

Traditionally, the identification of parasitic *Kudoa thyrsites* infection, associated with *post-mortem* myoliquefaction in fish, has been done by observing myxospores (mature spores) by microscopic methods using wet mounts or histological preparations of muscle tissue (Moran *et al*., 1999a). These methods, however, result in damage of the fish musculature (St-Hilaire *et al*., 1997). In addition, successful detection and identification of infection requires the parasite to have sporulated and contains myxospores. Myxospores are species specific and thus used for identification of infection (Moran *et al*., 1999a). Microscopic methods require a composite sample from several anatomical locations within the fish fillet. The reason being that infection may vary significantly among individual fish and among tissue samples within a single fish (Dawson-Coates *et al*., 2003). Lightly infected fish may thus be wrongly classified as uninfected. However, Samaranayaka *et al*. (2006) demonstrated that the average number of *Kudoa* spores from the nape (medial dorsal somatic) locations in Pacific hake was similar to the average number of spores for minced fillet samples. Mince from fish fillets can thus be used to count the average number of *Kudoa* spores in Pacific hake. These authors also reported that, although the number of spores differed between the different anatomical locations, the number of *Kudoa* spores were generally distributed in all anatomical locations tested.

Polymerase chain reaction (PCR) techniques have been widely used in food quality control as a fast, sensitive and accurate analytical method (Szalai, 1996). PCR assays can detect early stages of *Kudoa* infection, even in lightly infected fish (Hervio *et al*., 1997). Analysis of the ribosomal DNA (rDNA) sequence was used to study the phylogenetics of the Myxozoa as early as the 1994's by Smothers *et al*. (1994). Since then, several researchers have used the small subunit (SSU) rDNA sequence of myxozoans for systematics and life cycle studies and have developed highly sensitive and specific diagnostic PCR tests. Partial sequences of the 18 SSU rDNA have been published for *K. thyrsites* and for *Kudoa paniformis* (Hervio *et al*., 1997; Kent *et al*., 2001; Meng & Li-Chan, 2007). PCR assays based on the SSU rDNA sequences is sensitive enough to detect all life stages of these parasites and is a useful diagnostic tool for identification of *Kudoa* species (Kent *et al*., 2001).

Hervio *et al*. (1997) developed a PCR protocol with a primer pair specifically for *K. thyrsites* that amplified a 909 base pare (bp) region of the SSU rDNA. This PCR primer did not amplify the SSU rDNA region of other myxosporean *Kudoa* species, such as *K. amamiensis*, *K. paniformis*, and *K. miniauriculata*. The developed PCR protocol detected all stages of the *Kudoa* parasite, whereas microscopic methods can only detect the myxospore phase. It was suggested by Hervio *et al*. (1997), when using PCR methods, to use a large sample of tissue from several anatomical areas from a single fish in order to avoid false negative results.

Meng and Li-Chan (2007) developed PCR primer pairs specifically for *K. thyrsites* and *K. paniformis*. The primer pair for *K. paniformis* was based on a specific region of 1562 bp of the 18 SSU rDNA of *K. paniformis* and was able to distinguish between *K. thyrsites* and *K. paniformis*. The PCR of the sequence of the SSU rDNA of *K. thyrsites* and *K. paniformis* was shown to be a sensitive

and specific method with the ability of detecting low levels of *Kudoa* infection and capable of detecting low levels of *K. thyrsites* in the presence of high infection levels of *K. paniformis*. In comparison to real-time quantitative PCR (qPCR), PCR is labour-intensive and show poor sensitivity. It requires post-PCR processing that adds the risk of carryover contamination of PCR products (Löfström *et al*., 2015).

Funk *et al*. (2007) and Piazzon *et al*. (2012) developed qPCR assays for the detection and quantification of the Myxozoan parasites, *K. thyrsites* and *Enteromyxum scophthalmi*, respectively. These authors demonstrated that qPCR is a valuable tool with good sensitivity and repeatability for detection and amplification of Myxozoan parasites. It was also shown that qPCR assays were useful to monitor infection in fish throughout the entire life cycle of the parasite (Piazzon *et al*., 2012).

Partial sequences of the 18 SSU rDNA have been published for *K. thyrsites* (GenBank accession number; AF031412) and for *K. paniformis* (AF034640) (Hervio *et al*., 1997; Kent *et al*., 2001; Meng & Li-Chan, 2007). Partial sequence of the 28S large subunit (LSU) rDNA have also been reported for *K. thyrsites* and for *K. paniformis*. However, Whipps *et al*. (2004) reported that the phylogenetic analysis of LSU sequence produced trees that were similar to those derived from SSU sequence analysis, but with poor resolution regarding interrelationships of *K. thyrsites*, *K. quadricornis*, *K. miniauriculata*, and *K. paniformis*. This was due to LSU partial sequences being only about half the length of the SSU rDNA sequences. The partial sequence of the genomic DNA encoding the SSU rRNA is typically used to estimate relationships between lineages, since the SSU rRNA cistron evolves slowly over time (Sogin, 1989).

South African (SA) sardines (*Sardinops sagax ocellatus*) and Cape snoek (*Thyrsites atun*) are known to be two of the most heavily *K. thyrsites* infected fish within SA waters (Gilchrist, 1924; Langdon *et al*., 1992; Webb, 1990). The South African sardine is a commercially important resource as it contributes to the largest fishery (in terms on landed mass) in the country and is mainly canned for human and pet consumption (DAFF, 2014; Weston *et al*., 2015). Cape snoek is a popular eating fish in South Africa (Cawthorn *et al*., 2011), most commonly available in the Western Cape, and has a long eco-sociological, commercial and fishing history (Isaacs, 2013). From personal observations, both SA sardine and Cape snoek are evident to develop severe myoliquefaction as early as 24 h *post-mortem.* Several studies (Tsuyuki *et al*., 1982; Samaranayaka *et al*., 2008; Zhou & Li-Chan, 2009) indicated that severe myoliquefaction in Pacific hake (*Merluccius productus*) is associated with co-infection of *K. thyrsites* and *K. paniformis* due to higher proteolytic enzyme activities. While Zhou and Li-Chan (2009) suggested that infection by *K. thyrsites* may result in a greater adverse effect on texture than *K. paniformis* at the same level of infection, Tsuyuki *et al*. (1982) showed that unacceptable muscle texture was associated mainly with *K. paniformis* infection. Pacific hake infected with *K. thyrsites* showed increased proteolytic activity, however, the enzyme was described as heat labile with increased activity in the acid pH range and thus not responsible for the degradation of structural proteins, while the enzyme associated with *K. paniformis* infection was shown to be heat stable (optimal activity between 55 and 60˚C) with increased activity in both the acid and neutral pH

ranges. Funk *et al*. (2008) identified the protease from *K. thyrsites* to be a cysteine protease of the cathepsin L type. These researchers have shown that the cathepsin L protease are translated as preproenzymes from *K. thyrsites* and are not derived from a host response to the parasite. Endogenous cathepsins are found in cellular lysosomes and are generally referred to as acid or aspartyl proteases since cathepsins exhibit optimum activity at low pH values (Ashie & Simpson, 1997). The optimum pH for maximum activity of the cathepsin L protease from *K. thyrsites* is at 5.5; which is the iso-electric point for most of the main muscle proteins, where the net charge on is zero, and where denatured proteins are liable to attach by proteolytic enzymes (Lawrie, 1998). Noticeable loss of activity of the cathepsin L protease from *K. thyrsites* was observed at pH 6.5 (An *et al*., 1994; Funk *et al*., 2008), with increased stability at alkaline pH values (pH 8.8). The pH in fish muscle drops from 7.0 to 6.3 during the early *post-mortem* storage (Ofstad *et al*., 1996; Chéret *et al*., 2007; Françoise, 2010). Strategies to manipulate the *post-mortem* pH of muscle tissue in farm-reared Atlantic salmon (*Salmo salar*) have been suggested by several researchers (Watson, 1995; Funk *et al*., 2008) as a possible means to reduce the activities of cathepsin proteases. However, these strategies are impractical for wild caught fish.

At the time of writing this dissertation, there was a lack of knowledge whether SA sardine and Cape snoek are also infected with *K. paniformis*. Since the proteolytic enzymes associated with *K. thyrsites* and *K. paniformis*, respectively, differ in pH and temperature optima, it would be of interest to the fishing and fish processing industry to have knowledge about which *Kudoa* species is present in a particular fish species. Baseline information like this may assist in the development of future pH and temperature regulated processing strategies and/or technologies for the prevention of myoliquefaction development in infected fish.

Other SA marine fish species also infected by *K. thyrsites* include; Cape horse mackerel (*Trachurus capensis*), redeye (*Arnoldichthys spilopterus*), Cape dory (*Zeus capensis*), chub mackerel (*Scomber japonicus*), and Cape hake (*Merluccius capensis*) (Webb, 1990; Webb, 1993).

The aim of this study was to develop a qPCR method to be used as a fast, sensitive and qualitative measurement tool for the analysis of infection by *K. thyrsites* and *K. paniformis* in fish musculature, since the primers used in the conventional PCR study showed poor alignment to the reference sequence obtained from a nucleotide similarity search. The developed qPCR method was to serve as a fast diagnostic tool to be used in the research studies which follows Chapter 4 for determining the presence or absence of *K. thyrsites* and *K. paniformis* in the musculature of up to 300 fish samples during one analytic run (time and cost saving). Subsequently, it was determined whether Cape snoek and SA sardines were infected with *K. thyrsites* and/or *K. paniformis* with the use of existing PCR primer pairs (Meng & Li-Chan, 2007) and custom newly designed qPCR primers pairs.

4.2 Materials and methods

4.2.1 Samples and sample preparations

All fish samples were kindly supplied by the Department of Agriculture, Fisheries and Forestry (DAFF, Cape Town) (Addendum B). Six Cape snoek (during 2014) and 323 SA sardine samples (during 2015) were received as individually frozen fillets. Bad weather conditions resulted in poor Cape snoek runs during 2014, resulting in limited availability of samples. The Cape snoek samples were flecked and individually vacuum-sealed in plastic vacuum bags. The SA sardine samples were individually wrapped in tin-foil. All fish samples were kept frozen at -18ºC until microscopic, PCR and qPCR analyses were performed. All fish samples were thawed at room temperature (21-22ºC) for 20 min before DNA extractions were performed.

The six Cape snoek samples were used for microscopic (wet-mount preparations) and conventional PCR detection of *K. thyrsites* and *K. paniformis* with PCR specific primers as described by Meng and Li-Chan (2007). The PCR products from this PCR amplifications were sequenced and aligned on GenBank. Based on unsatisfactory GenBank results from the above-mentioned PCR primer set, two new primer sets, using single nucleotide polymorphism (SNP), consisting of two probes, specific to two alleles, were designed for a qPCR method (described in more detail below). This qPCR method was used for qualitative analysis of *K. thyrsites* and *K. paniformis*, respectively, in SA sardine samples. The extracted DNA from the Cape snoek samples were not used in the qPCR protocol because of insufficient DNA product after use in the PCR protocol.

It is noteworthy that thie SA sardine samples were not analysed with the use of the conventional PCR protocol but only with the qPCR protocol. The reason for this was; once the primers for the qPCR protocol were designed and their specificity were tested, the qPCR protocol served as a rapid, cost-effective (i.e. the potential of 384 samples to be analysed in one analytica run due to the use of 384-well templates) and qualitative (i.e. diagnostic) method (Funk *et al*., 2007) for detection of *K. thyrsites* and *K. paniformis*.

4.2.2 Spore images by light microscopy

The presence of *K. thyrsites* and *K. paniformis* spores were microscopically examined by taking a composite sample from the muscle tissue of the six Cape snoek samples. The tissue sample was placed on a glass slide, moistened with physiological saline solution (0.85%), and shredded with the use of a sterile scalpel blade. A glass cover slide was placed on top of the suspension. The wet-mounts were prepared in duplicate for each Cape snoek sample. Wet-mount preparations were also prepared from the liquid (drip) that accumulated in each vacuum bag in which each Cape snoek sample was stored. The wet-mount preparations were examined with the use of a compound light microscope (Olympus CX31, Japan) at 1000X magnification for the presence of *K. thyrsites* and *K. paniformis* spores. Descriptions and images of *K. thyrsites* and *K. paniformis* spores, as illustrated and described previously (Gilchrist, 1924; Kabata & Whitaker, 1981; Langdon, 1991; Whipps & Kent, 2006; Levsen *et al*., 2008), were used to identify *K. thyrsites* and *K. paniformis* mature spores

(myxospores) from images taken at 1000X magnification. Images of the different spores were taken using a Nikon camera (DS-Fi1) connected to a compound light microscope (Olympus CX31, Japan) at 1000X magnification. The images were saved in electronic format with the use of Nikon NisElements Imaging software (version 3.22, Nikon Instruments). These images were used to identify *K. thyrsites* and *K. paniformis* infected Cape snoek samples and to correlate infection with PCR results for the presence of these two *Kudoa* parasites. The PCR products from the amplified DNA of verified infected Cape snoek samples were sequenced and used as positive controls for analysis of the presence of *K. thyrsites* and *K. paniformis* in the SA sardine samples using the designed qPCR protocol.

4.2.3 Deoxyribonucleic acid (DNA) extractions

Deoxyribonucleic acid (DNA) was extracted with the use of the Qiagen DNeasy Mini Kit (Qiagen, Whitehead Scientific Pty., Ltd., Cape Town) from composite muscle tissue samples of approximately 10 to 20 mg. The composite muscle tissue samples were obtained by removing muscle tissue from three different locations (anterior, centre, and posterior) per Cape snoek and SA sardine fillet with the use of sterile scalpels. Composite tissue samples assist in improvement of data accuracy and prevention of false negatives (Samaranayaka *et al*., 2006; Funk *et al*., 2007; Marshall *et al*., 2016).

In addition to composite tissue samples, DNA was also extracted from accumulated tissue liquid from the Cape snoek samples, with the exception of one sample (no. 2), where there was no tissue liquid. The DNA from the tissue liquid was treated as duplicate samples for the DNA muscle tissue samples. The 11 DNA extracts from the muscle and accumulated fluid of the six individual Cape snoek samples were used for the detection of *K. thyrsites* and *K. paniformis* by PCR analysis as described by Meng and Li-Chan (2007). These DNA snoek samples were numbered 1a, 1b, 2b, 3a, 3b, 4a, 4b, 5a, 5b and 8a, 8b; where “a” indicated DNA from tissue liquid samples, and “b” indicated DNA from muscle tissue samples. Extracted DNA from SA sardine samples were used for the detection of *K. thyrsites* and *K. paniformis* by the designed qPCR protocol.

Prior to PCR and qPCR analyses, the concentration and purity of the extracted DNA were determined at the Centre for Proteomic and Genomic Research (CPGR, Cape Town, South Africa) using the NanoDrop ND-1000 (Thermo Fisher Scientific, USA). The concentration of extracted DNA was calculated based on the absorbance ratios of 260/280 and 260/230 nm. Agarose gel electrophoresis was used to evaluate the quality and molecular weight of the DNA samples. The GeneRuler 1 kilobases (kb) DNA ladder [250-10000 base pairs (bp), Thermo Fisher Scientific] was used as size reference. The DNA extracts were kept frozen at -80ºC until PCR and qPCR analysis to detect for the presence of *K. paniformis* and *K. thyrsites*.

4.2.4 Existing primers for conventional PCR

Partial 18 SSU rDNA was amplified by PCR with the following primer pairs (Meng & Li-Chan, 2007): KP18S4_f (GCTCAAAGCAGGCGTTACGTC) and KP18S4_r (CAAGATTCCCCATCCCTCTCG) for *Kudoa paniformis*, and KT18S6_f (CTCAACCAACTGGCCTCG) and KT18S1_r (CGTCAATTTCTTTAAATTTGG) for *K. thyrsites*. Primers for PCR were synthesised by The Synthetic DNA Laboratory (Department of Molecular and Cell Biology, University of Cape Town, Cape Town, South Africa) by using a Beckman 1000 M DNA synthesizer (Beckman Coulter, USA). The concentration (µM = nmole·ml^{-1}) of the primers were calculated using the formula:

$$\frac{(OD \times 37)}{(length\ of\ primer \times 325)} \times 1000$$

Where OD is the optical density at 260 nm, provided by The Synthetic DNA Laboratory (Table 4.1). The OD of each primer was multiplied by 37 (1 OD_{260} of single strand DNA = 37 µg DNA). This value was then divided by the length of the primer (nmer) multiplied by the average molecular weight of a nucleotide (i.e. 325 Daltons). Stock solutions of 100 µM for each primer were prepared (Table 4.1); which were subsequently diluted to 10 µM working solutions by mixing 20 µL of the 100 µM stock solutions with 180 µL water.

Due to the low specificity of these primers pairs for PCR, further development was performed by designing new primers pairs for qPCR as described below.

Table 4.1 Calculations of the concentrations for primers used for the conventional PCR

Primer information				**Concentration (nmole·ml^{-1}) = (µM)**	**100 µM stock solutions ($C_1V_1 = C_2V_2$)**		**10 µM working stock*** **($C_1V_1 = C_2V_2$)**	
Primer name	Sequence 5'-3'	Optical density (OD)	Length of primer (nmer)	$\frac{(OD \times 37)}{(nmer \times 325)} \times 1000$	Primer (µL)	Water (µL)	Stock (µL)	Water (µL)
KP18S4_f	GCTCAAAGCAGGCGTTACGTC	458.4	21	2485.099	8.0	192.0	20	180
KP18S4_r	CAAGATTCCCCATCCCTCTCG	453.3	21	2457.451	8.1	191.9	20	180
KT18S6_f	CTCAACCAACTGGCCTCG	357.3	18	2260.846	8.9	191.1	20	180
KT18S1_r	CGTCAATTTCTTTAAATTTGG	455.9	21	2472.546	8.1	191.9	20	180

*A final concentration of 0.3 µM of each primer was used in the PCR reactions

4.2.5 New primers designed for qPCR

Two custom TaqMan® single nucleotide polymorphism (SNP) genotyping assays were designed with the assistance of CPGR (Cape Town). Both assays were designed from the nucleotide sequence of the ssRNA genes of *K. paniformis* and *K. thyrsites* (Addendums C and D). This was done by aligning the *K. paniformis* 18 SSU rDNA partial sequence (AF034640 GenBank accession number) (Meng & Li-Chan, 2007) published in the National Centre for Biotechnology Information (NCBI) database with other *Kudoa* species, including: *K. inornata* (FJ790311.1), *K. megacapsula* (AB188529.1), *K. dianae* (AF414629.1), *K. paraquadricornis* (FJ792719.1), *K. trifolia*, (AM183300.2), *K. quadricornis* (FJ792721.1 and AY078428.1.1), and *K. thyrsites* (AY542481.1, AY54482.1 and AY941819.1). The same was done for the 18 SSU rDNA partial sequence of *K. thyrsites*. The alignment allowed for identification of unique sequences that was used for *K. paniformis* and *K. thyrsites* specific primers, as well as unique SNP sites (Addendum D), used as probe sites in the TaqMan® assays.

The *K. paniformis* assay mix contained two PCR primers (forward primer: 5'-CATGGATAACTGTGGTAAATCTAGAGCTAA-3' and reverse primer: 5'-AGAAATTCG-ATCGGACTATCGACAA-3') that flanked the region containing the single SNP site specific to *K. paniformis* (5'-ACTCTT-3'; Addendum D) and two TaqMan® probes. The one probed matched the heterologous allelic variant *K. paniformis* (5'-CTCAACCAACTGACTCTT-3'; Addendum C) and was labelled with the fluorescent dye, VIC*, while the second probe was designed for the homologous allelic variant: *K. thyrsites*/*K. inornata*/*K. megacapsula* (5'-CTGGCCTCGGTCATT-3') and was labelled with the fluorescent reporter dye, 6-carboxyfluorescein (FAM). Both probes contained a fluorescent quencher (NRQ). The *K. thyrsites* assay mix contained two PCR primers (5'-CCTATCAACTAGTTGGTGAGGTAGTG-3' and 5'-TCTCCGGAATCGAACCCTGAT-3') that flanked the region containing the SNP site specific to *K. thyrsites* (5'-GTTA-3'; Addendum D). In addition, the *K. thyrsites* assay mix contained two TaqMan® probes, one that matched the heterologous allelic variant *K. thyrsites* and labelled with VIC (5'-ACCCGTTAACACCTTG-3'), the other probe (5'-CGTCACAACCTTG-3'; Addendum C) matched the homologous allelic variant *K. paniformis*/*K. miniauriculata*/*K. dianae* and was labelled with FAM. The assay mixes also contained a fluorescent quencher (NRQ). Both assays are summarised in Table 4.2. A no template control (NTC), containing no DNA, was included in each assay.

* VIC = a fluorescent dye, proprietary to Life Technologies. Its chemical structure is currently (August, 2017) not publicly available (Anon., 2017).

Table 4.2 Single nucleotide polymorphism (SNP) assay information for *Kudoa thyrsites* and *K. paniformis,* respectively

Assay ID	***Kudoa thyrsites***	***Kudoa paniformis***
Assay mix concentration*	40x / 36 µM	40x / 36 µM
Forward primer sequence	5'-CCTATCAACTAGTTGGTGAGGTAGTG-3' Oligo ID: 17-1394	5'-CATGGATAACTGTGGTAAATCTAGAGCTAA-3' Oligo ID: 17-1396
Reverse primer sequence	5'-TCTCCGGAATCGAACCCTGAT-3' Oligo ID: 17-1395	5'-AGAAATTCGATCGGACTATCGACAA-3' Oligo ID: 17-1397
Reporter 1 sequence	5'- ACCCGTTAACACCTTG-3'	5'-CTCAACCAACTGACTCTT-3'
Reporter 1 dye	VIC	VIC
Reporter 2 sequence	5'-CGTCACAACCTTG-3'	5'-CTGGCCTCGGTCATT-3'
Reporter 2 dye	FAM	FAM
Reporter concentration	8 µM	8 µM
Reporter quencher	NRQ	NRQ
PCR product size	77 base pairs	161 base pairs

*Assay mixes were supplied as 40X stocks and stored at -20°C. A 20x working solution of each assay was prepared by diluting 50 µL of the 40X stock with 50 µL of TE (Tris-EDTA) buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA) and stored at -20°C as 20 µL aliquots.

ID = Identification
Oligo ID = refers to the oligomer identification number used in the primer development and specificity test (Table 4.3)
VIC = a fluorescent dye, proprietary to Life Technologies. Its chemical structure is currently (August, 2017) not publicly available (Anon., 2017)
FAM = 6-carboxyfluorescein (McGuigan & Ralston, 2002)
NRQ = fluorescent quencher

4.2.6 Primer specificity

4.2.6.1 Conventional PCR primers

The primer pairs used for the PCR assays were subjected to sequencing using homology searches using the Basic Local Alignment Search Tool (BLAST) on the National Centre for Biotechnology Information (NCBI; https://ncbi.nlm.nih.gov/) website.

4.2.6.1 qPCR primers

The specificity of the two primer pairs (17-1396 and 17-1397, and 17-1394 and 17-1395; Table, 4.3; Figure 4.1), which were designed to target both *K. thyrsites* and *K. paniformis* by amplifying two fragments representing *Kudoa* 18 SSU rRNA gene sequences, were tested by amplifying twenty randomly selected sardine DNA samples previously analysed with the qPCR assays (according to qPCR assay results: 16 samples were positive for *K. thyrsites* and four were negative; while none were positive for *K. paniformis*).

Table 4.3 PCR primers used for the amplification of *K. thyrsites* and *K. paniformis* DNA 18 SSU rRNA gene

Oligo ID	Oligo sequence	PCR product size (base pairs)	Used in qPCR assay mix
17-1394	CCT ATC ACC TAG TTG GTG AGG TAG TG	77	*K. thyrsites*
17-1395	TCT CCG GAA TCG AAC CCT GAT		*K. thyrsites*
17-1396	CAT GGA TAA CTG TGG TAA ATC TAG AGC TAA	161	*K. paniformis*
17-1397	AGA AAT TCG ATC GGA CTA TCG ACA A		*K. paniformis*

Primers 17-1396 and 17-1397 targeted a fragment of 161 base pairs (bp) from both *Kudoa* species. A partial nucleotide sequence alignment of both targets (Figure 4.1) showed that each species had a unique six nucleotide sequence (GCCTCG/ACTCCT; *K. thyrsites*/*K. paniformis*) that could be used to distinguish *K. thyrsites* from *K. paniformis*. Primers 17-1394 and 17-1395 targeted a fragment of 77 bp from both *K. thyrsites* and *K. paniformis*. A partial nucleotide sequence of this fragment showed that each species had a unique four nucleotide sequence (GTTA/TGTG; *K. thyrsites*/*K. paniformis*) that could be used to distinguish between the two *Kudoa* species. These two regions (GCCTCG/ACTCCT and GTAA/TGTG) were used for the genotyping qPCR assays to distinguish between *K. thyrsites* and *K. paniformis* as described above. PCR amplifications were performed in 100 µL total reaction volume using the KAPA HiFi PCR kit (KAPA, Biosystems) as per the manufacturer's instructions. The PCR cycling parameters were: 95°C for 3 min, followed by 98°C for 20 s, 60°C for 15 s, 35 cycles of 72°C for 15 s, 72°C for 1 min and stored at 4°C. Ten µL of each PCR product was visualised by agarose gel electrophoresis along with DNA ladders. Four PCR products of two samples were submitted to the

Central Analytical Facility (CAF, Stellenbosch University) for purification (removal of PCR reaction components) and Sanger sequencing. Both strands of each PCR product were sequenced using each PCR primer. The resulting sequences were compared with published nucleotide sequences using BLAST on the NCBI website.

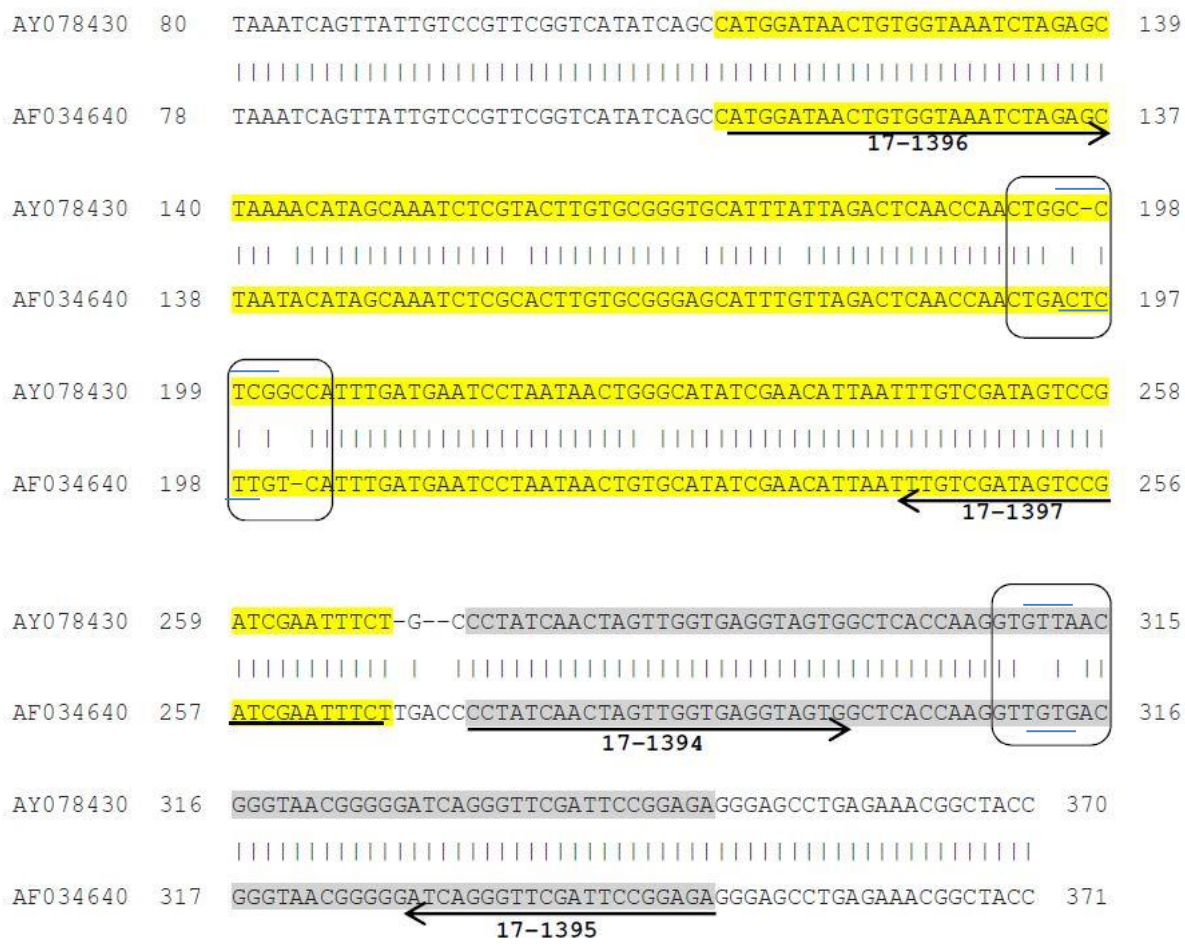

Figure 4.1 Partial sequence alignment of the *K. thyrsites* (GenBank AY078430) and *K. paniformis* (GenBank AF034640) SSU rRNA genes showing the nucleotide regions amplified by the two sets of PCR primers for the qPCR assays. Primers 17-1396 and 17-1397 amplify a fragment of 161 base pairs (shaded in yellow) that showed six nucleotide differences (GCCTCG/ACTCTT, positions 196-203) between the two sequences (boxed). Primers 17-1394 and 17-1395 amplified a fragment of 77 base pairs (shaded in grey) that contained three nucleotide differences (GTTA/TGTG, positions 310-313) between the two sequences (boxed).

4.2.7 DNA amplification, identification and sequencing

4.2.7.1 Conventional PCR

For PCR amplification, KAPA HiFi™ Taq (KAPA HiFi™ PCR Kit, KAPABiosystems, LASECSA) was used. The PCR of the DNA extracted from the Cape snoek samples were performed in a volume of 25 µL containing 5 µL KAPA HiFi buffer (with 2 mM $MgCl_2$), 0.75 µL dNTP mixture (0.3 mM), 0.75 µL of each primer (final concentration of 0.3 µM), 1 µL of extracted DNA, and 0.5 µL KAPA HiFi DNA

polymerase (0.5 U/25 µL). For PCR with *K. paniformis* primers, DNA was denatured at 95ºC for 5 min, followed by 35 cycles of amplification (98ºC for 20 s, 50ºC for 15 s and 72ºC for 60 s), and a final extension at 72ºC for 5 min. For PCR with *K. thyrsites* primers, an annealing temperature of 55ºC was used (instead of 50ºC), while all other conditions remained the same. A positive control for *K. thyrsites*, based on microscopic examinations and identification of mature spores (section 4.2.2), was included in each assay and a negative control, i.e. water, was included to monitor contamination. The amplified DNA segments were loaded onto a 1.5% agarose gel for electrophoresis. A low-range 1 kilobases (kb) DNA ladder (MassRuler Low Range DNA ladder, Inqaba Biotec) was used as a molecular weight marker. The corresponding PCR products at the estimated product sizes (728 bp for *K. paniformis*, and 908 bp for *K. thyrsites*; Meng & Li-Chan, 2007) were cut out of the gel for DNA extractions and Sanger sequencing analyses. Sequencing was done by the DNA sequencing unit, Central Analytical Facility (CAF, Stellenbosch). The resulting sequences were compared with sequences reported on NCBI using BLAST.

4.2.7.2 qPCR

The snoek DNA samples shown to express *K. thyrsites* from PCR analysis and DNA sequencing were used to prepare two sample pools and were used as positive controls for *K. thyrsites* in the qPCR assays. The concentration and purity of the two control samples were analysed with the use of a Nanodrop spectrophotometer (NanoDrop ND-1000, Thermo Fisher Scientific, USA). The control samples were then diluted with distilled water to prepare the DNA at 10 ng·µL^{-1} working concentrations.

It is notewothy that the extracted DNA from the six Cape snoek samples were not sequenced with the use of the qPCR assays due to unforeseen loss of the samples. Extracted DNA from the SA sardine samples in this research chapter were not amplified with the confentional PCR because of time and funding constraints. In addition, this study did not attempt to compare conventional PCR with qPCR. The DNA extracted from the SA sardine samples were analysed by qPCR using TaqMan® assays. All TaqMan® assays were performed in 384-well plates by amplifying 20 ng DNA in a 5 µL volume PCR reaction mix containing 0.9 µM primers and 0.2 µM probes. The assay mixes (containing the primers and probes) were supplied as 40x stock solutions (Table 4.2) and stored at -20°C. A 20x working solution of each assay was prepared by diluting 50 µL of the 40x stock solution with 50 µL of TE buffer (10 mM Tris-HCl pH 8.0; 1 mM EDTA) and stored at -20°C as 20 µL aliquots). A working master mix for each sample in each assay was prepared by mixing 0.25 µL of TaqMan® assay mix (20x), 2.5 µL TaqMan® Genotyping Master Mix (Thermo Fisher) and 0.25 µL of water (2.25 µL of water for the no template controls) and 2 µL of genomic DNA at 20 ng. Reactions were performed with the following protocol on the 7900HT Fast Real-Time PCR System: a pre-reading of the plate to record the background fluorescence; followed by the standard PCR cycling parameters: 95°C for 10 min, followed by 40 cycles

of 95°C for 15 s and 60°C for 1 min and lastly, the post-reading of the plate. End-point fluorescence was read on the 7900HT Fast Real-Time PCR System using the SDS version 2.3 software (Applied Biosystems, USA). The TaqMan® Genotyper software version 1.3 (Life Technologies Corporation, USA) was used to generate allelic discrimination plots for the alleles: *K. thyrsites* (allele 1 - labelled with VIC) and *K. paniformis*/*K. miniauriculata*/*K. dianae* (allele 2 - labelled with FAM), and for the heterologous alleles: *K. paniformis* (allele 1 - labelled with VIC) and *K. thyrsites*/*K. inornata*/*K. megacapsula*, (allele 2 - labelled with FAM), respectively. Within the allelic discrimination plots, the fluorescent signal from each individual DNA sample was represented as an independent data point on the plot. The x-axis was used to plot the VIC fluorescence value (allele 1) and the y-axis was used to plot the FAM fluorescence value (allele 2) for each sample within the two respective assays. Samples that showed presence of *K. thyrsites* only generated VIC fluorescence during the SNP assay for *K. thyrsites* (allele 1 - labelled with VIC) and *K. paniformis*/*K. miniauriculata*/*K. dianae* (allele 2 - labelled with FAM), while samples positive for *K. paniformis* only generated VIC fluorescence during the SNP assay for *K. paniformis* (allele 1 - labelled with VIC) and *K. thyrsites*, *K. inornata* and *K. megacapsula*, (allele 2 - labelled with FAM). The SDS software (version 2.3) analyses the before (pre-read) and the after (post-read) fluorescence levels and calculates normalised dye fluorescence (∆Rn) as a function of the cycle number for allele 1 and allele 2 within each SNP assay. The normalised dye fluorescence (∆Rn) is the amount of fluorescence generated from the amplified reaction normalised for the fluorescence from the unreacted probe (McGuigan & Ralston, 2002).

4.3 Results and discussion

4.3.1 Microscopic and PCR analysis of Cape snoek samples

Mature spores of *K. thyrsites* (Figure 4.2A) was observed in three of the six Cape snoek samples. No spores resembling those associated with *K. paniformis* was observed in any of the Cape snoek samples. The observed spores were similar in shape to spores of *K. thyrsites* as described by Langdon (1991), Moran *et al*. (1999a) and Levsen *et al*. (2008). The mature spores showed one large, one opposed small, and two intermediate-sized polar capsules arranged with convergent apices within four valves (Figure 4.2A).

Microscopic examination of wet-mounts from the Cape snoek samples (no. 4 and 8) with severe myoliquefaction (Figure 4.2C and D) showed many *K. thyrsites* spores (Figure 4.2A). Based on the microscopic evaluations, 50% of the Cape snoek samples were identified as being infected with *K. thyrsites*; while PCR results (Figure 4.3) showed that five of the six (83%) Cape snoek samples were infected with *K. thyrsites*. PCR methods are capable of detecting infection by *Kudoa* species at stages when no or low levels of spores are present, or when the microscopic method is unable to detect the spores. Studies by several researchers (Tsuyuki *et al*., 1982; Hervio *et al*., 1997; Moran *et al*. 1999b;

Moran, Morgalis *et al*., 1999; Young & Jones, 2005) have suggested that early stages of *Kudoa* parasites may be detected by PCR when mature spores are not present.

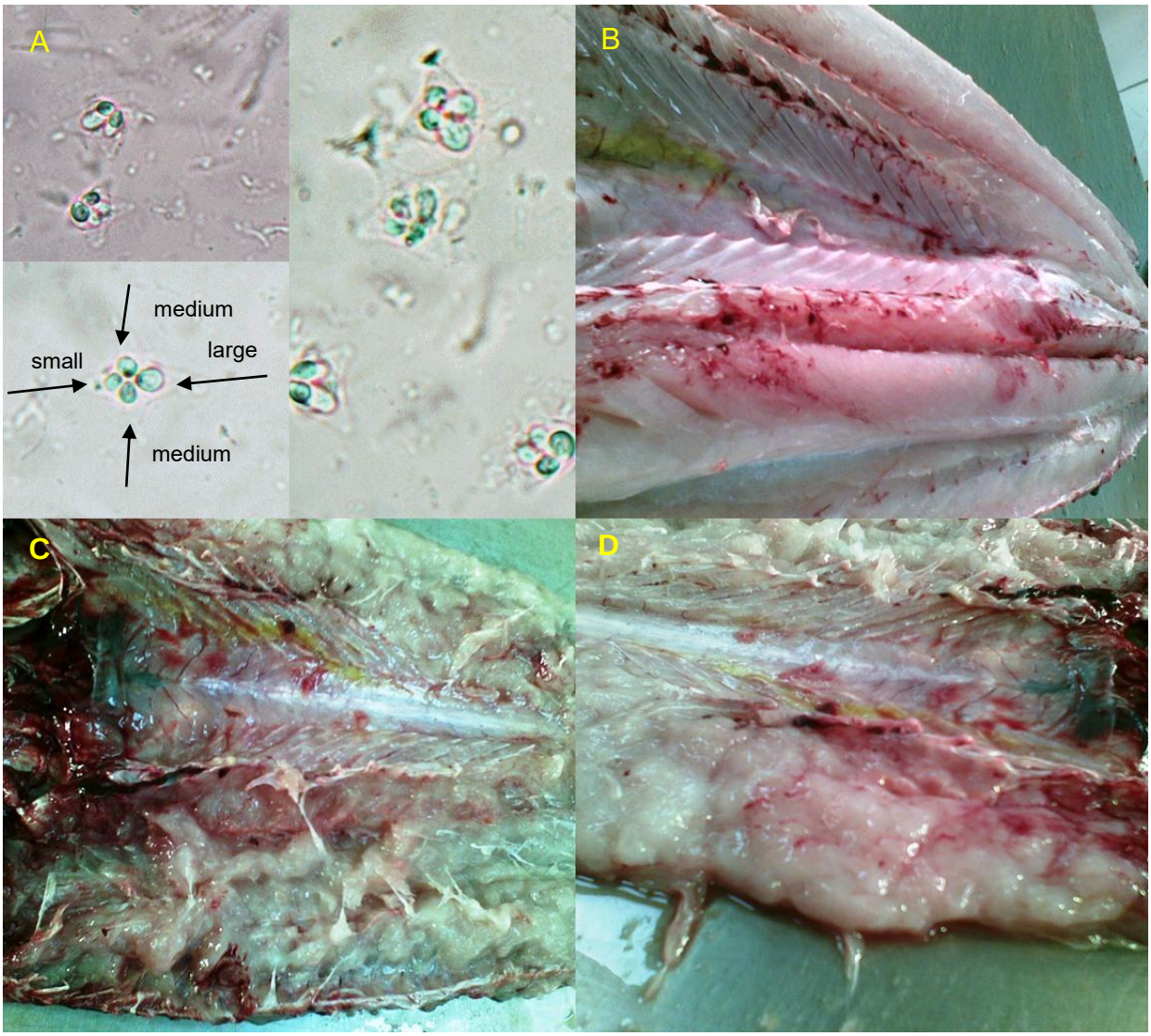

Figure 4.2 (A) Mature *Kudoa thyrsites* spores from Cape snoek samples (images taken with an Olympus CX31, Nikon DS-Fi1 camera at 1000X magnification, image captured with Nikon NisElements Imaging software version 3.22). (B) uninfected Cape snoek, (C) severely myoliquefied Cape snoek muscle (sample no. 8), and (D) less severe myoliquefied Cape snoek muscle (sample no. 4). Photos were taken with a Canon PowerShot S2 IS camera. The arrows indicate one large, one opposed small, and two intermediate-sized polar capsules containing mature spores.

In contrast to the microscopic examinations, PCR results (Figure 4.3) showed the presence of *K. paniformis* in one (no. 8b) of the Cape snoek samples. PCR results indicated that this one sample was also positive for the presence of *K. thyrsites* (Figure 4.3). This sample showed severe myoliquefaction (Figure 4.2C; sample no. 8) compared to the other Cape snoek samples which tested positive for only *K. thyrsites* (Figure 4.2D). The absence of *K. paniformis* spores in sample no. 8 could have been simply

because they were missed during examination with the use of the microscope (Hervio *et al*., 1997; Kent *et al*., 2001). Morado and Sparks (1986) documented that *K. paniformis* sometimes occurs simultaneously with *K. thyrsites* in Pacific hake. It is suggested that fish infected with *K. paniformis* undergo accelerated myoliquefaction because *K. paniformis* produces both acidic and neutral proteolytic enzymes (Tsuyuki *et al*., 1982), while *K. thyrsites* only produces acidic proteolytic enzymes (Stehr & Whitaker, 1986). Since no positive DNA controls (DNA for *K. thyrsites* and *K. paniformis*) were available when this PCR analysis was performed, Sanger sequencing analyses of the PCR products were analysed.

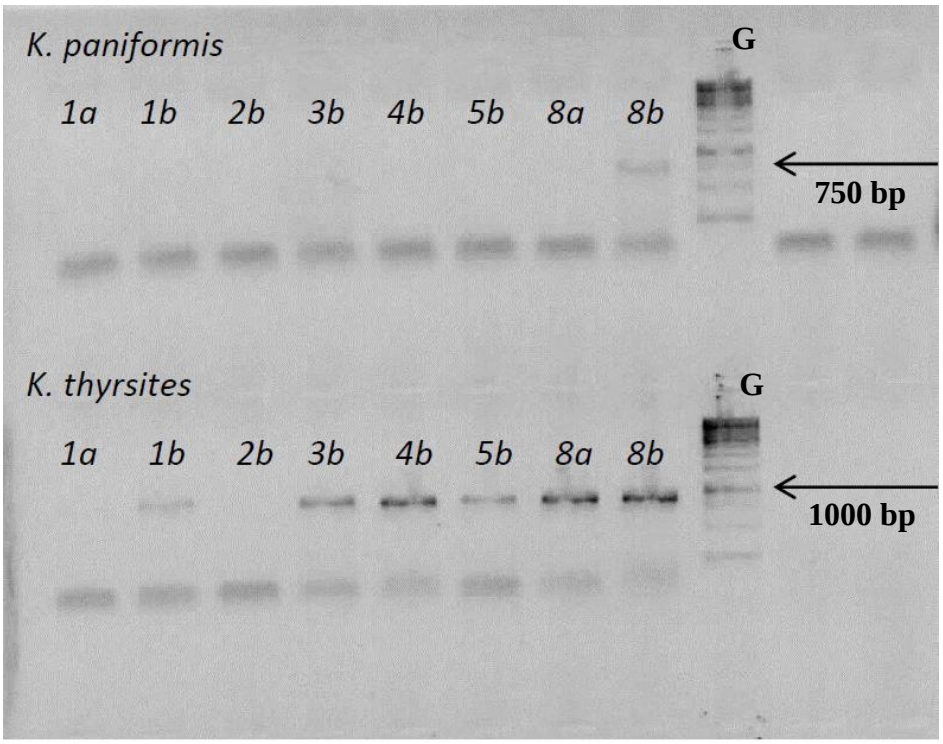

Figure 4.3 Agarose gel electrophoresis image of PCR results for *Kudoa paniformis* and *K. thyrsites* in Cape snoek samples 1a, 1b, 2b, 3b, 4b, 5b, 8a, and 8b (a = DNA extract from fish liquid, b = DNA extract from fish tissue). The black arrows points to 750 and 1000 bp bands, respectively. Amplification of *K. paniformis* and *K. thyrsites* DNA result in a 728 and 908 base pair product, respectively. G indicates the GeneRuler 1 kb DNA ladder (250-10000 bp).

The sequence of the PCR product amplified with the *K. thyrsites* specific PCR primers (Meng & Li-Chan, 2007) displayed high (99%, Table 4.4) homology to *K. thyrsites* entries on GenBank (NCBI nucleotide similarity search). However, sequencing of the PCR products (from sample 8b) amplified with the *K. paniformis* primers did not display homology to any sequence available in the GenBank database (Table 4.4). It could therefore not be stated whether snoek sample no. 8 (Figure 4.2C) was indeed infected with *K. paniformis*.

It is noteworthy to mention here that the number of cycles for amplification during the PCR assays was reduced to 25 (Table 4.4) in order to maximize sequence reliability and reduce the error rate of the *Taq* polymerase enzyme (Lorenz, 2012). However, at low cycle numbers there may have been insufficient product available for sequencing as a very faint product was visualized after gel electrophoresis. This might have contributed to the failed sequencing runs of the *K. paniformis* PCR products.

The PCR was, however, repeated with increased number of cycles (35). The PCR products, along with the DNA ladder, were electrophoresed on a 1% (w/v) agarose gel and the corresponding products at the estimated product sizes were cut out of the gel to ensure that only the product of interest was sequenced. Amplification of *K. paniformis* and *K. thyrsites* DNA resulted in 728 and 908 base pair products, respectively (Figure 4.3). In preliminary studies, wherein temperature gradient PCR amplifications were performed (data not shown), with the aim of optimizing the PCR thermal cycling parameters, annealing temperatures of 50-53˚C resulted in a PCR product of the correct size for *K. paniformis* (728 bp), while an annealing temperature of 55˚C resulted in the correct sized PCR product for *K. thyrsites* (908 bp). If the annealing temperature is too low, it could result in annealing to a number of species and this will result in a mixed PCR population which cannot be resolved properly, consequently producing a non-specific sequence (Lorenz, 2012).

Table 4.4 Sanger sequencing and BLAST results from GenBAnk for *Kudoa thyrsites* and *K. paniformis* PCR products (from Cape snoek muscle tissue)

Primer pair	Cycles*	Sequencing run	Homology on GenBAnk
K. thyrsites	25	>F07_3_KT_F_1.ab1 GSYSSGAAYCTTWWTAACTGGGCATATCGAACATTAATTTGTCGATAGTCCGATCGAATTTCTGCCCTATCAACTAGTT GGTGAGGTAGTGGCTCACCAAGGTGTTAACGGGTAACGGGGGATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAA ACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCAGACTTTGGGAGGTAGTGACGAGAAATA CCGGAGTAGACCGTATATTGGTTCACTATCGGAATGAACGTAATTTAATACCTTCGATGAGTAGCTACTGGAGGGCAAG TCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGTAGTGTATATCAAAATTGTTGCGGTTAAAACGCTCGTAGTTGG ATTACAAAAGCTCTCTGGCGGCCAAATCTAGGTTTGGTCGCCGTGGGGTTTTTTTATCGCGAGAGCCGCATGTGGGAT TAACTTCTTGTGTGCGGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTCAAAGCAGGCGATAGCTCGAATGT TATAGCATGGAACGATYATGTTGATCTTGTCGACTGTTGGTTGTTGACATTGGTCTCKATTAAAAGGGACATTTGAGGG CGTTAGTACTTGGTGGCGAGGGGTGACATYCTWAGACCCATCAAAKACTAACTAATGCGAAAGCCTTCRCCAAGAGTG GTTTKTKTTMTCCAAGT	Sequence ID: gb|AY941819.1| *Kudoa thyrsites* 18S small subunit ribosomal RNA gene, partial sequence *Identities: 634/636(99%)*
K. thyrsites	25	>G07_4_KT_F_1.ab1 GKSSGTTCMYTWWTWACTGGGCATATCGAACATTAATTTGTCGATAGTCCGATCGAATTTCTGCCCTATCAACTAGTT GGTGAGGTAGTGGCTCACCAAGGTGTTAACGGGTAACGGGGGATCAGGGTTCGATTCCGGAGAGGGAGCCTGAGAA ACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATCCAGACTTTGGGAGGTAGTGACGAGAAATA CCGGAGTAGACCGTATATTGGTTCACTATCGGAATGAACGTAATTTAATACCTTCGATGAGTAGCTACTGGAGGGCAAG TCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGTAGTGTATATCAAAATTGTTGCGGTTAAAACGCTCGTAGTTGG ATTACAAAAGCTCTCTGGCGGCCAAATCTAGGTTTGGTCGCCGTGGGGTTTTTTTATCGCKAGAGCCGCATGTGGGAT TAACTTCTTGTGTGCGGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTCAAAGCAGGCRATAGCTCGAATGT TATAGCATGGAACSATTATGTTGATCTTGTCKACTGTTGGTTGTTGACATTGGTCTCGATTAAAAGGGACATTTGARGGC GTTAGAACTTTGGTGGYKAGGGGTGAKCTCCCTACACCCATCAAAGTCTAACTRG	Sequence ID: gb|AY941819.1| *Kudoa thyrsites* 18S small subunit ribosomal RNA gene, partial sequence Identities: 680/688(99%)
K. paniformis	25	>E07_2_KP_F_1.ab1 NNNNN	*Not applicable*
K. paniformis	25	>D07_1_KP_F _1.ab1 NNNNN	*Not applicable*
K. paniformis	35	>B03_2_KP_F_1.ab1 NNNNN	*Not applicable*
K. paniformis	35	>A03_1_KP_F_1.ab1 GGCCCGGGAGGTACCCCCTGGTTATCGRATSCCTCGAGAGCMGGAACAATGGTCTCTAAAKATTGAMTTGTAGGGCC KAGTGWGMCCGGMTRGGGTGAWSCCWCTAATATTTACACKGAGAATGGCCGCTTTWAAKGCTGTTSCCGAATGAGA TCGAAGGTTRATTTCAAAGACAACAAYTTGRAGCATTTGCTCTCCCCGAAGCCMCTTGGTGCCCGGGTTATTTCGTGTC GCCACTGGKTTMTGGCTTCCTCCAGGGGACGATCCTWTCTGCCAKTYCGGCTATTACCTTTCCTTACGTGCTAAT	None

*Cycles refer to the number of annealing cycles used to amplify the PCR products

The primer pairs used for the PCR assays were also subjected to sequencing using BLAST homology searches. Results from the BLAST search tool indicated that the PCR primers for *K. paniformis* may also amplify *K. megacapsula* and *K. inornata* (Table 4.5; Addendum C) and that of *K. thyrsites* could potentially also amplify *K. megacapsula*, *K. quadricornis* and *K. paraquadricornis* (Table 4.6). However, the sequencing results of the PCR products from the Cape snoek DNA confirmed that *K. thyrsites* was present in the samples and none of the other above-mentioned *Kudoa* species. According to Dyková *et al*. (in McElroy *et al*., 2015), the myxozoan, *K. inornata*, commonly infects the white myotomal muscle of spotted seatrout (*Cynoscian nebulosus*) and forms plasmodia containing myxospores. These spores are released when the fish dies and the muscle starts to degrade. McElroy *et al*. (2015) reported that infection by *K. inornata* enhanced the swimming performance of spotted seatrout. *Kudoa megacapsula* is also associated with *post-mortem* myoliquefaction in marine fish species and has been reported by Yokoyama and Itoh (2005) to be present in SA splendid alfonso (*Beryx splendens*). *Kudoa megacapsula* can be distinguished from other *Kudoa* species described to date by the presence of the very large polar capsule and the distinct four wing-like projections (Yokoyama & Itoh, 2005).

Based on the above alignment of primer sequences and the theoretical low specificity of the PCR primers used in this study, two custom TaqMan® SNP genotyping assays were designed to be used as a fast, cost-saving, and qualitative analysis of *K. thyrsites* and *K. paniformis* in SA sardine samples.

Table 4.5 Comparison of *Kudoa paniformis* primer sequences relative to other species indicated it may also amplify *K. megacapsula* and *K. inornata.* Yellow highlighted areas indicate base pair differences between the primer sequences relative to *K. paniformis*. The 3' end of the primer is important to prevent mispriming as it creates a clamp for the *Taq* polymerase to begin synthesise of the sequence

Kudoa species	Accession	Forward Primer (5'-3')	Reverse Primer (reverse comp, 3'-5')
K. paniformis	AF034640.1	GCTCAAAGCAGGCGTTACGTC	CGAGAGGGATGGGGAATCTTG
K. miniauriculata	AF034639.1	GCTCAAAGCAGGCGTTACGTC	tGAGAGGGgTGGGaAATCTTa
K. inornata	FJ790311.1	GCTCAAAGCAGGCGTTAgcTC	CGAGAGGGgTGGGGAATCTTG
Kudoa sp. CMW-2003	AY302723.1	GCTCAAAGCAGGCGTTAgcTC	tGAGAGGGgTGGGaAATCTTa
K. dianae	AF414692.1	GCTCAAAGCAGGCGTTAgcTC	tGAGAGGGgTGGGGAATCTTa
K. paraquadricornis	FJ792719.1	GCTCAAAGCAGGCGTTAgcTt	tGAGAGGGgTGGGGAATCTTG
K. trifolia	AM183300.2	GCTCAAAGCAGGCGTTAgcTt	tGAGAGGGgTGGGGAATCTTG
K. quadricornis	FJ792721.1	GCTCAAAGCAGGCGTTAgcTt	tGAGAGGGgTGGGGAATCTTG
K. megacapsula	AB188529.1	GCTCAAAGCAGGCGaTAgcTt	tGAGAGGagTGGGrAATCTTG
K. thyrsites	AY542481.1	GCTCAAAGCAGGCGaTAgcTC	tGAGAGGagTGGGGAATCTTG

Table 4.6 Comparison of *Kudoa thyrsites* primer sequences relative to other species indicated that it may be able to also amplify *K. megacapsula*, *K. quadricornis*, and *K. paraquadricornis.* Yellow highlighting indicates base pair differences between the primer sequences relative to *K. thyrsites*. The 3' end of the primer is important to prevent mispriming as it creates a clamp for the *Taq* polymerase to begin synthesis of the sequence

Kudoa species	Accession	Forward Primer (5'-3')	Reverse Primer (reverse comp, 3'-5')
K. thyrsites	AY542481.1	CTCAACCAACTGgCctcg	ccAAATTTAAAGAAATTGACG
K. megacapsula	AB188529.1	CTCAACCAACTGgCctcg	ccAAATTTAAAGAAATTGACG
K. quadricornis	FJ792721.1	CTCAACCAACTGgCTtcg	cTAAATTTAAAGAAATTGACG
K. trifolia	AM183300.2	CTCAACCAACTGgCcCTT	cTAAATTTAAAGAAATTGACG
K. paraquadricornis	FJ792719.1	CTCAACCAACTGgCTtcg	cTAAATTTAAAGAAATTGACG
K. dianae	AF414692.1	CTCAACCAACTGACctcg	GTAAATTTAAAGAAATTGACG
Kudoa sp. CMW-2003	AY302723.1	CTCAACCAACTGACTtTg	GTAAATTTAAAGAAATTGACG
K. inornata	FJ790311.1	CTCAACCAACTGgCctcg	GTAAATTTAAAGAAATTGACG
K. miniauriculata	AF034639.1	CTCAACCAACTGACTtTg	GTAAATTTAAAGAAATTGACG
K. paniformis	AF034640.1	CTCAACCAACTGACTCTT	GTAAATTTAAAGAAATTGACG

4.3.2 Analyses of qPCR results for SA sardine samples

4.3.2.1 Verifying qPCR primers specificity

The PCR products of *K. thyrsites* and *K. paniformis* amplifications, using the two qPCR primer sets, on the selected 20 SA sardine DNA samples, are illustrated in Figure 4.4. PCR amplification using the 17-1396 and 17-1397 primers yielded the expected fragment of 161 bp from two of the SA sardine DNA samples (samples no. 2 and 5; Figure 4.5A).

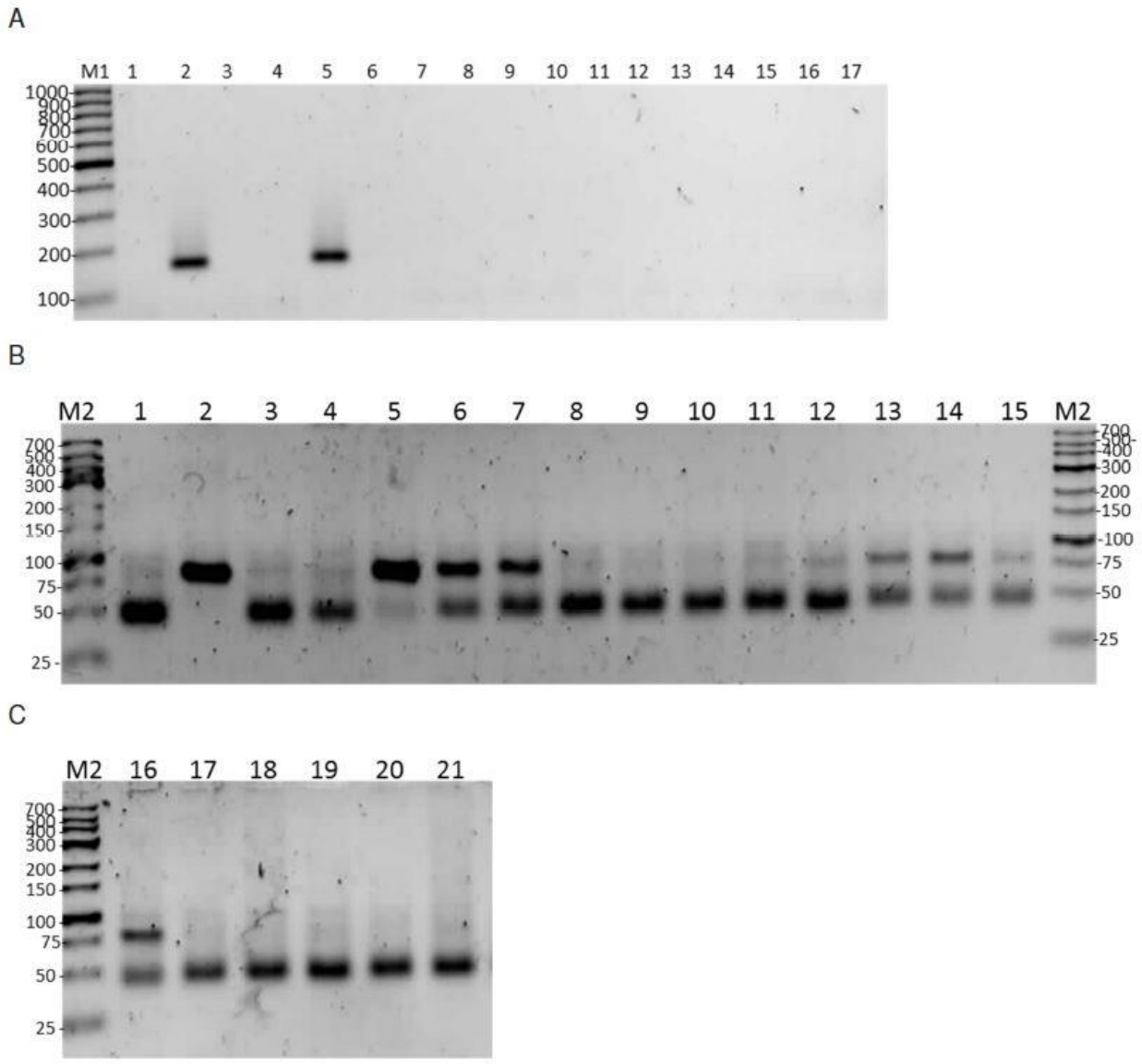

Figure 4.4 PCR amplifications of the *K. thyrsites* and *K. paniformis* SSU rRNA genes with primers 17-1396 and 17-1397 (A) and 17-1394 and 17-1395 (B, C). The PCR products with primers 17-1396 and 17-1397 (A) were analysed on a 2% agarose gel along with a 100 base pare DNA ladder (M1) and the PCR products with primer 17-1394 and 17-1395 (B, C) were electrophoresed on a 4% agarose gel along with a low range DNA ladder (M2). Sample no. 21 represented the no template control (NTC).

Seven of the SA sardine DNA samples showed positive results for the PCR amplification using the second primer set, 17-1394 and 17-1395 (Figure 4.5B). These primers yielded the expected fragment of 77 bp. However, with the exception of two samples (no. 2 and 5), the majority of the

samples also showed the amplification of primer dimers. The PCR products of samples no. 2 and 5 were therefore selected for further characterisation by DNA sequencing and identification with the use of the BLAST tool on the NCBI website.

The two 161 bp PCR products obtained using the 17-1396/17-1397 primer pair were each sequenced in both directions using each of these two primers for each direction. Similarly, for the two 77 bp PCR products obtained with the 17-1394/17-1395 primer pair, sequence analysis was done using each of the two primers for each direction. This analysis resulted in eight different DNA sequence segments (two from primer 17-1396, two from primer 17-1397, two from primer 17-1394, and two from primer 17-1395). These segments were compared to published nucleotide sequences using BLAST on the NBCI website.

All the nucleotide sequences of the 161 bp PCR products showed highly homologous identities (99%) to the *K. thyrsites* 18 SSU rRNA gene sequence (GenBank AY078430; Figure 4.5A) and to the *K. megacapsula* (98% homology) gene for 18 SSU rRNA (GenBank AB263074; Figure 4.5B). These results are consistent with the data obtained from the qPCR genotyping assays, verifying the presence of *K. thyrsites* in 204 SA sardine samples (Figures 4.6 and 4.7). The primer pair 17-1396/17-1397, yielding a PCR product of 161 bp, was therefore sensitive towards *K. thyrsites*. The primer pair used in the *K. paniformis* assay was therefore able to distinguish between the homologous allele *K. thyrsites*/*K. inornata*/*K. megacapsula* and the heterologous allele *K. paniformis*.

In contrast, all the nucleotide sequences of the 77 bp PCR products had no significant homologies to any nucleotide sequences in the NCBI database. The sequence data for this PCR product were of poor quality which could explain the NCBI results. In addition, the small size (77 bp) of the PCR product may also have contributed to the poor quality of the sequencing data. This region, however, could be amplified using primers 17-1396 and 17-1395 to produce a 240 bp PCR product (Figure 4.1) which will be large enough for sequencing. It is noteworthy that amplification of the 77 bp PCR product using primers 17-1396 and 17-1395 was not performed due to unforeseen loss of the samples.

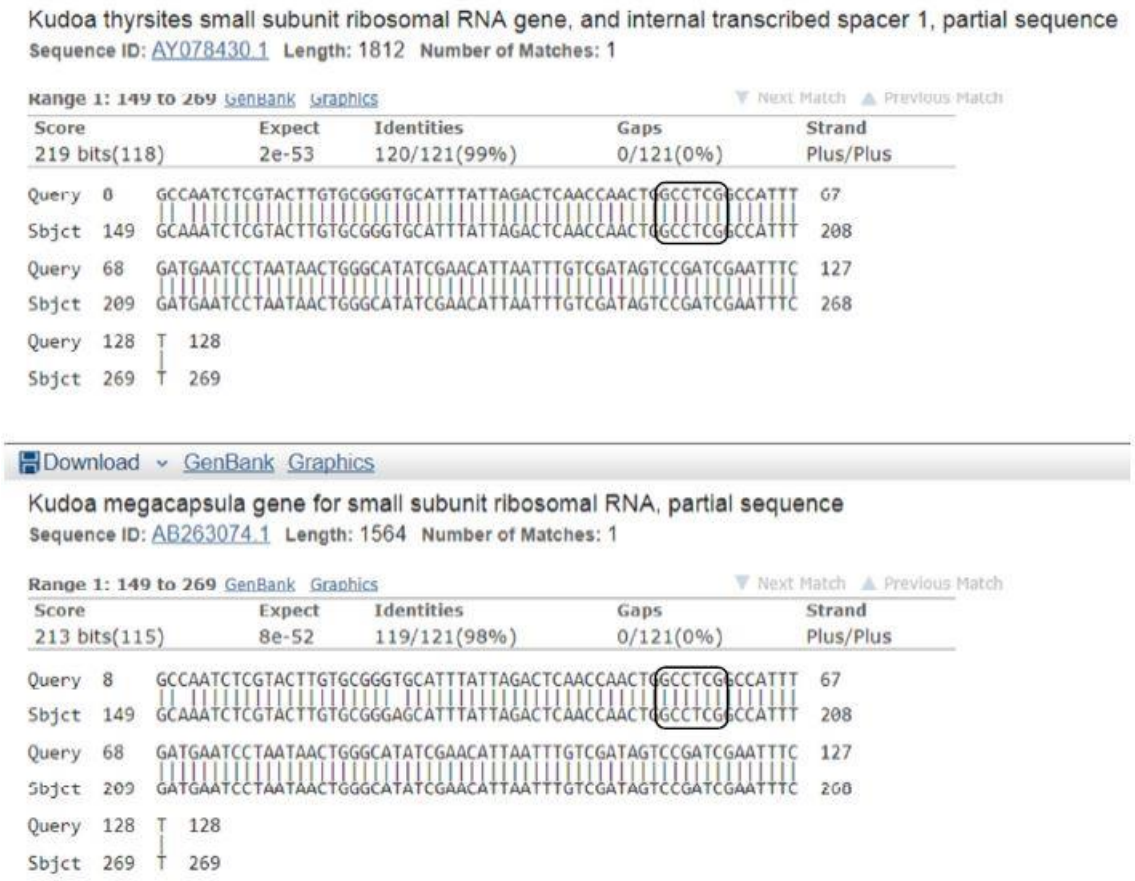

Figure 4.5 Representative result for the BLAST analysis of the 161 bp PCR product (from sample no. 2) sequenced using the 17-1396 primer. Shown are sequence alignments of the top two matches ('Query' represents the sample no. 2 sequence; 'Sbjct' represents the matching sequence in the NCBI database). The boxed regions of the sequence alignments indicated the sex nucleotide sequence (GCCTCG) that distinguishes the *K. thyrsites* SSU rRNA gene sequence from the *K. paniformis* SSU rRNA gene. Please note: BLAST analysis using the sample no. 5 sequence gave exactly the same result.

4.3.2.2 Presence of K. thyrsites *in SA sardine samples*

When analysing the allelic discrimination plots for the qPCR analyses of the DNA samples extracted from the SA sardines, 204 samples (88%) tested positive for the *K. thyrsites* allele (Figure 4.6). These 204 samples also tested positive for the *K. thyrsites*/*K. inornata*/*K. megacapsula* allele within the *K. paniformis* assay (Figures 4.7). None of the SA sardine samples tested positive for the homologous allele *K. paniformis*/*K. miniauriculata*/*K. dianae* (Figure 4.6) in the *K. thyrsites* assay. This indicated that the primer pairs for these qPCR assays were able to distinguish between *K. thyrsites* and *K. miniauriculata*, which has been a challenge in studies (Funk *et al*., 2007) using 18S rDNA primers. Analysis of the 18S rDNA sequences of *K. thyrsites* often cluster it together with *K. miniauriculata* and *K. paniformis* (Kent *et al*., 2001; Whipps *et al*., 2004; Tables 4.4 and 4.5).

Five of the SA sardine samples (2%) were indicated to be infected with *K. paniformis* (Figure 4.7) in the *K. paniformis* assay. It is noted that, due to the shortcoming of not verifying the presence of *K. paniformis* in these five sardine samples, the presence of this *Kudoa* species could therefore not be stated with certainty. In addition, results from the *K. thyrsites* assay indicated absence of the *K. paniformis*/*K. miniauriculata*/*K. dianae* allele for these five sardine samples. It was therefore

uncertain whether the positive results for *K. paniformis* in these five samples were due to primer dimers, or due to the presence of other *Kudoa* species. It would have been valuable to have amplified the DNA of these five sardine samples by PCR, using the 17-1396/17-1397 primer set, and to verify the PCR products by nucleotide sequencing and comparison with published nucleotide sequences using BLAST on the NCBI website. It is noteworthy that amplification and subsequent sequencing of the above-mentioned five sardine samples were not performed due to unforeseen loss of the samples.

The primer pair (17-1394/17-1395), which was used in the *K. thyrsites* assay mix, yielded a PCR product of 77 bp in size (Figure 4.4 B, C). Although comparison of the nucleotide sequencing results of this small PCR product (section 4.3.2.1) on the NCBI website did not show homology to *K. thyrsites*, the primer pair (17-1396/17-1397), which was used in the *K. paniformis* assay mixed, showed 99% homology to *K. thyrsites* (Figure 4.5). Therefore, the SA sardine samples that tested positive for both the *K. thyrsites*/*K. inornata*/*K. megacapsula* allele in the *K. paniformis* assay, and the *K. thyrsites* allele in the *K. thyrsites* assay, were indeed infected with *K. thyrsites* and not another *Kudoa* species.

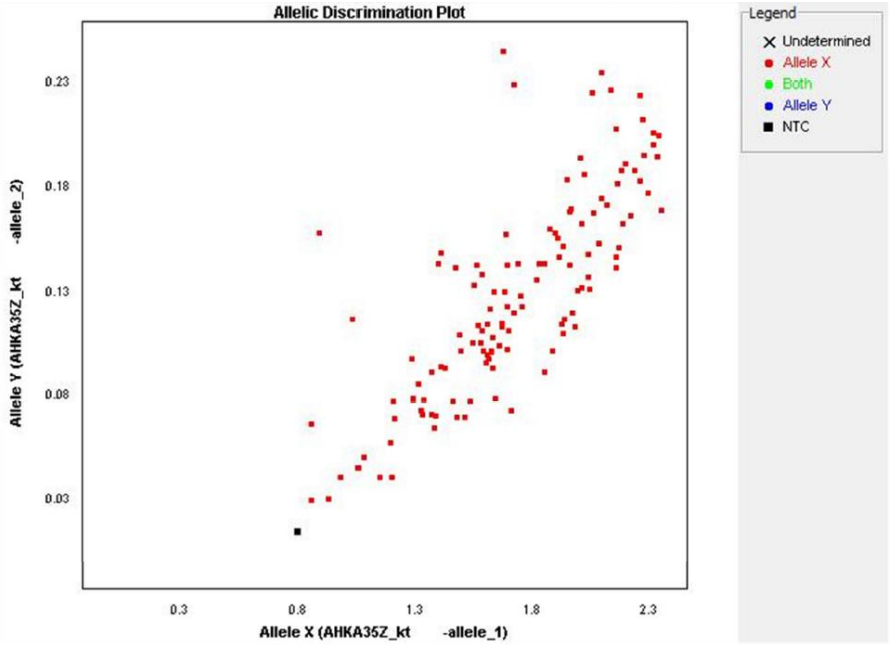

Figure 4.6 Representative allelic discrimination plot for *Kudoa thyrsites* SNP assay for SA sardine samples. Red dots (allele 1) represent samples infected with *K. thyrsites*. Blue dots (allele 2) represent samples that are infected with *Kudoa* parasites: *K. paniformis*/*K. miniauriculata*/*K. dianae*. The black dot represents the no template control (NTC).

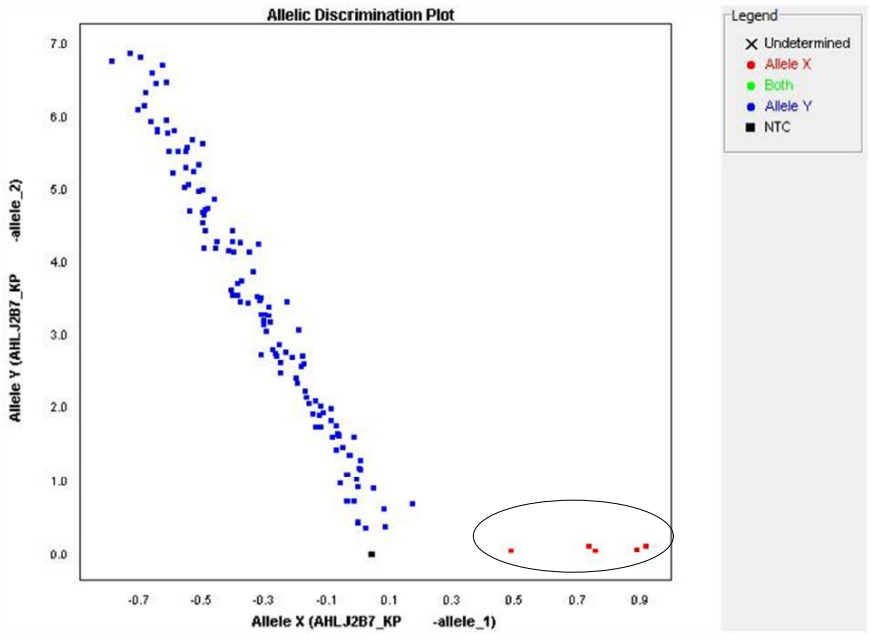

Figure 4.7 Representative allelic discrimination plot for the *Kudoa paniformis* SNP assay for SA sardine samples. The circled red dots (allele 1) represent samples infected with *K. paniformis*. Blue dots (allele 2) represent samples that are infected with *K. thyrsites*/*K. inornata*/*K. megacapsula*. The black dot represents the no template control (NTC).

The percentage (88%) of SA sardines infected with *K. thyrsites* in this study was higher than the 17% (n = 102) reported by Reed *et al*. (2012). These authors, however, used microscopic methods to examine *K. thyrsites* infection of the musculature of SA sardines. PCR is more effective in detecting low levels and early stages (other than mature spores) of infection, which may easily be missed when using microscopic methods (Hervio *et al*., 1997; Kent *et al*., 2001; Funk *et al*., 2007).

4.4 Conclusions

In conclusion, this study confirmed the presence of *K. thyrsites* in Cape snoek and SA sardine samples. This study showed that 83% of the six Cape snoek and 88% of the 204 SA sardine samples tested during this study was positive for *K. thyrsites* infection. The presence of *K. paniformis* could not be verified in one of the Cape snoek samples since sequencing of the PCR products did not display homology to any sequences available in the GenBank database. The qPCR primer pairs, designed for this study to distinguish between *K. thyrsites* and *K. paniformis*, showed to be sensitive for the detection of *K. thyrsites*. The qPCR assays designed for this study showed to be an improvement over the published conventional PCR assays since it demonstrated the ability to distinguish between *K. thyrsites* and the homologous allele *K. paniformis*/*K. miniauriculata*/*K. dianae*. Due to incomplete laboratory analysis and the absence of positive controls for *K. paniformis*, this study was unable to verify sensitivity of the qPCR primers towards the detection of *K. paniformis*. However, it is concluded that the qPCR primers and protocol, as developed for this study, could be used as a fast, cost-effective, qualitive screening tool for the presence of *K. thyrsites*.

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Chapter 5

Relationships between biological factors, season and the prevalence of *Kudoa thyrsites* in South African sardine (*Sardinops sagax ocellatus*), kingklip (*Genypterus capensis*), and Cape hake (*Merluccius capensis* and *M. paradoxus*)

Abstract

The prevalence of *Kudoa thyrsites*, responsible for *post-mortem* myoliquefaction in marine fish species, may be influenced by season, area of capture, size, sex, and species of fish. The prevalence of *K. thyrsites* in South African (SA) sardine, Cape hake, and SA kingklip is not well documented. With the use of custom designed quantitative polymerase chain reaction (qPCR) TaqMan® single nucleotide polymorphism (SNP) genotyping assays the qualitative prevalence of *K. thyrsites* was determined in 267 SA sardines, 57 Cape hake, and 70 SA kingklip samples. Relationships between *K. thyrsites* prevalence and area, season, sex and size were determined with the use of Chi-square tests. *K. thyrsites* prevalence was highest in SA sardines (91%), followed by Cape hake (83%), and then SA kingklip (40%). The prevalence of *K. thyrsites* was independent of sex, season, and area of capture for SA sardine, Cape hake and SA kingklip samples. No significant relationship ($P > 0.05$) between prevalence and size was found for SA sardine and SA kingklip samples. The average size of *K. thyrsites* infected Cape hake samples was significantly ($P < 0.05$) smaller (39.63 ± 10.88 cm) than that of uninfected Cape hake samples (49.46 ± 13.94 cm), however, larger sized (> 60 cm) fish were not well represented in this study. Larger sampling sizes are suggested for further studies into the relationship of *K. thyrsites* prevalence and variables, such as size and season, for Cape hake and SA kingklip. It was concluded that sex was not useful in the prediction of *K. thyrsites* infection in SA sardine, Cape hake and SA kingklip samples. In addition, area of capture, season, and size were not useful indicators of *K. thyrsites* infection in the SA sardine samples.

Keywords: Black pseudocysts, Cape hake, kingklip, *Kudoa thyrsites*, myoliquefaction, prevalence, sardine stocks, season, South African sardine, west coast

5.1 Introduction

The term, “prevalence”, is used to describe the level of parasitic infection as defined by Bush *et al*. (1997): “prevalence is the number of fish infected by a parasite divided by the number of fish examined, and typically expressed as a percentage”. South African (SA) Cape snoek (*Thyrsites atun*) and SA sardine (*Sardinops sagax ocellatus*), also known as pilchards (Grant *et al*., 1998), are documented to be heavily infected and appear to be major host reservoir species for *Kudoa thyrsites* (Webb, 1990; Langdon *et al*., 1992). South African Cape hake (*Merluccius capensis*) has also been reported to be infected with *K. thyrsites* (Webb, 1993). The presence of *K. thyrsites* is linked to the development of black pseudocysts and/or *post-mortem* myoliquefaction of the fish musculature due to the release of cysteine proteases (Funk *et al*., 2008) from this *Kudoa* parasite species. The development of myoliquefaction results in fish to become unfit for processing and resulting in economic losses. The prevalence and level of *K. thyrsites* infection may be influenced by several factors. These factors include: i) age, ii) sexual maturity of the fish (Shaw *et al*., 1997; Levsen *et al*., 2008), iii) season, where infection tends to be higher during summer than winter months (Munday *et al*., 1998; St-Hilaire *et al*., 1998; Moran, Kent *et al*., 1999; Moran, Whitaker *et al*., 1999), and iv) size of the fish, where larger sized fish are typically more heavily infected than medium to smaller sized fish (St-Hilaire *et al*., 1998; Levsen *et al*., 2008).

Visible myoliquefaction may be influenced by factors such as i) time of evaluation *post-mortem*, ii) level of *K. thyrsites* infection, iii) temperature during storage, and iv) the inherent quality of the muscle (Dawson-Coates *et al*., 2003). The prevalence of *K. thyrsites* in SA economically important fish species, such as SA sardine, Cape hake species (*Merluccius capensis* and *M. paradoxus*), and SA kingklip (*Genypterus capensis*), is not well documented. In addition, the effects of sex, size, and season of capture on the prevalence of *K. thyrsites* in these SA fish species are unknown.

The SA hake fishery, although not the largest in terms of tonnage, is the most valuable in South Africa, providing up to more than 30 000 jobs and an annual landed value of more than R5.2 billion (Branch & Clark, 2006; DAFF, 2014; DAFF, 2016). South African hake comprises of two species: shallow-water (*Merluccius capensis*) and deep-water hake (*M. paradoxus*). *Merluccius capensis* is found along southern and southwestern Africa, from Baie Farte (12.5°S), over the Agulhas Bank in the south, to Kwazulu-Natal (28°S) (Cohen *et al*., 1990). *Merluccius paradoxus* is found along the southern and southwestern African coast, from Cape Frio (18°S) south to the Agulhas Bank, and east to East London; as well as on the Madagascar Ridge (33°S 44°E). The west coast of South Africa provides the main habitat for the deep-water hake species (Strømme *et al*., 2016). These two species of hake are not easily distinguished from one another, and are marketed as a single species and commonly referred to as Cape hake.

Cape hake is one of the more popular fish species for human consumption and are available in most of South Africa’s restaurants and retail outlets (Cawthorn *et al*., 2011). Demersal longline fishing has been practiced in SA waters since 1983, with the main target species being SA kingklip (*Genypterus capensis*), shallow-water and deep-water hake (Badenhorst, 1988).

South African kingklip is a deep-water, relatively slow-growing, long-lived fish and is found in rocky areas from north of Walvis Bay on the west coast to east of Port Elizabeth on the south coast of South Africa (Punt & Japp, 1994; Anon., 2017). Kingklip is a SA commercially important species and a popular eating fish in restaurants (Cawthorn *et al*., 2011). Currently SA kingklip is rated orange on the South African Sustainable Seafood Initiative (SASSI) status and assessments indicate that stocks are slowly recovering and are being harvested at sustainable levels (Anon., 2017). Between 1983 and 1986, SA kingklip remained the principal target species (Badenhorst, 1988), however, since 1990 the capture of SA kingklip was closed (Punt & Japp, 1994) due to a sharp decline of the stocks (Badenhorst & Smale, 1991). Since no fishing method is entirely selective, SA kingklip is still captured along with targeted fish species and are now considered "bycatch", which means "unintended catch of non-target fish species" (WWF-SA, 2011). Since 2007 the Department of Agriculture, Forestry and Fisheries (DAFF) has implemented bycatch management measures, such as precautionary catch limits in the trawl industry for SA kingklip, and a seasonal closed area to protect the spawning grounds for SA kingklip (WWF-SA, 2011). During 2010 Cawthorn *et al*. (2011) conducted a survey evaluating the availability of fish species in SA restaurants (n = 215) and retail outlets (n = 200). From this survey, it was found that SA kingklip was the most commonly available fish species in restaurants, occurring in 88% of the restaurants surveyed, while Cape hake was the third most available (58% occurrence). Kingklip was the second most commonly available fish species in retail outlets, occurring in 75% of outlets surveyed, with Cape hake occurring in 92%.

The small pelagic purse fishery, that targets SA sardine, anchovy (*Engraulis encrasicolus*) and redeye round herring (*Etrumeus whiteheadi*), is the largest fishery in South Africa in terms of tonnage (DAFF, 2014). These species of fish are typically harvested off the west and south coast of South Africa (Beckley & Van der Lingen, 1999). The SA sardine is an economically valuable (second to the hake fishery) and ecologically important fish species and occur along the entire coast of South Africa (Beckley & Van der Lingen, 1999; DAFF, 2014, DAFF, 2016). In terms of ecological importance, SA sardine is one of the key fish species in the marine food web, where they play an important role in the transfer of energy produced by plankton to large-bodied predatory fish (DAFF, 2014), including Cape snoek (*Thyrsites atun*) (Griffiths, 2002) and Cape hake (Pillar & Wilkinson, 1995). Anchovy and round herring are mostly reduced to fish-meal, while sardine is mainly used for canning for human consumption and for pet food (DAFF, 2014). In 2013, surveys indicated a depleted state of the SA sardine resource, which had failed to recover since 2006 (DAFF, 2016). In 2014, only 700 ton, and in 2015, only 3 ton, of sardine was landed in the Port Elizabeth area, as compared to more than 5000 ton in the previous 15 years (with exception of 2013; during which about 2000 ton was landed). Due to the reduced sardine catches in 2014 and poor fishing season off Port Elizabeth, right holders started canning their catch rather than freezing it. This could not be done in Port Elizabeth, resulting in SA sardine fishing moving to Mossel Bay in 2015.

South African sardine may be divided into three distinct sub-populations: western, southern, and eastern stocks (Van der Lingen *et al*., 2015). The western and southern stocks occur to the west and

east of Cape Agulhas, respectively. The eastern stock occurs off the coast of Kwazulu-Natal in winter, but migrates southwards in summer (Weston *et al*., 2015). Reed *et al*. (2012) studied the potential of *K. thyrsites* as a biological tag for the purpose of sardine stock identification and studies concerning SA sardine stock structure. *Kudoa thyrsites* was not suitable to be used as a biological tag (Reed *et al*., 2012) as it did not fulfil the criteria (MacKenzie & Abaunza, 2005).

Since the prevalence of *K. thyrsites* in SA sardine, Cape hake and SA kingklip is not well documented, the aim of this study was to investigate the prevalence (i.e. presence or absence) of *K. thyrsites* and relationships thereof with species of fish, season, area, sex (male and female), sexual maturity (sardines only), size (caudal length in cm) and weight (g) of the fish in SA sardines, Cape hake and SA kingklip samples.

5.2 Materials and methods

5.2.1 Fish samples and sample preparations

A total of 828 SA fish samples of four different species, namely SA sardine (*Sardinops sagax ocellatus*), Cape hakes (*Merluccius capensis* and 82 *M. paradoxus*) and SA kingklip (*Genypterus capensis*), were analysed for the presence of *K. thyrsites*. All fish samples, which included: 561 SA sardines, 197 Cape hake (144 *M. capensis* and 83 *M. paradoxus*), and 70 SA kingklip, were kindly provided by the DAFF (Addendum B). With the exception of SA kingklip, fish samples were received as individually frozen, wrapped in tin-foil, single fillets. For the SA kingklip, only small portions (between 32 and 40 mm^3) of fillets from individual fish were received as individually, tin-foil-wrapped samples. Fish were harvested during 2014 and 2015. The SA sardines were harvested by the DAFF during pelagic surveys using midwater trawl nets. Catches were typically less than 1 ton in weight. Cape hake and SA kingklip were harvested by the DAFF during demersal surveys using bottom trawl nets. A total of 212 fish samples were analysed from 2014 and a total of 616 from 2015.

The biological data of each fish sample was provided by the DAFF. This included: sex (male and female), size (cm), weight (g), area of harvest (east, south and west coast, Figure 5.1) and date of harvest. In the case of SA sardine samples, the biological data also included gonad stage (Table 5.1) and fat stage (Table 5.2). Gonad stages are indications of sexually maturity of fish. The gonad maturity stages and fat stages of sardines were assigned by trained DAFF technicians as described by Van der Lingen *et al*. (2006) and Van der Lingen and Hutchings (2005). In the case of Cape hake, the data included difference between *M. capensis* and *M. paradoxus*. Area of harvest for the SA sardine samples included east, south, and west coast. This was used to determine whether the suggested SA sardine stocks (Van der Lingen *et al*., 2015) differ in the prevalence of *K. thyrsites* infection (specific interest to the DAFF). The Cape hake and SA kingklip samples were harvested from only the west coast area.

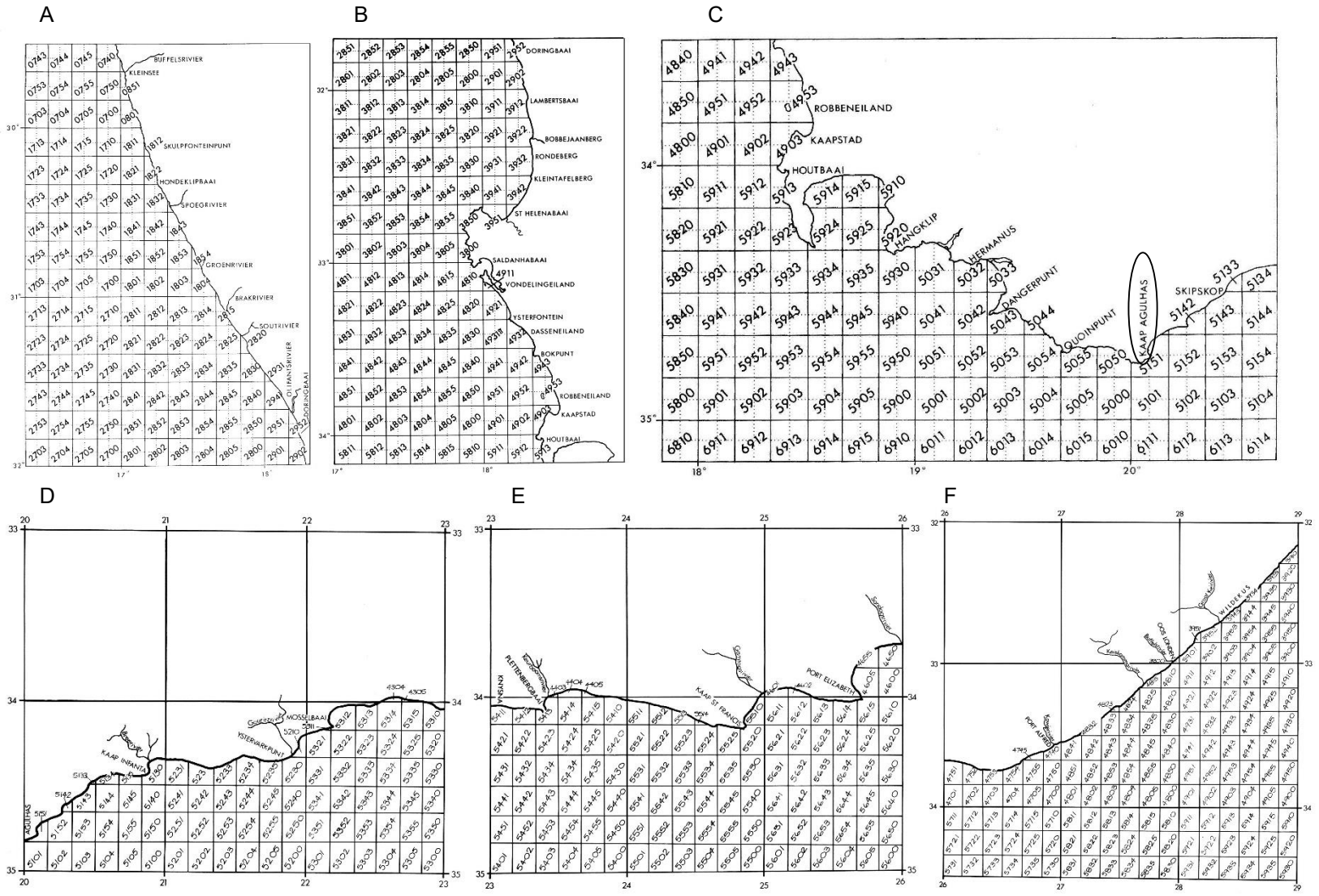

Figure 5.1 Area codes indicating Cape Agulhas (indicated by a circle at 20º longitude, map C) as the boundary between west and south coast where all fish harvested in area codes at longitude <20º were classified as west coast samples, all fish harvested in area codes between 20-28ºE (maps D-F) were classified as south coast, and all samples harvested in Kwazulu-Natal (KZN; >28ºE; map not shown) were classified as east coast (Figure of map curtesy of C. van der Lingen, DAFF).

The size of the fish samples was given as caudal length (cm), namely the length of a fish measured from the tip of the snout to the posterior end of the last vertebra of the mid-lateral portion of the hypural plate, excluding the length of the caudal fin; which is equivalent to standard length. The dates of harvest were used to determine the seasons of harvest. The seasons of harvest included summer (November till February), autumn (March and April), winter (June till August), and spring (September and October).

Table 5.1 Descriptions of the gonad maturity stages for SA sardines (shortened from Van der Lingen *et al*., 2006)

Stage	Description
1	Ovaries either immature or inactive. Testis flat and leaf-like, pink or transparent.
2	Ovaries inactive, but with the beginnings of enlargement. Testis beginning to thicken and become elongated with slight white colouration.
3	Ovaries elongated and filling over half the body cavity. Testis elongated, thickened and filling over half the body cavity.
4	Ovaries elongated, distended, filling approximately two-thirds of the body cavity. Colour is bright yellow. Testis further elongated, filling approximately two-thirds of the body cavity, with colour opaque white.
5	Ovaries at maximum size, almost filling body cavity. Dark in colour, no longer opaque. Testis at maximum size, colour opaque white, posterior half of gonad milky.
6	Same as stage 5, but greater enlargement has taken place. Fish in this condition almost in the act of spawning.
7	Ovaries elongated, but flat due to recent evacuation of ova, very bloodshot. Testis elongated, flat, strap-like and very bloodshot.
8	Ovaries much like stage 7, but obvious signs of recovery and reversion to stage 1. Testis – as for ovaries
9	Ovaries showing signs of having been recently spent, recovery to stage 1 apparent, however, evident that reversion to stage 1 is only temporary as signs of changing to stage 3 already evident. Testis – as for ovaries.
10	Ovaries still showing signs of having been recently spent, but evidence that gonads are about to become active almost immediately by changing to stage 3. Testis – as for ovaries.

Table 5.2 Description of fat stages for SA sardine (shortened from Van der Lingen, 2005)

Fat stage	Description
1	Fat lines are invisible or thin and intact.
2	Depth of fat greater than width or one or more fat lines.
3	Pyloric fat line noticeably thicker than the other fat lines, and about one-third the thickness of the *pyloric junction.
4	Depth greater than width for all fat lines but no fat lobes present
5	All fat slightly lobed, but no overlap between lobes
6	Fat line lobes obvious and show some overlap
7	Fat line lobes are large, lots of overlap.

*Pyloric junction is the area which connects the stomach to the duodenum.

Table 5.3 summarises sample information for all fish samples analysed. Fish samples were kept frozen at -18ºC until DNA extractions for analysis of the presence or absence of *K. thyrsites*.

Table 5.3 Summary of sample information for SA sardine, Cape hake and SA kingklip used to determine the prevalence of *Kudoa thyrsites* with season, geological area, size (caudal length in cm), weight (g), and sex (male and female) as variables

	SA sardines (*Sardinops sagax*)	**Cape hake** (*M. capensis* and *M. paradoxus*)	**SA kingklip** (*Genypterus capensis*)
Total number of samples (n)	n* = 561	*M. capensis*: n = 114 *M. paradoxus*: n = 83 Total n = 197	n = 70
Method of analysis**	PCR: n = 265 qPCR: n = 296	PCR: n = 35 qPCR: n = 162	PCR: n = 0 qPCR: n = 70
Year of harvest***	2014 and 2015	2014 and 2015	2015
Season of harvest	Summer: n = 214 Autumn: n = 183 Winter: n = 136 Spring: n = 28	Summer: n = 48 Autumn: n = 93 Winter: n = 54	Winter: n = 40 Spring: n = 30
Geological areas from where fish were harvested#	East Coast: n = 70**** South Coast: n = 167 West Coast: n = 302	West Coast	West Coast
Average size (cm) ± standard deviation	18.42 ± 1.57	43.13 ± 12.90	75.95 ±10.10
Range of size (cm)	13.20 – 21.50	23.00 – 75.20	58.10 – 100.30
Average weight (g) ± standard deviation	88.67 ± 22.70	No weight data available	2 205 ± 1 123
Sex classification	Male: n = 239 Female: n = 256	Male: n = 60 Female: n = 98 Unknown: = 38	Male: n = 20 Female: n = 50

*n - number of samples

** Method of analysis refers to the methodology used for determination of the presence of *K. thyrsites*. The two methodologies included PCR and qPCR as described under section 5.2.2 of this chapter.

***All fish samples were harvested by several recruitment survey vessels owned by DAFF (Department of Agriculture, Forestry and Fisheries, South Africa) during 2014 and 2015

****All east coast sardine samples were captured during June and July 2015.

#Figure 5.1 shows Cape Agulhas as the boundary between west and south coast where all fish harvested in area codes at longitude <20º were classified as west coast samples, all fish harvested in area codes between 20-28ºE were classified as south coast, and all samples harvested in Kwazulu-Natal (KZN) were classified as east coast.

5.2.2 Determination of Kudoa thyrsites *and* K. paniformis *presence*

5.2.2.1 DNA extractions and genomic amplification

Deoxyribonucleic acid (DNA) from all fish samples were extracted with the use of the Qiagen DNeasy Mini Kit (Qiagen, Whitehead Scientific Pty., Ltd., Cape Town). Composite muscle tissue samples of approximately 10 to 20 mg were removed from three different locations (anterior, centre, and posterior) per fish sample with the use of sterile scalpels. Amplification of *K. thyrsites* DNA was performed at the Centre for Proteomic and Genomic Research (CPGR, Cape Town, South Africa)

by the use of two genomic protocols: polymerase chain reaction (PCR) and real-time quantitative PCR (qPCR), as described in more detail in Chapter 4 (pp. 50-74) to be used as a qualitative diagnostic tool. The PCR protocol was primarily used for the 2014 analyses when the qPCR protocol was not fully developed yet. The majority of the 2015 samples were analyses with the use of the qPCR protocol (cost effective and time saving; i.e. 384 samples could be analysed in one analytical run using 384-well templates).

A total of 300 DNA samples were analysed with the use of the PCR protocol for amplification of the 18 SSU rDNA. The following PCR primer pair was used *for K. thyrsites*: KT18S6_f (CTCAACCAACTGGCCTCG) and KT18S1_r (CGTCAATTTCTTTAAATTTGG) (Meng & Li-Chan, 2007).

A total of 528 DNA samples were analysed with the use of the qPCR protocol. The primers and TaqMan® probes used in the qPCR assay for *K. thyrsites* were as follow: PCR primers (5'-CCTATCAACTAGTTGGTGAGGTAGTG-3' and 5'-TCTCCGGAATCGAACCCTGAT-3') that flanked the region containing the SNP site specific to *K. thyrsites* (5'-GTTA-3', Addendum D) were used. The two TaqMan® probes for *K. thyrsites* included one that matched the heterologous allelic variant *K. thyrsites* (5'-ACCCGTTAACACCTTG-3', Addendum C) and the second that matched the homologous allelic variant: *K. paniformis*/*K. miniauriculata*/*K. dianae* (5'-CGTCACAACCTTG-3'). Samples shown to be positive for *K. thyrsites* from previous studies (Chapter 4) were pooled for preparation of positive control samples (DNA at 10 ng·μL^{-1} working concentrations).

Amplification according to the PCR protocol was performed in a volume of 25 μL containing 5 μL KAPA HiFi buffer (with 2 mM $MgCl_2$), 0.75 μL dNTP mixture (0.3 mM), 0.75 μL of each primer (0.3 μM), 1 μL of extracted DNA, and 0.5 μL KAPA HiFi DNA polymerase (0.5 U/25 μL). For PCR with *K. thyrsites* primers, an annealing temperature of 55ºC was used, while all other conditions remained the same.

Amplification according to the qPCR protocol was performed by amplifying 20 ng DNA in a 5 μL volume PCR reaction mix containing 0.9 μM primers and 0.2 μM probes. A working master mix for each assay was prepared by mixing 0.25 μL of TaqMan® assay mix (20x), 2.5 μL TaqMan® Genotyping Master Mix (Thermo Fisher) and 0.25 μL water (2.25 μL of water for the no template controls) and 2 μL of genomic DNA at 20 ng. Reactions were performed by the following standard PCR cycling parameters: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, and post-reading of the reaction plate. End-point fluorescence was read on the 7900HT Fast Real-Time PCR System using the SDS version 2.3 software (Applied Biosystems, USA).

5.2.3 Statistical analyses

5.2.3.1 Exploratory data analyses

All data analyses were performed with the use of XLSTAT, version 19.01 (Addinsoft, 2017, Paris) and SAS statistical software, version 9.2 (SAS Institute Inc. Cary, NC, USA). Means, standard deviations and normality (skewness and kurtosis) of size and weight data per species of fish were

calculated and visualised by means of boxplots to identify outliers and dispersion of the data. The frequencies of the presence and absence of *K. thyrsites* were calculated for all fish samples analysed. Classification and regression trees, with the use of Chi-square automatic interaction detection (CHAID), and Chi-square frequency tables, were calculated for genomic method (namely the protocols: PCR and qPCR). This was done in order to determine whether results from the PCR protocol and results from the qPCR protocol may be pooled for analysis of relationships between the presence and absence of *K. thyrsites* and season of harvest, area of harvest, sex, and size of fish. It is noteworthy that samples with any missing biological data (including sex, size, area, and season of harvest) were excluded from statistical calculations for Chi-square relationships, Pearson's correlations and ANOVA's. Therefore, the total number of samples indicated in Table 5.3 may differ from the number of samples in tables under the results and discussion section of this chapter.

Classification and regression (CHAID) trees were generated for gonad stage and fat stage for SA sardines with area, season and sex as variables in order to determine whether gonad and fat stage may be included in Chi-square dependent tests with the presence of *K. thyrsites* without generating bias conclusions. Classification and regression (CHAID) trees were presented as box charts. Chi-square test were presented in tables with associated P-values calculated at the significance level of 0.05. Pearson's correlation tests were performed for size and weight data for the SA sardine and SA kingklip samples (weight data for Cape hake was not available) in an attempt to decide whether the relationship between *K. thyrsites* infection and both these variables needed to be investigate, or whether a relationship between *K. thyrsites* and only size was sufficient. Pearson correlation coefficients [R-values at the 5% significance level (P)] were calculated for the size and weight data for SA sardine and SA kingklip (hake only had size data).

In order to investigate the relationships between the prevalence of *K. thyrsites* and the variables: size of fish, weight of fish, sex (male and female), sexual maturity (sardines only), season of harvest, and area of harvest, the influence of these variables, and their interactions on the size of fish were investigated for each species of fish.

Analyses of variance (ANOVAs), a method belonging to Generalised Linear Models (GLM), were used to investigate how size of SA sardines, Cape hake, and SA kingklip, respectively, varies with sex, season, and area of capture. In the case of Cape hake; the species of hake (mc represented *M. capensis*, and mp represented *M. paradoxus*) was also included as one of the qualitative variables. Interactions between these qualitative variables were investigated before Chi-square tests were performed.

5.2.3.2 Chi-square tests

Frequency tables with Chi-square tests (confidence level at $P < 0.05$) were calculated for classifications of sex (male and female) area (east, south, and west coast), season (summer, autumn, winter, spring), species of Cape hake (hake data only) and size (cm) by *K. thyrsites* to test the dependence of the presence of *K. thyrsites*. It is notworthy that limitations in data collection

included unequal number of samples per fish species, sex, area, size and per month (season). South African sardine from the Eastern Cape (Kwazulu-Natal) only included samples harvested during June and July 2015. In addition, several samples had some missing biological data, for example unknown sex, or unknown size, and were not included in all statistical data analyses. Therefore, statistical analyses were performed on samples without any gaps in data, resulting in the total number of samples (n) as indicated in Table 5.3 to differ from the total of number for samples indicated in Tables below.

5.3 Results and discussion

5.3.1 Descriptive analyses

5.3.1.1 Prevalence and genomic methods (PCR and qPCR)

The CHAID classification tree (Figure 5.2) for the presence and absence of *K. thyrsites* with year of harvest (2014 and 2015) and method (PCR and qPCR protocols) showed a split between the years ($P > 0.05$) and significant splits ($P = 0.000$) for method within each year.

The percentage of samples that tested positive for the presence of *K. thyrsites* was higher for 2015 (79%) than for 2014 (31%). Since 2015 included SA sardine, Cape hake and SA kingklip samples, while 2014 only included SA sardine and Cape hake samples (Table 5.3), it is difficult to discuss why the prevalence was higher for 2015 than for 2014. In addition, 2015 included SA sardine samples from the east coast, of which all tested positive for the presence of *K. thyrsites*, while during 2014 no samples were collected from the east costs. The samples from the east coast during 2015 could possibly have contributed to the higher prevalence for 2015. Too many of the independent factors (such as area of capture, season, sex, and size), as well as the number of samples collected per factor, were difficult to control during sample collection.

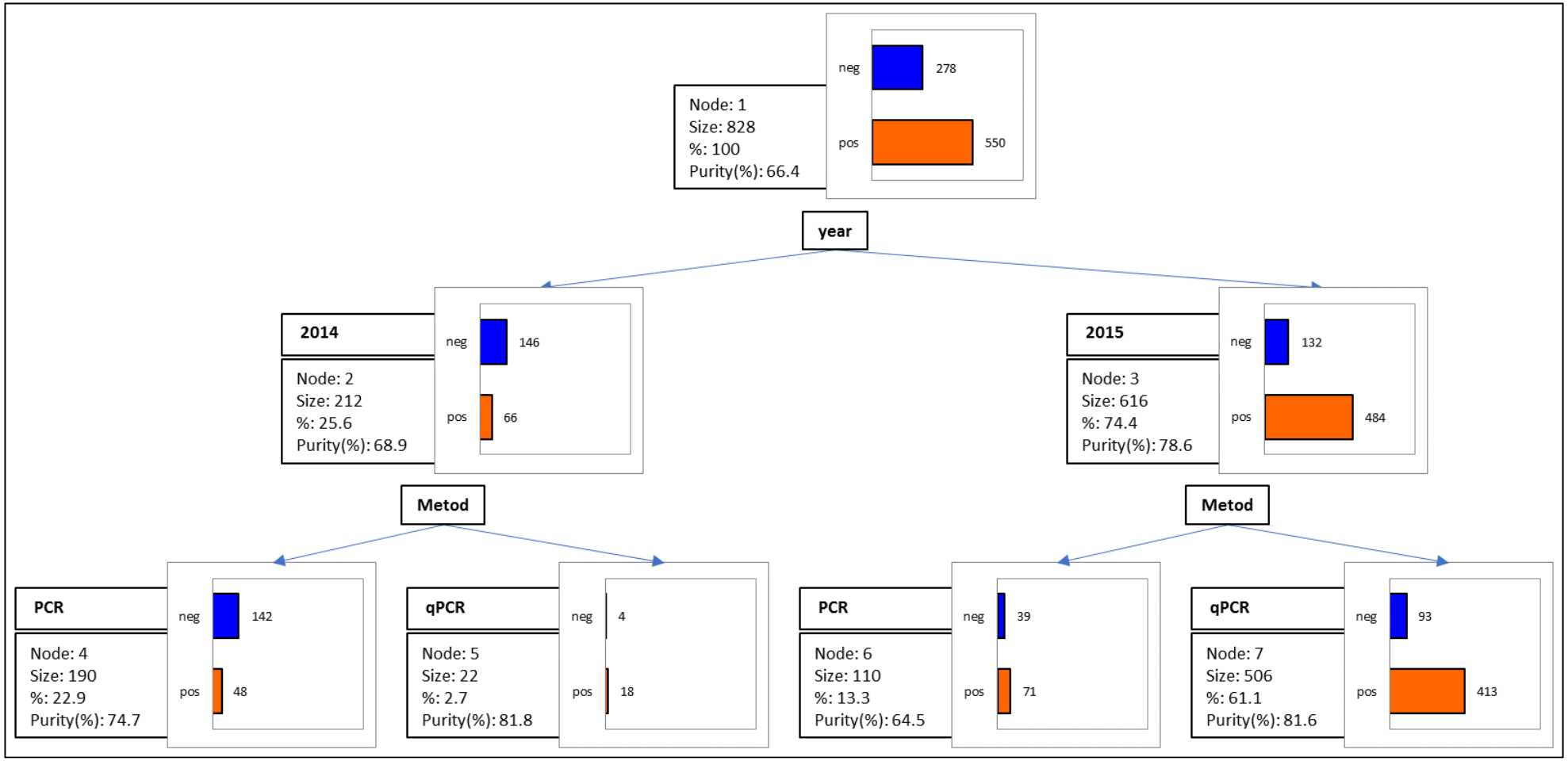

Figure 5.2 CHAID classification and regression tree for the presence and absence for *Kudoa thyrsites* with genomic method (protocols: PCR and qPCR) and year of harvest (2014 and 2015) as classification variables for all species of fish investigated.

Interruptions of sample collections occurred due to low sardine catches during 2014 and 2015, poor condition (lower than expected weight-at-length ratio) of sardines in the inshore and shelf waters between Danger Point and Mossel Bay, and in Algoa Bay during 2015 (DAFF, 2016) and survey vessels not going out to collect samples, resulting in unforeseen gaps in data collection.

Comparing the results from the PCR and qPCR methods (for both 2014 and 2015), a significant higher percentage of prevalence for *K. thyrsites* was found when the qPCR protocol was followed, compared to the PCR protocol. The qPCR protocol indicated that 82% of the samples (including SA sardines, Cape hake, and SA kingklip) fish that were analysed tested positive for the presence of *K. thyrsites*, while only 40% of the samples analysed with the PCR protocol tested positive. From these results, the qPCR protocol seemed to be more sensitive for detection of *K. thyrsites* than the PCR protocol. Although Löfström *et al*. (2015) stated that qPCR is a more sensitive method compared to PCR, such a conclusion cannot be made from these results since, in this study, the same samples were not analysed with both genomic methods (PCR and qPCR protocols). The qPCR protocol used in this study was developed (Chapter 4) mainly to be used as a sensitive, fast, cost-saving diagnostic tool for the analysis of the presence of *K. thyrsites* in a large number of samples. Since the qPCR protocol was developed specifically for this study as a diagnostic tool, all PCR data for 2014 and 2015 were excluded from further statistical analysis. Therefore, further discussions of results for this study are only pertaining to results obtained with the qPCR protocol for the 2014 and 2015 samples.

5.3.1.2 Prevalence of K. thyrsites *in SA sardine, Cape hakes and SA kingklip samples*

Table 5.4 shows a summary of the percentage of *K. thyrsites* infected samples for the SA sardine, Cape hake, SA kingklip and all fish samples analysed with the qPCR protocol during 2014 and 2015 (total samples of 528). Eighty two percent (82%) of the total 528 samples were infected. When investigating the prevalence of *K. thyrsites* per species of fish, first the independence (no relationship) between the Cape hake species (*M. capensis* and *M. paradoxus*) and the presence of *K. thyrsites* was determined by calculating the P-value for the Chi-square test. Figure 5.3 shows that there was no significant difference (P = 0.366) in *K. thyrsites* prevalence between the *M. capensis* (80%) and *M. paradoxus* (85%) samples.

Table 5.4 Prevalence (% infection) of *K. thyrsites* in SA sardines, Cape hake (*M. capensis* and *M. paradoxus*), SA kingklip, and total number of fish samples analysed

Fish species	Number of samples (n)	% of *K. thyrsites* infected samples **
Sa sardines	296	91
***Cape hake**	162	83
SA kingklip	70	40
Total samples	528	82

P-value < 0.0001 for Chi-square test at the significance level of 0.05
*Cape hake includes both *M. capensis* and *M. paradoxus*
**Only qPCR results

The Cape hake samples used in this study were collected from the same geological area along the west coast, however from different depths of the ocean. *Merluccius capensis*, which is the shallow-water hake species, are distributed from southern Angola to northern Kwazulu-Natal, while the deep-water hake, *M. paradoxus*, are distribute from northern Namibia to southern Mozambique (Cohen *et al*., 1990; DAFF, 2014). Although the distribution of the two species of hake differ with depth, there is significant overlap in their depth ranges. *Merluccius capensis* are distributed over a depth range of 30 to 500 m with the majority of the population occurring between 100 and 300 m. On the other hand, *M. paradoxus* are distributed over a depth range of 110 m to deeper than 1000 m with most of the population occurring between 200 and 800 m. The size of both species of hake increase with depth, allowing large *M. capensis* to co-exist and feed on smaller *M. paradoxus*. This co-existence and the extensive feeding of larger deep-water hake on smaller shallow-water hake may explain why the prevalence of *K. thyrsites* did not differ between the two species of hake. Further discussions of the prevalence of *K. thyrsites* related to Cape hake includes the pooled qPCR results for the *M. capensis* and *M. paradoxus* samples.

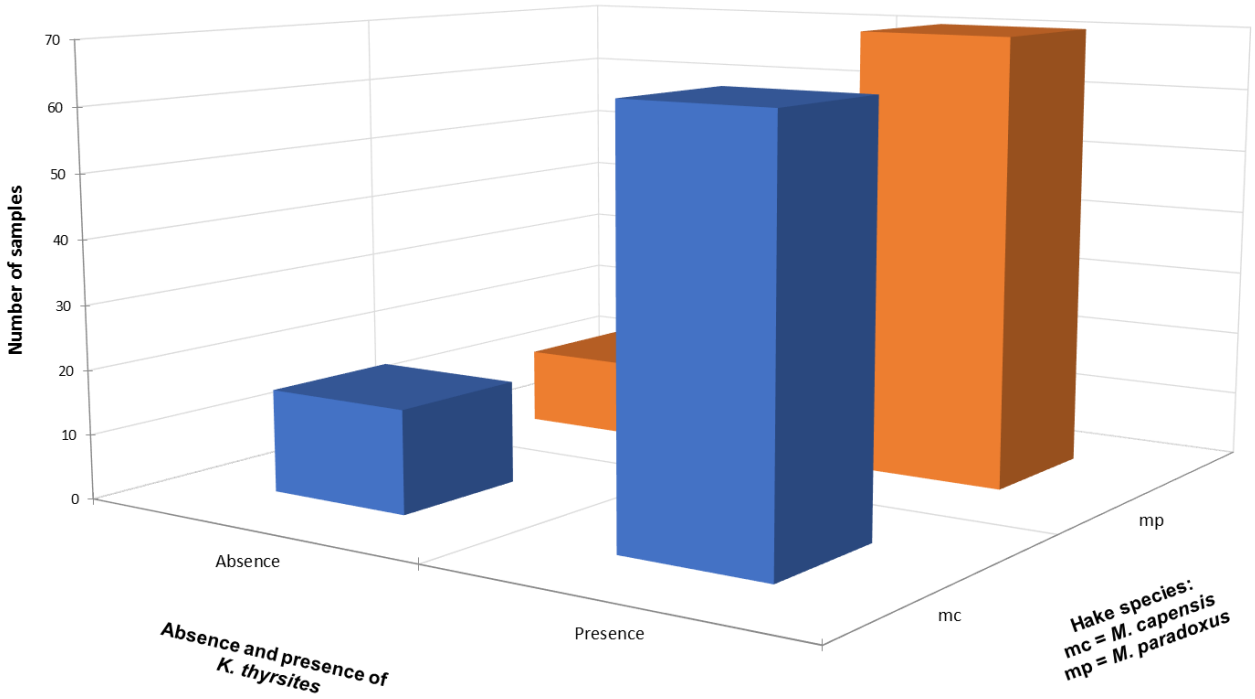

Figure 5.3 Three-dimensional view of the independence (Chi-square P = 0.366) between Cape hake species (mc = *M. capensis*, mp = *M. paradoxus*) and the presence and absence of *K. thyrsites*.

The SA sardine samples showed the highest percentage of infection (91%), followed by Cape hake samples with 83% (*M. capensis* and *M. paradoxus* pooled) and then the SA kingklip samples (40%). The P-value ($P < 0.0001$) for the Chi-square test indicated that there was a significant difference in prevalence of infection between SA sardines, Cape hake and SA kingklip samples. In a study by Reed *et al*. (2012), where 102 SA sardine samples were examined for the presence *K. thyrsites*, a prevalence of only 17% was documented. The study, however, made use of only light microscopy

to determine *K. thyrsites* infection. Microscopic methods are unable to detect early stages of infection (Tsuyuki *et al*., 1982; Hevio *et al*., 1997; Moran, Morgalis *et al*., 1999; Young & Jones, 2005). This may explain the large difference in prevalence between the present study and the study by Reed *et al*. (2012). No scientific literature could be found about the prevalence of *K. thyrsites* in Cape hake. A previous study by Webb (1993) only mentioned the presence of *K. thyrsites* in Cape hake (*M. capensis*). Also, no literature could be found about the prevalence of *K. thyrsites* in SA kingklip.

Although the mode of transmission of *Kudoa* parasites are not fully understood (Swearer & Robertson, 1999; Young & Jones, 2005), Swearer and Robertson (1999) demonstrated that fish may become infected by ingestion of infected eggs, while Moran, Whitaker *et al*. (1999) showed that *K. thyrsites* could be transmitted by injecting blood from an infected fish (Atlantic salmon) to healthy fish, suggesting that an extra-sporogonic stage may occur in the blood of salmonids. Webb (1993) described an extra-sporogonic stage as a reproductive cell called an amoebula. Although the life cycle of *K. thyrsites* is still unknow, it may be useful at this point to give a summary of the basic life cycle of *K. thyrsites* in Cape hake as suggested by Webb (1993). The basic life cycle starts with the transmission stage, where *K. thyrsites* spores are released into the water upon disintegration of the dead host. The spores are ingested by the new host, either through the water or through cannibalism. Once the spores are inside the new host, they attach themselves to the gut by discharging anchoring threads from their polar capsules, and then release amoebulae which crosses over the gut wall and enters into the blood stream. The amoebulae are carried through the blood stream to the muscles where they penetrate the cells and develop into cysts. Spores are then formed within these cysts. At maturity, the spores remain in the cysts where they are suspended in a fluid which contains proteolytic enzymes. When the host dies, the proteolytic enzymes leak out of the cysts and start to digest surrounding tissue, allowing the spores to spread through the liquefied tissue to eventually be released back into the water.

However, Langdon *et al*. (1992) argued that it cannot be assumed that transmission of *K. thyrsites* occurs through ingestion of infected fish by other predatory fishes, firstly, because the life cycle and route of transmission and infection is unknown. Secondly, because the Australian pilchard (*Sardinops sagax neopilchardus*), a planktivorous fish species, is also heavily infected with *K. thyrsites* and is suggested to be a major reservoir host in Southwest Australian waters. These authors suggested that infection of planktivorous fish is through ingestion of infected invertebrate hosts. In contrast, Webb (1990; 1993) suggested that there is no intermediate host for South African sardines and infection simply occurs by transmission of spores through the water upon the death of infected hosts. However, SA sardines are omnivorous and consume both phytoplankton and zooplankton, including anchovy eggs, dinoflagellates, crustacean eggs and copepods (Van der Lingen, 2002). Therefore, if there is an intermediate host (which still needs to be discovered), SA sardines are exposed to several sources which may contribute to *K. thyrsites* infection. It may thus be hypothesised that the higher prevalence of *K. thyrsites* in SA sardines, as compared to Cape

hake and SA kingklip, could be linked to their diet and the fact that SA sardines always occur together in huge schools. The risk of SA sardines to become infected is thus high.

The relatively high *K. thyrsites* prevalence of 83% for the Cape hake samples may be due to the diet of Cape hake, which largely consist of smaller hake, anchovies, round herring, and SA sardines (Pillar & Wilkinson, 1995). South African sardines were shown to be heavily infected with *K. thyrsites* and appeared to be a major host reservoir species for *K. thyrsites* (Webb, 1990). Hake often swim together in small schools and catch prey together (DAFF, 2014). With the death of infected hosts and the hypothesised release of spores into the water (Webb, 1993), there is a constant presence of *K. thyrsites* spores. The presence of *K. thyrsites* spores within the water, the co-existence of deep-water and shallow-water hake, and their diet of sardines and smaller hake, may all contribute to the seemingly high prevalence of *K. thyrsites* in the Cape hake samples.

The presence of *K. thyrsites* in the Cape hake species, *M. capensis*, has been reported as early as 1947 by Davies and Beyers (1947) and again by Webb in 1993 (Webb, 1993). qPCR results from the present study confirms the presence of *K. thyrsites* in *M. capensis* and *M. paradoxus*, while *K. paniformis* was not present in any of the Cape hake samples analysed. Results from the two qPCR assays indicated absence of the allele for *K. paniformis* (from the *K. paniformis* assay) and for the allele *K. paniformis*/*K. miniauriculata*/*K. dianae* (from the *K. thyrsites* assay). Although *K. paniformis* was not found to be present in the Cape hake samples analysed in this study, King *et al*. (2012) similarly reported that the prevalence of *K. thyrsites* in Pacific hake (*M. productus*) was higher than that of *K. paniformis*. These authors also reported that the level of infection was higher for *K. thyrsites* than for *K. paniformis*. The findings by King *et al*. (2012) was in contrast to results reported by Meng and Li-Chan (2007) and Zhou and Li-Chan (2009); where these authors reported that the major *Kudoa* species for Pacific hake appeared to be *K. paniformis*. Morado and Sparks (1986) also reported significantly higher infections of *K. paniformis* than *K. thyrsites* in Pacific hake. Tsuyuki *et al*. (1982) reported the presence of both *K. thyrsites* and *K. paniformis* in Pacific hake (n = 322), with a prevalence of 32% for *K. thyrsites*, and 39% for *K. paniformis*. The overall prevalence was 90%. Tamkee (2003) reported a much higher prevalence of *K. thyrsites* of 78% and 80% in Pacific hake samples obtained during 2001 and 2002, respectively. Tsuyuki *et al*. (1982) documented that 18% of the Pacific hake samples investigated was co-infected with *K. thyrsites* and *K. paniformis*. The prevalence of *K. thyrsites* and *K. paniformis* are clearly changing from year to year, but also expanding in their distribution. King *et al*. (2012) noted that the presence of *K. paniformis* became well-established in areas within Canadian waters where previously it was rare and undetected. Although transmission of *K. thyrsites* and *K. paniformis* may not occur between different stocks (for example inshore, resident stocks vs. offshore, migratory stocks) King *et al*. (2012) suggested that due to changes in the ecosystem, which may involve marine invertebrate as intermediate hosts, the distribution of these parasites eventually expands. Whipps and Kent (2006) investigated the global distribution of *K. thyrsites* from several global areas, including South Africa, British Columbia, England, Oregon, Japan, and Australia, to represent the major geographical regions of the Atlantic,

Eastern Pacific, Japan and Australia. These authors concluded that, although *K. thyrsites* has low host specificity, regional genetic differentiation exist, suggesting that this parasite does not move easily from one oceanic region to the next. However, within a specific geographic region, there was little evidence of genetic differentiation, supporting the low host specificity of *K. thyrsites*. This may explain why *K. thyrsites* was found to be present in samples from all the fish species (SA sardine, Cape hake, and SA kingklip) analysed in the present study.

South African kingklip is a bottom-dwelling fish, found at depths of 50-500 m with the majority found at depths of 250-350 m (Punt & Japp, 1994). They are predatory fish, feeding on hake, shrimp, squid, and other fish species, and therefore may become infected with *K. thyrsites* through their diet. The low prevalence of *K. thyrsites* found in the SA kingklip samples investigate in this study may be because SA kingklip do not occur in schools, but hunt for prey by themselves.

It was noted during this study that out of the 296 SA sardine samples analysed for the presence of *K. thyrsites* with the use of the qPCR protocol, 53 (18%) samples showed severe myoliquefaction (Figure 5.4). Therefore, in the majority (82%) of the infected SA sardine fillets, myoliquefaction were not apparent, even though the samples were infected with *K. thyrsites* as determined by qPCR. Uninfected SA sardine samples did not show signs of myoliquefaction (i.e. milkyness and/or jelly-like appearance; Figure 5.4; Patashnik & Groninger, 1964) although some degree of muscle disintegration was evident; most probably associated with endogenous proteolysis (Huss, 1995) and damage from pro-longed frozen storage (Huss, 1995; Samples, 2015; Cheng *et al*., 2017) of the samples.

It is noteworthy that although an attempt was made to judge the level of myoliquefaction in the SA sardine samples by noting visual appearance (i.e. milkyness and/or jelly-like) and softness (via pressure by the finger; Figure 5.4), quantification of the degree of myoliquefaction was not achieved. However, the difference between severely myoliquefied and normal samples was visually apparent (Figure 5.4).

St-Hilaire *et al*. (1997) and Zhou and Li-Chan (2009) demonstrated a significant negative correlation between the number of *K. thyrsites* spores per gram of fish tissue (Atlantic salmon) and the firmness of the infected muscle. Fish muscle with high intensity infections resulted in severe myoliquefaction (decline in firmness). These authors also reported that fish muscle with less than 20 000 spores per gram of muscle tissue did not show signs of myoliquefaction and appeared to have the same muscle texture as uninfected fish. The qPCR method used in this study was not used to determine the level of *K. thyrsites* infection. However, qPCR is typically used for the quantification of DNA of a specific organism (Löfström *et al*., 2015) and the potential, therefore, exists to use qPCR as a predictive tool for the level of *K. thyrsites* infection and consequently myoliquefaction. This may present the opportunity to remove severely myoliquefied fish from firm fish without having to cut the fish into fillets. However, the use of qPCR as a predictive method of myoliquefaction would be impractical for the fish processing industry, since qPCR is an expensive and time-consuming method.

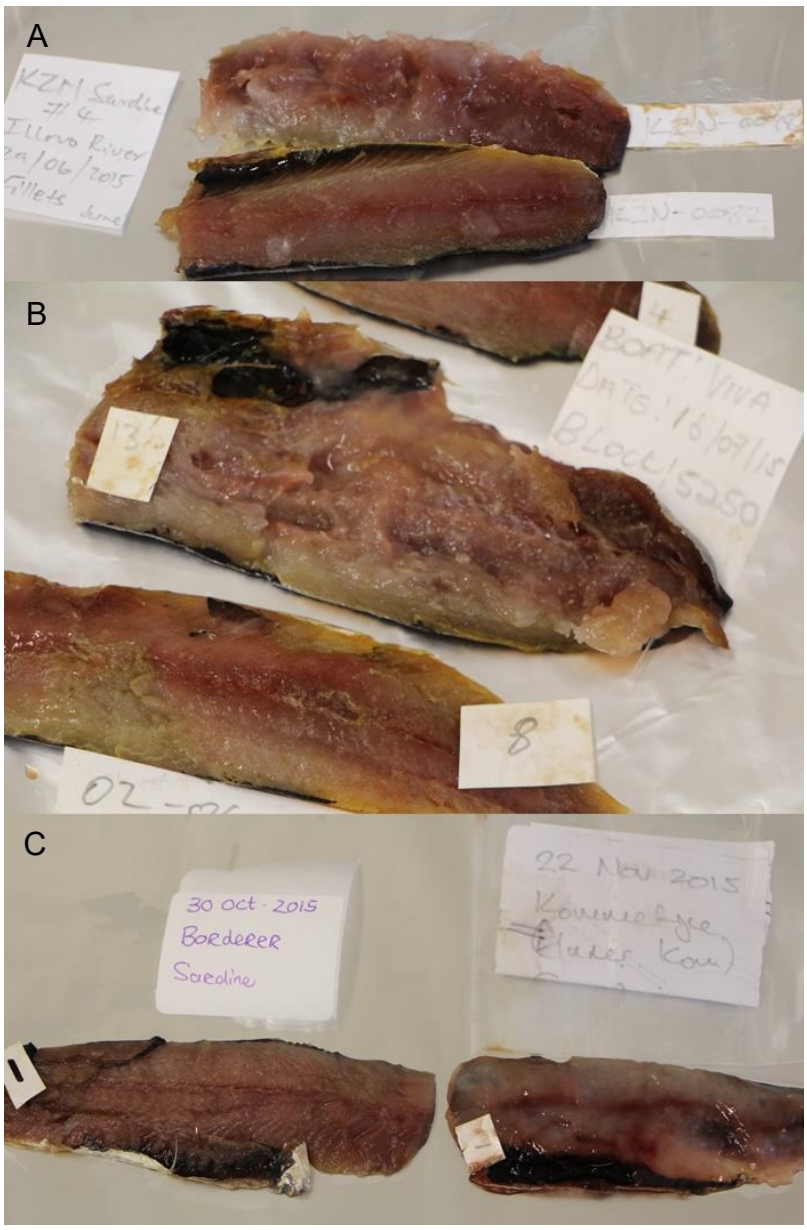

Figure 5.4 Photos (A and B) of frozen-thawed SA sardine fillets showing heavy myoliquefaction (top fillet) vs. no signs of myoliquefaction (bottom fillet). Photo C showing the typical milky appearance (fillet on right hand side) of myoliquefied fish muscle tissue. Photos were taken with a Canon PowerShot S2 IS camera.

Although the present study did not investigate the level of infection within a sample, it is noteworthy that the presence of black infected muscle fibres, or cysts, (Figure 5.5) were visible in several, but not all, of the Cape hake samples infected with *K. thyrsites*. This is in accordance with the findings of Webb (1993), who documented that *K. thyrsites* produce unsightly black streaks in the flesh of Cape hake fillets due to an immune response from the fish. Webb (1993) suggested that, instead of spores, a life stage called schizonts, grow within the cysts. Schizonts are cells which divide through the process of schizogony (similar to schizonts in the malaria parasite) to form daughter cells. The schizonts have similar infective capacities to the amoebulae and when released from the cyst, infection of adjacent tissue occurs in the life host. Schizonts would then form concentrations of secondary infections close to their release site, resulting in clumps of blackened cysts.

Figure 5.5 Black infected fibres (some black pseudocysts are indicated by cycles) in Cape hake fillet. Photo was taken with a Canon PowerShot S2 IS camera.

Before the immune response, the cysts are white in colour and not easily observed. Black pseudocysts (muscle fibres filled with *Kudoa* spores) seems to be a result the deposition of melanin granules around the infected area (Kabata & Whitaker, 1981; Tsuyuki *et al*., 1982). The accumulation of melanin seems to be gradual and result in a change of colour of the pseudocyst from pale brown, to dark brown and eventually black. The melanin depositions result in *Kudoa* spores to become compressed and eventually destroyed. This may explain why black pseudocysts were not evident in all of the infected Cape hake samples. In the case of the SA kingklip, it was difficult to observe any visible damage or immune response within the muscle tissue since only small sections of whole fillets from individual fish were used as samples in this study. Visible evidence of cellular immune response to *K. thyrsites* are host specific; where some fish species show consistent inflammatory responses (Morado & Sparks, 1986; Langdon *et al*., 1992; Webb, 1993; Moran, Margolis *et al*., 1999), while others do not show any response (Langdon *et al*., 1992; Shaw *et al*., 1997; Levsen *et al*., 2008; Marshall *et al*., 2016). Some fish species, for example Atlantic salmon (*Salmo salar*) may even recover from initial infection and develop a form of resistance to *K. thyrsites* (Moran, Margolis *et al*., 1999; Moran, Whitaker *et al*., 1999; Jones *et al*., 2016).

The presence of pale pseudocysts within the musculature of the Cape hake samples was not observed in the present study since detection of the white pseudocysts require careful examination (Samaranayaka *et al*., 2007). The present study used a qPCR protocol as a qualitative diagnostic tool and did not attempt at correlating visual tissue damage with the level of infection and the presence *K. thyrsites*. In addition, host response may differ from no response at all to black coloured pseudocysts (Morado & Sparks, 1986). Host response also varies with the size and age of the fish; where a host response is more common and pronounced in larger and older fish than smaller and

younger fish. Visual white pseudocyst and black pseudocyst counts in Pacific hake muscle tissue have shown positive correlations with protease activity and reduced texture quality of the fish (Morrissey *et al*., 1995). However, it has been documented that Pacific hake infected with the old black pseudocysts of *K. thyrsites* do not develop a mushy texture upon cooking (Tsuyuki *et al*., 1982).

In the present study, the appearance of myoliquefaction was not as apparent in the Cape hake samples as it was in the SA sardine samples, however, severe gaping was observed in many of the *K. thyrsites* infected Cape hake samples. Zhou and Li-Chan (2009) documented that although Pacific hake (*M. productus*) was typically infected with higher levels (10^6 to 10^8 spores·g^{-1} of fish tissue) of *K. paniformis* than *K. thyrsites* (10^6 to 10^7 spores·g^{-1} of fish tissue), *K. thyrsites* infection resulted in greater adverse effects on the cooked texture (maximum force < 150 g) of fish mince. It might be argued that the absence of severe myoliquefaction in the Cape hake samples was due to long frozen storage of the samples, however, freezing does not inhibit the activity of the proteolytic enzyme released by *K. thyrsites* (Tsuyuki *et al*., 1982; Zhou & Li-Chan, 2009). In addition, the SA sardine samples were also kept frozen for several months before analysis commenced. It is hypothesised that the extent of myoliquefaction between the SA sardine and the Cape hake samples may be related to differences in connective tissue (contents of collagen) of the myocommata and the size of the myotomes (Sikorski *et al*., 1984) between SA sardines and Cape hake and the level of *K. thyrsites* infection, as well as the amount and activity of the parasite-released proteolytic enzyme. Langdon (1991) documented that the collagen component of the myocommata connective tissue in *K. thyrsites* infected mahi mahi (*Coryphaena hippurus*) was more degraded than the myofibres 24 h *post-mortem*.

On the other hand, the absence of severe myoliquefaction in the Cape hake samples, compared to that of the SA sardine, may have been related to the effective stages of *K. thyrsites*. The level of proteolytic activity associated with *K. thyrsites* is related to the infective stage of the parasite (Tsuyuki *et al*., 1982; Zhou & Li-Chan, 2009) where hake infected with mainly young, white pseudocyst may have higher proteolytic activities than fish with black pseudocysts at acidic pH values.

5.3.1.3 Prevalence of K. thyrsites *and SA sardine gonad and fat stages*

The CHAIC classification tree tested the efficiency of the variables; area of harvest, season, and sex to predict the gonad and fat stages of SA sardines. Figure 5.6 represents the bar chart for the gonad stages (as defined in Table 5.1) of SA sardines, showing the split at each node where separation took place. It was observed that at each split, the majority of samples were classified into gonad stage 3 (early development of gonads), followed by stages 2 (inactive gonads) and 4 (gonads close to maturity). These stages were represented by 85% of the samples, while only 8% of the samples were classified into gonad stage 1 (immature or inactive gonads), and 9% into gonad stages 5, 6 and 7 (stages 5 and 6 = maximum gonad maturity before spawning, stage 7 = condition of gonads just after spawning). Due to limited number of samples showing maximum gonad maturity, these

gonad stages were poorly represented in this study. This may result into bias conclusions about the relationships between *K. thyrsites* presence and gonad maturity stage of sardines.

For the fat stages (as defined in Table 5.2), 77% of the samples were classified into fat stages 1, 2 and 3, with fat stage 4 represented by only 11% of the samples, and fat stages 5, 6 and 7 by only 12% of the SA sardine samples. This trend continued with each split (area, season and sex) through the CHAIC classification tree for fat stage.

The independent association between the prevalence of *K. thyrsites* (taking only qPCR results into account) and gonad and fat stage, respectively, was investigated. It was found that *K. thyrsites* presence was independent of both gonad and fat stage (P-values for Chi-square tests were > 0.05). In contrast to the gonad results for SA sardines, studies by St-Hilaire *et al*. (1998) and Levsen *et al.* (2008) showed that sexually mature Atlantic salmon (*Salmo salar*) are more likely to be infected with *K. thyrsites* than their sexually immature counterparts, possibly due to depressed host immunity (St-Hilaire *et al*., 1998; Jones *et al*., 2016).

The independence of the prevalence of *K. thyrsites* and gonad and fat stages could in part be explained by the high prevalence of *K. thyrsites* in the SA sardine samples used in this study. With a prevalence of 91% for the sardine samples, only a small number of samples were not infected, therefore, the number of infected samples in the different gonad and fat stages was not significantly different. However, in the present study, the stages within the gonad and fat stage classifications were not represented by equal number of samples. The gonad and fat stages of SA sardines were therefore not considered in further statistical analysis by GLMs.

Figure 5.6 CHAID classification and regression tree for gonad stage (g1 to g7 represents gonad stages as explained in Table 5.1) of SA sardines with area (east, south and west coast), season (summer, autumn, winter and spring) and sex (m – male and f – female) as classification variables.

5.3.1.4 Correlation between size and weight of fish samples

Table 5.5 summarises the Pearson's correlations between size and weight for the SA sardine and SA kingklip samples, respectively. For both species, a significant ($P < 0.0001$) linear positive correlation ($r = 0.928$ for sardines, $r = 0.957$ for kingklip) was found between size and weight. This indicated that as the size of the fish increased, the weight also increased. Therefore, the relationship between *K. thyrsites* prevalence was investigated for size only, allowing for better comparison between the three species of fish investigated in this study.

Figure 5.7 shows that the average (± standard deviation) size of fish was significantly different ($P < 0.001$) for the four species of fish. Kingklip was significantly larger in size (75.94 ± 10.10 cm) compared to SA sardine (17.97 ± 1.72 cm), *M. capensis* (38.61 ± 1.22 cm) and *M. paradoxus* (39.88 ± 1.00 cm). The average size for sardines was the smallest amongst the four species. The significant difference in size between SA kingklip, SA sardine, and Cape hake was expected since SA sardine is small pelagic fish typically ranging in caudal length between 12 and 25 cm (Van der Lingen *et al*., 2006; Reed *et al*., 2012). Cape hake and SA kingklip are demersal fish species; where Cape hake may range in length between 10 and > 95 cm (Botha, 1986), and SA kingklip between 20 and > 130 cm (Payne, 1986). There was, however, no significant difference in the average size between *M. capensis* and *M. paradoxus*. The average size for all the Cape hake samples combined was calculated as 39.37 ± 9.97 cm, which fell within the typical size range for Cape hake of 40 to 60 cm (Cohen, 1990). The relationship between *K. thyrsites* prevalence and the size was therefore calculated for the combined Cape hake size data.

Table 5.5 Mean (± standard deviations) size (cm) and weight (g) of SA sardine and SA kingklip samples, with Pearson's correlation coefficient (R-value), correlation coefficient of determination (R^2-value) and P-value for correlation between size and weight

	SA sardine		**SA kingklip**	
Variables	**Means and standard deviation**	**Pearson's correlation test**	**Means and standard deviation**	**Pearson's correlation test**
***Size (cm)**	17.97 ± 1.72	R = 0.928	75.95 ± 10.10	R = 0.957
Weight (g)	88.67 ± 22.70	R^2 = 0.860	2 258.09 ± 1 078.97	R^2 = 0.915
P-value		P < 0.0001		P < 0.0001

*Size refers to caudal length in cm
R - correlation coefficient measures the strength and the direction of a linear relationship between the two variables size and weight
R^2 - correlation coefficient of determination represents the percent of the data that is the closest to the regression equation (linear association between size and weight)
P-value with a significance level at 0.05

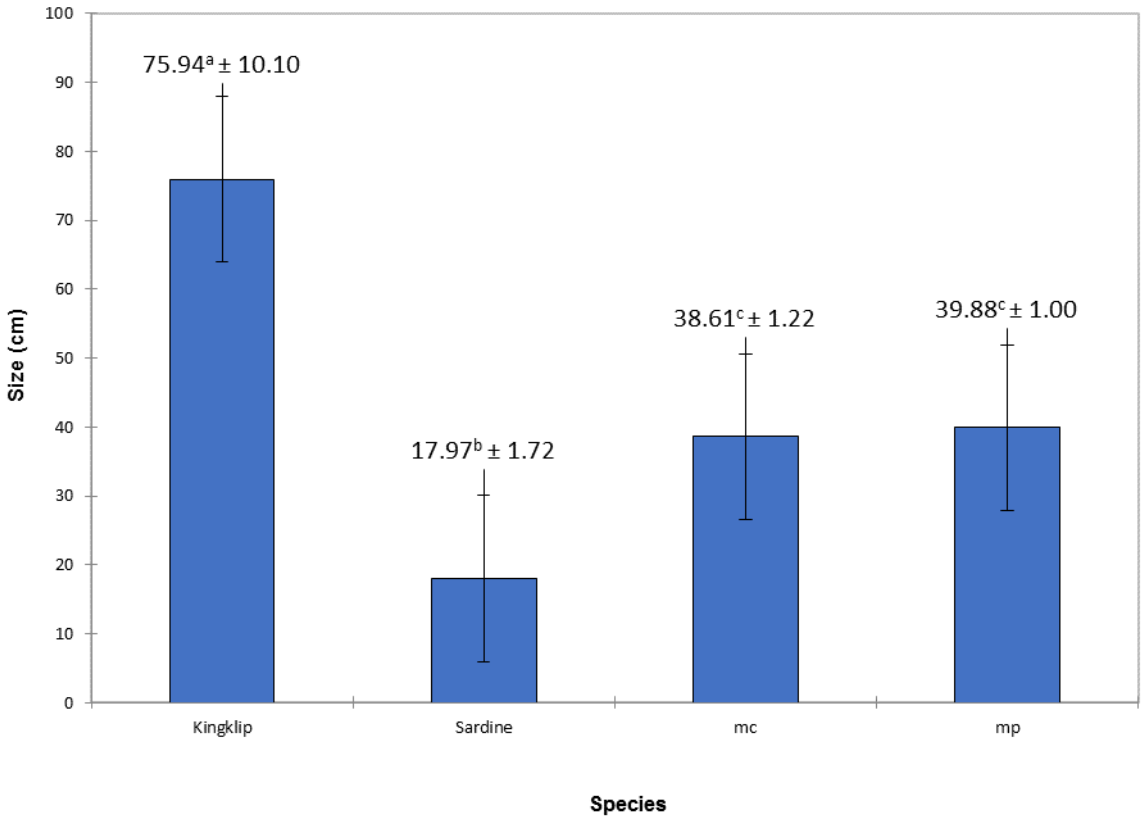

Figure 5.7 Means (± standard deviation), with standard error bars, for the size (cm) of the four species of fish: SA kingklip, SA sardine, and Cape hake (mc - *M. capensis* and mp - *M. paradoxus*). [abc]Different superscripts indicate significant differences at $P < 0.05$. Size refers to caudal length in cm. Total number of samples: SA kingklip = 70, SA sardine = 267, Cape hake = 57 (number of samples of mc = 22 and number of samples of mp = 35).

5.3.1.5 Effects of season, area, and sex on size of fish samples

The main purpose of this study was to investigate relationships between the prevalence of *K. thyrsites* in SA sardine, Cape hake, and SA kingklip and biological factors (sex and size), area of capture, and season. Since SA kingklip is not represented in 2014, all qPCR data from 2014 (22 sardine samples) were excluded from further statistical analysis and discussions. Further results and discussions pertain only to the qPCR 2015 data.

Table 5.6 summarises the generalised linear models used to investigate the influence of season (summer, autumn, winter and spring), area of harvest (east, south and west coast), and sex (male and female) on the size of the SA sardine samples used to study the relationship between *K. thyrsites* prevalence and these variables. For the SA sardine size data, no significant two-way interactions ($P > 0.05$) between the independent variables season, area and sex, were observed. There were significant differences in the size of SA sardines between the seasons ($P = 0.001$), areas of harvest ($P < 0.0001$), and between male and female samples ($P = 0.026$). Sardine samples from the east coast were significantly ($P < 0.05$) smaller (15.98 ± 1.64 cm) than samples from the south (19.69 ± 0.65 cm) and west coast (18.28 ± 1.35 cm). South African sardine samples from the south and west coast did not differ significantly ($P > 0.05$) in average size. The average size of female (18.46 ± 1.64

cm) SA sardine samples was significantly (P < 0.05) larger than male samples (17.54 ± 1.68 cm). Larger SA sardine samples were harvested during summer (18.52 ± 1.25 cm) and autumn (18.72 ± 1.31 cm), compared to winter (17.40 ± 1.96 cm) and spring months (17.56 ± 1.46 cm).

The results showed no significant interaction between the variables, season, area and sex for size. Therefore, the relationships between *K. thyrsites* prevalence and the variables season, area, sex, and size for SA sardines were separately investigated with the use of Chi-square tests.

Table 5.6 Analysis of variance (ANOVA) for the dependent variable SA sardine size (cm) with the variables season (summer, autumn, winter and spring), area of harvest (east, south and west coast), and sex (male and female) as independent variables, as well as two-way interactions

	Size (cm) of SA sardine samples		
Source	**df**	**MS**	**P-value**
Season	3	6.655	0.001
Area	2	142.154	< 0.0001
Sex	1	5.667	0.026
Season*Area	1	2.230	0.162
Season*Sex	3	0.332	0.830
Area*Sex	2	0.346	0.737
Error	254	1.133	

df - degree of freedom
MS - mean square
P-value - probability value of F-ratio test

Table 5.7 summarises the generalised linear models used to investigate the influence of season (spring and winter for SA kingklip, autumn and winter for Cape hake) and sex (male and female) on the size of the SA kingklip and Cape hake samples used to study the relationship between *K. thyrsites* prevalence and these variables. All SA kingklip and Cape hake samples were harvested along the west coast. For the kingklip samples, the variables season and sex showed no significant (P = 0.601) interaction. The size of the SA kingklip samples was significantly (P = 0.042) larger for samples harvested during spring (77.22 ± 8.91 cm) compared to winter (71.11 ± 9.49 cm) and significantly (P = 0.006) larger for female (78.06 ± 10.14) than male (70.27 ± 6.27) samples. As there was no significant interaction between the variables season and sex for the SA kingklip size data, the relationships between *K. thyrsites* prevalence and the variables season, sex, and size for the SA kingklip samples were investigated with the use of Chi-square tests.

In contrast to SA kingklip, the size data for the Cape hake samples showed a significant (P = 0.031) interaction between the variables season and sex, where female samples were larger in size than male samples for both autumn and winter (Figure 5.8). The size for the female samples was significantly (P < 0.05) different between autumn and winter, with larger sized samples harvested during winter than autumn. The opposite was evident for the male samples, where larger sized males were harvested during autumn than during winter. Although there was a significant interaction between season and sex for the size of hake, the relationships between *K. thyrsites* prevalence and the variables; season, sex, and size, respectively, were calculated.

Table 5.7 Analysis of variance (ANOVA) for the dependent variables SA kingklip size (cm) and Cape hake size (cm), respectively, with the variables season (autumn, winter and spring) and sex (male and female) as independent variables, as well as two-way interactions

	Size (cm) of SA kingklip samples			Size (cm) of Cape hake samples		
Source	**df**	**MS**	**P-value**	**df**	**MS**	**P-value**
Season	1	334.169	0.042	1	6.011	0.796
Sex	1	631.006	0.006	1	537.970	0.017
Season*Sex	1	21.416	0.601	1	439.151	0.031
Error	65	77.474		51	76.606	

df - degree of freedom
MS - mean square
P-value - probability value of F-ratio test

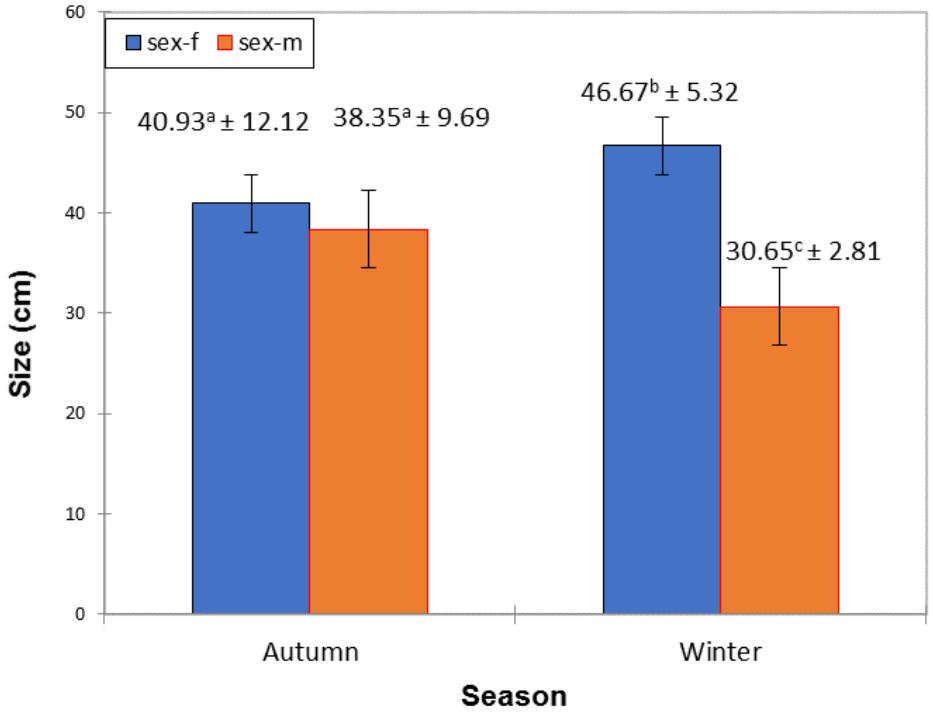

Figure 5.8 Means (± standard deviation) with error bars for the size (cm) of female (sex-f) and male (sex-m) Cape hake harvested during autumn and winter. [abc]Different superscripts indicate significant differences at $P < 0.05$. Size refers to caudal length in cm. Total number of samples for Cape hake size was 57.

5.3.2 Relationships between K. thyrsites *prevalence and variables*

5.3.2.1 Area of harvest

There was no significant (P = 0.075) difference in the prevalence *of K. thyrsites* between the three areas from which SA sardine samples were harvested (Table 5.8). All SA sardine samples (n = 70) from the east coast were infected with *K. thyrsites* (Table 5.8). The south coast samples (n = 56) had a *K. thyrsites* prevalence of 98.21%, while SA sardine samples from the west coast had the lowest *K. thyrsites* prevalence of 94.33%. This data is an indication that there may be no significant difference in *Kudoa* prevalence between the eastern, southern, and western SA sardine stocks. It is suggested that fish become infection with *K. thyrsites* by either directly ingestion of spores that was released into the water upon the death of infected hosts (Webb, 1990; Webb, 1993) and/or by ingestion of infected fish or eggs (Swearer & Robertson, 1999). The diet of SA sardine includes phytoplankton, zooplankton and anchovy eggs (Van der Lingen, 2002). Although a literature study

was unable to reveal whether anchovy (*Engraulis encrasicolus*) is also infected with *K. thyrsites*, it is suggested that infection is possible since anchovies also feed on phytoplankton and zooplankton (Bacha & Amara, 2009) and occur alongside sardines in the ocean (DAFF, 2014). Anchovies spawn along the whole Agulhas Bank, including on the western Agulhas Bank, as well as along the central and eastern Bank (Hutchings *et al*., 2002). Spawning can take place between 14-20 times a year, with spawning intervals of 7-11 days, releasing up to 160 000 eggs per female per season (Melo, 1994). South African sardines do not have a fixed spawning area (Hutchings *et al*., 2002; Coetzee *et al*., 2008). With the eastward shift in the SA sardine population (Coetzee *et al*., 2008), they spawn over similar areas to anchovy with the majority of spawning occurring on the west coast (Hutchings *et al*., 2002; Coetzee *et al*., 2008). SA sardine and anchovy juveniles (young fish developing into mature adults) school together (DAFF, 2014), increasing the potential of infection and re-infection by *K. thyrsites*. It is therefore argued that the potential for the eastern, southern, and western SA sardine stocks to become infected with *K. thyrsites* is equal and that sardines from one geological area are not more likely to be infected than those from another area.

The Cape hake and SA kingklip samples were harvested only from the west coast, thus no relationship between *K. thyrsites* prevalence and area was investigated for these two species of fish.

Table 5.8 Prevalence (%) of *Kudoa thyrsites* for SA sardine samples per areas: east, south, and west coast

Area	*K. thyrsites* prevalence (%)	Number of samples
East coast	100	70
South coast	98.21	56
West coast	94.33	141
***P-value**	0.075	Total n = 267**

*P-value of Chi-square test at confidence level 0.05.
** Total number of samples differs here from the total number of samples indicated in Table 5.3 (qPCR: n = 296) since only 2015 qPCR data was used, and all samples with any missing data (including sex, area, size, and season) were excluded from statistical analysis.

5.3.2.2 Season of harvest

When investigating the prevalence of *K. thyrsites* per season for the SA sardine samples, the P-value (P = 0.000) for the Chi-square test indicated that the prevalence of *K. thyrsites* was dependent on season (Table 5.9). The prevalence was lowest during spring (82.14%) while highest during autumn (100%), winter (98.28%) and summer (97.22%). However, in this study, spring was represented by only 28 SA sardine samples, as compared to more than half as many samples for summer, autumn and winter. When the data for spring was removed, the Chi-square test showed no significant difference (P = 0.496) in prevalence between summer, autumn and winter. These findings are in contrast to studies from the Pacific Ocean. In a study by Moran, Whitaker *et al*. (1999), where Atlantic salmon (*Salmo salar*) were held in seawater net-pens in the Pacific Ocean (British Columbia) to investigate the seasonality of the infective stage of *K. thyrsites*, it was found that higher prevalence occurred during the summer and autumn months compared to the winter and early spring months. Studies by Munday *et al*. (1998), St-Hilaire *et al*. (1998) and Moran, Whitaker *et al*. (1999) also reported that the level of infection tend to be higher during summer than during winter months.

However, results from a similar study (Jones *et al*., 2016) to that of Moran, Whitaker *et al*. (1999), indicated that infections in Atlantic salmon occurred throughout the year. Jones *et al*. (2016) suggested that warmer water temperatures during the winter months of 2013-2014 compared to 1995-1996 may explain the differences in results between these two similar studies.

The South African coast is flanked by two different oceanic currents; the cold Benguela Current from the South Atlantic Ocean along the west coast, and the warm Agulhas Currents from the Indian Ocean along the east coast (Teske *et al*., 2011). The water temperature of the warm Agulhas Currents range between 22 and 27°C, while that of the cold Benguela Currents is typically 4 to 7°C colder throughout the year (Teske *et al*., 2011; Forbes, 2017; Anon., 2017b). In contrast to the South African coast, the difference in water temperatures between winter and summer months for the Pacific Ocean along British Columbia range between 5 and 14°C (Jones *et al*., 2016; Anon., 2017c; Anon., 2017d). The larger difference in water temperatures between winter and summer months for British Columbia compared to that along the South African coast, may explain in part the difference in prevalence of *K. thyrsites* in marine fish species from those parts of the world compared to the South African coast. The infection of SA sardines and Cape hake with *K. thyrsites* may therefore not differ between warmer summer and colder winter months.

Table 5.9 Prevalence (%) of *K. thyrsites* for SA sardine samples per season: summer, autumn, winter and spring

Season	*K. thyrsites* prevalence (%)	Number of samples
Summer	97.22	72
Autumn	100	51
Winter	98.28	116
Spring	82.14	28
***P-value**	0.000	Total n = 267**

*P-value of Chi-square test at confidence level 0.05.
** Total number of samples differs here from the total number of samples indicated in Table 5.3 (qPCR: n = 296) since only 2015 qPCR data was used, and all samples with any missing data (including sex, area, size, and season) were excluded from statistical analysis.

For the Cape hake (P = 0.055) and SA kingklip (P = 0.366) samples, no significant relationships were found between *K. thyrsites* prevalence and season (Table 5.10). However, with the limited number of samples for Cape hake and SA kingklip, in addition to the limitation in that only two seasons were represented within each fish species, it was difficult to make any conclusion about the relationship between *K. thyrsites* prevalence and season for these two species of fish. It would be worthwhile for future studies to collect equal number of samples per fish species per season over a one- or two-year period in order to investigate whether there are seasonal changes for the prevalence of *K. thyrsites*. It is further suggested to also monitor the seasonal abundance of the parasite in the environment between different locations of capture and spawning grounds along the South African coast. Knowledge of the distribution and onset of *K. thyrsites* infection in economically important marine fish species, such as SA sardines and Cape hake, may be of value in developing means of control during annual catches by fisheries.

Table 5.10 Prevalence (%) of *K. thyrsites* for Cape hake and SA kingklip samples, respectively, per season

Cape hake			SA kingklip		
Season	***K. thyrsites* prevalence (%)**	**Number of samples (n)**	**Season**	***K. thyrsites* prevalence (%)**	**Number of samples (n)**
Autumn	82.05	39	Spring	46.66	30
Winter	100	18	Winter	35.90	39
Total n		57			69
*P-value		0.055			0.366

*P-value of Chi-square test at confidence level 0.05.

5.3.2.3 Sex (male and female)

The prevalence of *K. thyrsites* was independent on sex of fish (Table 5.11) for SA sardine (P = 0.133), Cape hake (P = 0.117) and SA kingklip (P = 0.874). Morado and Sparks (1986) also reported no difference in *K. thyrsites* prevalence between male and female samples for Pacific hake.

Table 5.11 Prevalence of (%) *K. thyrsites* in female and male samples for SA sardine, Cape hake and SA kingklip, respectively

	***K. thyrsites* prevalence (%)**			**Number of samples****		
Sex	**SA sardines**	**Cape hake**	**SA kingklip**	**SA sardines**	**Cape hake**	**SA kingklip**
Male	95.07	93.75	42.11	142	32	19
Female	98.40	80.00	40.00	125	25	50
***P-value**	0.133	0.117	0.874	Total n = 267	Total n = 57	Total n = 69

*P-value of Chi-square test at confidence level 0.05

** The numbers of samples for SA sardines, Cape hake and SAS kingklip differ here from the number of samples for qPCR as indicated in Table 5.3 since all samples with any missing data (including sex, area, size, and season) were excluded from statistical analysis.

5.3.2.4 Size (caudal length)

Table 5.12 summarises the P-values for the Chi-square tests performed to test the independence of the presence of *K. thyrsites* and size of the fish. This was done for SA sardine, Cape hake and SA kingklip samples, respectively. For SA sardine (P = 0.080) and SA kingklip (P = 0.526), the P-values indicated that there was no significant relationship between the size of the fish and the presence of *K. thyrsites*.

Table 5.12 Number of samples, means (± standard deviations) of the size (cm) of infected and uninfected samples, the range of size are the P-values for Chi-square test for the independence between *K. thyrsites* prevalence (%) and size (cm) for SA sardine, Cape hake and SA kingklip samples, respectively

Descriptive analyses	***K. thyrsites* prevalence (%)**		
	SA sardine	**Cape hake**	**Sa kingklip**
Number of samples (n)	267	57	69
Mean size (cm) ± standard deviation for infected samples	18.00[a] ± 1.74	39.63[a] ± 10.88	74.40[a] ± 9.47
Mean size (cm) ± standard deviation for uninfected samples	17.19[a] ± 1.01	49.46[b] ± 13.94	77.00[a] ± 10.49
Range of size (cm)	13.2 – 21.1	23.0 – 75.2	58.1 – 100.3
***P-value for Chi-square tests**	0.080	0.050	0.526

*P-value of Chi-square test at confidence level 0.05.
Size refers to caudal length in cm
[ab]Different superscripts indicate significant differences at P < 0.05 within a column.

In contrast to SA sardine and SA kingklip, the P-value (P = 0.050) for the Cape hake samples indicated a significant dependent relationship between the size and the presence of *K. thyrsites*. Infected Cape hake samples were on average smaller in size (39.63 ± 10.88 cm) than uninfected

(49.46 ± 13.94 cm) hake samples. This is in contrast to Morado and Sparks (1986) who reported that the prevalence of both *K. thyrsites* and *K. paniformis* increased with an increase in the size and age of Pacific hake. St-Hilaire *et al*. (1998) and Levsen *et al*. (2008) also reported that larger sized Atlantic salmon are typically more heavily infected than medium to smaller sized fish. Morado and Sparks (1986) investigated 178 Pacific hake samples, ranging in size from 10 cm to 90 cm, over a two-month period. The prevalence of *K. thyrsites* and *K. paniformis* were highest in fish of size 31 cm to 60 cm. In the present study, Cape hake samples of size 31 to 60 cm also showed the highest prevalence compared to samples between 23 to 30 cm and 61 to 72.5 cm. Typically the majority of Cape hake reach a size of 40 to 60 cm; with a maximum of 120 cm for *M. capensis*, and 82 cm for female and 53 cm for male of the *M. paradoxus* species (Cohen *et al*., 1990). This means that during survey sampling of Cape hake from the ocean, the size range 40-60 cm may be over represented, resulting in smaller and larger sizes not being part of the sample. The sample size of the population is therefore too small, consequently, the prevalence of *K. thyrsites* in smaller and larger sized hake is therefore not well measured. The size of the Cape hake samples used in this study ranged from 23.0 to 72.5 cm, with only one sample of 72.5 cm in size (Table 5.12). This sample tested negative for the presence of *K. thyrsites*. Large sized Cape hake was therefore not well represented. In addition, qPCR results from only 57 Cape hake samples were used in this study.

5.4 Conclusions

Using only qPCR results, this study showed that SA sardine, Cape hake and SA kingklip were infected with *K. thyrsites*, with the highest prevalence in SA sardines, followed by Cape hake and SA kingklip (91%, 83% and 40%, respectively) samples. This is the first report of the presence of *K. thyrsites* in SA kingklip. Based on the results from the two qPCR assays used in this study to test for the presence of several *Kudoa* alleles, it was concluded that *K. thyrsites* seems to be the major *Kudoa* species present in SA sardine, Cape hake and SA kingklip.

This study showed that although larger sized (> 16.0 cm) SA sardine samples were more likely to be infected than those of smaller size (13.0-16.0 cm), no significant relationship between prevalence and size were found for the SA sardine samples. The prevalence of *K. thyrsites* was also independent of size for the SA kingklip samples, while for the Cape hake samples, the average size (39.63 ± 10.88 cm) of infected fish was significantly smaller than the average size (49.46 ± 13.94 cm) of uninfected fish. Smaller sized (20-25 cm) and larger sized (> 60 cm) Cape hake were not well represented in this study, therefore the results not reflect true relationships between size and prevalence of *K. thyrsites*. It is concluded that the size of fish is not a useful prediction of *K. thyrsites* infection in SA sardines and SA kingklip. However, it is suggested that a larger number of Cape hake samples, where size-ranges are represented by an equal number of samples, be used to investigate the relationship between size and the prevalence of *K. thyrsites*.

The prevalence of *K. thyrsites* was independent of sex, and season for SA sardines, Cape hake and SA kingklip. Although SA sardine samples from the west coast showed a lower prevalence of

K. thyrsites compared to samples from the east and south coast, there was no significant difference in prevalence between samples from these different areas. It was therefore concluded that sex is not useful for the prediction of *K. thyrsites* infection in SA sardines, Cape hake and SA kingklip. It is further concluded that area of capture for SA sardines is not useful to the fishery and fish processing industries to predict the prevalence of *K. thyrsites* in sardine catches.

Although 91% of the SA sardine samples from this study were infected with *K. thyrsit*es, only 13% showed severe myoliquefaction. It was therefore concluded that, although the majority of SA sardines might be infected with *K. thyrsites*, severe myoliquefaction may occur in only a small percentage of the infected population. This may be related to the level of infection among individual fish. It is suggested for future research to investigate, quantify and correlate the level of infection (number of *K. thyrsites* spores) with the severity of *post-mortem* myoliquefaction in an attempt to assist the SA sardine canning industry to separate heavily infected fish from lightly infected fish as part of their quality control system. Lightly infected SA sardines may still be canned before severe myoliquefaction is evident. In contrast to the SA sardine samples, the Cape hake samples did not show signs of myoliquefaction, however, black pseudocysts were evident in some of the *K. thyrsites* infected samples. Further studies into the relationships between the extent of myoliquefaction and level of *K. thyrsites* between different species of fish are also suggested.

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Chapter 6

Moisture and ash content of South African sardine (*Sardinops sagax ocellatus*) and moisture, ash and protein content of Cape hake (*Merluccius capensis* and *M. paradoxus*) infected by *Kudoa thyrsites*

Abstract

Kudoa thyrsites infection is associated with *post-mortem* myoliquefaction in marine fish species, resulting in protein breakdown and loss of muscle texture. Myoliquefied fish is rejected by consumers and is unfit for further processing such as canning and/or filleting. However, since myoliquefaction is evident only 38 to 56 hours *post-mortem*, it is accepted that *K. thyrsites* infected fish may reach processors and be consumed by consumers. The consumption of *K. thyrsites* infected fish is harmless, however, the moisture, ash, and protein content of infected of South African (SA) sardine and Cape hake is unknown. This study investigated the moisture and ash content of SA sardine and Cape hake infected with *K. thyrsites*. In addition, the crude protein content of Cape hake samples, infected with *K. thyrsites,* was determined. The Association of Official Agricultural Chemists (AOAC) official methods were used to determine the moisture, ash and crude protein contents. Real-time quantitative polymerase chain reaction (qPCR), with the use of two TaqMan® single nucleotide polymorphism (SNP) assays, was used as a qualitative, diagnostic tool for identifying *K. thyrsites* infected fish samples. The moisture and ash contents between *K. thyrsites* infected and uninfected SA sardine and Cape hake samples were not significantly ($P > 0.05$) different. The crude protein content of *K. thyrsites* infected Cape hake muscle also did not differ significantly ($P > 0.05$) from uninfected samples. The overall moisture and ash content for the SA sardine samples were 70.83 ± 3.36% and 1.87 ± 0.83%, respectively. The overall percentage moisture, ash and crude protein content for the Cape hake samples were, 72.18 ± 3.53%, 1.16 ± 0.25% and 20.83 ± 1.81%, respectively. It was concluded that moisture, ash, and protein content were not useful in distinguishing between infected and uninfected samples. Since the protein content of infected hake did not differ significantly from uninfected samples, further research into the preparation of fish protein hydrolysates (FPH) from *K. thyrsites* infected Cape hake is suggested.

Keywords: Ash content, Cape hake, crude protein, *Kudoa thyrsites*, moisture content, SA sardine

6.1 Introduction

The major constituents in the edible portion of fish are protein, fat (lipids), and moisture, while ash (minerals and vitamins) and carbohydrates make up the minor components (Huss, 1995). These constituents are collectively referred to as the proximate composition of foodstuffs. The ash content refers to the inorganic residue, or amount of minerals, after complete oxidation of the organic matter (Ismail, 2017). The ash content of fish muscle typically ranges between 0.4 to 4.0% (Güner *et al*., 1998; Koubaa *et al*., 2010; Usydus *et al*., 2011; Dhaneesh *et al*., 2012). Carbohydrates make up only a very small part of fish muscle tissue, ranging from 0.2% to 1.5% (Françoise, 2010). Among the protein-rich food resources of the world, fish and fishery products are important sources of animal protein. Fish is considered as a source of high-quality protein because of the balanced content in amino acids, especially the essential amino acids necessary for physical and mental well-being (Swaisgood & Catignani, 1991; Schiepers *et al*., 2010). Raw marine and fresh water fish muscle typically contain between 12% and 22% protein (Table 6.1). The lipid and water content typically together make up about 80% of fish muscle (Ofstad *et al*., 1996), with moisture ranging between 28 and 96% (Huss, 1995; Weber *et al*., 2008; Koubaa *et al*., 2010; Marimuthu *et al*., 2012; Aberoumand, 2014).

Table 6.1 Average protein (%) content of the raw muscle tissue of several marine and fresh water fish species

Fish species	Average % protein of raw fish muscle	Reference
Rainbow trout (*Oncorhynchus mykiss*)	19.80 ± 0.03	Gokoglu *et al*. (2004)
Silver catfish (*Rhamdia quelen*)	15.50 ± 0.19	Weber *et al*. (2008)
Sandsteenbras (*Lithognatus mormyrus*)	18.92 ± 0.05	Koubaa *et al*. (2010)
Red mullet (*Mullus barbatus*)	20.27 ± 0.06	
Commmon pandora (*Pagellus erythrinus*)	19.52 ± 0.08	
Bluespotted seabream (*Pagrus caeruleostictus*)	18.17 ± 0.04	
Morocco dentex (*Dentex maroccanus*)	19.39 ± 0.05	
Catfish (*Clarias gariepinus*)	16.24	Foline *et al*. (2011)
Cod (*Gadus morhua callaries*)	17.40 ± 0.90	Usydus *et al*. (2011)
Herring (*Clupea harengus membras*)	18.10 ± 0.50	
Baltic salmon (*Salmo salar*)	18.40 ± 0.70	
Carp (*Cyprinus carpio*)	16.70 ± 0.80	
Trout (*Oncorhynchus mykiss*)	18.90 ± 0.80	
Walleye pollock (*Theragra chalcogramma*)	12.20 ± 2.00	
Sole (*Limanda aspera*)	13.40 ± 1.30	
Sutchi catfish (*Pangasius hypophthalmus*)	12.90 ± 0.80	
Tilapia (*Oreochromis niloticus*)	16.40 ± 0.60	

Knowledge of the proximate composition of fish and seafood products is important for several reasons. It helps food technologists to define the optimum processing and storage conditions to ensure the quality (for example, prevention of lipid oxidation and protein denaturation during frozen storage) and food safety (for example, carbohydrate concentrations influence the ultimate pH and consequently bacterial spoilage) of the food products. Knowledge of the proximate composition

assist consumers in making informed decisions about nutritional and energy values, and, in addition, assist food technologists in the accurate nutritional labelling of the food product. The high nutritional quality and health benefits of fish and fishery products have been well documented over many years (Steffens, 1997; Simopoulos, 2002; Moyad, 2005; Domingo, 2007; Usydus *et al*., 2011). Fish and fishery by-products are used in animal feeds, surimi-based products, and manufacturing of fish protein hydrolysates (FPH) (Chalamaiah *et al*., 2012). Proper information about the proximate composition of the raw materials are therefore important for formulating such products, as well as for calculating product yield. It is well documented that the proximate composition of fish (muscle as well as other organs, such as the gonads, ovaries, and liver) differ between species and show variation depending on seasonal changes, migratory behaviour, sexual maturity, and feeding and spawning cycles (Huss, 1995; Petit-Domínquez *et al*., 2010; Vollenweider *et al*., 2011). These variations are particularly obvious for wild, free-living fish; however, fish raised in aquaculture may show less variations since the fish farmer can control several of these factors, therefore, design the proximate composition of the fish by selecting certain farming conditions (Huss, 1995).

The infection of marine fish species with *K. thyrsites* and *K. paniformis* is associated with protein breakdown of muscle tissue due to the activity of *Kudoa* specific cathepsin proteolytic enzymes (An *et al*., 1994; Funk *et al*., 2008). Although infection by the parasite *K. thyrsites* in fish is considered harmless if consumed by humans (Alvarez-Pellitero & Sitja-Bobadilla, 1993), it poses an important concern with regard to wastage of animal protein and economic losses.

It has been noted in preliminary studies related to this study, that fresh South African (SA) sardine fillets (24 h *post-mortem*) infected with *K. thyrsites* and showing extensive myoliquefaction appeared moistier, (Figure 6.1A) compared to uninfected fillets (Figure 6.1B). Structural changes of muscle proteins results in the release of water molecules (Ofstad *et al*., 1996). Fish muscle lose most of its moisture during the early hours (three) *post-mortem* due to rigor contractions (Ofstad *et al*., 1996), reduction in muscle pH (Ofstad *et al*., 1996; Lawrie, 1998) and *post-mortem* changes in the muscle ultrastructure (Ofstad *et al*., 1996). Most of the water in muscle tissue exist within the myofibrils, in the spaces between the myosin and actin (tropomyosin) (Lawrie, 1998) and is referred to as “free water”. About 5% of the total water in muscle is directly bound to hydrophilic groups on proteins and is referred to as “bound water”. Protein denaturation results in the release of bound water, which may increase the amount of water released from the muscle tissue.

Figure 6.1 Photos of representative samples of *Kudoa thyrsites* infected and uninfected South African sardine fillets (24 hours *post-mortem*). (A) Extensively myoliquefied SA sardine fillet. (B) SA sardine fillet not yet showing signs of myoliquefaction. Photos were taken with a Canon PowerShot S2 IS camera.

The effects of *Kudao* parasites, associated with myoliquefaction (also referred to as “milky” or “jellied” flesh) on the chemical content of marine fish species were documented in as early as 1954 (Tibbo *et al*., 1961; Patashnik & Groninger, 1964). During 1954, Tsuchiya and Tatsukawa (in Tibbo *et al*., 1961) mentioned that “jellied” meat from swordfish (*Xiphias gladius* L.) contained a large amount of moisture. In 1957, Tsuchiya and Kudo (in Tibbo *et al*., 1961) found a higher content of water-soluble nitrogen in “jellied” swordfish. A study in 1964 (Patashnik & Groninger, 1964) analysed the moisture, oil, protein, ash, sodium and potassium content of milky (varying degrees of continuous to discrete milky appearances) halibut (*Hippoglossus stenolepis*) since previous findings from the same authors (unpublished data) indicated that the moisture and sodium content of Dover sole (*Microstomus pacificus*) with severe milky appearance increased, while the protein and potassium content decreased compared to normal fish. However, their results indicated that the milky halibut samples had similar moisture, oil, protein, ash, sodium and potassium contents compared to normal halibut.

Up to date, no additional published research could be found on the effects of *Kudoa* species, associated with myoliquefaction, on the chemical content of fish musculature. It was therefore of interest to investigate the effects of *K. thyrsites* infection (presence of the parasite) on the moisture, ash and protein content of SA sardine, Cape hake and SA kingklip. From a nutritional and quality

viewpoint, such information would be of importance to the fishing and fish processing industries, as well as to the consumer.

Since myoliquefaction associated with *K. thyrsites* becomes apparent only several hours (38–56) *post-mortem* (Levsen *et al*., 2008), infected fish inevitably reach processors and/or consumers. The extent of myoliquefaction is positively related to the level of infection and endogenous proteolytic activity (Dawson-Coates *et al*., 2003; Samaranayaka *et al*., 2006; Zhou & Li-Chan, 2009). If a method of prediction of infection by *K. thyrsites* could be developed, heavy infected samples, prone to develop severe myoliquefaction, could be removed and channelled into alternative process, such as fish meal and/or FPH (Samaranayaka & Li-Chan, 2008). Some studies (Samaranayaka & Li-Chan, 2008; Chalamaiah *et al*., 2012) investigated the effects of the proteolytic enzyme from *K. paniformis* on fish protein hydrolysates (FPH) prepared from Pacific hake. The proximate composition of Pacific hake muscle tissue infected with *K. paniformis* at an infection level of 20 x 10^6 spores·g^{-1} fish mince was 82.99 ± 0.05% moisture, 15.16 ± 0.06% crude protein, 1.20 ± 0.03% ash, and 1.78 ± 0.05% crude lipid (Samaranayaka & Li-Chan, 2008). The proximate composition of the Pacific hake muscle tissue with such a high level of *K. paniformis* infection was not compared to muscle tissue with lower levels of infection, or with fish without any infection. The reason for this was that the purpose of the study conducted by Samaranayaka and Li-Chan (2008) was to investigate the possibility of preparing antioxidative FPH from *K. paniformis* infected Pacific hake fillet mince without the addition of exogenous enzymes. Their study showed that FPH with antioxidant properties could be prepared from *K. paniformis* infected (required level of infection of ~10^7 spores·g^{-1}) Pacific hake fish fillet mince through one-hour autolysis at 52ºC at a pH value of 5.50 without addition of commercial proteolytic enzymes.

Near-infrared (NIR) spectroscopy has been shown to be a reliable, non-destructive method to determine the proximate composition (ash, moisture, fat and protein content) of fish and seafood products (Nortvedt *et al*., 1998; Khodabux *et al*., 2007; Folkestad *et al*., 2008; Nilsen *et al*., 2010; Malcolm *et al*., 2012). By correlating the moisture, ash and/or protein content of fish to the presence of *K. thyrsites*, (or level of infection), NIR has the potential to be used as a tool to identify infected fish and to predict the levels of infection, and consequently the degree of myoliquefaction. This would be of great benefit to the fishery and fish processing industry to aid in development of quality control and processing systems.

The aim of this study was to investigate the effects of *K. thyrsites* infection (presence of the parasite) on the moisture and ash content of South African sardine (*Sardinops sagax ocellatus*) and the moisture, ash and protein content of Cape hake (*Merluccius capensis* and *M. paradoxus*) raw muscle tissue, respectively. This research served to obtain base-line knowledge about whether fish infected with *K. thyrsites* differ in moisture, ash, and protein from uninfected fish. This information could aid in future research wherein the application of NIR as a predictive tool for the level of *K. thyrsites* infection and/or level of myoliquefaction, is investigated.

6.2 Materials and methods

6.2.1 Samples and sample preparations

A total of 106 SA sardine (*S. sagax ocellatus*) samples, 44 male and 62 female, were kindly supplied by the Department of Agriculture, Forestry and Fisheries (DAFF) (Addendum B). The fish samples were harvested from the western coast of SA at different months during 2015. South African sardines were harvested during pelagic surveys using midwater trawl nets. The SA sardine samples were received as individually tin-foil-wrapped, frozen fillets (a single fillet per sardine sample). All biological information, including size (caudal length in cm), weight (g), and sex (male and female) for each sardine fillet was provided by the DAFF. The average size of the SA sardine samples was 18.71 ± 1.45 cm and the average weight was 93.83 ± 21.18 g.

A total of 122 Cape hake (39 male, 46 females, 37 unknown sex; average size of 43.4 ± 15.4 cm) samples were kindly supplied by the DAFF during 2015 in the form of individually tin-foil-wrapped, frozen fillets (a single fillet per Cape hake sample). The Cape hake samples were harvested by the DAFF during demersal surveys using bottom trawl nets. All biological information, including size (caudal length in cm), sex (male and female) and species (*M. capensis* and *M. paradoxus*) for each hake fillet was provided by the DAFF.

Thirty-five of the Cape hake samples, with an average size of 55.29 ± 11.54 cm (weight data was not available), were used for moisture and ash analysis. The remainder of the Cape hake samples (average size 37 ± 9.41 cm) were used for crude protein analysis.

It is noteworthy that there were unavoidable gaps in analysis of protein for the SA sardine samples due to interruptions of laboratory work during 2015 and 2016, resulting in loss of the samples. These interruptions also resulted in the loss of some Cape hake samples; consequently, having two incomplete datasets for the Cape hake samples, where the one set lacked protein data, and the other set moisture and ash data.

6.2.2 DNA-based identification of Kudoa *infection*

The presence of *K. thyrsites* was determined according to the real-time quantitative polymerase chain reaction (qPCR) protocol, used as a qualitative diagnostic tool, as described in more detail in Chapter 4 of this dissertation (pp. 50-68).

6.2.2.1 DNA extractions

The extractions of deoxyribonucleic acid (DNA) from the fish samples had been performed before proximate composition analyses commenced in order to prevent carry-over of DNA between individual fish samples. DNA from each fish fillet was extracted with the use of the Qiagen DNeasy Mini Kit (Qiagen, Whitehead Scientific Pty., Ltd., Cape Town). The DNA samples consisted of DNA extracted from a composite muscle tissue sample of approximately 10 to 20 mg. The composite tissue samples were collected from the anterior, centre, and posterior regions of each fillet in order to prevent false negatives (Samaranayaka *et al*., 2006; Funk *et al*., 2007; Marshall *et al*., 2016), while

sterile scalpels per fish sample were used in order to prevent DNA carry-over and false positives. Prior to the TaqMan® SNP genotyping assays, the concentration and purity of the extracted DNA was determined at the Centre for Proteomic and Genomic Research (CPGR, Cape Town) using the NanoDrop ND-1000. The DNA extracts were kept frozen at -80ºC until qPCR analyses at CPGR.

6.2.2.2 Primers used and DNA amplifications

The custom TaqMan® single nucleotide polymorphism (SNP) genotyping assays, as described in Chapter 4 (pp. 50-74), was used for the determination of the presence of *K. thyrsites*. The *K. thyrsites* assay mix contained two PCR primers (5'-CCTATCAACTAGTTGGTGAGGTAGTG-3' and 5'-TCTCCGGAATCGAACCCTGAT-3') that flank the region containing the single nucleotide polymorphism (SNP) site specific to *K. thyrsites*. The *K. thyrsites* assay mix also contained two TaqMan® probes. The one probe matched the allelic variant *K. thyrsites* and labelled with VIC, while the second probe matched the allelic variant: *K. paniformis*/*K. miniauriculata*/*K. dianae*. The second probe was labelled with FAM (6-carboxyfluorescein).

All the qPCR TaqMan® assays were performed in 384-well plates. Twenty nano-grams (ng) of sample DNA was amplified in a 5 µL volume PCR reaction mix containing 0.9 µM primers and 0.2 µM probes. A working master mix for each assay was prepared by mixing 0.25 µL of TaqMan® assay mix (20x), 2.5 µL TaqMan® Genotyping Master Mix (Thermo Fisher), 0.25 µL water and 2 µL of genomic DNA at 20 ng. Samples shown to be positive for *K. thyrsites* from previous studies (Chapter 4) were pooled for preparation of positive control samples containing DNA concentrations of 10 $ng \cdot \mu L^{-1}$.

Reactions were performed according to the protocol as described in Chapter 4, which included a pre-reading of the plate, followed by the standard PCR cycling parameters of 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, and finally, the post-reading of the plate. End-point fluorescence was read on the 7900HT Fast Real-Time PCR System using the SDS version 2.3 software (Applied Biosystems, USA) and the TaqMan® Genotyper software version 1.3 was used to generate allelic discrimination plots.

6.2.3 Moisture, ash and protein analyses

Before moisture, ash and protein analyses, all Cape hake samples were thawed over night at refrigerator temperatures (4-6ºC) and conditioned at room temperature (21-22ºC) for 10 min before analysis. The SA sardine samples were thawed for 20 min at refrigerator temperatures due to the small size of the samples, and conditioned at room temperature for 5 min before analysis. For moisture and ash analyses, the skin and bones were removed from all Cape hake fillet samples before being homogenised (Russel Hobbs handheld blender). The bones of the SA sardine fillet samples were also removed, but the skin was kept intact and formed part of the homogenised sample. All moisture and ash analyses were done in duplicate. Moisture content was determined by drying the homogenised sample in a drying oven at 100°C for 24 h. The dried samples were allowed

to cool in a desiccator for 30 min. The samples were weighed and the moisture content determined according to the Association of Official Agricultural Chemists (AOAC) official method 934.01 (AOAC, 2002a). The ash content was determined according to the AOAC official method 942.05 (AOAC, 2002b). The oven dried samples from the moisture analysis, in the original crucibles, were placed in an incinerator oven at 500ºC for 6 h. After the oven had been turned off, the samples were left in the oven for a further 2 h and then transferred to a desiccator to cool for 30 min. The crucibles containing the ash were then weighed and the percentage ash was calculated.

Crude protein was determined in triplicate from intact Cape hake fillet muscle tissue, excluding bone and skin tissue. Samples were thawed overnight at refrigeration temperatures, and conditioned to room temperature for 20 min before analyses. Composite muscle tissue samples of 0.150 g were collected from three anatomical locations (anterior, middle, and posterior) from each fillet with the use of tweezers. Each composite sample was inserted into a foil-cup designed for the Leco protein analyser (LECO FP-428, USA). Nitrogen was determined according to the Dumas combustion method 992.15 (AOAC, 2002c) using the Leco FP-428 nitrogen and protein determinator (Leco, Saint Joseph, MI, USA). The Leco was calibrated with the internal standard ALFALFA (LECO, calibration sample for CHNS, 502-273, nitrogen of 3.38% ± 0.04). The nitrogen content was multiplied by 6.25 according to the AOAC official method 992.15 (AOAC, 2002c) to calculate the protein concentration as a percentage for each sample.

6.2.4 Statistical analyses

Statistical analyses were performed with the use of XLSTAT, version 19.01 (Addinsoft, 2017, Paris), and SAS statistical software version 9.2 (SAS Institute Inc. Cary, NC, USA). Means, standard deviations and normality (skewness and kurtosis) of the size (cm) data for each sample group of fish, (namely SA sardine, Cape hake samples used for moisture and ash, and Cape hake samples used for protein content analysis) were calculated and visualised by means of boxplots to identify outliers and dispersion of the size data. Pearson's correlation coefficient (at the 5% significance level) was calculated for the size and weight data for the SA sardine samples. Analyses of variance (ANOVAs) were used to investigate relationships and interactions of the independent variables size (cm) and sex (male and female), with the dependent variables moisture, ash and protein content. This was done for each sample group separately.

The frequency table with Chi-square tests (confidence level at $P < 0.05$) was calculated for classifications of Cape hake species (*M. capensis* and *M. paradoxus*) by using *K. thyrsites* as classification variable to test the dependence of the presence of *K. thyrsites* on classifications. Frequency tables with Chi-square tests (confidence level at $P < 0.05$) were also calculated for classification of sex (male and female) and size (cm) by using *K. thyrsites* as classification variable to test the dependence of the presence of *K. thyrsites* on classifications for each sample group of fish.

Analyses of variance (ANOVA) for the dependent variables moisture, ash and protein content with *K. thyrsites* independent variables, were calculated for each group of fish samples. Student's t-Least Significant Difference was calculated at the 5% confidence level to compare means for significant effects. Shapiro-Wilk tests were performed for testing non-normality (Shapiro & Wilk, 1965).

6.3 Results and discussion

qPCR results from this study indicated the presence of *K. thyrsites* in 65 of the 106 SA sardine samples (prevalence of as 64%) analysed. None of the Cape hake samples tested positive for the alleles *K. paniformis*/*K. miniauriculata*/*K. dianae* and *K. paniformis*, respectively. The Cape hake samples showed 66% (80 of the total 122 Cape hake samples: 17% for the moisture and ash sample group, and 85% for the protein sample group) prevalence for *K. thyrsites*.

6.3.1 Descriptive analyses

The Pearson's correlation coefficient (r) and coefficient of determination (R^2) between size (cm) and weight (g) for the SA sardine samples were calculated as r = 0.954 and R^2 = 0.909, showing a significant (P < 0.0001) positive linear relationship. Therefore, further discussion pertaining to SA sardine data is related to size data only. Pearson's correlation was calculated only for the SA sardine samples, since the Cape hake sample groups did not have weight data. The average moisture and ash content for all SA sardine samples was 70.83 ± 3.36% and 1.87 ± 0.83%, respectively. Table 6.2 summarises the ANOVA for the dependent variables; moisture and ash content (%) with the independent variables, size and sex for the SA sardine samples. There was no significant interaction between size and sex for moisture (P = 0.623) and ash (P = 0.484) content. The independent variables, size and sex also had no significant effect (P > 0.05) on moisture and ash content. Male samples had an average moisture and ash content of 71.24 ± 3.12% and 2.05 ± 0.87%, respectively. Female samples had an average moisture and ash content of 70.54 ± 3.49% and 1.74 ± 0.78%, respectively.

Table 6.2 Analysis of variance (ANOVA) for the dependent variables moisture (%) and ash (%), with the variables size (cm) and sex (male and female) as independent variables, as well as two-way interactions, for SA sardine samples

	Moisture (%)			Ash (%)		
Source	**df**	**MS**	**P-value**	**df**	**MS**	**P-value**
Size	1	27.950	0.115	1	0.042	0.805
Sex	1	2.048	0.668	1	0.501	0.392
Size*Sex	1	2.685	0.623	1	0.335	0.484
Error	102	11.054		105	0.678	

df - degree of freedom
MS - mean square
P-value - probability value of F-ratio test

Tables 6.3 and 6.4 summarise the ANOVAs for the Cape hake samples with moisture and ash, and protein content, respectively, as dependent variables, and sex and size of the fish samples as

independent variables. Similar to the SA sardine data, there was no significant interaction between sex and size for moisture (P = 0.166), ash (P = 0.592) and protein (P = 0.051) contents. There were also no significant differences in moisture (P = 0.356), ash (P = 0.944) and protein (P = 0.089) content between male and female Cape hake samples. Male hake samples had an average moisture, ash and protein contents of 72.13 ± 2.72%, 1.19 ± 0.14% and 20.91 ± 2.02%, respectively. Female hake samples had an average moisture, ash and protein contents of 72.91 ± 3.79%, 1.16 ± 0.33% and 20.73 ± 1.54%, respectively. These findings are similar to a study (Marais, 1990) wherein it was also demonstrated that no differences in moisture, ash, and protein content between males and females of the species *Myxus capensis*, *Diplodus sargus*, and *Monadactylus falciformis* of the Eastern Cape estuaries, South Africa, were found. The size of the Cape hake samples used for the determination of moisture and ash content had no significant (P = 0.944) effect on these dependent variables. The average moisture and ash contents for *K. thyrsites* infected and uninfected Cape hake samples combined were 72.18 ± 3.53% and 1.16 ± 0.25%, respectively.

Table 6.3 Analysis of variance (ANOVA) for the dependent variables moisture (%) and ash (%), with the variables size (cm) and sex (male and female) as independent variables, as well as two-way interactions, for Cape hake samples used for moisture and ash determinations

	Moisture (%)			Ash (%)		
Source	**df**	**MS**	**P-value**	**df**	**MS**	**P-value**
Size	1	10.787	0.356	1	0.000	0.944
Sex	1	21.639	0.196	1	0.023	0.617
Size*Sex	1	25.041	0.166	1	0.295	0.592
Error	21	12.133		21	0.087	

df - degree of freedom
MS - mean square
P-value - probability value of F-ratio test

Table 6.4 Analysis of variance (ANOVA) for the dependent variable protein (%) with the variables size (cm) and sex (male and female) as independent variables, as well as two-way interactions, for Cape hake samples used for protein determinations

		Protein content (%)	
Source	**df**	**MS**	**P-value**
Size	1	15.240	0.030
Sex	1	9.165	0.089
Size*Sex	1	12.258	0.051
Error	55	3.059	

df - degree of freedom
MS - mean square
P-value - probability value of F-ratio test

Size had a significant (P = 0.030) influence on protein content. Cape hake samples of larger size had a higher protein content than smaller sized samples. Cape hake samples smaller than the average size of 38.90 ± 10.42 cm had protein content of 20.24 ±1.88% and samples larger than 38.90 ± 10.42 cm had an average protein content of 21.08 ± 2.17%. The effect of body size on the proximate composition of different fish species is contradicting; where for some fish species no significant differences between various size groups were found, results for other species showed significant differences for some of the proximate components, while not for others (Hussain *et al*., 2016). However, several studies documented lower protein content for young and smaller fish

compared to adult and larger fish of the same species (Marais, 1990; Salam & Davies, 1994). These differences may be attributed to several intrinsic and extrinsic factors, such as, health status of the fish, feeding, metabolism, breeding period, and season (Huss, 1995; Guinea & Fernandez, 1997; Moss *et al*., 2009; Küçükgülmez *et al*., 2010).

The presence of *K. thyrsites* was independent of size for both the SA sardine (Chi-square P = 0.247) and Cape hake samples (Chi-square P = 0.053). The independence for the Cape hake samples in this study was in contrast to the findings reported in Chapter 5, where the presence of *K. thyrsites* was significantly (P = 0.05) dependent of size. The size range for the Cape hake samples analysed in Chapter 5 was 23.0 to 75.2 cm, where samples in the size range of 31.0 to 60.0 cm showed the highest prevalence. For the present study, the size range for the Cape hake samples was 23.4 to 75.1 cm (average 43.4 ± 15.4 cm), with the majority (78%) of samples falling within the 31.0 to 60.0 cm range. However, in contrast to the samples used in Chapter 5, several of the smaller (23-28 cm) and larger (61-75 cm) sized samples in this study tested positive for the presence of *K. thyrsites*, therefore, contributing to the insignificant Chi-square P-value (P = 0.053) indicated independences between the two factors.

Similar to the results described in Chapter 5, there was no significant difference (P = 0.3224) in the prevalence of *K. thyrsites* between the hake species; *M. capensis* (80.56%) and *M. paradoxus* (88.24%) (Table 6.5). There was also no significant difference (P = 0.0844) in prevalence of *K. thyrsites* (Table 6.5) between male and female for the SA sardine and the Cape hake samples. Morado and Sparks (1986) also reported no difference in *K. thyrsites* infection between male and female Pacific hake (*M. productus*) samples.

Table 6.5 Frequency (%) of infection by *K. thyrsites* between *M. capensis* and *M. paradoxus*, and between male and female samples for the SA sardine and Cape hake samples, respectively

	Hake species		**Sex**			
			Hake		**Sardines**	
Frequency	***M. capensis***	***M. paradoxus***	**Male**	**Female**	**Male**	**Female**
% of samples infected with *K. thyrsites*	80.56	88.24	93.75	78.57	56.82	69.35
Chi-square P-value	0.3224		0.0844		0.1848	

6.3.2 Sardines: Effects of Kudoa thyrsites *infection on moisture and ash content*

ANOVA for the dependent variables moisture (%) and ash (%) for the SA sardine samples, with *Kudoa* infection as independent variable, is summarised in Table 6.6. The Shapiro-Wilk test (P < 0.05) indicated that all data were normally distributed and did not require data transformation. Infection by *K. thyrsites* had no significant effect (P > 0.05) on the moisture or ash content of SA sardine muscle tissue. The SA sardine samples infected with *Kudoa thyrsites* had a moisture content of 70.98 ± 3.89%, while uninfected samples had a moisture content of 70.57 ± 2.13% (Table 6.7). This is similar to the findings by Patashnik and Groninger (1964) for halibut in that the moisture and ash contents of milky samples did not differ from values for normal fish. These authors argued that since the areas of the milky appearances were only a small fraction of the total weight of the samples,

the analytical results applied mainly to the predominantly normal tissue, and therefore no differences were observed. However, for the current study, the appearance of myoliquefaction within a sardine fillet was throughout the whole fillet and the same type of argument could not be followed (Figure 6.1). In addition, the sardine samples infected with *K. thyrsites* showing myoliquefaction were not compared with infected samples that did not show myoliquefaction. Since Tsuchiya and Tatsukawa (in Tibbo *et al*., 1961) found chemical changes in swordfish exhibiting myoliquefaction, it would be of interest to compare the proximate composition of myoliquefied with normal-looking fish muscle of infected fish; subsequently correlating it to the level of infection.

Table 6.6 Analysis of variance (ANOVA) of the dependent variables moisture (%) and ash (%) for SA sardine samples with *K. thyrsites* infection as independent variable

		Moisture (%)		Ash (%)	
Source	**df**	**MS**	**P-value**	**MS**	**P-value**
K. thyrsites	1	4.0632	0.5511	0.0866	0.7249
Error	104	11.3603		0.6955	
Shapiro-Wilk			< 0.0001		0.0017

df – degree of freedom
MS – mean square
P-value – probability value of F-ratio test

Table 6.7 Mean (± standard deviation) moisture (%) and ash (%) for *K. thyrsites* infected and uninfected SA sardine samples, respectively

Level of *K. thyrsites*	**Moisture (%)**	**Ash (%)**	**Number of samples**
Present	70.98[a] ± 3.89	1.89[a] ± 0.84	68
Absent	70.57[a] ± 2.13	1.83[a] ± 0.81	38
All sardine samples	70.83 ± 3.36	1.87 ± 0.83	106

[ab]Different superscripts for moisture (%) and ash (%), respectively, differ at $P < 0.05$.

In the case of this study, the sardine samples were kept frozen between 6 to 18 months before analysis commenced. The prolonged frozen storage might have resulted in moisture loss upon thawing because of cell damage and protein denaturation from ice crystal formation (Huss, 1994; Sultanbawa & Li-Chan, 2001; Careche *et al*., 2002; Leygonie *et al*., 2012; Syamaladevi *et al*., 2012; Cheng *et al*., 2017), consequently masking the effects of *K. thyrsites* induced proteolysis. A similar argument could be followed for the ash content; water soluble minerals and vitamins would leach out during the thawing of the samples due to cell damage from ice crystal formation during prolonged freezing-thawing cycles within the freezer (Sampels, 2015), while non-water-soluble minerals and vitamins would become more concentrated (Johnston *et al*., 1994). It is suggested to repeat this current study with the use of fresh samples and/or to homogenise fresh samples and keep the homogenised samples frozen until analysis. In addition, the frozen storage period of samples should be kept to a minimum.

Several studies have reported on the proximate composition of other small pelagic fish species (sardine, anchovy, and round herring) from other countries; European anchovies (Boran *et al*., 2008; Kaya & Turan, 2010; Roncarati *et al*., 2012), sardines from Brazil (Bulle *et al*., 2011), and round herring (*Etrumeus teres*) from the Mediterranean Sea (Küçükgülmez *et al*., 2010). However, no studies could be found for the proximate composition of SA sardine, anchovy (*Engraulis*

encrasicolus) and round herring (*Etrumeus whiteheadi*). The overall moisture content of 70.83 ± 3.36% for the SA sardine samples was similar to that of Brazilian sardines (*Sardinella brasiliensis*) of 70.41 ± 0.72% (Bulle *et al*., 2011), but lower compared to the moisture content (77.20 ± 0.40%) of European anchovy (*Engraulis encrasicholus* L.) from the south Adriatic seas (Roncarati *et al*., 2012) and Mediterranean round herring (75.0 to 76.0%; Küçükgülmez *et al*., 2010). However, Boran *et al*. (2008) and Küçükgülmez *et al*. (2010) reported significant seasonal variations in the moisture content (65.9 to 73.9%) for European anchovy and round herring, respectively, illustrating that seasonal changes, which are linked to spawning cycles, significantly influences the chemical compositions of fish. It is further noteworthy that the proximate and chemical composition of fish show further variation with respect to geographic location, size and sex (Huss, 1995; Güner *et al*., 1998) and it is therefore difficult to make direct comparisons between different fish species without keeping these factors in mind.

The ash content for *K. thyrsites* infected SA sardine samples was 1.89 ± 0.84%, and 1.83 ± 0.81% for the uninfected samples (Tables 6.7). The average ash content for the infected and uninfected samples combined was 1.87 ± 0.83%. Boran *et al*. (2008) reported the ash content of European anchovy to range from 1.7% to 2.2%, depending on the month of capture, illustrating that proximate composition of anchovies significantly varies during the catching season.

6.3.3 Cape hake: Effects of Kudoa thyrsites *infection on moisture, ash and protein content*

A total of 35 Cape hake samples were used for the investigation of the effect of *K. thyrsites* infection on moisture and ash content of Cape hake muscle tissue. Only 17% of these 35 Cape hake samples were infected with *K. thyrsites*. ANOVA for the dependent variables moisture and ash content, with *K. thyrsites* infection as independent variable, showed that *K. thyrsites* infection had no significant effect on the moisture content ($P = 0.5606$) or on the ash content ($P = 0.9190$) of muscle tissue from the Cape hake samples (Table 6.8). The Shapiro-Wilk test ($P < 0.05$) indicated that all data were normally distributed and that data transformation was not required (Table 6.8). The moisture content for infected fish was 72.97 ± 2.92% and for uninfected fish was 72.02 ± 3.68% (Table 6.9). Similar to the argument for the SA sardine samples, prolonged frozen storage of the Cape hake samples may have resulted in the alleviation of chemical changes (Patashnik & Groninger, 1964) associated with *K. thyrsites* induced proteases.

The overall moisture content (72.18 ± 3.53%) for the infected and uninfected Cape hake samples combined (Table 6.8) was lower compared to the moisture content (80.40%) of *M. capensis* (Cape hake) as reported by Simmonds *et al*. (in Pérez-Villareal & Howgate, 1987), as well as compared to European hake (*M. merluccius*) from the south Adriatic seas, with a moisture content of 78.34 ± 1.0% (Roncarati *et al*., 2012).

The ash content (1.16 ± 0.25%) of the Cape hake samples was lower than what was reported for European hake (1.61 ± 0.3%) (Roncarati *et al*., 2012), but higher than what was reported by

Simmonds *et al*. (in Pérez-Villareal & Howgate, 1987) for Cape hake (*M. capensis*) (1.10%). Pérez-Villareal and Howgate (1987) also reported a higher moisture content of 79.04% for European hake fillets (skinless and deboned) and a higher ash content of 1.31%. The difference in moisture and ash content among different hake species are to be expected since the proximate competition of raw fish muscle differs greatly among different fish species and is influenced by season and area of harvest (Gokoglu *et al*., 2004; Weber *et al*., 2008; Boran *et al*., 2008; Koubaa *et al*., 2010; Usydus *et al*., 2011; Roncarati *et al*. 2012).

Table 6.8 Analysis of variance (ANOVA) of dependent variables moisture (%) and ash (%) for raw Cape hake with *K. thyrsites* infection as independent variable

		Moisture (%)		Ash (%)	
Source	**df**	**MS**	**P-value**	**MS**	**P-value**
K. thyrsites	1	4.4164	0.5606	0.00065	0.9190
Error	33	12.7517		0.06258	
Shapiro-Wilk			0.0009		<0.0001

df – degree of freedom
MS – mean square
P – probability value of F-ratio test

Table 6.9 Means (± standard deviation) for moisture (%) and ash (%) content for *Kudoa thyrsites* infected, uninfected and all Cape hake samples

Level of *K. thyrsites*	Moisture (%)	Ash (%)	Number of samples
Present	72.97[a] ± 2.92	1.17[a] ± 0.08	6
Absent	72.02[a] ± 3.68	1.16[a] ± 0.27	29
All hake samples	72.18 ± 3.53	1.16 ± 0.25	35

[ab]Different superscripts for moisture (%) and ash (%), respectively, differ at $P < 0.05$.

A total of 87 Cape hake samples were used to investigate the effect of *K. thyrsites* infection on crude protein content of raw muscle tissue. Only 13 of the 87 Cape hake samples were not infected (prevalence of 85%). ANOVA for the dependent variable protein content, with *K. thyrsites* infection as independent variable, showed that *K. thyrsites* infection had no significant effect (P = 0.4602) on the protein content of Cape hake muscle tissue (Table 6.10). The percentage protein content for infected samples was 20.69 ± 2.06% and for uninfected samples was 20.24 ± 1.79% (Table 6.11). Once again, prolonged frozen storage for the Cape hake samples may have resulted in reduced chemical differences (Patashnik & Groninger, 1964) between *K. thyrsites* infected and uninfected samples.

In contrast to the protein content of 20.83 ± 1.81% for all the Cape hake samples, Simmonds *et al*. (in Pérez-Villareal & Howgate, 1987) reported a protein content of 18.00% (total nitrogen content of 2.88%) for Cape hake (*M. capensis*). The protein content of the Cape hake samples used in this study was also higher than the protein content (19.50 ± 1.10%) of raw European hake (*M. merluccius*) from the south Adriatic seas as reported by Roncarati *et al*. (2012). The higher protein content of the Cape hake samples in this study compared to the above two mentioned studies could be due to the differences in season, year, and area of capture. It is well documented that season, year, and area of capture influence the proximate composition of different fish species (Gokoglu *et al*., 2004; Weber *et al*., 2008; Boran *et al*., 2008; Koubaa *et al*., 2010; Usydus *et al*., 2011; Roncarati

et al. 2012). However, it may also be argued that due to the prolonged frozen storage of the samples before analyses commenced, resulted in moisture loss and the concentration of protein content.

Table 6.10 Analysis of variance (ANOVA) of dependent variable protein (%) for Cape hake muscle tissue with *K. thyrsites* infection as independent variable

	Protein (%)		
Source	**df**	**MS**	**P-value**
K. thyrsites	1	2.25832	0.4602
Error	85	4.10369	
Shapiro-Wilk			0.0037

df – degree of freedom
MS – mean square
P – probability value of F-ratio test

Table 6.11 Means (± standard deviation) for crude protein (%) content for *K. thyrsites* infected, uninfected, and for all Cape hake samples

Level of *K. thyrsites*	Protein (%)	Number of samples
Present	20.69[a] ± 2.06	74
Absent	20.24[a] ± 1.79	13
All hake samples	20.83 ± 1.81	87

[ab]Different superscripts for protein content (%) differ at $P < 0.05$.

6.4 Conclusions

This is the first report of moisture (70.83 ± 3.36%) and ash (1.87 ± 0.83%) content for SA sardines and are in range of what has been reported for small pelagic fish (sardine, anchovy, and round herring) from other countries. Infection by *K. thyrsites* had no significant effect ($P > 0.05$) on the percentage moisture and ash content of SA sardines. Similarly, the percentage moisture and ash content between *K. thyrsites* infected and uninfected Cape hake samples, did not differ significantly ($P > 0.05$). The protein content of *K. thyrsites* infected Cape hake also did not differ significantly ($P > 0.05$) from uninfected samples. *Kudoa thyrsites* infected SA sardine and Cape hake, despite its lower quality levels, are therefore still acceptable as nutritious food sources in terms of moisture, ash, and protein. The preparation of fish protein hydrolysates (FPH) from *K. thyrsites* infected fish is an alternative possibility to recover protein from fish with low market value. For future studies, it is therefore suggested to investigate the usefulness of *K. thyrsites* infected SA sardine and Cape hake to produce FPH.

Results from this study indicated that the proximate components; moisture, ash and protein, would not serve well as chemical composition indicators for *K. thyrsites* infection. These components, therefore, may not be useful for the development of a NIR method as a predictive tool. However, it is important to acknowledge that prolonged frozen storage (between 6 to 18 months) of the samples may have resulted in reduced chemical differences between *K. thyrsites* infected and uninfected samples. It is suggested for further research studies to obtain and analyse fresh fish samples.

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Chapter 7

Near-infrared classification of *Kudoa thyrsites* infected and uninfected fish using a handheld spectrophotometer, SIMCA and PLS-DA

Abstract

Kudoa thyrsites infection of marine fish typically results in myoliquefaction, which is only apparent 24 to 56 h *post-mortem*. The traditional methods of detection of *K. thyrsites* infected fish is time-consuming and destructive, reducing its marketability. This poses a challenge for the South African (SA) fish industry to remove infected fish before it reaches the market or further processing activities. This study investigated the use of near-infrared (NIR) spectroscopy, in combination with soft independent modelling of class analogy (SIMCA) and partial least square discriminant analysis (PLS-DA), for discriminating *K. thyrsites* infected fish from uninfected fish. A total of 334 fish samples (200 SA sardines, 64 Cape hake, and 70 SA kingklip) were used for this study. Infection of *K. thyrsites* was determined with the use of qPCR assays. Ninety percent (90%) of the SA sardine samples, 78% of the Cape hake samples, and 37% of the SA kingklip samples were infected. Class groups of infected and uninfected fish samples were created for the purpose of generating SIMCA and PLS-DA classification models for each species of fish, as well as for a species independent data set. Principal component analysis (PCA) of NIR spectra did not show any clustering for infected and uninfected samples. Calibration and test sample sets were generated for the purpose of building and testing the SIMCA and PLD-DA classification models. SIMCA and PLS-DA were unable to classify test samples correctly into the two classes. The number of misclassifications (NMC) was higher for the SIMCA models than for the PLS-DA models. SIMCA classified the majority of the samples in the test set into both classes. PLS-DA classified the majority of samples in the test set as infected. The use of NIR spectroscopy and classification models such as SIMCA and PLS-DA show limited use as a method to distinguish between *K. thyrsites* infected and uninfected fish samples. Textural and chemical changes during extended frozen storage of the fish samples may have masked the effects associated with *K. thyrsites* infection. Further studies are suggested where NIR spectroscopy is used in combination with texture analysis and image spectroscopy.

Key words: Cape hake, *Kudoa thyrsites*, kingklip, non-destructive, quality control, multivariate classification, sardines, SIMCA, PLS-DA

7.1 Introduction

The presence of parasites in fish fillets is a serious quality and food safety concern (Huss, 1994). Although the parasite *Kudoa thyrsites* does not pose a consumer health risk (Alvarez-Pellitero & Sitja-Bobadilla, 1993), the infection of marine fish species by this Myxozoan parasite is associated with quality defects such as the development of *post-mortem* myoliquefaction and unsightly cysts (Gilchrist, 1924; Webb, 1990; Dawson-Coates *et al*., 2003; Samaranayaka, *et al*., 2006; Levsen *et al*., 2008). The development of myoliquefaction is a result of a cysteine cathepsin L protease released by *K. thyrsites* (Tsuyuki *et al*., 1982; Funk *et al*., 2008). Myoliquefaction is typically apparent only at 24 h (in SA sardines) to several hours (38–56) *post-mortem* (Levsen *et al*., 2008). This poses a quality risk to the fishing and fish processing industries. The inability to identify infected fish early *post-mortem* and separate infected from uninfected fish, makes it difficult to manage processing and quality control.

The most common method for the detection of parasites in fish and removal thereof, is by manual visual inspection on a light table (candling) and by manual trimming of fillets (Cunningham, 2002; Cheng *et al*., 2013). This method is, however, most effective for parasitic nematodes, and not applicable to *K. thyrsites*, since the spores of *K. thyrsites* vary between 12.0 µm (Kabata & Whitaker, 1981) and 16.7 µm (Cunningham, 2002; Lom & Dyková, 2006) in diameter. Traditional methods for detection of *Kudoa* infection, such as counting of visible pseudocysts, microscopic examinations, and polymerase chain reaction (PCR), are time-consuming and destructive to the sample, making these methods impractical for the fishing and fish processing industries.

Several optical or spectroscopic systems, either as stand-alone or in combination, are used for online, non-invasive, and non-destructive, rapid food quality monitoring. These optical systems include for example: near-infrared (NIR), visible (VIS), mid-infrared (MIR), Raman, nuclear magnetic resonance (NMR) spectroscopy, hyperspectral imaging (HSI) and nuclear magnetic resonance imaging (NMRI) (Mathiassen, *et al*., 2011; Cheng *et al*., 2013). All of these optical systems have been applied in fish quality monitoring, including proximate composition and lipid characteristics (Khodabux *et al*., 2007; Brown *et al*., 2012; Karlsdottir *et al*., 2014), bacterial spoilage (Lin *et al*., 2006; Weeranantanaphan *et al*., 2011; Cheng *et al*., 2013), freshness (Bøknæs *et al*., 2002; Uddin & Okazaki, 2004; Sivertsen, Kimiya *et al*., 2011; Ottavian *et al*., 2013; Reis *et al*., 2017), texture analyses (Isaksson *et al*., 2001; Careche *et al*., 2002; Cheng *et al*., 2014) and presence of nematode parasites (Wold *et al*., 2001; Heia *et al*., 2007; Sivertsen, Heia *et al*., 2011; Sivertsen *et al*., 2012). Due to the impracticality of using manual sorting methods to separate parasite infected fish from uninfected fish, several researchers have demonstrated the use of imaging spectroscopy as a rapid, non-destructive method separating parasite infected from uninfected fish (Table 7.1) based on the optical properties of fish muscle and parasites (Stormo *et al*., 2004; Stormo *et al*., 2007).

Table 7.1 Summary of studies demonstrating the application of visible and near-infrared spectra (VIS/NIR) imaging and hyperspectral imaging (HSI) for detecting nematodes in cod (*Gadus morhua*)

Aim of study	Sample presentation	Wavelength ranges (nm)	Calibration performance	Reference
To detect nematodes by visible and near-infrared spectra (VIS/NIR) with soft independent modelling by class analogy (SIMCA)	Fish flesh of circular dimensions (thickness = 20 mm, D = 50 mm)	400-1100	Detect parasites as deep as 0.6 cm with SIMCA classification based on image pixel differences	Wold *et al*. (2001)
To detect nematodes by imaging and discriminant partial least square (DPLS) regression	De-skinned, intact fillets	350-950	DPLS regression models gave some false positives, but could detect parasites as deep as 0.8 cm	Heia *et al*. (2007)
To detect nematodes with imaging spectroscopy and selection of single wavelengths	De-skinned fillets	404-1026	Spectral images with significant higher contrast were produced when dividing the spectral data at two different wavelengths instead of using single-wavelength scans	Stormo *et al*. (2007)
To detect nematodes by trans-illumination hyperspectral imaging spectroscopy	Intact fillets	400-1000	Overall detection rate of 58% of all nematodes (n=922) Detection rate of 71% of dark nematodes Detection rate of 46% of pale nematodes False alarm rate of 60%	Sivertsen, Heia *et al*. (2011)
To detect nematodes by a hyperspectral inter-actance imaging (HSI) system	Intact fillets	448-752	Accepting 60% false alarm rate in fillets after the trimming station, Gaussian maximum likelihood classifier detected 61.5% for all nematodes, 70.8% of dark nematodes, and 60.3% of pale nematodes	Sivertsen *et al*. (2012)

Heia *et al*. (2007) used VIS/NIR imaging in transmission mode to identify nematode parasites on the surface of cod (*Gadus morhau*) fillets, as well as those embedded as deep as 0.8 cm into the fillet. This enabled detection of nematodes deeper into the fillet than typically possible by manual inspection. Wold *et al*. (2001) demonstrated the ability of multispectral imaging (400 to 1000 nm) in combination with soft independent modelling of class analogy (SIMCA) to distinguish nematode parasite infected from uninfected cod fillets.

SIMCA is a flexible, problem-dependent, multi-application use of principal component analyses (PCA)-modelling, where objects in one class or group show similar rather than identical behaviour (Wold *et al*., 1987). A SIMCA model is typically based on separate principal component (PC) models, one for each class or classification group. A calibration samples set is then used to build the SIMCA model by calculating PC models for the separate class groups. An (ideally) independent test sample set can then be subjected to the same PC model projections for classification into the separate class groups.

Partial least squares discriminant analysis (PLS-DA) is another type of multivariate data analysis method that is used for classification of samples into different classes or groups based on similarity (Szymańska *et al*., 2012; Ballabio & Consonni, 2013; Brereton & Lloyd, 2014; Grootveld, 2014; Gromski *et al*., 2015). PLS-DA is based on the PLS regression model where the dependent variable1: (Y) represents membership of a particular classification or class. In a two-class PLS-DA model, discrete numbers are assigned as dependent variables to samples at two levels, typically -1 and +1, representing the two different classes. Although 0 and +1 are sometimes employed, using -1 and +1 results in centring the values of Y and therefore simplifying the algebraic derivations (Brereton & Lloyd, 2014). Classification of expired and non-expired vacuum packaged smoked salmon based on PLS-DA models and hyperspectral imaging spectroscopy in the short-wave NIR has been demonstrated (Ivorra *et al*., 2013). Ottavian *et al*. (2013) investigated the use of different PLS-DA models for classification of frozen-thawed fish samples independent of fish species. These authors demonstrated overall classification accuracies between 80% and 91%, depending on the PLS-DA strategies used.

The MicroNIR™ 1700 (spectral range 908-1680 nm) spectrophotometer is a light-weight, hand-held, portable instrument; developed, manufactured and commercialised by VIAVI Solutions (previously JDSU, USA) and is applicable for on-line and off-line measurements (Alcalà *et al*., 2013). In a recent study investigating polyamide II (Unger *et al*., 2016), where the MicroNIR 1700 spectrophotometer was compared to a benchtop Fourier transform Bruker VECTOR 22/N FT-NIR spectrophotometer, it was concluded that the results derived from the spectra measured with these two instruments were the same. In a study by O'Brien *et al*. (2013), the VIAVI Solutions (previously JDSU, USA) MicroNIR 1700 spectrophotometer was used to discriminate between three pairs of fish species which are typically mislabelled in restaurants in the United States. PCA and SIMCA classification models were used to discriminate between winter cod and cod, samlet and salmon trout, and red mullet and mullet, respectively. Although a small sample set was used (30 fish in total),

it was demonstrated that, based on the spectra measured with the use of the miniaturised, hand-held MicroNIR spectrophotometer, SIMCA proved to be a suitable analytical tool for correctly assigning test fish to the corresponding fish species.

The possibility of using NIR spectroscopy as a rapid, non-invasive quality control method for detection of *Kudoa*-parasite infection before myoliquefaction is visible, especially in SA sardines (*Sardinops sagax ocellatus*) and Cape hake (*Merluccius capensis* and *M. paradoxus*), would be of great value to the fish processing industry (D. Brickles, 2016, Group Quality Assurance Manager, Irvin and Johnson Ltd., Cape Town, South Africa). NIR spectroscopic classification of fish as either being “parasite free” or “parasite infected” will enable informed decision making during quality control regarding further storage or immediate processing. The infection by *K. thyrsites* and *K. paniformis* is considered a quality risk, rather than a consumer health risk (Alvarez-Pellitero & Sitja-Bobadilla, 1993). The development of a method than can rapidly identify *Kudoa* infected fish is crucial for optimum utilisation of *Kudoa* infected fish (Samaranayaka *et al*., 2006). Alternative utilisation of *K. thyrsites* fish may include production of fish protein hydrolysates (FPH), therefore increasing its market value. However, up to date, no such rapid detection method, specifically for detection of *Kudoa* infection, has been reported. Although several studies (Table 7.1) have demonstrated the application of VIS/NIR (Wold *et al*., 2001) and hyperspectral imaging (HSI) (Sivertsen, Heia *et al*., 2011; Sivertsen *et al*., 2012) as effective quality control methods for industrial, non-destructive detection of parasitic nematodes in fish and fish fillets, no research has been documented about the use of NIR spectroscopy for detection of specifically *Kudoa* species. Neither has a handheld instrument been considered. The aim of this study was to investigate the use of a handheld, MicroNIR spectrophotometer, in combination with SIMCA and PLS-DA classification techniques, as a non-destructive, rapid method for the discrimination of *K. thyrsites* infected and uninfected fish species, including: SA sardines (*Sardinops sagax ocellatus*), Cape hake (*Merluccius capensis* and *M. paradoxus*) and SA kingklip (*Genypterus capensis*).

7.2 Materials and methods

7.2.1 Samples

A total of 200 SA sardine (*Sardinops sagax ocellatus*), 64 Cape hake (*Merluccius capensis* and *M. paradoxus*), and 70 SA kingklip (*Genypterus capensis*) samples were kindly supplied by the Department of Agriculture, Forestry, and Fisheries (DAFF, Cape Town) from several batches of pelagic and demersal surveys during 2015. With the exception of the SA kingklip samples, all samples were received as individually tinfoil-wrapped frozen fillets. The frozen SA kingklip samples were not in the form of whole fillets, but consisted of individually tinfoil-wrapped small portions of fillets. All samples were kept frozen at -18ºC until the presence of *Kudoa thyrsites* was determined with the use of the real-time quantitative polymerase chain reaction (qPCR) protocol as a qualitative diagnostic tool, as described in detail in Chapter 4 (pp. 50-74). The extraction of DNA for qPCR

analysis was performed before the NIR spectra have been recorded in order to ensure no DNA transfer between samples and prevention of false positives.

7.2.2 DNA-based identification of Kudoa thyrsites *infection*

DNA from composite muscle tissue samples were extracted with the use of the Qiagen DNeasy Mini Kit (Qiagen, Whitehead Scientific Pty., Ltd., Cape Town). The composite tissue samples were collected from the anterior, centre and posterior location of each fish fillet. Sterile scalpels were used for each sample to prevent DNA transfer between samples. A custom TaqMan® single nucleotide polymorphism (SNP) genotyping assay was used for the determination of the presence of *K. thyrsites*, as described in detail in Chapter 4 (pp. 50-74), at the Centre for Proteomic and Genomic Research (CPGR, Cape Town, South Africa).

The *Kudoa thyrsites* assay mix contained two PCR primers (5'-CCTATCAACTAGTT-GGTGAGGTAGTG-3' and 5'-TCTCCGGAATCGAACCCTGAT-3') that flanked the region containing the SNP site specific to *K. thyrsites* (5'-GTTA-3'; Addendum D). The assay mixes also contained two TaqMan® probes, one that matched the heterologous allelic variant *K. thyrsites* (5'-ACCCGTTAACACCTTG-3'), labelled with VIC (a fluorescent dye, proprietary to Life Technologies; Anon., 2017) and another that matched the homologous allelic variant: *K. paniformis*/*K. miniauriculata*/*K. dianae* (5'-CGTCACAACCTTG-3'), labelled with FAM (6-carboxyfluorescein). A template control (NTC) containing no DNA, a positive control (samples shown to be positive for *K. thyrsites* from previous studies, Chapter 4) and a fluorescent quencher (NRQ) were included in each assay. All TaqMan® assays were performed in 384-well plates by amplifying 20 ng DNA in a 5 µL volume PCR reaction mix containing 0.9 µM primers and 0.2 µM probes. A working master mix for each sample in each assay was prepared by mixing 0.25 µL of TaqMan® assay mix (20x), 2.5 µL TaqMan® Genotyping Master Mix (Thermo Fisher), 0.25 µL water and 2 µL of genomic DNA at 20 ng. Reactions were performed with the following protocol on the 7900HT Fast Real-Time PCR System: a pre-reading of the plate to record the background fluorescence; followed by the standard PCR cycle. The PCR cycle was performed with the following parameters: 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min, and post-reading of the plate. End-point fluorescence was read on the 7900HT Fast Real-Time PCR System using the SDS version 2.3 software (Applied Biosystems, USA). The TaqMan® Genotyper software 1.3 was used to generate allelic discrimination plots for the alleles: *K. thyrsites* (allele 1 - labelled with VIC) and *K. paniformis*/*K. miniauriculata*/*K. dianae* (allele 2 - labelled with FAM).

7.2.3 MicroNIR spectral acquisition

NIR absorbance spectra were collected using a MicroNIR 1700 (VIAVI Solutions, formerly JSDU, Santa Rosa CA, USA) miniature NIR spectrophotometer. The MicroNIR instrument consists of a linear variable filter as the dispersing element, that is coupled to a linear detector array (128-pixel

uncooled InGaAs photodiode array), containing a pair of integrated vacuum tungsten lamps as light source, and a 16-bit analog-to-digital converter for analog conversion.

NIR spectra were collected in the NIR region (908 to 1680 nm) of the electromagnetic spectrum at 6.2 nm intervals, resulting in 125 wavelength bands. The MicroNIR spectrophotometer was used in diffuse reflection mode and measurement parameters of integration time and number of scans were set to 14600 μs and 50 scans. All fish samples were thawed and conditioned at room temperature for 20 min before being scanned. Three spectra were collected per fish sample at the posterior, centre and anterior anatomical locations, and the average for each sample was calculated. Spectra were collected from only the flesh side (inside of the fillet) of each fish sample.

7.2.4 Multivariate data analyses

All multivariate data analyses were performed with the use of The Unscrambler software, version 10.4.1 (CAMO software, 2017, Oslo, Norway). Graphs and figures were generated with use of The Unscramble software and Microsoft Excel (Microsoft Office, 2016). Multiplicative scatter correction (MSC) was applied to the NIR spectra to enhance spectral characteristics and to reduce undesirable sources of variation. Savitzky-Golay first and second-derivatives were also tested as pre-treatment techniques to investigate any improvement in classification results.

7.2.4.1 Principal component analysis (PCA)

Exploratory PCA was performed on pre-treated spectra for each fish species, respectively, as well as for all fish samples combined (species independent sample set) to determine any degree of clustering between *K. thyrsites* infected and uninfected samples.

7.2.4.2 Calibration and test sets

SIMCA and PLS-DA models for classification of *K. thyrsites* infected and uninfected fish samples were developed for SA sardine, Cape hake and SA kingklip, respectively, as well as for all the samples together (species independent sample set). For the purpose of building and testing the classification models, calibration and test samples sets (Table 7.2) were generated. This was done by randomly placing one third of the infected and uninfected samples for each respective fish species into the test set; thus, leaving two-thirds of the data set as the calibration set. In the case of the species independent sample set, it was ensured that both the calibration and test sets contained SA sardine, Cape hake and SA kingklip samples for both the infected and uninfected classes. Full cross-validation (CV) was used as validation method when classification models were developed which were then tested on the independent test sets. The number of samples used in the calibration and test sets, respectively, are summarised in Table 7.2.

Table 7.2 Number of samples in calibration and test sets for the classification classes: *K. thyrsites* infected and uninfected fish samples (SA sardines, Cape hake and SA kingklip), used for the development of SIMCA and PLS-DA models and independent testing

Sample sets		Classification classes		Total number of samples
		Infected fish	Uninfected fish	
Calibration set	Sardines	120	14	134
	Hake	33	10	43
	Kingklip	17	29	46
Test set	Sardines	59	07	66
	Hake	17	04	21
	Kingklip	09	15	24
Total number of samples		255	79	334

7.2.4.3 Generating classification models: SIMCA and PLS-DA

SIMCA, a pattern recognition method that describes each class model separately after applying PCA, was used to develop SIMCA classification models for each fish species and for the species independent data set. The statistical significance level for the SIMCA classification was set at 5%. This assumed that there was a 5% risk that a particular test sample would fall outside the class, even if it actually belonged to the class; while 95% of the test samples which truly belonged to the class would fall inside the class (Esbensen, 1994). Explained variance plots for the PCA class models were studied to determine the number of factors to be used for prediction of the test set samples.

Graphical interpretation of the SIMCA classification results were done with the use of Coomans plots where the transverse distances from all the test samples to the two classes were visualised. To investigate correct classification of test samples, the leverages were also studied in the distance vs. leverage plot (Si vs. Hi), also called the membership plot. The membership plot showed the limits used in the classification for both the distance to the model (the residual standard deviation) and the leverage (distance to model centre) measured for each sample. Test samples that fell inside these limits were highly likely to belong to the model or class at the chosen significance level of 5%. The model distance plot was studied to visualise the distance between the two class models, namely *K. thyrsites* infected and uninfected, and to quantify whether the models were really different. The variable discrimination power plots were evaluated to determine the discrimination power of each variable in the two-model comparison. A value near 1 indicated no discrimination power, while a value greater than 3 indicated good discrimination for a particular variable (Esbensen, 1994).

A PLS-DA model was developed for each fish species separately, as well as for all fish species together (species independent data set) in order to discriminate fish samples with *K. thyrsites* present (infected samples) from uninfected samples. PLS-DA is a classification technique used to separate different groups of samples by linking two data matrices, namely the independent variables, X (NIR spectra) and the dependent variables, Y (class membership) (Szymańska *et al*., 2012; Brereton & Lloyd, 2014; Gromski *et al*., 2015). The differences between the two groups of samples were modelled with the partial least squares (PLS) regression algorithm, but coding for class membership using the response variable -1 for members in one class, and +1 for members in the second class. In this study, fish samples that were identified as *K. thyrsites* infected were placed in the “infected”

class with response variable +1, while those samples not infected were placed in the "uninfected" class with response variable -1. The calibration set was used to run the PLS regression (with full cross-validation). The explained variance plots were studied to determine the number of factors to be used for prediction of the test set samples. The developed model was then used to classify the samples in the test set. Test samples with predicted values ≥ 0 were assigned to the infected class, while test samples < 0 were assigned to the uninfected class.

Classification performances of SIMCA and PLS-DA were evaluated in terms of i) sensitivity; which represents the confidence of the class space, ii) specificity; which is the fraction of samples not belonging to the modelled class that is correctly rejected by the model, and iii) precision; which is the ratio of the number of samples correctly accepted and the total number of samples accepted by the model (Oliveri & Downey, 2012; Szymańska *et al*., 2012). Calculations of these were as follow:

$$\text{Sensitivity} = \frac{TP}{TP+FN}$$

$$\text{Specificity} = \frac{TN}{TN+FP}$$

$$\text{Precision} = \frac{TP}{TP+FP}$$

where i) TP is the true positive samples correctly classified inside the class, ii) FN is the false negative samples falling outside the classified class, iii) FP is the false positive samples extraneous to that class but classified within the class, and iv) TN is the true negative samples classified correctly outside the class. In the case of SIMCA, test samples classified into both classes and test samples not classified in any of the two classes, were counted as misclassifications. The number of misclassifications (NMC) was calculated as the sum of FP and FN (Szymańska *et al*., 2012). Confusion matrices were generated for the PLS-DA classification results.

The number of samples in the two classes were unequal for all the data sets. In order to compensate for this, the X-matrix for the PLS model was weight centred by subtracting the average of the means of the two class groups from the column values (Brereton & Lloyd, 2014). This was done using Microsoft Excel (Microsoft Office, 2016) to investigate any improvement of the classification performance of the PLS-DA models.

7.2.4.4 Means, standard deviations, and difference spectra calculations

The means and standard deviations for the MSC pre-treated NIR spectral data for infected and uninfected samples were calculated in an attempt to visualise any differences between infected and uninfected fish samples. Differences in mean spectra were calculated by subtracting mean values for infected samples from mean values for uninfected samples. This was done for the SA sardine, Cape hake, SA kingklip, and species independent data sets, respectively.

7.3 Results and discussion

7.3.1 Quantitative polymerase chain reaction (qPCR) for qualitative analysis

Results from the TaqMan® qPCR assays for *Kudoa thyrsites* showed that 179 SA sardines (prevalence of 90%), 50 Cape hake (prevalence of 78%), and 26 SA kingklip (prevalence of 37%) samples were infected with *K. thyrsites*. None of the samples tested positive for the presence of allele: *K. paniformis*/*K.* miniauriculata/K*. dianae*. The prevalence of *K. thyrsites* for all 334 samples analysed was 76%. Therefore, the total 255 samples that tested positive for *K. thyrsites* infection (infected samples), and the 79 samples that tested negative for *K. thyrsites* infection (uninfected samples) were used (Table 7.2) to generate classification models. Due to the high prevalence of *K. thyrsites* in SA sardines, the calibration sample set for the SA sardines contained 120 samples in the infected class, and only 14 in the uninfected class. This resulted in the number of samples for the two classes to be unequal, which is not ideal for generating classification models based on NIR spectra (Brereton & Lloyd, 2014; Grootveld, 2014). If the one class is represented by a much larger number of samples compared to the second class, it may result in a classification model to be biased towards the class being represented by the larger number of samples. The number of samples in the two classes for the Cape hake and SA kingklip calibration sets were also unequal, however, the difference in number of samples was much smaller compared to that of the SA sardine calibration set.

7.3.2 Spectral characterisation and PCA

Figure 7.1 shows some typical absorbance spectra for the raw and MSC pre-treated NIR spectral data for representative samples from the SA sardine, Cape hake, and SA kingklip data sets. Visually it was not possible to observe significant differences between the spectra for individual samples. PCA were performed on the MSC pre-treated (Savitzky-Golay first and second-derivatives pre-treatment did not improve PCA models) spectra for all fish species separately, as well as for all fish samples together, to investigate any form of clustering associated with the presence of *K. thyrsites* (Figure 7.2).

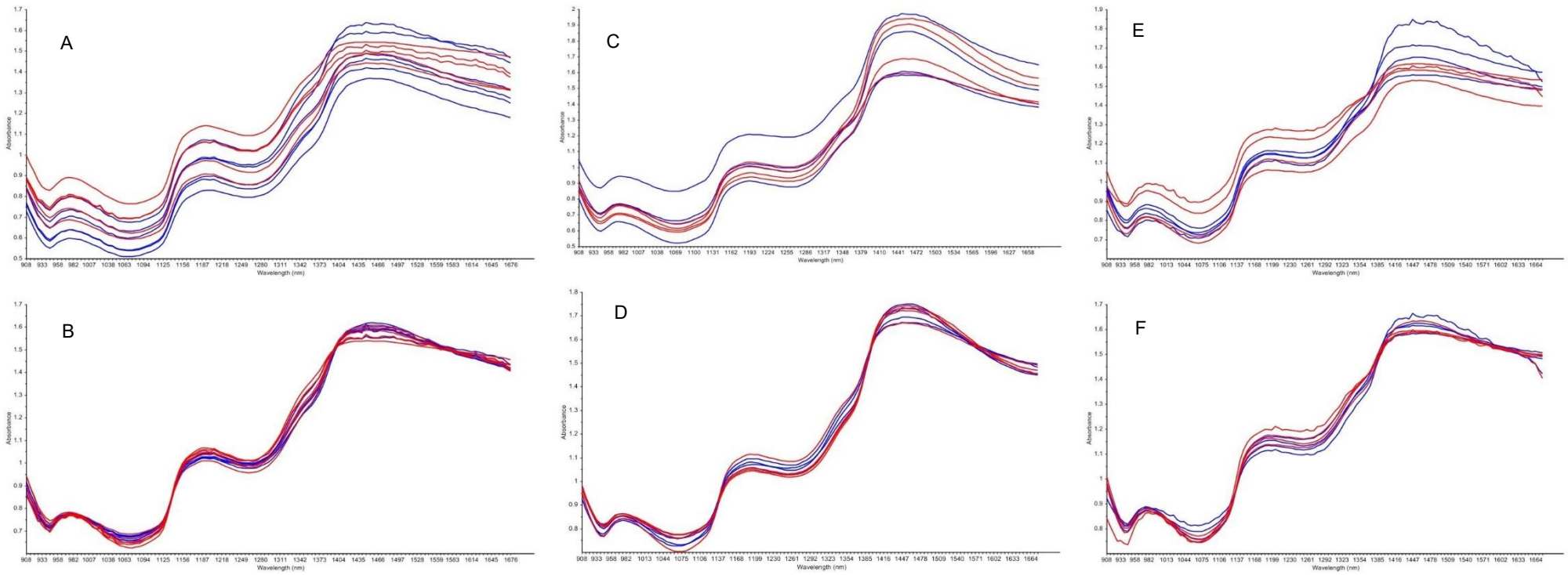

Figure 7.1 Representative raw NIR spectra for SA sardine (A), Cape hake (C) and SA kingklip (E) data sets, and MSC pre-treated spectra for sardine (B), Cape hake (D) and SA kingklip (F) data sets, respectively. Blue lines represent *K. thyrsites* infected samples and red lines represent uninfected samples.

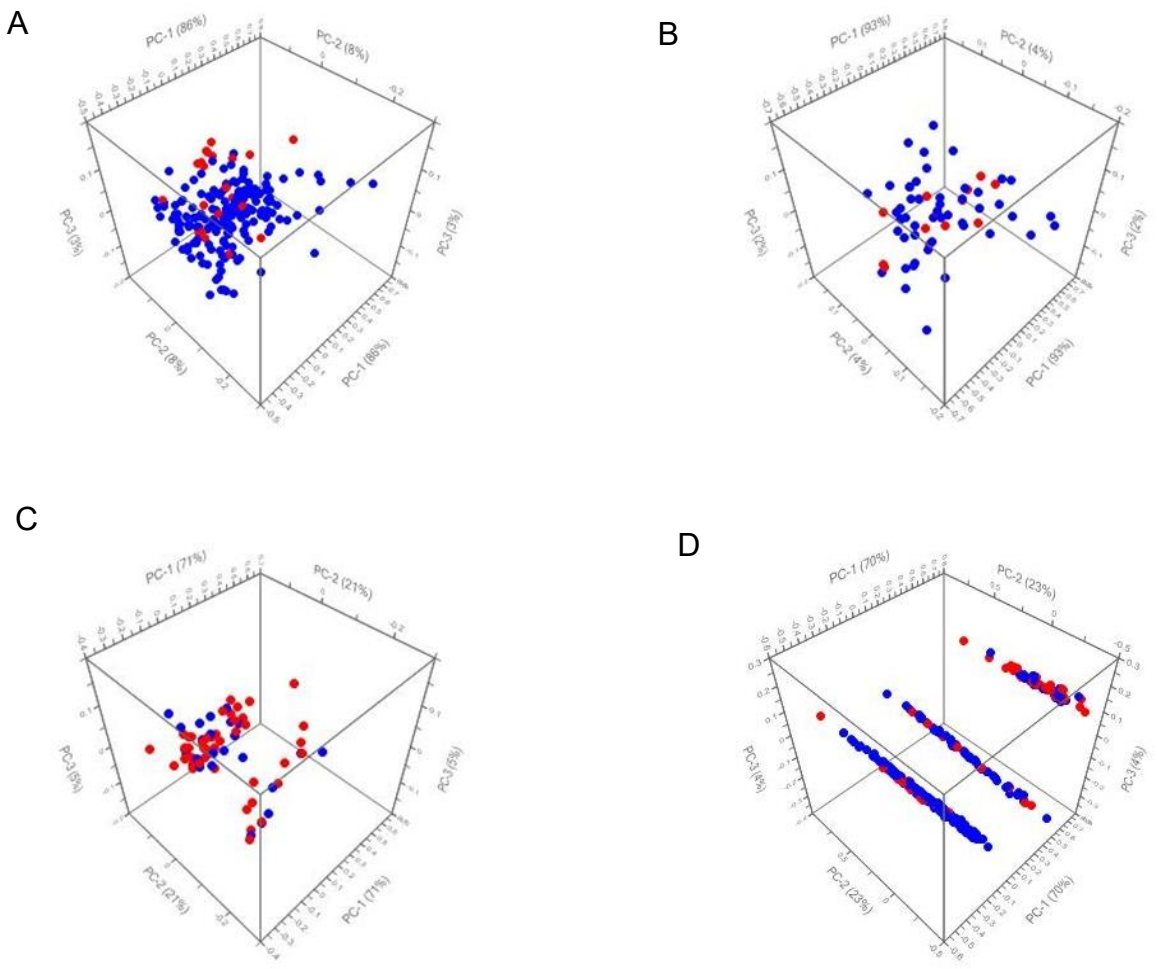

Figure 7.2 Three-dimensional (3D) PCA score plots for MSC pre-treated data, showing PC1 vs. PC2 vs. PC3 for the SA sardine (A), Cape hake (B), SA kingklip (C), and species independent (D) data sets. *Kudoa thyrsites* infected samples are represented as blue dots, and uninfected samples as red dots.

The three-dimensional score plots showing PC1 vs. PC2 vs. PC3 for SA sardines, Cape hake, and SA kingklip (Figures 7.2A, B and C) showed no clustering of *K. thyrsites* infected and uninfected samples. This was also the case for the species independent sample set. The score plots for PC4, PC5, and PC6 were also investigated. There was also no clear clustering of infected and uninfected samples when these principal components were considered. Since there was no clustering between infected and uninfected samples, the loading plots were not investigated.

Clear clustering of the different fish species (Figure 7.2D) was evident, with separation between SA sardine, Cape hake and SA kingklip samples. This grouping based on species of fish was expected since the fatty acid profile, fat, protein and moisture contents differ significantly between fish species (Özogul *et al*., 2008; Koubaa *et al*., 2010; Usydus *et al*., 2011; Chapter 6 of this dissertation). In addition, visual differences in the muscle structure between SA sardine, Cape hake and SA kingklip samples were evident due to differences in texture and size of the myotomes (muscle segments).

7.3.3. Prediction ability of SIMCA and PLS-DA models

Comparing the use of Savitzky-Golay first and second-derivatives as pre-treatment techniques with that of MSC, no improvement in the performance of the generated SIMCA and PLS-DA classification models were obtained. Pertaining to the SIMCA classification, the number of factors used for the SA sardine test set were two for the PCA infected model, and three for the PCA uninfected model. For Cape hake, two factors were used for the PCA infected model and one factor for the uninfected PCA model. For SA kingklip and the species independent test sets, three factors were used for both the infected and uninfected PCA models. Figure 7.3 shows the Coomans plots for the results of the SIMCA classification for the SA sardine and the species independent test sets, respectively.

In both the SA sardine and species independent SIMCA classification models, there were poor separation between the infected and uninfected calibration sample sets (Figure 7.3). This was expected, as a clear separation of the two classes was not seen in the overall PCA models of the calibration sample sets. Classifications of the samples in the test sets were poor, since samples mostly fell outside both classes (Figure 7.3A for SA sardine) or inside both classes (Figure 7.3B for species independent set). Similar results were obtained from the Coomans plots for SIMCA classification of the Cape hake and SA kingklip test sample sets. The number of samples in both the Cape hake and SA kingklip total data sets were low (total samples of 64 and 70, respectively). Theoretically the number of samples in a data set should be more than 100, or 5 times the number of X-variables (Grootveld, 2014).

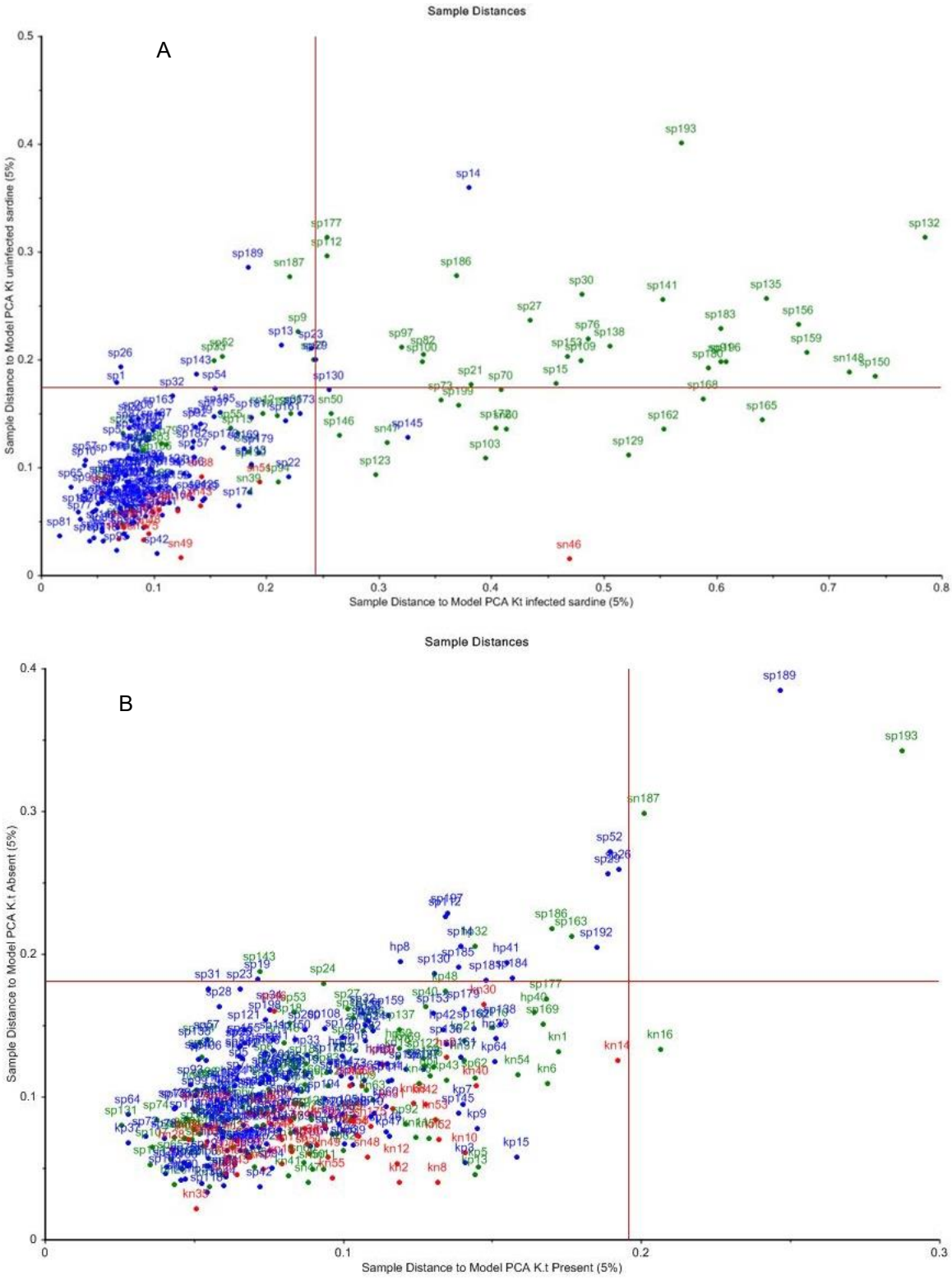

Figure 7.3 Coomans plots for SIMCA classification of the (A) SA sardine and (B) species independent test sample sets. Test samples are indicated in green, calibration samples for the *K. thyrsites* infected class are indicated in blue; while the samples for the uninfected calibration class are indicated in red.

Classification results for the SIMCA models for SA sardines, Cape hake, SA kingklip, and the species independent test sample sets are given in terms of the number of misclassifications (NMC) (Table 7.3), which is the sum of false positive (FP) and false negative (FN) (Szymańska *et al*., 2012). Samples classified as belonging to both classes were counted as false positives (FP). Samples not classified at all were counted as FN. The number of true positives (TP) and true negatives (TN) are also indicated in Table 7.3.

Table 7.3 Number of misclassifications (NMC) for SIMCA models for SA sardines, Cape hake, SA kingklip, and species independent test sample sets, respectively

SIMCA models	NMC	Total n	TP	TN
SA sardines	61	66	5	0
Cape hake	18	21	3	0
SA kingklip	23	24	1	0
Species independent data set	102	111	7	2

NMC - number of misclassifications = FP + FN
n - number of samples
TP = true positives, TN = true negatives

None of the SIMCA models showed acceptable prediction ability as more than 60% of the samples in the test sets were misclassified in all the models. The species independent SIMCA classification model classified only 8% of the test set samples correctly. Seven infected samples were correctly classified as infected (TP), while two uninfected samples were correctly classified as uninfected (TN). The total NMC was 102, where 98 samples were classified into both groups, 3 samples were not classified at all, and one infected sample was wrongly classified as uninfected (FN). SIMCA models are based on PC's (Oliveri & Downey, 2012). There was no clear clustering of infected from uninfected samples in the PCA models for SA sardine, Cape hake, SA kingklip, and the species independent data sets (Figure 7.2). This may therefore explain the low prediction ability of the SIMCA models.

Seven factors were used for the SA sardine, Cape hake, and SA kingklip PLS-DA models; while for the species independent PLS-DA model, five factors were used. Classification results for the PLS-DA models for the SA sardine, Cape hake, SA kingklip, and the species independent test sample sets are summarised as confusion matrixes in Table 7.4. In the case of the SA sardines, all test samples were predicted as infected, with the number of misclassifications calculated as 7 (all being FP). Similar classification results were obtained in the case of the Cape hake test sample set. All the hake test samples (n = 21) were classified as being infected, with 4 false positives and a NMC of 4. For both the SA sardine and Cape hake PLS-DA models, the sensitivity was calculated as 1, while the specificity was calculated as zero (since TN = 0 in both cases). The precision was calculated as 0.89 for SA sardines and 0.81 for Cape hake. These values, however, do not indicate acceptable classification ability of the models, since none of the uninfected test samples were correctly classified as uninfected.

Table 7.4 Confusion matrixes for the SA sardine, Cape hake, SA kingklip, and species independent PLS-DA models, respectively

	Predicted uninfected	**Predicted infected**	**Total n**
SA sardines:			
Test set sample number n = 66			
Actual uninfected	0 (= TN)	7 (= FP)	7
Actual infected	0 (= FN)	59 (= TP)	59
Total n	0	66	66
Cape hake:			
Test set sample number n = 21			
Actual uninfected	0 (= TN)	4 (= FP)	4
Actual infected	0 (= FN)	17 (= TP)	17
Total n	0	21	21
SA kingklip:			
Test set sample number n = 24			
Actual uninfected	12 (= TN)	3 (= FP)	15
Actual infected	9 (= FN)	0 (= TP)	9
Total n	21	3	24
Species independent sample set:			
Test set sample number n = 111			
Actual uninfected	15 (= TN)	11 (= FP)	26
Actual infected	9 (= FN)	76 (= TP)	85
Total n	23	88	111

n – number of samples
TN = true negative, FP = false positive, FN = false negative, TP = true positive

The SA kingklip PLS-DA model (Table 7.4) gave a NMC of 12 (FP + FN), where 3 samples were wrongly classified as infected (FP) and 9 samples were wrongly classified as uninfected (FN). For the SA kingklip PLS-DA model, sensitivity was calculated as zero (due to TP = 0), specificity as 0.80, and precision as zero (since TP = 0).

The species independent PLS-DA model correctly classified 91% (77 of 85 samples) of the infected test samples into the infected class, and 58% (15 of 26 samples) of the uninfected samples into the uninfected class (Table 7.4). The sensitivity of this PLS-DA model was calculated as 0.89, the specificity as 0.58, and the precision as 0.87. Although the calculated sensitivity and precision values seem acceptable, the correctly classified uninfected samples were all SA kingklip samples (TN = 15), while none of the uninfected SA sardine and Cape hake test samples were correctly classified as uninfected (FP = 11). The 76 (TP) samples correctly classified as being infected were all SA sardine and Cape hake samples, while none of the infected SA kingklip test samples were correctly classified as infected. It seemed though as if the species independent PLS-DA model distinguished between SA kingklip samples, and SA sardine and Cape hake samples. Although a species independent classification model would be ideal, it seems as though species-specific models might be more useful to distinguish between *K. thyrsites* infected and uninfected fish samples. It also seemed as though the PLS-DA models (species dependent and independent) classified the majority of the samples in the test sets as infected, thus favouring the class with the larger number of representative samples (Brereton & Lloyd, 2014; Grootveld, 2014). It may be argued that greater

importance was given to the infected class since the number of samples in the different classes was unbalanced, where the infected class had a much higher number of samples than the uninfected class. When dealing with unbalanced classes, it is recommended to perform weight centring on the X-matrix for the PLS model by subtracting the average of the means of the two class groups from the column values (Brereton & Lloyd, 2014). In an attempt to compensate for the unbalanced classes in this study, this was done for all the PLS-DA models. This, however, did not improve the classification ability of any of the PLS-DA models.

Similar to the SIMCA classification models, the PLS-DA models did not show acceptable classification results. Classification of samples into both classes is not seen with the use of the PLS-DA model since the predicted values for the Y-variable of the test samples are discriminated based on the threshold of zero (0). Predicted samples are simply classified either as belonging to the one class (predicted $y \geq 0$) or the other class (predicted $y < 0$). However, PLS-DA does not take into account within-class variability (Brereton & Lloyd, 2014), making it difficult to assign samples with values close to zero (with 1 as the level of the infected class, and -1 as the level of the uninfected class) into the correct class. For the purpose of evaluating the performance of the PLS-DA models in this study, all test set samples with predicted y-values ≥ 0 were classified as infected, and those samples < 0 were classified as uninfected.

7.3.4 Means, standard deviations, and difference in mean spectra

The PLS-DA and SIMCA models were unsuccessful in classifying infected and uninfected fish samples into the two separate classes. To further understand this unsuccessful classification ability of the models, the means, standard deviations and difference in mean MSC pre-treated NIR absorbance spectra for the SA sardine, Cape hake, SA kingklip, and species independent data sets were investigated. This was also done in an attempt to identify specific wavelengths where differences between infected and uninfected fish samples may be evident.

Figures 7.4, 7.5, 7.6 and 7.7 show the mean MSC pre-treated absorbance values and standard deviations for *K. thyrsites* infected (blue line graphs) and uninfected (red line graph) samples for SA sardine, Cape hake, SA kingklip, and the species independent data set, respectively. Visually, no spectral differences between infected and uninfected samples were observed for SA sardines, Cape hake or SA kingklip (Figures 7.4, 7.5 and 7.6).

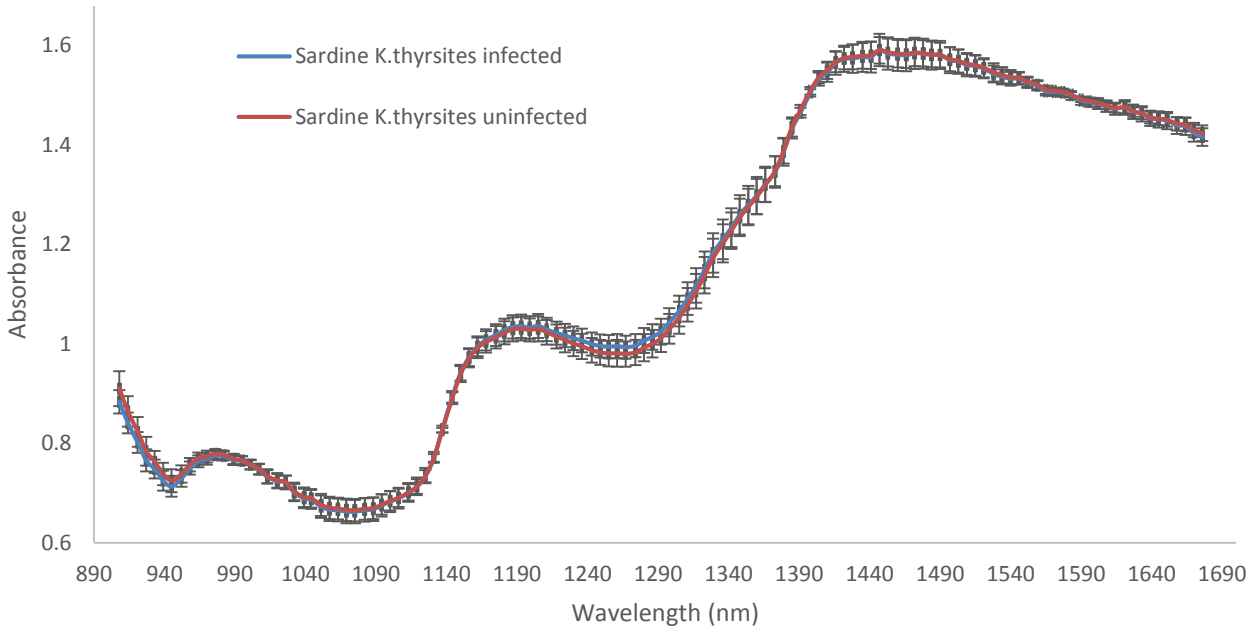

Figure 7.4 Mean MSC pre-treated NIR spectra and standard deviations (as error bars) for *K. thyrsites* infected (blue graph) and uninfected (red graph) SA sardine samples.

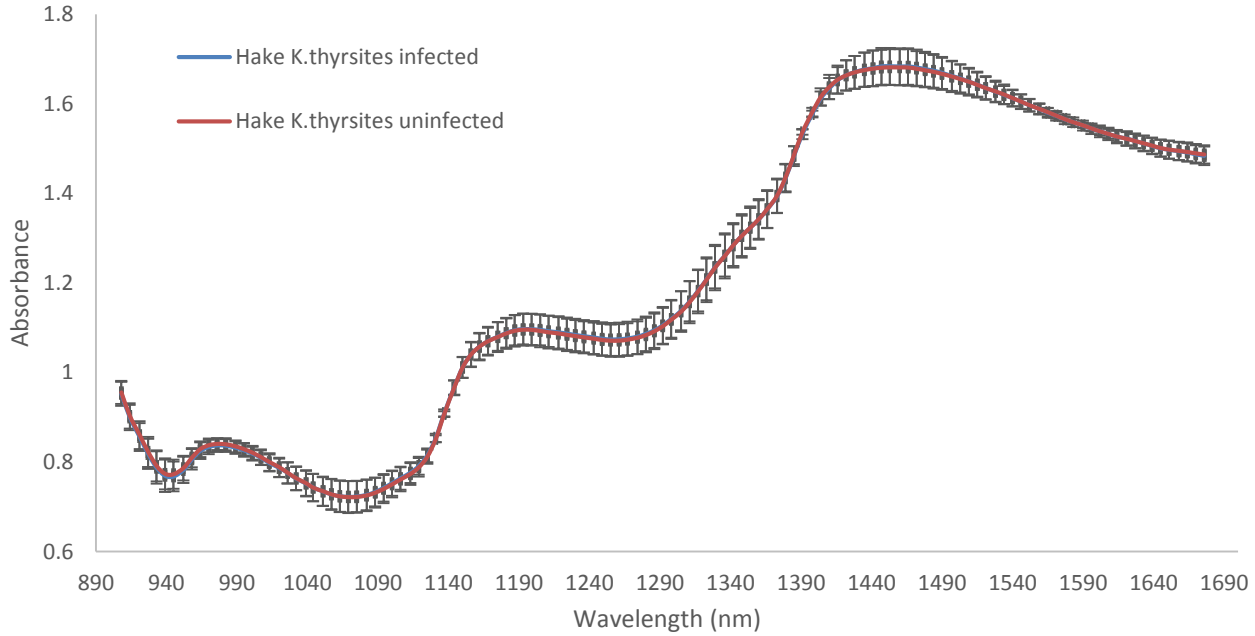

Figure 7.5 Mean MSC pre-treated NIR spectra and standard deviations (as error bars) for *K. thyrsites* infected (blue graph) and uninfected (red graph) Cape hake samples.

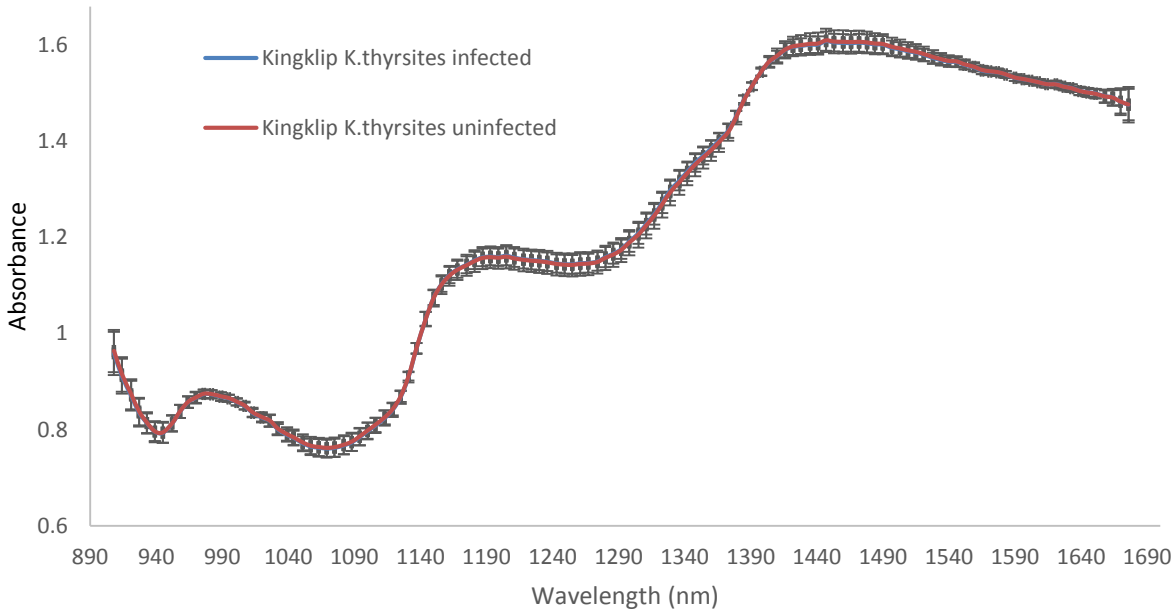

Figure 7.6 Mean MSC pre-treated NIR spectra and standard deviations (as error bars) for *K. thyrsites* infected (blue graph) and uninfected (red graph) SA kingklip samples.

The MSC pre-treated spectra (Figure 7.8) for the species independent data set showed a difference in the mean absorbance values between infected and uninfected samples. The infected samples had lower mean values between 890 and 1380 nm compared to the uninfected samples. The functional groups of components such as proteins, water, fatty acids, and carbohydrates, absorb at several NIR wavelength ranges and can overlap with other functional groups' absorption (Osborne *et al*., 1993.) This make it difficult to identify specific absorption regions from raw spectra.

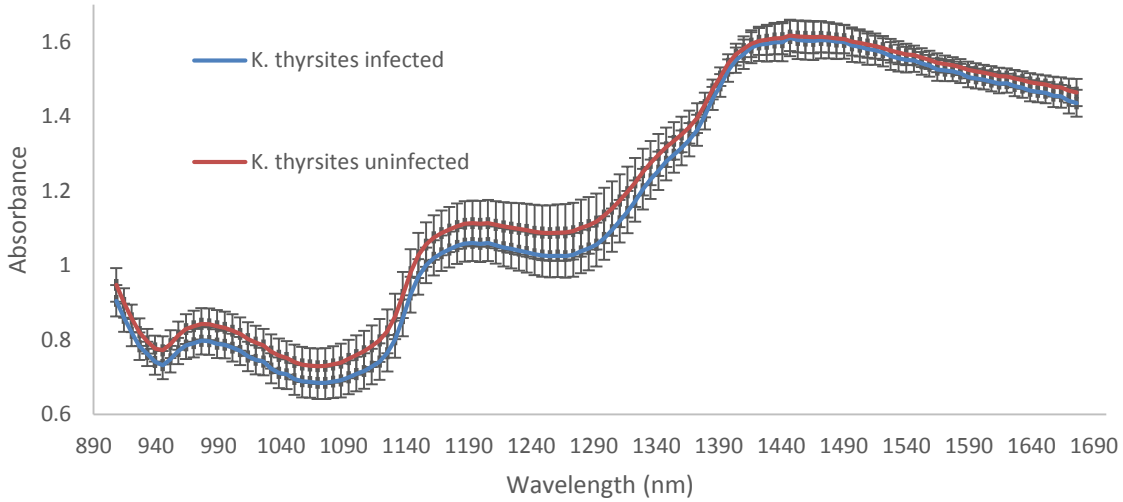

Figure 7.7 Mean MSC pre-treated NIR spectra and standard deviations (as error bars) for *K. thyrsites* infected (blue graph) and uninfected (red graph) fish (species independent) samples.

The difference in mean spectra between infected and uninfected SA sardine samples (Figure 7.8, blue line graph) showed an absorption band between 1218-1342 nm, and a peak at 1390 nm. These may have been related to CH_2 (C-H stretch second overtone, and 2 x C-H stretch and C-H deformation) (Osborne *et al*., 1993); possibly associated with fatty acids (Khodabux *et al.*, 2007). No significant absorption band or peaks were evident in the Cape hake and SA kingklip difference spectra (Figure 7.8, red and green line graphs, respectively).

When visualising the difference in spectra between the mean MSC pre-treated NIR spectra values for infected and uninfected samples for the species independent data set (Figure 7.8, orange line graph), absorption peaks at 933 nm and 1137 nm, and a band at 1206-1290 nm, were observed. The peak at 933 nm may be related to C-H stretch, third overtone of CH_2 (Osborne *et al*., 1993) which may be associated with fatty acids (Khodabux *et al.*, 2007). The peak at 1137 nm and the band at 1260-1290 nm could also be associated with fatty acids (2 x C-H stretch and C-H deformation modes associated with CH_3) (Osborne *et al*., 1993; Khodabux *et al.*, 2007). Absorption peaks related to fatty acids are mainly attributed to the C-H and CH_2 vibrations with the first overtone of C-H stretch in the range of 1718-1760 nm, and the second overtone in the range 1100-1390 nm.

In this study, it was expected to observe differences in NIR spectra between infected and uninfected samples at wavelengths associated with water, proteins, peptides and amino acids because of the extensive protein breakdown associated with *K. thyrsites* infection (Tsuyuki *et al*., 1982; Langdon, 1991; Samaranayaka *et al*., 2006; Funk *et al*., 2008). Extensive protein breakdown (hydrolyses) as a result of the activity of the cysteine cathepsin L protease excreted by *K. thyrsites* (Tsuyuki *et al*., 1982; Samaranayaka *et al*., 2006; Funk *et al*., 2008) result in release of moisture (Ofstad *et al*., 1996) and peptide fragments. In general, N-H stretch first overtone of $CONH_2$ of proteins and amino acids are typically visible at 1430 nm (Osborne *et al*., 1993) and O-H first overtone associated with water is typically visible at 1440-1450 nm. Proteins and peptides have broad, strong bands that are difficult to differentiate due to considerable overlap of the bands. Near-infrared spectra of fish may be dominated by absorption peaks attributed to protein at 1510 nm, 1700 nm and 1738 nm (Uddin & Okazaki, 2004).

Raw fish muscle typically contains between 12 and 22% protein (Gokoglu *et al*., 2004; Weber *et al*., 2008; Koubaa *et al*., 2010; Usydus *et al*., 2011; Foline *et al*., 2011), while lipids and water together make up about 80% of fish muscle (Ofstad *et al*., 1996). The moisture content of fish muscle ranges from 65 to 80% (Gokoglu *et al*., 2004; Bulla *et al*., 2011; Roncarati *et al*. 2012) as influenced by catching season (Boran *et al*., 2008). Fish muscle has a very low carbohydrate content, ranging between 0.2 to 1.5% (Françoise, 2010). The observed differences in difference spectra for the fish species independent data set, as compared to that of the respective species, could be due to changes in moisture content, fatty acids and protein structure between the difference fish species. These changes probably occurred during frozen storage of the samples.

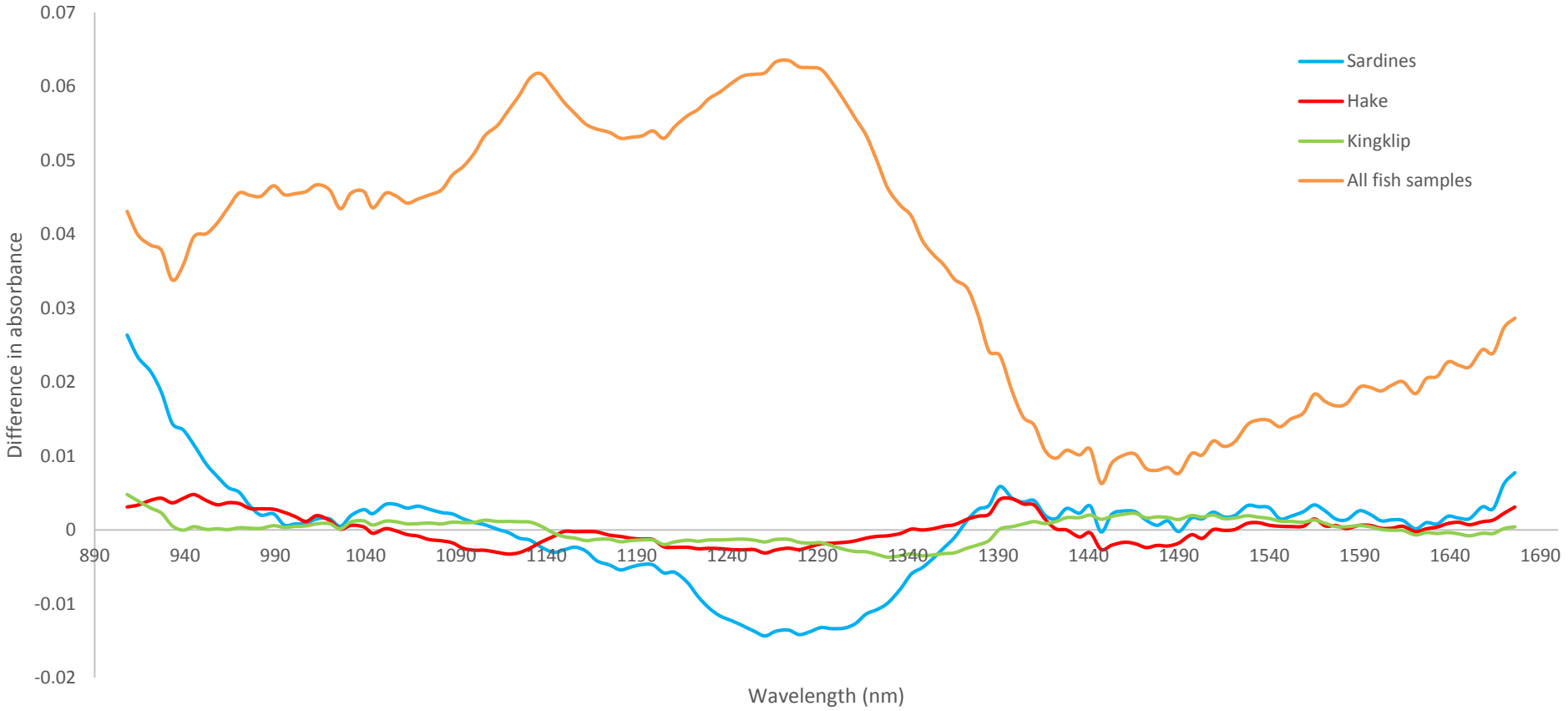

Figure 7.8 Difference in mean MSC pre-treated NIR spectra for SA sardines, Cape hake, SA kingklip, and species independent data sets, respectively. Differences in mean spectra were calculated by subtracting mean values for infected samples from mean values for uninfected samples.

The fish samples used for this study were kept frozen at -18ºC for up to eight months before analyses commenced due to time constraints. In addition, DAFF survey trawlers harvested fish samples once per month, or sometimes only every second month, over an extended period of time. The prolonged frozen storage resulted in water loss, protein degradation and aggregation (Sultanbawa & Li-Chan, 2001; Careche *et al*., 2002) due to autolysis (in addition to *K. thyrsites* associated proteolysis) and water crystal formation during freeze-thaw cycles (Huss, 1994).

Differences in NIR spectra associated with proteins and water were not evident between infected and uninfected fish samples. It is hypothesised that structural changes due to prolonged frozen storage of the fish samples used in this study reduced or masked the effects of the *K. thyrsites* associated proteolytic activity (Zhou & Li-Chan, 2009). Acquisition of NIR spectra of fresh fish samples earlier than 24 h *post-mortem* is suggested. Textural changes (myoliquefaction) in the muscle tissue of fresh SA sardine samples (Figure 7.9, before being frozen for use in this study) infected with *K. thyrsites* were observed as early as 24 h *post-mortem*. However, textural changes as were observed in the SA sardine samples, were not evident in the Cape hake and SA kingklip samples. Since the hand-held NIR spectrophotometer is light-weight, small, and portable, it would be possible to acquire spectra as sooner than 24 h *post-mortem*. The hand-held NIR spectrophotometer only requires a laptop or tablet with the software installed. Such an instrument-system can be taken onto trawlers with ease.

Figure 7.9 *Kudoa thyrsites* infected fresh SA sardine samples at 24 h *post-mortem*. (A) Extensively myoliquefied SA sardine samples. (B) SA sardine sample not yet showing myoliquefaction at 24 h *post-mortem*.

Black pseudocysts were visible in some of the Cape hake samples. Textural changes such as gaping, and chemical (accumulation of melanin and formation of black pseudocysts) changes associated with *K. thyrsites* infection have been demonstrated for hake species (Kabata & Whitaker, 1981; Tsuyuki *et al*., 1982). In the case of Cape hake species, where white, light brown and/or black pseudocysts may be evident due to host specific response towards *K. thyrsites* (Morado & Sparks, 1986; Samaranayaka *et al*., 2007), it is suggested to investigate the use of imaging spectroscopy (Stormo *et al*., 2004; Heia *et al*., 2007; Stormo *et al*., 2007; Sivertsen, Heia *et al*., 2011; Sivertsen *et al*., 2012) in an attempt to differentiate between *K. thyrsites* infected and uninfected Cape hake samples based on optical properties of fish muscle and pseudocysts.

In the case of SA sardine, it might be possible to correlate the level of *K. thyrsites* infection and texture analyses (such as compression, firmness, and/or shear force) with NIR spectroscopy (Isaksson *et al*., 2001; Cheng *et al*., 2014) in an attempt to separate samples with the potential of becoming extensive myoliquefied from samples that might still be suitable for canning.

7.4 Conclusions

In this study, NIR spectroscopy, in combination with SIMCA and PLS-DA, was unable to distinguish between *K. thyrsites* infected and uninfected fish samples. The fish samples used for this study were frozen for up to eight months before analysed. Structural and chemical changes due to frozen storage may have masked the chemical changes associated with *K. thyrsites* infection, thus, differences in NIR spectra and PCA were not evident between infected and uninfected samples. Further species dependent studies are recommended. In the case of SA sardine, it is suggested to investigate the use of NIR spectroscopy and texture analyses, in combination with the level of *K. thyrsites* infection (namely the number of *Kudoa* spores per gram of muscle tissue), to distinguish between infected samples with the potential of developing extensive myoliquefaction and those that may still be firm and suitable for canning. It is recommended to collect NIR spectra of samples earlier than 24 h *post-mortem*. This can be done by taking the portable, hand-held, MicroNIR spectrophotometer onto fishing vessels. NIR spectra of the same samples, in addition to texture and prevalence analyses, should then to be acquired again at 24 h *post-mortem* in an attempt to study differences in textural properties between early *post-mortem* and 24 h *post-mortem* samples. *Kudoa thyrsites* infected fish which may not become extensively myoliquefied can then be separated and used for canning. Infected fish which may develop extensive myoliquefaction, and not suitable for canning processing, may be channelled to alternative processing, such as fish meal and/or fish oil manufacturing. However, since the prevalence of *K. thyrsites* (90% in this study, and 91% in Chapter 5) in SA sardine is very high, it will be a challenge to analyse a sufficient number of SA sardine samples in order to obtain a calibration set with two classes of about 50 samples each. A quantity of at least 500 SA sardine samples may be required in order to obtain 50 uninfected samples.

For Cape hake, it is suggested to investigate the use of imaging spectroscopy in an attempt to differentiate between *K. thyrsites* infected and uninfected samples based on the presence of *K.*

thyrsites associated pseudocysts. Differences in optical spectra between pseudocysts and fish muscle tissue may be significant enough in order to generate classification models. It is further recommended to ensure equal number of infected and uninfected samples in the data sets, although this may require analysis of large numbers of fish samples over an extended period of time.

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Chapter 8
General discussion and conclusions

8.1 General discussion and conclusions

The infection of marine fish species with the Myxozoan parasites, *Kudoa thyrsites* and *K. paniformis*, result in unsightly cyst formation and/or *post-mortem* myoliquefaction, leading to economic losses (Stehr & Whitaker, 1986; Moran, Whitaker *et al*., 1999; Marshall *et al*., 2016). While infected fish appear unharmed while alive (Marshall *et al*., 2016), these quality defects are typically only apparent when the fish are filleted and/or processed several hours *post-mortem* (Levsen *et al*., 2008). Although *K. thyrsites* and *K. paniformis* infection does not pose any health risk when infected fish are consumed (Alvarez-Pellitero & Sitja-Bobadilla, 1993), it does result in customer complaints and economic losses (Moran, Whitaker *et al*., 1999; D. Brickles, 2016, Group Quality Assurance Manager, Irvin and Johnson Ltd., Cape Town, South Africa, personal communication). Several methods have been used to identify infected fish. Methods of detection included ultraviolet (UV) fluorescence with the use of UV light (Webb, 1990), counting of white and/or black pseudocysts as a potential method for sorting Pacific hake according to level of infection (Morrissey *et al*., 1995; St-Hilaire *et al*., 1997), microscopic (wet mounts and/or histological sectioning and staining of muscle tissue) and polymerase chain reaction (PCR) technologies (Cunningham, 2002; Young & Jones, 2005; Meng & Li-Chan, 2007; Yokoyama & Itoh, 2005; Piazzon *et al*., 2012; Jones *et al*., 2016), and antigen-capture enzyme-linked immunosorbent assays (ELISA) (Taylor & Jones, 2005; Jones *et al*., 2012). While some of these methods has potential for application in aquaculture, many of these methods are not applicable for use in the fishing industry because they require careful examination, are time-consuming, expensive, and are destructive to the fish samples. Near-infrared (NIR) spectroscopy is a rapid, non-destructive, economical (once calibration has been completed), and environmental friendly method that has been shown to be useful for the classification of parasitic nematode infected and uninfected fish fillets (Heia *et al*., 2007; Sivertsen, Kimiya *et al*., 2011; Sivertsen *et al*., 2012). If a NIR method can be developed for fast and non-destructive classification of *K. thyrsites* infected and uninfected fish, it may be further developed into a routine quality control protocol for use by the fish industry. In addition, such a NIR method will be economically beneficial and time-saving for further research studies pertaining to *K. thyrsites* infected fish samples.

The objectives of this study were: i) to develop a quantitative PCR (qPCR) method to be used as a routine, qualitative diagnostic analytical tool for the detection of the presence of *K. thyrsites* and *K. paniformis* in fish muscle tissue, ii) to investigate the prevalence of these two *Kudoa* parasites in South African (SA) sardine, Cape hake, and SA kingklip as influenced by factors such as season

and area of capture, size and sex of the fish samples, iii) to investigate effects of *K. thyrsites* infection on the moisture and ash content of SA sardines and on the moisture, ash and protein content of Cape hake, and iv) to investigate the usefulness of NIR spectroscopy to discriminate between *K. thyrsites* infected and uninfected SA sardine, Cape hake, and SA kingklip samples.

In this study, qPCR assays, that targeted a single nucleotide polymorphism (SNP) of the nucleotide sequence of the 18 SSU ribosomal RNA genes of *K. thyrsites* and *K. paniformis*, respectively, were developed. The qPCR assays were used as a qualitative diagnostic tool for distinguishing between fish samples infected with *K. thyrsites* and uninfected samples. Two qPCR primer sets and probes were designed to be used in the two separate assays, where in the one assay (*K. thyrsites* assay) it distinguished between an allele for *K. thyrsites* and an allele for *K. paniformis*/*K. miniauriculata*/*K. dianae*, and in the second assay (*K. paniformis* assay) between an allele for *K. paniformis* and an allele for *K. thyrsites*/*K. inornata*/*K. megacapsula*. The qPCR primer set designed to yield a fragment of 161 base pairs (used in the *K. paniformis* assay) was specific towards *K. thyrsites*, verifying the presence of this *Kudoa* parasite in SA sardine, Cape hake, and SA kingklip samples analysed throughout this study. This also indicated that this primer set was able to distinguish between *K. thyrsites* and the allele *K. paniformis*/*K. miniauriculata*/*K. dianae*. The *K. thyrsites* assay was therefore useful as a qualitative diagnostic tool for the detection of *K. thyrsites* and was used in subsequent analyses throughout this study.

A noticeable limitation experienced throughout this study was the inability to verify the presence of *K. paniformis* in one Cape snoek sample, 12 SA sardine samples analysed with PCR (total number of SA sardine samples for PCR = 265) and five SA sardine samples analysed with the qPCR assays (total number of SA sardine samples for qPCR = 296). Reasons for this include the inability of the study to verify the specificity of the qPCR primers towards *K. paniformis* and failed attempts to successfully sequence PCR and qPCR products from amplifications of samples that indicated the presence of *K. paniformis*. It is therefore still unknown whether *K. paniformis* is present in marine fish species caught along the South African coast. Further studies are suggested to determine the presence of *K. paniformis* in Cape snoek and SA sardine samples, as well as in water samples from areas where fish are harvested by fishing boats and trawlers. However, based on literature (Morado & Sparks, 1986; St-Hilaire *et al*., 1998; Moran, Whitaker *et* al., 1999; Jones *et al*., 2016) the presence of *K. paniformis* has been shown to be mainly in fish from the Pacific Ocean. It is therefore hypothesised that this parasite is not present in South African waters.

This study presents the first report of *K. thyrsites* in SA kingklip. A total of 70 SA kingklip samples were analysed and 40% were infected. Throughout this study, a total of 296 SA sardine and 170 Cape hake samples were analysed for the presence of *K. thyrsites* using the qPCR method. qPCR results showed that the SA sardine samples had a higher prevalence (92%) of *K. thyrsites* compared to Cape hake (79%) samples.

The prevalence of *K. thyrsites* in the SA sardine samples used in this study was much higher than what was reported by previous studies (17%, n = 102; Reed *et al*., 2012). The reason for this

could be because these authors used light microscopy to detect the presence of *K. thyrsites* spores. Light microscopy is able to only detect myxospores, while qPCR methods are useful for detecting less severe and early stages of infections when no myxospores are present (Grabner *et al*., 2012). qPCR is therefore a more sensitive method compared to microscopic examinations for the detection of *K. thyrsites* infection. This study is the first attempt to study the prevalence of *K. thyrsites* in Cape hake species. This study showed that samples of both Cape hake species, *M. capensis* and *M. paradoxus*, were infected with *K. thyrsites*, with no significant difference in prevalence between the two species. The reason for this may be related to the diet of Cake hake. Cape hake are opportunistic feeders, and on the west coast, mature *M. capensis* feed on smaller deep-water hake and SA sardines (Pillar & Wilkinson, 1995). In addition, deep- (*M. paradoxus*) and shallow-water (*M. capensis*) hake often occur together and are therefore captured together during demersal fishing activities (DAFF, 2014). It is therefore concluded that separating deep-water from shallow-water hake on a processing line is not a useful means of predicting *K. thyrsites* infection in Cape hake.

Eighteen percent (18%) of the SA sardine samples (n = 296) used in the prevalence study of this dissertation showed severe myoliquefaction. This showed that, although the prevalence of *K. thyrsites* was high for the SA sardine samples investigated in this study, not all infected samples became degraded to such an extent that it may not be suitable for canning. Severely myoliquefied sardines are not suitable for canning as it will result in complete degradation of the product during processing (Webb, 1990). If infected samples not showing myoliquefaction could be separated from those already severely myoliquefied, the former could still be of acceptable quality for further processing. Further studies are suggested where the relationship between the level of infection (number of *K. thyrsites* spores) and the severity of *post-mortem* myoliquefaction in SA sardines is investigated. It is acknowledged that, because this study failed to quantify the level of *K. thyrsites* infection within individual samples, this study was limited in that correlations between the level of infection and myoliquefaction (particularly in the SA sardine samples) could not be investigated. Future studies are suggested where the qPCR assay used in this study are further developed as to be used for quantification of infection (Jones *et al*., 2016) within a fish sample. The severity of myoliquefaction in SA sardines (and other fish species) can then be correlated to the level of infection, providing baseline knowledge for future studies.

qPCR results from this study showed that there were no significant differences in the prevalence of *K. thyrsites* between male and female samples (SA sardine, Cape hake and SA kingklip), season of capture (SA sardine), and area of capture (SA sardine). The prevalence of *K. thyrsites* was independent of size (cm) for the SA sardine and the SA kingklip samples. A possible reason why the prevalence of infection showed no relationship with sex, area of capture, and size of the SA sardine samples is because fish occur together in the ocean; sharing feeding and breeding grounds. Although the mode of infection is not yet fully understood, it is suggested that the *K. thyrsites* parasite is naturally released into the water upon death of infected fish (Webb, 1993). This result in the possibility of healthy fish to become infected. In conclusion, the use of sex was not a viable solution

to predict *K. thyrsites* infection in SA sardines, Cape hake and SA kingklip samples. In addition, it is suggested that using the area of capture, and/or size of SA sardines to separate infected from uninfected fish before canning are not viable solutions to prevent myoliquefied fish from entering the canning process.

Analysis of the Cape hake samples during two independent studies showed contradicting results for the relationship between size of fish and prevalence of *K. thyrsites*. In the first study, higher prevalence for smaller sized (39.63 ± 10.88 cm) than for larger sized (49.46 ± 13.94 cm) samples was observed, while in the second, results showed no significant (Chi-square P-value > 0.05) relationship between size and prevalence. The contradicting results may have been related to the availability of smaller and larger sized Cape hake as compared to the typical size of 40 to 60 cm. The Cape hake samples ranged in size from 23.0 to 72.5 cm, with only two sample of 72.5 cm (one tested positive and the other negative for *K. thyrsites*) and the majority of samples between 30 and 60 cm (the average size for all Cape hake samples was 38.34 ± 10. 08 cm). The smaller and larger sizes were therefore not well represented in this study. The importance of sampling size and the use of representative samples during research studies has been noted. Results from the present study showed that Cape hake samples of size 31 to 60 cm showed the highest prevalence, coinciding with the size range represented by the majority of the samples. If a larger number of sample in the size ranges of 23 to 30 cm and 65 to 80 cm were analysed, the prevalence of *K. thyrsites* infection for larger sized Cape cake might be higher than for smaller sized samples, or vice versa. Several researchers (St-Hilaire *et al*., 1998; Levsen *et al*., 2008) documented that the prevalence of infection and level of *K. thyrsites* infection are higher in larger than smaller sized hake species. Although it is known that Cape hake develops immune response against *K. thyrsites* (Morado & Sparks, 1986; Langdon *et al*., 1992; Webb, 1993; Moran, Margolis *et al*., 1999) it is, however, unknown whether this defence response result in some resistance, resulting in reduced prevalence and severity of infection (Jones *et al*., 2016) in larger sized fish. Knowledge of the host defence response over the life cycle of the fish may be of importance in order to further understand differences in visual damage of the muscular tissue in fish with different infection levels, as well as between different fish species. Such information may also be useful to the fishing industries for further developing control strategies for which size of fish should be caught. While defence responses towards *K. thyrsites* have been studied in hake species (Webb, 1993; Tsuyuki *et al*., 1982) and Atlantic salmon (Jones *et al*., 2016; Moran, Kent *et al*., 1999), it is unknown whether the SA sardine also has a defence response.

The data from this study showed that infection by *K. thyrsites* had no significant effect (P > 0.05) on the moisture and ash contents of SA sardine and Cape hake samples, and on protein content of Cape hake samples. The overall moisture and ash contents for the SA sardine samples were 70.83 ± 3.36% and 1.87 ± 0.83%, respectively. The overall moisture content for the Cape hake samples was 72.18 ± 3.53%, the ash content was 1.16 ± 0.25%, and the crude protein was 20.62 ± 2.02%. It is therefore concluded that the consumption of uninfected SA sardines and Cape hake is not superior in terms of moisture, ash and protein content compared to infected fish. It is further concluded that

the concentrations of the proximate components; moisture, ash, and protein, showed to be not useful as chemical indictors of *K. thyrsites* infection and therefore would not serve well in development of a NIR spectroscopic method for the prediction of infected fish samples.

Protein breakdown as a result of cysteine cathepsin L protease excreted by *K. thyrsites* (Tsuyuki *et al*., 1982; Samaranayaka *et al*., 2006; Funk *et al*., 2008) result in myoliquefaction and structural changes. It was expected to observe NIR spectral differences between *K. thyrsites* infected and uninfected fish sample due to these structural differences. However, in this study, NIR spectroscopy, in combination with SIMCA and PLS-DA, respectively, was unable to distinguish between *K. thyrsites* infected and uninfected fish samples. One drawback of this study was the lack of obtaining NIR spectral data from fresh fish samples as early as 24 h *post-mortem*. The fish samples used in this study were frozen for up to eight months before analysed. Pro-longed frozen storage of fish result in water loss, protein degradation and aggregation (Sultanbawa & Li-Chan, 2001; Careche *et al*., 2002) due to endogenous autolysis and water crystal formation. Therefore, the structural and chemical changes due to frozen storage may have masked the chemical changes associated with *K. thyrsites* infection in the fish samples analysed during this study. It is therefore concluded that NIR spectroscopy, in combination with SIMCA and PLS-DA, respectively, was not a useful method to predict *K. thyrsites* infection in frozen fish samples.

In the case of SA sardine, it is suggested to investigate the use of NIR spectroscopy and texture analyses, such as Warner-Bratzler, puncture, compression, or textured profile analysis (TPA), to distinguish between extensively myoliquefied and fish not yet showing myoliquefaction, although also infected with *K. thyrsites*. This may be of value to the SA sardine canning industry in order to separate fish unfit for canning from fish suitable for canning, even though those fit for canning are infected. High temperature (such as used during canning processing: 115-121ºC; Anon., 1981) will denature and inactive any proteolytic enzymes associated with texture degradation. However, since this study showed that about 90-91% of SA sardines analysed during this study were infected, such a study may require more than 500 SA sardine samples in order to ensure an equal amount of infected and uninfected samples (50 of each) for the generation of reliable NIR calibration models (Grootveld, 2014).

The Cape hake samples did not show any signs of myoliquefaction, although gaping and black pseudocysts were evident in some of the infected samples. The presence of white, brown, and/or black pseudocysts is common in *K. thyrsites* and/or *K. paniformis* infected hake species before myoliquefaction is evident (Tsuyuki *et al*., 1982; Morado & Sparks, 1986; Webb, 1993; Tamkee, 2003; Samaranayaka *et al*., 2007). The use of hyperspectral imaging (HSI) and/or visible and near-infrared (VIS/NIR) spectroscopy are useful technologies to separate nematode infected from uninfected fish (Wold *et al*., 2001; Heia *et al*., 2007; Sivertsen, Kimiya *et al*., 2011; Sivertsen *et al*., 2012). This is possible because of optical differences between fish flesh and parasitic worms (Stormo *et al*., 2004; Stormo *et al*., 2007). It is suggested, for future studies, to investigate the use of HSI and VIS/NIR spectroscopy in an attempt to distinguish between *K. thyrsites* infected and uninfected Cape

hake samples based on optical properties of fish muscle and pseudocysts. If these technologies can show that optical properties between fish flesh and pseudocysts are significantly different as to allow the generation of classification models, on-line automatic detection systems could be developed as has been demonstrated for nematode detection (Sivertsen, Heia *et al*., 2011; Sivertsen *et al*., 2012).

In conclusion, it is clear from this study that the development of strategies to predict infection of *K. thyrsites* in wild-caught marine fish species requires large sampling sizes and cost-effective and time-saving analytical methods (such as qPCR and/or NIR spectroscopy). This study also showed that the prevalence of *K. thyrsites* is quite high (79-92%) in the two most economically important marine fish species (SA sardine in terms of tonnage, and Cape hake in terms of value) of South Africa. It is therefore unavoidable that the majority of fish caught will be infected. Development of control strategies to identify or predict heavily infected and/or severely myoliquefied fish would be of greater use to the fish processing industry. On the other hand, the use of processing technologies, such as ionizing radiation, to prevent the development of severe myoliquefaction by inhibiting proteolytic activities (Saha *et al*., 1995; Hwang & Hau, 1995; Hultmann & Rustad, 2004), would be of great interest. Further studies investigating the use of freezing in combination with or without ionizing radiation as possible preventative methods of severe myoliquefaction development are suggested.

8.2 References

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Addendum A

A review of *Kudoa*-induced myoliquefaction of marine fish species in South Africa and other countries

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Myoliquefaction of fish musculature results in customer quality complaints and in huge economic losses, especially with regard to Pacific hake (*Merluccius productus*), farm-reared Atlantic salmon (*Salmo salar*), South African pilchards (*Sardinops ocellatus*) and Cape snoek (*Thyrsites atun*). Myoliquefaction, or 'jelly flesh', is caused by proteolytic enzymes released by the marine myxosporean parasite, *Kudoa thyrsites*, after the death of the fish. Currently there are no fast methods of detection for this microscopic parasite, and because myoliquefaction is evident only after 38–56 h post-mortem, infected fish inevitably reach the processor and/or consumer. Several methods of detection have been investigated, but most of these methods are time-consuming and/or result in destruction of the fish, and are thus impractical for fishing vessels and fish processors. Limited research is available on possible means of destroying or inhibiting the post-mortem activity of the parasitic proteolytic enzyme. Means such as manipulating post-mortem pH and temperature control have been suggested; leaving opportunities for research into food technology applications such as cold-chain management and ionising radiation.

Introduction

Post-mortem myoliquefaction of fish muscle, commonly known as 'milky flesh', 'soft flesh' or 'jelly flesh', is a phenomenon associated with parasitic infection by *Kudoa thyrsites* and/or *K. paniformis. Kudoa* infection has been researched extensively[1] as a result of the economic implications to marine and aquaculture industries. Pacific hake (*Merluccius productus*)[2] and farm-reared Atlantic salmon (*Salmo salar*)[3-5] have been studied more extensively. Parasites from the family Kudoidae Maglitsch, 1960, result in post-mortem myoliquefaction, and, in certain fish species, in visible cysts in the musculature.[6] The economic implications are of great concern for the salmon aquaculture industry, as infected fish show no external signs of infection. Even after death, myoliquefaction becomes apparent only after several hours (38–56 h) post-mortem.[7] Currently there is no rapid method to identify and quantify *Kudoa* infection. A visual counting method, in which visible white pseudocysts are counted, has been suggested for Pacific hake. Although this method is time consuming,[8] as it requires careful examination, it could be implemented as a means to remove heavily infected fillets with black streaks caused by black pseudocysts. White pseudocysts are only one of the few stages in the *Kudoa* life cycle. In some fish host species, black pseudocysts are present, while in other host species, such as salmonids, there is no formation of black pseudocysts. The muscle fibre can thus become completely infected and rupture without any macroscopic evidence of infection. While some kudoid species are responsible for post-mortem myoliquefaction and cyst formation, others seem to have relatively little effect in host species.[9] Many kudoid species show low host specificity, for example, *K. thyrsites* has been recorded from 18 different fish families representing nine fish orders.[10] Taxonomies of the class Myxosporea[11,12] and the genus *Kudoa*[11], have been elaborated in previous review articles, and are not the focus of this review. Information about host species-specific kudoids, level of infection and effects on host musculature, in commercially important fish species, is of great economic importance. However, limited information about *Kudoa* infections and the effects thereof on marine fish quality and the economic impact thereof in South Africa is available. Here we review some of the global research on *K. thyrsites* and *K. paniformis* in several marine fish species of economic importance, with reference, where applicable, to South African marine fish species.

Myoliquefaction in marine fish in South Africa and other countries

Myoliquefaction is a common phenomenon in South African Cape snoek (*Thyrsites atun*). The musculature of infected fish becomes completely soft and jelly-like (Figure 1) and is commonly known as 'pap snoek'. Soft flesh was described for the first time in *T. atun* in Australia and South Africa as early as 1910 and 1924, respectively.[13] Myoliquefaction in Cape snoek is caused by the myxosporean parasite *K. thyrsites*. This parasite was shown to have infected up to 27 fish species worldwide[11], including mahi-mahi (*Coryphaena hippurus*)[14], Atlantic salmon and Pacific hake[3,10,11]. South African pilchard (*Sardinops ocellatus*), Cape hake (*Merluccius capensis*) and Cape dory (*Zeus capensis*) are also known to be infected by *K. thyrsites* (Table 1), with pilchards being among the most heavily infected fish species in South African waters.[15]

The presence of *K. thyrsites*, associated with myoliquefaction in dried fillets exported to Japan, was reported for the first time in 2005 in splendid alfonso (*Beryx splendens*) from South Africa.[16] In the Northwest Pacific of North America, the occurrence of *K. thyrsites* is of great economic importance as it has a significant impact on farmed Atlantic salmon and, to a lesser extent, in coho salmon (*Oncorhynchus kisutch*).[17]

Kudoa paniformis often occurs simultaneously with *K. thyrsites* in Pacific hake.[18] South African hake[19] is believed to undergo accelerated myoliquefaction because *K. paniformis* produces both acidic and neutral proteolytic enzymes[19]; *K. thyrsites* only produces acidic enzymes[1]. Several studies[2,20,21] have indicated that the optimum conditions for endogenous enzymes from *K. paniformis* in Pacific hake are a pH range of 5.25–5.50 and a temperature range of 52–55 °C.

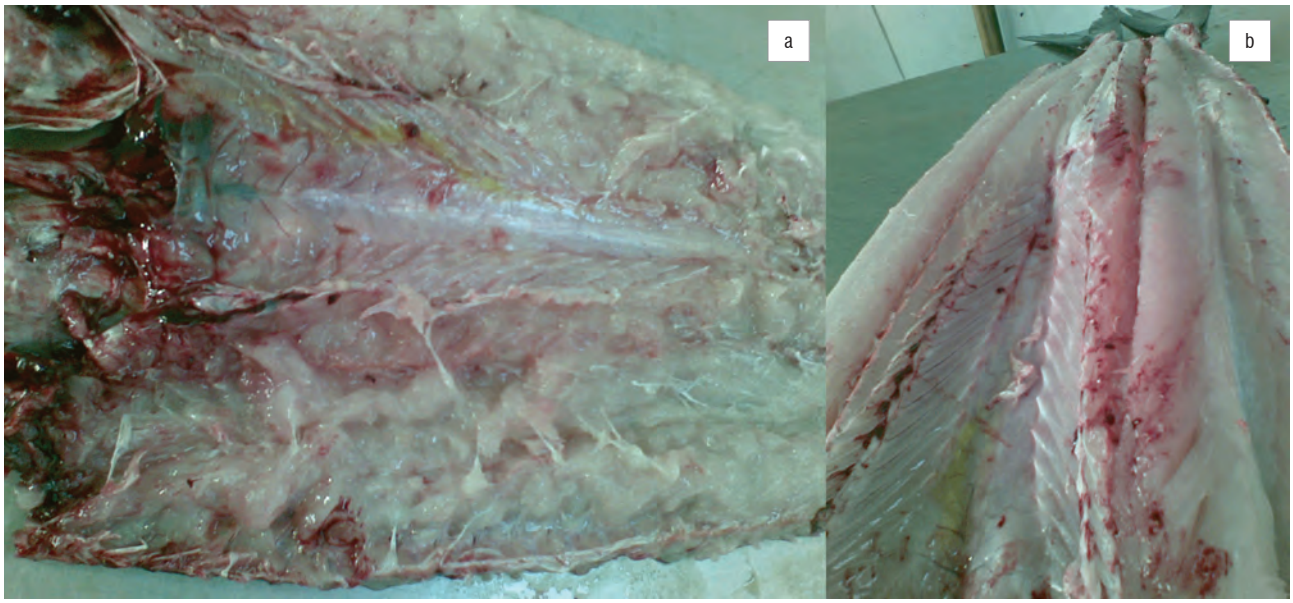

Figure 1: Refrigerated (5 °C) flecked Cape snoek (*Thyrsites atun*) musculature at 48 h post-mortem (a) with and (b) without myoliquefaction.

Table 1: A summary of reports of marine fish species, from different geological areas, infected by *Kudoa thyrsites*

Geographical region	Fish species	Common/regional name
South Africa[10,13,14]	*Sardinops ocellatus*	South African pilchard
	Thyrsites atun	Cape snoek
	Lepidopus caudatus	Silver scabbardfish (beltfish)
	Merluccius capensis	Cape hake
	Zeus capensis	Cape dory
	Beryx splendens	Splendid alfonso
Southern Australia[14]	*Thyrsites atun*	Barracouta (snoek)
Western Australia[10]	*Coryphaena hippurus*	Common dolphinfish
	Sardinops sagax neopilchardus	Pilchard
	Spratelloides robustus	Blue sprat (round herring)
Canadian Pacific[10, 17,19]	*Merluccius productus*	Pacific hake
	Oncorhynchus kisutch	Coho salmon
Washington Pacific[10]	*Salmo salar*	Atlantic salmon
Japanese sea[10]	*Cypsilurus agoo*	
	Engraulis japonicus	Japanese anchovy
Mauritania (Atlantic)[10]	*Zeus faber*	John dory
North Sea[6]	*Scomber scombrus* L.	Atlantic mackerel

The cytoplasmic cysteine proteases from *K. thyrsites* and *K. paniformis* have been identified and characterised in Pacific hake, and it was found that *K. paniformis* was the predominant parasitic species in the muscles.[21] Pacific hake off the coast of Vancouver Island (British Columbia, Canada) was also mainly infected with *K. paniformis*, while only a small number of *K. thyrsites* spores was detected.[2] In a study in which 322 Pacific hake from the west coast of British Columbia (offshore) were investigated for the presence of both *K. thyrsites* and *K. paniformis*, *K. paniformis* was found to be the predominant infection, with 32.3% and 38.8% of fish samples infected with either *K. thyrsites* or *K. paniformis*, respectively, and 18.3% infected with both parasitic species.[18] Only 10.5% of the 322 samples was uninfected; however, the intensity of infection in the 89.5% infected samples was not uniform between samples or within a sample. Infection was generally more concentrated in the anterior part of the fillets and diminished toward the posterior area.

Mode of action of *Kudoa thyrsites* on fish tissue

Kudoa thyrsites releases pro-enzymes from the pre-sporogenic plasmodia[1,20] that support its development by breaking down fish tissue without causing harm to the live fish. Ultra-structural evidence presented by Stehr and Whitaker[1] suggested that while the fish is alive, neighbouring muscle fibres, and myofibrils of the infected muscle fibre that are not in direct contact with *K. thyrsites* plasmodia, are unaffected by the presence of the parasite. However, when these enzymes continue to be released by the parasite after the fish is killed, myoliquefaction of the musculature occurs. The level of proteolytic activity seems to be linked to the stage of parasitic infection[19] as well as to the level of infection[2]. Proteolytic activity in hake muscle infected with the young parasite stage (white pseudocysts) was on average higher (7-fold) at an acidic pH range than at a neutral pH range. Muscle infected with older black pseudocysts showed a smaller difference in proteolytic activity between acidic and neutral pH ranges.[19] However, fish muscle infected with black pseudocysts showed higher proteolytic activity, even though the correlation of black pseudocysts with proteolytic activity was low.[8] A strong linear relationship was observed between spore counts and endogenous proteolytic activity in Pacific hake infected mainly by *K. paniformis*.[2]

Cathepsin L proteases are responsible for myoliquefaction in infected fish and it is clear this cysteine protease is derived from the parasite and not from a host response to the parasite.[21] Cathepsin L from *K. thyrsites* differs from other cathepsin L proteases in that it contains only four of the six cysteine residues believed to be involved in disulphide bonds. *K. thyrsites* cathepsin L is translated as a pro-enzyme which is then chemically altered to an active enzyme by removal of the pro-region during limited proteolysis.[20,21] This process is regulated by pH, where a decrease in pH results in the destabilisation of the pro-region, thus exposing the cleavage site. Cathepsin L protease from *K. thyrsites* shows maximum activity at pH 5.5, the iso-electric point of muscle proteins, and a marked loss of activity at pH 6.5.[19] As the pH in fish muscle drops from 7.0 to 6.5 during early post-mortem storage,[22,23]

myoliquefaction is not evident early post-mortem. Reducing the amount of glycogen in Atlantic salmon muscle cells prior to harvesting might possibly reduce the amount of lactic acid generated in the muscle post-mortem and consequently reduce the activity of cathepsin L proteases released from the parasite.[2]

Characteristics of *Kudoa thyrsites* infection

Kudoa thyrsites is a multivalvulid myxosporean parasite of marine fishes and has a complex life cycle with more than one host; very little is known about the life cycle of this parasite.[11,19,24]

Based on the life cycle of freshwater myxosporean species, a life cycle for *K. thyrsites* has been hypothesised.[24,25] Parasitic plasmodia (cysts containing many spores) grow within the myocytes and sporulation of plasmodia within the somatic musculature of the host fish results in many myxospores inducing a chronic inflammatory response, but without any apparent harm to the health of the fish. The parasite penetrates the core of muscle fibre and spreads along myocytes without damaging the sarcolemma, resulting in individual muscle fibres being filled with spores without involving the connective tissue.[19] This aggregate of spores within the somatic muscle fibre is referred to as the pseudocyst.[26] The myxospores are released from the pseudocyst upon death of the host fish, and are then taken up by an intermediate host. Within the intermediate host, a second sporulation results in the development of actinospores. These actinospores are released to infect a new host fish and continue the parasite's life cycle. Whilst the parasite remains within the muscle fibre (where it contains both developing and mature spores), infected fibres appear white[1]; however, if the sarcolemma is destroyed by the parasite, there is a rapid development of a fibroblast layer around the parasite and the pseudocyst becomes black in appearance. In certain fish host species, such as salmonids, there is no formation of black pseudocysts[26] and the muscle fibre can be completely infected and rupture without any macroscopic evidence of infection.

Although the stage of *K. thyrsites* that is infective to fish has not yet been identified,[4] the presence of plasmodia in myocytes of Atlantic salmon has been observed after only 9 weeks of exposure to contaminated seawater[27]. *K. thyrsites* is different from other members of the myxozoan species in that it infects a wide variety of fish species around the world (Table 1). The route of infection is unknown; however, transmission of the parasite does not occur directly between fish. Infection by *K. thyrsites* occurs only in seawater and not in fresh water.[6] South African snoek and pilchards appear to be major host reservoir species for *K. thyrsites*[12,15] in South African waters. It cannot, however, be assumed that transmission of *K. thyrsites* occurs through ingestion of infected fish by other predatory fishes, firstly, because the life cycle and route of transmission and infection is unknown, and, secondly, because the Australian pilchard (*Sardinops sagax neopilchardus*), a planktivorous fish species, is also heavily infected with *K. thyrsites* and is suggested to be a major reservoir host in Southwest Australian waters. It is thus suggested that infection of plantivorous fish is through ingestion of infected invertebrate hosts.[12]

Factors affecting the level of *Kudoa thyrsites* infection

The degree of visible myoliquefaction from *K. thyrsites* infection may be influenced by several factors, such as time to evaluation post-mortem, the number of *K. thyrsites* present in the flesh, temperature during storage, and the inherent quality of the muscle.[4] The latter is affected by factors such as growth and feeding condition of the fish, water temperature, and processing conditions. From a correlation between post-mortem myoliquefaction and intensity of *K. thyrsites* infection in Atlantic salmon, it was found that high intensity of infection resulted in severe autolysis of the somatic musculature.[3] A mean plasmodia count of 0.3 mm^{-2} or a mean spore count of 4 x 10^5/g tissue is suggested to be indicative of severe myoliquefaction.[4]

The age and size[5,7] of fish as factors affecting the degree of post-mortem myoliquefaction was demonstrated in 2008[7], when large (>600 g) Atlantic mackerel had a significantly greater prevalence of myoliquefaction than medium-sized (400–600 g) mackerel. Pen-reared sexually mature Atlantic salmon (*Salmo salar*) are also more likely to be infected with *K. thyrsites* than their sexually immature counterparts.[5] A local snoek processor (Theron R 2012, oral communication, June) reported that large Cape snoek tend to develop myoliquefaction to a greater extent than do smaller fish. Levsen et al.[7] hypothesised that mackerel become infected at a late stage of their lifespan, or, that the parasite develops relatively slowly. Previous studies[19,27] have indicated that *K. thyrsites* has a slow plasmodial development in fish and develops into microscopically visible plasmodia in adult fish, although a histologically undetectable parasite stage may be present in younger fish. Early stages of the parasite may be detected by PCR[11,28,29] and immunohistochemistry[29] techniques. *K. thyrsites* was identified in young tube-snouts (*Aulorhynchus flavidus*) using PCR when visual or histological methods were negative.[25] Similarly to results of an earlier study[7], it was also found that the prevalence of infection was greater in adult tube-snouts (up to 100%) than in young tube-snouts (about 50%)[25].

The prevalence of *Kudoa* infection is also affected by season, with the level of infection tending to be higher during summer than during winter months.[5,6,11,30,31] The effect of season on *K. thyrsites* and *K. paniformis* infection levels in South African marine fish species, such as Cape snoek, Cape hake, Cape dory and South African pilchard, is not well documented.

Detection and identification of *Kudoa thyrsites* infection

Kudoa thyrsites myxospores (Figure 2) are the most characteristic stage of infection and are suggested to be the infective stage of invertebrate hosts or vertebrate predatory fish species.[7,12,14,24] *K. thyrsites* myxospores vary between 12.0 μm[32] and 16.7 μm[10,33] in diameter, and, in the absence of visible pseudocysts, infection with *K. thyrsites* is not macroscopically visible. Infection is thus not macroscopically detected until myoliquefaction, several hours post-mortem. Examination of both muscle and blood samples of Atlantic mackerel (*Scomber scombrus* L.) immediately after catch was unsuccessful in identifying infection by *K. thyrsites*.[7] Several researchers[8,34] have suggested visual counting of white pseudocysts as a potential method for sorting Pacific hake according to level of infection; a positive correlation between the number of white pseudocysts, protease activity and texture of Pacific hake has been found. The drawback of this method is that it requires careful and time-consuming examination, because white pseudocysts are not readily detectable, and is therefore not feasible for a production line.

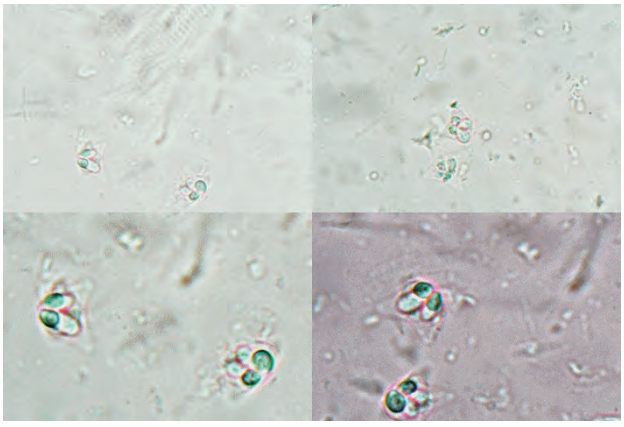

Figure 2: Light micrographs (1000X magnification, Olympus CX31, Nikon DS-Fi1 camera, Nikon NisElements Imaging software version 3.22) of *Kudoa thyrsites* spores from infected Cape dory (*Zeus capensis*) muscle.

Detection of the parasite by means of ultraviolet fluorescence has been investigated,[15] but this method is unreliable because the parasitic cysts do not glow in dark-coloured muscles or in dead flesh that is more than a few hours old.

Identification of parasitic infections relies on traditional skills such as microscopy and taxonomy. Modern methods, such as DNA (PCR) technologies, have made important contributions to the screening, diagnosis and taxonomy of parasitic infections in fish species.[33] Primers have been designed for amplification of the SSU rDNA region for detection of specific *K. thyrsites* where the primer pair Kt18S6f and Kt18S1r amplifies a 909-bp region of the *K. thyrsites* SSU rDNA and does not produce a PCR product from host DNA.[29]

The identification of infection by *K. thyrsites* is usually done by microscopy, using either wet mounts or histological sections of muscle tissue, which results in disfiguration of the fish and thus negatively influences its market value. Sampling from the hyohyoideus ventralis muscle (striated muscle) from under the operculum of Atlantic salmon, from which a wet mount is prepared and examined under phase contrast microscopy provides a non-destructive method for assessing fish for the presence or absence of *K. thyrsites* spores as only a small amount of fillet is needed.[34] Counting the number of plasmodia in the opercular mandibular muscle of Atlantic salmon did not reflect the number of plasmodia in the fillets and was not a good indicator of fillet quality (i.e. it did not correlate with the number of pits in the fillet).[4] The most reliable predictive parasite counts were those from somatic and opercular samples.[4] Because sampling from somatic muscle tissue damages the fillet, this sampling method has been considered undesirable; however, as only a small sample (25 mg) is required, the sample could be removed from the fillet without any visual damage to the fillet.[4] Because the number of plasmodia and spores vary significantly among individual fish and among tissue samples within a fish, it is recommended that multiple samples be taken from a single fish. To avoid false negatives and to improve detection when using the PCR test method, a large sample of tissue from several anatomical areas from a single fish should be included in the initial homogenate.[34] An alternative quantitative method is an antigen-capture enzyme-linked immunosorbent assay[36] which utilises uncharacterised soluble parasite antigens to estimate the severity of the infection. This assay is similar in sensitivity to PCR and may be used, with an appropriate sampling plan, as an early diagnostic tool.

In comparison with microscopic methods, in which only myxospores (Figure 2) are detected, the PCR method is useful for detecting less severe and early stage infections. Although PCR testing for *K. thyrsites* is specific and more sensitive than wet mount and histological examinations, it provides limited information on parasite locality in specific tissues. Although histological staining provides for localisation of recognisable mature infections, it does not allow characterisation of obscure developmental stages of the parasite. By combining methods of in-situ hybridisation and immunohistochemistry, previously undescribed developmental stages of *K. thyrsites* may be characterised.[29] In-situ hybridisation can be used as a tool to localise *K. thyrsites* in specific tissues and to visualise cryptic stages, whereas immunohistochemistry can be used to detect early developmental stages, but not pre-sprogonic stages or mature spores of *K. thyrsites*.

A recent study[36] investigated the efficacy of dietary nicarbazin, an equimolar complex of 4,4′-dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine used in poultry feeds for the prevention of coccidiosis[37], against *K. thyrsites* in seawater-reared Atlantic salmon post-smolts. Dietary nicarbazin was found to significantly reduce the prevalence and severity of *K. thyrsites* in fish. Effects on mortality of fish and of high residues of nicarbazin in skin, liver and muscle warrant further studies. Development of treatment regimes using medicated diets is possible for farmed-reared fish, but such strategies are not possible for wild fish.

Conclusions

Although the parasite *K. thyrsites* is considered harmless if consumed by humans[38], there has been a report[39] of an immunological response after consumption of fish infected with *Kudoa*. No conclusive relationship with any pathological disorder has, however, been found.

In relation to global food security issues, the most important concern with regard to *Kudoa* infections is that it results in waste of animal protein and economic losses. In the case of wild-caught fish, rapid methods of identifying infected fish as soon as possible after harvest or capture at sea will be of benefit to the fishery industry. If infected fish can be identified at an early stage post-mortem, the infected fish may be used for alternative processing. Alternatively, opportunities exist for investigating post-harvest technologies, such as ionising irradiation[40] to inhibit the development of myoliquefaction, and, consequently, reduce waste and economic losses. Such possibilities for South African conditions will enable the fishing industry, especially the small-scale fishing industry, to minimise the wastage experienced currently.

Authors' contributions

All authors contributed to the writing of the manuscript.

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Addendum B

agriculture,
forestry & fisheries

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19th April 2016

To the Cape Peninsula University of Technology Ethics Committee

Re: Collection of hake and sardine samples by DAFF for research conducted by Ms S Henning and her M. Tech student Ms Z. Phike

Dear Madam/Sir,

The Branch: Fisheries Management is responsible for advising on the sustainable management of commercially exploited marine fishery species, including hake *Merluccius* spp sardine *Sardinops sagax*. The Resources Research Directorate of the Branch regularly collects samples of both of these species from research surveys and commercial catches in order to conduct research to provide such advice. These samples are collected under Research Permits issued each year by the Chief Director, Fisheries Research and Development, DAFF, which allow the collection, transportation and possession of marine species for research purposes. Biological data including length and mass, sex and gonad maturity stage, and fat stage are collected and the fish are examined for selected parasites. Tissue samples are also taken, including fillets which are sent for processing by Ms Henning and her students in order to determine *Kudoa* spp infection levels. Such data is of interest and

utility to DAFF given the negative impacts of *Kudoa* infection on product quality of these harvested resources, and the department values this collaboration with Ms Henning and CPUT.

Yours sincerely,

C.D. van der Lingen
Specialist Scientist, Offshore Resources Research
Research and Development
Branch: Fisheries Management
Department of Agriculture, Forestry and Fisheries
South Africa

Addendum C

```
FILE: Multiple_Sequence_Alignment
PROJECT:
NUMBER: 13
MAXLENGTH: 6298
NAMES: pani AF034640.1 mini AF034639.1 ino FJ790311.1 kudoa AY302723.1 dia AF414692.1 para FJ792719.1
       tri AM183300.2 quad FJ792721.1 quad AY078428.1 mega AB188529.1 thy AY542481.1 thy AY542482.1
       thy AY941819.1
MAXNAMELEN: 16

ORIGIN
pani AF034640.1  ..................GGTCATATGCTCGTCTCAAA     20
mini AF034639.1  ..................GGTCATATGCTCGTCTCAAA     20
ino FJ790311.1   acctggttgatcctgccagtGGTCATATGCTCGTCTCAAA     40
kudoa AY302723.1 ..................GGTCATATGCTCGTCTCAAA     20
dia AF414692.1   ..................GGTCATATGCTCGTCTCAAA     20
para FJ792719.1  ........gattctgccagtGGTCATATGCTCGTCTCAAA     32
tri AM183300.2   ............tgccagtGGTCATATGCTCGTCTCAAA     27
quad FJ792721.1  ........gattctgccagtGGTCATATGCTCGTCTCAAA     32
quad AY078428.1  ..................GGTCATATGCTCGTCTCAAA     20
mega AB188529.1  ..................GGTCATATGCTCGTCTCAAA     20
thy AY542481.1   ..................GGTCATATGCTCGTCTCAAA     20
thy AY542482.1   ..................GGTCATATGCTCGTCTCAAA     20
thy AY941819.1   ..................GGTCATATGCTCGTCTCAAA     20
Consensus                         ggtcatatgctcgtctcaaa

pani AF034640.1  GATTAAGCCATGCAAGTCTAAGTCCACATCATATAAAGAT     60
mini AF034639.1  GATTAAGCCATGCAAGTCTAAGTtCAtATCAcATAAAGAT     60
ino FJ790311.1   GATTAAGCCATGCAAGTCTAAGTtCAtATCATATAAAGAT     80
kudoa AY302723.1 GATTAAGCCATGCAAGTCTAAGTtCAtATCATATAAAGAT     60
dia AF414692.1   GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     60
para FJ792719.1  GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     72
tri AM183300.2   GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     67
quad FJ792721.1  GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     72
quad AY078428.1  GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     60
mega AB188529.1  GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     60
thy AY542481.1   GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     60
thy AY542482.1   GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     60
thy AY941819.1   GATTAAGCCATGCAAGTCTAAGTtCAtATCATcTAAAGAT     60
Consensus        gattaagccatgcaagtctaagt ca atca  taaagat
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Commented [NA1]: *K. paniformis* specific only

Commented [NA2]: *K. paniformis* specific only

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pani AF034640.1  GAAACTGCGA..CGCTCAGTAAATCAGTTATTGTCCGTTC     98
mini AF034639.1  GAAACTGCGA..CGCTCAGTAAATCAGTTATTGTCCGTTC     98
ino FJ790311.1   GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    120
kudoa AY302723.1 GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    100
dia AF414692.1   GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    100
para FJ792719.1  GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    112
tri AM183300.2   GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    107
quad FJ792721.1  GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    112
quad AY078428.1  GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    100
mega AB188529.1  GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    100
thy AY542481.1   GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    100
thy AY542482.1   GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    100
thy AY941819.1   GAAACTGCGAagCGCTCAGTAAATCAGTTATTGTCCGTTC    100
Consensus        gaaactgcga  cgctcagtaaatcagttattgtccgttc

pani AF034640.1  GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    138
mini AF034639.1  GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    138
ino FJ790311.1   GGTCATATaAaCCATGGATAACTGTGGTAAATCTAGAGCT    160
kudoa AY302723.1 GGTCATATaAGCCATGGATAACTGTGGTAAATCTAGAGCT    140
dia AF414692.1   GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    140
para FJ792719.1  GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    152
tri AM183300.2   GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    147
quad FJ792721.1  GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    152
quad AY078428.1  GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    140
mega AB188529.1  GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    140
thy AY542481.1   GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    140
thy AY542482.1   GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    140
thy AY941819.1   GGTCATATCAGCCATGGATAACTGTGGTAAATCTAGAGCT    140
Consensus        ggtcatat a ccatggataactgtggtaaatctagagct

pani AF034640.1  AATACATAGCAAATCTCGCACT....TGTGCGGGAGCATT    174
mini AF034639.1  AATACATAGCAAATCTCGtACT....TGTaCGGGAGCATT    174
ino FJ790311.1   AATACATAGCAAATCcCGCACT....TGTGCGGGAGCATT    196
kudoa AY302723.1 AATACATAGCAAATCTCGtACT....TGTaCGGGAGCATT    176
dia AF414692.1   AATACATAGCAAATCTCGtACacgtgTGTGCGGGAGCATT    180
para FJ792719.1  AATACATAGCAAATCTCaCgCT....TGcGtGGGAGCATT    188
tri AM183300.2   AATACATAGCAAATCTCGtACT....TGTaCGGGAGCATT    183
quad FJ792721.1  AATACATAGCAAATCTCaCgCT....TGcGtGGGAGCATT    188
quad AY078428.1  AATACATAGCAAATCTCaCgCT....TGcGtGGGAGCATT    176
mega AB188529.1  AATACATAGCAAATCTCGtACT....TGTGCGGGAGCATT    176
thy AY542481.1   AAaACATAGCAAATCTCGtACT....TGTGCGGGAGCATT    176
thy AY542482.1   AAaACATAGCAAATCTCGtACT....TGTGCGGGAGCATT    176
thy AY941819.1   AAaACATAGCAAATCTCGtACT....TGTGCGGGtGCATT    176
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Commented [NA3]: *K. thyrsites* specific

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Consensus        aa acatagcaaatc c   c     tg   ggg gcatt

pani AF034640.1  TGTTAGACTCAACCAACTGACTCTT....GTCATTTGATG    210
mini AF034639.1  TaTTAGACTCAACCAACTGACTtTggt....CATTTGATG    210
ino FJ790311.1   TaTTAGACTCAACCAACTGgCctcg....GcCATTTGATG    232
kudoa AY302723.1 TaTTAGACTCAACCAACTGACTtTggt....CATTTGATG    212
dia AF414692.1   TaTTAGACTCAACCAACTGACctcggt....CATTTGATG    216
para FJ792719.1  TaTTAGACTCAACCAACTGgCTtcg....GcCATTTGAcG    224
tri AM183300.2   TaTTAGACTCAACCAACTGgCcCTTaaaaGcCATTTGATG    223
quad FJ792721.1  TaTTAGACTCAACCAACTGgCTtcg....GcCATTTGAcG    224
quad AY078428.1  TaTTAGACTCAACCAACTGgCTtcg....GcCATTTGAcG    212
mega AB188529.1  TaTTAGACTCAACCAACTGgCctcg....GcCATTTGATG    212
thy AY542481.1   TaTTAGACTCAACCAACTGgCctcg....GcCATTTGATG    212
thy AY542482.1   TaTTAGACTCAACCAACTGgCctcg....GcCATTTGATG    212
thy AY941819.1   TaTTAGACTCAACCAACTGgCctcg....GcCATTTGATG    212
Consensus        t ttagactcaaccaactg c          catttga g

pani AF034640.1  AATCCTAATAACTGTGCATATCGAACATTAATTTGTCGAT    250
mini AF034639.1  AATCCTAATAACTGTGCATATCGAACATTAATTTGTCGAT    250
ino FJ790311.1   AATCCTAATAACTGTGCATATCGAACATTAATTTGTCGAT    272
kudoa AY302723.1 AATCCTAATAACTGTGCATATCGAACATTAATTTGTCGAT    252
dia AF414692.1   AATCCTAATAACTGTGCATATCGAACATTAAcTTGTCGAT    256
para FJ792719.1  AATCCTAATAACTGaGCATATCGAACATTAATTTGTCGAT    264
tri AM183300.2   AATCCTAATAACTGaGCATATCGAACATTAATTTGTCGAT    263
quad FJ792721.1  AATCCTAATAACTGaGCATATCGAACATTAATTTGTCGAT    264
quad AY078428.1  AATCCTAATAACTGaGCATATCGAACATTAATTTGTCGAT    252
mega AB188529.1  AATCCTAATAACTGgGCATATCGAACATTAATTTGTCGAT    252
thy AY542481.1   AATCCTAATAACTGgGCATATCGAACATTAATTTGTCGAT    252
thy AY542482.1   AATCCTAATAACTGgGCATATCGAACATTAATTTGTCGAT    252
thy AY941819.1   AATCCTAATAACTGgGCATATCGAACATTAATTTGTCGAT    252
Consensus        aatcctaataactg gcatatcgaacattaa ttgtcgat

pani AF034640.1  AGTCCGATCGAATTTCTTGACCCCTATCAACTAGTTGGTG    290
mini AF034639.1  AGTCCGATCGAATTTCTTGACCCCTATCAACTAGTTGGTG    290
ino FJ790311.1   AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    309
kudoa AY302723.1 AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    289
dia AF414692.1   AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    293
para FJ792719.1  AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    301
tri AM183300.2   AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    300
quad FJ792721.1  AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    301
quad AY078428.1  AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    289
mega AB188529.1  AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    289
thy AY542481.1   AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    289
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Commented [NA4]: *K. paniformis* specific

Commented [NA5]: *K. paniformis* specific

Commented [NA6]: *K. thyrsites* Forward primer also picks up *Kudoa inornata* and *K. megacapsula*

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thy AY542482.1   AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    289
thy AY941819.1   AGTCCGATCGAATTTCTg...CCCTATCAACTAGTTGGTG    289
Consensus        agtccgatcgaatttct    ccctatcaactagttggtg

pani AF034640.1  AGGTAGTGGCTCACCAAGGTTGTGACGGGTAACGGGGGAT    330
mini AF034639.1  AGGTAGTGGCTCACCAAGGTTGTGACGGGTAACGGGGGAT    330
ino FJ790311.1   AGGTAGTGGCTCACCAAGGTcGTGACGGGTAACGGGGGAT    349
kudoa AY302723.1 AGGTAGTGGCTCACCAAGGTTGTGACGGGTAACGGGGGAT    329
dia AF414692.1   AGGTAGTGGCTCACCAAGGTTGTGACGGGTAACGGGGaAT    333
para FJ792719.1  AGGTAGTGGCTCACCAAGGTTGcGACGGGTAACGGGGGAT    341
tri AM183300.2   AGGTAGTGGCTCACCAAGGTaGTGACGGGTAACGGGGGAT    340
quad FJ792721.1  AGGTAGTGGCTCACCAAGGTTGcGACGGGTAACGGGGGAT    341
quad AY078428.1  AGGTAGTGGCTCACCAAGGTTGcGACGGGTAACGGGGGAT    329
mega AB188529.1  AGGTAGTGGCTCACCAAGGTTTtTGACGGGTAACGGGGGAT    329
thy AY542481.1   AGGTAGTGGCTCACCAAGGTgtTaACGGGTAACGGGGGAT    329
thy AY542482.1   AGGTAGTGGCTCACCAAGGTgtTaACGGGTAACGGGGGAT    329
thy AY941819.1   AGGTAGTGGCTCACCAAGGTgtTaACGGGTAACGGGGGAT    329
Consensus        aggtagtggctcaccaaggt    acgggtaacgggg at

pani AF034640.1  CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    370
mini AF034639.1  CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    370
ino FJ790311.1   CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    389
kudoa AY302723.1 CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    369
dia AF414692.1   CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    373
para FJ792719.1  CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    381
tri AM183300.2   CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    380
quad FJ792721.1  CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    381
quad AY078428.1  CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    369
mega AB188529.1  CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    369
thy AY542481.1   CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    369
thy AY542482.1   CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    369
thy AY941819.1   CAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTAC    369
Consensus        cagggttcgattccggagagggagcctgagaaacggctac

pani AF034640.1  CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    410
mini AF034639.1  CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    410
ino FJ790311.1   CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    429
kudoa AY302723.1 CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    409
dia AF414692.1   CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    413
para FJ792719.1  CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    421
tri AM183300.2   CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    420
quad FJ792721.1  CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    421
quad AY078428.1  CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    409
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Commented [NA7]: *K. thyrsites* specific

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mega AB188529.1  CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    409
thy AY542481.1   CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    409
thy AY542482.1   CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    409
thy AY941819.1   CACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATC    409
Consensus        cacatctaaggaaggcagcaggcgcgcaaattacccaatc

pani AF034640.1  CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    450
mini AF034639.1  CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    450
ino FJ790311.1   CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    469
kudoa AY302723.1 CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    449
dia AF414692.1   CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    453
para FJ792719.1  CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    461
tri AM183300.2   CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    460
quad FJ792721.1  CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    461
quad AY078428.1  CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    449
mega AB188529.1  CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    449
thy AY542481.1   CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    449
thy AY542482.1   CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    449
thy AY941819.1   CAGACTTTGGGAGGTAGTGACGAGAAATACCGGAGTAGAC    449
Consensus        cagactttgggaggtagtgacgagaaataccggagtagac


pani AF034640.1  CATA.ATTGGTTCACTATCGGAATGAACGTAATCTAATAC    489
mini AF034639.1  CtTtaATTGGTTCACTATCGGAATGAACGTAATCTAATAC    490
ino FJ790311.1   CATA.ATTGGTTCACTATCGGAATGAACGTAATCTAATAC    508
kudoa AY302723.1 CtTtaATTGGTTCACTATCGGAATGAACGTAATCTAATAC    489
dia AF414692.1   CtTttta.GGTTCACTATCGGAATGAACGTAATCTAATAC    492
para FJ792719.1  CgTtaATTGGTTCACTATCGGAATGAACGTAATCTAATAC    501
tri AM183300.2   CgTtaATTGGTTCACTATCGGAATGAACGTAATCTAATAC    500
quad FJ792721.1  CgTtaATTGGTTCACTATCGGAATGAACGTAATCTAATAC    501
quad AY078428.1  CgTtaATTGGTTCACTATCGGAATGAACGTAATCTAATAC    489
mega AB188529.1  CgTAtATTGGTTCACTATCGGAATGAACGTAATtTAATAC    489
thy AY542481.1   CgTAtATTGGTTCACTATCGGAATGAACGTAATtTAATAC    489
thy AY542482.1   CgTAtATTGGTTCACTATCGGAATGAACGTAATtTAATAC    489
thy AY941819.1   CgTAtATTGGTTCACTATCGGAATGAACGTAATtTAATAC    489
Consensus        c t     ggttcactatcggaatgaacgtaat taatac

pani AF034640.1  CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    529
mini AF034639.1  CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    530
ino FJ790311.1   CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    548
kudoa AY302723.1 CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    529
dia AF414692.1   CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    532
para FJ792719.1  CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    541
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tri AM183300.2   CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    540
quad FJ792721.1  CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    541
quad AY078428.1  CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    529
mega AB188529.1  CTTCGATGAGTAaCaACTGGAGGGCAAGTCTGGTGCCAGC    529
thy AY542481.1   CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    529
thy AY542482.1   CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    529
thy AY941819.1   CTTCGATGAGTAGCTACTGGAGGGCAAGTCTGGTGCCAGC    529
Consensus        cttcgatgagta c actggagggcaagtctggtgccagc

pani AF034640.1  AGCCGC.GTAATTCCAGCTCCAGTAGTGTATATCAAAATT    568
mini AF034639.1  AGCCGC.GTAATTCCAGCTCCAGTAGTGTATATCAAAATT    569
ino FJ790311.1   AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCttAATT    588
kudoa AY302723.1 AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    569
dia AF414692.1   AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    572
para FJ792719.1  AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    581
tri AM183300.2   AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    580
quad FJ792721.1  AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    581
quad AY078428.1  AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    569
mega AB188529.1  AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    569
thy AY542481.1   AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    569
thy AY542482.1   AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    569
thy AY941819.1   AGCCGCgGTAATTCCAGCTCCAGTAGTGTATATCAAAATT    569
Consensus        agccgc gtaattccagctccagtagtgtatatc  aatt

pani AF034640.1  GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    608
mini AF034639.1  GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    609
ino FJ790311.1   GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    628
kudoa AY302723.1 GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    609
dia AF414692.1   GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    612
para FJ792719.1  GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    621
tri AM183300.2   GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    620
quad FJ792721.1  GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    621
quad AY078428.1  GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    609
mega AB188529.1  GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    609
thy AY542481.1   GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    609
thy AY542482.1   GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    609
thy AY941819.1   GTTGCGGTTAAAACGCTCGTAGTTGGATTACAAAAGCTCT    609
Consensus        gttgcggttaaaacgctcgtagttggattacaaaagctct

pani AF034640.1  TTGATGGCCAAATCTAGGTTTGGTCGTCGTGGGGTTTTTT    648
mini AF034639.1  TTGATGGCCAAATCaAGGTTTGGTtaTtGTGGGGTTTTTT    649
ino FJ790311.1   TTGAcGGCCAAATCaAGGTTTGGTCGTCGTGGGGTTTTTT    668
kudoa AY302723.1 TTGATGGCCAAATCaAGGTTTGGTCGTtGTGGGGTTTTTT    649
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dia AF414692.1    TTGATGGCCAAATtTAGGTTTGGTCGTCGTGGGGTTTTTT    652
para FJ792719.1   TTGgcaGCCAAATCTAGGTTTGGTtGgtGTGGGGTTTTTT    661
tri AM183300.2    TTGgTGGCCAAATCTAGGTTTGGTCGcCGTGGtGTTTTTT    660
quad FJ792721.1   TTGgcaaCCAAATCTAGGTTTGGTtGgtGTGGGGTTTTTT    661
quad AY078428.1   TTGgcaaCCAAATCTAGGTTTGGTtGgtGTGGGGTTTTTT    649
mega AB188529.1   cTGgcGGCCAAATCaAGGTTTGGTCGcCGTGGGGTTTTTT    649
thy AY542481.1    cTGgyGGCCAAATCTAGGTTTGGTCGcCGTGGGGTTTTTT    649
thy AY542482.1    cTGgcGGCCAAATCTAGGTTTGGTCGcCGTGGGGTTTTTT    649
thy AY941819.1    cTGgcGGCCAAATCTAGGTTTGGTCGcCGTGGGGTTTTTT    649
Consensus          tg    ccaaat  aggtttggt    gtgg gtttttt

pani AF034640.1   TAT.CGCGAGAGCCGCATGTGGGATTAAATTCTTGTGTGC    687
mini AF034639.1   TAT.CGCGAGAGCCGtATGTGGGATTAAATTCTTGTGTGC    688
ino FJ790311.1    TAT.CGCGAGAGCCGtATGTGGGATTAAATTCTTGTGTGC    707
kudoa AY302723.1  TAT.CGCGAGAGCCGtATGTGGGcTTAAATTCTTGTGTGC    688
dia AF414692.1    TATgCGCGAGAGCCGtAcGTGGGATTAAATTCTTGTGTaC    692
para FJ792719.1   TAT.CGCGAGAGCCGtATGTGGGATTAAATTCTTGTGTGC    700
tri AM183300.2    TAT.CGCGAGAGCCaCAcGTGGGATTAAATTCTTGTGTGt    699
quad FJ792721.1   TAT.CGCGAGAGCCGtATGTGGGATTAAATTCTTGTGTGC    700
quad AY078428.1   TAT.CGCGAGAGCCGtATGTGGGATTAAATTCTTGTGTGC    688
mega AB188529.1   TAT.CGCGAGAGCCGtATGTGGGATTAAATTCTTGTGTGC    688
thy AY542481.1    TAT.CGCGAGAGCCGCATGTGGGATTAAcTTCTTGTGTGC    688
thy AY542482.1    TAT.CGCGAGAGCCGCATGTGGGATTAAcTTCTTGTGTGC    688
thy AY941819.1    TAT.CGCGAGAGCCGCATGTGGGATTAAcTTCTTGTGTGC    688
Consensus         tat cgcgagagcc  a gtggg ttaa ttcttgtgt

pani AF034640.1   GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    727
mini AF034639.1   GGTCACTTGCaAGGTGTGCCTTaAATAAAGCACAGTGCTC    728
ino FJ790311.1    GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    747
kudoa AY302723.1  GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    728
dia AF414692.1    GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    732
para FJ792719.1   GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    740
tri AM183300.2    GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    739
quad FJ792721.1   GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    740
quad AY078428.1   GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    728
mega AB188529.1   GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    728
thy AY542481.1    GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    728
thy AY542482.1    GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    728
thy AY941819.1    GGTCACTTGCGAGGTGTGCCTTGAATAAAGCACAGTGCTC    728
Consensus         ggtcacttgc aggtgtgcctt aataaagcacagtgctc

pani AF034640.1   AAAGCAGGCGTTACGTCGAATGTTATAGCATGGAACGATT    767
mini AF034639.1   AAAGCAGGCGTTACGTCGAATGTTATAGCATGGAACGATT    768
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Commented [NA8]: *K. thyrsites* specific

Commented [NA9]: *K. paniformis* forward Primer also picks up *K. minicapsula*

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ino FJ790311.1   AAAGCAGGCGTTAgcTCGAATGTTATAGCATGGAACGATT    787
kudoa AY302723.1 AAAGCAGGCGTTAgcTCGAATGTTATAGCATGGAACGATT    768
dia AF414692.1   AAAGCAGGCGTTAgcTCGAATGTTATAGCATGGAACGATT    772
para FJ792719.1  AAAGCAGGCGTTAgcTtGAATGTTATAGCATGGAACGATT    780
tri AM183300.2   AAAGCAGGCGTTAgcTtGAATGTTATAGCATGGAACGATT    779
quad FJ792721.1  AAAGCAGGCGTTAgcTtGAATGTTATAGCATGGAACGATT    780
quad AY078428.1  AAAGCAGGCGTTAgcTtGAATGTTATAGCATGGAACGATT    768
mega AB188529.1  AAAGCAGGCGaTAgcTtGAATGTTATAGCATGGAACGATT    768
thy AY542481.1   AAAGCAGGCGaTAgcTCGAATGTTATAGCATGGAACGATT    768
thy AY542482.1   AAAGCAGGCGaTAgcTCGAATGTTATAGCATGGAACGATT    768
thy AY941819.1   AAAGCAGGCGaTAgcTCGAATGTTATAGCATGGAACGATT    768
Consensus        aaagcaggcg ta  t gaatgttatagcatggaacgatt

pani AF034640.1  ATGTTGATCTTGTCGACTGTTGGTTGTTGGCATTGGTCCC    807
mini AF034639.1  ATGTTGAcCTTGTtGACTGTTGGTTGTTGaCAgTGGTCCC    808
ino FJ790311.1   ATGTTGAcCTTGTCGACTGTTGGTTGTTGGCAgTGGTCCC    827
kudoa AY302723.1 ATGTTGAcCTTGTtGACTGTTGGTTGTTGaCAgTGGTCCC    808
dia AF414692.1   ATGTTGAcCTTGTCGACTGTTGGTTGTTGaCAgTGGTCCC    812
para FJ792719.1  ATGTTGATCTTGTCGACTGTTGGTTGTTGaCATTGGTCtC    820
tri AM183300.2   ATGTTGAcCTTGTCGACTGTTGGTTGTTGGCAgTGGTCCC    819
quad FJ792721.1  ATGTTGATCTTGTCGACTGTTGGTTGTTGaCATTGGTCtC    820
quad AY078428.1  ATGTTGATCTTGTCGACTGTTGGTTGTTGaCATTGGTCtC    808
mega AB188529.1  ATGTTGATCTTGTCGACTGTTGGTTGTTGaCATTGGTCtC    808
thy AY542481.1   ATGTTGATCTTGTCGACTGTTGGTTGTTGaCATTGGTCtC    808
thy AY542482.1   ATGTTGATCTTGTCGACTGTTGGTTGTTGaCATTGGTCtC    808
thy AY941819.1   ATGTTGATCTTGTCGACTGTTGGTTGTTGaCATTGGTCtC    808
Consensus        atgttga cttgt gactgttggttgttg ca tggtc c

pani AF034640.1  GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGCGGCG    847
mini AF034639.1  GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    848
ino FJ790311.1   sATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    867
kudoa AY302723.1 GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    848
dia AF414692.1   GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    852
para FJ792719.1  GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    860
tri AM183300.2   GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    859
quad FJ792721.1  GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    860
quad AY078428.1  GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    848
mega AB188529.1  GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    848
thy AY542481.1   GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    848
thy AY542482.1   GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    848
thy AY941819.1   GATTAAAAGGGACATTTGAGGGCGTTAGTACTTGGtGGCG    848
Consensus         attaaaagggacatttgagggcgttagtacttgg ggcg
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Commented [NA10]: *K. paniformis* specific

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pani AF034640.1  AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    887
mini AF034639.1  AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    888
ino FJ790311.1   AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    907
kudoa AY302723.1 AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    888
dia AF414692.1   AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    892
para FJ792719.1  AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    900
tri AM183300.2   AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    899
quad FJ792721.1  AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    900
quad AY078428.1  AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    888
mega AB188529.1  AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    888
thy AY542481.1   AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    888
thy AY542482.1   AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    888
thy AY941819.1   AGGGGTGAAATCCTTAGACCCATCAAAGACTAACTAATGC    888
Consensus        aggggtgaaatccttagacccatcaaagactaactaatgc

pani AF034640.1  GAAACGATTCGCCAAGAATGTTTTCATTAATCAAGAACGA    927
mini AF034639.1  GAAAgcATTCGCCAAGAATGTTTTCATTAATCAAGAACGA    928
ino FJ790311.1   GAAAgcATTCGCCAAGAATGTTTTCATTAATCAAGAACGA    947
kudoa AY302723.1 GAAAgcATTCGCCAAGAATGTTTTCATTAATCAAGAACGA    928
dia AF414692.1   GAAAgcATTCGCCAAGAATGTTTTCATTAATCAAGAACGA    932
para FJ792719.1  GAAAgcATTCGCCAAGAgTGTTTTCATTAATCAAGAACGA    940
tri AM183300.2   GAAAgcATTCGCCAAGAgTGTTTTCATTAATCAAGAACGA    939
quad FJ792721.1  GAAAgcATTCGCCAAGAgTGTTTTCATTAATCAAGAACGA    940
quad AY078428.1  GAAAgcATTCGCCAAGAgTGTTTTCATTAATCAAGAACGA    928
mega AB188529.1  GAAAgcATTCGCCAAGAgTGTTTTCATTAATCAAGAACGA    928
thy AY542481.1   GAAAgcATTCGCCAAGAgTGTTTTCATTAATCAAGAACGA    928
thy AY542482.1   GAAAgcATTCGCCAAGAgTGTTTTCATTAATCAAGAACGA    928
thy AY941819.1   GAAAgcATTCGCCAAGAgTGTTTTCATTAATCAAGAACGA    928
Consensus        gaaa  attcgccaaga tgttttcattaatcaagaacga

pani AF034640.1  AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    967
mini AF034639.1  AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    968
ino FJ790311.1   AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    987
kudoa AY302723.1 AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    968
dia AF414692.1   AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    972
para FJ792719.1  AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    980
tri AM183300.2   AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    979
quad FJ792721.1  AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    980
quad AY078428.1  AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    968
mega AB188529.1  AAGTTGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    968
thy AY542481.1   AAGTaGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    968
thy AY542482.1   AAGTaGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    968
thy AY941819.1   AAGTaGGAGGTTCGAAGACGATCAGATACCGTCCTAGTTC    968
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Commented [NA11]: *K. paniformis* specific

Commented [NA12]: *K. thyrsites* specific

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Consensus        aagt ggaggttcgaagacgatcagataccgtcctagttc

pani AF034640.1  CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1007
mini AF034639.1  CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1008
ino FJ790311.1   CATACAGTAAACTATGCCAACgTGGGATcAGtCCGGTTTA   1027
kudoa AY302723.1 CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1008
dia AF414692.1   CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1012
para FJ792719.1  CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1020
tri AM183300.2   CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1019
quad FJ792721.1  CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1020
quad AY078428.1  CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1008
mega AB188529.1  CATACAGTAAACTATGCCAACATGGGATTAGCCCGGTTaA   1008
thy AY542481.1   CtTACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1008
thy AY542482.1   CtTACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1008
thy AY941819.1   CtTACAGTAAACTATGCCAACATGGGATTAGCCCGGTTTA   1008
Consensus        c tacagtaaactatgccaac tgggat ag ccggtt a

pani AF034640.1  ATCCAGGTTGGGCCCTCGGTGAAAATAAGTGTTTCGGTTC   1047
mini AF034639.1  ATCCAGGTTGGGCCCTCaGTGAAAAccAGTGTTTCGGTTC   1048
ino FJ790311.1   ATCCAGGTTGGGCCCTCGGTGAAAAcAAGTGTTTCGGcTC   1067
kudoa AY302723.1 ATCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1048
dia AF414692.1   ATCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1052
para FJ792719.1  ATCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1060
tri AM183300.2   ATCCAGGTTGGGCCCTCGGTGAAAATcAGTGTTTCGGTTC   1059
quad FJ792721.1  ATCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1060
quad AY078428.1  ATCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1048
mega AB188529.1  AaCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1048
thy AY542481.1   AaCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1048
thy AY542482.1   AaCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1048
thy AY941819.1   AaCCAGGTTGGGCCCTCaGTGAAAATAAGTGTTTCGGTTC   1048
Consensus        a ccaggttgggccctc gtgaaaa  agtgtttcgg tc

pani AF034640.1  CGGGGAGAGTGCGCACGCAAGTGGTAAATTTAAAGAAATT   1087
mini AF034639.1  CGGGGAGAGTGCGCACGCAAGTGGTAAATTTAAAGAAATT   1088
ino FJ790311.1   CGGGGAGAGTGCGCACGCAAGTGGTAAATTTAAAGAAATT   1107
kudoa AY302723.1 tGGGGAGAGTGCGCACGCAAGTGGTAAATTTAAAGAAATT   1088
dia AF414692.1   CGGGGAGAGTGCGCgCGCAAGcGGTAAATTTAAAGAAATT   1092
para FJ792719.1  tGGGGAGAGTGCGCACGCAAGTGcTAAATTTAAAGAAATT   1100
tri AM183300.2   CGGGGAGAGTGCGCACGCAAGTGcTAAATTTAAAGAAATT   1099
quad FJ792721.1  tGGGGAGAGTGCGCACGCAAGTGcTAAATTTAAAGAAATT   1100
quad AY078428.1  tGGGGAGAGTGCGCACGCAAGTGcTAAATTTAAAGAAATT   1088
mega AB188529.1  tGGGGAGAGTGCGtACGCAAGTGccAAATTTAAAGAAATT   1088
thy AY542481.1   tGGGGAGAGTGCGtACGCAAGTGccAAATTTAAAGAAATT   1088
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Commented [NA13]: *K. thyrsites* specific

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thy AY542482.1   tGGGGAGAGTGCGtACGCAAGTGccAAATTTAAAGAAATT   1088
thy AY941819.1   tGGGGAGAGTGCGtACGCAAGTGccAAATTTAAAGAAATT   1088
Consensus         ggggagagtgcg  cgcaag g  aaatttaaagaaatt

pani AF034640.1  GACGGAATGGCACCACCAGGAGTGGAGCCTGCtGCTTAAT   1126
mini AF034639.1  GACGGAATGGCACCACCAGGAGTGGAGCCTGC.GCTTAAT   1127
ino FJ790311.1   GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1147
kudoa AY302723.1 GACGGAAaGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1128
dia AF414692.1   GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1132
para FJ792719.1  GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1140
tri AM183300.2   GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1139
quad FJ792721.1  GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1140
quad AY078428.1  GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1128
mega AB188529.1  GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1128
thy AY542481.1   GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1128
thy AY542482.1   GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1128
thy AY941819.1   GACGGAATGGCACCACCAGGAGTGGAGCCTGCgGCTTAAT   1128
Consensus        gacggaa ggcaccaccaggagtggagcctgc gcttaat

pani AF034640.1  TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGA   1166
mini AF034639.1  TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGA   1167
ino FJ790311.1   TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGA   1187
kudoa AY302723.1 TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGA   1168
dia AF414692.1   TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGA   1172
para FJ792719.1  TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGg   1180
tri AM183300.2   TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGg   1179
quad FJ792721.1  TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGg   1180
quad AY078428.1  TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGg   1168
mega AB188529.1  TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGg   1168
thy AY542481.1   TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGg   1168
thy AY542482.1   TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGg   1168
thy AY941819.1   TTGATTCAACACGGGGAAACTCACCAGGTCCAGACATTGg   1168
Consensus        ttgattcaacacggggaaactcaccaggtccagacattg

pani AF034640.1  TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1206
mini AF034639.1  TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1207
ino FJ790311.1   TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1227
kudoa AY302723.1 TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1208
dia AF414692.1   TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1212
para FJ792719.1  TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1220
tri AM183300.2   TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1219
quad FJ792721.1  TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1220
quad AY078428.1  TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1208
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Commented [NA14]: *K. thyrsites* reverse primer also picks up *K. megacapsula*

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mega AB188529.1  TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1208
thy AY542481.1   TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1208
thy AY542482.1   TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1208
thy AY941819.1   TAGGATTGACAGACTGAGAGATCTTTCATGATTTGATGAT   1208
Consensus        taggattgacagactgagagatctttcatgatttgatgat


pani AF034640.1  TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1246
mini AF034639.1  TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1247
ino FJ790311.1   TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1267
kudoa AY302723.1 TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1248
dia AF414692.1   TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1252
para FJ792719.1  TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1260
tri AM183300.2   TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1259
quad FJ792721.1  TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1260
quad AY078428.1  TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1248
mega AB188529.1  TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1248
thy AY542481.1   TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1248
thy AY542482.1   TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1248
thy AY941819.1   TGGTGGTGCATGGCCGTTCTTAGTTGGTGGAGTGATCTGT   1248
Consensus        tggtggtgcatggccgttcttagttggtggagtgatctgt

pani AF034640.1  CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1286
mini AF034639.1  CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1287
ino FJ790311.1   CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1307
kudoa AY302723.1 CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1288
dia AF414692.1   CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1292
para FJ792719.1  CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1300
tri AM183300.2   CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1299
quad FJ792721.1  CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1300
quad AY078428.1  CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1288
mega AB188529.1  CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1288
thy AY542481.1   CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1288
thy AY542482.1   CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1288
thy AY941819.1   CAGGTTTATTCCGGTAACGAGCGAGACCACGATCTTTAAT   1288
Consensus        caggtttattccggtaacgagcgagaccacgatctttaat

pani AF034640.1  TGATTACGGTTGAATTGTCTTGACCGATCTTAAAGAGACC   1326
mini AF034639.1  TGATTACGGTTaAAaTGTCTTGACCGATCTTAAAGAGACC   1327
ino FJ790311.1   TGATTACGGTTaAATTGTCTTGACCGATCTTAAAGAGACC   1347
kudoa AY302723.1 TGATTACGGTTaAAaTGTCTTGACCGATCTTAAAGAGACC   1328
dia AF414692.1   TGATTACGGTTaAATTGTCTTGACCGATCTTAAAGAGACC   1332
para FJ792719.1  TGATTACGGTTGAATTGTCTTGACCGATCTTAAAGAGACC   1340
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tri AM183300.2    TGATTACGGTTaAAaTGTCTTGACCGATCTTAAAGAGACC   1339
quad FJ792721.1   TGATTACGGTTGAATTGTCTTGACCGATCTTAAAGAGACC   1340
quad AY078428.1   TGATTACGGTTGAATTGTCTTGACCGATCTTAAAGAGACC   1328
mega AB188529.1   TGATTACGGTTaAATTGTCTTGACCGATCTTAAAGAGACC   1328
thy AY542481.1    TGATTACGGTTaAATTGTCTTGACCGATCTTAAAGAGACC   1328
thy AY542482.1    TGATTACGGTTaAATTGTCTTGACCGATCTTAAAGAGACC   1328
thy AY941819.1    TGATTACGGTTaAATTGTCTTGACCGATCTTAAAGAGACC   1328
Consensus         tgattacggtt aa tgtcttgaccgatcttaaagagacc

pani AF034640.1   ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1366
mini AF034639.1   ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1367
ino FJ790311.1    ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1387
kudoa AY302723.1  ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1368
dia AF414692.1    ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1372
para FJ792719.1   ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1380
tri AM183300.2    ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1379
quad FJ792721.1   ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1380
quad AY078428.1   ACCGGAGTTAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1368
mega AB188529.1   ACCGGAGTgAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1368
thy AY542481.1    ACCGGAGTgAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1368
thy AY542482.1    ACCGGAGTgAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1368
thy AY941819.1    ACCGGAGTgAAGCCGGGGGAAGCGTGGCAATAACAGGTCT   1368
Consensus         accggagt aagccgggggaagcgtggcaataacaggtct

pani AF034640.1   GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1406
mini AF034639.1   GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1407
ino FJ790311.1    GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1427
kudoa AY302723.1  GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1408
dia AF414692.1    GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1412
para FJ792719.1   GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1420
tri AM183300.2    GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1419
quad FJ792721.1   GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1420
quad AY078428.1   GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1408
mega AB188529.1   GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1408
thy AY542481.1    GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1408
thy AY542482.1    GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1408
thy AY941819.1    GTGATGCCCTTCGATGTTCTGGGCTGCACGTGTGCTACAA   1408
Consensus         gtgatgcccttcgatgttctgggctgcacgtgtgctacaa

pani AF034640.1   TGATAGTGACAACAAGTTCCTGCTCCGAGAGGGATGGGGA   1446
mini AF034639.1   TGATAGTGACAACAAGTaCCTGCTCtGAGAGGGgTGGGaA   1447
ino FJ790311.1    TGATAGTGACAACAAGTTCCTGCTCCGAGAGGGgTGGGGA   1467
kudoa AY302723.1  TGATAGTGACAACAAGTaCCTGCTCtGAGAGGGgTGGGaA   1448
```

Commented [NA15]: *K. paniformis* specific

Commented [NA16]: *K. paniformis* reverse primer is specific to *K. paniformis*

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dia AF414692.1    TGATAGTGACAACAAGTgCCTGCcCtGAGAGGGgTGGGGA   1452
para FJ792719.1   TGATAGTGACAACAAGTTCCTGCcCtGAGAGGGgTGGGGA   1460
tri AM183300.2    TGATAGTGACAACAAGTTCCTGCcCtGAGAGGGgTGGGGA   1459
quad FJ792721.1   TGATAGTGACAACAAGTTCCTGCcCtGAGAGGGgTGGGGA   1460
quad AY078428.1   TGATAGTGACAACAAGTTCCTGCcCtGAGAGGGgTGGGGA   1448
mega AB188529.1   TGATAGTGACAACAAGTTCCTGCTCtGAGAGGagTGGGrA   1448
thy AY542481.1    TGATAGTGACAACAAGTTCCTGCTCtGAGAGGagTGGGGA   1448
thy AY542482.1    TGATAGTGACAACAAGTTCCTGCTCtGAGAGGagTGGGGA   1448
thy AY941819.1    TGATAGTGACAACAAGTTCCTGCTCtGAGAGGagTGGGGA   1448
Consensus         tgatagtgacaacaagt cctgc c gagagg  tggg a

pani AF034640.1   ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1486
mini AF034639.1   ATCTTaAAAATCGCTaTCTTGCTTTGGACTGAGCCTTGTA   1487
ino FJ790311.1    ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1507
kudoa AY302723.1  ATCTTaAAAATCGCTaTCTTGCTTTGGACTGAGCCTTGTA   1488
dia AF414692.1    ATCTTaAAAATCGCTaTCTTGCTTTGGACTGAGCCTTGTA   1492
para FJ792719.1   ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1500
tri AM183300.2    ATCTTGAAAATCGCTaTCTTGCTTTGGACTGAGCCTTGTA   1499
quad FJ792721.1   ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1500
quad AY078428.1   ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1488
mega AB188529.1   ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1488
thy AY542481.1    ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1488
thy AY542482.1    ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1488
thy AY941819.1    ATCTTGAAAATCGCTGTCTTGCTTTGGACTGAGCCTTGTA   1488
Consensus         atctt aaaatcgct tcttgctttggactgagccttgta

pani AF034640.1   ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1526
mini AF034639.1   ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1527
ino FJ790311.1    ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1547
kudoa AY302723.1  ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1528
dia AF414692.1    ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1532
para FJ792719.1   ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1540
tri AM183300.2    ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1539
quad FJ792721.1   ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1540
quad AY078428.1   ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1528
mega AB188529.1   ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1528
thy AY542481.1    ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1528
thy AY542482.1    ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1528
thy AY941819.1    ATAATTGCTCACGAAAGAGGAATTCCTCGTAAGCGCGAGT   1528
Consensus         ataattgctcacgaaagaggaattcctcgtaagcgcgagt

pani AF034640.1   CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTA....   1562
mini AF034639.1   CATCAGCTCGTGTTGAATAaGTCTCTGCCCTTTGTA....   1563
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Commented [NA17]: This is *K. thyrsites* and *K. megacapsula* specific

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ino FJ790311.1   CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTAcaca   1587
kudoa AY302723.1 CATCAGCTCGTGTTGAATAaGTCTCTGCCtTTTGTA....   1564
dia AF414692.1   CATCAGCTCGTGTTGAATAaGTCTCTGCCCTTTGTA....   1568
para FJ792719.1  CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTAcaca   1580
tri AM183300.2   CATCAGCTCGTGTTGAATAaGTCTCTGCCCTTTGTAcaca   1579
quad FJ792721.1  CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTAcaca   1580
quad AY078428.1  CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTA....   1564
mega AB188529.1  CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTA....   1564
thy AY542481.1   CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTAcaca   1568
thy AY542482.1   CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTAcaca   1568
thy AY941819.1   CATCAGCTCGTGTTGAATACGTCTCTGCCCTTTGTAcaca   1568
Consensus        catcagctcgtgttgaata gtctctgcc tttgta
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Addendum D

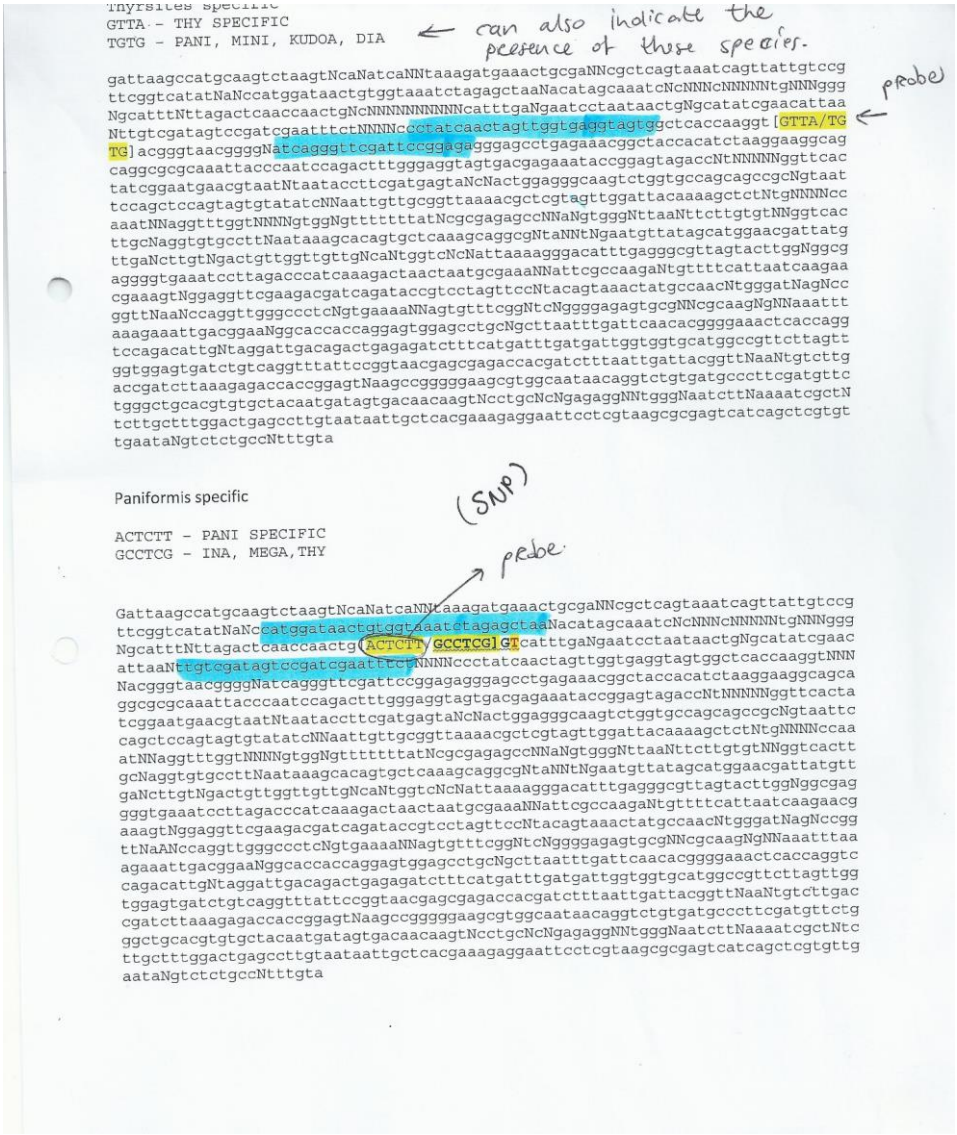

Figure 1. Nucleotide BLAST search showing primer, SNP, and probe design for TaqMan® *Kudoa paniformis* and *K. thyrsites* specific assays.