# Comparative phylogeography of the catshark, *Haploblepharus pictus* and its nematode parasite, *Proleptus obtusus*

by
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**Abstract**

The comparative phylogeography of the host-parasite relationship of the southern African endemic dark shyshark, *Haploblepharus pictus* and its nematode parasite, *Proleptus obtusus* was investigated. To date, no studies have been conducted on the population structure of catsharks and their species specific parasites and little is known about the population dynamics of these species. A total of 116 catsharks and 201 parasites were analysed from seven South African localities. The mitochondrial marker COI was used and species specific primers were designed for both the host and parasite. Haplotype networks were constructed and no strong geographically structured groupings were found for either species. Pairwise $\Phi_{st}$ values for the parasite and host found Gansbaai to be significantly differentiated from the other sites. Fu's $F_s$ were significantly negative for both host and parasite indicating population disequilibrium. *Proleptus obtusus* displayed a pattern of population expansion which was confirmed by the mismatch distribution. Mismatch distributions failed to indicate population expansion for the sharks. Other factors such as selection, migration or genetic drift are likely the cause of the population disequilibrium detected. Interestingly, no barrier to gene flow was found around Cape Point, a known break for other species such as the clinid, *Clinus cottoides* and the caridean shrimp *Palaemon peringueyi*. The outcome of this study suggests that levels of gene flow in *H. pictus* are high enough to suggest that the documented site fidelity is not as strong as originally proposed. The parasite, being dependent on the host, shows a similarly high level of gene flow among sampling sites.

## Opsomming

Die vergelykende filogeografie van die gasheer-parasiet verhouding tussen die endemiese suider-Afrikaanse donker skaamhaai, *Haploblepharus pictus* en sy nematode parasiet, *Proleptus obtusus* is ondersoek. Huidiglik is daar nog geen ander studies uitgevoer met betrekking tot die populasie struktuur van skaamhaaie en hul spesies-spesefieke parasiete nie en min is bekend oor die populasie dinamiek van hierdie spesies. In hierdies studie is ‘n totaal van 116 skaamhaaie en 201 parasiete vanaf sewe lokaliteite geanaliseer. Die mitikondriale merker COI is hiervoor gebruik en spesie spesefieke inleiers is vir beide gasheer en parasiet ontwerp. Haplotipe netwerke is saamgestel vir beide spesies en het geen duidelike geografies gestruktureerde groepe aangedui nie. Paarsgewyse $\phi_{st}$ waardes van beide parasiet en gasheer het daarop gedui dat Gansbaai geneties gedifferensieerd is van alle ander lokaliteite. Fu se $F_s$ was statisties betekenisvol met ‘n negatiewe waarde vir beide spesies, wat dui op populasie disekwilibrium. *Proleptus obtusus* het ‘n patroon van populasie groei getoon, wat deur Fu se Fs en die misparing verspreiding bevestig is. Die misparing verspreiding het nie populasie toename vir die skaamhaaie aangedui nie. Die waargeneemde populasie disekwilibrium is waarskynlik die gevolg van seleksie, migrasie of genetiese drywing. Geen genetiese breuk is by Kaap Punt, wat ‘n genetiese breuk vir verskeie ander spesies soos *Clinus cottoides* en *Palaemon peringueyi* is, gevind nie. Die uitkomstes van hierdie studie stel voor dat vlakke van geen vloei in *H. picuts* hoog genoeg is om ‘n patroon van genetiese vermenging tussen lokaliteite, op die mitokondriale DNS vlak, tot gevolg te hê. Dit beteken moontlik dat die gedokumenteerde gebied gebondenheid van hierdie spesie nie so sterk, soos oorspronklik voorgestel, is nie. Die parasiet, waarskynlik aangesien hy van sy gasheer afhanklik is, toon ‘n soortgelyke hoë vlak van geen vloei tussen lokaliteite. Dus toon beide spesies ‘n algehele afwesigheid van genetiese struktuur, met die isolasie van Gansbaai van alle ander lokaliteite.

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# Table of Contents

## List of Tables

## List of figures

**Figure 8:** Map of the South African coastline showing the direction of gene flow for *Proleptus obtusus* between sampling localities. Sampling localities are represented by circles: 1. Jacobsbaai; 2. Blouberg; 3. Kommetjie; 4. Wooley's Pool; 5. Rooiels; 6. Betty's Bay; 7. Gansbaai. The arrows between the circles represent gene flow. The large dashed lines represent the Benguela and Agulhas currents.

# CHAPTER 1: Introduction

## The South African marine environment

The South African coastline has been extensively studied, with research focussing on the sandy beach environment (McLachlan & Brown, 2006), the dynamics of rocky shores, specifically that of the intertidal zone and its inhabitants (Bustamante & Branch, 2004) as well as species inhabiting estuaries and the factors affecting them (Branch, 1999; Harrison & Whitfield, 2006). These studies, amongst others, significantly contributed towards understanding the biology of coastal species as well as their interactions with other species and the environment.

The South African coastal waters are host to an extraordinarily wide variety of marine species (Awad *et al*., 2002). The two main factors contributing to this high species richness are the long, variable coastline along with two major currents systems (Awad *et al*., 2002). The cold Benguela current flows along the west coast and the warmer Agulhas current borders the east coast (Griffiths *et al*., 2010). This together with additional specific oceanographic features contributed towards the description of five biogeographical regions mainly based on faunal and floral composition as well as water temperatures, these are: the Namaqua Bioregion, the South-western Cape Bioregion, the Agulhas Bioregion, the Natal Bioregion and the Delagoa Bioregion (Lombard *et al*., 2004; Fig. 1).

Species richness decreases from the east to west coast for many taxa, with the highest number of endemic species found on the east coast (Turpie *et al*., 2000; Awad *et al*., 2002). This is a consequence of the warmer waters and the closer connectivity between this region and the species rich tropical Indo-Pacific and western Indian Ocean (Turpie *et al*., 2000). Cape Point, situated at the western end of the south coast, represents a unique location where a sudden increase in species diversity is again found. It marks the border between the east and west coasts and forms the boundary of two bioregions, the Namaqua and the South-western Cape bioregions (Turpie *et al*., 2000; Awad *et al*., 2002; Lombard *et al*., 2004). Cape Point is not only documented as a ecological break-point; several studies have also found decreased gene flow across this area (Emanuel *et al*., 1992; Evans *et al*., 2004; Teske *et al*., 2007; Turpie *et al*., 2000; Matthee *et al*., 2007; Neethling *et al*., 2008; von der Heyden *et al*., 2008).

Despite the few studies mentioned above, genealogical data on the geographic genetic structuring (phylogeography) of coastal South African species is still limited. Phylogeography involves the study of genetic lineages and the processes structuring their

geographical distribution, and is usually at the intraspecific level (Avise, 1998; Nieberding *et al*., 2004). It attempts to determine the evolutionary and ecological processes responsible for the spatial and temporal genetic patterns that exist, thereby providing a solid approach to understanding the structuring of populations along the coast as well as their genetic diversity (Beheregaray, 2008). The outcome of these is critical for the further identification of cryptic species and or genetic lineages that represent important components of marine biodiversity.

Phylogeography is a fast growing field in South Africa. Several marine phylogeographical studies have been conducted along the South African coastline involving a variety of different organisms. Numerous species have shown structure in accordance with the two known breaks, around Cape Point and Cape Agulhas and/or the five major bioregions: the perlemoen, *Haliotis midae* (van der Merwe, 2009); the clinid, *Clinus cottoides* (von der Heyden *et al*., 2008); the shrimp, *Palaemon peringueyi* (Teske *et al*., 2007); and the mussel, *Perna perna* (Zardi *et al*., 2007); the isopod, *Exosphaeroma hylecoetes* (Teske *et al*., 2006); the cumacean, *Iphinoe truncata (*Teske *et al.,* 2006); the mudprawn, *Upogebia africana* (Teske *et al*., 2006) and the limpet, *Patella granularis* (Ridgeway *et al*., 1998). Interestingly, all of the above species display large differences in terms of life histories, from the isopods, which are brooders releasing their planktonic larvae into the oceanic currents, to the klipvis which are live bearers. Life history is therefore not as good a predictor of population structuring as originally thought. Teske *et al.,* (2007) showed that the nature, duration and mobility of larvae have a limited effect on population connectivity.

Recent trends in the field indicate that comparative phylogeographic studies can provide more in depth analyses and understanding of species structuring and interactions as congruence between co-distributed species can be used to indicate the common processes which have created genetic divergence (Teske *et al*., in press). The comparative method is one of the newer developments in the field of phylogeography (Beheregaray, 2008). It involves the comparison of the phylogeographic patterns of multiple sympatric species (Arbogast & Kenagy, 2001) and provides useful information on the congruency of sympatric species lineages, landscape evolution, adaptive radiation, the dispersal of taxa, speciations and extinctions as well as revealing cryptic vicariance (Bermingham & Moritz, 1998; Arbogast & Kenagy, 2001; Nieberding *et al*., 2004). These studies go hand in hand with ecosystem studies that should ideally be incorporated into genetic outcomes. A number of comparative phylogeographic studies have been conducted on closely related species and describe the outcome briefly (Bermingham &

Martin, 1998; Bernatchez & Wilson, 1998; Ditchfield, 2000; Dawson *et al.*, 2002; Michaux *et al.*, 2005)

Comparative analyses of host and parasite phylogeography can provide another fine scale dimension to the interpretation of phylogeographic patterns (Nieberding *et al.*, 2004; Kaliszewska *et al.*, 2005; Glennon *et al.*, 2008). Although many of the factors affecting the presence and distribution on host species by parasites are well understood, the effect of a single parasite species on multiple hosts is not well understood (Banks & Paterson, 2005). The host-parasite relationship under investigation in this study focuses on a species of catshark, *Haploblepharus pictus,* which is endemic to the southern African region, and on its nematode parasite, *Proleptus obtusus*. Moravec *et al.*, (2002) first recorded the presence of *P. obtusus* in *H. edwardsii*. To date, no studies have been conducted on the population structure of catsharks or their species specific parasites and little is known about the population dynamics of these species.

The host parasite system involving the South African endemic catshark*, H. pictus*, and its multi-host nematode, *P. obtusus*, provide a unique opportunity to further our current knowledge on comparative phylogeography in the marine context. This host-parasite system is of interest for several reasons. The distribution of the two species overlap at Cape Point, which acts as a significant barrier to gene flow in other marine species (Emanuel *et al.*, 1992; Turpie *et al.*, 2000; Evans *et al.*, 2004; Teske *et al.*, 2007; von der Heyden *et al.*, 2008). It will thus be of interest to investigate the effect of this known barrier on each species. In addition, *Proleptus obtusus* displays several traits that could contribute to decreased congruence between host and parasite geographic evolution. Firstly, *P. obtusus* is not dependent on a single host as it develops first in an intermediate host before being passed to the definitive host (Williams & Richards, 1978). Secondly, host specificity is also decreased as *P. obtusus* has also been found in three species of South African catshark as well as one species of Mediterranean catshark (Sanmartin *et al.*, 1989). It is unclear as to whether it is the same species of parasite found in all four sharks as only morphological identifications have been carried out so far (Moravec *et al.*, 2002; Moravec, 2007). Thirdly, a decrease in host specificity and local adaptation leads to decreased chances of vertical transmission (Nieberding & Olivieri, 2006). One of the factors that could increase the degree of congruence between *P. obtusus* and *H. pictus* is its ability to become well established on its host (Moravec, 2007).

Given the above and the possibility that *H. pictus* shows strong site fidelity (Escobar-Porras, 2009), it is possible that the host may show a strong phylogeographic structure among sampling sites while the multi-host system of *P. obtusus* suggests that

the parasite may show genetic panmixia. However, should the parasite and the host show a disjunct distribution around Cape Point, then it is reasonable to suggest that the multiple hosts will also show a similar disjunct distribution. It can thus be argued that parasites are very powerful indicators with which to study comparative phylogeography.

## Host-parasite systems

Parasitism is an intimate association between two organisms, one being the parasite and the other the host (Rohde, 1993). Parasites use their hosts as a source of nourishment and as a habitat, thereby living at their hosts expense (Grabda, 1991). The relationship between parasite and host evolved from free-living organisms with phylogenetically "older" parasites tending to be more host specific (Grabda, 1991). Therefore, the close association between parasites and their hosts can be used to determine the history of the parasite, as well as the host and give insight into their co-evolution (Nieberding & Olivieri, 2006).

Parasites can be separated into several different categories with regard to their level of dependence on their host, host specificity, position on the host and host life stage they infect (Rohde, 1993). Hosts can be distinguished based on the role they play in the parasite's life, such as; intermediate host, definitive or final host, paratenic or transport host and vector host (Rohde, 1993). As mentioned above *P. obtusus* is not host specific having both an intermediate and several definitive hosts, one of which is *H. pictus*. The host specificity of *P. obtusus* can provide information about its distribution among its hosts population and can provide information about the possibility of a common history shared with *H. pictus* (Clayton *et al.*, 2004; Nieberding *et al.*, 2008).

Over the last few decades parasitologists have tried to determine what influences species richness for parasite communities (Timi & Poulin, 2003). Host characteristics and environmental factors both play a major role in determining parasite colonisation and extinction rates (Timi & Poulin, 2003). A major factor structuring parasite populations is the movements of their hosts (Wielgoss *et al.*, 2008). Parasites can display unique phylogeographical patterns relative to their hosts. This is dependent on their, host specificity, dispersal abilities as well as their host-dependent dispersal abilities (Nieberding *et al.*, 2005).

A parasites' ability to adapt to its host and speciate is largely affected by gene flow occurring among populations and by the genetic drift within populations (Criscione *et al.*, 2005). Population structure, effective population sizes (Ne) and migration rate are

important when trying to link host and parasite phylogenies (Criscione *et al*., 2005; Nieberding & Olivieri, 2006). Effective population size will greatly influence the local adaptive potential of the population as well as the amount of genetic diversity in the population (Criscione *et al*., 2005). The genetic diversity of a population is important to its success and can be strongly affected by the relationship between mode of reproduction, transmission of the parasite to the host and the mating system (Criscione *et al*., 2005).

The majority of studies that have been conducted on marine fish parasites in southern Africa have given insight into their life cycles (Davies & Smit, 2001; Smit et al., 2003) or taxonomy (Basson *et al*., 1983; Moravec *et al*., 2002; Dippenaar & Jordaan, 2008; Hadfield & Smit, 2008). Particular attention has been placed on certain groups of parasites such as the copepods (Basson & Van As, 1991; Grobler *et al*., 2002; Dippenaar *et al*., 2008), haemogregarines (Davies & Smit, 2001; Smit *et al*., 2002; Davies *et al*, 2003; Hayes *et al*, 2006;) and gnathiid isopods (*et al*., 1999; Smit & Van As, 2000; Davies & Smit, 2001; Smit & Basson, 2002). Few studies have dealt with both the macro and micro-evolutionary impacts of the intimate interaction in a single host-parasite system (for example see Clayton & Johnson, 2003).

*Haploblepharus pictus*

*Haploblepharus pictus* is a small brown shark reaching ~57cm at full length (Compagno, 1984). Several distinct dark saddles extend over the entire body, giving rise to the common name, the dark shyshark or catshark. It is abundant in shallow inshore areas. Until recently, its distribution was believed to span the entire west coast up till Cape Agulhas on the south coast (Branch *et al*., 2010). The distribution has now been extended as far east as Port Elizabeth (Branch *et al*., 2010). It feeds on bottom-dwelling invertebrates and reaches sexual maturatity at ~40cm (Smith & Heemstra, 2003). *Haploblepharus pictus* is oviparous with females on each occasion producing two egg cases at a time which they attach to the seaweed (Branch *et al*., 2010).

Few studies have been conducted on the movement patterns of catsharks. A local study conducted by Escobar-Porras *et al*., (2009), on the movement of *Haploblepharus edwardsii*, *Haploblepharus fuscus* as well as two species from the genus *Poroderma*, found *H. edwardsii* to be a fairly resident species. *Haploblepharus pictus* is similar in size, movement abilities and general behaviour to *H. fuscus* so it is likely that the dark catshark shows some site fidelity.

Chondrichthyian exploitation

The potential site fidelity of catsharks may contribute to genetic population differentiation at local scales and, should this pattern emerge for *H. pictus,* it may have important conservation implications. Sharks are remarkably vulnerable to over-exploitation, specifically in areas where their exploitation is localized (Stevens *et al.*, 2000). The extinction of such localized populations could lead to the loss of unique genetic lineages which may significantly alter the evolutionary potential of the species. The ability to recover from low numbers is hampered by their life-history strategy of slow growth. Sharks only reach maturity later in life (Stevens *et al.*, 2000) and generally have a low number of offspring per generation. Sharks have long life spans but low fecundity, therefore requiring many years to decades before they are able to recover from a drop in population numbers (Stevens *et al.*, 2000).

Despite the direct pressures on local populations, it is very difficult to obtain statistics on the by-catch of South African catsharks. Records have shown, however, that the total percentage of by-catch of *H. edwardsii* from demersal long line activities was 2.2% for 2005 – 2007 (Petersen *et al.*, 2008). This figure may not seem high, however, it only represents a small fraction of the total by-catch since data for other species or from other commercial fisheries are lacking. Although recreational fishing does contribute to total chondrichthyian mortality, the overall percentage is minimal in comparison to commercial fishing (Stevens *et al.*, 2000).

Population structure

Shark mitochondrial DNA mutates slowly which can lead to low genetic variation creating difficulty in studying shark population genetics (Martin *et al.*, 1992). Shark dispersal patterns are also not well understood as barriers in the marine environment are poorly defined (Lewallen *et al.*, 2007). The majority of studies conducted on catsharks, or the family they belong to, have focused on their reproductive biology (Ebert *et al.*, 2006), feeding ecology (Ebert *et al.*, 1996; Lechanteur & Griffiths, 2003), distribution (Compagno *et al.*, 1991), phylogeny and systematics (Human *et al.,* 2006) and mortality factors (Martin, 2004).

Significant genetic structure has, however, been found for certain species of shark using several mitochondrial makers, such as the control region for the great white shark (*Carcharodon carcharias*), the D-loop, COI, ATPase and NADH genes for the gummy shark (*Mustelus antarcticus*), the control region and D-loop region for the blacktip shark (*Carcharhinus limbatus*), and the control region for the leopard shark (*Triakis*

*semifasciata*) (Gardner & Ward, 1998; Pardini *et al*., 2001; Duncan *et al*., 2006; Lewallen *et al*., 2007). This is most likely due to their reproductive behaviour which lacks a pelagic larval stage, as well as the philopatric nature of many shark species (Duncan *et al*., 2006). It is unknown whether catsharks will show significant population structure. However, based on their life history and the findings of the above-mentioned studies, catsharks could likely show population structure.

*Proleptus obtusus*

*Proleptus obtusus* was first discovered by Dujardin (1845) as a specimen collected inside the catshark *Scyllium catulus* which was found in the Mediterranean. It belongs to the Phylum Nematoda, order Spirurida, family Physalopteridae and is known to inhabit several different elasmobranch species (Moravec, 2007). Both a definitive and intermediate host are required in order for *P. obtusus* to complete its life cycle (Moravec *et al*., 2002). Three definitive hosts are known to be infected by *P. obtusus*: *H. pictus*, *H. edwardsii* and *P. africanum* (Yeld, 2008). There are very few papers to date that discuss the genus *Proleptus* and those that do describe the morphology (Williams & Richards, 1978; Moravec *et al*., 2002) or account for their presence (Duran *et al*., 1989).

The identification of different species of *Proleptus* has created much debate as the majority of taxonomic identifications are based on features such as the length and angle of the spicules, positioning of the vulva and the nature of the genital papillae (Moravec, 2007). Morphologically, species of the genus *Proleptus* are highly similar and minor differences are used to distinguish between species. *Proleptus obtusus,* for example, differs from other species based on the positioning and angle of the right spicule (Moravec *et al*., 2002).

The order Spirurida consists of nematodes that are oviparous, producing fully formed first stage larvae (Anderson, 1988). Fish spirurines have life cycles that often require aquatic arthropods as obligate intermediate hosts (Moravec, 2007). An intermediate host serves as a site in or on which the parasite will experience changes in its development. However, sexual maturity is not reached (Criscione *et al*., 2005). It has been proposed that *Carcinus maenas* (common shore-crab) and *Eupagurus bernhardus* (common hermit-crab) play roles as intermediate hosts for *P. obtusus* in the catshark species *Scyliorhinus caniculus* (Lloyd, 1920). For the Chilean catshark, *Schroederichthys chilensis*, the intermediate host was also found to be a crab, *Cancer plebejus* (George-

Nascimento *et al.*, 1993). The intermediate host for *H. pictus* has not yet been established, although it is most likely a crustacean or even multiple crustacean species.

Population structure

Nematodes display a great diversity of life cycles (Blouin *et al.,* 1999). They have been extensively studied but fewer studies have dealt with the genetic aspect of the species. The genetic structure of a parasite can give insight into the evolutionary processes affecting parasite populations, such as host-race formation, adjustment to host defences and speciation (Blouin *et al.,* 1999). The majority of nematode species for which population genetic studies have been conducted are either human or agricultural parasites (Blouin *et al*., 1999; Wu *et al*., 2009). Parasites of domestic organisms show rapid recent demographic expansions (Blouin *et al*, 1995; Donnelly *et al*., 2001; Mes, 2003). Studies of terrestrial nematodes have found host movement to be a key determinant of genetic population structure of a parasite (Blouin *et al*., 1995). A study conducted by Blouin et al. (1995) found that parasites inhabiting sheep and cattle had a pattern of high gene flow between populations whilst deer parasites had significant population structuring and isolation by distance. However, these parasites have only a single definitive host unlike *Proleptus obtusus*, which has three known host species in South Africa (Blouin *et al.,* 1995; Reed & Hafner, 1997; Johnson *et al*., 2002; Bruyndonckx *et al*., 2010). A study on the phylogeography of *P. obtusus* would therefore provide interesting insight into the structure of a generalist parasite.

## Aims and Predictions

There are three aims important to this study. Firstly, the phylogeography of *H. pictus* will be investigated. It is predicted that significant population structure will be found due to catshark life history characteristics as well as the presence of a barrier to gene flow along the southern African coastline. Secondly, the phylogeography of the parasite, *Proleptus obtusus* will be investigated. It is predicted that little structure will be found due to the presence of several definitive and intermediate hosts. Lastly, the co-phylogeography of the host and parasite will be investigated. It is expected that a lower degree of congruence between host and parasite phylogenies will be established due to the presence of more than one definitive host and of an intermediate host as well as due to the difference in reproductive strategy, with the shark being a slow breeder with low fecundity where as the parasite produces large numbers of offspring.

# CHAPTER 2: Material and Methods

## Sampling protocol

Samples were collected from seven localities along the South African coast; from Jacobsbaai on the West coast to Gansbaai on the South coast (Fig. 1). Between three to twenty three catsharks were caught at each location in shallow rocky bays no deeper than three meters. Netting was filled with bait consisting of mackerel heads and pilchards and tied with cable ties. A large rock was placed in the bait bag so as to keep it anchored to the sediment. Once the sharks were attracted to the bait they were caught by hand by a person snorkelling overhead.

Catsharks were then taken to the shore where they were sexed, measured, identified (Compagno *et al.*, 1989; Smith & Smith, 1966; Smith & Heemstra, 2003; Branch *et al.*, 2010) and a fin clip was taken for further DNA analyses. Most individuals were released after non-destructive sampling. The location of the fin clipping was noted to avoid duplicate samples taken at each site and the tissue was placed in a 5 ml tube containing 100% ethanol. The tube contained a label with the site abbreviation and sample number. To obtain parasite samples, two to seven catsharks from each site were kept and taken back to the laboratory for dissection. The catsharks taken for dissection were killed on site by the addition of ethyleneglycolmonophenylether (Merck) and placed in plastic bags.

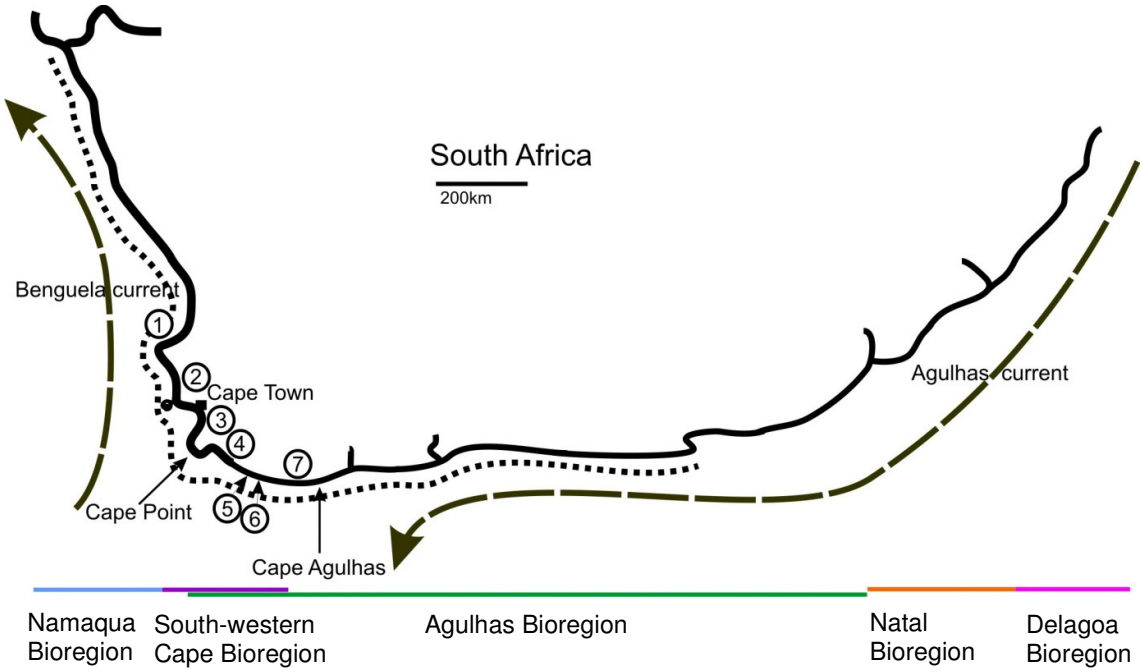



**Figure 1:** Map showing the seven sampling localities and the distribution of *Haploblepharus pictus* (represented by the dotted line). The coloured lines represent the different biogeographic regions (see Lombard 2004). The dashed lines with arrows represent the two major current systems. Sampling localities: 1. Jacobsbaai; 2. Blouberg; 3. Kommetjie; 4. Wooley's Pool; 5. Rooiels; 6. Betty's Bay; 7. Gansbaai.

## Laboratory procedures

### Parasite removal

Catsharks were dissected using a standard LASEC SA dissection kit and the stomachs removed. The stomach contents were emptied and sorted. The parasite, *P. obtusus* was removed and placed in 5 ml tubes containing 100% ethanol. Each tube contained a label with the site abbreviation and sample number. Dissection and parasite removal occurred within 48 hours after the host was killed.

### Molecular techniques

Total genomic DNA was extracted from *P. obtusus* and *H. pictus.* The DNeasy® Blood and Tissue Qiagen kit was used for the DNA extraction procedure following standard protocols. Where possible, 15 parasites were extracted and sequenced from each shark and 30 parasites from each site. If 15 parasites could not be obtained from one individual then more sharks were caught and dissected from the same site.

Universal COI primers (COI CI-J-1718 and COIH7005) were used for both species and PCR setups were run as specified by Reed *et al*., 2004. The PCR setup consisted of; 14.4µl PCR water, 2.5µl buffer, 2.5µl dNTPs, 2µl MgCl, 1.25µl of each primer, 0.2µl taq and 2µl DNA. The sequences generated for each species were aligned in BioEdit and to optimize amplification success species specific primers were designed based on these; for the sharks (COISF – 5' TGG TGC ATG AGC AGG AAT AGT 3' and COISR – 5' GCC TGC TGG ATC AAA GAA TGT ACC ACG TAC TCG 3') and for the parasites (Pob1F – 5' TGC TYT ATC TTW TTC RRT TAC 3' and Pob620R – 5' GGA ATA GCA ATA ATA ACA GTA 3'). The following nuclear primer pair was used: ATPSβ (ATPSbf1 – 5' CGT GAG GGH AAY GAT TTH TAC CAT GAG ATG AT 3' and 5' CGG GCA CGG GCR CCD GGN GGT TCG TTC AT 3') (Worheide *et al*., 2008). Eight sequences were obtained however, no variation was found, and therefore further investigation of the nuclear gene was not seen to be necessary.

The PCR protocol used for COISF and COISR was as follows; a preliminary denaturation for three minutes at 94°C, followed by 35 cycles (30 seconds at 94°C; 45 seconds at 49 – 51°C (temperature varied between samples), 45 seconds at 72°C) and a final extension for five minutes at 72°C. The PCR protocol for ATPSβ was the same but with an annealing temperature of 60°C. The same protocol was also used for the parasites however, the annealing temperature varied between 43 – 45°C depending on the sample.

The PCR products were separated with the use of gel electrophoresis on a 1% agarose gel, excised and captured by hand and then purified with a commercial purification kit Promega, GE Healthcare. Lastly, cycle-sequencing reactions were performed with the use of BigDye Terminator Chemistry (Applied Biosystems) and the products analyzed on an automated sequencer (AB 3100, Applied Biosystems). Catshark sequences were compared to other Scyliorhinid sequences and parasite sequences were compared to other sequences from the order Spirurida on GenBank to confirm that the correct region had been amplified.

## Data analyses

### Haplotype networks

TCS 1.21 (Clement *et al.,* 2000) was used to construct haplotype networks with a 95% confidence interval. All the haplotypes were connected within the 95% confidence limit. CorelDRAW X3 was used to redraw the coalescent relationships that exist between the different mitochondrial haplotypes.

Population structure

Arlequin 3.1 (Excoffier *et al.,* 2005) was used to perform an AMOVA (Analysis of Molecular Variance) (Excoffier *et al.,* 1992) to test for differentiation between and within predefined groups and to produce standard molecular diversity indices. The groups were defined by sampling localities chosen from previous marine phylogeographical studies in South Africa (von der Heyden et al., 2008 & Neethling et al., 2008). This allowed for phylogeographical comparisons between species Pairwise $\Phi_{ST}$ of differentiation, which accounts for the degree of differentiation between haplotypes, was estimated in Arlequin 3.1. A Mantel test was also run in Arlequin 3.1 with 10000 permutations to determine whether isolation by distance has occurred. SAMOVA (Spatial Analysis of Molecular Variance) was performed to calculate the genetic structure of the sampled populations (Dupanloup *et al*., 2002). SAMOVA can be used to identify possible genetic barriers between groups of populations, without having pre-defined populations. Bayesian Analysis of Population Structure (BAPS v.5.3) was also performed as it is a more sensitive test that determines the distribution of mitochondrial variation along the sampled geographic range without the use of a predefined number of groups (Corander *et al*., 2003).

Demographic history

Fu's Fs was calculated to test the assumption that mutation-drift equilibrium exists within groups (Fu, 1997). Mismatch distributions of pairwise differences were performed between haplotypes to examine past population demography and to determine whether the population show a signature of expansion (Harpending, 1994) in Arlequin 3.1 (Excoffier *et al.,* 2005). The smoothness of the mismatch distribution was measured by a raggedness statistic (Ramos-Onsins and Rozas, 2002).

Migration between sampling sites

Migrate-n (version 3.1.6) was used to estimate effective population sizes and past migration rates as well as to determine the directionality and degree of gene flow between sampling sites (Beerli, 2008). Maximum likelihood inferences were tested and a stepping-stone model with asymmetrical gene flow was used as it accounted for the coastal habitat of the study species. The first two runs were set at two repeats, 10 short-chains with 25000 generations and two long-chains with 1250000 generations. Discrepancies were found among runs so further runs were performed with slight adjustments to the

parameters (number of repeats and long chain length) so as to determine the most accurate gene flow estimates.

# CHAPTER 3: Results

## Sequence variation and diversity estimates

A total of 116 catsharks were obtained from the seven localities. Sequences with a final length of 553 base pairs were obtained. The COI data set consisted of 27 polymorphic sites with 17 transitions and 11 transversions. Two hundred and one parasites were extracted from 23 catsharks. Sequences were aligned and edited and a total of 201 sequences of 554 base pairs were obtained.

A total of 42 haplotypes were recovered for the sharks with 29 haplotypes represented by single individuals (Fig. 2a). The two haplotypes representing the greatest number of individuals were shared by 11% of catsharks. The majority of haplotypes differed by one site change. Haplotype diversity was high, 0.9490 ± 0.0080 and varied only slightly between sites. Nucleotide diversity was very low, 0.008882 ± 0.005093. Further, the nuclear marker ATPSß was amplified for ten individuals that showed the greatest variation at the mitochondrial level, but showed no variation.

Seventy three haplotypes were recovered in total for the parasite with 58 unique haplotypes (Fig. 2b). The majority of differences between haplotypes involved one site change, with the exception of three haplotypes that differed by 10 site changes from the common haplotype. Haplotype diversity was high for the parasites (0.8892 ± 0.0395) but lower than that found in the sharks and varied only slightly between sites. Nucleotide diversity was an order of magnitude higher than that of the sharks (0.0533 ± 0.0267).

**Table 1:** The numbers of sharks, *Haploblepharus pictus,* dissected. The numbers of *Proleptus obtusus*, parasites removed and the location from where they were removed.

| Location | Sample | Number of parasites | Number of parasites sequenced | Sex |
|---|---|---|---|---|
| Betty's Bay | BB6 | >20 | 8 | M |
| Betty's Bay | BB8 | >40 | 13 | F |
| Betty's Bay | BB9 | <20 | 9 | F |
| Rooiels | R 12 | >30 | 11 | M |
| Rooiels | R 13 | >50 | 14 | F |
| Wooley's Pool | FB1 | >20 | 15 | M |
| Wooley's Pool | FB2 | <20 | 13 | M |
| Jacobsbaai | JA5 | 20 | 10 | M |
| Jacobsbaai | JA7 | 2 | 2 | F |
| Jacobsbaai | JA9 | 5 | 5 | M |
| Jacobsbaai | JA11 | 1 | 1 | M |
| Jacobsbaai | JA12 | 2 | 1 | F |
| Jacobsbaai | JA13 | 8 | 6 | F |
| Jacobsbaai | JA15 | 5 | 5 | F |
| Blouberg | BL11 | 2 | 2 | F |
| Blouberg | BL14 | >20 | 17 | F |
| Blouberg | BL15 | 10 | 8 | M |
| Blouberg | BL17 | 2 | 2 | M |
| Kommetjie | KO20 | 15 | 15 | F |
| Kommetjie | KO21 | <20 | 15 | M |
| Gansbaai | GB9 | 15 | 12 | F |
| Gansbaai | GB15 | >20 | 15 | M |
| Gansbaai | GB21 | 5 | 2 | M |

Haplotype networks

The haplotype network constructed for the catsharks displayed no clear phylogeographic structure as there was extensive haplotype sharing between localities (Fig. 2a). Sharks from Gansbaai and Wooley's Pool had the lowest haplotype diversity ($h$ = 0.757, $h$ = 0.667) and neither had any unique haplotypes. The highest number of unique haplotypes was found for sharks at Betty's Bay characterized by the highest haplotype and nucleotide diversity ($h$ = 0.968, $\pi$ = 0.013). Several of the more variable sequences found for the catsharks are presented in Appendix I.

The haplotype network for *Proleptus obtusus* did not yield significant structure either (Fig. 2b). There were, however, a high number of unique haplotypes connected to two main ancestral haplotypes producing star-like clusters, which were both connected by a single step to a third smaller ancestral haplotype. Three individuals were separated from the rest by ten mutational steps and showed sequence variation of >2%. The sequences for these three parasites can be found in Appendix I. These individuals may, however, represent a different species, but it is unclear thus far. The individuals were from three different localities (Rooiels, Betty's Bay and Wooley's Pool). In sharp contrast to the intra-populational pattern found for the sharks, Gansbaai parasites had the fewest unique haplotypes (four) with the lowest haplotype and nucleotide diversity ($h$ = 0.823, $\pi$ = 0.003) and Rooiels was found to have the highest haplotype diversity ($h$ = 0.937).

Variation between and within populations of parasite and host

A distance matrix was run in BioEdit to determine the level of variation between individuals from the same and different localities. Less than 2% variation was found for individual catsharks between and among localities. Less than 1% variation was found for the parasites with the exception of three individuals which had >2% variation.

Analysis of Molecular Variance

Significant overall population differentiation ($\Phi_{st}$ = 0.149, P = 0.001) was revealed for the sharks. Pairwise $\Phi_{st}$ of differentiation revealed several interesting finds. Firstly, Gansbaai sharks were significantly differentiated from all other sampling populations ($\Phi_{st}$; 0.2-0.4), which may suggest the presence of a gene flow barrier (Table 3). Secondly, significant differences were found between Kommetjie sharks and the four other sites; Gansbaai,

Rooiels, Blouberg and Jacobsbaai. Lastly, significant $\Phi_{st}$ values were found between Wooley's Pool and Blouberg as well as Wooley's Pool and Jacobsbaai. This may be indicative of shallow but significant population structure between some sampling localities for the catsharks. This may however, be attributed to the low sample number (n = 3) at Wooley's Pool, creating a skew in analysis. The percentage of variation among populations (14%) was low in comparison to that within populations (85%). The Mantel test did not detect a significant relationship between geographical and genetic distance (0.449, $p > 0.05$) for the catsharks.

The overall $\Phi_{st}$ values for the parasites among the sampling sites was not significant ($\Phi_{st} = 0.00$, $p = 0.333$). As was the case for the sharks, pairwise $\Phi_{st}$ analyses revealed significant differences between Gansbaai and all the other sites ($\Phi_{st}$; 0.01 – 0.06) (Table 4). Percentage variation was negligible for among-population variation but there was significant within-population variation. The Mantel test found a significant relationship between geographical and genetic distance (0.484, $p < 0.05$) for the parasites.

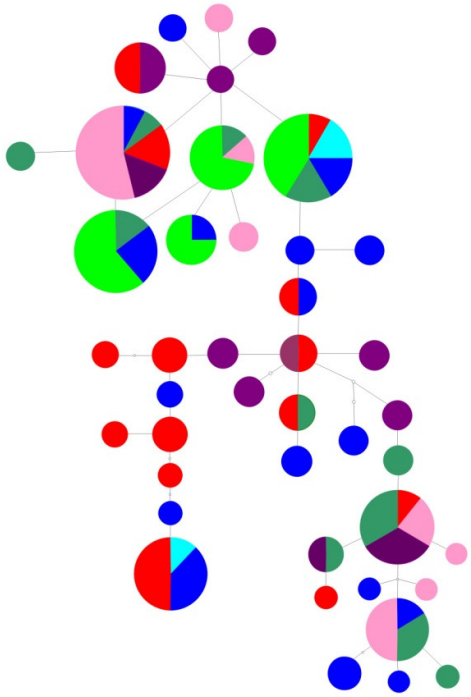
Spatial analysis of molecular variance and Bayesian analysis of population structure

SAMOVA revealed the highest $F_{CT}$ ($F_{CT} = 0.1863$) at two groups for the sharks. One group consisted of Gansbaai and the other included all of the remaining sites. This supports the pairwise $\Phi_{st}$ pattern outlined above.

SAMOVA was run for the parasites and revealed the highest $F_{CT}$ ($F_{CT} = 0.04244$) at two groups. One group consisted of Kommetjie and Blouberg and the other Gansbaai, Betty's Bay, Rooiels, Wooley's Pool and Jacobsbaai. This represents different groups to those found for the sharks and was in sharp contrast to the $\Phi_{st}$ pattern among sampling sites outlined above. $F_{CT}$ was very low, however, showing shallow population structure.

BAPS was run for the sharks and the parasites separately and did not identify any barriers to gene flow or separation of populations. As is shown by Figures 3 and 4, all localities grouped together, as indicated by the uniformity in colour between sights, thus contradicting the two groups found by SAMOVA and pairwise $\Phi_{st}$.

2a)



| Key | |
|---|---|
| Gansbaai | |
| Rooiels | |
| Betty's Bay | |
| Wooley's Pool | |
| Blouberg | |
| Kommetjie | |
| Jacobsbaai | |

2b)

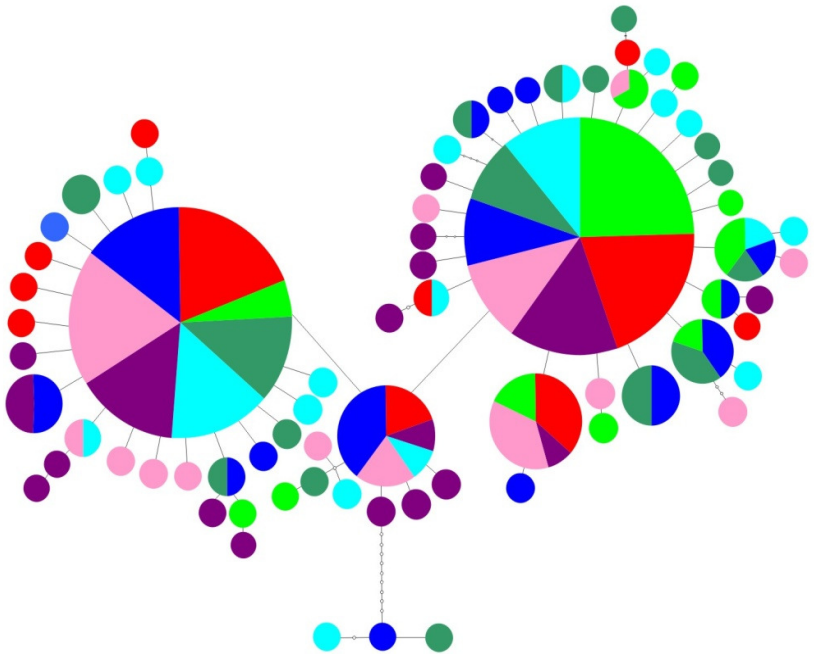


**Figure 2a):** Haplotype network based on 42 haplotypes of *Haploblepharus pictus* based on 116 sequences of COI from seven localities. Each circle represents a unique haplotype and the size is relative to the frequency of the haplotype. The smallest circles represent a missing haplotype.

**b)** Haplotype network based on 73 haplotypes of *Proleptus obtusus* based on 201 sequences of COI from seven localities. Each circle represents a unique haplotype and the size is relative to the frequency of the haplotype. The smallest circles represent a missing haplotype.

**Table 2:** Population specific diversity indices and haplotype frequencies of *Haploblepharus pictus* and *Proleptus obtusus* for the seven sampling sites. Significant Fu's $F_s$ values in bold.

| Sampling site | Gansbaai | Betty's Bay | Rooiels | Wooley's Pool | Kommetjie | Blouberg | Jacobsbaai |
|---|---|---|---|---|---|---|---|
| **Population (n)** | | | | | | | |
| *P. obtusus:* | 29 | 30 | 25 | 28 | 30 | 29 | 30 |
| *H. pictus:* | 21 | 23 | 16 | 3 | 21 | 17 | 15 |
| **# of Haplotypes** | | | | | | | |
| *P. obtusus:* | 13 | 16 | 15 | 19 | 11 | 14 | 18 |
| *H. pictus:* | 4 | 17 | 11 | 2 | 14 | 8 | 11 |
| **# of unique haplotypes** | | | | | | | |
| *P. obtusus:* | 4 | 6 | 7 | 12 | 6 | 8 | 13 |
| *H. pictus:* | 0 | 10 | 3 | 0 | 4 | 4 | 6 |
| **# of polymorphic sites** | | | | | | | |
| *P. obtusus:* | 17 | 27 | 26 | 32 | 13 | 18 | 19 |
| *H. pictus:* | 4 | 22 | 12 | 8 | 14 | 12 | 10 |
| **Nucleotide diversity** | | | | | | | |
| *P. obtusus:* | 0.003 ± 0.002 | 0.006 ± 0.003 | 0.006 ± 0.004 | 0.006 ± 0.004 | 0.050 ± 0.025 | 0.053 ± 0.027 | 0.006 ± 0.003 |
| *H. pictus:* | 0.003 ± 0.002 | 0.013 ± 0.007 | 0.009 ± 0.005 | 0.010 ± 0.008 | 0.009 ± 0.005 | 0.009 ± 0.005 | 0.007 ± 0.004 |
| **Haplotype diversity** | | | | | | | |
| *P. obtusus:* | 0.823 ± 0.067 | 0.931 ± 0.027 | 0.937 ± 0.031 | 0.934 ± 0.034 | 0.837 ± 0.044 | 0.889 ± 0.040 | 0.915 ± 0.036 |
| *H. pictus:* | 0.757 ± 0.048 | 0.968 ± 0.022 | 0.950 ± 0.036 | 0.667± 0.314 | 0.952 ± 0.030 | 0.816 ± 0.082 | 0.952 ± 0.040 |
| **Fu's Fs** | | | | | | | |
| *P. obtusus:* | **-7.471, p = 0** | **-8.024, p = 0** | **-7.266, p = 0.002** | **-12.442, p = 0** | 10.771, p = 0.997 | 6.228, p = 0.972 | **-11.010, p = 0** |
| *H. pictus:* | 1.071, p = 0.737 | **-5.717, p = 0.016** | -2.943, p = 0.066 | 3.101, p = 0.850 | **-4.668, p = 0.024** | 0.191, p = 0.557 | **-4.552, p = 0.013** |

**Table 3:** Pairwise $\Phi_{st}$ values among sampling localities for *Haploblepharus pictus.* The $\Phi_{st}$ values are given below the diagonal and the significance above.

| Location | Gansbaai | Betty's Bay | Rooiels | Wooley's Pool | Kommetjie | Blouberg | Jacobsbaai |
|---|---|---|---|---|---|---|---|
| **Gansbaai** | - | P < 0.05 | P < 0.05 | P < 0.05 | P < 0.05 | P < 0.05 | P < 0.05 |
| **Betty's Bay** | 0.216 | - | ns | ns | ns | ns | ns |
| **Rooiels** | 0.369 | 0.014 | - | ns | P < 0.05 | ns | ns |
| **Wooley's Pool** | 0.371 | 0 | 0.196 | - | ns | P < 0.05 | P < 0.05 |
| **Kommetjie** | 0.391 | 0.033 | 0.157 | 0.029 | - | P < 0.05 | P < 0.05 |
| **Blouberg** | 0.329 | 0.012 | 0 | 0.173 | 0.158 | - | ns |
| **Jacobsbaai** | 0.413 | 0.044 | 0.008 | 0.223 | 0.139 | 0.031 | - |

**Table 4:** Pairwise $\Phi_{st}$ values among sampling localities for *Proleptus obtusus.* The $\Phi_{st}$ values are given below the diagonal and the significance above.

| Location | Gansbaai | Betty's Bay | Rooiels | Wooley's Pool | Kommetjie | Blouberg | Jacobsbaai |
|---|---|---|---|---|---|---|---|
| **Gansbaai** | - | P < 0.05 | P < 0.05 | P < 0.05 | P < 0.05 | P < 0.05 | P < 0.05 |
| **Betty's Bay** | 0.034 | - | ns | ns | ns | ns | ns |
| **Rooiels** | 0.036 | 0 | - | ns | ns | ns | ns |
| **Wooley's Pool** | 0.049 | 0 | 0 | - | ns | ns | ns |
| **Kommetjie** | 0.013 | 0 | 0 | 0 | - | ns | ns |
| **Blouberg** | 0.010 | 0 | 0 | 0 | 0 | - | ns |
| **Jacobsbaai** | 0.062 | 0 | 0.006 | 0 | 0.001 | 0.001 | - |

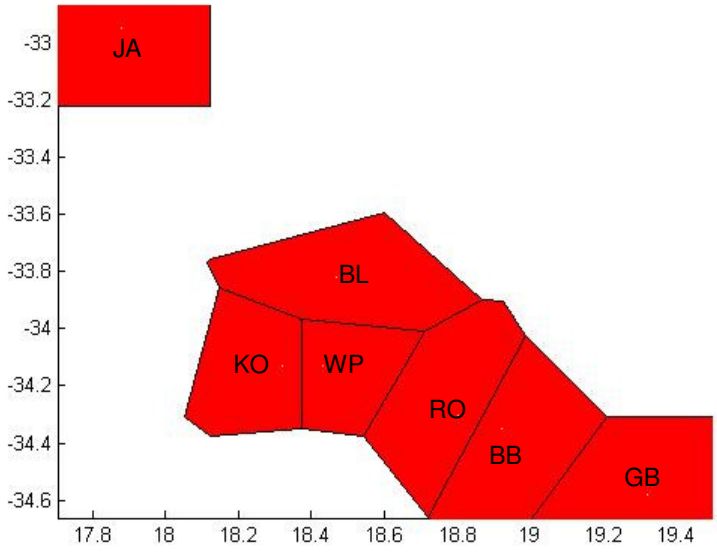



**Figure 3:** The estimated spatial genetic structure, as was established by BAPS, for the seven sampling localities for *Haploblepharus pictus*. (JA: Jacobsbaai, BL: Blouberg, KO: Kommetjie, WP: Wooley's Pool, RO: Rooiels, BB: Betty's Bay and GB: Gansbaai).

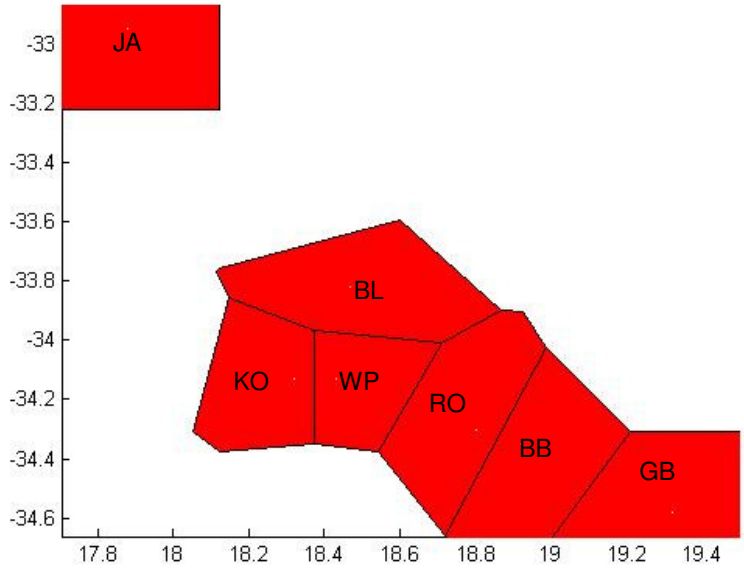



**Figure 4:** The estimated spatial genetic structure, as was established by BAPS, for the seven sampling localities for *Proleptus obtusus*. (JA: Jacobsbaai, BL: Blouberg, KO: Kommetjie, WP: Wooley's Pool, RO: Rooiels, BB: Betty's Bay and GB: Gansbaai).

Mismatch distributions and test for neutrality

Fu's $F_s$ overall were highly negative and significant (-22.2, p = 0), which suggests that the catshark population is not in genetic equilibrium (Table 2). Fu's $F_s$ for Betty's Bay, Kommetjie and Jacobsbaai were significantly negative for the sharks at each of these localities. Gansbaai, Rooiels, Wooley's Pool and Blouberg were, however, not significantly negative, indicating stable populations. The observed data for the overall mismatch distribution differs significantly from the expected distribution which, along with the low raggedness statistic (0.011) and non-significant SSD (p > 0.05) statistic, indicates population expansion. The mismatch distribution (Fig. 5), however, suggests the catsharks have a stable population (p > 0.05). This indicates that the disequilibrium of the population is not due to population growth.

Fu's $F_s$ overall (-24.1, p = 0.001) as well as individually were significantly negative indicating that the *Proleptus obtusus* population is not in equilibrium. Fu's $F_s$ were found to be significantly negative, for all populations except Blouberg and Kommetjie, which appear to have had stable populations. The mismatch distribution showed a strong signal of population expansion (Fig. 6). The observed data does not differ from the expected distribution. Along with the low raggedness statistic (0.023) and non-significant SSD (p > 0.05) statistic, this indicates population expansion.

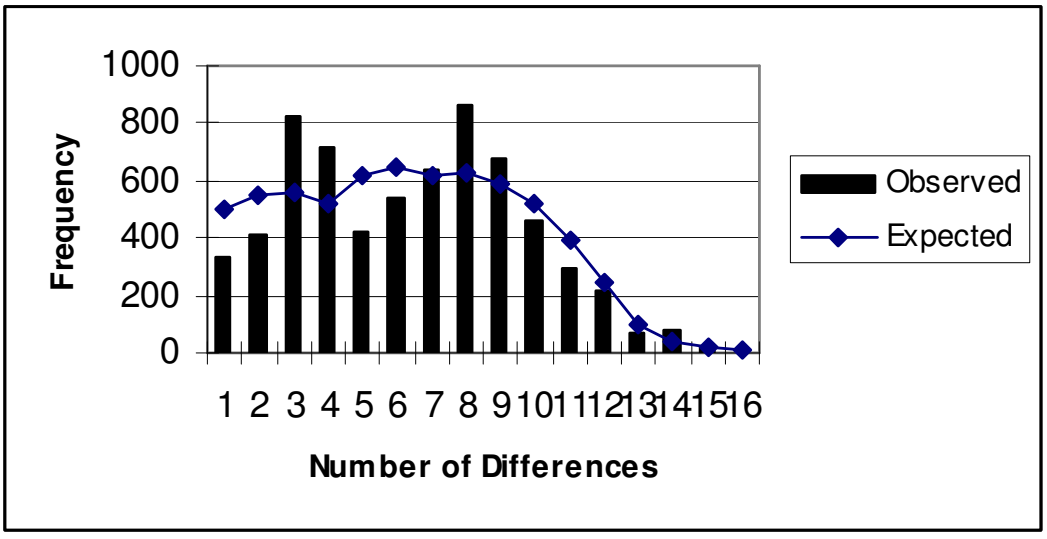



**Figure 5:** Mismatch distribution for 116 *Haploblepharus pictus* mtDNA sequences.

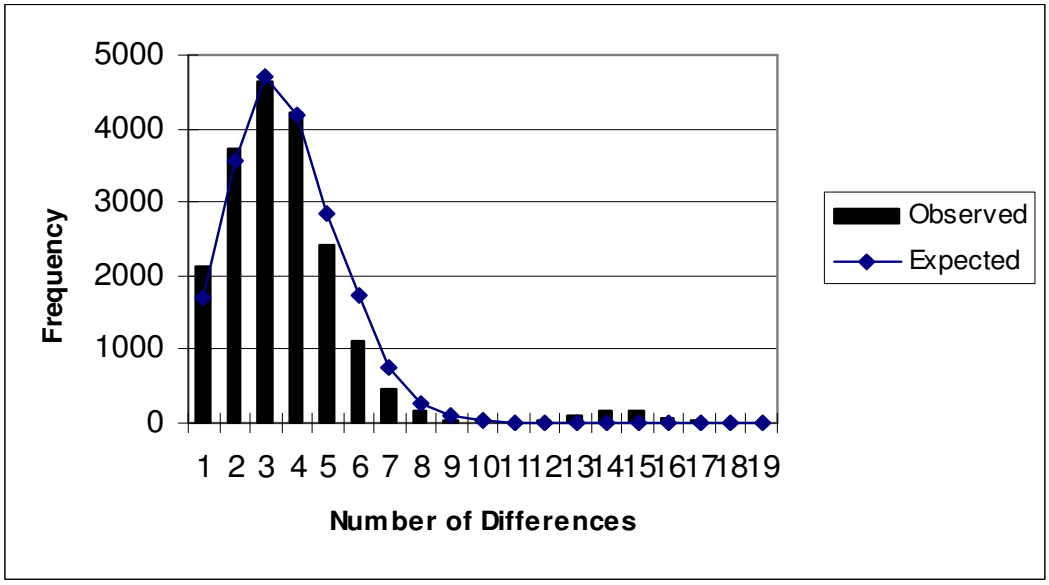



**Figure 6:** Mismatch distribution for 201 *Proleptus obtusus* mtDNA sequences

Migration between sampling sites

Although no two runs produced the same values, the dominant direction of gene flow remained the same. The overall pattern suggests that gene flow for the sharks was strongest from the east to west, from Gansbaai to Jacobsbaai (Fig. 7). The higher gene flow was between Gansbaai and Betty's Bay as well as Blouberg and Jacobsbaai. Gene flow from the west to the east coast was for the most part limited. There was, however, considerable gene flow between Wooley's Pool and Rooiels as well as between Rooiels and Betty's Bay. The parasites displayed bidirectional gene flow, with the exception of Jacobsbaai to Blouberg (Fig. 8). The problems and downfalls of Migrate-n will be dealt with in the discussion.

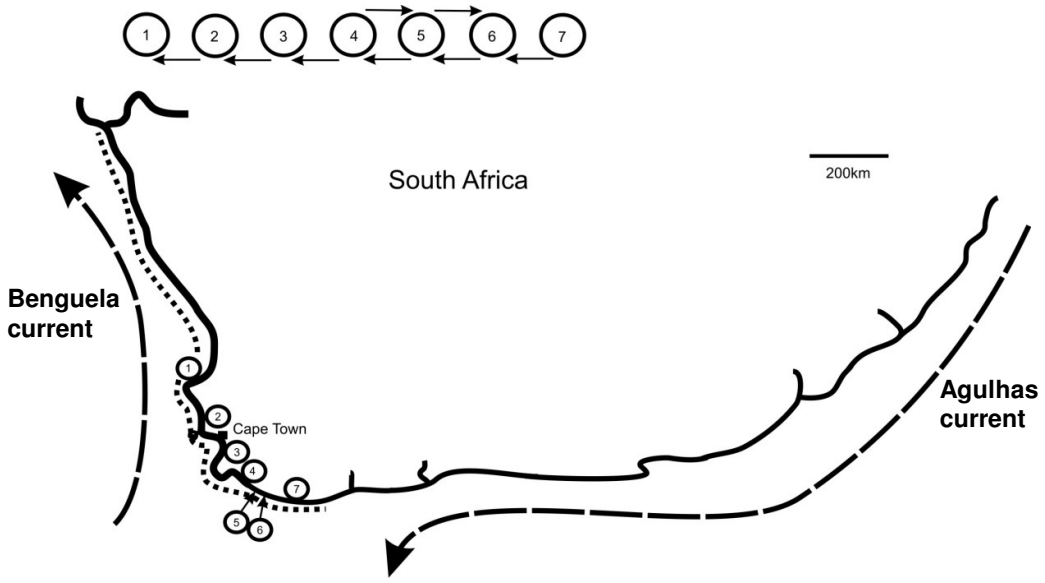



**Figure 7:** Map of the South African coastline showing the direction of gene flow for *Haploblepharus pictus* between sampling localities. Sampling localities are represented by circles: 1. Jacobsbaai; 2. Blouberg; 3. Kommetjie; 4. Wooley's Pool; 5. Rooiels; 6. Betty's Bay; 7. Gansbaai. The arrows between the circles represent gene flow. The Agulhas and Benguela Currents are shown.

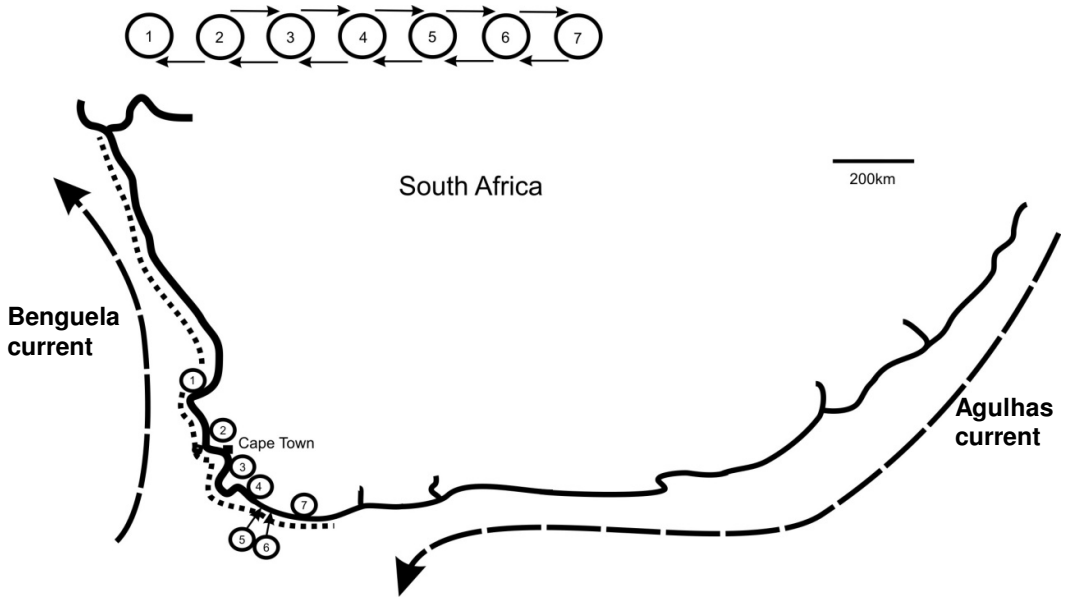



**Figure 8:** Map of the South African coastline showing the direction of gene flow for *Proleptus obtusus* between sampling localities. Sampling localities are represented by circles: 1. Jacobsbaai; 2. Blouberg; 3. Kommetjie; 4. Wooley's Pool; 5. Rooiels; 6. Betty's Bay; 7. Gansbaai. The arrows between the circles represent gene flow. The Agulhas and Benguela Currents are shown.

# CHAPTER 4: Discussion

## Population structure of *Haploblepharus pictus*

### Population structure and gene flow patterns

A species dispersal ability is of critical importance to its population growth rates, gene flow as well as the persistence of the species (Lowe & Allendorf, 2010). The behavioural characteristics of marine organisms, along with the topography of their environment, ocean currents and sea floor topology can present barriers to gene flow creating isolation (Gaida, 1997, Waples, 1998). A study conducted by Escobar-Porras (2009) found adult catsharks to show strong site fidelity but also suggest that they have the potential to move long distances along the coastline. The genetic data generated by this study is useful in indicating the levels of historical connectivity between populations.

SAMOVA maximised at two groups for the catsharks, Gansbaai and the other six sites. This was also found to be the case for pairwise $\Phi_{ST}$, which showed a significant difference between Gansbaai and all the other sites (Table 2). One explanation for the separation of the catsharks at Gansbaai is the lack of unique haplotypes at Gansbaai, indicating it as a possible source population. Secondly, environmental conditions may also differ along the species range and this can effect the genetic signature obtained (Hewitt, 1996). Although the sampled range did not cover the entire species distribution, differences may have been encountered on a micro-scale due to habitat changes along the coast (sandy beach, rocky shore, estuary mouths) as well as the influence of the smaller inshore currents. Microhabitats have been known to play an important role in reproductive success and survival rate from many species (Haramura, 2007). This could explain the decreased numbers of sharks at some sights as well as the genetic differences between sights.

Individuals from Gansbaai represent the most southern population sampled. *Haploblepharus pictus,* however, has extended its distribution from Cape Agulhas on the south coast to near Port Elizabeth on the south eastern coast (Branch *et al*., 2010). The range expansion may have been recent, resulting in the potential representation of Gansbaai as a peripheral population. Other studies have also found significant differences between outlying populations and central populations (Tolley *et al*., 2005; von der Heyden *et al*., 2008; Reynolds *et al*., unpublished), a pattern known as the edge effect (Cook, 1961). According to the edge effect, changes may have occurred more rapidly for the

peripheral populations than the central population, or vice versa (Cook, 1961). The range expansion may however, have occurred some time ago thereby creating established populations along the coast. Gansbaai would then be considered a central population as it falls in the middle of *H. pictus'* distribution.

As previously mentioned, a break has been established for several species near Cape Agulhas. The break is supported by the limited gene flow across Cape Agulhas (von der Heyden *et al*., 2008). It is unlikely that the separation at Gansbaai is representative of a break as it was not detected by BAPS or migrate and there was clearly gene flow taking place between Gansbaai and the other localities.

Fu's $F_s$ indicated that overall the *H. pictus* population is not in equilibrium. However, the mismatch distribution established that this was not due to population expansion. This was further confirmed by the haplotype network which did not show the traditional star-like signature of expansion. The disequilibrium may be due to certain haplotypes having a selective advantage, genetic drift, selection, migration, or a recent genetic bottleneck (Cantanhede *et al*., 2005; Wu *et al*., 2010).

The population structure of marine organisms in relation to their distribution can provide insight into the geographical breaks found along a coastline. As previously mentioned, studies have found a genetic break at Cape Point for several marine species (von der Heyden *et al*., 2008; Evans *et al*., 2004; Teske *et al.,* 2007). No barrier to gene flow was found for the catshark at Cape Point. This is most likely due to the increased mobility of the species and in particular that of the juveniles. Marine species that have a larval dispersal phase make frequent use of the major current systems for transport around the coast (Neethling *et al*., 2008; Pattrick & Strydom, 2008). Although catsharks do not have a larval phase, the juveniles may make use of some of the smaller inshore currents for movement around the coast.

Migration between sampling sites

Although the values for Migrate were inconsistent (from 114 to $1.62 \times 10^{11}$ individuals between two localities), the direction of gene flow remained the same with every analysis. Gene flow was from the east to west coast, i.e. from Gansbaai to Jacobsbaai. This westerly direction of gene flow has also been recorded for several other species, such as the clinid fish, *Clinus cottoides* (von der Heyden *et al*., 2008) and the goby, *Caffrogobius caffer* (Neethling *et al*., 2008). There was an eastward flow of genes from Wooley's Pool to Rooiels and Rooiels to Betty's Bay. *Clinus cottoides* showed a similar pattern, with gene

flow from Rooiels to Betty's Bay. This may be due to smaller counter currents present along that stretch of the coast as well as the close proximity of the sites. A report on South African inshore currents in this region found a strong westward flow in both winter and summer. However, there was also a fairly strong eastward current in winter (Harris, 1978).

Determining the gene flow of a species is important as it enables a better understanding of the structure and movement of its population. Direct measures of migration (e.g. mark-recapture) however, present many difficulties as they are expensive, time-consuming and usually very technically challenging (Whitlock & McCauley, 1999). In response to these problems, analyses, such as migrate-n have been developed that use data of gene frequency to indirectly determine the degree of gene flow in natural populations (Slatkin, 1985). As can be seen from the results discussed above, problems do arise when using such analysis (Whitlock & McCauley, 1999, Bohonak *et al.*, 1998 , Bossart & Prowell, 1998).

Specifically, the estimates produced by indirect analyses rely on a mathematical partnership between genetic structure and the rate of gene flow (Whitlock & McCauley, 1999). These estimates therefore assume that the assumptions of the mathematical model are representative of the ecological components of the natural populations (Whitlock & McCauley, 1999). This is unlikely to be the case. A recent paper by Marko & Hart (2011) further addressed the problems with the use of such analyses. The study emphasized the importance of several mitochondrial or nuclear markers in order to get an accurate idea of a populations gene flow.

## Population structure of *Proleptus obtusus*

### Population structure and gene flow patterns

The COI gene showed limited variation, with less than 1.5% sequence variation between most individuals. Even with minimal variation, Gansbaai was found to separate out from the rest of the sites according to the pairwise $\Phi_{st}$ (Table 4). This may have been due to the small number of unique haplotypes at Gansbaai. If this pattern holds then the separation of Gansbaai for both parasite and host indicates that *P. obtusus* may be more host specific than currently recognised. According to many studies, generalist parasites are expected to show little or no genetic structure (Archie & Ezenwa, 2011). This however, is not always the case in natural systems as gene flow between hosts can be limited by differences behaviourally or ecologically thereby creating structure (Archie & Ezenwa, 2011). This may explain the separation of Gansbaai for both species

The other known definitive hosts, *P. africanum* and *H. edwardsii,* both have distributions that extend beyond that of *H. pictus* (Branch *et al*., 2010). The extension of the other shark species distributions past that of *H. pictus* effectively increases the distribution of *P. obtusus,* which in turn would expose the parasite to other factors that may act as a barrier to gene flow along the coastline, thereby creating alternative structuring to that of *H. pictus*. *Poroderma africanum* for example, has a distribution that extends the entire length of the South African coastline (Branch *et al*., 2010). It therefore crosses through all major bioregions, which has been found to create structuring in other species (Teske *et al*., 2006; Evans *et al*., 2004).

*Proleptus obtusus*, as mentioned above, is a multi-host parasite which has most likely under gone several host switching events (Moravec *et al*., 2002; Yeld, 2009). The ability to switch between hosts may account for the increased genetic diversity of the parasites' compared to the sharks as the likelihood of a population bottleneck is reduced. The nucleotide diversity was found to be much higher for the parasite than the sharks possibly indicating greater diversity for the parasites. Parasites display faster evolutionary rates than hosts which could influence the genetic diversity (Kaltz & Shykoff, 1998; Hafner *et al*., 1994; Page, 1998; Nieberding *et al*., 2004). It is unclear as to when host switching may have occurred. However, if it was fairly recently then a newly established population would have been the product of a founding event in which a new host species was colonised by several individuals (Banks & Paterson, 2005). This would lead to a signature of a population bottleneck. High haplotypic diversity (0.8892 ± 0.0395) and low nucleotide

diversity (0.053258 ± 0.026683) were found for *P. obtusus*. When linked to the star-like haplotype network this indicates a recent population expansion from a small number of ancestors (Grant & Bowen, 1998). Fu's $F_s$ indicated population disequilibrium, and the mismatch test showed that this could be due to population expansion which further supports the expansion, shown by the star-like haplotype network (Fig 2b; Slatkin & Hudson, 1991; D'Amato & Carvalho, 2005).

Interestingly, subtle differences were not only found between localities and between hosts, but also within an individual host. Little genetic variation was found between parasites from the different sharks between localities. Three individuals were found to be significantly different to the rest of the parasites found within the same shark. The overall variation between parasites was found to be very low, less than 1%, with the exception of these three outliers, which all showed over 2% variation with the other parasites. *Haploblepharus pictus* has a varied diet which includes different species of crab (Lechanteur & Griffiths, 2003). *Proleptus obtusus* is known to use crabs as intermediate hosts but it is unclear as to which and how many crabs species it utilises (Lloyd, 1920; George-Nascimento *et al.*, 1993). The host is likely to feed on several infected hosts thereby being infected multiple times by the parasite, increasing the genetic variation of the parasites within a single host. It could also be possible that the three different parasites represent another species. However, no data exists on the sequence distances between species of *Proleptus*. It is plausible that cryptic speciation may have taken place within the *P. obtusus* species inhabiting the sharks. Cryptic speciation has been found in several other parasites (Hung *et al.*, 1999; Bartosova & Fiala, 2010). The three individuals found to be significantly different were not examined morphologically as they were presumed to be *P. obtusus* based on findings from a previous study (Yeld, 2009). To resolve this matter, future studies should aim to include both morphological and genetic identifications.

Isolation by distance was found for the parasites as indicated by the Mantel test (0.484, $p < 0.05$). This is most likely due to the small sample size as parasite samples were only taken from a few sharks at each sight thereby limiting the amount of variation that could be found. As mentioned above, little variation was found between parasites within a single host possibly representing clonal reproduction. It is therefore probable that greater variation may have been encountered had parasites been taken from more catsharks.

SAMOVA was run and maximized at two groups, Kommetjie and Blouberg, and the rest of the sites (Gansbaai, Rooiels, Betty's Bay, Wooley's Pool and Jacobsbaai). It is

unclear why those two sites would separate out. However, one possible explanation could be the increased nucleotide diversity of parasites of Kommetjie and Blouberg over the other sites (0.006 – 0.05) (Table 2). The $F_{CT}$ value as well as the pairwise Φst values were very low ($F_{CT}$ = 0.04244, Φst < 0.06), which indicate very shallow population structure. Both tests involve the allocation of the data into predefined populations, which may create a bias towards the formation of groups.

BAPS was run and no sites were found to separate from the rest, indicating a lack of genetic structure. This was expected as little variation was found between parasites within and between sharks. The haplotype network (Fig. 6) confirmed this as no geographic separation was found. Helminths parasitizing fish in marine systems are usually generalists with several intermediate and definitive hosts (Marcogliese, 2002). It was expected that limited structuring would be found as they are likely to be moving between hosts and therefore are not so restricted in their dispersal abilities or as greatly affected by gene flow barriers. This further emphasizes the significant effect that host specificity has on the structuring of parasite populations, as less host specific parasites generally exhibit less population structure (Nieberding *et al.*, 2008).

Migration between sampling sites

Although the values for Migrate were inconsistent (from 4 to 182 individuals between two localities), the direction of gene flow remained the same for every run. Gene flow was bidirectional with the exception of Jacobsbaai to Blouberg. Parasite movement is largely restricted to movements of the host and intermediate host. It is unclear what species of crab the intermediate host is, thereby limiting how much is known of the parasite movement. However, previous studies on other catsharks inhabited by a *Proleptus* species have found it to be a crab (Lloyd, 1920; George-Nascimento *et al.*, 1993). The intermediate hosts' movement may play a major role in gene flow patterns of the parasite (Rauch *et al.*, 2005).

*Proleptus obtusus* abundance

Considerable differences were found in the number of parasites per catshark from the different localities (Table 4). A study conducted by Marques & Cabral, (2007) found that underestimation of mean abundance and intensity occurred when dealing with small sample sizes (<40). Less than seven catsharks were dissected from each site for removal

of parasites; therefore the probability of finding the catsharks that have high numbers of parasites is lower (Marques & Cabral, 2007). Parasite prevalence was, however, 100% as was also the case for a study conducted by Yeld (2008). Marques & Cabral (2007) found that sample size should, not affect the prevalence of parasites. Endoparasites are also considered to be less aggregated than ectoparasites, which could account for the lower numbers of parasites found in some of the sharks (Marques & Cabral, 2007).

## Host-parasite comparison

### Analysis of Molecular Variance and Spatial Analysis of Molecular Variance

Analyses revealed interesting finds for the host and parasite. Overall haplotype diversity was high for both species, with nucleotide diversity being lower (Table 2). Such a pattern is found when population expansion has taken place after a time of low effective population size (Grant & Bowen, 1998). Such rapid population growth leads to the retention of new mutations, thus explaining the high number of unique haplotypes (Grant & Bowen, 1998). Nucleotide diversity was found to be slighter higher for the parasite when compared to the host. This may be due to several factors, such as increased mutation rate, smaller effective population size and shorter generation times for the parasite (Grant & Bowen, 1998).

Effective population size is an important component of the population genetics of a species. Many species characteristics can be used to predict its effective population size such as selection, population size changes, sex ratio, pattern of inheritance, mating system and population subdivision (Caballero, 1994). In general it can be said that parasites have smaller effective population sizes than their hosts (Morand *et al*., 2006). A smaller effective population size leads to a decrease in the amount of genetic drift as well as the slower spread of beneficial alleles (Poulin, 2007).

For both species, no structure was found amongst most of the sites. Gansbaai, however, presented an interesting result when the pairwise $\Phi_{st}$ values are compared for both species as it provide circumstantial evidence for similar population histories. This is likely representative of the break found near Cape Agulhas however, more sampling is required.

Although some structural similarity, around Gansbaai, was found for the two species, it was however, to a lesser degree than has been found in other host-parasite studies. A study of the phylogeography of two species of seastar and their parasites found little genetic structuring for a generalist parasite, where as a specialist showed a trend of regional structure according to its host (Crandall *et al.,* 2008). Another study dealing with three species of parasite found on rays in southern Australia also found a generalist parasite to display no population structure but a more host specific parasites did (Glennon *et al*., 2008). As was expected, host specificity plays a major role in determining the structure of parasite populations. Thus the limited congruence found between *P. obtusus* and *H. pictus* can be explained by the decreased host specificity of the parasite.

Fu's $F_s$ were significantly negative for both host and parasite, suggesting recent demographic change and population disequilibrium. Five of the seven localities were significantly negative for *P. obtusus;* however, only three were significantly negative for *H. pictus*. This was supported by the mismatch distribution as the parasites revealed population expansion where as the shark population was found to be stable. Other studies have found a similar pattern, with both host and parasites having significantly negative Fu's $F_{s,}$ indicating population disequilibrium (Crandall *et al*., 2008; Kaliszewska *et al*., 2005).

Haplotype network

No structure was found for either the host or parasite by the Haplotype network with no geographic separation apparent. Two-star like clusters were constructed for the parasite population, indicating population expansion. It is interesting to note that the parasite has a markedly higher number of haplotypes than the host. Parasites are known to have shorter generation times and faster mutation rates, therefore showing faster evolutionary rates of molecular sequences than their hosts (Kaltz & Shykoff, 1998; Hafner *et al*., 1994; Page, 1998; Nieberding *et al*., 2004). This could lead to an increase in the number of haplotypes found. The sharks may also be infected multiple times from parasites reproducing in the other definitive host, these would then be passed on to the intermediate host and ingested by *H. pictus*.

## Conclusions

Overall weak congruence was established between *H. pictus* and *P. obtusus,* with both species displaying shallow structure among sampling sites. *Proleptus obtusus* inhabits several species of catshark and is known to a have an intermediate host, possibly limiting the amount of structure found. The separation of Gansbaai for both host and parasite indicates some level of congruence as well as the possibility that *P. obtusus* may be more host specific than currently recognised. Cape Point was not found to be a barrier to gene flow for either species, presenting an interesting find as most species previously studied have shown limited gene flow around Cape Point.

Few cophylogeographic studies have focused on one parasite species found on multiple definitive hosts or investigated the effects on coevolutionary history (Banks & Paterson, 2005). Host specificity plays a major role in determining congruence between parasite and host populations. Although *P. obtusus* is not species specific it can still provide insight into some of the factors affecting *H. pictus* population structure. The increase in gene flow for *P. obtusus* in comparison to *H. pictus* reveals greater barriers to dispersal and a strong influence of coastal currents on *H. pictus*.

The weak congruence established between host and parasite in this study could be a result of several factors. The presence of an intermediate host can have a significant impact on the congruency of the parasite-host phylogenies as an intermediate host will enable the parasite to move and diversify away from the host thereby decreasing the degree of congruence (Nieberding & Olivieri, 2006). It is clear that the level of congruence is highly dependent on which of the hosts in the parasite's life cycle is being compared (Criscione *et al*., 2005).

Multi-host parasites present several challenges to a cophylogeographic study due to their complex life history and dependence on several hosts. If possible, such a system should be studied as part of a larger study incorporating all host species. This would provide clarity as to which hosts play the most significant role in forming the parasite genetic structure as well as which hosts have been most recently colonised.

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## Appendix I

Unique sequences for *Haploblepharus pictus.*

BB15-
CTACTGATTCGAGCAGAACTTGGTCAACCCGGCTCACTCTTAGGAGATGATCAGATTTACAATGTAATC
GTAACAGCCCATGCCTTCGTAATAATTTTTTTCATGGTTATACCAGTCATAATCGGAGGATTCGGTAATT
GACTTATCCCATTAATAATTGGTGCACCGGATATAGCCTTCCCTCGAATAAATAATATAAGTTTCTGACTT
CTTCCTCCTTCTTTCTTACTCCTTCTAGCTTCCGCAGGGGTTGAAGCTGGAGCTGGAACAGGATGAACT
GTTTACCCACCATTAGCTGGTAATTTAGCACACGCCGGACCATCTGTTGACTTGGCTATCTTTTCCCTTC
ATTTAGCCGGTATTTCTTCAATTTTAGCCTCAATCAATTTTATTACAACCATTATTAATATAAAACCCCCAG
CCATTTCTCAATATCAAACTCCACTATTTGTTTGGTCTATTCTTATCACCACTGTTCTCCTACTTCTGGCA
CTGCCAGTGCTGGCAGCTGGAATTACAATATTACTTACGGACCGTAATCTTAATACCACATTCTTTG
BL13:
CTACTGATTCGAGCAGAACTTGGTCAACCCGGCTCACTCTTAGGAGATGATCAGATTTACAATGTAATC
GTAACAGCCCATGCCTTCGTAATAATTTTTTTCATGGTTATACCAGTCATAATCGGAGGATTCGGTAATT
GACTTATCCCATTAATAATTGGTGCACCGGATATAGCCTTCCCTCGAATAAATAATATAAGTTTCTGACTT
CTTCCTCCTTCTTTCTTACTCCTTCTAGCTTCCGCAGGGGTTGAAGCTGGAGCTGGAACAGGATGAACT
GTTTACCCACCATTAGCTGGTAATTTAGCACACGCCGGACCATCTGTTGACTTGGCTATCTTTTCCCTTC
ATTTAGCCGGTATTTCTTCAATTTTAGCCTCAATCAATTTTATTACAACCATTATTAATATAAAACCCCCAG
CCATTTCTCAATATCAAACTCCACTATTTGTTTGGTCTATTCTTATCACCACTGTTCTCCTACTTCTGGCA
CTGCCAGTGCTGGCAGCTGGAATTACAATATTACTTACGGACCGTAATCTTAATACCACATTCTTTG
FB1:
CTACTGATTCGAGCAGAACTTGGTCAACCCGGCTCACTCTTAGGAGATGATCAGATTTACAATGTAATC
GTAACAGCCCATGCCTTCGTAATAATTTTTTTCATGGTTATACCAGTCATAATCGGAGGATTCGGTAATT
GACTTATCCCATTAATAATTGGTGCACCGGATATAGCCTTCCCTCGAATAAATAATATAAGTTTCTGACTT
CTTCCTCCTTCTTTCTTACTCCTTCTAGCTTCCGCAGGGGTTGAAGCTGGAGCTGGAACAGGATGAACT
GTTTACCCACCATTAGCTGGTAATTTAGCACACGCCGGACCATCTGTTGACTTGGCTATCTTTTCCCTTC
ATTTAGCCGGTATTTCTTCAATTTTAGCCTCAATCAATTTTATTACAACCATTATTAATATAAAACCCCCAG
CCATTTCTCAATATCAAACTCCACTATTTGTTTGGTCTATTCTTATCACCACTGTTCTCCTACTTCTGGCA
CTGCCAGTGCTGGCAGCTGGAATTACAATATTACTTACGGACCGTAATCTTAATACCACATTCTTTG
GB12:
CTACTGATTCGAGCAGAACTTGGTCAACCCGGCTCACTCTTAGGAGATGATCAGATTTACAATGTAATC
GTAACAGCCCATGCCTTCGTAATAATTTTTTTCATGGTTATACCAGTCATAATCGGAGGATTCGGTAATT
GACTTATCCCATTAATAATTGGTGCACCGGATATAGCCTTCCCTCGAATAAATAATATAAGTTTCTGACTT
CTTCCTCCTTCTTTCTTACTCCTTCTAGCTTCCGCAGGGGTTGAAGCTGGAGCTGGAACAGGATGAACT
GTTTACCCACCATTAGCTGGTAATTTAGCACACGCCGGACCATCTGTTGACTTGGCTATCTTTTCCCTTC
ATTTAGCCGGTATTTCTTCAATTTTAGCCTCAATCAATTTTATTACAACCATTATTAATATAAAACCCCCAG
CCATTTCTCAATATCAAACTCCACTATTTGTTTGGTCTATTCTTATCACCACTGTTCTCCTACTTCTGGCA
CTGCCAGTGCTGGCAGCTGGAATTACAATATTACTTACGGACCGTAATCTTAATACCACATTCTTTG
JA5:

CTACTGATTCGAGCAGAACTTGGTCAACCCGGCTCACTCTTAGGAGATGATCAGATTTACAATGTAATC
GTAACAGCCCATGCCTTCGTAATAATTTTTTTCATGGTTATACCAGTCATAATCGGAGGATTCGGTAATT
GACTTATCCCATTAATAATTGGTGCACCGGATATAGCCTTCCCTCGAATAAATAATATAAGTTTCTGACTT
CTTCCTCCTTCTTTCTTACTCCTTCTAGCTTCCGCAGGGGTTGAAGCTGGAGCTGGAACAGGATGAACT
GTTTACCCACCATTAGCTGGTAATTTAGCACACGCCGGACCATCTGTTGACTTGGCTATCTTTTCCCTTC
ATTTAGCCGGTATTTCTTCAATTTTAGCCTCAATCAATTTTATTACAACCATTATTAATATAAAACCCCCAG
CCATTTCTCAATATCAAACTCCACTATTTGTTTGGTCTATTCTTATCACCACTGTTCTCCTACTTCTGGCA
CTGCCAGTGCTGGCAGCTGGAATTACAATATTACTTACGGACCGTAATCTTAATACCACATTCTTTG

KO1:

ATTCGAGCAGAACTTGGTCAACCCGGCTCACTCTTAGGAGATGATCAGATTTACAATGTGATCGTAACA
GCCCATGCCTTCGTAATAATTTTTTTCATGGTTATACCCGTCATAATCGGAGGATTCGGTAATTGACTTA
TCCCATTAATAATTGGTGCACCGGATATAGCCTTCCCTCGAATAAATAATATAAGTTTCTGACTTCTTCCT
CCTTCTTTCTTACTCCTTCTAGCTTCCGCAGGAGTTGAAGCTGGAGCTGGAACAGGATGAACTGTTTAC
CCACCATTAGCGGGTAATTTAGCACACGCCGGACCATCTGTTGATTTAGCTATCTTTTCCCTTCATTTAG
CCGGTATTTCTTCAATTTTAGCCTCAATCAATTTTATTACAACCATTATTAACATAAAACCCCCAGCCATT
TCTCAATATCAAACTCCACTATTTGTTTGGTCTATTCTTATCACCACTGTTCTCCTTCTTCTGGCACTACC
AGTGCTGGCAGCTGGAATTACAATATTACTTACGGACCGTAATCTTAATACCACATTCTTTG

RO25:

ATTCGAGCAGAACTTGGTCAACCCGGCTCACTCTTAGGAGATGATCAGATTTACAATGTAATCGTAACA
GCCCATGCCTTCGTAATAATTTTTTTCATGGTTATACCAGTCATAATCGGAGGATTCGGTAATTGACTTAT
CCCATTAATAATTGGTGCACCGGATATAGCCTTCCCTCGAATAAATAATATAAGTTTCTGACTTCTTCCTC
CTTCTTTCTTACTCCTTCTAGCTTCCGCAGGGGTTGAAGCTGGAGCTGGAACAGGATGAACTGTTTACC
CACCATTAGCTGGTAATTTAGCACACGCCGGACCATCTGTTGATTTGGCTATCTTTTCCCTTCATTTAGC
CGGTATTTCTTCAATTTTAGCCTCAATCAATTTTATTACAACCATTATTAATATAAAACCCCCAGCCATTTC
TCAATATCAAACTCCACTATTTGTTTGGTCTATTCTTATCACCACTGTTCTCCTACTTCTGGCACTGCCAG
TGCTGGCAGCTGGAATTACAATATTACTTACGGACCGTAATCTTAATACCACATTCTTTG

The three unique sequences for the outlying species of *Proleptus.*

B8j:

TGCTGGTAATAGTTGAACTTTTTATCCTCCTTTAAGGGTAGAGGGTCAACCTGATATATCTACAGATATT
ATAATTTTAGGTTTACATATAGTGGGTATTGGTTCTCTTTTAGGAGCTATTAATTTTGTTGTTACTGTTCAA
AATATACGTGCTACTACTATATGTTTTGAACAATTAAGGATATTTGTTTGGACGATTTATTTGACTTCTTTG
TTATTAGTTTTAAGAGTGCCTGTGTTAGCGGGAGCTTTATTATTTTTGTTATTAGATCGAAATTTTAATTCT
TCTTTTTTTGATGCTAAAAAAGGAGGGAGTCCTTTACTTTATCAACATTTATTTTGATTTTTTGGACATCCA
GAAGTTTATATTATTATTTTACCAGCTTTTGGTATTATTAGAGAATGTGTTTTATATTTATCTGATAAGGAA
CGTTTATTTGGTCAAATAACTATAACTTTTGCTTCTATTTGAATTGCTATCTTAGGTTGTAGTGTATGGGT
TCATCACATATATACTTCAGGTGCGGATGTAGATACTCGTAGTTATTTTGGGG

R13o:

TGCTGGTAATAGTTGAACTTTTTATCCTCCTTTAAGGGTAGAGGGTCAACCTGATATATCTACAGATATT
ATAATTTTAGGTTTACATATAGTGGGTATTGGTTCTCTTTTAGGAGCTATTAATTTTGTTGTTACTGTTCAA
AATATACGTGCTACTACTATATGTTTTGAACAATTAAGGATATTTGTTTGGACGATTTATTTGACTTCTTTG
TTATTAGTTTTAAGAGTGCCTGTGTTAGCGGGAGCTTTATTATTTTTGTTATTAGATCGAAATTTTAATTCT
TCTTTTTTTGATGCTAAAAAAGGAGGGAGTCCTTTACTTTATCAACATTTATTTTGATTTTTTGGACATCCA
GAAGTTTATATTATTATTTTACCAGCTTTTGGTATTATTAGAGAATGTGTTTTATATTTATCTGATAAGGAA
CGTTTATTTGGTCAAATAACTATAACTTTTGCTTCTATTTGAATTGCTATCTTAGGTTGTAGTGTATGGGT
TCATCACATATATACTTCAGGTGCGGATGTAGATACTCGTATTTATTTTGGGG

F1a:

TGCTGGTAATAGCTGAACTTTTTATCCTCCTTTAAGGGTAGAGGGTCAACCTGATATATCTACAGATATT
ATAATTTTAGGTTTACATATAGTGGGTATTGGTTCTCTTTTAGGAGCTATTAATTTTGTTGTTACTGTCCA
AAATATACGTGCTACTACTATATGTTTTGAACAATTAAGGATATTTGTTTGGACGATTTATTTGACTTCTTT
GTTATTAGTTTTAAGAGTGCCTGTGTTAGCGGGAGCTTTATTATTTTTGTTATTAGATCGAAATTTTAATT
CTTCTTTTTTTGATGCTAAAAAAGGAGGGAGTCCTTTACTTTATCAACATTTATTTTGATTTTTTGGACATC
CAGAAGTTTATATTATTATTTTACCAGCTTTTGGTATTATTAGAGAATGTGTTTTATATTTATCTGATAAGG
AACGTTTATTTGGTCAAATAACTATAACTTTTGCTTCTATTTGAATTGCTATCTTAGGTTGTAGTGTATGG
GTTCATCACATATATACTTCAGGTGCGGATGTAGATACTCGTAGTTATTTTGGGG