THE BIOLOGY AND SYSTEMATICS OF SOUTH AFRICAN PIPEFISHES OF THE GENUS *SYNGNATHUS*

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ABSTRACT

Syngnathus the most speciose genus in the family Syngnathidae is widely in the Atlantic and Eastern Pacific oceans. However, it is poorly represented in the Indian Ocean with the only two species, *Syngnathus temminckii* and *S. watermeyeri* occurring in Southern African estuaries and coastal areas. *Syngnathus temminckii* the most common South African pipefish has been synonymised with *S. acus*, as the morphological and genetic divergence between these two populations has not been documented. There is also uncertainty in the taxonomic status of *S. watermeyeri*, an endemic estuarine pipefish that is restricted to two Eastern Cape estuaries. The purpose of this study was therefore to compare biological, morphological and genetic variation of South African *Syngnathus* species among different populations/locations, and with European populations of *S. acus*.

Sixteen meristic and ten morphometric characteristics were quantified from specimens obtained from field as well as various international natural history museum collections. Univariate (ANOVA) and multivariate (principal component analysis and discriminant analyses) analyses were used to assess morphological differences among the species. Morphometric variables were adjusted as ratios of the standard length and using an allometric procedure. ANCOVA analysis indicated significant differences between *S. acus* and *S. temminckii* for the relationships of the standard length (SL) and all morphological characters. There was no significant correlation between SL and snout length, snout depth, inter-orbital width and trunk depth for *S. watermeyeri.* The analyses provided evidence for distinct populations of *S. acus, S. temminckii* and *S. watermeyeri* although morphological character differentiation was greater between *S. watermeyeri* and the other two larger species. Although, significant differences were observed for meristic characters, pairwise comparisons did not reflect a clear pattern of variability. Most of the measured morphological characters contributed more than 70% to the morphological variation between the populations. Plot of the canonical scores for the variables resulted in the specimens clustering according to species groups and locations of *S. temminckii*.

Sequences of 750 base pairs of the mitochondrial cytochrome gene from 11 localities were compared with published sequences of other species of *Syngnathus*. Phylogenetic analysis was performed using parsimony, maximum likelihood (ML) and Bayesian inference (BI). The South African species were revealed to be sister-taxa with about 6 % divergence, while *S. temminckii* and *S. acus* had about 11% sequence divergence. 20 haplotypes among 46 total specimens from the three species. Gene flow was estimated at approximately 3 migrants per generation between the two South African populations and about 1 per generation between *S. temminckii* and *S. acus.* Such strong stock structuring among presumably recently established post-Pliocene (< 2 Million years ago) populations suggests that these species are reproductively isolated. Morphological and genetic variation observed in this study combined with current knowledge of life history attributes of the South African pipefishes indicate that conservative management decisions are necessary until the patterns and extent of differentiation among populations species-wide can be investigated further. It is thus being proposed that the name of the South African population of *S. acus* be changed to S*yngnathus temminckii* (Kaup, 1856).

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Declaration

This dissertation is my own unaided work and is being submitted for the Doctor of Philosophy in Fisheries Science at the Department of Ichthyology and Fisheries Science, Rhodes University in Grahamstown. It has not been submitted in whole or part for any degree or examination in any university.

Monica Mwale

Chapter One

General Introduction

1.1 Introduction

Pipefishes belong to the family Syngnathidae (meaning 'fused jaw') that also includes pipehorses, seadragons and seahorses. The family is distributed worldwide across a wide range of sea-grass and coral reef habitats from boreal regions to southern cold waters (Dawson, 1986). It has 55 described genera with more than 320 species that differ in various morphological characters (Kuiter, 2000; Dawson, 1985). Most of the species in the family are found in marine waters, though fresh water species are not uncommon (Vincent, 1995). Breeding populations of syngnathids have been recorded throughout the salinity range from freshwater to hyper-saline waters (Casey *et al.*, 2004; Rosenqvist & Johansson, 1995; Gordina *et al.*, 1991; Dawson, 1985; Gill, 1905). Species abundance of Syngnathidae is probably highest in temperate and subtropical waters while the greatest diversity occurs in the subtropical/tropical Indo-Pacific where about 70% of the recognized species are found (Kuiter, 2000; Dawson, 1985). The distribution and abundance of sea grasses influences the life history of syngnathids since grass beds are used as nursery areas, feeding grounds and shelter from predators (Teixeira & Vieira, 1995; Vincent, 1995a). Dead sea grass or detached algae may also be used as shelter and may transport fishes to shallow or deeper waters (Vincent, 1995).

Syngnathidae is generally recognized as a monophyletic taxon comprising an evolutionary gradient with pipefishes being the most primitive and the seahorses (*Hippocampus*) being the most derived (Grosberg & Cunningham, 2001; Helfman *et al.*, 1997; Vincent, 1996b; Vincent, 1995a). While there is great morphological variation within the genera of this family, the distinctions among genera are still based largely the emphasis of brood pouch closure type and location as defined by Duncker (1915). Herald (1959) further classified these fishes into two groups, with the Urophori being species where brooding may occur on the tail (A-type) and the Gastrophori with brooding on the abdomen (B-type). This evolutionary gradient is based on the extent of brood pouch

development and the degree of male parental care among genera. The brood pouch types range from simple attachment of the eggs to the ventral body surface in primitive forms (e.g., *Nerophis* and *Entelurus*), flaps protecting the eggs (e.g., *Corythoichthys*), an enclosed pouch in *Syngnathus*, and to the more advanced sealed pouch of the seahorse genus *Hippocampus* (Wilson *et al.*, 2001; Helfman *et al.*, 1997; Vincent, 1995b; Herald, 1941; Herald, 1940). This has provided the foundation for examining syngnathid evolution in a biogeographic framework.

1.2 Characteristics of pipefishes

There are 300 species of pipefishes in 35 genera whose taxonomy is in urgent need of revision (Kuiter, 2000). Southern Africa has 14 genera of syngnathids (with six subgenera) with at least 25 species of pipefishes and six species of seahorses (Dawson, 1986). Pipefishes are slender and elongate, with the head and snout aligned with the long axis of the body that is encased in a series of bony rings (McEachran & Fechhelm, 1998). Like all syngnathids, pipefishes have lobate gills, a pore-like gill opening located above the opercle, lack pelvic fins and true jaw teeth, have a body protected by a ring-like arrangement of bony dermal plates and other specialized features (Fritzsche, 1980). Pipefishes are sexually dimorphic and males carry the eggs and nurse their offspring on the body surface or inside a brood pouch under the trunk or tail. In terms of reproductive investment, females contribute eggs, while males fertilize them and provide nutrition and oxygen to the developing embryos (Berglund, 1993; Berglund, 1991).

Pipefishes, like most other syngnathids are characterized by restricted distributions, low mobility, small home ranges, low fecundity, lengthy parental care and mate fidelity in most species (Vincent, 1996). A gradient of paternal care is also characteristic of pipefishes. Eggs may be brooded openly on the male's abdomen, in individual membranous egg compartments, in a partly closed pouch consisting of pouch plates, in a fully closed pouch consisting of two folds, or in a fully closed saclike pouch (Figure 1.1). This gradient has been used to construct a phylogeny of pipefish genera, largely confirmed by molecular evidence (Vincent *et al.*, 2001; Helfman *et al.*, 1997).

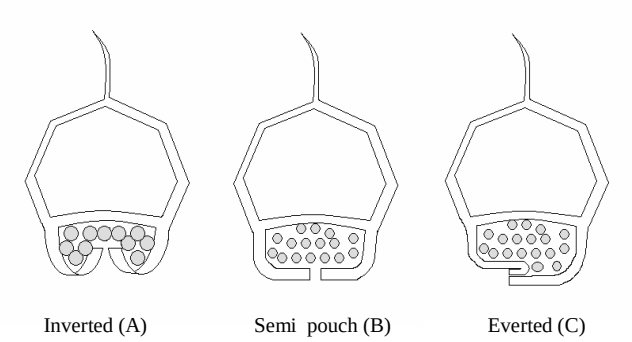


Figure 1.1: Cross-sectional diagrams of brood pouch closures of pipefish genera with bilateral membranous folds. Examples of pipefish genera with these brood pouch types are *Syngnathus* (A), *Corythoichthys* (B), & *Hippichthys* (C) (redrawn from Dawson, 1985).

1.3 Taxonomic status - *Syngnathus*

Syngnathus is the most speciose genus in the family Syngnathidae (Kuiter, 2000) with 32 recognized species (Appendix I). The genus is distributed widely in the Atlantic and Eastern Pacific oceans but rare in the Indo-West Pacific, where only one species occurs (Figure 1.2). It is a highly diverse genus of freshwater, estuarine and marine pipefishes (Dawson, 1985). Pipefishes of the genus *Syngnathus* can be distinguished from other species of the family by various characteristics. They have discontinuous superior trunk and tail ridges and the lateral trunk ridge ends near the anal ring or is confluent with the lateral and superior tail ridges. The brood pouch is under the tail, with pouch plates and folds with an inverted pouch closure (Dawson 1985). However phylogenetic relationships based on morphological characters are not fully resolved (Kuiter, 2000). Taxonomic problems persist among some pipefish species in the genus *Syngnathus*, that have been provisionally included but may belong to other genera (Kuiter, 2000). The lateral body ridge configuration is highly variable in some species while several species (e.g. *S. acus*, *S. leptorhynchus* and *S. schlegeli*) are very similar in meristic values and general morphology. Examples of such taxonomic problems are the two poorly known pipefish species of *Syngnathus, S. acus* and *S. watermeyeri* occurring in Southern Africa.

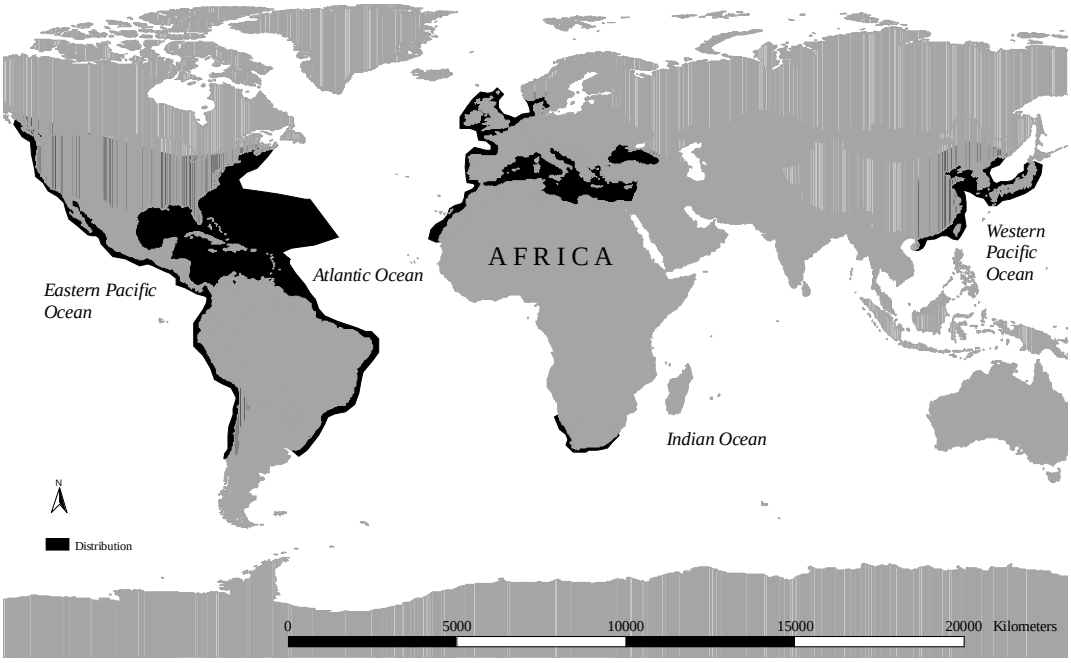


Figure 1.2: The distribution of species of the genus *Syngnathus* from Dawson (1985).

1.3.1 *Syngnathus acus* Linnaeus 1758

S*yngnathus acus*, also locally known as the long snout pipefish, is the most common pipefish species in South African estuaries (Whitfield, 1998). It is pale greenish to brown in coloration with variable markings (Figure 1.3 and Plate I.1) and is associated with aquatic vegetation such as *Zostera* species. The distribution of the species (Figure 1.4) ranges from the Eastern North Atlantic: Norway, Faroes and British isles to western Sahara, Senegal-Gambia, and from Namibia to the Cape of Good Hope and northward to the coast of Zululand in the Western Indian Ocean (Kuiter, 2000; Dawson, 1985).

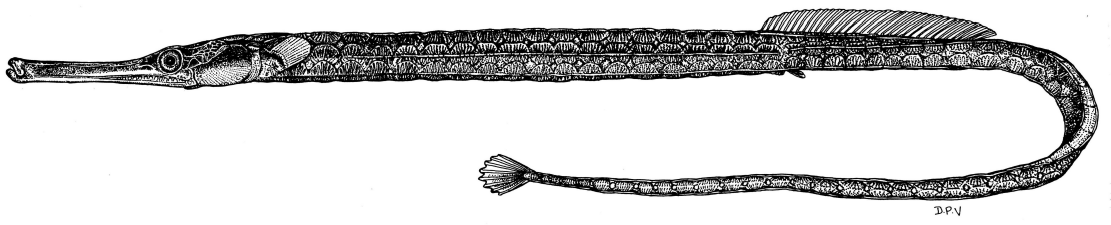


Figure 1.3: *Syngnathus acus* (South Africa), the long snout pipefish (Whitfield, 1998).

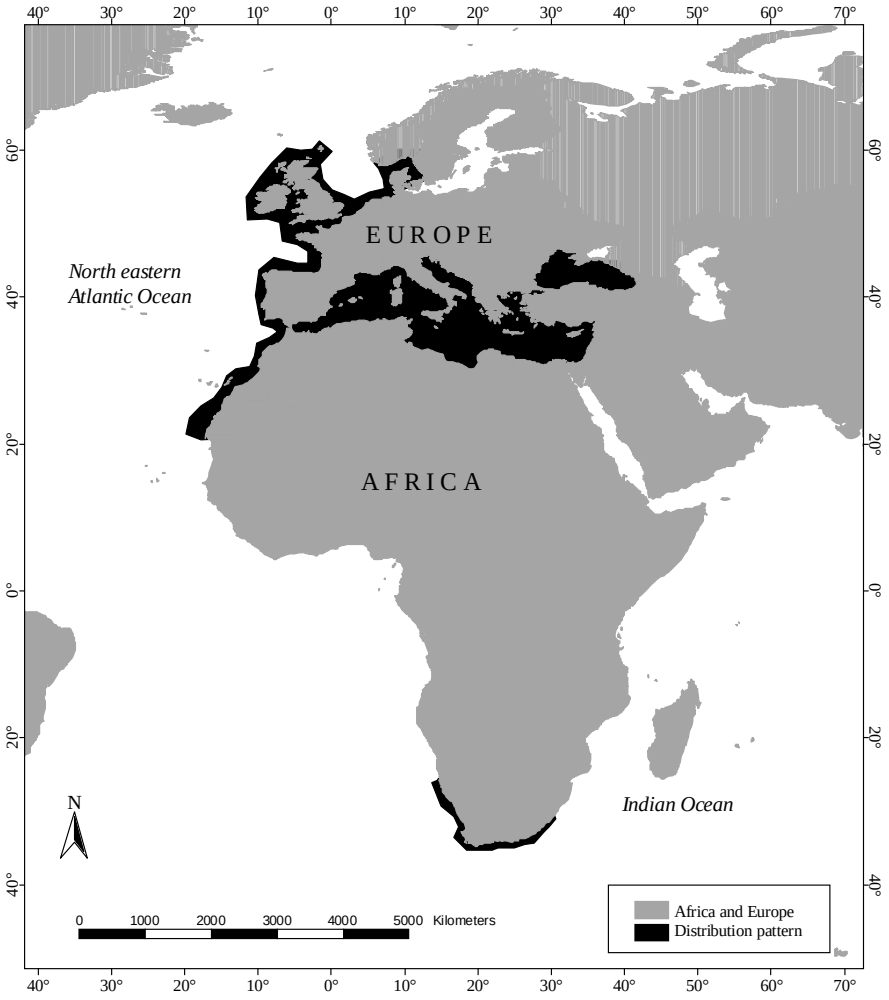


Figure 1.4: Putative distribution of *Syngnathus acus* (Dawson, 1986).

However, despite literature records (Dawson, 1986), occurrence of the long snout pipefish in the Atlantic outside South African waters lacks conclusive evidence. There are currently five nominal species that have been synonymised with *S. acus* (Dawson, 1985). Only two of these descriptions *S. temminckii* (Kaup, 1856) and *S. delalandii* (Kaup, 1856) were based on South African populations (Appendix II). Most of the data available on the long snout pipefish is based on information of *S.acus* in Europe and various Mediterranean regions, where it is commonly known as the greater pipefish (Kuiter, 2000). Furthermore, Linnaeus originally described the species *S. acus* based on European specimens, and Kuiter (2000) has pointed out that the long snout pipefish of

southern Africa or "*S. temminckii*" differs considerably from *S. acus* of the Mediterranean and North-eastern Atlantic. The lateral body ridge configuration is highly variable in the *S. acus* group as the lateral trunk and tail ridges may be unilaterally or bilaterally continuous or discontinuous (Kuiter, 2000: Dawson, 1985). Thus *S. acus* appears to be a species complex that requires taxonomic revision, in order to define its geographic limits.

1.3.2 *Syngnathus watermeyeri* Smith 1963

Taxonomic confusion is also evident for the poorly known estuarine pipefish, *S. watermeyeri*. Dawson (1986) stated that the retention of this pipefish in *Syngnathus* was provisional as its status within the genus was uncertain. *Syngnathus watermeyeri* differs from all the other species of the genus by having a short snout and fewer pectoral fin rays (6-8 as opposed to 10-14). The species is greenish brown in colouration with close-set dark lines on the head and pale lines on the body (Figure 1.5 and Plate I.2).

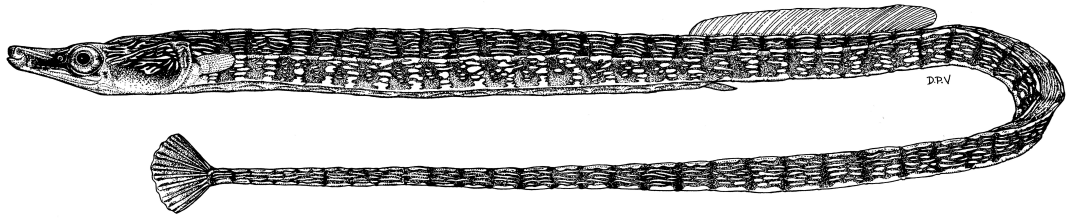


Figure 1.5: *Syngnathus watermeyeri*, the estuarine pipefish (Whitfield, 1998).

The species has a very restricted distribution and is endemic to South Africa (Dawson 1986; Skelton, 1987). It is presently restricted to the East and West Kleinemonde estuaries (33°32'28.2"S 27°02'51.7"E), although it was previously recorded in three adjacent estuaries (Kasouga, Bushmans and Kariega, Figure 1.6). Smith first described the species in 1963 using specimens from the Bushmans river estuary in South Africa. The disappearance of the species from these systems (Kariega and Kasouga) is disconcerting (Whitfield, 1998; Skelton, 1987). As a species listed as Critically Endangered (CR - B1+2abd) on the IUCN Red List 2002, understanding the phylogenetic distinctiveness of *S. watermeyeri* is therefore important (Reed *et al.*, 1998). Although a

population of *S. watermeyeri* has been found in the Kleinemonde system and a single specimen has been recently collected in the Bushman's river, the causes that might have pushed this pipefish to the verge of extinction need to be identified. Understanding this would be essential to prevent future threats to this and other pipefish populations. The decline in this pipefish's distribution is probably due to habitat losses resulting from reservoir construction, surface water diversion projects, ground water depletion and other factors. The most important threat for the survival of most species of Syngnathidae is the disturbance of their habitat by human factors (Vincent, 1995a).

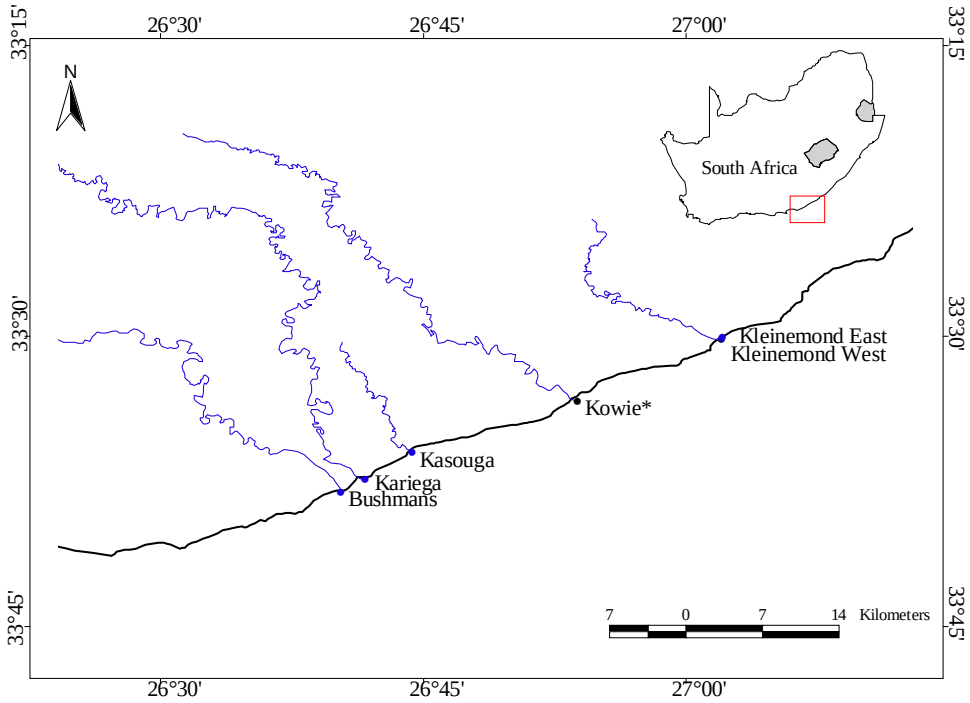


Figure 1.6: Distribution of *Syngnathus watermeyeri* in South Africa (Excluding the Kowie estuary).

1.3.3 Current concerns

According to Kuiter (2000), Dawson (1986) and Fritzsche (1980) the taxonomy of the genus *Syngnathus* is not well defined. Aspects and details of the phylogeny of species are contentious, many taxonomic issues are unresolved and the status of some species is uncertain. In pipefish taxonomy, morphometric and meristic characters have been used to

delineate population structure and species. However, pipefish species have been difficult to analyze phylogenetically because they have few useful morphological characters (Kuiter, 2000). Pipefishes exhibit great morphological plasticity so that defining characters for species descriptions and establishing keys can be difficult. In cladistic taxonomy, monophyletic taxa are recognized by the presence of apomorphic character states (more recently evolved, derived or advanced) whereas the plesiomorphic character state (more ancestral, primitive or generalized) is shared among members of a taxon presumed to be ancestral, if it is found with members of sister taxa or the outgroup during classification (Staton *et al.*, 1997; Helfman *et al.*, 1997). Defining the character states of extant species and those of their ancestors is necessary to understand the process of evolution and species divergence. Living species are presumed to represent the most recent manifestations of adaptations and lineages that have survived over time (Avise, 2000; Dobzhansky, 1951).

Dobzhansky (1951) wrote that "biological classification is simultaneously a man-made system of pigeon holes devised for the purpose of recording observations in a convenient manner as well as an acknowledgement of the fact of organic discontinuity." Today systematic studies explicitly aim to infer phylogenetic history and relatedness among living organisms and among living and extinct organisms. Points in the history of the species when the defining traits appeared can be inferred by comparison of character states and their distribution among taxa. Inconsistent findings with most morphological interpretations of fish phylogeny depict the importance and significance of ancestral morph types in defining taxa and the need for finding other methods of clarifying phylogenetic order. Although fossils have been used in designating apomorphic and plesiomorphic morphological characters in pipefishes, this is not adequate for many species and character states**.** The morphological characters that have been used to differentiate pipefish species exhibit extensive variation among and within species. Several species, *S. acus*, *S. schlegeli*, *S. leptorhynchus* and *S. euchrons* are very similar in meristics and general morphology. There is also much between-population variation in widespread species. Therefore, identifying specimens without locality data can be very difficult for most species of the genus (Dawson, 1985). Some pipefish species have such

variable descriptions that they can be keyed out in two different places using the available identification keys (e.g., *S. acus* in Dawson's 1985 descriptions).

1.4 Systematic methods

1.4.1 Morphological systematics

For decades, the approaches available for describing relationships of fishes have depended on data from comparative morphology, physiology, and other measurable phenotypic features (Wiens, 2001; Bennetts *et al.*, 1999; Avise, 1994). Morphological analysis was chosen as one of the systematic tools to study South African pipefishes because morphological studies have been especially useful in defining and in organizing fish species into genera (Stepien & Kocher, 1997). However, it has become clear that these traditional taxonomic methods of analysis are unable to examine taxonomic diversity with high resolution. It is rare to find morphological studies of syngnathids that present a hypothesis of relationship above the level of species comprising a genus, primarily due to a lack of congruence of characters (Stepien & Kocher, 1997). Various research results have led to questions about the status of several morphological characters used in piscine phylogenetic construction (Rasmussen & Arnasson, 1999).

The similarity in morphological characters or homology is one of the major limitations associated with taxonomic techniques. This is because it makes population-specific differences difficult to interpret such that the elucidation of the structure of populations may become problematic (Smedbol *et al.*, 2003; Goldstein *et al.*, 2000). Similarity between organisms is not always the result of shared phylogenetic history and may result from independent adaptations to similar environments (Wiens *et al.*, 2003; Brooks & McLennan, 2002; Reed *et al.*, 1998). That is, organisms that live in similar habitats may resemble each other in outward appearance but have quite different phylogenetic relationships and evolutionary origins. There are different types of similarities among organisms and these can be due to convergence, reversal and similarities in general, or homologous traits (Brooks & McLennan, 2002). Although morphological and genetic differentiation can proceed at different rates, convergence can be a confounding factor for both molecular and morphological analyses especially in the analysis of closely related

taxa (Bennetts *et al.*, 1999; Avise, 1994). However, these problems are not identical for molecular and morphological data and ambiguity can be minimized by doing outgroup analysis and by checking the frequency and distribution of character states within the study group as well as ontogeny, function, biogeography and fossil evidence. Despite the need for alternative methods, morphology is still the most widely used method in answering systematic and taxonomic questions regarding the status of fish species (Wiens, 2000).

1.4.2 Molecular systematics

Although systematic work has been done on pipefishes (Prein, 1995; Vincent, 1995b; Orr & Fritzsche, 1993; Dawson, 1985; Fritzsche, 1980; Herald, 1965), very little is known about their evolution and genetic structure (Avise *et al.*, 2002; Wilson *et al.*, 2001; Jones *et al.*, 2001; Helfman *et al.*, 1997; Herald, 1941). Molecular analysis was used in this study mainly because of its potential to provide information about the fundamental genetic basis of evolutionary change that is generally inaccessible through traditional taxonomic techniques (Bennetts *et al.*, 1999; Avise, 1994). Since the emergence of molecular techniques like the polymerase chain reaction (PCR) and gel electrophoresis (Meyer, 1993), DNA analysis has become a useful tool for the study of evolutionary relationships among fishes (Mattern, 2004; Alvarez-Valin & Gomez N., 2002; Bloomer & Impson, 2002; Sullivan *et al.*, 2000; Caldara *et al.*, 1996). The relative ease with which DNA sequences of mitochondrial genes can be determined through PCR, universal primers, and direct sequencing has led to large DNA sequence databases that have aided our understanding of relationships between organisms (Mattern & McLennan, 2004; Stephens & Wiens, 2003; de Queiroz *et al.*, 2002; Santini & Tyler, 2002; Rosenbaum *et al.*, 2000; Emerson *et al.*, 1999; Georges *et al.*, 1998; Caldara *et al.*, 1996).

Molecular markers have been used for diagnostic purposes to identify unique groups of populations and can tell us a lot about the relationships of organisms that cannot otherwise be inferred from morphology, behaviour or biochemical studies (Goldstein *et al.*, 2000). Analysis of DNA molecules provides a large number of observable characters for use in phylogenetic analysis (Smouse *et al.*, 1991). Theoretical and empirical studies

have shown that a certain number of characters is crucial in estimating phylogenetic relationships (Wiens, 2001; Wiens, 2000). Molecular studies can include up to several hundred or thousand characters as opposed to morphological analysis, which rarely includes more than a few hundred characters with an average of three characters per taxon (Hillis & Wiens, 2000). Comparisons of the DNA sequences of various genes between different organisms has also led to the recognition of high levels of genetic variation in a wide range of natural populations (Knaepkens *et al.*, 2004; Knapen *et al.*, 2003; Grosberg & Cunningham, 2001; Swain *et al.*, 2001; Thomas *et al.*, 1999). For example, a molecular study of a circumtropically distributed damselfish species group (*Abudefduf sordidus)* using a fragment of the mitochondrial cytochrome oxidase I region showed that the recognized taxonomy underestimated the diversity of the group by one species (Bermingham *et al.*, 1997). It has been observed that genetic structure can reveal a great deal about a population's history of subdivision and gene flow, in particular the history of its interactions with other species when set in a phylogenetic context (Grosberg & Cunningham, 2001; Goldstein *et al.*, 2000; Near *et al.*, 2000; Miyanohara *et al.*, 1999; Meyer, 1993). Findings of the *Abudefduf sordidus* group by Bermingham et al. (1997) elaborated the history of phylogenetic relationships for the Caribbean and eastern Pacific populations with regards to a biogeographic and vicariant event (the closure of the central America seaway). Therefore, genetic data and analyses permit species and geographical populations to be placed in a phylogenetic context for historical biogeographical analysis.

1.4.2.1 Mitochondrial DNA

Early studies of molecular phylogeny were based on whole molecule restriction fragment length polymorphism (RFLP) analysis with some studies including comprehensive restriction-site mapping. More recently the trend has been to use PCR to amplify and sequence portions of specific genes (Billington, 2003). The rapidly evolving mitochondrial DNA (mtDNA) region was chosen for this study because it is one of the most frequently used DNA regions for both intra and inter-specific genetic studies of fishes (Casey *et al.*, 2004; Brant & Orti, 2002; Caldara *et al.*, 1996; Bowen *et al.*, 1994; Meyer, 1993; Avise *et al.*, 1987). Mitochondrial DNA is particularly attractive for phylogenetic analysis because of its high mutation rate (Meyer, 1993). This high

mutation rate coupled with a generally accepted maternal mode of inheritance that prevents recombination of the mutations arising in different individuals during sexual reproduction have made mtDNA analysis popular in phylogenetic studies. It has been widely used in fish studies on gene flow, hybrid zones, population structure and other population level studies for various organism groups (Wilson *et al.*, 2001; Santini, 2000; Walker & Avise, 1998; Stepien & Rosenblatt, 1996; Stepien & Rosenblatt, 1991; White, 1986). As a result, extensive genetic diversity in mtDNA has been documented in both fish and other organisms (Behrmann-Godel *et al.*, 2004; Randall *et al.*, 2003; Knapen *et al.*, 2003; Borsa, 2002; Rees *et al.*, 2001; Tabata & Taniguchi, 2000; Echelle *et al.*, 2000; Avise & Walker, 1999; Avise & Walker, 1998; Echelle & Dowling, 1992).

The fast rate of evolution of mtDNA compared to nuclear DNA makes it useful in obtaining high resolution when analyzing recent evolutionary events (Weibel & Moore, 2002; Meyer 1993). The rapid substitution rates of mtDNA mark points of ancestry without obliterating accrued synapomorphies and give high resolution among recently diverged or closely related species (Bloomer & Impson, 2002; Fuch *et al.*, 2000; Doukakis *et al.*, 1999). The similar substitution rates that are typical among lineages are crucial because unchanging bias in nucleotide composition among lineages is important for accuracy in molecular genetic analysis. Furthermore, mtDNA is easy to extract and isolate, so that sequences can be analysed in various ways and even converted to genetic distances for phylogeographic analysis. The fact that organisms are united by a single phylogenetic history makes it possible to track the relationships among them using characters that are homologous (the same because they are derived from the same common ancestor), independently evolving and heritable (Behrmann-Godel *et al.*, 2004; Avise & Wollenberg, 1997). The phylogenies inferred from mtDNA comparisons represent the presumed historical sequences of mutational events accompanying the differentiation of maternal lines (Avise *et al.*, 1987). Any DNA sequence is therefore a snapshot representation of the substitutional (evolutionary) history in an evolutionary lineage (Meyer, 1993). Therefore, genetic variation between species or populations is a direct consequence of mtDNA and nuclear mutations at the intra individual level (Meyer, 1993).

The mitochondrial genome shows a 5 to 10-fold higher nucleotide substitution rate than protein-coding nuclear genes (Billington, 2003; Meyer, 1993; Brown *et al.*, 1979). This high rate of substitution is probably due to the sensitivity of the mitochondrial genome to exogenous mutagens and a high spontaneous mutation rate. A recent study has revealed a several hundred-fold higher rate of somatic mutation both *in-vivo* and *in-vitro* in human mtDNA than in nuclear genes (Junjian & Herbert, 1999). As a result, it has been suggested that mutations resulting from replication errors and oxidative damage are the major sources of mtDNA mutation (Meyer 1993). However, divergence rates specific for mitochondrial control regions in fishes have not been established (Donaldson & Wilson, 1999). Although it is now possible to investigate DNA sequence changes in genes from virtually any organism, it is critical to clarify the extent and type of mutations and their rate of DNA evolution among any lineages being studied (Junjian & Herbert, 1999). This is because suggestions have been made by various studies on the heterogeneous nature of the mutation rates along DNA sequences of specific genes (Edwards & Beerli, 2000; Bermingham *et al.*, 1997; Kornfield & Parker, 1997). The rate of substitution is the result of a delicate interplay of forces, including mutation, selective constraints arising at both molecular and organismal levels, and population level events (Kocher & Carleton, 1997). However, studies of closely related species will not be affected by multiple substitutions and should provide a better representation of evolutionary events (Kocher & Carleton, 1997; Meyer, 1993).

The complete mitochondrial genome has been sequenced and the gene order has been determined for several vertebrates and invertebrates. Mitochondrial DNA is a single double-stranded and typically circular molecule that has a variable sequence and is contained in multiple copies of mitochondria per cell (Billington, 2003; Taanman, 1999; Meyer, 1993). The organization of the molecule is very simple compared to nuclear DNA (nDNA). Mitochondrial DNA encodes 37 genes, two for ribosomal RNAs (12sRNA and 16sRNA), 13 genes coding for protein sub-units and 22 genes coding for transfer RNAs (Taanman, 1999; Avise *et al.*, 1987). In addition, there are one or more regions that appear to be non-coding regions such as the control region (which contains the D-loop) in vertebrates (Staton *et al.*, 1997; Meyer, 1993; Avise *et al.*, 1987). Though the gene order

of animals is slightly different in every phylum that has been studied, the piscine gene order (Figure 1.7) does not differ from the consensus gene order of vertebrates (Meyer 1993). While gene order appears to be very stable across fish taxa the most often observed differences are due to tRNA gene transpositions (Meyer, 1993; Avise *et al.*, 1987).

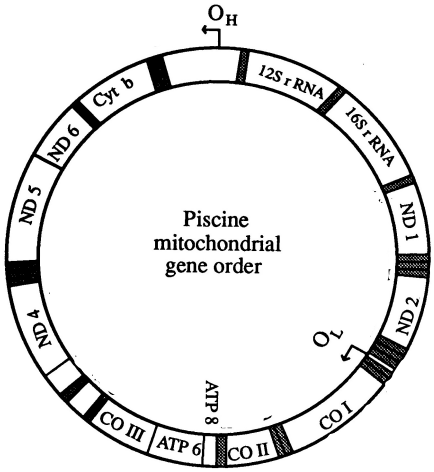


Figure 1.7: The mitochondrial gene order of fish edited from Meyer (1993). The origins of the H (O_H) and L-strand (O_L) replication are indicated in the figure. The origin of the H-strand is in the control region. Transfer RNA genes are shown in shaded boxes and not defined for this study.

The order of these 37 genes in mtDNA has been hypothesized to contain phylogenetic information that is useful in determining the pattern of ancient and recent divergences (Billington, 2003). These genes range from rapidly evolving sequences (e.g., D-loop, ND1, ND3/4 and ND5/6) for examining intra-specific and inter-specific relationships between closely related organisms, through moderately evolving genes (e.g., cytochrome *b*) for examination of relationships among genera and finally to slowly evolving 12s and 16s RNA genes for family level comparison (Billington, 2003; Meyer 1993). Therefore, the choice of sequence or gene used in a phylogenetic study depends on the nature of the investigations, that is, whether the questions posed are at the population or phylogenetic level.

1.4.2.1.1 Cytochrome *b*

The mitochondrial cytochrome *b* (cyt *b*) region is the most widely used region in studies of molecular phylogeny of fishes (Billington, 2003). It was selected to study the two pipefishes of *Syngnathus* because it exhibits both conserved and variable regions and thus contains phylogenetic signals at many different divergence levels. Cytochrome *b* sequences have been used to resolve phylogenetic relationships among syngnathids (Casey *et al.*, 2004; Teske *et al.*, 2004; Near *et al.*, 2000) over a wide range of taxonomic levels and deep cladogenetic events involving ancient divergences (Naylor & Brown, 1998; Zardoya *et al.*, 1998; Van de Peer & De Wachter, 1997; Mindell & Honeycutt, 1990) and to assess population or low-level taxonomic relationships (Tsigenopoulos *et al.*, 2003; Lovejoy & de Araújo, 2000; Rocha-Olivares & Vetter, 1999; Waters & Burridge, 1999; Meyer, 1993). Furthermore, cyt-*b* sequence divergences have been estimated for various animals and seldom exceed a few percent within fish species (Waters & Burridge, 1999; Irwin *et al.*, 1991).

1.4.3 Phylogeography

Mitochondrial DNA genealogies have also been used extensively to trace population level processes for within species variation for fish and various organisms in relation to their geographic distribution (Hrbek *et al.*, 2004; Avise, 2000; Meyer, 1993; Avise *et al.*, 1987). The observation that most species are divided into spatially and demographically distinct populations is one of the most fundamental observations in nature and is the basis for phylogeographic work (Altman & Taylor, 2003; Cox & Moore, 2000; Avise *et al.*, 1987; Mayr, 1963; Dobzhansky, 1951). Molecular data are the primary means of assessing phylogeographic relationships among organisms and examining questions of zoogeographic subdivisions and relationships among areas (Lovejoy & de Araújo, 2000; Stepien & Kocher, 1997). Species generally have geographic populations that have a particular distribution that is determined by barriers to dispersal. These barriers determine the frequency of occurrence and the degree of geographical continuity among populations (Humphries & Parenti, 1986). It will be possible to study the phylogeography of *S. acus* because it has spatially separated populations (Europe and Africa) and therefore a dramatically disjunct distribution pattern. Evolutionary and geological processes occur

over large temporal and spatial scales and may have resulted in the distribution patterns observed for *S. acus* (Avise 1998). Various fish species have phylogenies that cannot be fully understood without reference to the evolutionary history of their phylogeographic structure (Cox & Moore, 2000; Avise, 2000; Avise, 1998; Bermingham *et al.*, 1997; Morrone & Crisci, 1995; Meyer, 1993; Humphries & Parenti, 1986). For example, Bernardi & Talley (2000) suggested that the speciation event between two sister species of top minnows (Fundilidae) *Fundulus nottii* and *F. escambiae* might be correlated with tectonic events occurring in the northern gulf region of California that had apparently shifted drainage patterns from a northeast southwest direction to a north-south direction.

The role ecological zones have played in developing the variation among populations of South African species of *Syngnathus* can be determined by studying the comparative phylogeography of different species community compositions (Altman & Taylor, 2003; Cox & Moore, 2000; Avise, 2000; Avise *et al.*, 1987; Mayr, 1963; Dobzhansky, 1951). Understanding the extent of population subdivisions and its causes is important in the study of evolutionary histories and ecological research of species diversity (Altman & Taylor, 2003). Some of this work has been done for within species variation for South African terrestrial and freshwater habitats because the biogeographical barriers that are responsible for the current distributions of their fauna are more obvious and have been well documented (deMenocal, 2004; Vences *et al.*, 2004; Bloomer & Impson, 2002; Turner *et al.*, 2001; Sullivan *et al.*, 2000; Knight *et al.*, 1999). However, population subdivisions of South African marine and estuarine organisms have been less studied. This is due to a variety of factors such as the presumed high dispersal potential of organisms with pelagic larvae and the large population sizes of marine and coastal fish populations (Mattern & McLennan, 2004; Fletcher *et al.*, 2004; Altman & Taylor, 2003; Gill & Kemp, 2002; Grosberg & Cunningham, 2001; Pyle & Randall, 1994). However, there are several reasons why pipefish occurring in southern African estuaries and coastal environments, unlike typical marine species, might be thought to exhibit population structure. Firstly, estuaries undergo cyclical isolation and reconnection with the marine environment due to irregular opening and closing of estuary mouths, and therefore may represent a habitat that is periodically isolated from the surrounding marine environment

(Whitfield, 1998). Pipefishes show some degree of site fidelity (Howard & Koehn, 1985) and this isolation may result in population differentiation and structuring. Secondly, it has been observed that the occurrence and diversity of fishes in South African estuaries varies according to latitude (biogeography) and the individual characteristics of each estuary (Allanson & Baird, 1999; Blaber, 1985; Day, 1981). The 3100 km South African coastline is characterised by variable geomorphology as well as complex climatic and oceanographic conditions that have a profound influence on estuarine environments and coastal waters (Whitfield, 1998; Day, 1981). South African estuaries are affected by variables such as water quality, quantity and movement, which in turn affect the substratum or habitat in different parts of each system (Whitfield, 1998; Whitfield 1992). Changes in these variables affect the distribution and abundance of fishes in estuaries. Estimating the level of genetic diversity of South African species of *Syngnathus* among various estuaries will help us understand how the communities and biological interactions have developed over time. These interactions and variations may vary geographically as a result of ecological influence, and recent species associations (Wares & Cunningham, 2001).

1.5 Thesis outline

The aim of this research was to characterize the South African pipefishes of the genus *Syngnathus* by examining inter- and intra-specific variation between locations by integrating biological, morphological and molecular data. Although estuarine ecosystems of South Africa have been widely studied and support diverse communities of vertebrates and invertebrates, the phylogenetic history and patterns of species populations within estuaries are poorly documented. Studying the population structure of widely distributed fish species like *S. acus* could unravel phylogeographic relationships and gene flow connections among estuaries. There are various systematic procedures available today that can be applied to study such issues. Morphological and molecular approaches to systematics both have different strengths as well as difficulties and problems with regards to data analysis. Therefore, this thesis aims to use and compare different methods and procedures to understand the systematics of *Syngnathus* species in South Africa.

The first research objective of this thesis was to assess the distribution and biology of South African pipefishes of the genus *Syngnathus*. This was done in chapter two with an emphasis on the distribution of the South African population of *S. acus* (*S. temminckii*[*]) using museum specimens and routine collections. More work was done with this species because it is widely distributed and the most common pipefish in southern Africa (*Syngnathus watermeyeri,* though endemic, has a very restricted distribution). Aspects of the biology were addressed with emphasis on the specimens of *S. temminckii* from the Swartvlei estuary population where routine sampling had been done for a year. This was done to estimate its reproductive season and aspects of the biology of South African populations of *S. temminckii.*

Chapter three focuses on the morphological variation of species of South African *Syngnathus*. This variation was also studied with regard to the potential influence of sexual dimorphism, biogeographical regions and localities on morphology. The following key questions were addressed in this chapter:

- To what extent are the two South African species of *Syngnathus* morphologically different from each other?
- Is the population of *S. temminckii* (South African *S. acus*) morphologically different from the populations of *S. acus* in the North-eastern Atlantic, Mediterranean and North Seas?
- What is the morphological variation of *Syngnathus* specimens from different South African locations*?*

The fourth chapter focuses on the molecular analysis of specimens of *S. acus, S. temminckii* and *S. watermeyeri* using mitochondrial DNA for species discrimination. The two South African species were also compared to other species of the genus using GenBank sequences to investigate their placement within the genus. Sequences of a seahorse *Hippocampus hippocampus,* a snake pipefish, *Entelurus aequoreus* and a stickleback *Gasterosteus aculeatus* were used as outgroups (Wilson *et al.*, 2001). These

[*] The South Africa "*S. acus"* population will be referred to as *S. temminckii* while the European population will still be referred to as *S. acus* from this chapter onwards and throughout this thesis for ease of description during comparisons.

species were selected as additional taxa because they have been shown from independent evidence (Brooks & McLennan, 2002; Wilson *et al.*, 2001) to be distantly related at different taxonomic levels to the ingroup members. The seahorse and snake pipefish have been suggested as potential sister groups to *Syngnathus* (Wilson *et al.*, 2003; Jones *et al.*, 2003; Avise *et al.*, 2002) while the stickleback is a distantly related taxon. The main objective of this chapter is to formulate a hypothesis of the evolution of the two South African species of *Syngnathus,* and their relations to the *S. acus* species population as well as other species in the genus using mitochondrial cytochrome *b* sequences. The following key questions were asked:

- Is the South African population of *S. temminckii* genetically different from the European populations of *S. acus*?
- Is the placement of *S. watermeyeri* in the genus *Syngnathus* valid?
- What is the level of genetic difference between the two South African species?
- What is the divergence time estimate for the radiation of the genus and the two South African species?

The fifth chapter studies the phylogeographic relationships of South African specimens of *Syngnathus temminckii* to better understand the historical events that may have led to speciation and distribution. The same species used in the chapter 5 were used as outgroups to study these historical speciation events. The evolutionary history of this fish species in generating the observed biodiversity may help us to understand the current species richness. It could also have strong implications for conservation management of endemic species and morphologically different forms or species (Bennetts *et al.*, 1999). Population differences among South African populations of *S. temminckii* were also examined to estimate the level of genetic diversity within this species. The following key questions were investigated:

- What is the genetic diversity within and among South African sampling sites of *S. temminckii*?
- Do the South African populations of *S. temminckii* exhibit geographic differences?

- What are the events that have might have led to the separation and current distribution of South African species of *Syngnathus*?

The final chapter will be a synthesis and summary of the implications from all the systematic analyses done on the South African species. This chapter also attempts to show the advantages of combining biological, morphological and molecular data in phylogenetic analysis. Information on the systematics and biology of South African pipefishes should be essential for understanding the events that have resulted in speciation and the geographic distribution patterns within this genus and the isolating mechanisms that may have led to the genetic divergence of the South African *Syngnathus* populations.

Chapter Two

Biology and distribution of *Syngnathus* in Southern Africa

2.1 Introduction

The coast of South Africa is subject to a range of complex climatic and oceanographic conditions (Beckley *et al.*, 2002; Allanson & Baird, 1999). These complex conditions are linked to the South Atlantic Ocean and South Indian Ocean anti-cyclones that cause the cyclonic weather systems of the 'roaring forties' which in turn affect the oceanographic characteristics and hence the coastal environment of the region (Beckley *et al.*, 2002). As a result of this variable environment southern Africa supports a remarkable diversity of marine and estuarine fish with about 227 endemic coastal species and 14 genera with at least 25 species of pipefishes (Beckley *et al.*, 2002: Dawson, 1986). The occurrence and diversity of South African fishes varies according to latitude and the individual characteristics of the coastal area or estuary (Turpie *et al.*, 2000; Blaber, 1985). This is due to the effect of environmental variables, such as temperature or food availability that influence the maximum abundance a fish species can attain and its distribution range. Species richness therefore increases from west to east with Indo–Pacific species dominating the warmer Agulhas current waters whose temperatures are generally >22°C off the east coast (Whitfield, 1998; Beckley & Van Ballegooyen, 1992). Although the highest number of endemic fishes is found on the south coast near Port Elizabeth, the west coast (with a lower species diversity) is more important economically because of a higher biomass of fish (Beckley *et al.*, 2002; Turpie *et al.*, 2000; Beckley, 1988). The higher productivity of this region is due to southeasterly and southerly winds which result in the large-scale upwelling of nutrient rich cooler water off the Benguela current upwelling region (Olivar & Beckley, 1994; Shillington, 1986).

South African coastal areas, lagoons and estuaries are highly productive ecosystems and support a variety of marine fishes including pipefishes that use them as refuge from hydrodynamic forces and tidal regimes, shelter against predation and as feeding and nursery grounds (Hiddink & Jager, 2002; Teixeira & Vieira, 1995; Vincent, 1995b).

Pipefishes are an important ichthyofauna trophic component of the sub-aquatic vegetated and reef habitats in these ecosystems and can be found at depths from about 5 to 30 m (Dawson, 1985; Teixeira & Musick, 1995). Pipefishes share an interesting behavioural aspect in their reproductive strategy. The usual sex-roles are reversed, as males may limit the females reproductive success and/or the females compete with each other for access to mates (Wilson *et al.*, 2003; Berglund & Ahnesjö, 2003; Ahnesjö, 1996; Svensson, 1988). It has been observed that females of some species of *Syngnathus*, such as *S. typhle* may provide eggs to several males while males may receive eggs to fill their brood pouch from one or several females (Avise *et al.*, 2002; Berglund, 1991). The females will usually have oocytes at different developmental stages and will therefore have mature eggs continuously throughout the breeding season (Ahnesjö, 1996). The females therefore produce more eggs than the males can brood during an equivalent period of time (Berglund & Ahnesjö, 2003; Ahnesjö, 1996). The incubation period of *S. typhle* may extend to one month (Berglund & Ahnesjö, 2003; Ahnesjö, 1996), and male pipefishes release offspring that do not need any further brood care. Colouration can be variable within species for camouflage to match certain algae or sponges or between males and females during courtship or after mating (Kuiter, 2000). Pipefishes are generally diurnal and use their snout for suction feeding primarily on small crustaceans such as mysids or on larval fishes (Campbell & Able, 1998; Svensson, 1988). Pipefishes are poor swimmers and therefore catch prey by lying along the edges of reefs or attaching themselves to seagrass beds in strategic areas with moderate currents (Kuiter, 2000). Thus, the dependence of pipefishes on vegetation, reef and sheltered estuarine and coastal habitats makes them vulnerable to habitat alteration.

Ecological studies of South African estuarine and coastal systems through regional surveys have provided site-specific data for various South African fish species (Harrison, 2004; Whitfield, 1998; Day, 1981). These studies have also been useful in deriving local and regional spatial patterns of coastal and marine fish communities and the factors affecting them (Beckley *et al.*, 2002; Turpie *et al.*, 2000). However, most of these advanced ichthyofaunal surveys have not been uniformly spread along the coast and have been restricted to important commercial fishes (Turpie *et al.*, 2000). The ecology of non-

important commercial fishes has been neglected though they are major components of the community structure of reef and vegetative habitats. Estuarine fish communities have generally been studied comparatively to quantify species richness (Santoul *et al.*, 2005) and species assemblage patterns (Mbande *et al.*, 2005; Beckley *et al.*, 2002; Turpie *et al.*, 2000). Several factors and processes linked to exchanges between estuarine and coastal zones (e.g. vegetation, nutrient inputs, temperature, salinity, dissolved oxygen and river discharge) have been shown to affect the spatial and temporal changes in abundance and distribution patterns of estuarine fishes (Ramos-Miranda *et al.*, 2005; Harrison, 2004). For example, Harrison (2000) carried out a long-term survey of ichthyofaunal communities of South African estuaries at the regional scale and concluded that the geomorphology, opening phases and biogeographical region shape fish species richness and community assemblages. Although our understanding of the patterns and processes underlying the distribution of pipefish communities has made much progress in the past decade (Hiddink & Jager, 2002; Teixeira & Vieira, 1995; Gordina *et al.*, 1991; Ryer & Orth, 1987; Howard & Koehn, 1985) little is known about the spatial patterns of biological or life history traits in specific fish assemblages. Various studies done of fish distributions indirectly support the habitat template concept of ecosystems, which relates trends in species traits to habitat variability (Santoul *et al.*, 2005; Townsend *et al.*, 1997) as a theoretical framework for assessing and predicting the functional organization of river or estuarine communities (Schlosser, 1987). Consequently, the link between a species biological traits and environmental variability should be studied to explain the distribution of fish species.

The ecology and biology of some species of *Syngnathus* are well studied (Wilson *et al.*, 2003; Hiddink & Jager, 2002; Campbell & Able, 1998; Gordina *et al.*, 1991; Howard & Koehn, 1985; Gronell, 1984). However, published details of the life histories and field studies of South African pipefishes of *Syngnathus* are not available. Biological information on the two South African species is restricted to records of occurrence in broad-spectrum studies on specific estuaries (Whitfield, 1998) and offshore environments. Understanding South African pipefish life history becomes particularly important now that one of the species, *S. watermeyeri* has been added to Appendix II of

CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) (Foster & Vincent, 2004; Whitfield, 1995). Poorly planned and badly situated coastal development in sensitive areas due to expanding human populations, often without regard to the environment and river degradation (Whitfield, 1998) are the main causes for this pipefish's vulnerability. Pipefishes are among the many groups of fishes whose life histories and restriction to narrow strips of coastline or eelgrass habitats in the estuaries or rivers, making them highly vulnerable to habitat alteration. Comparative studies on seahorses have indicated that species with low rates of natural population increase, complex social behaviour and distributions in vulnerable habitats are more likely to be threatened by changes in habitats (Foster & Vincent, 2004). A thorough understanding of South African pipefish populations of *Syngnathus* and their spatial and temporal structure is therefore needed. Knowledge of the reproduction and development of these pipefishes is crucial in developing responsible management strategies for their conservation. Therefore, the purpose of this chapter is to summarize the distribution of South African pipefish of *Syngnathus* based on historical and recent survey data. Understanding the distribution of a species is one of the most important and basic requirements for effective conservation and ecological and evolutionary research. Emphasis will be placed on the South African population of *S. temminckii* ("*S. acus*") as this had a larger data set due to its wider distribution and therefore presence in ichthyofaunal surveys.

2.2 Materials and methods

2.2.1 Study area

The study area was the South African region where the two pipefishes of *Syngnathus* (*S. temminckii* and *S. watermeyeri*) have been previously recorded and were collected for this study. The South African coastline contains a wide variety of ecosystems, including 465 estuaries (Figure 2.1). It stretches for some 3100 km from the Orange River mouth (28° 38'S; 16° 27'E) on the west coast (Atlantic Ocean) to Kosi Bay (26° 54'S; 32° 48'E) on the east coast (Indian Ocean) (Harrison, 2004). Although numerous, South African estuaries are generally small and cover only some 600 km^2 of this coastline. The

characteristics of the estuarine types are dependent on their position along the coastline and thus on the climate of the region (Allanson & Baird, 1999; Whitfield, 1998).

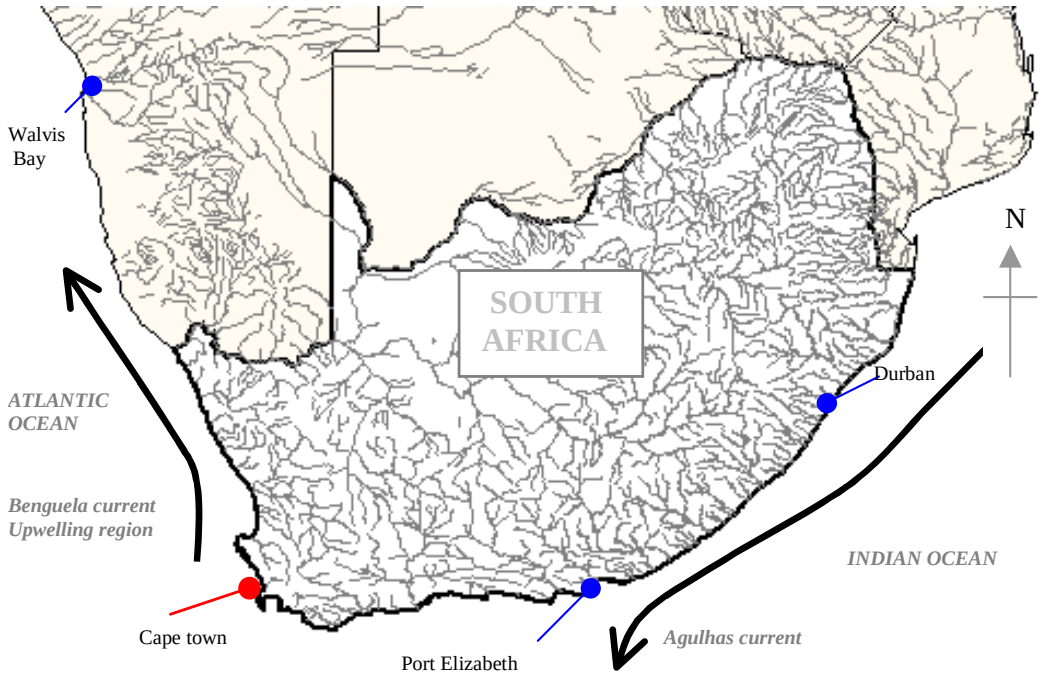


Figure 2.1: Map of South Africa showing estuaries with their associated river systems, the major offshore currents and the position of major coastal towns.

2.2.2 Data collection and sampling

Locality and distributional data for specimens of the two South African species of *Syngnathus* were determined from historical museum specimens in fish collections held in the South African Institute for Aquatic Biodiversity (SAIAB/RUSI) and the South African Museum - Iziko (SAM) collected between 1950 and 2003. The latitudes and longitudes for most localities of the collections were taken directly from the specimen's catalogue data. Details of the date of collection, locality and number of individuals per collection were also recorded. Field sampling covering the distributions of the two pipefishes was undertaken throughout the South African coastline (June 2003) including

six surveys in the Swartvlei estuary, every 2nd month from 2002 to 2003. The estuaries were sampled using a 2 m seine net during daylight hours. Seine netting was limited to shallow (<1.5 m deep), unobstructed areas of estuaries with gently sloping banks. Additional data on *S. watermeyeri* were incorporated from recently collected field data from the East Kleinemonde estuary between 1996-2003. Most of the specimens that were collected in these field surveys were returned alive to the habitat. Any specimens retained were preserved in 70% ethanol for DNA analyses. The available specimens from collections and surveys were identified and measured. The pipefishes captured were counted and also sexed to determine sex and size classes. All the collected specimens will be deposited in the SAIAB fish collection. Some specimens were dissected to get the numbers of eggs/oocytes in males and females.

2.2.3 Data analysis

2.2.3.1 Length-frequency distributions of South Africa species of *Syngnathus*

The standard length (SL) of all animals from museum specimens and routine collections were measured to the nearest mm using dial calipers. The pipefish were grouped by the date of collection and locality and divided into six length-classes (0-50, 51-100, 101-150, 151-200, 201-250 and > 250 mm) to obtain the length frequency distributions of each species. Juvenile specimens whose sex could not be determined were not included in the statistical analysis. Male pipefishes were examined for the presence of a brood pouch and the presence of eggs and embryos (if pigmented eyes were visible) in the brood pouch. Developing brood pouches were distinguished from fully developed pouches to identify immature and mature males (Plate I.3). Females were examined for the presence of eggs to determine the number of breeding and non-breeding individuals. The specimen data were coded for a given month of the year to determine the number breeding animals in that month for each species. The length-frequency distributions of the individuals of each species were also plotted with regard to sex and maturity. Abundances of the two species per given year or locality were not compared because different sampling gear was used by the different collectors depending on locations and collection dates.

2.2.3.2 Reproductive biology

The historical museum collections and routine sampling data from the Swartvlei estuary were analysed to estimate the duration of the reproductive season and time and details of spawning (size at maturity, fecundity and mating types). The reproductive season was estimated by considering the number of breeding individuals that were collected for each season among collection years. Sex and size classes (sex-age structures) and sex ratios were also determined to estimate the fecundity. Size frequency distributions were used as they describe the percentage of individuals in a population or assemblage that fall within defined size categories (Stergiou & Karpouzi, 2003). Skewness of these distributions from known normal distributions can be a sensitive indicator of deviations of observed from expected values. Chi-square (X^2) analysis was used to detect significant deviations from the expected 1:1 sex ratio at a type I error level with $\propto = 0.05$. These analyses were performed using the STATISTICA software package (StatSoft, 2002).

The length at maturity was taken as the size at which the percentage of the number of males and females in the sample that were pregnant or gravid was 50% of the number of analysed specimens. The traditional way of describing maturation in fisheries science is based on maturity ogives, which describe how the probability of an organism maturing during a given time interval depends on its age and size (Stearns & Koella, 1986; FAO, 1984). The size at first sexual maturity (L_{M50} = length at which 50% of individuals were mature) was estimated by fitting the fraction of mature individuals to a two-parameter logistic curve function estimated from the cumulative percentage of mature individuals from the length–frequency data. The curve was defined by the following equation (Weyl & Booth, 1999):

$$P(L) = \frac{1}{1 + e^{-(L-L_{M50})/\delta}}$$

where $P(L)$ is the percentage of mature fish at length L, L_{M50} is the length at (50%) maturity and δ is the width of the ogive.

2.2.3.3 Reproductive biology of *S. temminckii*

This analysis was based on data for *S. temminckii* because there were too few specimens of adult *S. watermeyeri*. Males and females were dissected to get counts of mature oocytes (those hydrated) as well as numbers of eggs/embryos in the male brood pouch. The average number of eggs/embryos in the male brood pouches and hydrated oocytes found in gravid females were compared using T-tests (STATISTICA) by defining sex as the independent variable and the numbers of eggs/embryos and hydrated oocytes as dependent variables. The obtained results were compared with information from field guides, published literature and other books, grey literature (reports, theses), and communications with syngnathid researchers on other species of *Syngnathus* (Foster & Vincent, 2004; Wilson *et al.*, 2003; Berglund & Ahnesjö, 2003; Jones *et al.*, 2003; Campbell & Able, 1998; Gordina *et al.*, 1991; Ryer & Orth, 1987; Howard & Koehn, 1985).

2.3 Results

2.3.1 Distribution data

A total of 545 specimens of *S. temminckii* and 134 specimens of *S. watermeyeri* were available from historical museum collections and routine survey collections conducted between April 1950 and December 2003 (Figure 2.2). The list of the museum specimen catalogue numbers used in the study is given in appendix III. The long snout pipefish *S. temminckii* was consistently present in collections throughout the 53-year collection record, while *S. watermeyeri* was absent from collections for 21 years between 1963-1994. All records of this species occurrence and distribution after 1995 are from the East Kleinemonde estuary. The species has not been collected in the other three estuaries although routine ichthyological surveys had been done on these systems (Whitfield, 1998). The Catch per Unit Effort (CPUE) over most of the estuaries ranged from 0 to 5 fish per seine (2 m).

Table 2.1: Distribution records of specimens of South African *S. temminckii* and *S. watermeyeri* and the number of individuals used in the analysis. The * next to a location indicates that specimens were also collected during the recent surveys.

LOCALITY	Latitude	Longitude	Sea access	n
S. temminckii				*543*
Umkomaas (on pipeline)	30°12'00"S	30°48'00"E	Marine	1
Kwelera (1st tributary)	32°54'00"S	28°05'00"E	Temporally open	5
East London	33°01'42.9"S	27°54'57.3"E	Temporally open	15
Keiskamma	33°18'00"S	27°29'00"E	Temporally open	3
Great fish river*	33°29'37.3"S	27°08'10.0"E	Permanently open	101
Kowie*	33°36'11.2"S	26°54'10.2"E	Permanently open	7
Kariega*	33°40'55.9"S	26°41'15.6"E	Permanently open	104
Bushmans *	33°41'41.0"S	26°39'48.6"E	Permanently open	6
Sunday's	33°43'00"S	25°51'00"E	Permanently open	5
Swartkops*	33° 57'S	25 °28' E	Permanently open	23
Algoa bay	34°02'00"S	25°42'00"E	Marine	4
Gamtoos	33°58'00"S	25°03'00"E	Temporally open	1
Cape St Francis	34°13'00"S	24°50'00"E	Temporally open	1
Kromme*	34°08'27.9"S	24°50'36.7"E	Temporally open	4
Keurbooms	34°02'00"S	023°23'00"E	Marine	3
Knysna*	34°04'38.9"S	23°03'33.4"E	Temporally open	108
Swartvlei*	34°01'51.2"S	22°47'49.3"E	Temporally open	126
Breede river	34°24'00"S	20°50'00"E	Marine	4
Klein*	34°25'25.0"S	19°18'13.4"E	Temporally open	10
False Bay*	34°11'00"S	18°26'00"E	Marine	3
Great Berg*			Permanently open	2
Lamberts bay	32°05'00"S	18°18'00"E	Marine	3
Luderitz bay*			Marine	2
Walvis bay*	22°56' S	14°30' E	Marine	2
S. watermeyeri				*108*
East Kleinemonde*	33°32'21.8"S	27°02'55.2"E	Temporally open	67
West Kleinemonde*	33°32'28.2"S	27°02'51.7"E	Temporally open	3
Bushmans*	33°41'41.0"S	26°39'48.6"E	Permanently open	25
Kariega	33°40'55.9"S	26°41'15.6"E	Permanently open	11
Kasouga	33°39'00"S	26°44'00"E	Temporally open	2

The number of specimens per locality as well as access of the locality to the sea were also determined and are shown in Table 2.1. The Swartvlei estuary has the highest number of specimens due to the 229 juveniles that were sampled during the routine surveys of this estuary. The distribution map of *S. temminckii* based on the available latitude and longitude data of the specimens collected during the surveys or available from museums is given in figures 2.3. The distribution of *S watermeyeri* from the available museum

specimens and surveys done during this study was similar to that from historical literature (See figure 1.6 on page 7 in chapter 1 for the distribution map). The records confirm that this species is only known from the Kleinemonde, Kasouga, Bushmans and Kariega estuaries. The results suggest *S. temminckii* has a wide distribution in Southern Africa ranging from Walvis Bay in Namibia to as far as the coastal areas in the East (Umkomaas) in the Kwa-Zulu Natal (KZN) province of South Africa. The species has marine as well as estuarine populations. *Syngnathus watermeyeri* on the other hand is more restricted and was only collected from the East and West Kleinemonde and Bushmans estuaries during the recent surveys with no record or evidence of a marine population.

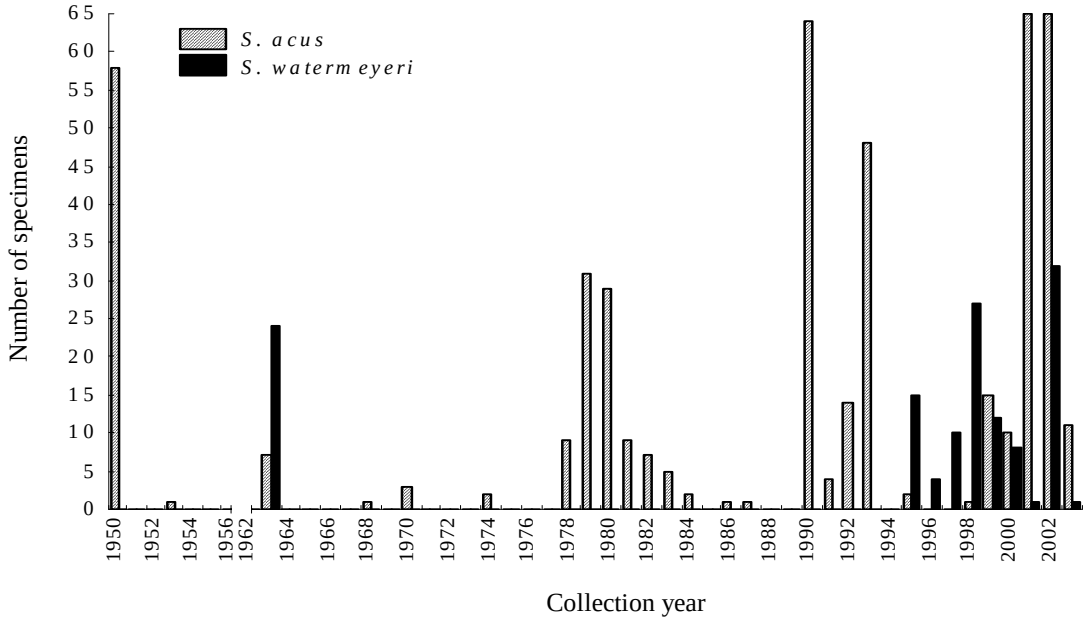


Figure 2.2: Numbers of South African pipefish specimens of *S. temminckii* and *S. watermeyeri* available from museum collections (1950-2003) and survey data.

The size-length frequencies for the two South African species are given in figure 2.4. The juveniles sampled from the Swartvlei routine surveys are not included in this graph. The standard length of *S. temminckii* specimens ranged from 10-300 mm. The standard

lengths of *S. watermeyeri* specimens were much lower for this smaller species with the smallest individual being 58 mm and the largest was 147 mm. There were more juvenile specimens of *S. temminckii* collected during the surveys than *S. watermeyeri*. The most abundant size class for adults of both species was the 101-150 mm category. The frequency distribution of monthly catches of the two species of *Syngnathus* from the collection data and sampling done as part of this study is shown in figure 2.5 where the numbers of catches of fish for each month were grouped for all the collection years. They therefore represent the entire period of available specimens from 1953-2003. The highest numbers of pipefishes were caught during the spring and summer period from September to May for *S. temminckii* and from August, April and February for *S. watermeyeri*. Peak number of individuals was in November for *S. temminckii*. There were very few fish caught during the winter months (June and July) for both species and the summer month of January for *S. watermeyeri* although there were surveys during these months.

Figure 2.3: Distribution map of *Syngnathus temminckii* in Southern Africa based on the SAIAB fish collection and recent sampling records, with the position of major coastal towns.

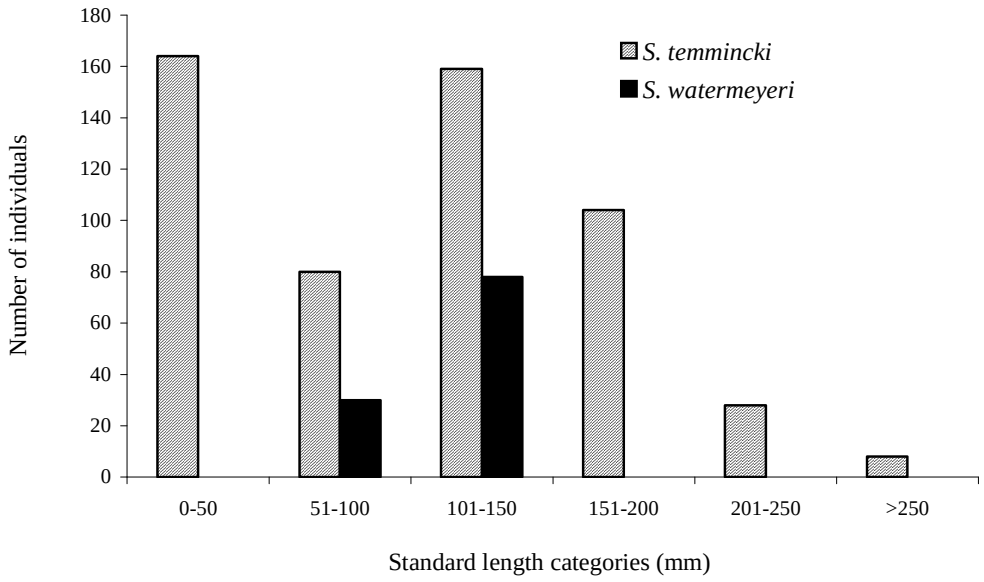


Figure 2.4: Size classes of all South African pipefish specimens of *S. temminckii* (n = 543) and *S. watermeyeri* (n =108) in the SAIAB fish collection and from routine surveys.

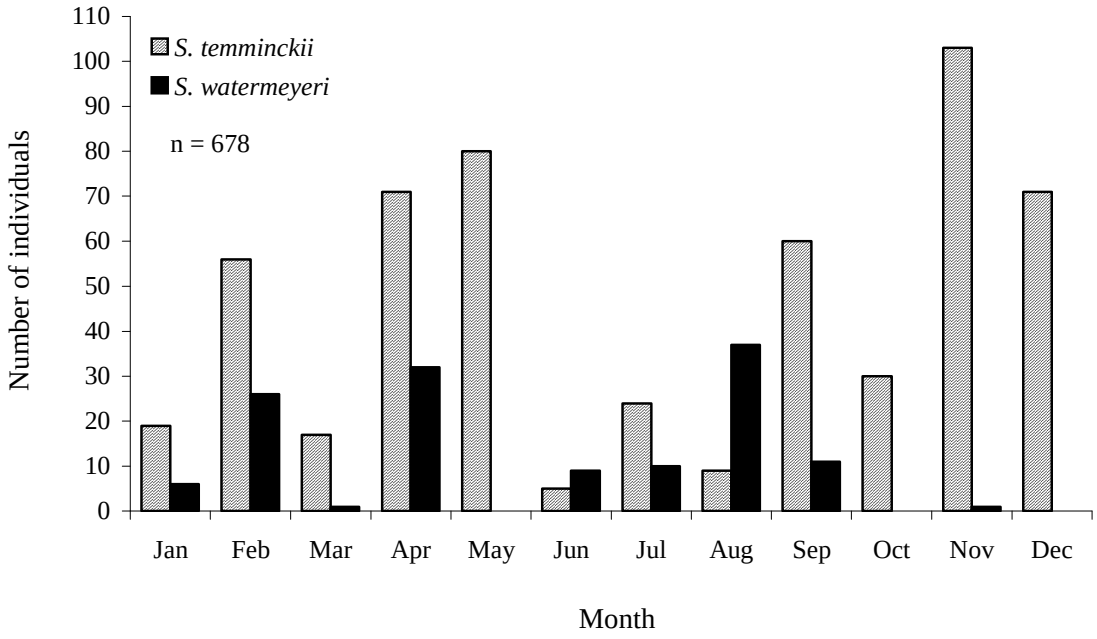


Figure 2.5: Total monthly catches of *S. temminckii* and *S. watermeyeri* compiled from the two Museum collection records and additional sampling data.

2.3.2 Reproductive biology

The length-frequency distributions of males and females for the two species are shown in figure 2.6. The observed sex ratio for specimens of *S. temminckii* of 0.6:1.0 (114 males to 203 females) was biased towards females (X^2 = 24.99, P < 0.05) and significantly different from the expected 1:1 ratio. The observed sex ratio for *S. watermeyeri* (0.7:1.0) was also biased towards females representing 32 females and 22 males (X^2 = 1.85, P < 0.05) and significant. Brooding males and gravid females of the two species were present almost throughout the year except for April and May for *S. temminckii* and June and July for *S. watermeyeri*. However, this may not be significant as it may be related to sampling effort.

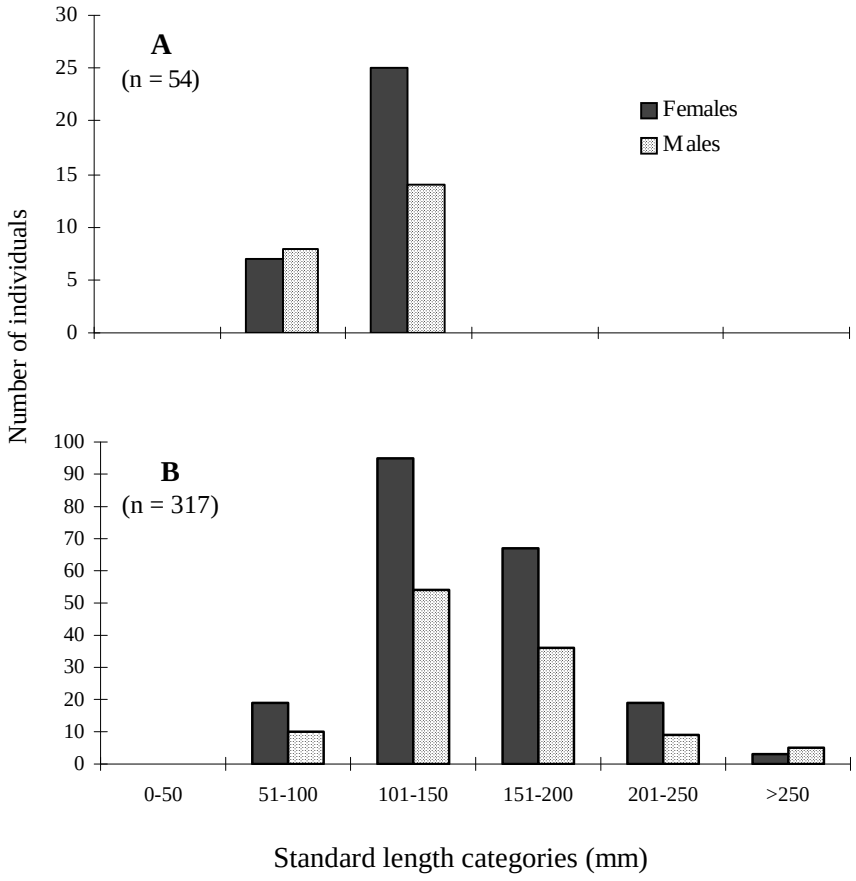


Figure 2.6: Length-frequency distribution of males and females for all the analysed specimens of *S. watermeyeri* (A) and South African *S. temminckii* (B).

The monthly length-frequency distribution (figure 2.7) suggests that the breeding season for *S. temminckii* population is during spring and summer from August to February with peaks from September to January. There was only one small museum collection in October, which may explain the lower numbers in this month.

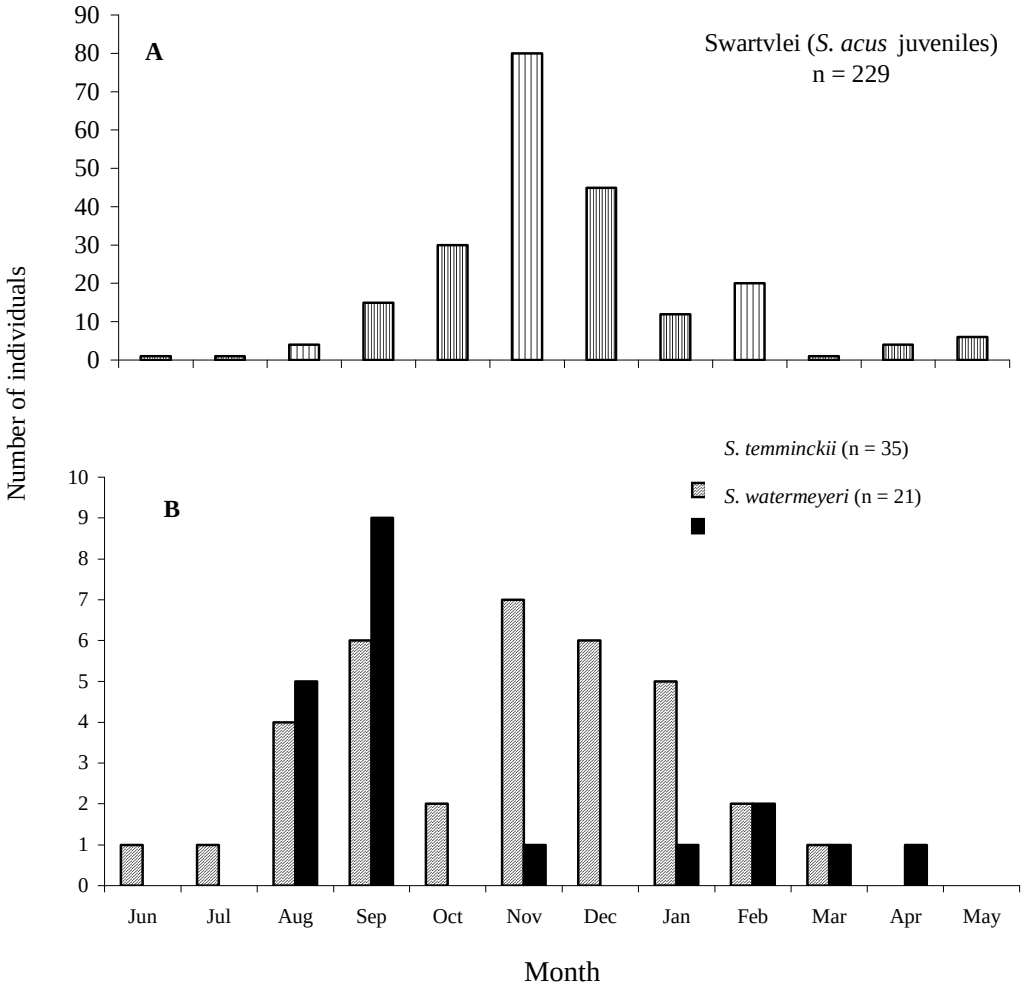


Figure 2.7: Monthly distribution of the number of juvenile pipefish (10-90 mm) of *S. temminckii* collected throughout the 2002 and 2003 study period in the Swartvlei estuary (A) and the number of gravid females and pregnant males collected throughout the year for all analysed specimens of South African *S. watermeyeri* and *S. temminckii* (B).

However, the sampling data from the Swartvlei, also suggested that the spring season is the peak of the breeding season for this species (figure 2.7) as juvenile pipefishes (< 50 cm) were collected throughout the year in all sampling localities with high densities during spring and early summer. The data on the monthly distribution of breeding adults of *S. watermeyeri* was too low to allow confident conclusions. It is hypothesized that this species breeds during spring, as the highest number of breeding adults was found between August and September.

The number of males and females in the sample that were pregnant or gravid for each size category are given in figure 2.8. The number of gravid females for each category appeared to be higher than that of pregnant males. However, this could not be tested. The smallest male and female of *S. watermeyeri* carrying eggs were 94 and 96 mm (SL) respectively. It appears most breeding animals are found between 100-150 mm standard length and that most of the males and females of this species mature in this size category (graph A in figure 2.8). There were also a higher number of gravid females than males for the 100-150 mm SL size category. The smallest male of *S. temminckii* carrying eggs was 108 mm, while the smallest female was 105 mm (SL).

The estimated size at 50% maturity for these two species (figure 2.9) were different, as can be expected due to the difference in maximum size between the species. Size at 50% maturity was estimated as 102 mm for females and 118 mm for males of *S. watermeyeri*. The estimated size at 50% maturity for *S. temminckii* was 120 and 129 mm SL for females and males respectively. Furthermore, sizes at 100% maturity were approximately 140 mm and 170 mm for *S. watermeyeri* and *S. temminckii*, respectively. It is important to note that all the males of *S. temminckii* in the sample that were greater than 250 mm in standard length were carrying eggs.

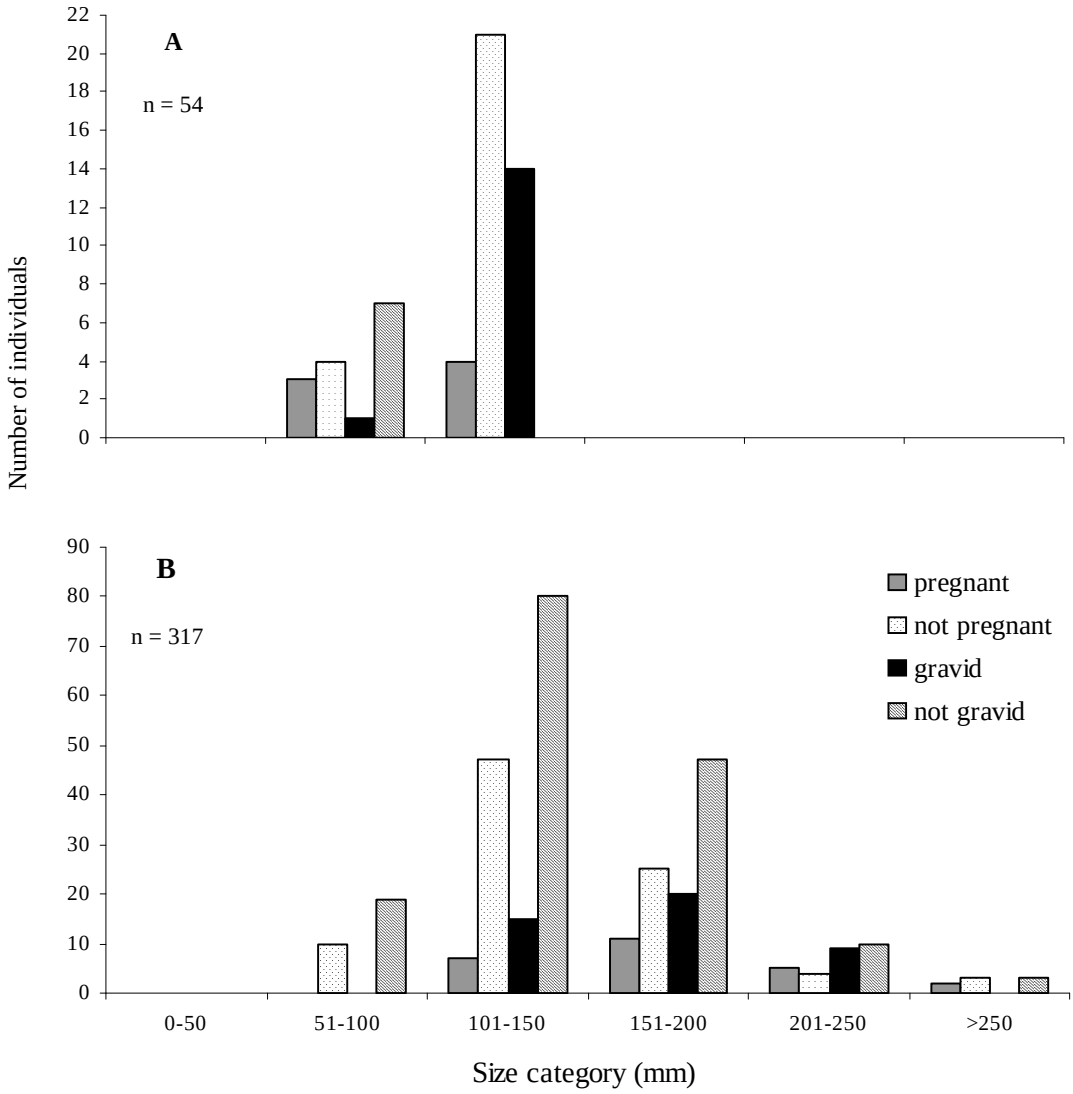


Figure 2.8: Length-frequency distribution of the comparison between gravid/not gravid females and pregnant/not pregnant males for all the analysed specimens of the two South African species of *Syngnathus.* A = *S. watermeyeri* and B = *S. temminckii*.

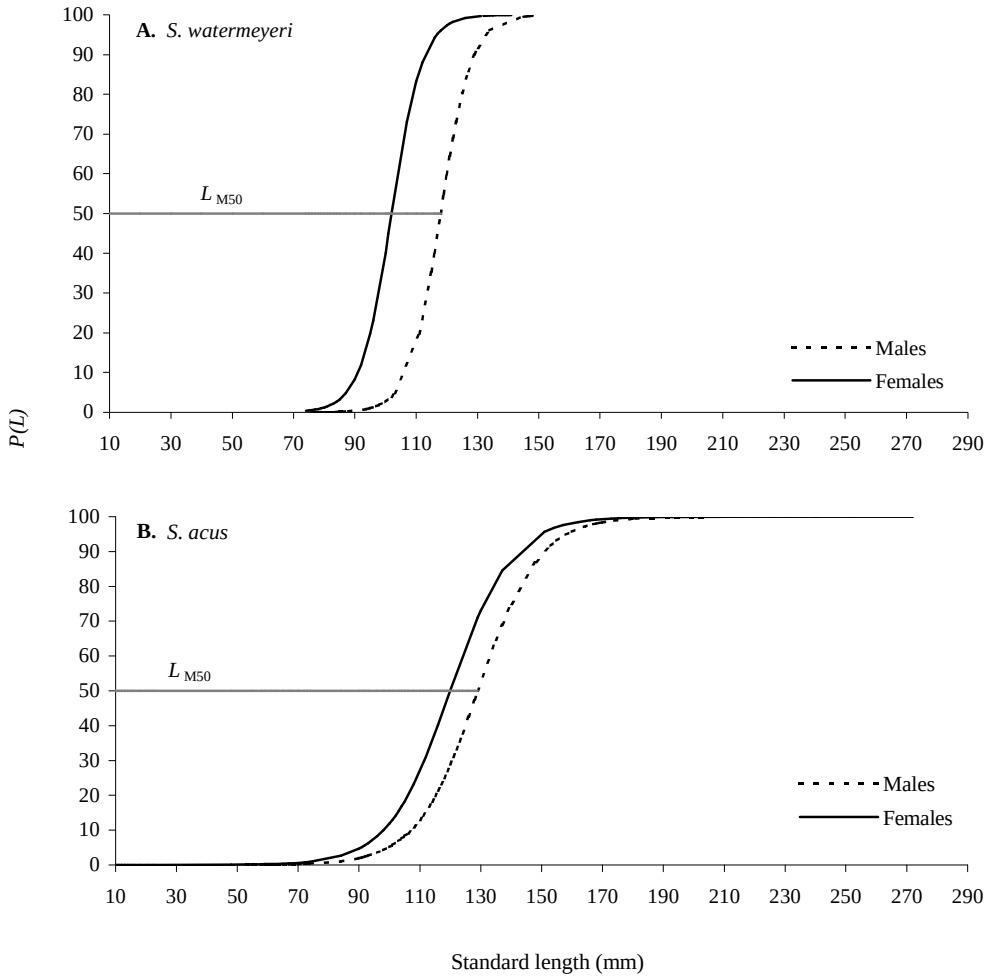


Figure 2.9: Logistic ogives fitted to the percentage of sexually mature males and females of *S. watermeyeri* (A) and *S. temminckii* (B) illustrating the average size at 50% maturity (L_{M50}).

2.3.2.1 *Syngnathus temminckii* (South Africa)

Seventeen females and 13 males of *S. temminckii* were dissected for reproductive analysis. The results of T-tests revealed that the mean number of mature oocytes in the females (379) was not significantly different at $P > 0.05$ ($P = 0.06$; $F = 2.61$) from the mean number of eggs/embryos in the male brood pouch (551) (Figure 2.10). The T-test was performed because the Levene test for homogeneous variances was not statistically

significant (P = 0.14; F = 2.35). The lowest numbers of oocytes and eggs carried by males and females were 86 and 105, respectively. The relationship between number of eggs/embryos and oocytes and standard length was linear and positively correlated for all males and females (eggs/oocytes = 6.13 SL – 778, r^2 = 0.56, n = 30) implying that fecundity increases with the size of the individual.

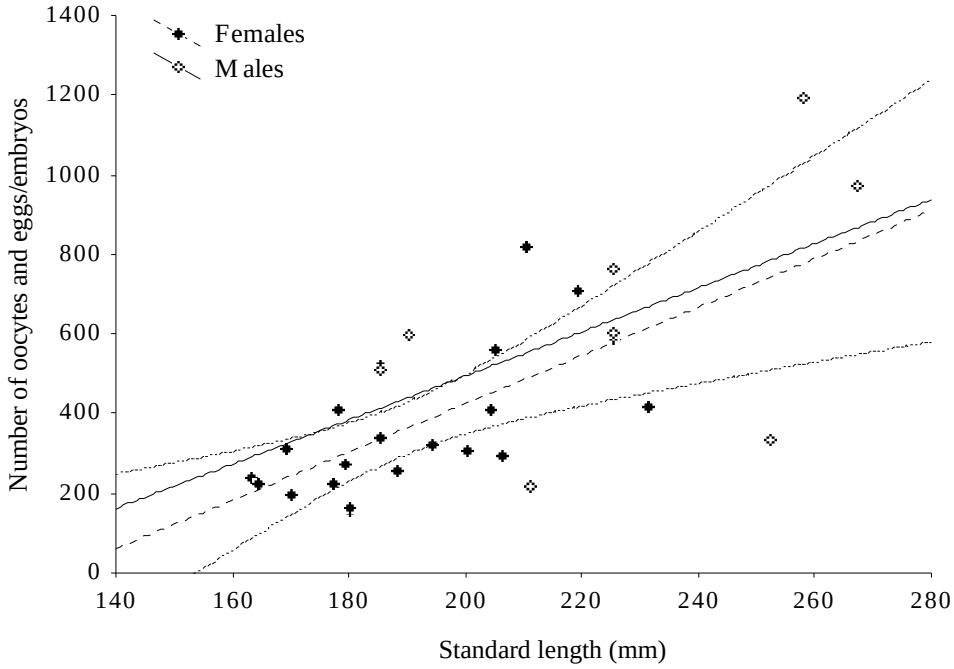


Figure 2.10: Regression of number of oocytes and eggs/embryos against standard length in the population of *S. temminckii*. The 95% confidence interval for the number of oocytes is also displayed.

The regression equations for both sexes between number of eggs/embryos or oocytes and the standard length were: number of eggs/embryos per male = 6.17 SL – 765 (n = 13; r^2 = 0.63) and number of oocytes per female = 6.35 SL – 845 (n = 17; r^2 = 0.47). The comparison of slopes, which appeared slightly higher for males than for females, did not provide evidence for significant differences between sexes (ANCOVA; P = 0.29)

indicating a similar increase in fecundity (number oocytes or eggs) with increasing length for both sexes. However, males were significantly larger than the females. The maximum number of eggs carried by males was outside the 95% confidence interval of the range carried by females suggesting that males may be receiving eggs from more than one female in this species. However this was not significantly different from the average number of eggs carried by the females ($F = 3.79$, $P = 0.06$).

2.4 Discussion

2.4.1 Population distribution

The results of the analysis suggest the existence of an established breeding population of *S. temminckii* in Southern Africa. There was a continued capture of *S. temminckii* in South Africa over a period of 50 years as a result of various surveys. Furthermore, the distribution range of this species and the continuous presence of juveniles and sexually mature males and females in various surveys is also an indication of an established species. As noted in the literature, this species can reproduce, stay within and complete its life cycle in estuarine environments (Whitfield, 1998). Coastal lagoons and estuaries are highly productive ecosystems that can serve as habitat and nursery areas for juvenile stages pipefishes (Ramos-Miranda *et al.*, 2005). However, the data on the occurrence of this species in coastal areas shows that the species is not totally dependent on estuaries, although *S. temminckii* individuals spend the juvenile and adult phases of their life cycle within open or closed estuarine systems. It has been suggested that most pipefishes associate strongly with seagrasses, but this is likely to be a reflection of researchers having easy access to pipefishes at shallow depths, or from collecting or trawling (Kuiter, 2000). Pipefishes are more difficult to find in deeper water and trawlers avoid reef habitats where they may be found. Survey results (Whitfield, 1998; Russell, 1994) have suggested that the upper salinity tolerance for *S. temminckii* is approximately 42‰. Thus, survival in, and dispersal via the marine environment is likely. Laboratory trials on salinity tolerance should be conducted to test this hypothesis. However, most of the field data were from seine netting surveys in estuaries, little trawling or collecting was done in coastal areas. It is therefore reasonable to expect that the South African *S. temminckii* populations have an even wider distribution range than reported.

The *S. temminckii* population was most prevalent in the warm temperate and cool temperate regions of South Africa. Thus this species may prefer water temperatures below 25ºC. For example, a mass mortality of pipefishes was recorded in the Swartvlei estuary when temperature exceeded 32ºC (Russell, 1994). Therefore, the two South African species of *Syngnathus* are restricted in their distribution to specific habitats and biogeographical zones. Even in the tropical zones such as Southeast Asia where a number of pipefish genera have evolved independently, species of pipefishes are generally restricted in their distribution to specific biogeographical regions (Kuiter, 2000). This restriction could be due to... However, the collection of this species in the sub-tropical area at Umkomaas demonstrates that this species is not only found in colder waters.

The distribution of *S. watermeyeri* unlike *S. temminckii* is, however, very restricted and no collections of the species have ever been made in other estuaries, apart from the four adjacent estuaries in the Eastern Cape of South Africa. Given the currently available records, we can hypothesise that either *S. watermeyeri* has been lost from various South African estuarine systems, or that the species has never colonised them. Although the historical distribution of this species covered adjacent estuaries over a distance of about 100 km, the species was never recorded from the Kowie estuary, a system that is in the middle of this distribution range and has a population of *S. temminckii*. We would expect to find *S. temminckii* and *S. watermeyeri* occurring together in this system as the two species are present in the other four estuaries. If *S. watermeyeri* has been lost from the Kowie then there is a need to find reasons. An environmental explanation is unlikely given that climatic conditions are fairly uniform throughout this region, and physicochemical characteristics of this estuary are very similar to those in estuaries where the species is extant or has been previously recorded. Another reason for the apparent loss could be through competition with other fishes, and in particular *S. temminckii*. However, the extent to which differences between closely related species are the evolved outcome of ecological interactions between them is not yet known for most organisms (Schluter, 2003). There is no evidence whether these two closely related species show differences in morphological traits that affect resource use to verify whether there is resource competition between them. Although, the two species currently occur in the

Kleinemonde estuary, there has been no record of *S. watermeyeri* specimens since 2000. The remaining hypothesis that would explain the restricted distribution of *S. watermeyeri* is that the species has never inhabited these estuaries from which it is absent, as the species just like other pipefishes may be a poor disperser and may have evolved within the Eastern Cape. However, this species occurs within the area near Port Elizabeth on the south coast where the highest number of endemic South African species has been recorded (Beckley *et al.*, 2002; Turpie *et al.*, 2000). This area is characterised by changes in ocean currents with variable weather patterns that may be significant in creating isolation and speciation among fish species and may be responsible for *S. watermeyeri* evolution (Teske *et al.*, 2005).

2.4.2 Reproductive biology

It appears that the spawning period of the two South African species covers several months. The occurrence in the collections and surveys of a large number of newly hatched young, males with eggs in the brood pouch and gravid females suggests that spawning continues from June to March for *S. temminckii* and August to May for *S. watermeyeri*. However, due to the small sample sizes of the endangered *S. watermeyeri* these apparent differences in spawning season were not analysed statistically. The spawning season for *S. temminckii* occurs mainly in later spring and early summer, with reproductive activity peaking in November and coinciding with higher water temperatures. Although no data on *S. temminckii* was available in October, the peak increase of breeding adults in November coincides with the observed increase from August to September and suggests that lack of specimens is merely a result of a lower sampling effort. These results are similar to the observations of larvae of this species in the Swartvlei estuary that were also most prevalent during this time.

The estimated spawning period of *S. temminckii* was longer than the periods reported for most species of *Syngnathus*. A Black Sea species, *S. schmidti*, was shown to have a spawning season that commenced in summer (May to October) and lasted for approximately six months (Gordina *et al.*, 1991). The long spawning period for *S. temminckii* is also supported by the fact that newly hatched fish, juveniles and breeding

adults were observed in every month. The results reflect reduced spawning activity during the colder months when the temperature may not be optimal. Although there was less data for *S. watermeyeri*, it appears that this species also spawns in spring and summer seasons at higher water temperatures. The food resources during the warmer months may play a significant role in supporting the observed seasonal patterns of reproduction within these two species. A recent study has observed that the highest abundance of larval fishes and zooplankton in some south and west coast estuaries of South Africa occurs during the summer months (Fortuin, 2005). A lack of growth and reduced breeding activity during winter months is common for other *Syngnathus* (Campbell & Able, 1998) and other temperate estuarine fishes (Blaber, 2000; Whitfield, 1998) and may also coincide with reduced food resources.

The sex ratio was estimated because it has been said to be the best predictor of sex-roles and can therefore be used to determine which sex competes more actively for potential mates (Wilson *et al.*, 2003; Berglund & Ahnesjö, 2003). The study found relatively more females than males for the two South African species. This skewed sex ratio with more abundant females is typical of pipefishes of *Syngnathus* and has been observed among various species. Targett (1984) and Herald (1941) observed a skewed ratio biased towards females in the gulf pipefish (*Syngnathus scovelli*) and kelp pipefish (*Syngnathus californiensis*) ranging from 1:15 and 1:3 male to females respectively. Berglund and Rosenqvist (1993) observed an excess number of reproducing females in *S. typhle* and concluded that males act as reproductive “bottlenecks” thereby skewing the operational sex ratio. A skewed sex ratio is expected to have an effect on the spawning system of a species since the sex in excess will compete actively for mates (Berglund, 1991; Svensson, 1988). Therefore, the two South African species of *Syngnathus* may exhibit sex-role reversal with females competing for males.

Males of South African *S. temminckii* may also exhibit “polygamous” behaviour, as the numbers of eggs/embryos in the male brood pouch were higher than the average number of oocytes found in gravid females. The number of eggs in one male was twice the number of eggs found in the females. This suggests that males can mate with more than

one female during the reproductive season, as the carrying capacity of a male brood pouch appears to be larger. It is possible that there is a difference between the time required by the male to brood the eggs and the time required for the female to develop hydrated oocytes. In such cases if the egg production is much faster than the male brooding period and if there is a male shortage, females can successfully mate with more than one male (Teixeira & Vieira, 1995; Gronell, 1984). Furthermore, there were differences in the number of eggs carried by the male with regard to size. The positive correlation between number of eggs in the brood pouch and the size of the individual suggests that larger males may be more reproductively efficient. This was also supported by the estimates for size at maturity (figure 2.10), which showed that males attain sexual maturity at a larger size than females for both *S. temminckii* and *S. watermeyeri*. Size at maturity was estimated as 102 mm and 118 mm for *S. watermeyeri* and 120mm and 129 mm for *S. temminckii* males and females respectively. The number of oocytes in a gravid female was also positively correlated with fish size.

The results therefore suggest that the size of males and females may play a major role in mating. For example, the effect of size on mating success has been observed among various species of *Syngnathus*, as larger females are preferred by males, possibly because they produce larger eggs, which improves the males reproductive success (Berglund & Rosenqvist, 1993; Berglund, 1991). It was observed that the oocytes of a gravid female were of different sizes and possibly different stages of development. The high proportion of reproductively active females, their presence over the whole study time and the different sizes of oocytes in females may indicate that *S. temminckii* is a batch spawner (Hesp *et al.*, 2002). An extended spawning period of several months, or during the whole year, with possible bimodal intensity is a character of an indeterminate spawning species (Hesp *et al.*, 2002). However, this hypothesis should be tested in further studies using histological data.

2.4.2.1 Reproductive strategy

The theory of r and K selection predicts that species in different environments will differ in life history traits such as size, fecundity, age at first reproduction, number of reproductive events during the lifetime, and total life span (MacArthur & Wilson, 1967). Most natural habitats offer intermediate conditions so that fish will tend either to the r-end of the spectrum or the K-end, where r refers to the strategy involving many small and fecund organisms, and K refers to the strategy involving larger and fewer organisms (Flegr, 1997; MacArthur & Wilson, 1967). The two South African species represent an interesting scenario with regards to their reproductive model. The two species exhibit some r-selection properties in that they occur in estuarine and coastal areas, which are very unstable habitats although highly productive (Appendix IV). *Syngnathus temminckii* has a high reproductive output (more than 500 eggs in one brood pouch) that is expected for r-selected species and with repeated breeding, which provides for a fast population increase. The estimated population sex ratios and sizes at maturity also suggest that males and females of *S. temminckii* and *S. watermeyeri* may have different distributions, activity or movements, and variation between sexes in growth, mortality and longevity. Furthermore, the males and females of pipefish species have been observed to mature within the first and second year of birth with a short life span of less than four years (Campbell & Able, 1998; Vincent, 1995a; Vincent, 1995b).

However, the two species also exhibit some K- selection properties in that the species are brooders, and spend time and resources ensuring survival during gestation, and produce young that are fully developed and independent. However they provide no parental care unlike most K- selected organisms. Many species of fish generally show a wide range of the r-K continuum (FAO, 1984). Although about 44 embryos (Whitfield, 1995) have been recorded in *S. watermeyeri*, no conclusion can be made about this specie's r-K range. Many r-selected species are associated with unstable environments that alternate between periods of abundance and periods of high mortality and therefore exist below the carrying capacity of an environment (FAO, 1984). *Syngnathus watermeyeri* inhabits such habitats and has limited dispersal capabilities just like other pipefish species. The absence of its juveniles in collections and its endangered status may also imply an unstable

environment. A mass mortality of *S. watermeyeri* was recorded during a survey by this study after an estuarine opening event when 40 dead pipefishes were collected after stranding in reed beds of the East Kleinemonde estuary. A population genetic analysis of *S. watermeyeri* may show a population demographic pattern of an expanding population following a bottleneck event. It appears therefore that a reproductive strategy that may fall within the r range of the r-K continuum may have evolved within these two South African species of *Syngnathus* employ. While the concept of r/K selection has drawn some criticism, it has generally proven useful in explaining how life history traits influence the ability of a species to respond to environmental changes (Begg *et al.*, 1999b; Campbell & Able, 1998).

2.4.3 Conclusions

In summary, this study shows that the knowledge about South African pipefishes is very useful in understanding life history and population dynamics. High priority should be afforded to understand and conserve these vulnerable fishes, particularly as they are part of the large endemic component of the South African ichthyofauna. Though these species may live in a variety of habitats with a moderately large geographical range, they are highly vulnerable due to the unstable environments they reside in and their low fecundity and limited dispersal capacity.

Chapter Three

Morphological variation among South African species of the genus *Syngnathus*

3.1 Introduction

Morphology has been the primary source of information for taxonomic studies and evolutionary relationships of fishes (Mattern & McLennan, 2004; Foster & Vincent, 2004; Dawson, 1985; Fritzsche, 1980; Gill, 1905). It is the basic fundamental observation in biology. Species of almost all groups of organisms have been described and identified on the basis of comparative anatomy or morphological data (MacLeod, 2004; Hillis & Wiens, 2000). The differences that exist today between species in morphology, physiology, ecology, behaviour and distribution have evolved in the course of phylogenesis (Avise, 2000; Hennig, 1965). Patterns of phenotypic variation have therefore been interpreted within an evolutionary history framework in attempts to elucidate how these factors are responsible for the differentiation and observed variations within and between species (Hillis & Wiens, 2000). Despite the availability of new methods like genetic and behavioral studies, systematic ichthyologists have continued to rely heavily on morphology for taxonomic characters (Boyd, 2002; Kuiter, 2000).

There are various reasons for this, one being that morphological studies allow for much more thorough taxon sampling than is generally possible with molecular data (Wiens, 2001; Stepien & Kocher, 1997). Morphologists can analyze large samples of each species from throughout its range, and can examine all known specimens for many groups of poorly known organisms where the only known specimens are represented by the holotype or type series (Hillis, 1987). Analyses of large sample sizes in molecular studies are often limited by availability of specimens (because of the inapplicability of museum specimens) and/or expense of analysis (Hillis & Wiens, 2000). Inclusion of sufficient taxa in systematic studies is important as it can improve accuracy of character reconstruction and phylogenetic estimation (MacLeod, 2004; Wiens, 2001; Avise & Wollenberg, 1997). Furthermore, ancestral character states can be used and easily scored for a variety of taxa in morphological analyses where fossils and/or suitable outgroups

are available. Other taxonomic methods such as behavioral studies are not easy to plan and obtain. Biological studies are also reliant on morphological data to determine the species being sampled (Brooks & McLennan, 2002).

Traditional morphometric analysis is still popular and has become more effective due to the use of improved statistical and multivariate analysis. However, many aspects of morphological analysis are still controversial, including the way in which characters are selected, whether intraspecific characters can be included, how within-species differences are coded, how character states are ordered and how morphological characters are weighted relative to each other (MacLeod, 2004; Brooks & McLennan, 2002; Wiens, 2001; Avise, 2000). Ideal characters are those that vary between the groups being studied but not within them (Wiens, 2001). A thorough understanding of a character's distribution is important because polymorphism not only occurs at the level of surface traits of a species but also at the level of underlying mechanisms. The mechanisms may involve effects of factors such as sex and growth stage that may in turn be potential confounding factors in the analysis (Ereshefsky & Matthen, 2005). A lack of difference between groups or species in one character does not overrule the differences observed in other characters (Hillis & Wiens, 2000; Hillis, 1987). A proper approach to morphological analysis must therefore recognize these differences and explore the mechanisms that produce and maintain them (Ereshefsky & Matthen, 2005).

3.1.1 The effect of size on morphological variation

Morphological characters describe variation that is fundamentally quantitative whether it is variation in relative size or shape or in counts of meristic characters (Wiens, 2001). Size and sex are the two main factors referred to in the context of non-geographic variation that have to be accounted for when distinguishing species (Swain & Foote, 1999). Size is critical because it can vary with developmental stage such that comparing groups with different average sizes can result in erroneous results in species distinctions. It is also necessary to check for the effect of sex on morphology because males and females can be very different morphologically within a species (Palma & Andrade, 2004). It has been shown that the size factor can account for up to 80 % or more of the

variation among a set of variables (Tzeng & Yeh, 2004), and may thus play a predominant role in creating bias if not removed from morphometric analysis. Variation in fish size is therefore usually assessed by several normalization methods that have been shown to eliminate the influence of size due to allometric growth (Doherty & McCarthy, 2004; Lleonhart *et al.*, 2000).These procedures involve allometric equations that not only quantify the size dependence of a trait, but also permit comparisons among individuals, populations, or species that differ in body size (Sinervo & Huey, 1990). The most common method to eliminate the effect of size and reduce all the individuals to the same size is by adjusting the ratio of every measurement to a selected independent variable. For example, most studies of fish have adjusted the length of variables such as head length using the standard length as the independent variable (Sinervo & Huey, 1990; Herald, 1941). However, this procedure is only valid if the species under study exhibits isometric growth, that is shape and ratios do not change with size. Several other methods like allometric models, regression analysis and multivariate analysis have also been used to correct for the size factor (Lleonhart *et al.*, 2000; Reist, 1985). These procedures have been shown to adequately achieve size and shape separation in fish analyses and reasonably meet statistical assumptions (Palma & Andrade, 2004; Langerhans *et al.*, 2003; Innes & Bates, 1999; Reist, 1986). For example, a clear morphological difference based on 18 adjusted morphometric characters for *Pagrus pagrus* samples, and 14 for both *Pagellus bogaraveo* and *Dentex dentex*, was displayed between Atlantic and Mediterranean samples (Palma & Andrade, 2004).

However, Swain and Foote (1999) have emphasized that body size variation is not simply a component that needs to be corrected for or even removed and ignored, but that it is instead a biologically important variable that needs to be considered in conjunction with variation in shape for some fish species. Body size has been shown to have a strong influence on an organism's life history (Knouft, 2003). Talk about cryptic species. The measures of size differences between species can be indicators of niche differentiation in similar coexisting species due to morphological or behavioral characteristic variation (Knouft, 2003; Guill *et al.*, 2003). For example, body shape has been correlated with the extent of stream residence among the species of *Oncorhynchus* (Swain & Foote, 1999).

Body shape has also been linked to the ecological characteristics and evolutionary relationships within *Etheostoma* (Knouft, 2003). Guill *et al.* (2003) found significant and considerably greater body depth of *Etheostoma caeruleum* as compared to other darter species (*E. nigrum* and *E. stigmaeum*) when comparing morphological variation.

3.1.2 South African species of *Syngnathus*

As previously stated (Dawson, 1986), taxonomic relationships within *Syngnathus* require clarification. Previous reviews of pipefish genera by Dawson (1985 and 1986), Herald (1941) and Fritzsche (1980 and 1981) were based on specimens from limited geographic areas such that the conclusions reached were often inaccurate or turned out to be invalid. Species within *Syngnathus* have been recognized on the basis of differences in morphological features that generally overlap among species within this genus (Dawson, 1985). Variation in morphology of different populations has been documented in various widely distributed species such as *Hippocampus kuda* and *Syngnathus leptorhynchus* (Kuiter, 2000; Fritzsche, 1980). Many localized color forms are present, and most specimens are lumped as a single species, due to inconclusive research from preserved specimens. Therefore, ranges and mean values of morphological characters have been important in defining group memberships and species designations. The differences in populations from different geographic areas of wide-ranging fishes are sometimes as substantial within species as they are within genera (Dawson, 1986). It has been suggested that most pipefish species with wide geographic ranges and which occur in different habitats may consist of genetically distinct forms or different species (Chenoweth *et al.*, 2002; Kuiter, 2000; Vincent, 1996). Wide geographic sampling is therefore critical for syngnathid descriptions.

However, phenotypic variation is particularly high in many kinds of fishes (Borsa, 2002; Tudela, 1999; Hillis, 1987), and more so in the family Syngnathidae (Teske *et al.*, 2004; Kuiter, 2000; Dawson, 1985), and should not necessarily be associated with high genetic variability. Morphological variation between geographically separated populations may be due to the interaction between the genetic structure of the population and environmental conditions. Aquatic environments exhibit great spatial and temporal

variability that affects fish distribution and dispersal and therefore plays a major role in shaping morphological variability among fishes (Grosberg & Cunningham, 2001). The South African coastline, which is very variable, is such an environment as it is characterised by complex climatic and oceanographic conditions with variable geomorphology. It is has been divided into three main biogeographical regions based largely on water temperatures namely cool temperate, warm temperate and sub-tropical regions (1970s; Whitfield, 1998). These different biogeographic regions have been shown to have a profound influence on estuarine environments and coastal waters and hence phenotypic variation among fishes (Turpie *et al.*, 2000; Whitfield, 1998). The major environmental factors (or variables) responsible for distribution and morphological variation are differences in both the abiotic and the biotic habitat characteristics like temperature, salinity, water depth, direction of ocean currents, substrates and vegetation types (Wiens, 2001). These variables result in differences in the availability and composition of resources. Species widely distributed in such heterogeneous environments may therefore be expected to exhibit differentiation in genetic and phenotypic characters or both (O'Reilly & Horn, 2004). For example, several populations of the freshwater three-spine stickleback (*Gasterosteus aculeatus*) appear to have originated largely as a result of this diversification after invasions of the marine population into freshwater habitats (Brooks & McLennan, 2002; McKinnon & Rundle, 2002; Bell & Foster, 1994).

The *S. acus* species complex is an ideal species for studying such variation because of its presumed broad distribution. The range of the species spans all three Southern African biogeographic regions (*S. temminckii*) and is stated as having a European population (Whitfield, 1998). It was also be interesting to verify whether there was morphological variation within *S. watermeyeri* as this species has a restricted distribution and presumably restricted dispersal capabilities. Phenotypic variation within a species increases if the species has limited powers of dispersal (O'Reilly & Horn, 2004). The assumption for the restricted dispersal of South African pipefishes of *Syngnathus* stems from the rare occurrence of the juveniles of these fishes in offshore ichthyoplanktonic tows (Dawson, 1986). Juveniles and adults of these two pipefishes have been mainly recorded from estuaries and estuarine and coastal bay reed beds in nearshore and shallow

water habitats (Whitfield, 1998; Vincent, 1995b). Since the individual characteristics of each estuary play an important role in determining its fish community structure (Harrison, 2004), the pipefish specimens of *Syngnathus* from this study will be grouped according to their location and biogeographic region. The assumption here is that estuaries in which similar physical/geo-morphological processes operate ought to produce similar habitats (Harrison, 2004; Allanson & Baird, 1999) and as a result similar morphological types. Any other differences in the pipefish communities of the estuaries of a particular type therefore could be due to biogeography or speciation. This chapter will attempt to clarify these issues using standard morphological procedures. Therefore, the aim of this chapter is to analyse and compare the morphological differences between the species of South African *Syngnathus* using large sample sizes and size-adjusted data. The different localities of *S. acus* (European and South African populations) will also be compared morphologically to verify whether they represent more than one species.

3.2 Materials and methods

3.2.1 Specimen collection

Fish used in this study were collected from various South African estuaries and coastal areas between the years 2001-2003 using a two-meter seine net. Specimens of *Syngnathus* from the following museums were also examined; South African Institute of Aquatic Biodiversity, Grahamstown (SAIAB/RUSI), South African Museum (SAM), Museum National d'Histoire Naturelle, Paris (MNHN), Natural History Museum of London (BMNH), National Museum of Natural History, Leiden (RMNH) and the Zoologisches Institut und Zoologisches Museum, Hamburg (ZMH). Sample sizes and localities for the specimens examined are shown in Table 3.1. Figures 3.1 and 3.2 are maps for the locations of European specimens of *S. acus* and South African specimens of *S. temminckii* available for analysis created using ArcView3.2 (ESRI, 1999). The details of the museum material examined and voucher numbers are given in appendices III and V. Photographs of some holotypes and specimens of some species are given in plate I.4-I-8. The holotype and syntypes of *S. acus, S. temminckii* and *S. delalandii* were included in the morphological analyses to verify whether the different locations of "*S. acus*" represent more than one species group.

Table 3.1: The list of locations and number of specimens (n) used in the study.

Location	GPS	Biogeographic region	n
S. acus			
United Kingdom, France, Spain & Morocco	Not available	North Eastern Atlantic	84
United Kingdom	Not available	North Sea	6
Cyprus, Greece & France	Not available	Mediterranean Sea	5
S. temminckii (Southern Africa)			
Walvis Bay (Namibia)	22º56' S 14º30' E	West coast	3
Lüderitz Bay (Namibia)	26°65' S 15°15' E	West coast	3
False Bay	34°09' S 22°07' E	West coast	2
Klein	34°25'25.0"S 19°18'13.4"E	West coast	10
Swartvlei	34°01'51.2"S 22°47'49.3"E	West coast	111
Knysna	34°04'38.9"S 23°03'33.4"E	West coast	34
Kromme	34°08'27.9"S 24°50'36.7"E	West coast	5
Kariega	33°40'55.9"S 26°41'15.6"E	East coast	86
Kowie	33°36'11.2"S 26°54'10.2"E	East coast	7
East London	33°01'42.9"S 27°54'57.3"E	East coast	15
S. watermeyeri			
Bushmans	33°41'41.0"S 26°39'48.6"E	East coast	10
Kariega	33°40'55.9"S 26°41'15.6"E	East coast	9
West Kleinemonde	33°32'28.2"S 27°02'51.7"E	East coast	3
East Kleinemonde	33°32'21.8"S 27°02'55.2"E	East coast	33

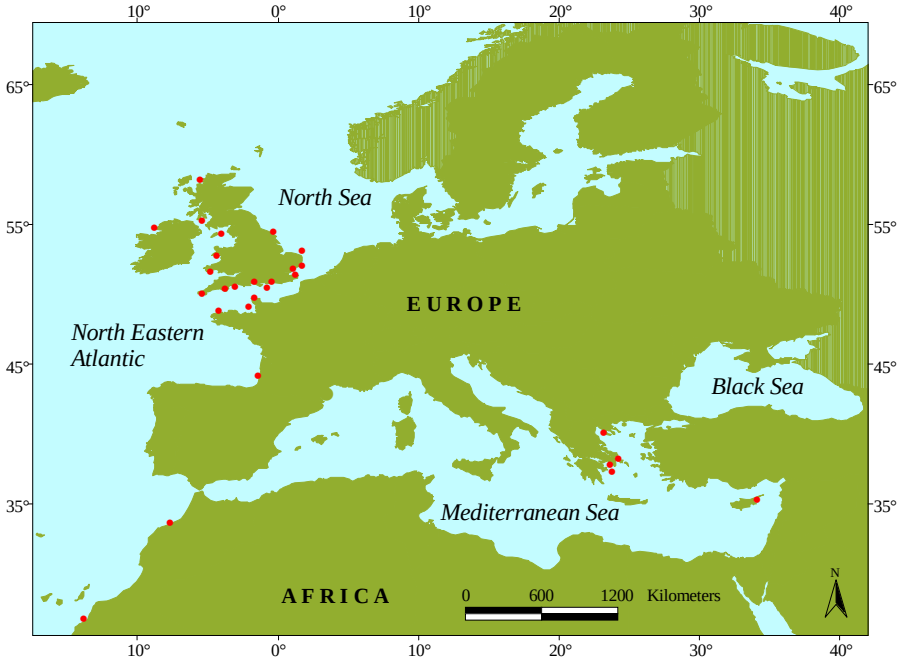


Figure 3.1. Localities (●) of museum specimens of European *S. acus* that were analysed morphologically. Details for exact location data are given in Appendix VI.

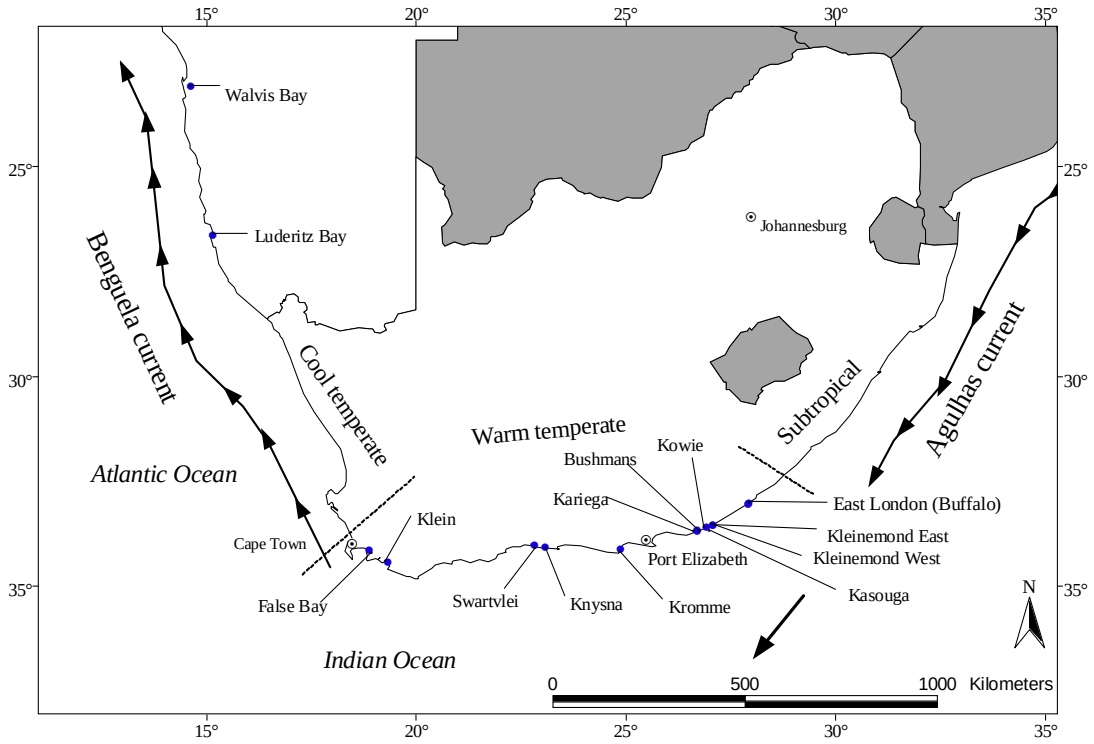


Figure 3.2. Biogeographical regions of South Africa showing the localities (●) of South African species specimens used in the morphological analysis. *Syngnathus watermeyeri* was only available from the Kariega, Bushmans, Kasouga and Kleinemonde estuaries.

3.2.2 Morphometric and meristic data

Counts and measurements follow the standard methods given by Dawson (1985) and Herald (1941). Measurements of body lengths were done on a measuring board graduated in 1.0 mm intervals. All other smaller measurements were taken using dial calipers and recorded to the nearest 0.1 mm. Fin rays were counted using a stereomicroscope. The rays of both pectoral fins were counted and given as an average. Some fin-ray counts could not be determined because of the poor condition of the specimen. In such instances the reported values were taken from the original description (for holotypes) or the specimen was discarded from the analysis. The number of subdorsal rings was estimated to the nearest fourth of the ring length and is the sum of subdorsal trunk and tail rings (Dawson, 1986). Table 3.2 and Figure 3.3 show and define the 16 meristic and morphometric characters examined for all specimens.

Table 3.2. Description of the morphological characters measured (Dawson, 1986).

No.	Character		Description
1	Standard length	SL	Distance from tip of lower jaw to base of median caudal fin rays
2	Head length	HL	Tip of lower jaw to posterior margin of opercle
3	Snout length	SnL	Anterior tip of snout to inner anterior margin of bony orbit
4	Snout depth	SnD	Smallest vertical dimension of snout
5	Caudal fin length	CFL	Length of caudal fin
6	Orbit diameter	OD	Bony orbit diameter
7	Inter-orbital width	IOW	The smallest bony width measured above centers of the eyes
8	Trunk depth	TD	Maximum depth of trunk between outer margins of superior and median ventral trunk ridges
9	Dorsal fin base	DFB	Dorsal fin base
10	Pectoral fin rays	PFR	Number of pectoral fin rays (average of both fins)
11	Dorsal fin rays	DFR	Number of dorsal fin rays
12	Tail rings	Tail	From 1st ring behind anus to penultimate ring excluding terminal element bearing caudal fin.
13	Trunk rings	Trunk	From ring bearing pectoral fin base to ring bearing the anus
14	Subdorsal ring	SDR1	Number of trunk rings covered by the DFB
15	Subdorsal ring	SDR2	Number of tail rings covered by the DFB
16	Subdorsal rings	SDR	SDR1 +SDR2

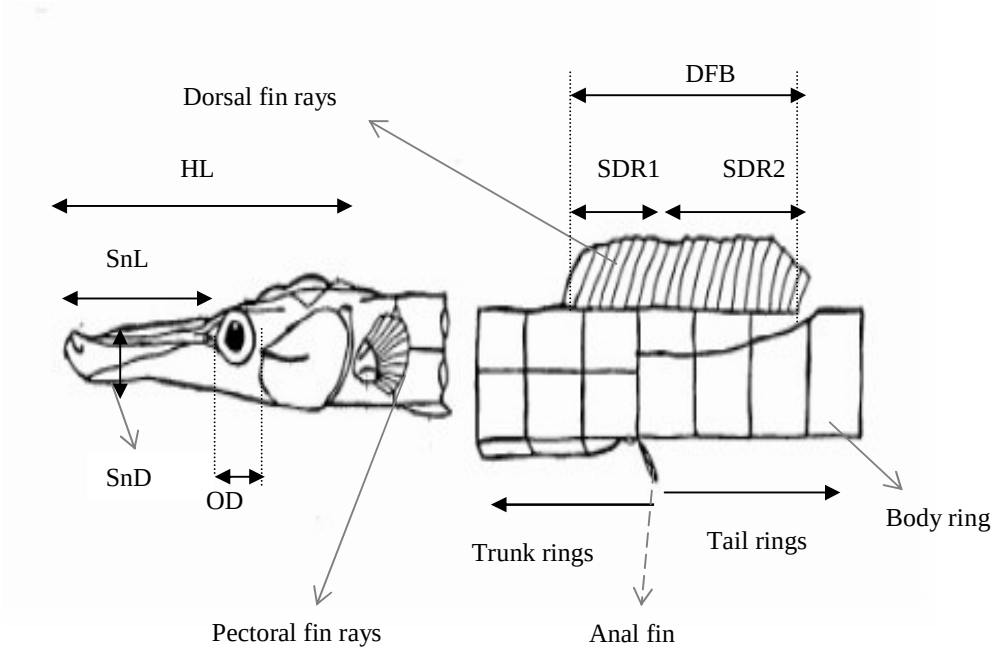


Figure 3.3: An illustration (Dawson, 1985) of some of the morphometric and meristic characters described in Table 3.2.

3.2.3 Data and their adjustments

Linear measurement characters were standardized prior to statistical analysis using two procedures. The first procedure arrived at proportions by standardizing all morphometric data as ratios of standard length (SL) except for the snout length, which was also standardized as the ratio of head length (SnL/HL). The second procedure adjusted the morphological measurements using the modified formula of Reist (1985) from Turan (2004) and Cabral *et al.* (2003) represented as:

$$M_{adj} = M\,(L_s / L_o)^{b}$$

where M_{adj} is the size-adjusted measurement for the character, L_o is the standard length of the fish, M is the original morphometric measurement and L_s is the mean standard length. The slope of the regression model of the logarithms of M and L_o, using all specimens is represented by *b*. For the analysis, specimens were grouped according to sex, species group (each population with holotype/syntype), biogeographical region (cool temperate and warm temperate) and locality. These various categories or effects were selected and analysed because they have been reported to have an effect on the phenotypic expression of fish (Casey *et al.*, 2004; Avise, 2000; Benzie, 1997). There were three species groups, a group of *S. watermeyeri* and two groups of *S. acus* specimens separated due to the spatial distance between populations, that is, South African (*S. temminckii*) versus European (*S. acus*). Specimens of *S. temminckii* were divided into two biogeographical groups: region 1 = West Coast (Kromme to Walvis Bay) and region 2 = East Coast (Bushmans to East London). Only two regions were included in the analysis of the South African samples because there were too few specimens available from the subtropical biogeographical region (Figure 3.2). The different localities of *S. temminckii* and *S. watermeyeri* were separated according to the locality data of the available specimens listed in Table 3.1.

Only locations with more than ten available specimens were included when analyzing the effect of locality on morphological variation. Therefore, the False Bay, Kromme and Kowie *S. temminckii* specimens were excluded while the specimens of *S. watermeyeri* from the neighboring East and West Kleinemonde estuaries were combined to make a

larger sample. This resulted in five localities (East London, Kariega, Knysna, Swartvlei and Klein) for *S. temminckii* and three localities (Bushmans, Kariega and Kleinemonde) for *S. watermeyeri*. Though there were a number of European specimens of *S. acus*, these were not divided into specific localities because of too few specimens (less than 10) from the North and Mediterranean seas. Each statistical model therefore tested for the effects of each factor (sex, species group, biogeographical region and locality) on a response variable that is the morphological character. The following null hypotheses were tested: (1) there is no difference in character means among sexes or (2) among species groups, (3) biogeographical region had no effect on morphological variation of *S. temminckii* populations, and (4) there is no morphological variation among the different localities of South African specimens of *S. temminckii* or *S. watermeyeri*.

3.2.4 Statistical tests

Analyses were performed using the STATISTICA software package (StatSoft, 2002). All analyses were performed on unadjusted, proportional data and size-adjusted data (M_{adj}). Exploratory analysis was done as an initial step to test for homogeneity of variance. Residuals were tested for normal distribution using the Shapiro-Wilk's W test. One-way ANOVA (*F* test) was used to check for phenotypic variation between species groups and their holotypes/syntypes among morphological characters. Statistical tests employed the Tukey's post-hoc HSD (honestly significantly different) test for unequal N to assess the relative importance of each character for group separation among categories. Separate analyses were initially done for morphometric and meristic characters based on the assumption that the two kinds of variables respond differently to environmental conditions and genetic make-up. However, as there were no significant differences in results ($P > 0.05$), all morphological characters were combined in subsequent analyses. Standard length and all other measurements were used for estimation of length-relationship correlations among the three species groups. The slopes of SL versus other morphometric characters were compared using analysis of covariance (ANCOVA) to check whether these were significantly different among species groups. Principal component analysis (PCA) and discriminate analysis (DA), both of which are useful in analysing intraspecific variation were also employed using STATISTICA. Principal

component analysis was performed to detect morphological differences between groups, to determine the contribution of each character to the differentiation and to choose the appropriate subset of variables to be used in DA. The DA was used to obtain a function for discriminating groups previously defined by the PCA using stepwise and forward procedures. All juveniles and adult specimens with missing values were excluded from PCA and DA for each specific analysis. The analyses were done using these two methods to determine the effects of sex, biogeographical regions and location on phenotypic variation among specimens as well as within the species groups.

3.3 Results

3.3.1 Basic statistics

A total of 430 specimens were analysed, representing South African (Indian and Atlantic oceans) and European populations (North East Atlantic, English Channel, the North Sea and Mediterranean Sea). Variation in morphological characteristics (especially the snout length, and counts of DFR and PFR) were sufficient to identify *S. watermeyeri* (Figure 3.4). The basic statistics and relationships of morphometric and meristics characters for all the specimens according to species group are summarized in Tables 3.3 and 3.4. The relationships among meristic characters for species groups are also presented in figure 3.5. The descriptive statistics are mean, standard deviation (std dev), standard error (Std err) and range. The number of subdorsal rings is estimated to the nearest fourth of ring length such that the point of dorsal fin origin is recorded in quarter-ring intervals (0.25) before or behind the anterior margin of the first tail ring (0-point) (Dawson, 1986). The morphological measurements of the holotypes and syntypes of nominal species conspecific with *S. acus* were segregated according to populations. The *S. acus* holotype being a larger specimen (434 mm) had morphological measurements similar to the European *S. acus* group but was completely out of the range of measurements for the South African *S. temminckii* group. The type specimens of the two South African types, *S. temminckii* and *S. delalandii*, were however much smaller in size and within the limits of the *S. temminckii* group. Morphological characters were positively correlated to standard length ($r^2 > 70\%$) for the two *S. acus* groups. However, there was no significant correlation ($r^2 < 70\%$) between SL and SnL, SnD, IOW and TD for *S. watermeyeri*.

Figure 3.4: Differences in snout length of South African specimens of *S. watermeyeri* (A) and *S. temminckii* (B).

Table 3.3: Variation in meristic characters for each of the species groups. Species group 1 = *S. temminckii* (South Africa), 2 = *S. acus* (Europe) and 3 = *S. watermeyeri.*

Character	Sp. Group	n	Mean	Std dev	Std err	Min	Max	***P*****
Dorsal fin rays	1	275	37	1.68	0.101	33	42	*
	2	83	39	2.69	0.295	33	44	
	3	53	30	1.29	0.177	28	33	
Pectoral fin rays	1	202	12	0.84	0.059	11	14	b & c
	2	45	12	0.80	0.120	10	14	
	3	21	7	0.58	0.126	6	8	
Trunk rings	1	277	20	0.76	0.046	17	20	b & c
	2	84	20	1.17	0.127	15	21	
	3	53	17	0.43	0.059	16	18	
Tail rings	1	277	39	1.28	0.077	36	43	*
	2	84	43	2.05	0.223	37	45	
	3	53	38	0.88	0.121	36	41	
Sub dorsal rings 1	1	277	1.17	0.35	0.021	0.25	2.00	b & c
	2	83	1.26	0.38	0.042	0.50	2.75	
	3	42	0.50	0.21	0.033	0.25	1.00	
Sub dorsal rings 2	1	277	8.18	0.55	0.033	6.75	9.50	*
	2	83	7.93	0.62	0.068	6.50	9.50	
	3	53	6.82	0.34	0.047	6.00	7.50	
Sub dorsal rings	1	277	9.36	0.55	0.033	7.75	10.75	
	2	83	9.19	0.71	0.078	7.50	11.25	
	3	53	7.22	0.35	0.049	6.25	8.00	

The abbreviations are number of specimens (n), standard deviation (Std dev), standard error (Std err) and range (minimum and maximum). Significant results (*P*** < 0.05) for differences between species groups are displayed as follows: * = all species groups different from each other while: b = 1 & 3 and c = 2 & 3 are significantly different from each other.

Table 3.4: Variation in morphometric characters (mm) among the species groups. Species group 1 = *S. temminckii*, 2 = *S. acus* and 3 = *S. watermeyeri.*

Character	Sp. Group	n	Mean	Std dev	Std err	Min	Max	*P***
Standard length	1	277	133.8	37.69	2.26	32.0	261.0	*
	2	84	244.1	90.89	9.92	69.0	412.0	
	3	53	111.5	14.65	2.01	85.0	144.0	
Head length	1	277	16.7	5.25	0.32	4.5	34.2	*
	2	84	34.0	12.22	1.33	10.1	58.0	
	3	53	10.5	1.07	0.15	7.9	12.7	
Snout length	1	277	8.6	3.68	0.22	1.8	21.2	*
	2	84	19.6	7.67	0.84	4.7	32.9	
	3	53	3.6	0.57	0.08	2.4	4.8	
Snout depth	1	80	1.6	0.39	0.04	0.8	2.7	a, c
	2	81	2.9	0.89	0.10	0.8	4.9	
	3	41	1.3	0.19	0.03	0.4	1.7	
Orbit diameter	1	272	2.5	0.55	0.03	0.9	3.9	*
	2	84	3.9	1.18	0.13	1.3	6.4	
	3	53	1.8	0.24	0.03	1.3	2.6	
Inter orbital width	1	272	1.2	0.24	0.02	0.4	2.3	a, c
	2	84	2.1	0.70	0.08	0.8	3.8	
	3	53	1.0	0.23	0.03	0.7	2.1	
Trunk depth	1	277	3.9	1.44	0.09	1.0	10.9	a, c
	2	84	8.4	3.64	0.40	1.7	18.6	
	3	53	4.1	0.75	0.10	2.7	6.0	
Dorsal fin base	1	277	16.8	4.7	0.28	4.5	35.1	*
	2	84	29.3	11.8	1.29	8.1	58.9	
	3	53	12.4	1.7	0.23	8.0	15.5	
Caudal fin length	1	271	4.6	1.19	0.07	1.50	8.1	*
	2	81	8.1	2.93	0.32	2.79	13.5	
	3	53	3.7	0.56	0.08	2.34	4.9	

Note:** Significant results ($P < 0.05$) for differences between species groups are displayed as follows: * = all; a = 1 & 2; b = 1 & 3 and c = 2 & 3 species groups are significantly different from each other.

ANCOVA revealed that most of the slopes of the regression models of the relationship between SL and the other morphometric characters were significantly different from 0 ($P < 0.05$) and significantly different among the species groups for unadjusted data (Table 3.5). The slopes were significantly different among species groups for SnD, OD and DFB (Table 3.5). The relationship of SnL to HL for European *S. acus* populations was significantly different from that of the two South African species groups. SnL and HL

regression models were significant with regard to comparisons with *S. watermeyeri* while TD and CFL were only different between *S. acus* and *S. temminckii*.

Table 3.5: Results of ANCOVA analysis on the relationship between standard length and other morphometric characters for each species group.

Morphometric variable	Species (slope intercept - *b*)			*P***
	S. temminckii (SA)	*S. acus* (Europe)	*S. watermeyeri*	
SnL/HL	0.68	0.59	0.42	a & b
SnL	0.09	0.08	0.03	b & c
SnD	0.01	0.01	0.01	
HL	0.13	0.13	0.06	b & c
TD	0.03	0.04	0.03	a
IOW	0.01	0.01	0.01	a & c
OD	0.01	0.01	0.01	
CFL	0.02	0.03	0.03	a
DFB	0.12	0.13	0.10	

Note: * = all species groups different from each other while; a = 1 & 2; b = 1 & 3 and c = 2 & 3 are significantly different from each other. Species groups 1 = *S. temminckii*, 2 = *S. acus* and 3 = *S. watermeyeri.*

3.3.2 Effects

3.3.2.1 Size-adjusted data (M_{adj})

ANOVA showed that there were differences between size-adjusted morphometric characters (Table 3.6). Size-adjusted characters were significantly different for species groups, sex, localities and the two SA biogeographic regions for *S. temminckii* ($P < 0.05$). Most meristic characters were different among the species groups except for trunk and SDR1 that were not different between *S. acus* and *S. temminckii*. Significant differences were also found among the three species groups of *Syngnathus* for most of the morphometric characters. Though the effect of sex on morphometric characters was significant only three characters (HL, SnL and TD) were significantly different among the species groups. The two South African biogeographical regions were also significantly different morphologically except for four meristic (DFR, SDR1, SDR2 and SDR) and one morphometric character (SnD). However, there were no significant differences for all meristics among the three locations of *S. watermeyeri*. There were

some differences in morphometric data between locations of *S. watermeyeri*. The Kleinemonde specimens were only significantly different from the other two locations in HL but also differed from the Kariega samples for the OD measurements. Average values for the Bushmans and Kariega specimens were only different with regards to the OD.

Table 3.6: Results of ANOVA of adjusted (M_{adj}) morphometric characters for the effects of species group (1 = *S. temminckii*, 2 = *S. acus* and 3 = *S. watermeyeri*), sex, biogeographical regions (*S. temminckii*) and locations (*S. watermeyeri*) on morphological variation. The * represents comparisons that are significant at $P < 0.05$.

Character	Species group			Sex	*S. temminckii*	*S. watermeyeri*		
	1 & 2	1 & 3	2&3		SA regions (East and West)	1 & 2	1 & 3	2&3
HL	*	*	*	*	*			*
SnL	*	*	*	*	*			
SnD	*	*		*				
OD		*	*		*	*		
IOW	*		*		*			
TD	*	*	*	*	*			
DFB		*	*		*			
CFL					*			

The results for different localities of the *S. temminckii* for size-adjusted data are presented in table 3.7. *Syngnathus temminckii* locations were morphologically distinct for most characters ($P < 0.05$) but not for three meristic characters (SDR1, SDR and PFR). Post-hoc pairwise comparisons (Tukey's HSD test) between the specimens did not show significant differences ($P > 0.05$) for all locations (Table 3.7). The Klein population was similar to the Swartvlei population for most morphometrics except in the SnL. The East London population was only different by two morphometric characters from the Knysna (SnD and IOW) and Kariega (OD and DFB) populations. The Swartvlei and Knysna individuals were very similar to each other apart from differences in SnL, DFB, Trunk and DFR. There were significant differences for most characters among the Kariega, Knysna, and Swartvlei populations. The major differences between these locations were for the morphometric characters SnL, OD, TD and DFB. The Kariega population was the most morphologically different population.

Table 3.7: Significant differences based on pairwise comparisons for size adjusted (M_{adj}) data between different localities of *S. temminckii* (1 = Klein, 2= Swartvlei, 3 = Knysna, 4 = Kariega and 5 = East London). The * represents comparisons that are significant at $P < 0.05$.

Morphometric	Pairwise comparison								
Character	1 & 3	1 & 4	1 & 5	2 & 3	2 & 4	2 & 5	3 & 4	3 & 5	4 & 5
HL		*	*		*	*	*		
SnL	*	*	*	*	*	*	*		
SnD			*					*	
OD				*	*	*	*		*
IOW		*	*		*	*	*	*	
TD		*		*	*	*	*		
DFB				*	*		*		*
CFL		*			*		*		

3.3.2.2 Proportional data

Proportional data were only slightly different from the size-adjusted data (M_{adj}). Morphometric characters also showed very high variation among the three species groups except for IOW. There were no significant differences between *S. watermeyeri* and *S. acus* for SnD and OD. The effect of sex on morphometrics was significant for three morphometric characters HL, OD and TD. The results for the three *S. watermeyeri* populations were similar to the trends found for the size-adjusted data. The characters responsible for variation were very similar except for the HL measurement. *Syngnathus temminckii* from different locations were morphologically distinct for most characters ($P < 0.05$) but not for three meristic characters (SDR1, SDR and PFR). The Klein population was similar to the Swartvlei population for all morphometric characters. The East London population was different from Knysna (SnL, SnD and IOW) and Kariega (OD) estuaries. The Swartvlei and Knysna populations were very similar to each other apart from differences in SnL, DFB, Trunk and DFR. Comparisons among locations often showed significant differences among the Kariega, Knysna, and Swartvlei populations. The major differences between these populations were for morphometric data, namely HL, SnL, OD, TD and DFB. The *S. watermeyeri* pipefish from the Kariega estuary were the most morphologically different population.

3.3.3 Multivariate analysis

3.3.3.1 Principal component analysis (PCA)

Sixteen characters were analysed in the PCA (Table 3.8). Only a few morphometric characters (HL, SnL and DFB) were significant in explaining phenotypic variation among groups. About 70% of the total variation associated with the 14 characters was accounted for by the first three factors with the first two factors accounting for approximately 63% of the total variation. DFB had the highest loading on factor 1 (0.84) while HL and PFR had the highest loadings on factor 2 (0.84 and 0.75, respectively). Factor 2 had a much smaller eigenvalue (1.36) and only explained 10% of the variance, while factor 3 (Eigenvalue = 1.06) explained 7% of the variance.

Table 3.8: Factor loadings as a result of the principal component analysis of all specimens analysed for proportional and size-adjusted (M_{adj}) data. Marked values contribute more than 70% to the observed variation and are significant at $P < 0.05$.

Character	Proportional data		Size-adjusted data		
	Factor 1	Factor 2	Factor 1	Factor 2	Factor 3
SL (SnL/HL*)	***0.86**	*0.16			
HL	**0.89**	-0.07	0.17	**0.84**	0.21
SnL	**0.93**	0.02	0.12	**0.75**	0.07
SnD	-0.01	-0.38	-0.19	0.10	0.66
OD	0.10	-0.14	0.03	0.64	0.52
IOW	-0.20	0.10	0.11	0.22	**0.78**
TD	-0.08	-0.60	-0.44	-0.50	0.32
DFB	0.05	**0.80**	**0.84**	0.05	0.14
CFL	-0.14	0.35	0.60	0.02	0.44
PFR	**0.82**	0.36	0.44	**0.82**	0.09
DFR	**0.82**	0.38	0.57	**0.71**	0.08
Trunk	**0.75**	0.31	0.37	**0.74**	0.14
Tail	**0.73**	-0.04	0.45	0.58	0.15
SDR1	0.59	0.32	0.55	0.49	0.23
SDR2	0.48	**0.71**	**0.78**	0.44	-0.07
SDR	0.64	**0.70**	**0.80**	0.54	0.05
Eigenvalue	6.84	1.94	7.46	1.36	1.06
% Variance	37	17	53	10	7

Note: * is for raw and proportional data sets only and refers to the ratio of SnL to HL. All other characters in the proportional data set are the ratios of morphometric characters with regard to SL.

However, factor 3 was disregarded in the scatter plots because it possessed only one variable (IOW) with a high factor loading and only contributed a low proportion to the observed variance. The highest factor loading (0.78) on this factor was for OD. The resultant scatter plots of factor 1 versus factor 2 also showed some considerable overlap between the *S. acus* and *S. temminckii* population groups (Figure 3.5). There was no overlap between *S. watermeyeri* and any of the two other species groups. The PCA only correctly classified 90% of the specimens designated as *S. temminckii*, 70% of the *S. acus* and 100% for those designated as *S. watermeyeri*. Inter versus intra-group distances were large enough to conclude confidently that there were three distinct species groups represented by the specimens.

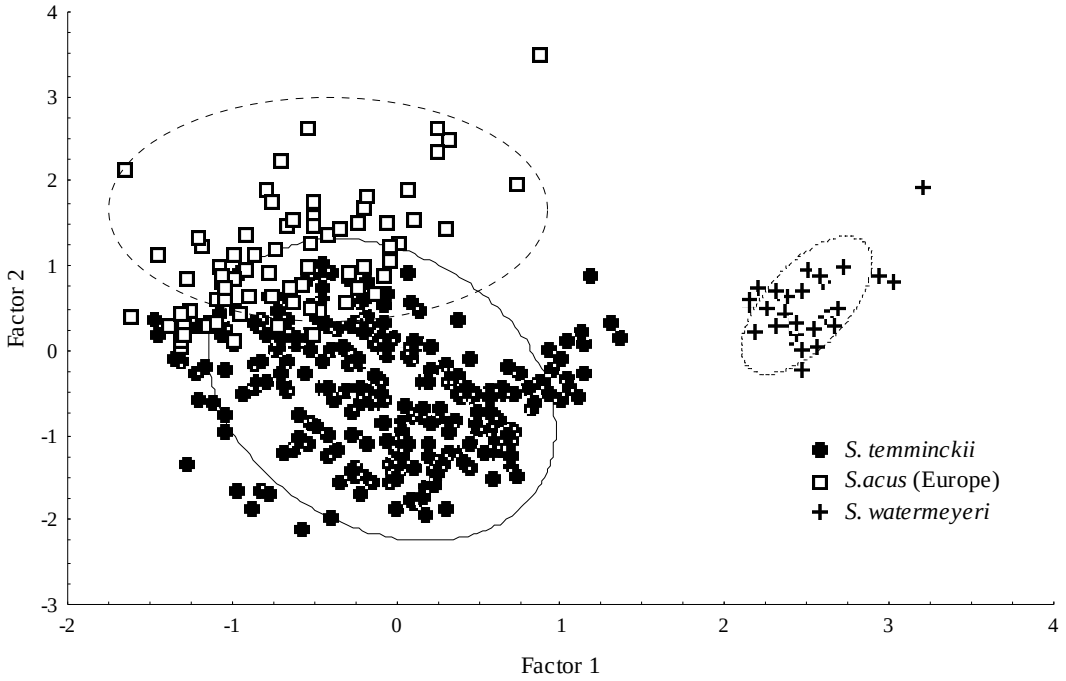


Figure 3.5: Factor scores with 95% confidence ellipsoids of PCA on the three species groups for size-adjusted morphometric characters.

Results for proportional data were slightly different from size-adjusted data, particularly with regard to the distribution of significant characters among factors. The two principle components that were extracted only explained about 54% of the variation among the

specimens. All meristic characters were responsible for explaining variation while only the ratios of three morphometrics (SnL, HL and DFB) were significant just like for size-adjusted data. Group distinctions and scatter plots for the different effects were however, similar those of the other data sets but defined by both meristic and morphometric character types. Although PCA analysis distinguished the three species groups, there was some overlap in the resultant clusters between specimens of *S. acus and S. temminckii* (similar to Figure 3.5). The proportion of specimens correctly classified into their populations was 97% overall, 97% for *S. temminckii*, 68% for *S. acus* (Europe) and 100% for *S. watermeyeri*.

3.3.3.1.1 Effects

There were some differences in population structure due to the biogeographic region of the specimens of *S. temminckii*. A scatter plot of the size-adjusted data revealed variation leading to extensive overlap in morphological characters between populations of the two biogeographical regions (Figure 3.6).

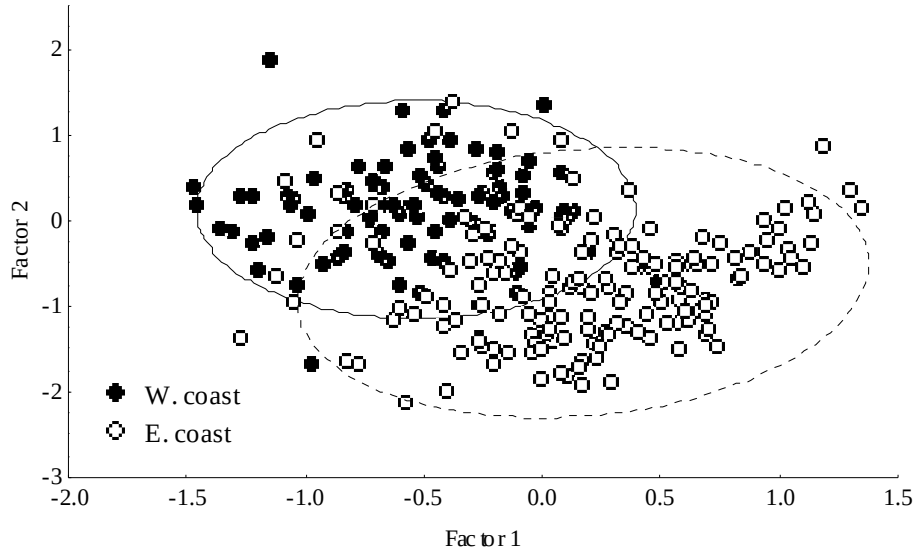


Figure 3.6: Factor scores of PCA with 95% confidence ellipsoids of the two South African biogeographic regions of *S. temminckii*.

Comparisons between the five populations (*S. temminckii*) were not significant, except between the Swartvlei and Kariega estuaries, which were the most morphologically distinct populations among locations (Figure 3.7). These estuaries had the highest percentage of correctly classified pipefish specimens, about 80% and 90% respectively.

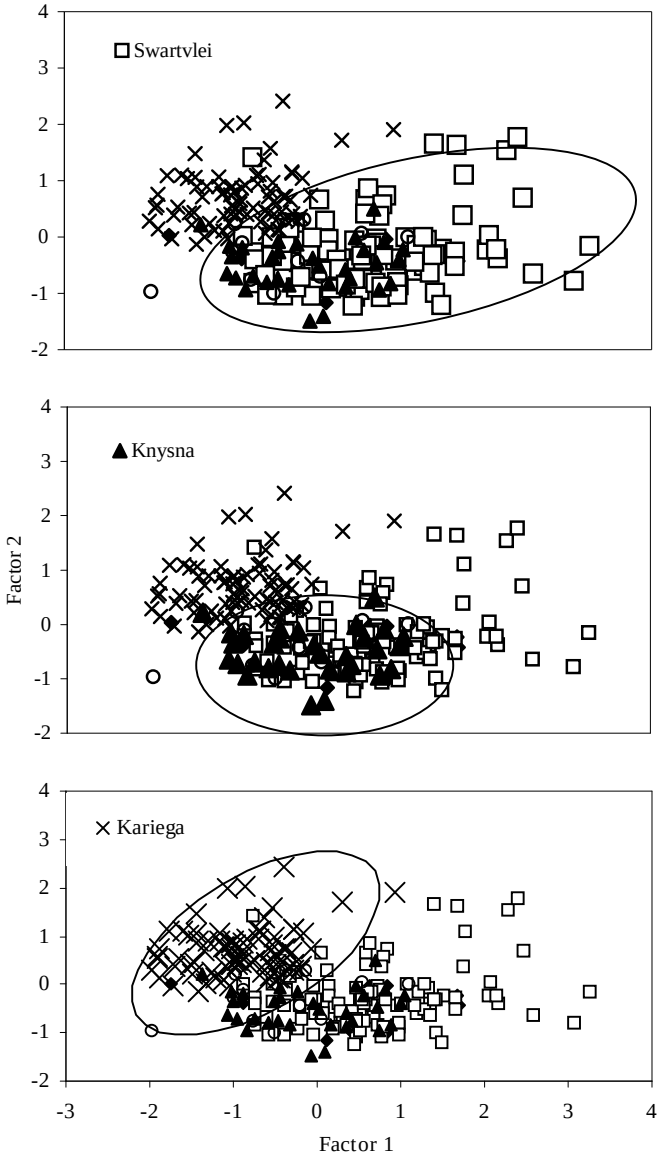


Figure 3.7: Factor scores of PCA for the three South African localities of *S. temminckii*. Localities are identified as follows: Klein ◆, Swartvlei □, Knysna ▲, Kariega ✖ and East London ○. The ellipsis on each graph indicates the specified population.

However, by comparison, *S. watermeyeri* specimens from different estuaries were morphologically more similar than *S. temminckii* specimens from different localities. PCA could not morphologically distinguish specimens between the three locations of *S. watermeyeri*. The PCA revealed that there was no phenotypic variation due to sex among the South African *Syngnathus* specimens examined. This suggests that sexual dimorphism may not be significant in defining morphological variation in this genus. The effect of sexual dimorphism in pipefishes is due to shape rather than size variation as a result of the presence of the brood pouch in males and the extended trunk in gravid females.

3.3.3.2 Discriminant Analysis (DA)

3.3.3.2.1 Proportional and size-adjusted data (M_{adj})

Discriminant function analysis on 16 size-adjusted variables showed that the effects of species group, sex, biogeographical region and location were significant ($P < 0.05$) in discriminating the specimens into distinct groups. Two characters (SL, and SDR) were redundant and therefore not included in the model. There were highly significant differences in morphological characters among the three species groups ($F = 142.6$, Wilk's $\lambda = 0.025$, $P < 0.05$). Partial Wilk's Lambda indicated that characters PFR, Tail, SDR2, Trunk and DFR contributed most to the overall discrimination (from first to fifth, respectively). The characters IOW, SDR1, DFB, CFL, OD and SnL contributed least to the overall discrimination among groups (Table 3.9). The smaller the Wilk's λ, the lesser the contribution it makes to the discrimination between species groups. Therefore, this analysis suggested that meristic characters are the most important variables responsible for discrimination amongst the three species groups. The first discriminant function discriminated mostly between *S. watermeyeri* and the other two groups. The canonical mean for *S. watermeyeri* was quite different from that of *S. acus* and *S. temminckii*. The second discriminant function distinguished mostly between specimens of the European *S. acus* from the two South African species groups. However, the magnitude of this discrimination was much smaller than that of the first function and it accounted for less of the observed morphological variation.

Table 3.9: Discriminant function analysis summary for morphological differences between the three species groups using size-adjusted data. Variables in model: 13; n = 366; Wilk's Lambda: 0.025; F (26,702) = 142.60; and $P < 0.05$.

Character	Wilk's λ	Partial λ	F-remove	P-level	Tolerance	Root 1	Root 2
PFR1	0.054	**0.47**	201.14	0.000	0.98	**-0.77**	0.14
Tail	0.049	**0.51**	166.33	0.000	0.61	0.14	**-1.03**
SDR2	0.029	0.88	23.64	0.000	0.53	-0.20	**0.50**
Trunk	0.029	0.86	28.05	0.000	0.71	**-0.34**	0.34
DFR	0.030	**0.84**	32.45	0.000	0.68	-0.26	**-0.48**
HL	0.030	**0.85**	30.28	0.000	0.55	-0.31	**-0.49**
TD	0.027	0.92	14.17	0.000	0.72	-0.02	-0.37
IOW	0.026	0.96	7.05	0.001	0.83	-0.05	-0.24
SDR1	0.026	0.97	6.20	0.002	0.82	-0.21	-0.05
DFB	0.026	0.97	6.11	0.002	0.63	0.05	0.26
CFL	0.026	0.98	4.31	0.014	0.80	-0.18	-0.04
OD	0.026	0.98	3.07	0.048	0.82	-0.04	0.16
SnL	0.026	0.99	2.12	0.121	0.57	0.02	0.17
Eigenvalue						9.14	2.89
Cum. Prop.						0.76	1.00
Canonical means							
S. temminckii						-1.07	1.02
S. acus (Europe)						-1.12	**-3.19**
S. watermeyeri						**8.36**	-0.03

Note: Values in bold indicate species groups that are responsible for the discrimination.

The scatter plot of the two discriminant functions (Figure 3.8) confirms the above interpretation and shows the three distinct species groups. In the figure, specimens of *S. watermeyeri* cluster together and are plotted in isolation. Thus, the first discriminant function mostly discriminates this group from the two *S. acus* groups. The second function shows evidence for discrimination between the *S. acus* and *S. temminckii* specimens. However, the discrimination is not as distinct as that provided by the first function. Although, the effect of the "sex" was also significant, the analysis summary revealed that only two characters (TD and SDR1) were significantly different among males and females (Appendix VII). The effect of biogeographic regions on morphological variation was also significant as populations of *S. temminckii* were morphologically different from each other (Appendix VII). The discrimination between these two biogeographic regions was based on differences in SnL, OD, IOW, and TD.

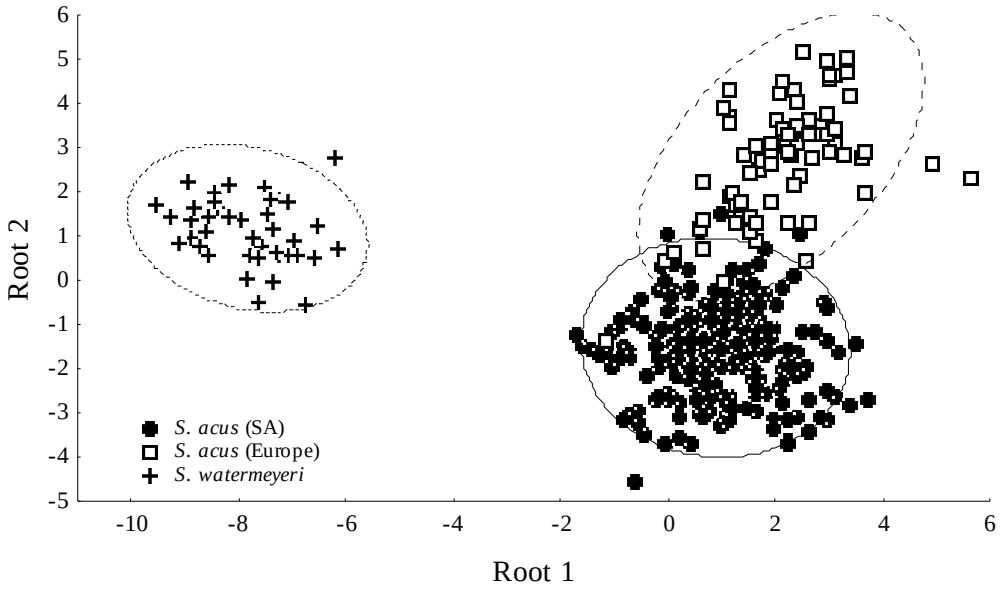


Figure 3.8: Canonical scores with 95% confidence ellipsoids of DA on the three species groups for size-adjusted morphometric characters.

Table 3.10: The classification matrix for the observed classifications according to DA for the effects of species, sex, biogeographical regions (*S. temminckii*), and localities of *S. temminckii* and *S. watermeyeri* on morphological variation using size-adjusted data.

Effect	Group	n	Percent correct in cluster Size adjusted
Species group	*S. temminckii*	250	97.30
	S. acus (Europe)	77	97.40
	S. watermeyeri	42	100.00
Sex	Female	95	75.26
	Male	79	71.43
Biogeographical region	Eastern Cape	96	96.67
(*S. temminckii*)	Western Cape	161	93.75
Locality - *S. temminckii*	East London	15	27.27
	Kariega	82	100.00
	Knysna	34	75.76
	Swartvlei	110	86.36
	Klein	10	30.00
Locality - *S. watermeyeri*	Bushmans	8	87.50
	Kariega	8	62.50
	Kleinemonde	26	92.30

There were some morphological differences in the population structure of *S. temminckii* due to the locations of the specimens (F = 12.1, Wilk's λ = 0.13, $P < 0.05$). The partial Wilk's λ indicated that the characters SnL and DFB contributed most to the overall discrimination (Table 3.11). All other characters though significant contributed very little to the differentiation explained by the discriminant function. The analysis extracted 4 discriminant functions that were mainly defined by morphometric characters, except for function 3, which was dependant on meristic characters (PFR and Trunk).

Table 3.11: Discriminant function analysis summary for morphological differences between the localities of *S. temminckii* using size-adjusted data. Variables in model: 12; n = 228; Wilk's Lambda: 0.13; F (48,818) =12.06; and $P < 0.05$.

Character	Wilk's λ	Partial λ	F-remove	P-level	Tolerance	Roots			
						1	2	3	4
SnL	0.140	**0.91**	5.20	0.001	0.32	0.28	**-0.64**	0.00	-0.70
OD	0.177	**0.72**	20.74	0.000	0.91	0.40	**0.67**	0.13	-0.01
IOW	0.150	**0.85**	9.60	0.000	0.78	**-0.44**	-0.22	-0.17	0.59
DFR	0.136	0.94	3.70	0.006	0.82	-0.01	-0.20	0.41	0.56
Trunk	0.139	0.92	4.63	0.001	0.78	0.10	-0.05	**-0.71**	-0.02
PFR	0.137	0.93	4.09	0.003	0.98	0.05	0.10	**0.59**	-0.12
TD	0.134	0.95	2.86	0.025	0.82	-0.26	0.14	0.10	-0.24
DFB	0.140	**0.91**	5.18	0.001	0.72	-0.21	-0.39	-0.00	-0.53
HL	0.136	0.93	3.76	0.006	0.30	0.46	0.35	0.04	0.463
Tail	0.131	0.97	1.56	0.185	0.75	0.11	-0.17	0.25	0.23
CFL	0.130	0.98	1.13	0.343	0.87	-0.12	0.11	0.08	-0.26
SDR	0.130	0.98	1.12	0.348	0.70	-0.07	0.18	0.20	0.20
Eigenvalue						2.25	0.77	0.23	0.11
Cumulative prop.						0.67	0.90	0.97	1.00
Canonical means									
Klein						-1.60	0.47	**-2.08**	-0.32
Swartvlei						-1.12	0.65	0.24	0.01
Knysna						-1.12	**-1.83**	-0.03	0.31
Kariega						**2.01**	0.10	-0.07	0.09
East London						0.45	-1.24	0.40	**-1.27**

Note: Values in bold indicate the locations that are responsible for the discrimination.

These results imply that the observed morphological differences among geographical areas were mainly due to the larger values of SnL, DFB and IOW of the East Coast specimens. The Kariega population was the most distinct grouping with 100 % reclassification. The Knysna population had 76% correct reclassifications respectively. High reclassifications for these populations suggest that these estuaries could be responsible for distinguishing the West coast biogeographic region of the South African *S. temminckii* population from the East coast. Since the number of individuals in the overlap region among the estuaries shows the degree of relatedness among the estuaries, it would appear that the Kariega and East London as well as the Klein and Knysna population groups seem to define two different groups of morphologically similar populations (Figure 3.9).

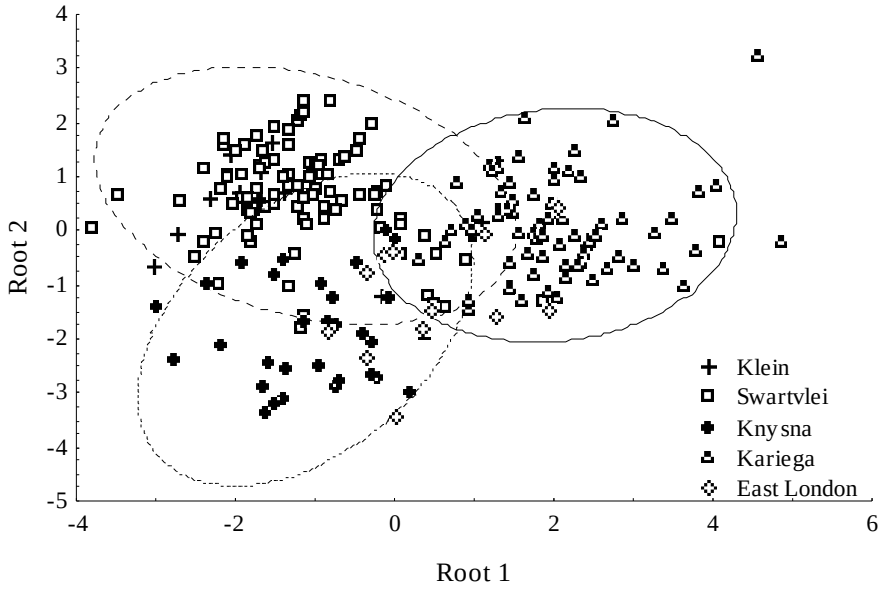


Figure 3.9: Canonical scores with 95% ellipsoids (---Swartvlei, —Kariega and ····Knysna) of selected localities of *S. temminckii* for size-adjusted morphometric characters.

Locations of *S. watermeyeri* were morphologically different from each other (F = 4.22, Wilk's λ = 0.24, $P < 0.05$) for the analysed characters. The three locations of this species were mainly differentiated from each other on the basis of morphometric characters (Table 3.12). Partial Wilk's Lambda indicated that variables DFB and OD contributed most to the discriminant function model for the effect of location. Although SnL, SDR and HL were included in the model they contributed little to the discrimination among population groups of *S. watermeyeri*. The first discriminant function discriminated the Bushmans estuary specimens from the other two locations. This function was mainly defined by differences in DFB, SnL, SDR and HL. The second discriminant function distinguished between Kariega and the two other estuaries by a much smaller magnitude and is explained by difference in OD and IOW (Figure 3.10). The canonical mean for the Kariega population in this second function was significantly different from that of the other population groups.

Table 3.12: Discriminant function analysis summary for morphological differences between the three locations of *S. watermeyeri* using size-adjusted data. Variables in model = 8; n = 42; Wilk's Lambda: 0.24; approx.; F (16,64) = 4.22; and $P < 0.05$.

Character	Wilk's λ	Partial λ	F-remove	P-level	Tolerance	Root 1	Root 2
OD	0.310	**0.76**	4.93	0.014	0.72	-0.27	**-0.85**
DFB	0.433	**0.55**	13.26	0.000	0.54	**-1.17**	0.07
HL	0.285	0.83	3.22	0.053	0.62	**0.60**	-0.36
SDR	0.319	**0.74**	5.57	0.008	0.76	**0.66**	0.43
SnL	0.312	**0.76**	5.04	0.012	0.59	**0.78**	0.32
PFR1	0.270	0.88	2.22	0.125	0.73	-0.52	0.04
IOW	0.263	0.90	1.74	0.191	0.80	-0.13	**0.54**
CFL	0.255	0.93	1.21	0.311	0.78	-0.35	-0.20
Eigenvalue						1.58	0.64
Cumulative prop.						0.71	1.00
Canonical means							
Bushmans						**2.37**	0.50
Kariega						0.20	**-1.58**
Kleinemonde						-0.79	0.34

Note: Values in bold indicate locations that are responsible for the discrimination.

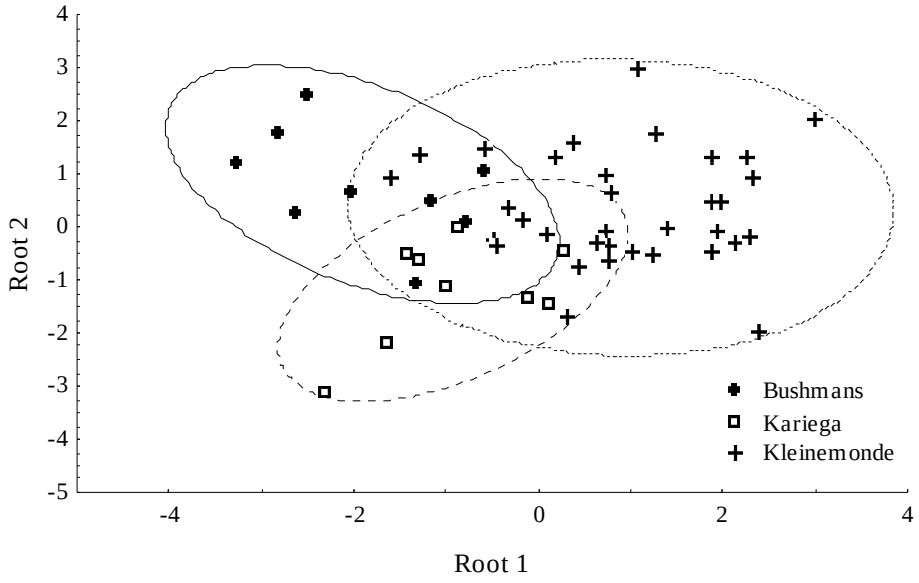


Figure 3.10: Canonical scores with 95% ellipsoids of the three localities of *S. watermeyeri* for size-adjusted morphometric characters.

3.4 Discussion

Morphological analysis of proportional and size-adjusted data sets consistently revealed the same species group segregations. The data differed in the variables responsible for the clustering of groups. The effect of sex on morphometrics was significant although the only important characters were the trunk and body length characters. There were no differences among meristic characters with regard to sex for the 95 females and 79 males that were analysed. Males were significantly larger than females for all species and populations examined. Adult male and female *Syngnathus* will have different morphology due to the presence of the brood pouch on the tail of the males while females will generally have a distended trunk when gravid. However, this did not affect discrimination because there was no interaction between sex and species, locality or biogeographical areas. Therefore, it was possible to distinguish between species groups without treating sexes separately. It appears that sex did not bias the results when analysing morphological variation among these species of *Syngnathus.*

3.4.1. Species groups

Both types of analyses (univariate and multivariate) supported the hypothesis that the three species groups are morphologically distinct from one another. The pectoral fin ray count was the most discriminating character among the species groups. The PFR count (6-8) segregated the specimens of *S. watermeyeri* from the two *S. acus* species groups (11-14). Consequently, specimens of *S. watermeyeri* were the easiest to identify when comparing groups. Other morphological differences were seen in the measurements of head characters as well as body rings. Head length characters (SnL and HL) accounted for much of the differences between groups and could be used for separating the European *S. acus* population from the South African specimens (Table 3.4). Though most of the characters positively correlated with SL, the removal of the size factor by the ratio method and normalization techniques did not greatly change the trends observed in the results. The only difference in the results of different treatments of the data sets was in the importance of different characters distinguishing groups. The characters responsible for variation or discrimination did not change when comparing species groups, sexes, biogeographical regions or localities. According to these results, size is a major factor of variation among these species groups. This is not surprising as *S. watermeyeri* is a very small species, while the European specimens of *S. acus* were significantly larger for most morphometric characters than the South African specimens of *Syngnathus*. Although the data sets were adjusted for proportions, it appears that the relationships among morphological characters and their proportion to the standard length are important in segregating species.

Based on all morphological characters examined, *S. watermeyeri* was the most divergent group. There was no overlap in the scatterplots of the PCA or DA analyses between this species and the two *S. acus* groupings. Compared to the *S. acus* populations, *S. watermeyeri* had a shorter snout, a lower number of fin rays (pectoral and dorsal) and body rings (trunk and tail) or sub-dorsal rings. Unlike the other *S. acus* populations, *S. watermeyeri* does not seem to have a coastal or marine population and is thought to be primarily estuarine (Whitfield, 1998; Dawson, 1986). There is no record of adult or juvenile specimens of the species in the sea. Restriction of *S. watermeyeri* to the estuarine

environment could imply that this species has been able to diverge more, as it has been reproductively isolated due to very limited gene flow between its locations as well as from other pipefish groups. Generally, marine and estuarine-dependent fish species with coastal populations will exhibit low genetic variation due to a high potential for gene flow in the ocean environment (Grosberg & Cunningham, 2001). Currents or locations that fishes are exposed to play an important role in spatial distribution, movement and isolation of fish species due to their effect on population mixing (Rocha-Olivares & Vetter, 1999). Other processes such as competition, predation, recruitment, disturbances and immigration are also important for local-scale assembly of communities and may affect the distribution of species (Mora *et al.*, 2003). The only extant records of *S. watermeyeri* are from the East and West Kleinemonde systems, estuaries that are temporary open to the sea (Whitfield, 1998). The species exhibits the attributes generally ascribed to narrowly distributed species like small body size and habitat specificity (Gill & Kemp, 2002). These attributes may affect dispersal and the colonisation of new areas by such species. Conversely, widely distributed species have larger body sizes, are habitat generalists and have long dispersal intervals (Gill & Kemp, 2002). These dispersal intervals relate to ontogeny and determine the length of survival of the larvae, juvenile or adult fish before they get a suitable habitat (Mora *et al.*, 2003). Dispersal and therefore population mixing among the estuaries that this species resides in is therefore limited.

However, the rediscovery of a specimen of *S. watermeyeri* in the permanently open Bushmans estuary could indicate that there is some dispersal occurring among the estuaries or that the distribution range of this pipefish species is not yet fully known. Furthermore, the historical record of the occurrence of the species in three adjacent estuaries (Kariega, Kasouga and Bushmans) suggests that though dispersal of *S. watermeyeri* among estuaries may occur, this could be limited to shorter distances (compared to *S. temminckii*). The species is primarily estuarine and will probably not survive for long periods in the marine environment (Kuiter, 2000). This pipefish could have disappeared from the other three estuaries due to an increase in the salinity of the estuaries. Anthropogenic changes to these rivers, including channeling, construction of dams and altered flow regimes resulted in the reduction of freshwater input into these

estuaries and affected habitats critical to the ecology of this species (Whitfield, 1998). This could have led to an increase in more salinity-tolerant fishes at the expense of obligate estuarine species. Timeframe of antropogenic events. Therefore, *S. temminckii* could have expanded its distribution and abundance at the expense of the more freshwater-dependent estuarine pipefish. Unfortunately, the life history information for this species is lacking, and the species is endangered, as it is presently limited to the East Kleinemonde estuary. It would be interesting to test the salinity requirement of the species in laboratory experiments to verify this hypothesis.

No single character can reliably separate the North Atlantic and South African *S. acus* population groups as there was considerable overlap in meristic and morphometric characters between the two groupings. The overlap in range values observed is normal among syngnathids (Kuiter, 2000; Fritzsche, 1980; Herald, 1965) and groups that are closely related or living in similar habitats. Meristic characters were more effective than morphometric characters in separating the *S. acus* and *S. temminckii* populations when large numbers of specimens were included. *Syngnathus acus* specimens from Europe differ from the South African specimens in having on average more dorsal fin rays, trunk and tail rings and subdorsal rings (Table 3.4). The lack of effective utility of the selected characters is due to the reported morphological similarity among different species of *Syngnathus* (Dawson, 1986; Vincent, 1995; Kuiter, 2000; Teske *et al.*, 2003). However, South African and Europeans populations were morphologically different and segregation by visually comparing their appearance is possible as the one species is larger with an angle in the head (Kuiter, 2000). The specimens can also be easily segregated based on locality data. Although there are some similarities between *S. acus* and *S. temminckii*, the differences observed between them have the potential to allow for species discrimination.

The disjunct distribution pattern of North-Eastern Atlantic and South African populations supports the recognition of *S. temminckii* as different from the European *S. acus*. These two populations are geographically isolated from each other. There are no confirmed records of *S. acus* in the tropics between Walvis Bay in Namibia and Morocco (Rabat)

suggesting the possibility of two different species. It was also interesting to note that the morphological measurements of the holotype of *S. acus* were similar and within the range of the European *S. acus* specimens (Appendix V), while the syntypes of *S. temminckii* had measurements within the range of the South African specimens. Though one could argue that *S. acus* displays an anti-tropical species distribution, the differences observed among the specimens were large enough to refute this possibility. The magnitude of the phenotypic variation observed suggests that the populations have been reproductively isolated from each other and that geographical separation could account for this. Large spatial distances among populations may affect gene flow and expose fishes to different environmental conditions (Jones *et al.*, 2003; Chenoweth *et al.*, 2002; Wilson *et al.*, 2001). Temperature could play a role in this phenotypic differentiation as European waters are characterised by lower temperatures and significantly higher levels of nutrient resource composition as opposed to our South African waters (Garibaldi & Limongelli, 2002). Fishes from cooler water European areas are generally larger as opposed to populations in the warmer Indian Ocean. For example, species distributed over broad geographic ranges often exhibit thermal clines in body size, with the majority of species exhibiting larger adult size in colder environments (Angilletta *et al.*, 2004).

The time of the separation between these populations could not be determined from these results. While small differences among populations may take place over hundreds of years, studies on North American freshwater fishes suggest that species level differences can take place over at least ten thousands of years (Schaeffer & Cavender, 1986). Further studies on these specimens using genetics should answer this question. However, the results of this morphological analysis demonstrate that there is enough morphological evidence to suggest that the South African population currently known as *S. acus* may be a different species than the European populations.

3.4.2 Locality

There were some differences in morphology among individuals of South African species of *Syngnathus* among the locations examined. These differences were mainly among the morphometric characters HL and SnL. Differences in morphology among locations are

common and have been observed among various fish species (O'Reilly & Horn, 2004; Langerhans *et al.*, 2003; Swain & Foote, 1999). Most locations including those that were geographically closest to each other were significantly different from each other in some characters. The mosaic of environments on the South African coastline, combined with dispersal and differential adaptation may explain some of the differences among South African localities. This is more so for *S. temminckii*, which is widely distributed in various habitats in permanently and temporarily open estuarine systems, as well as in coastal bay and reed bed areas. The environmental factors or local habitats and the species tolerances of these factors are critical in generating variation as they may either promote or limit gene flow and hence population mixing (Turan, 2004). Therefore, morphological variation observed within each species could merely be a reflection of environmentally induced differences. Furthermore, the analyses revealed variation among specimens from different localities in morphological characters such as trunk depth, head length, snout depth and snout length that are known to vary with environmental conditions (O'Reilly & Horn, 2004; Tzeng & Yeh, 2004; Chernoff, 1982; Barlow, 1961). Many fishes exhibit temperature-related variation in meristic data (Jørgensen *et al.*, 2005). Meristic traits are generally set early in ontogeny and remain stable throughout the life of an individual. Lower meristic data in warmer waters are due to the more rapid development at these temperatures (Jørgensen *et al.*, 2005; Tzeng & Yeh, 2004).

Therefore, the environmental conditions prevailing at the critical stage in the development of an individual are important in defining morphology. Phenotypic plasticity is common and has been observed in several freshwater and marine species with widespread populations for example *Menidia beryllina,* a freshwater atherinid (Chernoff *et al.*, 1981), and the Atlantic herring *Clupea harengus* (Jørgensen *et al.*, 2005). Although morphological divergence in body size among populations is not uncommon (Angilletta *et al.*, 2004; Partridge & Coyne, 1997), phenotypic plasticity is likely to be a major contributor to location differences. It appears that species of *Syngnathus* in South Africa may exhibit phenotypic plasticity as an adaptive response to divergent selection pressures. It would be interesting to determine whether these differences are also due to genetic divergence.

The magnitude of morphological divergence observed between samples from different localities tended to increase with increasing spatial distance. For example, the East London and Kariega locations which are closer to each other (< 150km) and had specimens that were very similar morphologically, while the Kariega and Swartvlei (> 450km) were more dissimilar. Various studies have shown that the magnitude of morphological divergence among population groups increases with increasing geographical distance (O'Reilly & Horn, 2004; Turan, 2004; Quilichini *et al.*, 2004; Lutjeharms *et al.*, 2003; Lydeard *et al.*, 1995). Geographical distance is important for variation because it has a great influence on species dispersal and therefore gene flow and population mixing (Gill & Kemp, 2002). The exchange between individuals in different environments increases the potential for adaptation in these different habitats and evolution of populations as independent biological entities (Turan, 2004). Generally, geographic groups are allopatric populations in various degrees of speciation. Complete or partial allopatry of populations usually precedes speciation and the level of morphological divergence between populations can indicate the evolutionary stage they are at. It is therefore hypothesized that the South African population of *S. temminckii* like most wide spread species exhibits phenotypic plasticity possibly because there has been geographic isolation within this population.

There was also a pattern of association between *S. temminckii* from the Klein, Swartvlei and Knysna estuaries, which are all temporary open systems. These pipefish were clearly similar to each other in size and slightly smaller than specimens from the other two localities. This could indicate that environmental conditions are important in phenotypic variation among pipefishes. Isolation by distance and environmental conditions may determine the population structure of *S. temminckii*. However, it is important to note that spatial and environmental barriers may not be necessarily the sole mechanism promoting differentiation among these populations. Genetic variation could be very important in this morphological divergence though it could not be quantified using this data set. Recent research favors a combination of genetics and morphology when using phenotypic diversity to distinguish species as morphological characters can be very plastic.

3.4.3 Biogeographical regions in South Africa

PCA and DA results revealed that there was population structuring between the two selected biogeographical regions (East and West) of *S. temminckii*. Most separation in the scatterplots of the PCA was clustered within the first factor, although there was considerable overlap between the two regions. Therefore individuals of *S. temminckii* in Southern African waters exhibit unique phenotypic attributes that can be distinguished among regions. The general assumption among researchers has been that widespread estuarine-dependent species exhibit little genetic variation throughout their geographical range (Mora *et al.*, 2003; Gill & Kemp, 2002; Grosberg & Cunningham, 2001). This is perhaps largely because of the high gene flow potential in the continuous ocean environment that can lead to high population mixing of individuals from geographically distant regions (Mora *et al.*, 2003).

Although *S. temminckii* is distributed throughout the South African coastline, dispersal of pipefish between the East and West coast may be restricted due to the oceanographic peculiarities in the area near Port Elizabeth where complex circulation occurs as the Agulhas current leaves the coast following the shelf break (Turpie *et al.*, 2000; Beckley & Van Ballegooyen, 1992; Beckley, 1988). A complex circulation pattern of cold water eddies, intrusions and large offshore meanders of the Agulhas Current all occur at this location with several gyre-like patterns (Lutjeharms, 2005; Lutjeharms *et al.*, 2003; Olivar & Beckley, 1994; Shillington, 1986). Wind-driven currents interact with the Agulhas Current, such that surface waters in the western part tend to drift to the northwest while there is often a clockwise movement of water on shore and to the east in the eastern part of the region (Turpie *et al.*, 2000; Lutjeharms *et al.*, 2003). These vigorous upwelling events induced by winds or the Agulhas Current introduce cold conditions that may interfere with the movement of juvenile and adult pipefish (Olivar & Beckley, 1994; Beckley & Van Ballegooyen, 1992; Shillington, 1986). They may place constraints on the reproductive strategies employed by a wide variety of fish species and most likely the *Syngnathus* that inhabit these diverse shores (Hutchings *et al.*, 2002). These mechanisms may retain eggs, larvae or juveniles of fish species onto the Agulhas bank or displace them far offshore. A sufficient degree of prolonged post-larval

separation among geographical areas may result in notable phenotypic and genetic differentiation among fish populations within a species (Turan, 2004) as could be seen with South African specimens of *S. temminckii*. The west coast of South Africa is influenced by north drifting cold water. Events of upwelling take place when surface water is blown offshore and cold deep water moves to the surface near the coast (Shillington, 1986). Warm temperate estuaries are characterised by high salinities and low turbidities due to low rainfall and runoff, high seawater input and evaporative loss, while cold temperate, and especially subtropical, estuaries have lower salinities and higher turbidity, due to relatively high runoff (Harrison, 2004).

3.4.4 Conclusions

Studies of phenotypic variation play a very important role in species identification. Phenotypic characters, like standard length, snout length and pectoral fin ray counts can be related to genetic or environmental effects and are subject to selection pressure. The challenge for taxonomic studies is to determine the cause of the observed variation among populations. This is because genotype and phenotype variation proceed at different rates and may have different causes and different effects (Wiens, 2001). The observed morphological patterns among species groups, sexes, localities and biogeographical regions are likely to be a result of a mixture of genotypic and phenotypic variation. Some morphological differences could be a result of short-term environmentally induced phenotypic variation (Begg *et al.*, 1999a). However, these morphological analyses provide an insight into the differences between the South African pipefish species of *Syngnathus*. The data indicate that South African *Syngnathus* species have diverged considerably in body morphology. The extent of differentiation is illustrated by the scatterplots of the PCA and DA of South African *Syngnathus* in which there is no overlap of individuals between *S. watermeyeri* and *S. temminckii*. Specimens of the South African *S. temminckii* species group are phenotypically more similar to the geographically distant population group of European *S. acus* than to *S. watermeyeri*. Furthermore, the South Africa population group of *S. acus* (including *S. temminckii* and *S. delalandii* syntypes) is morphologically distinct from the specimens from Europe. Recognition of the South African *S. acus* species group as a distinct species is supported

by these results. The South African *S. acus* population should be referred to as *S. temminckii*, Kaup 1856.

Chapter Four

Molecular phylogeny of South African pipefish of the genus *Syngnathus* using cytochrome *b* sequences of mitochondrial DNA

4.1 Introduction

The phylogenetic pathways of evolutionary change in various teleost fish taxa have been investigated extensively using morphological character states (Bell & Foster, 1994). Although the relationships among various species have been resolved with these methods, the data often provide insufficient information to resolve more complex phylogenetic relationships. Similarly, the morphological characters used in pipefish taxonomy are generally highly conserved and may be prone to convergence, and too similar among taxa such that phylogenetic resolution is difficult (Kuiter, 2000; Wiens, 2000; Herald, 1959). Molecular systematic methods therefore offer other approaches to compare levels of genetic divergence between taxa with few phenotypic differences (Stepien & Rosenblatt, 1996). It is possible to infer evolutionary mechanisms and relationships of all types of organisms from gene phylogenies constructed with molecular data (Wiens, 2000; Bermingham *et al.,* 1997; Bernardi, 1997). This is because molecular biology has shifted the analysis of mutations from indirect, phenotypic inferences to the direct characterization of mutations by DNA sequence analysis (Meyer, 1993).

Although many studies have been carried out on the systematics of the family Syngnathidae, estimations of the general phylogeny of the family has not been done. Taxonomic knowledge within the family is therefore considered incomplete (Kuiter, 2000; Dawson, 1985), and revisions are on-going (Casey *et al.,* 2004; Wilson *et al.*, 2003; Kuiter, 2001; Lourie *et al.,* 1999). Molecular phylogenies of the family (Wilson *et al.*, 2003) and genera have only appeared quite recently (Teske *et al.*, 2005; Teske *et al.*, 2004; Casey *et al.*, 2004; Jones *et al.*, 2003; Wilson *et al.*, 2001; Lourie *et al.*, 1999). However, most of these studies have concentrated on seahorse species *Hippocampus.* Other genera like *Syngnathus* and most of the species in the family still need to be studied. Wilson *et al*'s (2003) study showed good within-pouch-type resolution

suggesting that most genera were monophyletic with regards to pouch type. Their analyses also supported the monophyly of the Urophori (tail brooding) and Gastrophori (abdominal brooding) groups of syngnathid fishes suggested by Herald (1959). However, there were low levels of phylogenetic resolution of basal relationships between pouch types such that there is still uncertainty about the phylogenetic placement and taxonomic relationships of genera in the family (Wilson *et al.*, 2003; Kuiter, 2001). It has been suggested that the unresolved generic relationships within the family may reflect a period of rapid diversification (Jones *et al,* 2003; Wilson *et al.*, 2003; Lourie *et al,* 1999).

Recent molecular systematic investigations using mitochondrial 16S and 12S RNA, and cytochrome *b* sequences have suggested that *Hippocampus* and *Syngnathus* are sister-taxa and the more derived genera of Syngnathidae as they have the most advanced types of enclosed brood pouches (Wilson *et al.*, 2001). This result contradicts traditional morphological revisions by Herald (1959) and Kuiter (2000) that have placed *Hippocampus* in the subfamily Hippocampinae with three other sister genera of pygmy pipehorses. It was stated that most of the characters used in Herald's (1959) analysis have multiple independent origins in Syngnathidae and therefore may have confounded these previous attempts to identify the ancestors of seahorses. However, the work of Wilson *et al*. (2001) does not include any species from the other *Hippocampinae* genera to test this hypothesis. Although the three gene fragments provided good generic resolution there were low levels of phylogenetic resolution of basal relationships between genera for each gene fragment. Therefore, the reliability of the hypothesized relationship between *Hippocampus* and *Syngnathus* also needs testing. Morphological treatments of the family (Kuiter, 2000; Dawson, 1985; Herald, 1959) have suggested that the genus *Syngnathus* is part of the subfamily Syngnathinae and thus could be more closely related to the other 41 genera that are part of this group than possibly to *Hippocampus*. Therefore, taxon relationships among genera in Syngnathidae are still contentious and selection of suitable outgroups for *Syngnathus* is not clear. Further re-examinations of pouch development and/or taxonomic revision of the family may still be necessary.

However these molecular systematic investigations have helped to elucidate some relationships within *Syngnathus* (Wilson *et al.*, 2003 and 2001). These results clearly separated European and western Atlantic species and demonstrated a close relationship between eastern pacific, Atlantic Ocean and a disjunct Indo-Pacific lineage. However, like most of the morphological and genetic studies that have been done on the genus (Gordina *et al.*, 1991; Fritzsche, 1981; Herald, 1941), this study excluded the two South African species. Since it has been suggested in Chapter three that the South African population of *S. temminckii* (formally known as *S. acus*) is different morphologically from European *S. acus*, the genetic relationship between these two species needs to be established. It is necessary to establish whether the observed morphological differences are genetic or merely a consequence of the species geographical distributions. Genetic differences are sometimes not always detected, even in cases where morphological differences exist among fish species (Turner, 1999; Avise & Aquadro, 1982). For example in ciscoes, a single nucleotide difference was found between morphologically distinct individuals of *Coregonus artedi* and *C. zenithicus* from Lake Nipigon (Reed *et al.*, 1998). This is because closely related species can show significant divergence in morphology when species differences are due to morphological adaptation or if the species being studied have not been separated for a long period of time (Kornfield & Parker 1997). Although derived diagnostic characters may be of taxonomic utility, they are phylogenetically uninformative if they are autapomorphic (Kornfield & Parker, 1997). Furthermore, there is a need to clarify the placement of *S. watermeyeri* within *Syngnathus* due to its lower pectoral fin ray count compared to the other species in the genus (Dawson 1985).

4.1.1 Selection of DNA region for analysis

The cytochrome *b* gene of mitochondrial DNA was chosen for this study because it is the most frequently used region for the study of evolutionary relationships among vertebrates (Kocher *et al*, 1985). It has proven useful in phylogenetic studies of various fish species for example, syngnathids (Teske *et al.*, 2004; Casey *et al.*, 2004; Wilson *et al.*, 2003; Lourie *et al.*, 1999), cyprinids (Tsigenopoulos *et al.*, 2003; Liu, 2002; Durand *et al.*, 2002; Echelle & Dowling, 1992; Smouse *et al.*, 1991), and various other fish groups

(Behrmann-Godel *et al.*, 2004; Knapen *et al.*, 2003; Borsa, 2002; Sullivan *et al.*, 2000; Song *et al.*, 1998). The phylogenetic relationships established often describe species evolutionary histories and the historical relationships among species or genera (Grosberg & Cunningham, 2001; Echelle *et al.*, 2000; García *et al.*, 2000). Species phylogenies are important in that they not only reflect relationships between species and within taxon groupings, but may also define the limits of what constitutes a species (Goldstein *et al.*, 2000). These limits or boundaries of a phylogeny are crucial for taxonomy as phylogenetic studies in which the cytochrome *b* gene has been used have led to the proposition of new classification schemes that have better reflected the phylogenetic relationships among the species studied (Wiens, 2004; Castresana, 2001). Character-based methods have been typically used within the framework of a variety of species concepts to operationally delimit species boundaries (Wiens, 2004; Hillis, 1987). Incorporating mtDNA data could be useful, as a lack of diagnostic morphological characters has hindered the establishment taxonomic boundaries in reconstructing phylogenies of closely related species of *Syngnathus.* Therefore, cytochrome *b* phylogenies could help in the genus assignment of newly identified species (Briones *et al.*, 2005; Brandstatter *et al.*, 2005; Farias *et al.*, 2005; Yokoyama *et al.*, 2001; Castresana, 2001; Hillis, 1987).

The usefulness of a given gene in addressing phylogenetic questions depends on various factors, among which is the rate of evolution of that gene (Stepien & Kocher, 1997). For example, very slowly evolving highly constrained genes have been shown to be useful for reconstructing very old phylogenies (Bermingham *et al.*, 1997; Bernardi, 1997; Lydeard & Roe, 1997). The cytochrome *b* gene is often popular in phylogenetic analyses because the dynamics and rules of substitution for this protein-coding gene are somewhat clearer than in other mtDNA regions like tRNA and rRNA regions whose secondary structures may make the alignment process very difficult (Meyer, 1993). Like all genetic data, cytochrome *b* contains discrete character states, which can exhibit variable rates of mutation (ranging from rapid to conservative) making it useful for phylogenetic hypotheses for a wide range of divergences within taxa (Song *et al.*, 1998).

However, various problems have been encountered when using cytochrome *b* gene sequences and other mitochondrial genes as phylogenetic markers (Yoder *et al.*, 1996; Meyer 1994; Graybeal 1993). These include base compositional biases, rate variation between lineages, limited variation in the first and second codon position and saturation at third codon positions (Kocher & Carleton, 1997; Meyer, 1993). Generally, substitutions at third positions of protein coding regions accumulate quickly and become saturated (Kocher & Carleton, 1997). This saturation effect affects the amount of phylogenetic information available for resolving "deep" evolutionary questions and the number of informative sites for the third codon position for population level studies (Meyer, 1994). This transitional bias appears to decrease while transversions become more apparent with increasing sequence divergence and time since common ancestry (Meyer, 1993). However, studies of closely related species will not be as affected by multiple substitutions and should provide a good representation of evolutionary history (Meyer, 1993). Despite these issues, the cytochrome *b* region is still popular as a phylogenetic tool and has been more widely used than other mitochondrial genes. The advantages of abundant comparative sequence data and well-characterized gene function and protein structure therefore enhance its utility for evolutionary investigations (Bernardi, 1997; Yoder *et al.*, 1996).

It is important to note that the phylogenetic history of specific gene lineages (gene trees) can differ in topology and will not always reflect the phylogenetic history of the populations in which those gene trees are being studied (Degnan & Rosenburg, 2006). This is because the lineage sorting process is stochastic and largely independent at different genetic loci, such that gene trees derived from different loci may be incongruent (John & Avise, 1998). Hybridization, introgression, selection and incomplete lineage sorting can differ among interspecific gene phylogenies (Degnan & Rosenburg, 2006; Nichols, 2001). Thus, within a species, many interrelated gene trees can be found, one for each non-recombined locus in the genome. While complete genome sequences have enormous potential for addressing phylogenetic issues, their utility for reconstructing phylogeny is initially quite limited due to the requirement of thorough taxon sampling within a clade for accurate reconstruction of phylogenies (Lerat *et al.*, 2003). However,

gene trees not only can provide evidence for gene duplication events but can in some cases also reflect speciation events (Maddison, 1997). This occurs if the time intervals between species-branching events are much greater than the time intervals between lineage sorting events in each species, such that the gene and species trees are likely to be concurrent (Lerat *et al.*, 2003; Rosenberg & Nordborg, 2002; Nichols, 2001).

Although *Syngnathus* with some of the most developed male brooding structures in the Syngnathidae is the largest genus with 32 valid species, no studies have looked at the phylogeny of the two South African species (Lourie *et al.*, 1999; Dawson, 1985; Herald, 1959). The genus is widely distributed in the Atlantic Ocean with few species in the Pacific and Indian Oceans (Dawson, 1985). *Syngnathus* has been especially successful at achieving wide geographic distributions (Kuiter, 2001; Lourie *et al.* 1999; Dawson 1985), indicating that the long-distance dispersal capability in this genera may be higher than that of other members of the Syngnathidae. Therefore, both vicariance and dispersal may need to be considered to explain current distributions of the species of the genus. It will be essential to realize that dispersal has two components: the ability to move and the ability to become established. Biogeographical and genealogical data will be useful in providing insight on the origin and diversity in *Syngnathus* and the two South African species. The objective of this chapter is to establish the phylogenetic placement of the South African species, *S. temminckii* and *S. watermeyeri*, within the phylogeny of *Syngnathus* using cytochrome *b* sequences. The overall aim of the chapter is thus to examine the systematic status of South African species of *Syngnathus*.

4.2 Materials and methods

4.2.1 Sample collection

Specimens of *S. temminckii* and *S. watermeyeri* were obtained using a two-metre seine net from various estuaries along the Southern African coastline (Figure 4.1 and Table 4.1). However, specimens of *S. watermeyeri* were only available from the East Kleinemonde estuary of the Eastern Cape. Specimens of the European population of *S. acus* were obtained from the northeastern Atlantic (Dr Fabian Blanchard of the

Département Ressources Halieutiques - France) and Mediterranean Sea (Dr. Argyris Kallianiotis of the Fisheries Research Institute - Greece). Tissues and fin clips were removed from the tail part of the body from specimens caught in the field and preserved in 99% ethanol.

Table 4.1: Pipefish specimens used for DNA analysis and abbreviations used in phylogenetic trees.

Species/ Location	GPS	Code	Region	Sea access	n
S. temminckii					
Lüderitz Bay	26°65'00"S 15°15'00"E	LD	West coast	Open	2
Berg	32°46'10.8"S 18°08'44.2"E	BG	West coast	Permanently open	1
False Bay	34°09'S, 22°07'E	FB	West coast	Open	2
Klein	34°25'25.0"S 19°18'13.4"E	KE	West coast	Temporally open	3
Swartvlei	34°01'51.2"S 22°47'49.3"E	SW	West coast	Temporally open	8
Knysna	34°04'38.9"S 23°03'33.4"E	KN	West coast	Permanently open	3
Kromme	34°08'27.9"S 24°50'36.7"E	KR	West coast	Permanently open	4
Kabeljous	34°00'17.4"S 24°56'15.5"E	KB	West coast	Temporally open	1
Sundays	33°43'19.4"S 25°50'57.9"E	SN	East coast	Permanently open	1
Kariega	33°40'55.9"S 26°41'15.6"E	KA	East coast	Permanently open	4
Kowie	33°36'11.2"S 26°54'10.2"E	KO	East coast	Permanently open	5
West Kleinemonde	33°32'28.2"S 27°02'51.7"E	WK	East coast	Temporally open	2
Great fish	33°29'37.3"S 27°08'10.0"E	GF	East coast	Permanently open	4
S. watermeyeri					
East Kleinemonde	33°32'21.8"S 27°02'55.2"E	EK	East coast	Temporally open	5
S. acus					
Bay of Biscay	Not available	BB	Atlantic	Open	5
Thracian Sea	Not available	TH	Mediterranean	Open	5

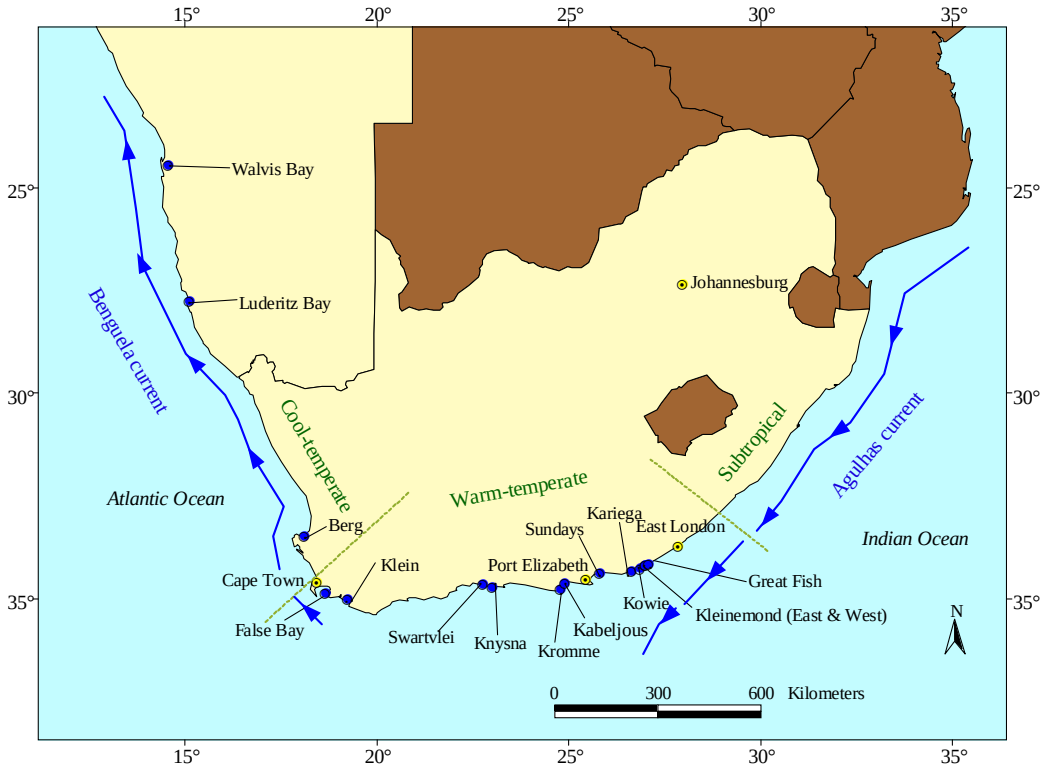


Figure 4.1: Map of South Africa showing transitional zones of the three biogeographical regions (green) and locations where the specimens of *S. temminckii* and *S. watermeyeri* (East Kleinemonde only) were collected. See Turpie et al., (2000) amongst others.

4.2.2 DNA extraction and amplification

DNA was extracted using a DNeasy QIAGEN tissue extracting kit (Qiagen, Chatsworth, CA) and the chelex extraction method (Walsh *et al.*, 1991). DNA was digested using proteinase K and purified by ethanol precipitation. The amount of DNA extracted was quantified by running 5 µl of each extraction on a 1 % agarose gel stained with ethidium bromide. A fragment of the cytochrome *b* gene was amplified by the polymerase chain reaction (PCR) with a combination of three primers (Table 4.2). Two of these primers were designed based on cytochrome *b* sequences of other pipefishes and closely related species deposited in GenBank by searching for highly conserved regions to amplify a 1107 bp sized fragment. The other primer (HI16091) was obtained from a study on Anabantid species (Roos, 20054). The primer combination of SynL and SynH was used

for the PCR step while SynL and Hcytb were used for sequencing to generate a sequence of 820 bp. DNA sequences of the cytochrome *b* region were amplified using standard PCR protocol (Innis *et al.*, 1988). This was performed using 1-2 µl of DNA in a 50 µl reaction, containing 1 unit of *Taq* DNA polymerase (BIOTAQ), with 5 µl of 10x buffer, 0.2mM of each dNTP and of 5mM of a 25mM $MgCl_2$ solution. The amplification procedure comprised of a denaturation step of 2 minutes at 94°C, 35 cycles including denaturing for 1 minute at 94°C, 60°C for annealing and 1 minute of extension at 72°C, and a final extension step at 72°C for 10 minutes. Thermal cycling was performed in either a Thermo Hybrid PCR Sprint Temperature Cycling system machine or a Corbett Research PC-960G Micro-plate Gradient thermal cycler. The PCR product was purified using the QIAquick (Qiagen) PCR purification Kit. Approximately 0.2 µg of the purified product was cycle-sequenced in both forward and reverse directions with the ABI Prism BigDye V3.1 terminator cycle sequencing kit (Applied Biosystems) with 5 pmol primer and 2 µl Terminator ready reaction mix. The cycle sequencing reaction comprised of 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. The Ethanol and EDTA procedure was used to precipitate the cycle sequence product, which was analysed on an ABI 3100 Genetic Analyser automated sequencer (Applied Biosystems).

Table 4.2: Primers used for PCR and sequencing reactions of the mitochondrial cytochrome *b* region.

Primer name	Sequence (5'-3')	Direction	Length base pairs
SynL	ATG ACC AAT TTA CGA AAA AC	forward	20
SynH	GGC TTT ATT TTC CGT TCA GC	reverse	20
HI16091	GTA TCA TTC TGG TTT GAT GTG	reverse	21

4.2.3 Genetic analyses

The reverse and forward sequence chromatogram outputs from the automated sequencer were assembled using Sequencher 3.1.1 (Gene Codes Corporation, Ann Arbor, Michigan), a program for DNA sequence assembling and editing. Sequences were aligned using clustal W (Thomson *et al.*, 1994; Chenna *et al.*, 2003) and BioEdit sequence alignment programs (Hall, 1999) using default parameters and verified

manually. Other sequences of closely related species in the genus were downloaded from GenBank and included in the analysis (Table 4.3). Various sequences of different genera in the family were also included as outgroups to investigate the relationship of *Syngnathus* with other syngnathid genera. These sequences were *Hippocampus hippocampus, Hippocampus zosterae, Hippocampus abdominalis, Solegnathus hardwickii, Doryrhamphus excisus, Doryrhamphus dactyliophorus, Hippichthys penicillus, Nerophis ophidion, Corythoichthys haematopterus* and *Entelurus aequoreus*. Sequences of the stickleback family Gasterosteidae (*Gasterosteus aculeatus* and *Pungitius pungitius*) and the flutemouth, *Aulichthys japonicus*, a member of the family Aulorhynchidae from the order Gasterosteiformes were also downloaded as they represent a more ancient divergence that is closely related to the family Syngnathidae.

Table 4.3: List of species whose GenBank sequences were included in the analysis.

Species	Collection locality	Biogeographical area	Accession numbers
S. acus	Britain, North Wales	eastern Atlantic	AF356073
S. rostellatus	Sweden	eastern Atlantic	AF356041
S. abaster	West Sicily, Italy	eastern Atlantic	AF356060
S. typhle	Sweden /Italy	eastern Atlantic	AF356059
S. taenionotus	Italy	eastern Atlantic	AF356061
S. floridae	Florida, USA	western Atlantic	AF356069
S. louisianae	Florida, USA	western Atlantic	AF356070
S. scovelli	Florida, USA	western Atlantic	AF356068
S. fuscus	Virginia, USA	western Atlantic	AF356056
S. leptorhynchus	Humboldt, USA	eastern Pacific	AF356064
S. schlegeli	Pearl River estuary, China	western Pacific	AF356051
H. hippocampus	unavailable	unavailable	AF192665
H. zosterae	unavailable	unavailable	AF192706.1
H. abdominalis	unavailable	unavailable	AF192640.1
D. excisus	unavailable	unavailable	AF356075.1
D. dactyliophorus	Philippines	eastern Pacific	AY787231.1
C. haematopterus	unavailable	unavailable	AY787230.
E. aequoreus	Sweden	western Atlantic	AF356044.1
N. ophidion	Sweden	western Atlantic	AF356043
P. pungitius	unavailable	unavailable	AF356080.1
G. aculeatus	unavailable	unavailable	AY283303
A. japonicus	unavailable	unavailable	AF356080.1
S. hardwickii	unavailable	unavailable	AY166829
H. penicillus	unavailable	unavailable	AF356053

4.2.3.1 Cytochrome *b* sequence variation

Nucleotide composition and bias as well as mtDNA diversity were examined using PAUP* version 4.0b10 (Swofford, 2003). The numbers of variable sites, parsimony-informative sites, and base frequencies were also estimated. The heterogeneity test X^2 as implemented in PAUP* was used to test the null hypothesis of base frequency stationarity among sequences. The possibility of saturation was examined by plotting pairwise numbers of observed changes (transitions and transversions treated separately) versus percent sequence divergence (Calculated as number of differences divided by the total number of nucleotides compared x 100) (Mattern, 2004; Meyer, 1993; Nei, 1987). Saturation occurs when multiple mutational events occur at the same nucleotide position such that they may obscure the correct interpretation of sequence differences (Meyer, 1993). The amount of sequence saturation is inferred from the shape of the trend line, with a linear relationship indicating that the sequence is unsaturated and non-linear relationship indicating the presence of saturation (Wilson *et al.*, 2001; Meyer, 1993).

4.2.4 Phylogenetic analyses

4.2.4.1 Parsimony analyses

Parsimony analyses using PAUP* were done for all available species of *Syngnathus* to evaluate the phylogeny as well as the placement of the three study species (*S. acus*, *S. temminckii* and *S. watermeyeri*) within this phylogeny. Parsimony analysis was performed with equally weighted characters using the heuristic search algorithm by the tree-bisection-reconnection (TBR) branch swapping method and 100 random addition replicate data sets. All analyses were rooted using sequences of other species from the family Syngnathidae (Table 4.3). This was done to verify whether there is a sister group relationship between *Hippocampus* and *Syngnathus* as has been previously suggested (Wilson *et al.*, 2001). Bootstrap analyses (Felsenstein 1985) were performed on the parsimony trees with 1000 replications. For all bootstrap support results, weak, moderate and strong support was defined as 50-69, 70-89, and 90-100 percentages, respectively. Bootstrap analysis is a statistical analysis commonly used to place confidence intervals on phylogenies and to test the reliability of clades in the evolutionary tree. It is the probability of obtaining at least the amount of evidence in favor of the clade in question if

that clade exists (Felsenstein, 1985). Although the statistical interpretation of support is still controversial, most practitioners have used the bootstrap as a measure of phylogenetic accuracy (Farias *et al.*, 2005; Mattern & McLennan, 2004; Near *et al.*, 2004; Santini & Tyler, 2002; Wilson *et al.*, 2001; Efron *et al.*, 1996). Branch support may also be assessed using the jackknife (Farris *et al.*, 1996) or Bayesian posterior probabilities (Huelsenbeck *et al.*, 2001; Larget & Simon, 1999). In contrast, Bayesian analyses estimate the posterior probability of each clade based on the frequency at which that clade is resolved among sampled trees once stationary log-likelihood values have been reached (Huelsenbeck *et al.*, 2001). It has been empirically observed that the latter is almost always larger than the former (Cummings *et al.*, 2003; Wilcox *et al.*, 2002; Suzuki *et al.*, 2002; Rannala & Yang, 1996). However, the available theoretical arguments for these comparisons are still not conclusive because these inferences were drawn from data using various methods and both simulated and empirical data (Huelsenbeck & Rannala, 2004; Simmons *et al.*, 2004; Douady *et al.*, 2003).

4.2.4.2 Maximum Likelihood analysis (ML)

Models of DNA substitution were tested using MODELTEST (ver.3.7) (Posada & Crandall, 1998) in PAUP* to determine the optimal model of molecular evolution for the ML phylogenetic analyses. MODELTEST uses a set of model selection frameworks: hierarchical likelihood ratio tests (hLRTs), Akaike information criterion (AIC; Akaike, 1973), and Bayesian information criterion (BIC) (Posada & Buckley, 2004; Posada & Crandall, 1998). Using distance/likelihood options both the gamma shape parameter and transition-transversion (Ti/Tv) ratio were interactively generated. The estimated likelihood scores and parameters were then used in the subsequent ML analysis. However it has been suggested that the hLRT, is not the optimal strategy for model selection in phylogenetics, and that approaches like the AIC and BIC methods offer important advantages (Sullivan & Joyce, 2005). We evaluated the fit of these models to the data using the Akaike information criterion (AIC) because the AIC allows non-nested models to be ranked and compared and facilitates the identification of groups of models that have similar fits to the data (Burnham and Anderson, 1998; Buckley & Cunningham, 2004). The popular Akaike's criterion is defined as: $AIC=-2\ln(L) + 2k$, where L is the

(maximum) likelihood, and k the number of parameters of certain model of interest. The model minimizing AIC optimizes the trade-off between fit and parameter number. The model favored is that model with the lowest AIC (Sullivan & Joyce, 2005). The best substitution/rate variation model and its respective model was used to search for the ML tree using a heuristic search, TBR and 10 random addition replicate data sets in PAUP*. Clade support was evaluated for the ML analysis through bootstrapping with the chosen model of sequence evolution. This was through the heuristic search mode and TBR branch swapping with 1000 replicates.

4.2.4.3 Bayesian Inference (BI)

Bayesian analyses were performed using MrBayes v3.0B (Ronquist & Huelsenbeck, 2003) and were initiated from random starting trees. Four Monte Carlo Markov chains (MCMC) were run for 1,000,000 generations. A longer chain was also run to ensure that stationary was reached by the 1,000,000 generation. This was to ensure that the algorithm was appropriate to provide convergence in the estimations of the tree topology with the best posterior probability, branch lengths, the parameter values of DNA substitution models and posterior probability estimates of node support (Ronquist & Huelsenbeck, 2003; Near *et al,* 2004). Trees were sampled every 100 generations to yield 10,001 trees. Examination of the maximum likelihood scores for variation identified that the burnin (stationary chain state) was reached by the 20,000th generation. In order to obtain posterior probability values, a 50% majority rule consensus tree was constructed using PAUP* from 95% of these trees after discarding 200 trees as burnin. To ensure that the Markov chain was sampling from the posterior distribution, this procedure was repeated two more times (Hillis & Wilcox, 2005). Node support expressed as posterior probabilities was interpreted as the frequency that a particular clade occurs within the collection of trees after the burnin. All posterior probabilities that were greater than 95% were considered to be significant and supported by the data. Bayesian posterior probabilities for branches in a phylogenetic analysis represent the probability that the corresponding clade is present in the true tree, given the data examined, the likelihood model, and the specified priors (Hillis & Wilcox, 2005; Huelsenbeck & Rannala, 2004; Larget & Simon, 1999).

4.2.5 Estimation of divergence times

The rate of gene evolution is governed by the overall mutation rate and the proportion of sites at which changes are neutral (Bromham & Penny, 2003). Because these factors can differ between species or over time, they can give rise to lineage effects and cause variation in substitution rates between species, such that some branches of the phylogeny may have a faster rate of molecular evolution than others (Bromham & Penny, 2003). Rate variation among sites is usually modeled by a gamma density function (Kumar *et al.,* 2004). Therefore, the rate heterogeneity among sites was investigated in these sequences using the likelihood ratio test (LRT) in PAUP*. As there was no significant rate variation among site of these sequences, equal rates were assumed for all sites. The results for testing for a molecular clock test also suggested such that a molecular clock should be accepted for this dataset. Therefore, divergence times within the genus for the 12 species were estimated by computing all uncorrected pairwise distances for PAUP*, using the gamma parameter estimated by using maximum likelihood (PAUP*).

4.2.5.1 Molecular clock calibration

The timing of major splits among the lineages or molecular clock rate of the available species of *Syngnathus* was estimated in two ways. The first estimation was done using a molecular clock calibration based on the geographically isolated populations of *S. acus* (north-eastern Atlantic and Mediterranean). These two populations were selected because they occur in two marine biogeographical regions, the Mediterranean Sea and the north-eastern Atlantic that are divided by the Strait of Gibraltar (Bargelloni *et al.*, 2003). The divergence date was based on the separation of the Mediterranean basin from the Atlantic Ocean with the rise of the Strait of Gibraltar during the late Miocene from about 6 to 4.5 million years ago (Mya) (Kumar *et al.*, 2004; Gantenbein & Keightley, 2004). The formation of the Rock of Gibraltar began (as far as the Jurassic Period), when shells of marine organisms accumulated in the Atlantic Ocean that was much narrower than it is today and formed a layer of limestone (Hsü *et al.*, 1977). Compression at the boundary of the African and Eurasian plates then uplifted this layer above sea level about 5.6 Mya (Duran *et al.*, 2004; Gantenbein & Largiad, 2003). The closing of the Strait created land bridges that together with a lower sea level (200m below present sea level) due to larger

polar ice caps, resulted in the closure of the Strait of Gibraltar and the desiccation of the Mediterranean Basin (known as the Mediterranean Salinity Crisis, MSC) (Hsü *et al.*, 1977). This event may have led to vicariance and genetic differentiation by preventing the exchange of individuals between Atlantic and Mediterranean species (Bremer *et al.*, 2005; Palma & Andrade, 2004; Lo Brutto *et al.*, 2004). Therefore if *S. acus* was distributed in both the Atlantic Ocean and Mediterranean basin before the MSC, its populations may have also subsequently became isolated during the establishment of the Gibraltar land bridge. If this isolation prevented the exchange of individuals, then these two populations offer an excellent opportunity to study evolutionary rates in *Syngnathus*.

The second calibration rate used in this study is more conservative and based on the widely accepted conservative mtDNA molecular clock benchmark of 2% per million years (Myr) estimated for fish mitochondrial DNA (Billington, 2003; Arbogast *et al.*, 2002; Durand *et al.*, 2002; Edwards & Beerli, 2000; Bermingham *et al.*, 1997). This clock is equivalent to a substitution rate of 0.01 nucleotide substitutions per site per lineage per Myr (Bermingham *et al.*, 1997). This rate was selected, as it was similar to the estimated molecular clock calibration for *Hippocampus*, a genus that is in the same family as *Syngnathus* (Jones *et al.*, 2003). A 1.4% cytochrome *b* sequence divergence rate per Myr (based on split between *Hippocampus ingens* and *H. reidi* across the Isthmus of Panama) has been used by various authors to estimate divergence times (Lourie *et al.*, 2005; Casey *et al.*, 2004; Lourie & Vincent, 2004).

4.3 Results

4.3.1 Cytochrome *b* sequence variation among species of *Syngnathus*

PCR successfully amplified about 743 bp the cytochrome *b* region for all the species examined (Sequences will be deposited in GenBank). Comparison of *Syngnathus* species and all available specimens of the three study species identified several conserved regions in their sequences. Of the 743 bp-aligned sequences, 496 sites were identical (66 %) while 254 (34 %) were different. Of the 254 sites that were different, only 125 (16.7%) sites were parsimony informative (shared by at least two individuals or species) while

129 (17.2%) were autapomorphic. Therefore, 625 (73.2%) characters were uninformative under the parsimony criterion. The dominant feature of this mtDNA region was the high rate of transitions, the majority of which were C→T changes relative to transversions. Consistent with the strong transition bias, the average nucleotide composition of the sequences was globally G-deficient (17%) whereas the other nucleotides were not very different from 25% with A, C and T making up 24%, 28% and 31% of the sequence nucleotide composition respectively. Although base composition was slightly variable among taxa, it was homogenous or not significantly different in the overall variable sites as tested by the heterogeneity test (X^2 = 76.195, d.f=186, P = 0.9).

Sequence divergence estimates among all the species (Table 4.4) varied from 3.1–20.5% (between species within *Syngnathus*) to a maximum of 25% between species of *Syngnathus* and other genera of Syngnathidae (average 21%). The range in differences between species was 3-21%, while individuals within a species differed by 0.1-7.5% substitutions. The nucleotide differences among individuals of a species were slightly higher among *S. acu*s (0.13-6.6%) than among *S. temminckii* (0.13-2.3%) and *S. watermeyeri* (1-3%) specimens. Except for the comparison between *S. typhle* and *S. taenionotus* (3.1%), and *S. floridae* and *S. louisianae* (3.4%), most pairwise sequence divergences between species had genetic values greater than 10%. The other low nucleotide divergences among species were observed between *S. abaster* versus *S. typhle* and *S. taenionotus* (3%) and between the two South African species (6.6%) indicating that these species are closely related and may share most recent common ancestors (MRCA).

Table 4.4: Percent pairwise sequence divergences among all the species (lower diagonal) and their standard errors (upper diagonal). Underlined numbers are the lowest and highest divergences among the analysed species.

Species	Code	1	2	3	4	5	6	7	8	9	10	11	12	13
S. watermeyeri	1		0.9	1.1	1.3	1.3	1.2	1.3	1.3	1.3	1.3	1.4	1.4	1.3
S. temminckii	2	6.6		1.1	1.2	1.2	1.2	1.2	1.4	1.4	1.3	1.4	1.4	1.4
S. acus	3	12.7	11.8		1.1	1.2	1.1	1.1	1.3	1.3	1.1	1.3	1.3	1.3
S. typhle	4	16.0	14.6	12.7		0.6	0.9	1.2	1.3	1.3	1.2	1.4	1.3	1.3
S. taenionotus	5	16.2	14.8	12.5	3.1		0.9	1.2	1.3	1.3	1.2	1.4	1.4	1.3
S. abaster	6	16.0	14.8	12.3	5.5	5.7		1.2	1.4	1.4	1.2	1.4	1.4	1.3
S. rostellatus	7	16.2	14.2	12.0	13.1	13.5	12.5		1.4	1.3	1.2	1.4	1.4	1.3
S. floridae	8	18.4	17.6	16.8	16.2	16.4	17.8	16.8		0.6	1.2	1.4	1.4	1.4
S. louisianae	9	18.2	17.8	16.5	15.9	15.9	17.5	16.7	3.4		1.2	1.4	1.4	1.4
S. schlegeli	10	16.1	16.0	12.2	14.2	13.9	14.6	13.6	15.9	15.4		1.3	1.4	1.3
S. scovelli	11	18.0	17.8	16.5	19.0	19.4	19.0	19.0	18.2	18.3	15.8		1.1	1.3
S. fuscus	12	18.3	17.2	15.9	17.5	18.2	17.8	19.1	17.8	17.8	17.9	10.8		1.4
S. leptorhynchus	13	19.6	19.6	18.5	17.9	18.3	18.3	19.4	19.5	19.1	18.3	19.3	20.5	

The plot of the number of substitutions versus percent sequence divergence showed that the sequences exhibited saturation after 21% sequence divergence (Figure 4.2). The graph displays saturation only when the nucleotide differences among sequences are not phylogenetically useful (Kocher & Carleton, 1997). This may imply that although the species are evolving and becoming genetically different, similarity is just as likely to reflect chance convergence, as it is to reflect ancestry (Kocher & Carleton, 1997; Meyer 1993). Therefore, no sequences were removed from these analyses because *Syngnathus* species showed a linear relationship for sequence divergences ranging from 3% to a maximum of 21% between most species and *S. leptorhynchus*. The graph only flattened out after pairwise comparisons of sequences of species of *Syngnathus* with the other species from other genera of Syngnathidae (> 21%). The DNA sequence divergences between the species of the genus were therefore phylogenetically useful as their comparisons were in the phase when the trend line was increasing. It was therefore assumed that saturation would not affect analysis of the ingroup (divergence < 21%).

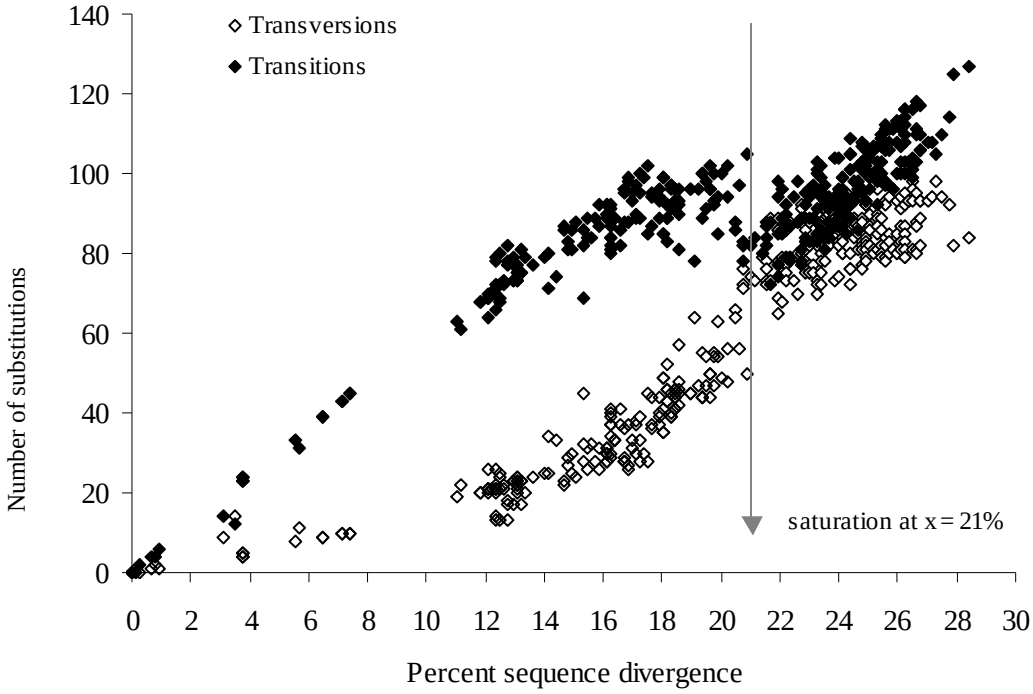


Figure 4.2: Transitions/transversions plotted against pairwise percent sequence divergence for 63 sequences of *Syngnathus* showing the point where sequences become saturated.

Syngnathus acus and *S. temminckii* individuals were very different genetically, with nucleotide divergences ranging from 11.7-15.0%. These sequence divergence values are within the ranges observed for congeners among fish species (Johns & Avise, 1998; Meyer, 1993). The plot of nucleotide divergences (Figure 4.3) of these two species shows three distinct groups. The first group (A) exhibits low sequence divergence between 0-3% and defines most of the differences between pairwise comparisons of individuals within *S. temminckii* and each of the two locations of *S. acus*. The next group labeled B (4-7%) defines nucleotide differences between *S. acus* specimens from the Bay of Biscay and the Thracian Sea. The last group (C) with the highest sequence divergence (11-15%) represents the difference between pairwise comparisons of *S. acus* and *S. temminckii* specimens, with the higher values being South African and Bay of Biscay specimens.

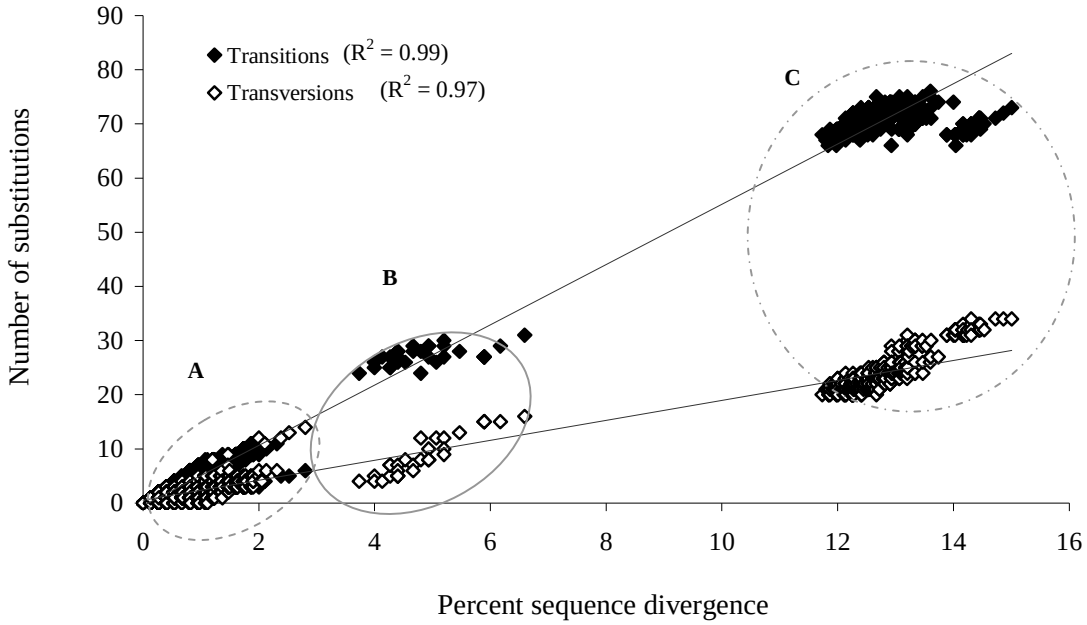


Figure 4.3: Transitions/transversions plotted against pairwise differences among sequences of *S. acus* and *S. temminckii* showing three distinct groups A, B and C.

4.3.2 Phylogenetic relationships within *Syngnathus*

4.3.2.1 Parsimony analysis

Initial parsimony analysis was done using all available sequences (with GenBank sequences). Although various specimens from each genus were also initially included the analysis, these were later reduced since they increased computation time and did not change the resultant topology of the *Syngnathus* phylogeny. *Solegnathus hardwickii* was only used in an initial analysis as the available sequence (420 bp) was much shorter than that of all specimens available (743 bp) and therefore considerably reduced the number of available characters for analysis. Furthermore, removing this species from the analysis did not greatly change the resultant topology of the *Syngnathus* tree or relationships among genera (Appendix VIII). Of important note were the seahorse species that all clustered together in a strongly supported clade such that using fewer representatives did not influence the relationships among taxa. Hence, 29 sequences were chosen for the final phylogenetic analysis after trying out various outgroups combinations. Of the 743

base pairs that were aligned, 383 positions were identical while 360 positions were different among taxa. Only 329 of the polymorphic sites were phylogenetically informative (Table 4.6). Unweighted parsimony analysis recovered one parsimonious tree with 1696 steps with values of 0.38 and 0.56 for the Consistency (CI) and Retention indices (RI), respectively. The resultant tree is given in figure 4.4 and nodes defining supported clades are labeled from A to M.

Table 4.6: Results of the unweighted parsimony analysis of 12 species of *Syngnathus* and 18 other species from other genera based on 743 base pairs of cytochrome *b*.

	Identical sites	Polymorphic sites	Parsimony informative sites	Autapomorphic sites
No. of bases	383	360	329	31
% of sequence	52	48	44	4

The phylogenetic tree of *Syngnathus* is not well resolved, as the clades defining relationships among species have no bootstrap support. However, the monophyly of the genus is moderately supported with 74% bootstrap support (Clade K). The tree has an unresolved polytomy that associates South African species with some species of the western Atlantic Ocean, and all the eastern Atlantic species and *S. schlegeli* from the western Pacific Ocean (clade J). Some of the eastern Atlantic/Mediterranean species namely, *S. abaster, S. typhle* and *S. taenionotus* form a strongly supported clade (Clade G) that is nested within this polytomy. Unlike the results of Wilson *et al.,* (2001), this clade was not paraphyletic as *S. typhle* specimens belonged to a supported clade (Appendix VIII). The tree also suggests that the two South African species, *S. watermeyeri* and *S. temminckii* are more closely related to each other than either is to *S. acus*. The two species pair up in a supported clade (100% bootstrap support) as sister taxa. These results like the morphological analysis show that *S. acus* and *S. temminckii* are two phylogenetically distinct species. Mean pairwise distance between the two species was 11%, which is within the range observed by Johns and Avise (1998) for congeneric fish species. Another feature of this tree is some divergence within the *S. acus* and *S. temminckii* groups. There were two well-supported clades (E and F) of *S. acus* with bootstrap values of 100%. These clades comprised of Atlantic Ocean (Bay of

Biscay) and Mediterranean Sea (Thracian sea) specimens. The tree also consists of moderately supported clades within the *S. temminckii* group though the relationships among the individuals are unresolved (Appendix VIII). While there have been relatively fewer genetic changes within *S. temminckii,* the *S. acus* seems to have undergone more intra-specific divergence based on the available data. The other main features of the tree are the basal position of *S. leptorhynchus,* which is an eastern Pacific Ocean taxon and was the most genetically different species based on pairwise sequence comparisons with other species (average 19% divergence). *Syngnathus scovelli* and *S. fuscus* appear to be more closely related to each other than to the other species in this polytomy. The two species appear to share a most recent common ancestor and as they had a strongly supported lineage (clade I with 99% bootstrap support). *Syngnathus floridae* and *S. louisianae* appear to share a most recent common ancestor (clade H) for this phylogenetic relationship. However, the *S. floridae* and *S. louisianae* clade was paraphyletic (Appendix VIII) and is therefore unresolved though strongly supported. Other species of *Syngnathus* from the northwestern Atlantic need to be added to this data to verify the phylogenetic relationships defined by these two clades (clades G and H).

The relationships among genera of Syngnathidae are unresolved as species all cluster together in an unresolved basal polytomy (clade M). The monophyly of the family was however strongly supported with 96% bootstrap in all parsimony analyses (Appendix VII). These results are however not in agreement with the results of Wilson *et al.,* (2001), which suggested a sister group relationship between *Hippocampus* and *Syngnathus*. The genus, *Hippocampus* is monophyletic with 98% bootstrap support (clade L) and part of the polytomy. The relationships between Hippocampinae and Syngnathinae were not tested, as there were insufficient species. Therefore, further examinations of pouch development and taxon relationships among genera in Syngnathidae are still necessary. Because our goal was to infer the placement of South African species within this phylogeny, this phylogeny can still serve as a valuable source of information for evaluating relationships among species of *Syngnathus*.

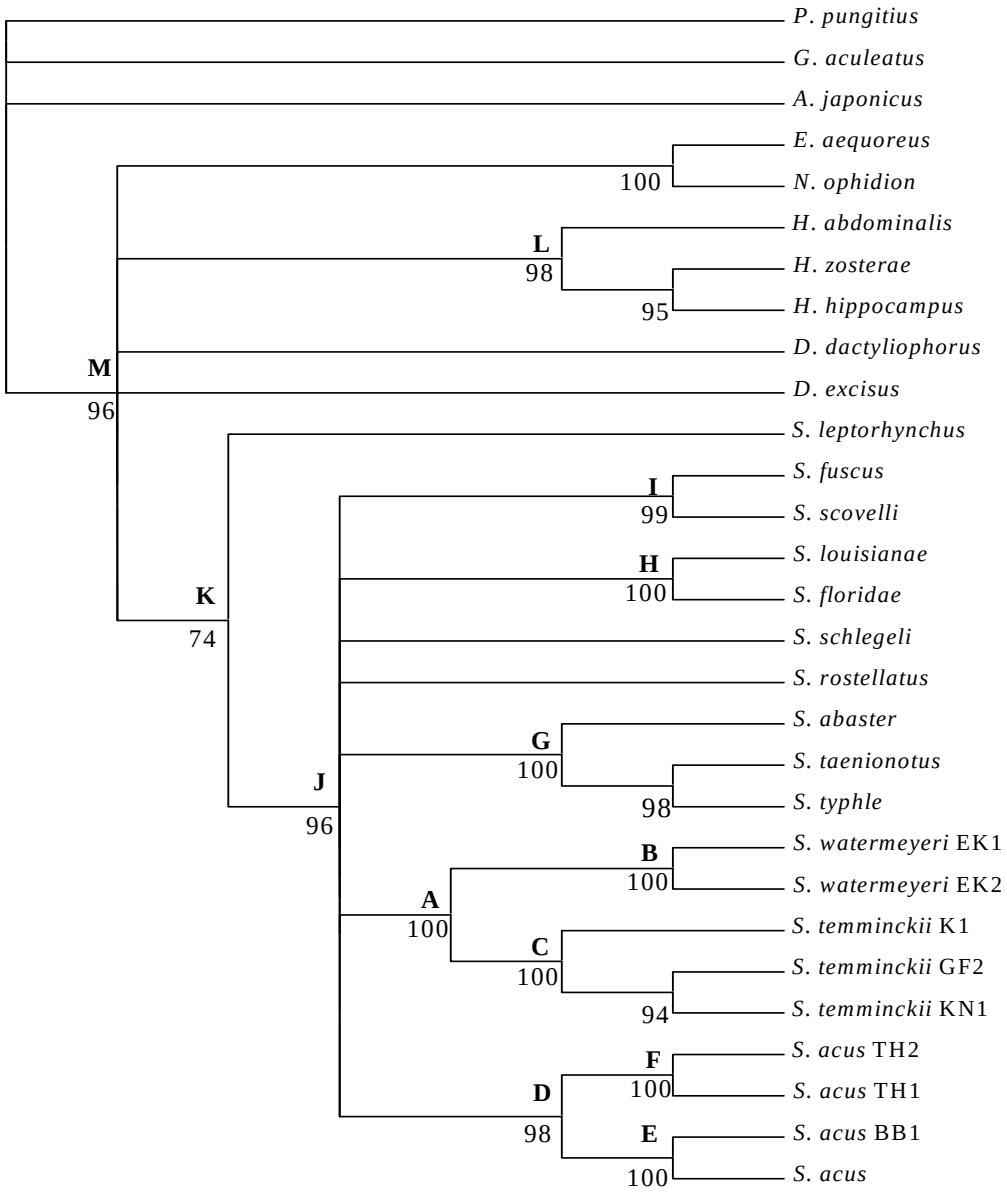


Figure 4.4: Phylogenetic relationships among species of *Syngnathus* inferred from the parsimony analysis of cytochrome *b* sequence data of 29 selected sequences. Codes for locations of *S. acus, S. watermeyeri* and *S. acus* are given in Table 4.1. Clades are labelled from A to M while the numbers below the branches are the associated bootstrap support values.

4.3.2.2 Maximum Likelihood analysis

ModelTest 3.06 recommended the variant model of the HKY (Hasegawa-Kishino-Yano) model (HKY+Γ+I) with invariable sites and gamma-distributed rate using the Akaike Information Criterion (AIC). The HKY model (Hasegawa *et al.*, 1985) has five model parameters and assumes different rates for transitions and transversions (Crandall, 2001; Posada and Crandall, 1998). This model estimated the proportion of invariable sites (Pinvar or I) as 0.48 and a gamma shape parameter (Γ) estimate of 0.96. The data were estimated as having base frequencies at 29%, 31%, 11% and 29% for A, C, G and T, respectively. The resultant tree obtained using this model, PAUP* is represented in figure 4.5 with associated bootstrap values for clades (A-N).

Although both parsimony and ML analyses generate the same tree topologies, clade N was only supported in the ML tree. This clade did not have significant support in the parsimony tree (52%) but was supported (76%) though moderately by the ML tree. The monophyly of the genus was also moderately supported (86%) while *S. leptorhynchus* and the two western Atlantic species (*S. scovelli* and *S. fuscus*) were basal in the genus. Both parsimony and ML trees indicate that *S. acus* and *S. temminckii* belong to two distinct and strongly supported clades (D and C). Other similarities between the topologies include the divergence within *S. acus* and a sister relationship between the two South African species. The monophyly of the family was also strongly supported (clade M) with species of *Hippocampus* also forming a strongly supported clade (L). Furthermore, this analysis also suggests that there is no sister-taxa relationship between *Hippocampus* and *Syngnathus* (clade M). Syngnathidae though strongly supported as monophyletic with 99% bootstrap support had an unresolved basal polytomy showing no phylogenetic relationships among genera. Otherwise, the ML tree was very similar to the results obtained by Wilson *et al.*, (2001).

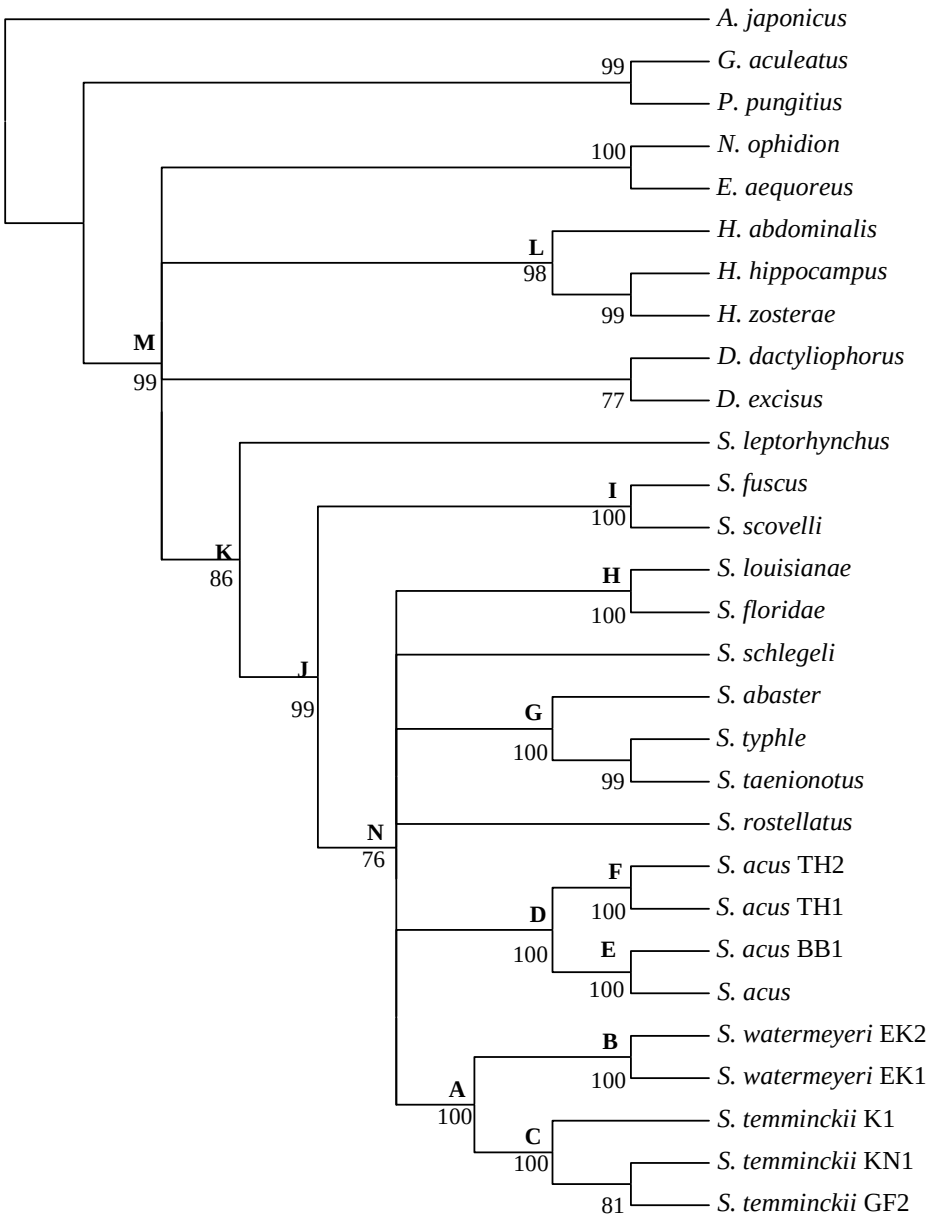


Figure 4.5: The maximum likelihood tree of cytochrome *b* sequences with bootstrap values based on the estimated model parameters of the HKY+Γ+I model of substitution. Branches are scaled to number of substitutions per site. Clades are labelled from A to N while the numbers below the branches are the associated bootstrap support values.

4.3.2.3 Bayesian Inference

The effective sample size of the Markov chain in the Bayesian analysis was 10001. The mean ln likelihood of these trees was –5624. For the Bayesian inference method, identical topologies were recovered for each of the four runs with the dataset, although posterior probabilities for some of the nodes differed only slightly (0.01-0.02) between each of the Bayesian runs. Burnin trees for each run were identified and excluded from the majority rule consensus tree calculations. Fourteen very strongly supported clades (A to M and O) were identified in the inferred phylogeny. Although both parsimony and ML analyses generate the same tree topology as BI, clade O was only supported in the BI tree (Table 4.7).

Bayesian inference suggests that South African species share a most recent common ancestor (clade O) with northeastern Atlantic species of the genus, a relationship that is not supported by the other analyses. All the other major nodes defining strongly and moderately supported clades in the parsimony tree were also significantly supported by Bayesian posterior probabilities estimates. Bayesian inference shows that *S. acus*, *S. temminckii* and *S. watermeyeri* belong to two phylogenetically distinct and strongly supported clades within *Syngnathus*. The monophyly of *Syngnathus* is supported by Bayesian analysis with 100% posterior probability (clade K). Furthermore, there is also no support for the suggested sister-group relationship between *Hippocampus* and *Syngnathus* according to the results of this analysis (Figure 4.6).

Table 4.7: Bootstrap support values for parsimony and maximum likelihood analyses and Bayesian probability estimates for the major supported clades expressed as percentages.

Analysis	Clade														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Parsimony	100	100	100	98	100	100	100	100	99	96	74	98	96	-	-
ML	100	100	100	100	100	100	100	100	100	99	86	98	99	76	-
BI	100	100	99	100	100	100	100	100	100	99	100	100	100	-	99

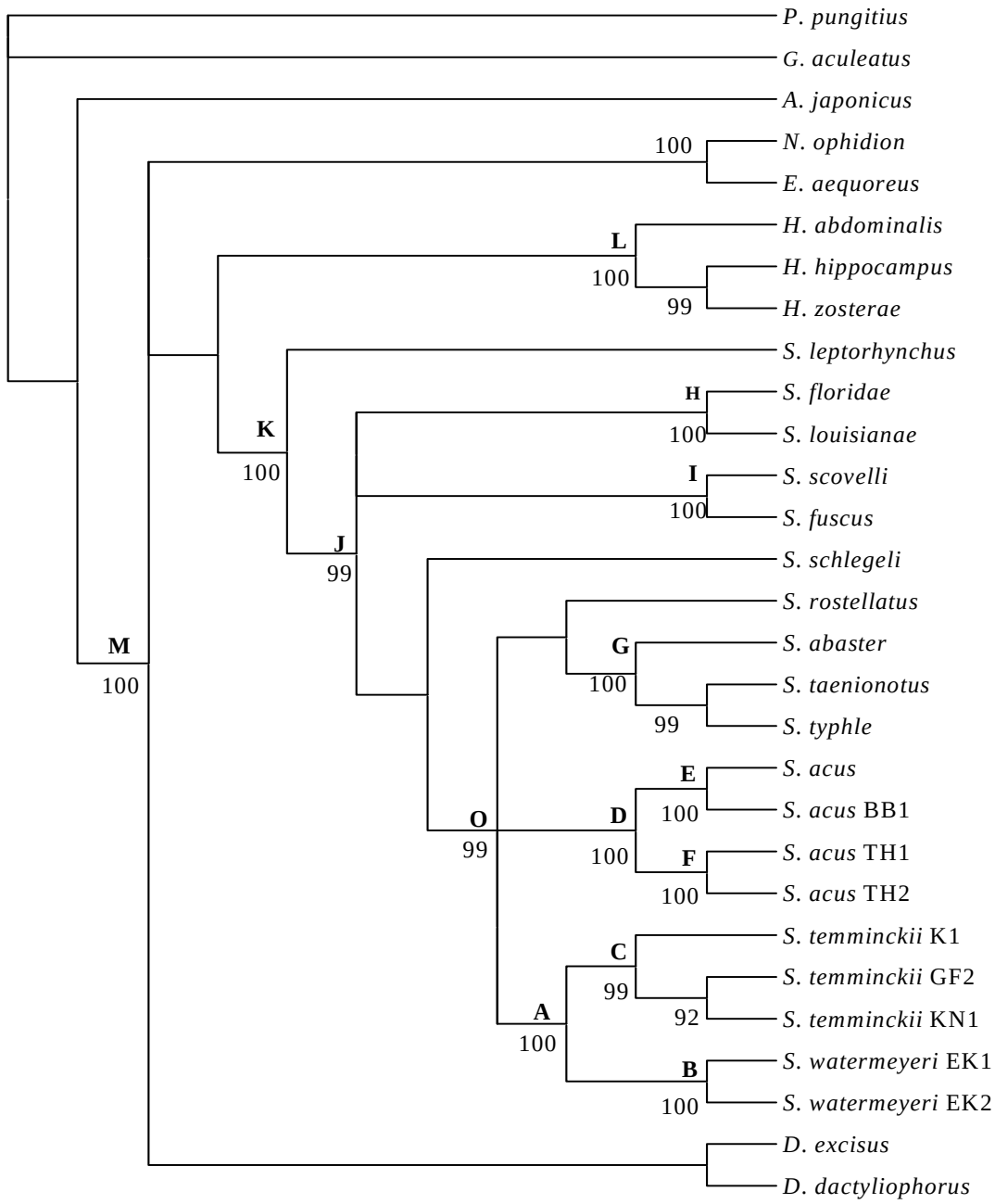


Figure 4.6: The resulting topology of a 10^6 generations Bayesian inference analysis of 29 selected cytochrome b sequences. Clades are labelled from A to M and O, while the numbers below the branches are the associated significant Bayesian posterior probabilities values.

4.3.3 Divergence time estimates

The molecular clock hypothesis was accepted for this data, as the rates of mutations across lineages of the species were not heterogeneous using the likelihood ratio test. The calculated likelihood ratio test statistic was assessed by reference to chi-square tables, and was found to be insignificant ($P < 0.05$). Two calibration points were used to get a conservative range for the time estimates for the radiation of the different clades. Molecular clock calibration based on the rise of the Strait of Gibraltar suggests that pipefishes of the genus *Syngnathus* arose more than 23 Mya during the Miocene period (23.8-5.3 Mya; Berggren *et al.*, 1995) (Figure 4.7). The average mitochondrial DNA molecular clock of 2% sequence divergence for fishes estimated the evolution of *Syngnathus* to be around 12 Mya (Figure 4.6). Therefore, both calibration procedures imply that pipefish species, which are almost distributed as widely as the genus *Hippocampus* may have a Miocene origin (Casey *et al.*, 2004; Fritzsche, 1980). Considering both calibration clocks, the first splitting event within the genus, i.e., the divergence between *S. leptorhynchus* and the remaining *Syngnathus* species could have occurred about 18-10 Mya (clade K).

The burst of divergence that produced a polytomy (J) among the Atlantic, Mediterranean and Indian Ocean clades probably due to major diversification of major lineages within the genus is estimated to have taken place some 13-7 Mya during the mid-Miocene period. Although the mtDNA clades of some species were easily recognized and separated from geographic and genetically different species, there was no phylogenetic resolution among species to determine the order in which these species diversified into these regions. However, ML analysis suggests that the earliest ancestor of this clade appears to be a species that gave rise to *S. fuscus* and *S. scovelli* and the rest of the species in clade N. The percent sequence divergence between *S. temminckii* and *S. watermeyeri* suggests that the two species diverged about 6.5-3.5 Mya during the Pliocene. On the other hand, diversification within species such as *S. temminckii* and *S. watermeyeri* is more recent and may have been influenced by Pleistocene conditions (1.8 Mya - 8000 years ago).

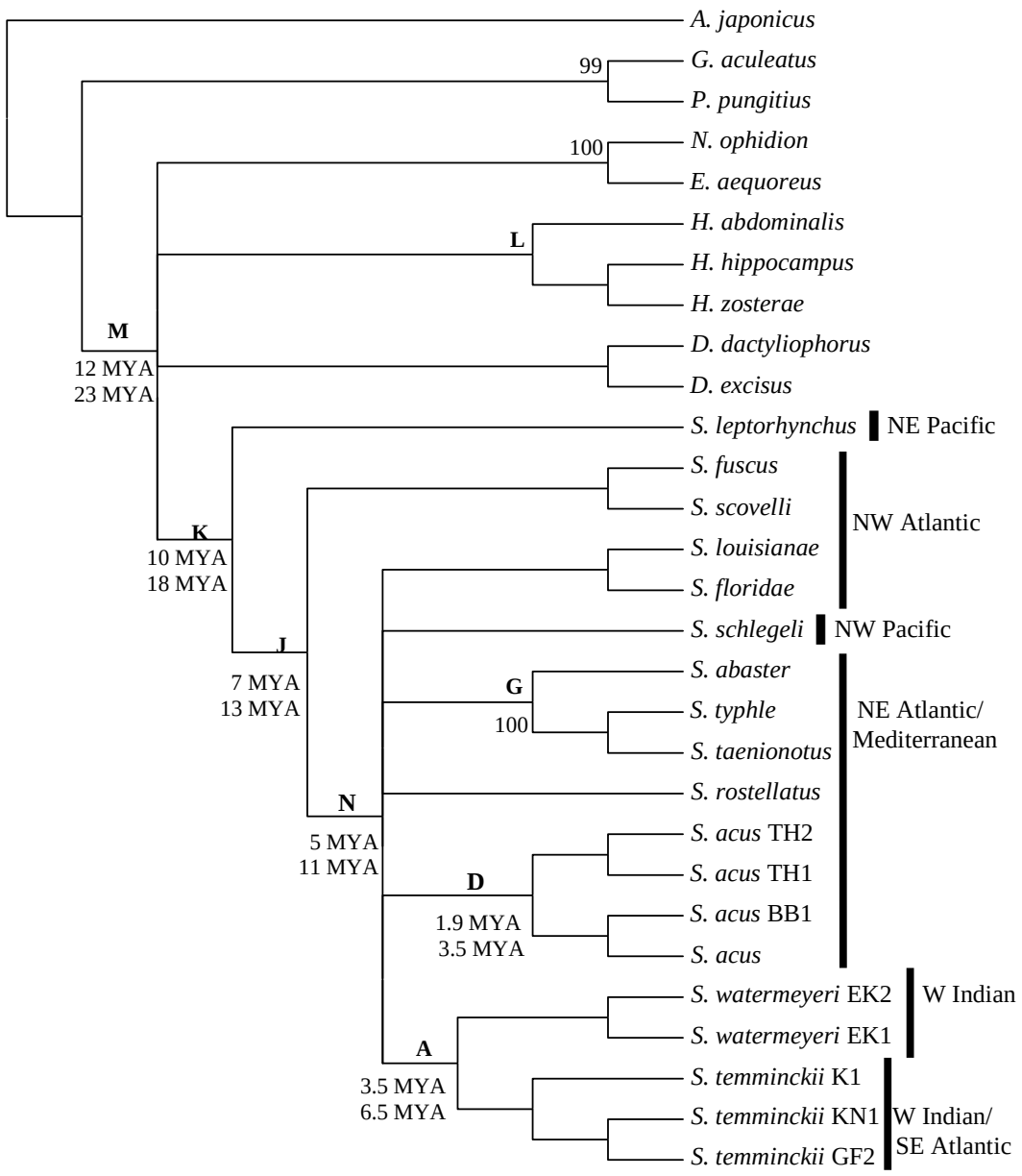


Figure 4.7: The results of the molecular clock analysis obtained from estimated p-distances using PAUP*. The lower age estimates for each clade that is represented are based on the average rate of 2% sequence divergence for mitochondrial DNA of fish, while the higher estimates on the bottom were estimated based on the rise of the Strait of Gibraltar.

4.4 Discussion

4.4.1 Phylogenetic relationships

The observed sequence divergences between individuals of all species of *Syngnathus* ranged from 3-20 % and are similar to values estimated for various other fish species using the cytochrome *b* region (Lourie *et al.*, 2005; Teske *et al.*, 2004). García *et al*., (2000) found a maximum uncorrected pairwise divergence of 28% (average ~ 17%) in killifishes, Reed *et al.,* (2005) found a maximum 18% among carangid fishes, while Casey *et al.* (2004) found > 24 % Tamura-Nei distance among seahorses. The highest sequence divergence (20%) occurred between *S. leptorhynchus* and *S. fuscus*. Based on the observed levels of sequence divergence, the data represent long-separated species and diverse evolutionary histories within *Syngnathus*. The high rate of sequence divergence between individuals of *S. acus, S. watermeyeri* and *S. temminckii* (7-11%) suggests that these taxa are distinct species. The phylogenetic analyses results using parsimony, ML and BI methods were not surprising given the morphological differences observed among these species.

The trees that were retrieved by all methods (Figure 4.4-4.6) were characterised by similar species groupings and generally agree with the results obtained by Wilson *et al.* (2001) for relationships within *Syngnathus*. *Syngnathus acus* and *S. temminckii* were each found to belong to genetically distinct monophyletic clades that were strongly supported among all tree topologies. The results show that *S. acus* and the two South African species have been reproductively isolated for a sufficiently long time such that they have diverged from each other. These interpretations are in agreement with the morphological results of the previous chapter. Therefore, the Southern African long snout pipefish that is currently recognized as *S. acus* (Dawson, 1986) is morphologically and genetically distinct from the *S. acus* occurring in European and Mediterranean waters, and should be referred to as *S. temminckii*. Although the generic status of *S. watermeyeri* was previously considered uncertain (Dawson, 1985), the cytochrome *b* analysis results show that this species is rooted within *Syngnathus*. The results showed that *S. watermeyeri* is closely related to *S. temminckii* as they share a most recent common ancestor. This phylogenetic relationship was strongly supported by the estimated Parsimony, ML and BI tree

topologies (Clade A). The relationship between the two species was not surprising as they geographically isolated from the other species in the genus. These two species are presently the only known Indian Ocean species and therefore represent a group that has diverged from other *Syngnathus* lineages.

The phylogenetic analysis also consistently demonstrated the existence of two distinct lineages within *S. acus*. Specimens of *Syngnathus acus* occurring in the Thracian Sea (Clade F) where genetically distinct from specimens collected from the northeastern Atlantic Ocean (clade E). These two clades were strongly supported by all analytical analyses with 100 percent bootstrap and posterior probabilities values. Whether these two clades correspond to two subspecies or merely haplotypes is still uncertain at this time. Subspecies taxonomy within fishes has been controversial such that closely related populations are therefore generally described as species complexes (Taylor, 1999; Turner, 1999). The relationships of clades E and F of the *S. acus* group therefore require further investigation and taxon sampling. They will be assumed to represent different haplotypes of the species. The distinct forms of *S. acus* may be as a result of genetic polymorphism within a species that is widespread and therefore exposed to different ecological and selection pressures that may cause genetic drift. These two forms could also imply the existence of two distinct gene pools in the two biogeographical regions that are have been geographically separated by the strait of Gibraltar and are now possibly reproductively isolated.

The remaining species (*S. abaster, S. typhle* and *S. taenionotus*) from the eastern Atlantic Ocean, North Sea and Mediterranean Seas all form clade G (with 100% bootstrap support). Although these three species are part of the unresolved polytomy (clade J), they share a most recent common ancestor and may therefore represent a northeastern Atlantic lineage. Furthermore, this northeastern Atlantic clade was also observed in the Wilson *et al* (2001) study and was also within a monophyletic clade with *S. rostellatus* and *S. acus*. While parsimony analysis and ML analysis from this study do not support this relationship with *S. rostellatus* and *S. acus*, BI inference analysis retrieved this clade but with the addition of the two South African species (clade O). In this study, the four

Western Atlantic species (*S. floridae, S. louisianae, S. fuscus* and *S. scovelli*) formed two clades that were strongly supported in all analyses. However, the *S. floridae* and *S. louisianae* clade was paraphyletic for *S. floridae* (Appendix VIII). These results were also recovered by Wilson *et al.,* (2001) and suggest diversification of distinct lineages within the northwestern *Syngnathus* species that requires further investigation. The western Pacific species *S. schlegeli* was also embedded within unresolved clade J and could represent dispersal to this region from the Atlantic. The long evolutionary history of *S. leptorhynchus,* an eastern Pacific species, suggests that this species that has undergone much diversification and could be the most basal species in the genus.

Analysis of the cytochrome *b* dataset supports the monophyly of *Syngnathus* regardless of the phylogenetic analyses employed. It is also important to note that support for the monophyly of *Syngnathus* was not as strong as that observed for basal, intergeneric clades (clade M) a result also observed by Wilson *et al* (2001). Poor resolution and weak support at the base of a phylogeny can be due to either lack of information necessary to resolve basal relationships or fast differentiation of lineages at the base of the tree (Poe & Chubb, 2004; Jackman *et al.*, 1999). The lack of resolution within the *Syngnathus* clade is probably due to the missing data even though the available datasets included more then 320 parsimony informative characters (Rydin & Källersjö, 2002). Although all the geographic distribution of the genus was reflected by this analysis, only a third of the species were incorporated since specimens of some species were unavailable. These results still suggest a strong correspondence between geographic origins of some species lineages. All the strongly supported clades of species or sister pairs within this genus were grouped according to geographic region as opposed to species occurring in different regions. The results suggest that morphological variation and genetic divergence in *Syngnathus* may be associated with the diverse ecological and distributional characteristics of the group. The trees shown in this study are likely to be the first to depict relationships among members of the genus with the inclusion of the two South African species. It is hoped that future inclusion of other taxa into this phylogeny will help resolve this genus. This study also showed coherence between molecular and morphological patterns of variation among the three target species (*S. acus, S. temminckii*

and *S. watermeyeri*). Therefore, it is suggested that although the species of *Syngnathus* are very similar morphologically, they possess a sufficient number of morphological characters that differ in such a way that are useful in the distinction of species.

4.4.2 Divergence time estimates

Species in the genus *Syngnathus* provide an ideal system to study phylogeographic patterns as they are distributed over a vast geographic range and throughout the three main world oceans. This distribution covers the major marine phylogeographical breaks defined by vicariant events that have been used to estimate divergence times for most marine fishes (Bernardi *et al.*, 2004; Gantenbein, 2004; Broderick *et al.*, 2003; Bermingham *et al.*, 1997). The pattern of cytochrome *b* sequence divergence has been shown to have implications for molecular clock calibration as this gene has been useful in providing phylogenetic utility only for taxa with relatively recent divergences of up to approximately 50 Mya (García *et al.*, 2000; Moritz *et al.*, 1987; Wilson *et al.*, 1985). Pipefish though considered to be the oldest group of syngnathids and the first to exhibit a reed-like body, are relatively recent as they appeared in the fossil record during the Eocene (54-37 Mya) (Fritzsche, 1980). The evolution of several groups of Syngnathidae during this period can be linked to the origin of intertidal vegetation during this time period. Presently, syngnathids are one of the major and highly diversified ichthyo-fauna inhabiting these intertidal vegetative habitats. These pipefish divergence time estimates also coincide with the “sudden” fall (in just 200,000 years) in global temperature of 3–4 °C that took place about 34 Mya at the end of the Eocene Period (Cavelier *et al.*, 1981). This cooling led to the expansion of ice on Antarctica and a corresponding 40m fall in global sea level (Cavelier *et al.*, 1981) that may have changed prevailing ocean current directions and hence marine fauna distribution patterns.

The results suggest that the diversification within Syngnathidae that may have led to the evolution of several genera took place approximately 23 and 12 Mya in the Miocene (23.8-5.3Mya). These divergences dates can be supported by Paleoclimate data that shows that there was a brief respite from the first cooling event in the Miocene between 20 and 16 Mya that could have allowed conditions in the northern Atlantic to become

warmer and hence more suitable for pipefish establishment. The analyses also indicate that diversification may have occurred in the genus *Syngnathus* about 18–10 Mya (clade K) during the mid and late Miocene. The separation of the South African (Indian Ocean) clade from other clades and most of the other species and the radiation of the Atlantic Ocean and Mediterranean Sea species occurred about 11-5 Mya. The timing of these radiation events also coincides with major Paleoclimate events that took place in the northern hemisphere. Around 5.6 Mya, the Mediterranean basin dried out and re-flooded several times, leaving salt deposits of more than 1500 m thickness (the MSC event). This created changes in ocean circulation patterns throughout the world and therefore fish movement mechanisms (Meijer & Krijgsman, 2005). This event occurred during the second major cooling period that was so intense such that south-eastern Greenland was completely covered with glaciers by 7 Mya (Sigman *et al.*, 2004; Cavelier *et al.*, 1981). During the coldest periods of the Ice Age, average global temperatures were probably 4 - 5° Celsius colder than they are today.

Therefore, these changes in sea level, ocean current directions and temperature could have forced fragmentation of some species to new areas (e.g., South Africa). By about 5-6 Mya, glaciers were extending into Scandinavia and the northern Pacific region (Sigman *et al.,* 2004). Changes such as the emergence of mountain chains and sea straits could have formed barriers to dispersal and directly isolated pipefishes, while islands and peninsulas may have provided important refuge during phases of habitat retraction and encouraged species divergence. The earth began to warm up again between 5 and 3 million years ago, such that the sea was much warmer around North America and the Antarctic than it is today (Sigman *et al.,* 2004). Warm-weather plants grew in Northern Europe where they cannot survive today and probably encouraged the dispersal of species to the colder Atlantic waters. Tropical records indicate that significant tropical climate reorganization occurred twice: once between 4.5-4.0 Mya, well before significant northern hemisphere glaciation (NHG), and once between 2.0-1.5 Mya, well after the onset of significant NHG (Bartolli *et al.*, 2005). Subtropical and tropical regions began to cool causing their upwelling regions to cool, invigorating the west to east temperature and pressure gradients and reinforcing the cooling trend (Bartolli *et al.*, 2005).

Circulation changes in the Atlantic Ocean were also possibly forced by tectonic events such as the restriction of the Central American seaway and the rise of the Strait of Gibraltar during this time. Thus, gradual changes in basin geometry, land-surface conditions and other events, occurring at least over the past ± 4−1.5 Mya, most probably forced global cooling and circulation pattern changes (Bartolli *et al.*, 2005; Ravelo *et al.*, 2004; Haug & Tiedemann, 1998). This could have led to the geographic expansion of species from the Atlantic and then perhaps a southward retreat to the South Atlantic and Indian Ocean in Southern Africa in response to cooler temperatures. Low-lying coastal areas and estuarine habitats the typical habitats for syngnathids are extremely vulnerable to sea level rises and marine incursion and were hence clearly affected during this time (Baxter & Meadows, 1999).

The *S. acus* clades E and F were also estimated (using 2% as sequence divergence) to have diverged during the period when the Atlantic Ocean and Mediterranean Sea split. Paleoclimate data has shown that the Atlantic Ocean broke through the Strait of Gibraltar and created the present Mediterranean Sea about 4.5 Mya (Bartolli *et al.*, 2005; Ravelo *et al.*, 2004; Haug & Tiedemann, 1998). It is generally admitted that normal marine conditions were restored at the beginning of the Pliocene by an abrupt re-flooding of the Mediterranean during the Zanclean deluge (Meijer & Krijgsman, 2005). Currently an encounter of the superficial inflow of Atlantic water and Mediterranean waters, which flow out at depth occurs in the most western part of the Alboran Sea and results in a frontal zone, the Almería-Oran Oceanic Front (AOOF) (Bartolli *et al.*, 2005; Gysels *et al.*, 2004; Ravelo *et al.*, 2004). The AOOF and the narrow oceanic strait together represent a natural barrier to gene flow between Atlantic and Mediterranean marine fauna. These barriers could cause and maintain allopatric separation in different marine species and could be responsible for the observed differentiation within *S. acus* populations. Although a few species show no differentiation between their Atlantic and Mediterranean populations, a clear phylogenetic break as is hypothesized for these two populations of *S. acus* has been observed among other marine species (Bremer *et al.*, 2005; Baus *et al.*, 2005; Palma & Andrade, 2004; Gantenbein & Largiad, 2003; Borsa, 2002). For example, large mtDNA divergence was observed between Atlantic and

Mediterranean samples of three sparids, *Lithognathus mormyrus, Spondyliosoma cantharus,* and *Dentex dentex* (Bargelloni *et al.*, 2003).

The rise of the Isthmus of Panama, which occurred approximately 3.5 Mya has been proposed as the major historical event responsible for the separation of Atlantic and Pacific faunas (Bernardi *et al.*, 2004; Bermingham *et al.*, 1997). The distinct phylogenetic break between these two regions observed for marine and freshwater species suggests that the Panamanian Isthmus is a long-term barrier to the flow of genes between the western and eastern Pacific and Atlantic fish species (Bermingham *et al.*, 1997). For example, Casey *et al.* (2004) observed that *Hippocampus ingens*, the only eastern Pacific species, was genetically closer to the Caribbean *H. reidi* than any of its western Pacific counterparts. Therefore, the eastern Pacific *S. leptorhynchus* may have been separated by the rise of the Isthmus of Panama from the Caribbean and Atlantic *Syngnathus* species. However, *S. leptorhynchus* is the most basal species in the phylogeny and may therefore have diverged earlier than the Atlantic pipefish species, despite the fact that the latter occur in greater diversity and numbers. The rise of the Panamanian Isthmus was not used for calibration in this study, as there was only one specimen from the eastern Pacific available for analysis. This geographic break could however become very useful once more species from this region are included in the phylogeny.

4.4.3 Conclusions

The results of this study though similar to the Wilson *et al* (2001) study in terms of the phylogeny of *Syngnathus,* were very different with regards to its generic placement of in Syngnathidae. While most of the genera were not available for analysis, various analyses using different combinations of outgroups, from the sub-families, Hippocampinae and Syngnathinae did not support a close relationship between *Syngnathus* and *Hippocampus*. There was however an unresolved polytomy between genera that requires further investigation. While the morphological relationships have been hypothesized and described (Kuiter; Herald, 1959) there is need for further revision and to incorporate more genetic data to resolve these relationships.

Although the results of this cytochrome *b* phylogenetic study could not resolve phylogeny of *Syngnathus*, the data identified a number of clades or supported relationships among some species and geographical regions. The study obtained relatively well-supported trees that are nearly identical across a variety of analyses apart from clade support values for two clades. It appears that species of the genus *Syngnathus* have the ability to disperse and become established, as species distribution ranges are either widespread (*S. acus* or *S. typhle*) or restricted (e.g. *S. watermeyeri*) in some instances. The two South African species and *S. schlegeli* are found at the periphery of the distribution range in the Indo-Pacific and could be a result of long distance dispersal, as the species of the genus are more abundant in the central region (Atlantic). There are 22 species, out of the 32 valid species of *Syngnathus*, distributed in the western and eastern Atlantic regions. According to Brooks and McLennan (2002), the larger central population is assumed to be the "principal source of evolutionary change," when determining species origins. This is because large populations have a higher probability of gaining new mutations and a lower probability of going extinct than small populations. Small populations if isolated will usually become more highly derived ecologically compared to the more widespread or larger populations.

The trees shown (Figure 4.4-4.5) are not resolved at internal clades, though some general species relationships could be deduced. This could an artifact of taxon sampling with only 12 species of *Syngnathus* and about 20 species having not been sampled. Increased resolution in these clades will require more extensive sampling of the other species in the genus. Although the cytochrome *b* data presented here does not enable us to resolve this phylogeny and maybe a reflection of the evolution of this gene, it has allowed us to suggest some testable hypothesis for future analysis. The data revealed distinct geographic lineages that could point to biogeographic breaks that may later define this phylogeny. Lanyon (1993) advises that systematists identify both a ''best estimate'' and a ''reliable estimate'' of phylogenetic relationships. The trees therefore represented in this study are only an estimate of the hypothetical relationships of South African species within *Syngnathus.*

Chapter Five

The phylogeography of South African species of *Syngnathus*

5.1 Introduction

Documenting the extent and causes of population subdivisions via phylogeography has been a major focus in the evolutionary and molecular ecological studies of various fish families (Brandstatter *et al.*, 2005; Durand *et al.*, 2005; Mattern, 2004). Phylogeography has contributed considerably to our understanding of the factors that have influenced population structure and species divergence among syngnathids (Teske *et al.*, 2004; Jones *et al.*, 2003; Wilson *et al.*, 2001; McCoy *et al.*, 2001; Jones *et al.*, 2001). Significant geographic differentiation can occur between populations that still occasionally exchange individuals, but are at equilibrium or when a historical event has permanently interrupted gene flow between the populations being considered (Grosberg & Cunningham, 2001). For example, genetic analysis revealed strong differentiation when comparing Atlantic and Mediterranean samples of sparid species, *Lithognathus mormyrus* and *Dentex dentex*, and moderate for *Pagrus pagrus* that have been geographically isolated (Bargelloni *et al.*, 2003). Whenever gene flow between populations is prevented, a combination of mutation and random extinction events of lineages (lineage sorting) will eventually occur (Grosberg & Cunningham, 2001; Avise, 2000). The result is that each population will have a unique most recent common ancestor (MRCA) and lineage (Wakeley, 2005; Grosberg & Cunningham, 2001). The alleles in this lineage will coalesce with each other before coalescing with alleles in the other subpopulation or lineages (Grosberg & Cunningham, 2001; Avise, 2000; Avise, 1998).

Phylogeographic inference is achieved by studying reconstructed genealogical lineages of individual genes (gene trees) sampled from different populations with an emphasis on the evolutionary and geological processes as well as the ecological pressures governing distribution (Knowles, 2004; Avise, 2000). These processes promote evolutionary mechanisms such as migration, admixture, isolation by distance, and gene flow that produce and maintain genetic differentiation (Knowles, 2004; Morrone & Crisci, 1995;

Endler, 1982). However, a species history might not be easily inferred from a gene genealogy because experimental evaluation of historical dispersal or vicariance is not feasible (Brown & Lomolino, 1998; Endler, 1982). Distinguishing between ancient vicariance and dispersal can be problematic. Though the divergence of populations from their ancestral populations is defined by the cessation of gene flow, the timing of this divergence, and whether it coincides with particular geological events or at some time after such historical events is not usually known (Edwards & Beerli, 2000). Furthermore, these vicariant events may have occurred such that some gene lineages may be lost by chance and may therefore not be accounted for (Knowles & Maddison, 2002; Brown & Lomolino, 2000). It is possible, however, to indirectly evaluate historical causes of a phylogeographical pattern by carefully analysing the distribution of variation of known organisms in light of known ecological requirements together with these historical vicariant events and other common barriers to dispersal (Mercer & Roth, 2003; Brown & Lomolino, 2000; Endler, 1982). The varying dispersal potential caused by ecological differences plays an important role in determining a species' phylogeographical structure due to its effect on the way a species may have responded to historical events (Lourie *et al.*, 2005). This diversity and potentially complex configuration of processes operating at the population level presents a major challenge to testing historical hypotheses (Knowles, 2004).

Presently, most of the phylogeographic studies of fishes have been for freshwater habitats because the biogeographical barriers that have resulted in current freshwater fish distributions are more obvious and have been well documented (Tsigenopoulos *et al.*, 2003; Sullivan *et al.*, 2002; Englbrecht *et al.*, 2000). Estuarine and coastal fish population subdivisions for species such as pipefishes have been less studied because there is limited information regarding the ecology and dynamics of marine systems that affect these species, and because their geographical boundaries are generally less clear (Turpie *et al.*, 2000). It is difficult to define boundaries or biogeographical zones in the marine environment due to the absence of physical barriers to fish movement and changes in habitat (Turpie *et al.*, 2000). Estuarine and marine fishes are therefore considered to have potentially high effective population sizes and wider distributions, with reduced genetic

structuring (Altman & Taylor, 2003). However, recent studies show that the genetic structure of estuarine and marine fishes can be evaluated (Avise *et al.*, 2002; Jones *et al.*, 2003). It has been observed that genetic structure in marine populations is affected by the historical and contemporary interactions of a complex set of ecological, demographic, genetic, behavioral, oceanographic, climatic and tectonic processes (Lourie & Vincent, 2004; Grosberg & Cunningham, 2001; Hewitt, 1996). These factors act across a range of spatial and temporal scales and determine rates and patterns of dispersal of gametes, zygotes, larvae and adults, and therefore promote genetic structure within and among populations (Grosberg & Cunningham, 2001). Some studies have also shown that historical and geological events like Pleistocene climatic changes, and associated glacial advances and retreats, caused range reductions that contributed to genetic diversification of some estuarine fishes (Teske *et al.*, 2004; Wilson *et al.*, 2001; Avise & Walker, 1998). The Pleistocene, which occurred about two million years ago (Mya), is characterised by the lowering of sea levels, which would have led to the drying of some estuaries and also limited connectivity among estuarine populations (Durand *et al.*, 2005). There was therefore limited migration of individuals and gene flow between rivers, resulting in DNA sequence divergence (Bernardi & Talley, 2000). This could have promoted the genetic differentiation and bottleneck events that can be observed in the genetic structure and genetic diversity of estuarine fish populations today.

Furthermore, the extreme fluctuations of physical and ecological conditions (e.g. turbidity, salinity and temperature) in estuarine environments can create genetic structure as they enable evolutionary mechanisms such as genetic drift and selection to interact within these habitats (Durand *et al.*, 2005; Altman & Taylor, 2003). These changing physical and ecological conditions may act as barriers and therefore prevent or limit gene flow. Thus fish species inhabiting these habitats must either be highly tolerant or adapt to these fluctuating conditions through natural selection which may be evidenced by DNA sequence divergence among these fish populations (Durand *et al.*, 2005; Bernardi & Talley, 2000). South African estuaries are even more interesting as the 3000 km coastline has some 300 rivers entering the coastal zone (Allanson & Baird, 1999). The influences on these estuaries as well as the species residing in them are not stable throughout the

year and depend mainly on rainfall as it affects the opening and closing of the estuarine mouth. Therefore a wide variety of estuarine environments that support pipefish populations can be found on our coastline. Most of the small estuaries are periodically open to the sea and remain closed for much of the year when sand bars are formed at the mouths during the drier periods of the year and may hence be isolated from the marine environment and from each other (Whitfield, 1998). On the other hand, the permanently open estuaries are generally characterised by reversed salinity gradients due to tidal influence and freshwater seepage (Whitfield, 1998; Day, 1981). A further common factor is that almost all estuaries in South Africa are located in incised bedrock valleys and thus are laterally confined. South African estuaries may therefore represent islands with moderate levels of gene flow or isolation among pipefish populations and hence population subdivisions in a phylogeographic sense.

Therefore, although estuarine environments are more diffuse, they exhibit the same phenomena i.e. dispersal and vicariance, as freshwater habitats (Cox & Moore, 2000). Although it is generally difficult to identify many of these biotic and physical environmental processes and their scale of operation, the nature and outcomes of species interactions may influence population structure. Of the two South African species, *S. temminckii* is an ideal species for studying phylogeography. Unlike *S. watermeyeri* that is restricted to the East Kleinemonde estuary, *S. temminckii* has a wide distribution that spans all three Southern African biogeographic regions (Cool temperate, warm temperate and subtropical) that have been divided based on temperature, geology and biological interactions. The boundaries or transitional zones between different biogeographic regions may effectively act as barriers to gene flow as they have been shown to have a profound influence on estuarine species composition and to govern distribution of fishes along the South African coastline (Turpie *et al.*, 2000; Whitfield, 1998). Secondly, although this species is widely distributed, it is typically only found in meadows of the sea grass, *Zostera capensis* and sheltered embayments in shallow water less than 10 m deep. Such habitats tend to be scattered along the coastline, often in sheltered bays that may be separated from each other by unsuitable habitats and ocean currents (Lourie *et al.*, 2005; Howard & Koehn, 1985). The lack of a dispersive egg and larval phase and the

weak swimming ability of adult pipefishes therefore suggest that movement within and among estuaries is limited and that the life cycle of this species commonly occurs over a very localized geographical scale (Chenoweth *et al.*, 2002; Howard & Koehn, 1985). Therefore, it could be predicted that dispersal and population mixing of *S. temminckii* among the estuaries that this species inhabits could be limiting resulting in the species showing genetic structuring.

Therefore, the main objective of this chapter was to determine the phylogeography of the two South African species of *Syngnathus* using mitochondrial cytochrome *b* sequences. This was done by examining the genetic variability and population structure among populations of *S. temminckii* and *S. watermeyeri*. The following specific questions were addressed:

1. What is the amount of genetic variation within and between the South African populations?
2. What is the amount of gene flow between the species populations?
3. Are the populations of *S. temminckii* structured geographically?
4. How could historical events have affected the present population structures and distribution of South African *Syngnathus* species?

The overall aim of this work was to present a hypothesis on the historical phylogeography of *Syngnathus* species in Southern Africa and to account for the current spatial distribution of cytochrome *b* variation in *S. temminckii*. Understanding how the South African species of *Syngnathus* have arisen should also increase our understanding of estuarine and near-shore oceanic environments and the role they play in fish dispersal and survival.

5.2 Materials and methods

5.2.1 Study area and sampling design

Fish samples for pipefish sequences (*Syngnathus acus* and *S. watermeyeri*) were obtained from locations along the Southern African coastline (see Figure 4.1 page 94). There were 40 individuals of *S. temminckii* and five individuals of *S. watermeyeri* available for

analysis. The study area covered most of the present distribution range of *S. temminckii* and *S. watermeyeri.* DNA specimens of *S. watermeyeri* were only available from the East Kleinemonde estuary because, specimens from other historical locations (originally preserved in formalin) could not be used. Other specimens of the European population of *S. acus* were obtained from the North East Atlantic and Mediterranean Sea. The sampling sites were identified according to their access to the marine environment that is whether permanently open or temporarily closed to the sea. These were also identified according to biogeographical regions based on the biogeographic species breaks observed by Turpie *et al's.* (2000) analysis of South African coastal fish species. *Syngnathus temminckii* specimens represented two groups, region 1 = West coast (Kabeljous estuary to Lüderitz bay) and region 2 = East coast (Sundays to the Great Fish) (see Table 4.1 page 94). Only two biogeographical regions were available for the analysis of *S. temminckii* due to the absence of specimens from the subtropical biogeographic region (see Figure 4.1). Most of the specimens recorded from this region have been caught offshore and could therefore represent drifters dispersed from estuaries or bays by strong ocean currents. There is currently no conclusive evidence of *Syngnathus* populations in the subtropical region. The Lüderitz Bay and Berg estuary specimens were included with the West coast specimens because of insufficient numbers from the cool-temperate region.

5.2.2 Molecular biology techniques

The DNA extraction and amplification of the cytochrome *b* mitochondrial region was performed according to the procedure described in chapter four (Section 4.2.2). Sequencing was performed in both directions using an ABI 3100 Genetic Analyser automated sequencer (Applied Biosystems). Sequence clean up and alignment procedures are also described in Chapter 4, section 4.2.3.

5.2.3 Nucleotide polymorphism

The level of mitochondrial DNA polymorphism within populations was estimated both as haplotype diversity (*h*: Nei, 1987 Nei, 1978b), nucleotide diversity (π: Nei & Tajima, 1981) and as percent sequence divergence between haplotypes using the statistical package ARLEQUIN version 2.000 (Schneider *et al.*, 2000; Nei, 1978a). The haplotype

diversity describes the number and frequency of different haplotypes (segregating sites) in the sample, while nucleotide diversity is the average number of nucleotide differences per site between sequences (Kvist, 2000). These diversity indices therefore describe the DNA sequence polymorphism, and thus the amount of genetic variation in the population. The number of haplotypes observed and diversity of haplotypes was calculated for each location of *S. temminckii* and for each species independently.

5.2.4 Intraspecific haplotype estimation

Intraspecific gene genealogies were inferred using two different network construction methods, all of them implemented in freely available software packages. A Minimum spanning tree (MST) (Kruskal, 1956; Prim, 1957) for *S. temminckii* specimens was constructed using TCS version 1.13 (Clement *et al.*, 2000; Templeton *et al.*, 1992) in order to explore the genealogical relationships among the mtDNA haplotypes. This method uses coalescent theory to determine the limits of parsimony to define a set of plausible connections among haplotypes that have a greater than 95 % cumulative probability of being true (Lourie *et al.*, 2005; Templeton, 1998;Templeton & Sing, 1993). Networks are appropriate for closely related species because they incorporate non-bifurcating genealogical information of population level divergences that is associated with reconstructing relationships of closely related haplotypes/populations (Clement *et al.*, 2000). A haplotype network of the two South African species and *S. acus* was also constructed using NETWORK version 2.0 (Bandelt *et al.*, 1999; Bandelt *et al.*, 1995). This program uses the median-joining network approach whereby all MST's are first combined within a single network following an algorithm analogous to that proposed by Excoffier & Smouse (1994). Then, using the parsimony criterion, inferred intermediate haplotypes are added to the network in order to reduce overall tree length (Cassens *et al.*, 2003). This program was selected for the species networks because it can account for distantly related sequences or connections among haplotypes that have a large number of mutational steps.

5.2.5 Population structure, gene flow and genetic divergence

Population structure (F_{ST}) and migration rate (N_m) were also calculated using ARLEQUIN. These analyses were performed to examine the distribution of genetic variation and level of divergence among individuals within a population, among sample locations, geographical regions and between the two South African species and *S. acus* (Europe). F_{ST} measures the effect of population subdivision, which is the reduction in heterozygosity in a subpopulation due to genetic drift (Wright, 1965). It is the most inclusive measure of population substructure and is most useful for examining the overall genetic divergence among subpopulations (Cassens *et al.,* 2003). F_{ST} values up to 0.05 indicate negligible genetic differentiation whereas values greater than 0.25 imply that there is a lot of genetic differentiation within the populations being analyzed. Seven populations of *S. temminckii* were defined for this analysis: Great fish, Kowie, Kariega, Kromme, Knysna, Swartvlei and Klein. Individuals from Lüderitz Bay and the Berg estuaries were excluded from these analyses because of insufficient sample sizes from each of these locations. The groupings that had higher values of F_{ST} and were significantly different from random distributions of individuals were assumed to be the most probable geographical subdivisions.

5.2.6 Neutrality and demographic history

The mean number of pairwise nucleotide divergences (*k*) or genetic distances, 'the mismatch distribution was also estimated. Mismatch distributions are useful in making inferences about the historical demography of species (Schneider & Excoffier, 1999). The distribution of the observed number of differences between pairs of haplotypes in populations at demographic equilibrium is usually multimodal, whereas it is unimodal in populations that have experienced a recent demographic expansion (Durand *et al.,* 2005; Excoffier, 2001). Harpending's (1994) raggedness test (ARLEQUIN) was used to identify whether an observed mismatch distribution was drawn from small expanded or stationary populations, which are characterised by small or large raggedness indices respectively. The DNA sequences were also analyzed to test for departures from mutation-drift equilibrium with Tajima's *D* test (Tajima, 1989) using ARLEQUIN. This test is based on the fact that under the neutral model, estimates of the number of

segregating sites or polymorphic nucleotides per site (θ) and of the average number of nucleotide differences (*k*) are correlated (Tajima, 1989). If the value of *D* is too large or too small, the neutral 'null' hypothesis is rejected. In a population with decreasing size, the expectation of Tajima's *D* is positive, while a negative Tajima's *D* is predicted for a population with increasing size (Aris-Brosou & Excoffier, 1996). Under assumptions of neutrality, the nucleotide diversity and number of polymorphic nucleotides per site (θ) should be equal. If this is not the case, populations maybe affected by selection, and other processes like population expansion, bottleneck, or mutation rate heterogeneity (Aris-Brosou & Excoffier, 1996; Tajima, 1989; Nei & Tajima, 1981).

5.2.7 Genetic diversity and distance

The relationship or the correlation between pairwise N_m values and geographical distance was performed to determine the extent of genetic differentiation among locations of *S. temminckii* that could be explained by geographic distance. Populations were geo-referenced and pairwise geographical distances were calculated in ArcView 3.2 (ESRI, 1999). Geographical distance was estimated as the coastal distance between pairs of populations without crossing land and was measured as linear distance in km. A significant association between genetic diversity and geographic distance indicates genetic structuring and that dispersal of individuals in populations is limited by spatial scale (Vilá *et al.*, 1999; Slatkin, 1985).

5.3 Results

5.3.1 Nucleotide polymorphism

There were a total of 36 haplotypes defined by 141 polymorphic sites identified across all three species for the 56 individuals analysed (Table 5.1). The nucleotide divergences among individuals of a species were slightly higher among *S. acus* (0.13-6.59 %) than among *S. temminckii* (0.13-2.3 %) and *S. watermeyeri* (1-3 %) specimens. There was substantial sequence divergence between the three species as is expected for well-differentiated and structured populations that have been genetically isolated. This high haplotype diversity value was due to the fact that most of the identified haplotypes were

unique for each species and not shared among population groups. *Syngnathus temminckii*, *S. watermeyeri* and *S. acus*, each had about 60%, 80% and 90% of their total haplotypes as unique and not shared haplotypes, respectively.

Table 5.1: The number of individuals analysed (n), number of polymorphic sites (N_{PS}), haplotypes detected (N_{hap}), cytochrome *b* haplotype diversity (*h*) and nucleotide diversity (π) indices for each species, all three species and for species locations of *S. temminckii* and *S. acus*. The values of segregating/polymorphic sites (θ) and of the mean number of nucleotide differences (*k*) are also indicated.

Species/location	n	N_{PS}	N_{hap}	θ	*k*	*h* (± SD)	π (± SD)
S. temminckii	40	23	22	5.17	4.59	0.96 ± 0.01	0.0062 ± 0.0002
S. watermeyeri	5	4	8	3.84	3.60	0.90 ± 0.16	0.0046 ± 0.0014
S. acus	11	9	32	10.92	15.31	0.96 ± 0.05	0.0206 ± 0.0024
All species	56	36	141	30.70	37.64	0.98 ± 0.01	0.0507 ± 0.0072
S. temminckii							
Klein	3	1	2	0.67	0.67	0.67 ± 0.31	0.0009 ± 0.0004
Swartvlei	8	8	7	3.08	3.32	0.96 ± 0.08	0.0045 ± 0.0042
Knysna	3	2	2	1.33	1.33	0.67 ± 0.31	0.0018 ± 0.0008
Kromme	5	5	3	2.73	2.83	1.00 ± 0.03	0.0038 ± 0.0037
Kariega	4	3	3	1.64	1.67	0.83 ± 0.22	0.0023 ± 0.0006
Kowie	5	1	2	0.48	0.40	0.40 ± 0.24	0.0005 ± 0.0003
Great Fish	4	0	1	-	-	-	-
S. acus							
Bay of Biscay	6	7	5	3.07	2.73	0.93 ± 0.12	0.0037 ± 0.0008
Thracian Sea	5	4	4	1.92	1.80	0.90 ± 0.16	0.0024 ± 0.0007

The nucleotide and haplotype diversities exhibited by the *S. acus* population were significantly higher than South African species values. Nucleotide diversities among the SA samples were estimated as 0.006 for *S. temminckii* and 0.004 for *S. watermeyeri*. The nucleotide diversity of the European *S. acus* species was approximately four times that of the two South African species. The higher value for *S. acus* (0.021) was due to the high sequence divergences observed between its two populations (Bay of Biscay and Thracian Sea) suggesting a strong phylogeographic structure. The estimated nucleotide diversity values for the locations of *S. temminckii* and *S. acus* (range 0.0005 - 0.0045) were also well within the ranges expected for populations except for Swartvlei specimens which exhibited a higher value (0.00448). It is also important to note that values for θ and *k*

were very similar for most comparisons within a species group indicating that there maybe selection occurring within species populations.

5.3.2 Intraspecific haplotype estimation for the three species

The minimum spanning network for the haplotypes of the three species constructed using NETWORK is shown in figure 5.1. A single line connects haplotypes, with the number of nucleotide substitutions within species ranging from 1-3. However all the species were separated by more than 37 mutations and connected via missing haplotypes (mV).

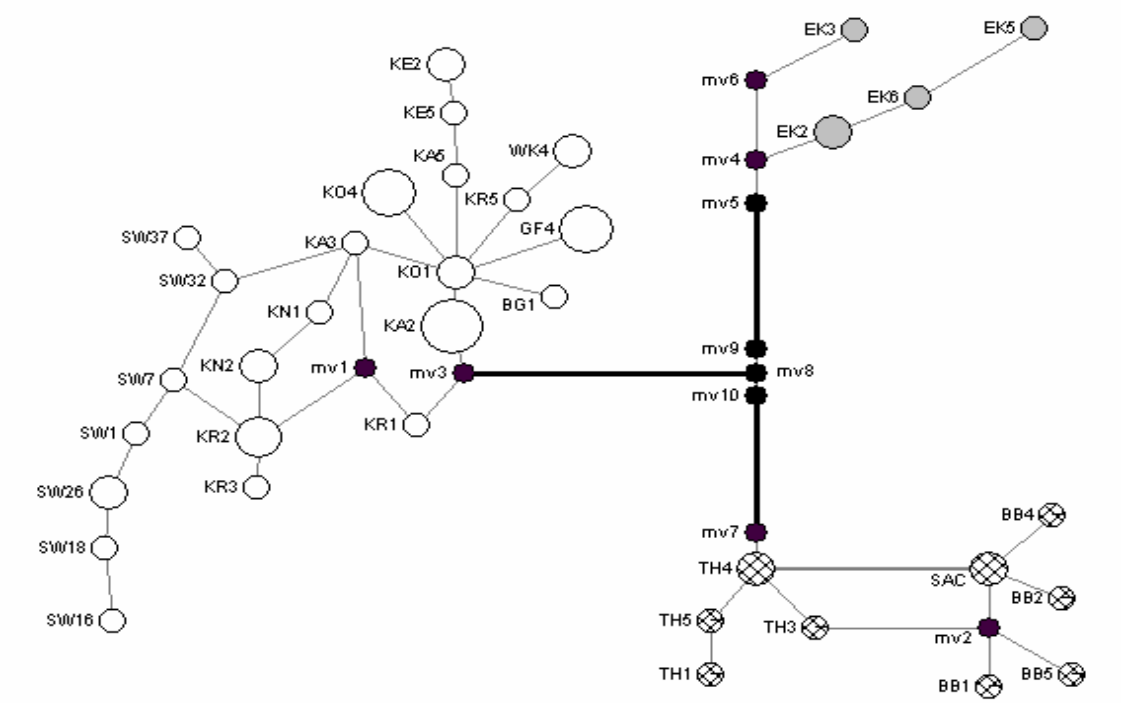


Figure 5.1: The haplotype network of cytochrome *b* sequences of the three selected species of *Syngnathus*. Circles indicate the haplotypes for each species as follows: missing haplotypes (●), *S. temminckii* (○), *S. watermeyeri* (●) and *S. acus* (⊗). Thick lines indicate the connections between the species (all number of substitutions > 37).

5.3.3 Intraspecific haplotype estimation for *S. temminckii*

A total of 23 haplotypes were detected for the 40 sequences that were analysed for *S. temminckii* (Table 5.2). Of the nine haplotypes that were shared among individuals, only

three were distributed in more than one locality: H1 was shared between the Kromme estuary and Lüderitz bay; H23 among Kariega and Kabeljous estuaries and False Bay; and H15 between the Kowie and Sundays river estuaries. The narrow geographical distribution of haplotypes among this species and number of haplotypes not shared indicates that there is very little contemporary gene flow between populations occupying different estuaries. However, the fact that the Kromme and Luderitz shared a haplotype may indicate retention of ancestral polymorphism. The minimum spanning network for the *S. temminckii* haplotypes constructed using TCS is shown in figure 5.2. A single line connects haplotypes, with the dash across the branch representing the number of nucleotide substitutions separating them (ranging from 1-3 in this network). An important feature of the network is that some extant haplotypes generally serve as internal nodes, and may be interpreted as surviving ancestral haplotypes that have differentiated into the descendant haplotypes. This is because haplotypes with the greatest degree of connectivity have the highest probability of being the oldest in the network (Crandall & Templeton, 1993). Therefore, the haplotypes with the highest probability of being ancestral were haplotypes H1, H15 and H18. Geographically these haplotypes are central relative to the others. Another important aspect is that there are many unique haplotypes among the individuals studied of *S. temminckii*. This could imply that the species has a large population size or that purifying selection is acting on the species (Innan & Stephan, 2000).

Another important feature of the network were the two closed-looped connections of haplotypes H1, H7, H8 & H18 and H1, H12, H13 & H18. Loops in network analysis define ambiguous, alternative and equally parsimonious pathways connecting haplotypes (Excoffier & Smouse, 1994). These could reflect convergence from independently arising mutational pathways or a direct evolutionary path that may not even have occurred (Crandall & Templeton, 1993). For example in the network represented above, haplotype H8 (Swartvlei) may have arisen by independent mutations in H7 (Swartvlei) and H18 (Kariega). However the most probable pathway for this haplotype would be H7 as it is most parsimonious being a basal haplotype from the same location (Swartvlei). For the other loop involving haplotype H13 (Knysna), the most probable and parsimonious link

would be with H12 (Knysna) than with H18 (Kariega) again due to the shared locality of the two haplotypes and the reduced number of substitutions between them (2 versus 3). There were two closed loops indicating ambiguous connections between some interior haplotypes. Despite these ambiguities, these haplotypes were resolved and instead nested in the next higher-level clade according to Templeton and Sing (1993) procedure for dealing with ambiguity in haplotype relatedness.

Table 5.2: Distribution and numbers of individuals for each identified haplotype (H1-H23) of *S. temminckii* among sample localities. Population abbreviations follow table 4.1.

Haplotype	**LD**	**BG**	**FB**	**KE**	**SW**	**KN**	**KR**	**KB**	**SN**	**KA**	**KO**	**WK**	**GF**
H1	2	-	-	-	-	-	1	-	-	-	-	-	-
H2	-	1	-	-	-	-	-	-	-	-	-	-	-
H3	-	-	-	-	3	-	-	-	-	-	-	-	-
H4	-	-	-	-	1	-	-	-	-	-	-	-	-
H5	-	-	-	-	1	-	-	-	-	-	-	-	-
H6	-	-	-	-	1	-	-	-	-	-	-	-	-
H7	-	-	-	-	1	-	-	-	-	-	-	-	-
H8	-	-	-	-	1	-	-	-	-	-	-	-	-
H9	-	-	-	-	1	-	-	-	-	-	-	-	-
H10	-	-	-	-	-	-	1	-	-	-	-	-	-
H11	-	-	-	-	-	-	1	-	-	-	-	-	-
H12	-	-	-	-	-	2	-	-	-	-	-	-	-
H13	-	-	-	-	-	1	-	-	-	-	-	-	-
H14	-	-	-	-	-	-	-	-	-	-	-	2	-
H15	-	-	-	-	-	-	-	-	1	-	1	-	-
H16	-	-	-	-	-	-	-	-	-	-	-	-	4
H17	-	-	-	-	-	-	-	-	-	-	4	-	-
H18	-	-	-	-	-	-	-	-	-	1	-	-	-
H19	-	-	-	-	-	-	1	-	-	-	-	-	-
H20	-	-	-	-	-	-	-	-	-	1	-	-	-
H21	-	-	-	1	-	-	-	-	-	-	-	-	-
H22	-	-	-	2	-	-	-	-	-	-	-	-	-
H23	-	-	2	-	-	-	-	1		2	-	-	-
N_{indiv}	2	1	2	3	8	3	4	1	1	4	5	2	4

Note: Haplotypes with the highest probability of being ancestral within the species are indicated in bold

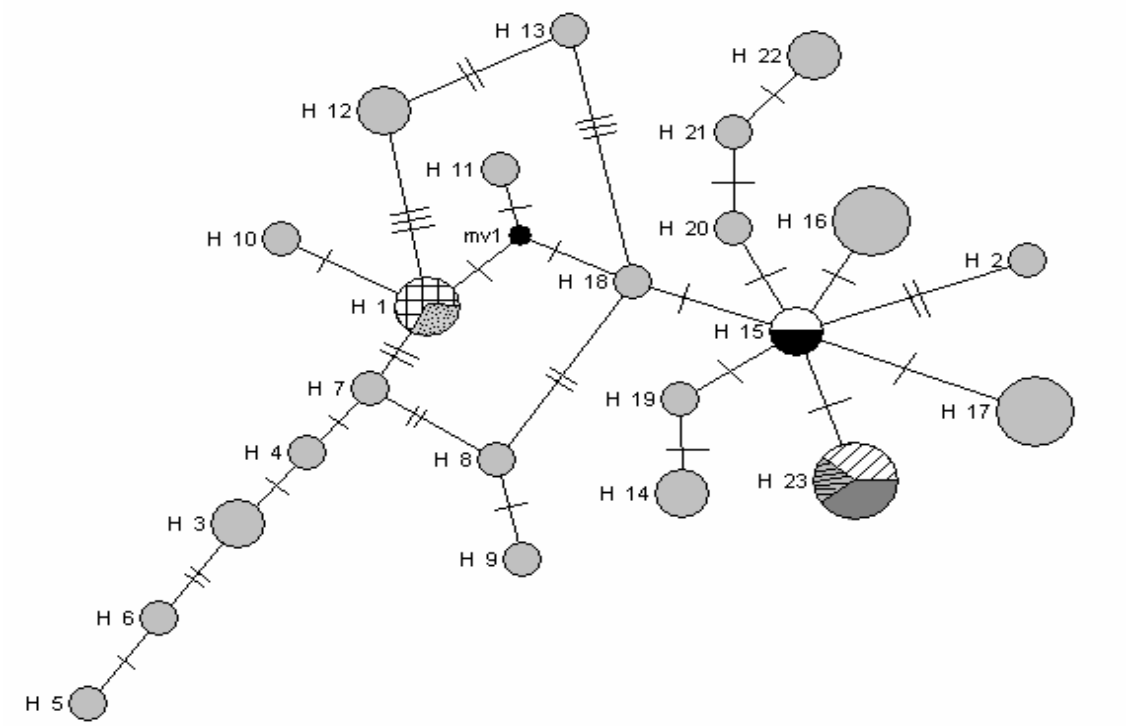


Figure 5.2: The haplotype network of unique (●) and shared (mixed shading) *S. temminckii* haplotypes. Circle sizes are proportional to the observed haplotype frequencies while lines on each branch are the number of fixed substitutions between haplotypes. The black circle mv1 (●) represents a missing haplotype and dashed lines in the network indicate ambiguous connections (haplotypes interconnected forming a closed loop that can be broken at several places).

5.3.3.1 Geographic variation

For the second level of the analysis, biogeographic details were added to the genealogical relationships of the network among haplotypes as specified in table 5.1 (Figure 5.3). The results show that that there was a trend in the distribution of haplotypes. Haplotypes specific to an area were more closely related to each other only separated by 1 to 3 mutations. However, the haplotypes that were common to both regions or shared by more than one individual were situated near the middle of the network and had many more descendants. Most of the haplotypes at the tips of the branches were represented by single individuals and confined to one locality as would be expected if these haplotypes

represent the most recent divergences. This phylogeographic analysis does not demonstrate geographical structuring between the two regions based on the available data. It however shows a trend of some geographical structure among the populations of *S. temminckii* in South Africa. The presence of West coast haplotypes (H2 & H21-23) within East coast lineages suggests that either there is some recent gene flow occurring with individuals occasionally mixing between the regions or that there is retention of ancestral polymorphisms.

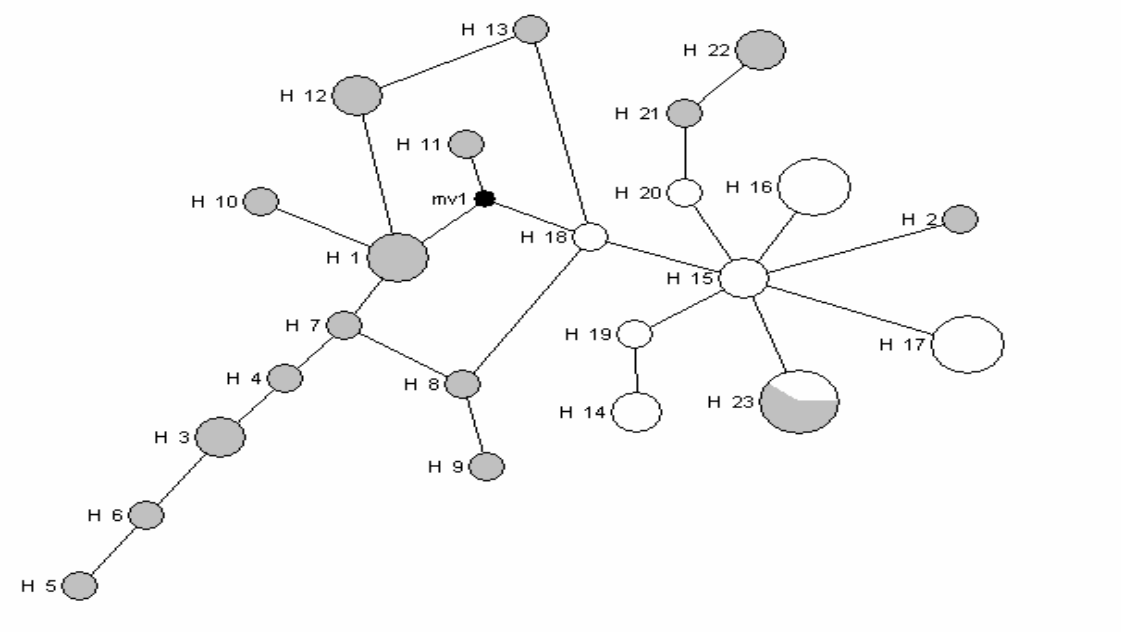


Figure 5.3: The haplotype network showing the distribution of *S. temminckii* haplotypes across biogeographic regions. East coast (○) and West coast (●). Circle sizes are proportional to the observed haplotype frequencies. Mv1 (●) is a missing haplotype.

5.3.3.2 Access to sea

The accessibility of the locality to the marine environment was plotted on to the network to identify whether this was significant in determining relationships through gene flow among populations of *S. temminckii* (Figure 5.4). It was also observed that most of the haplotypes at the tips of the branches were from temporary open systems that do not have

permanent access to the sea. Most of the internal haplotypes that could be classified as being basal or ancestral within the species were from permanently open systems or bays that have open access to the sea (e.g. Kariega, Kromme and Kowie). Of the three haplotypes that were shared among locations, two (H23 and H1) were shared between permanently open estuaries and the only two bay localities, False Bay and Lüderitz Bay. It may be hypothesized that historical dispersal of the species could have been from the marine environment into estuaries where subsequent diversification could have occurred. However, though the haplotypes show biogeographic structure, the small samples sizes for most locations limit the phylogeographical inference that can be made on this species

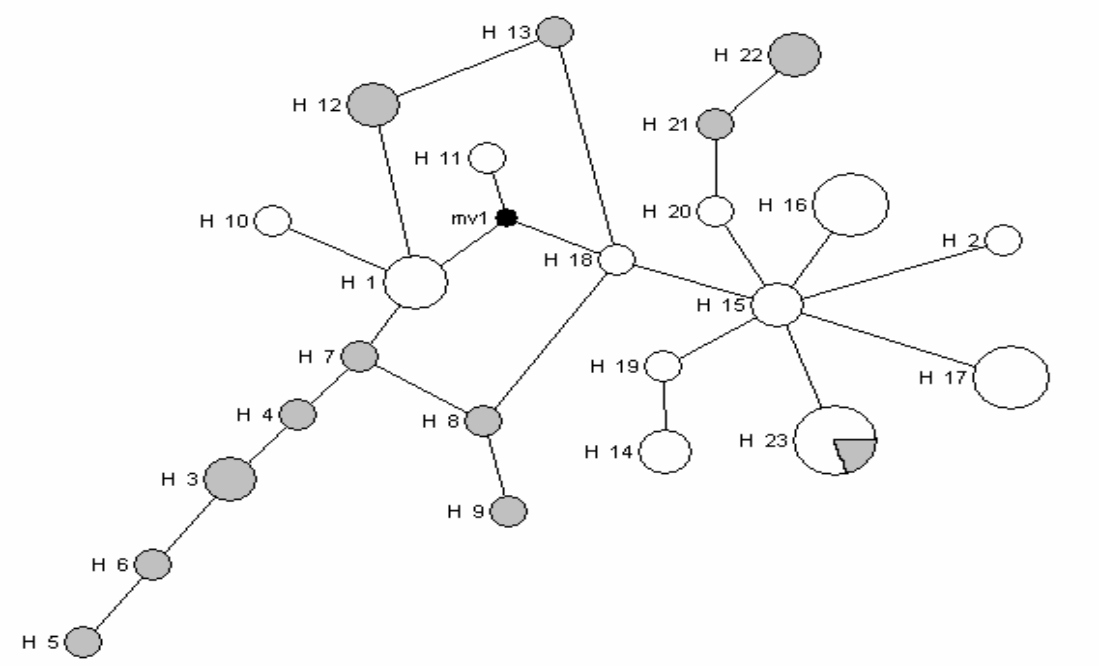


Figure 5.4: The haplotype network showing the distribution of haplotypes of *S. temminckii* according to access to the marine environment. Estuary types are indicated as follows: Temporary open systems (●) and Permanently open access (○).Mv1 (●) is a missing haplotype.

5.3.4 Population subdivisions

The estimated components of genetic structure inferred from dispersal parameters among populations of the three species were very high (average F_{ST} = 0.898 and N_m = 0.03) (Table 5.3). These observed high values of F_{ST} suggest incomplete homogenization of populations due to lack of gene flow among the species populations. The interspecific haplotype estimation of the three species also indicated that the amount of gene flow between the three species was very low. This suggests that the three species are genetically distinct and have been geographically and reproductively isolated from each other. The low N_m value also suggests moderate levels of gene flow and therefore some degree of isolation among the populations (Templeton, 1998). The number of fixed differences was high ranging from 45 – 76 also suggesting genetic differentiation among the three species.

Table 5.3: Nucleotide diversity for the three study species. FD = fixed differences; SM = shared mutations; *k* = average number of nucleotide differences between populations; D_A = number of net substitution per site; F_{ST} = genetic divergence among populations; and N_m = number of migrants per generation.

Species	SM	FD	*k*	D_A	F_{ST}	N_m
S. temminckii x *S. watermeyeri*	0	39	49.15	0.061	0.917	0.025
S. temminckii x *S. acus*	0	65	87.46	0.104	0.886	0.033
S. watermeyeri x *S. acus*	0	76	94.29	0.114	0.900	0.029
All three species					0.898	0.030

The amount of genetic variance partitioned among the populations and the genetic distance measures indicate significant population subdivisions within these species. F_{ST} was high for the two *S. acus* populations (0.9108) and ranged from 0.286 to 0.895 for *S. temminckii* populations. However, the overall F_{ST} value of 0.68 obtained for the six analysed populations of *S. temminckii* suggests that gene flow among the various locations of this species is restricted (Table 5.4). Gene flow levels among adjacent populations showed a trend with locations close to each other having related haplotypes.

Table 5.4: Population subdivision statistics for the overall genetic divergence among subpopulations (F_{ST}) and gene flow (N_m) for populations of two species. F_{ST} (upper right matrix) and N_m (lower left matrix).

Location	GF	KO	KA	KR	KN	SW	BB	TH
S. temminckii	(0.678)							
Great fish		0.889	0.583	0.622	0.895	0.775		
Kowie	0.031		0.294	0.545	0.634	0.740		
Kariega	0.179	0.337		0.333	0.743	0.630		
Kromme	0.152	0.209	0.500		0.286	0.453		
Knysna	0.029	0.041	0.086	0.184		0.698		
Swartvlei	0.073	0.088	0.146	0.302	0.108			
S. acus								
Atlantic ocean								0.911
Thracian Sea							0.0245	

5.3.4.1 Isolation by distance

An isolation by distance analysis was done to compare different estimates of gene flow between population pairs of *S. temminckii* (Table 5.5). There was a positive although statistically insignificant correlation (r^2 = 0.36: P > 0.05) between genetic and geographical distance among the six populations when all F_{ST} pairwise comparisons were included (Figure 5.5). However, this correlation was significant (r^2 = 0.86: P < 0.05) when only F_{ST} values between sites more than 50 km apart where included. This pattern of isolation by distance over such a small geographical scale suggests that movement by this species is generally restricted to very short distances. The high values observed for proximate estuaries are also not surprising since these estuaries may also be separated from each other due to temporal characteristics, like opening phases and local oceanographic factors. Therefore, the results reflect incomplete homogenization of populations due to incomplete flow of genes among *S. temminckii* populations.

Table 5.5: Estimated distances (Km) among the nine populations of *S. temminckii*. (Standard error ± 5 Km).

Location	LD	BG	FB	KE	SW	KN	KR	KB	SN	KA	KO	WK
Berg	712											
False bay	1132	420										
Klein	1224	512	92									
Swartvlei	1689	977	557	465								
Knysna	1719	1007	587	495	30							
Kromme	2041	1329	909	817	352	322						
Kabeljous	2081	1369	949	857	392	362	40					
Sundays	2331	1619	1199	1107	642	612	290	250				
Kariega	2416	1704	1284	1192	727	697	375	335	85			
Kowie	2439	1727	1307	1215	750	720	398	358	108	23		
W. Kleinemonde	2452	1740	1320	1228	763	733	411	371	121	36	13	
Great fish	2462	1750	1330	1238	773	743	421	381	131	46	23	10

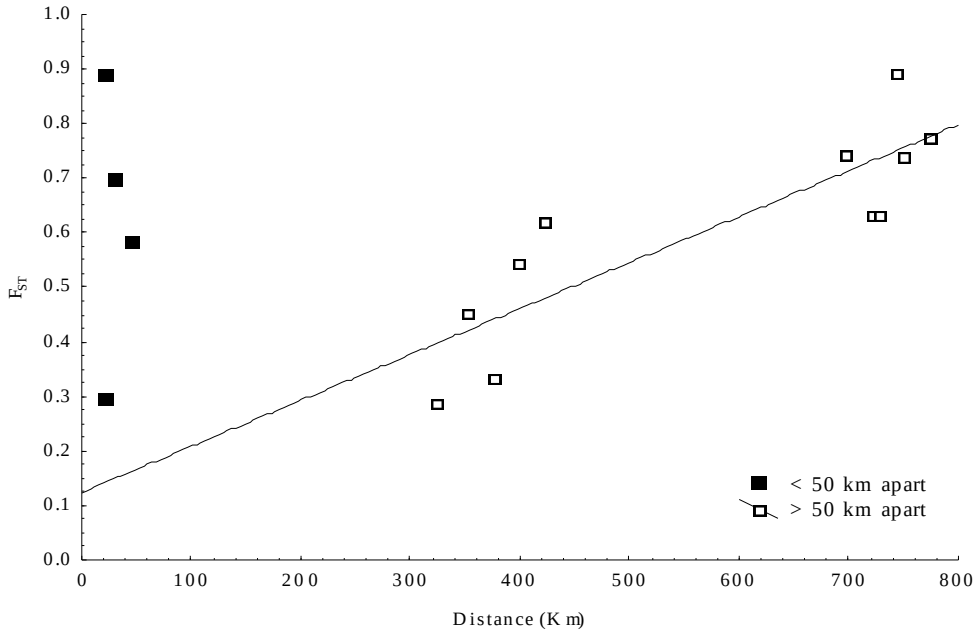


Figure 5.5: Isolation by distance among six *Syngnathus temminckii* populations. Note: pairwise comparisons between sites less than (■) or greater than (□) 50 Km apart. The Regression line is fitted for comparisons of populations more than 50 km apart.

5.3.5 Population demography of the three species

Pairwise mismatch distributions and results of Tajima's *D*-test for each species are given in figure 5.7. All *D* values obtained from the tests were negative and not significant ($P > 0.05$) ranging from –0.440 for *S. temminckii*, and –0.374 for *S. watermeyeri.* The negative values obtained indicate a high number of unique haplotypes in the populations. This negative value also suggests that either purifying selection is acting on the species or that both populations have experienced recent population expansions (Innan & Stephan, 2000). The estimated value for *S. acus* was also insignificant though positive but greater than zero (1.86). This suggests that this population may have undergone a bottleneck because most of the heterozygosity is due to alleles of moderate to high frequency, which are not those likely to be lost in a bottleneck. These results suggest that the hypothesis of neutral evolution of Tajima's *D*-test for these species can be rejected because all three species populations have undergone either selection and/or population expansion events.

The mismatch distribution (Figure 5.6) for the *S. temminckii* samples was unimodal, further suggesting population expansion of this species. However the observed mismatch distribution for *S. acus* was bimodal and covered a large range of pairwise differences (0-30). The first mode showed intra-site differences of BB and TH specimens, while the second mode with larger pairwise differences is due to pairwise comparisons between specimens from the two locations (inter-site). The apparent population expansion, suggests that the two populations have recently undergone range expansion on a sufficient scale after genetic differentiation. The mismatch distribution of *S. watermeyeri* also shows evidence of population expansion within the East Kleinemonde estuary though these results cannot be interpreted with confidence due to a low number of specimens available for this species to show pairwise comparisons.

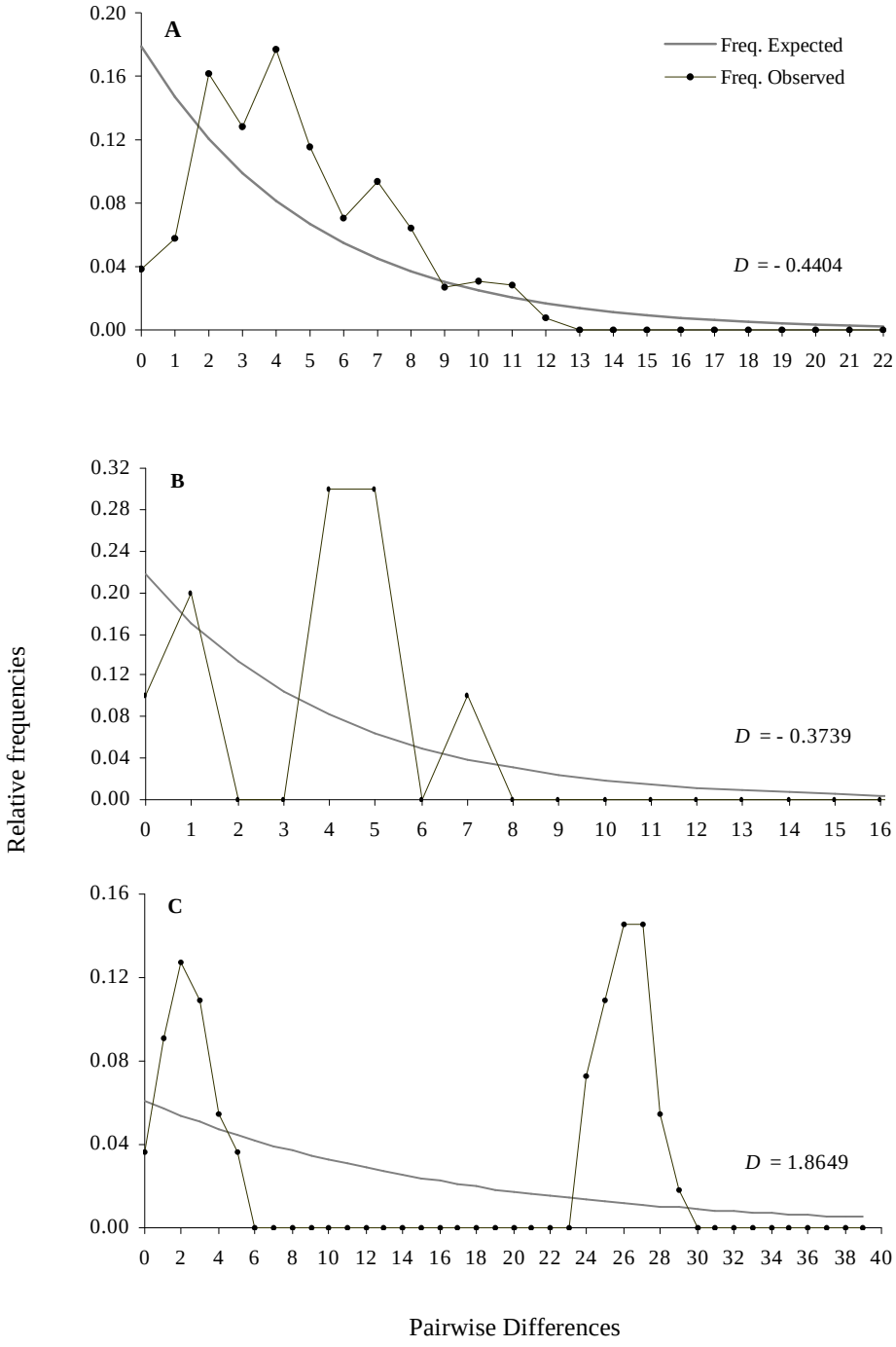


Figure 5.6: The observed pairwise mismatch distributions for cytochrome *b* sequences of *S. temminckii* (A), *S. watermeyeri* (B) and *S. acus* (C) for all of the individuals and the expected distribution model under the assumption a constant non expanding or declining population. Tajima's *D* value is given for each species ($P > 0.05$).

5.4 Discussion

5.4.1 Interspecific haplotype networks

This study found phylogeographic structure in the cytochrome *b* mitochondrial region of SA pipefishes and species of *Syngnathus*. The nucleotide and haplotype diversities exhibited among the SA samples were significantly higher than the *S. acus* population, but within the range reported for other species of Syngnathidae and mtDNA genes. The networks of the haplotypes exhibited significant support for distinct geographic clades and species. All the diversity indices and sequence divergence values obtained showed significant differences in mtDNA diversity among the three species. The minimum spanning network for the haplotypes of the three species showed that the number of nucleotide substitutions within species ranged from 1-3, while species were separated by more than 37 mutations or connected via missing haplotypes (Figure 5.1). There were no shared mutations among the three species with the number of fixed differences among them ranging from 45–76. A minimum threshold of approximately one-tenth of the average p-distance found between well-established species in a lineage where more than a single individual per species has been sequenced has been interpreted as intra-specific variation (Monaghan *et al.*, 2005). However, Davis & Nixon (1992) have suggested that evolutionary lineages become candidates for species when at least one character state is fixed in one group and absent in the other (Johnson & Jordan, 2000). However various authors have suggested that DNA sequence information should on be used for species delimitation if fixed DNA differences are supported by other source of taxonomic data such as geographical, morphological, ecological, reproductive and behavioural information (DeSalle *et al.*, 2005; Wiens & Penkrot, 2002). Therefore, as these values correspond to maximum intra-specific divergences in mtDNA of 1–2% between morphologically divergent groups, the specimens of *S. temminckii* and *S. acus* appear to represent reproductively isolated groups that warrant classification as distinct species (Monaghan *et al.*, 2005; Avise & Walker, 1999)

Although inadequate sampling can falsely designate species candidates (Templeton, 1998; Davies & Nixon, 1992), the sample sites for *S. temminckii* and *S. watermeyeri* are geographically representative of each species (Whitfield 1998; Dawson, 1985) and

therefore overwhelmingly satisfy the candidate criteria. Though the entire distribution range of *S. acus* was not available for analysis, the presence of two geographically distant populations was sufficient. Templeton (1994, 1989) emphasized that a population genetic approach must be used to identify species and understand the causes of speciation. Evolutionary lineages defined as species must (1) be phylogenetically distinct, (2) show no recent gene flow, and (3) demonstrate ecological or demographic limitations to reproduction and therefore genetic exchange (Johnson & Jordan, 2000; Templeton, 1989). Therefore, *S. acus, S. temminckii* and *S. watermeyeri* satisfy criteria 1 and 2 as they all have phylogenetically distinct evolutionally lineages with little or no gene flow among them. It could also be suggested that criterion 3 can be satisfied for *S. acus* and *S. temminckii* populations as these populations are spatially and therefore ecologically isolated. The observed values for θ and *k* were very similar and thus also indicated that selection maybe occurring within and between populations. This is expected as the distance between the two SA species and *S. acus* as well other species of *Syngnathus* may prevent juvenile dispersal and gene flow. Furthermore, the estimated component of genetic structure inferred from the dispersal parameter, N_m (number of migrants) among populations of the three species was very low (average $N_m = 0.03$) (Templeton, 1998). This low N_m value also suggests that species of *Syngnathus* are not effective dispersers over large spatial scales as there have been no random exchanges of fish or juveniles between these specie's populations.

The estimated nucleotide diversity values (range 0.0005-0.0045) for the locations of *S. temminckii* and *S. acus* were also well within the ranges expected among species populations that have been geographically isolated. The nucleotide diversity of the European *S. acus* was approximately four times that of the two South African species. The higher value for the two populations of *S. acus* (0.021) indicates a strong phylogeographic structure within this species. This phylogeographic structuring was evident in the minimum-spanning network as the haplotypes from the Bay of Biscay (Atlantic Ocean) formed a unique lineage from the Thracian Sea (Mediterranean) haplotypes. This deep phylogeographic break between these two populations that do not share haplotypes suggests that there has been a long-term barrier of gene flow between

them resulting in isolation. There were also two possible links connecting these two locations implying convergence or that there may have been two colonisation events to or from the Atlantic and the Mediterranean. The *S. watermeyeri* specimens were genetically diverse although only a few individuals were analysed for this species. Some of the haplotypes of this species (EK8 & EK5) were separated by 4 substitutions, also suggesting remarkable diversity within this species.

5.4.2 Intraspecific haplotype tree for *S. temminckii*

According to Hewitt (1999) "population structure is the distribution of genotypes in space and time, and is the result of both present processes and past historical events that limit gene flow between populations." The population structuring of *S. temminckii* was relatively structured when compared with *S. watermeyeri* but less structured than that of *S. acus*. The high F_{ST} values obtained for the two *S. acus* and *S. temminckii* populations suggests that gene flow among the various locations of these species is relatively restricted. The narrow geographical distribution of haplotypes among this species and number of haplotypes not shared indicates that there is very little contemporary gene flow between populations occupying different estuaries. This was expected as pipefishes are considered to be relatively sedentary as they are not capable of independent strong locomotion and are thus unable to actively disperse to other estuaries via the marine habitat. The mean home distance of pipefish can be estimated as 100-200 m based on the study of *S. typhle* (Vincent, 1995b). The results also suggest that there may be isolation by distance over that geographical scale occurring among the populations of *S. temminckii*. These six populations showed a positive significant correlation between geographical and genetic distance when only F_{ST} values between sites that were separated by more than 50 km were included (Figure 5.6). There was a significant association between haplotypes and sample sites as haplotypes specific to a region were more closely related to each other and were only separated by 1 to 3 fixed mutations. This pattern of isolation by distance over such a large geographical scale is common among syngnathids and suggests that movement of *S. temminckii* individuals is generally restricted to very short distances with little dispersal between distant estuaries. (Lourie *et al.*, 2005; Teske *et al.*, 2004).

An important feature of the network is that some extant haplotypes serve as internal nodes that are usually regarded as surviving ancestral haplotypes (Crandall & Templeton, 1993). These haplotypes could represent convergence from independently arising mutational pathways or a direct evolutionary path that may not even have occurred (Crandall & Templeton, 1993). Slight molecular separations in the network may reflect more recent population subdivisions, whereas deep subdivisions may be evidence of a major source of intraspecific evolutionary gene pool diversity (Avise 1992). The haplotypes that were common to the west and east coast regions or shared by more than one population were situated near the middle of the network and had many more descendants. Most of the haplotypes that were at the tips of the branches were represented by single individuals and confined to one locality as would be expected if these haplotypes represent the most recent divergences. However, there has been sufficient recent gene flow occurring within this species with individuals occasionally mixing between the regions, as there was no clear geographical pattern with some haplotypes being shared by the two regions. The high F_{ST} values observed for some proximate estuaries are likely to be a consequence of these estuaries being separated from each other due to temporal characteristics, like opening phases and local oceanographic factors.

The results also suggest that accessibility of the locality to the marine environment is significant in determining relationships among localities of *S. temminckii* (Figure 5.5). Though some estuaries are close to each other they may be affected by different opening phases as well as local currents and geography that may prevent gene flow between them. It was also observed that most of the haplotypes at the tips of the branches were from temporarily closed estuaries or systems that do not have permanent access to the sea. On the other hand most of the internal haplotypes that could be classified as being basal or ancestral within the species were generally from permanently open systems or bays that have open access to the sea (e.g. Kariega, Kromme and Kowie). If we assume that there is no difference in the dispersal ability of the larvae in the ocean among the localities, the factor affecting the isolation between the populations of *S. temminckii* may be a lack of estuaries and sheltered bays along the coast, different opening and closing phases, as well

as local currents and gyres in the oceans that will prevent fish from dispersing and settling. This suggests that historical dispersal of the species could have been from the marine environment into estuaries where subsequent diversification after isolation must have occurred. This pattern of genetic structure suggests that there may have been past fragmentation and/or long distance colonisation between regions followed by subsequent isolation among locations of *S. temminckii* (Lourie *et al.*, 2005). However, though the haplotypes show biogeographic structure, the small samples sizes for most locations limit the inference that can be made about the phylogeographic structure of this species as the potential causes of this genetic variation cannot be inferred. There is need for adequate sampling of all geographical locations in order to identify and distinguish the various factors influencing spatial distribution of genetic variation among populations (Templeton, 1998). Therefore, the major conclusions that can be made are that the results reflect incomplete homogenization of populations due to incomplete flow of genes among all populations of *S. temminckii*.

5.4.3 Demographic history

The mismatch distributions, N_m values, and the negative values obtained for Tajima's *D*-test for *S. acus* and *S. temminckii* are consistent with past population expansions and gene flow. Recently founded populations should show the signature of a rapidly expanding population, because they are unlikely to have reached an equilibrium between haplotype diversity (mutations) and genetic drift (demographic changes) (Wares & Cunningham, 2001). There was a high number of unique haplotypes in these two populations and this suggests that purifying selection may also be acting on these species (Innan & Stephan, 2000). The unimodal mismatch distribution (Figure 5.7) for *S. temminckii* further suggests demographic changes some time in the past through selection and/or population expansion events among populations of this species. It has been observed that the major global climatic and environmental changes of the quaternary (i.e. glacial periods and sea level changes) that induced habitat change appear to have affected the evolution and distribution and demographic history of various fish groups (Wares & Cunningham, 2001). This distributional concordance across fish taxa suggests that the geographic

ranges of extant lineages have generally gone through a pronounced shift through time in response to common vicariant events.

South African estuaries have almost all originated in formerly incised bedrock valleys cut during periods of lowered sea levels during the Pliocene and Pleistocene epochs (Patridge & Maud, 2000). The entire Pleistocene Epoch (1.8 Mya to 10,000 years ago) characterized by various climatic oscillations and cycles of glaciation and melting signifies such vicariant events (Appendix VIII). Indications are that sea level fluctuations have played an important role in the ecology, geomorphology and archaeology of the Southern Africa region, and that the resultant coastal environments may have been subject to rapid change (Baxter & Meadows, 1999; Cooper *et al.*, 1999). The sea level rise before the Holocene, by approximately 130 m about 13,000 years ago, during the maximum of the penultimate Glacial and the sea-level decline to about 120m below the present sea level during the Last Glacial Maximum (LGM) are important for the present geographical and environmental conditions of the surrounding landscape of South African estuaries (Patridge & Maud, 2000; Cooper *et al.*, 1999). Semi-enclosed estuarine conditions evidently occurred frequently during these periods as sea-level rise overtook deposition in the estuaries and created new habitats resulting in either temporary or permanent marine incursions by fish species (Baxter & Meadows, 1999).

These periods of estuarine formation combined with variable temperatures and precipitation levels, together with strong climate fluctuations, may have led to the diversification of the two South African pipefish species that were estimated to have evolved during the Pleistocene. While high sea levels may have improved connectivity and encouraged the dispersal of these pipefishes to various locations, low sea level changes may have affected the connectivity between estuaries and encouraged the isolation of South African populations of *Syngnathus*. These habitat changes may have also affected the viability of these populations as well as their chances for population expansion or reduction.

5.4.4 Conclusions

The South African species of *Syngnathus* are significantly differentiated genetically throughout their distribution range with the highest degree of differentiation found within *S. temminckii*. This implies that gene flow and dispersal are restricted among this species' populations. The positive correlation between genetic diversity and geographical distance between localities suggests diversity is linked to isolation. If the geographic range of a species is large relative to its dispersal potential, then isolation will lead to divergence between subpopulations (Grosberg & Cunningham, 2001). Gene flow may be restricted due to the oceanography of the South African coastline as well as the poor dispersal ability of pipefishes. The trend in the genetic diversity with the observed high gene flow values suggests restricted gene flow and isolation-by distance among South African populations. The mismatch distributions also support this hypothesis and point to population expansions in these two species after isolation. However, there is need for further molecular analysis of the two South African species populations to shed more light on the phylogeographic patterning of these species. These results are congruent with the morphological data in which no clear geographical pattern was evident.

Chapter Six

General Discussion

6.1 Introduction

The aim of this study was to analyse the South African species of *Syngnathus* using biological, morphological and genetic data. This was done by comparisons with closely related species of the genus in order to draw an inference about the evolutionary processes that may have led to the diversification of *Syngnathus* in South Africa. This comparative procedure was selected because inferences from comparing closely related species have provided the basis of the conceptual foundation of the present views on speciation among fishes (Helfman *et al.*, 1997; Bermingham *et al.*, 1997; Meyer, 1993; Smith, 1963). However, although the origin of species or 'speciation' is a central issue in evolutionary biology, there is no single recognized mechanism of speciation (Coyne & Orr, 2004; Brooks & McLennan, 2002; Mayr, 1963). Although species are fundamental units in studies of evolution, ecology, phylogeny, and conservation biology, surprisingly little attention has been paid to the methods and data used to recognize and delimit them. Questions on what species are and how they are formed, whether species exist as discrete, objective entities or as purely arbitrary constructs or whether they are an artificial term of convenience remain contentious (Coyne & Orr, 2004; Turner, 1999). This is more so for fish species for which basic assumptions of the various species concepts are violated. Clonal inheritance of nuclear genes occurs in several lineages and hybridizations are frequent and often lead to introgression (Turner *et al.*, 2001; Pyle & Randall, 1994). Furthermore, research into the ecological, genetic and behavioral changes during speciation is difficult because the process is usually only identifiable after observing divergence (McPhail, 1994).

The aim of this discussion is to summarize the patterns of divergence that were observed, the distinctiveness and position of South African pipefishes in the phylogeny of the genus *Syngnathus* using the various systematic procedures. Species concepts and their applications for fish studies have already been discussed by various authors (Coyne &

Orr, 2004; Brooks & McLennan, 2002; Turner, 1999). However, few specific criteria or methods for species delimitation have been proposed (Templeton, 2001; Wiens, 1999; Davis & Nixon, 1992), and these criteria are rarely stated explicitly by empirical workers. This discussion is based on an adjustment of the hypothetical speciation flow chart defined by Brooks and McLennan (2002) on the discovery and evaluation of species as taxonomic units (Appendix IX) and the steps defined by Templeton (1989) for evolutionary lineages defined as species. These procedures were selected as they encompass morphological, behavioural, reproductive or ecological information in testing the null hypothesis that there is no differentiation between two geographical entities. The question of how and why species are produced and their reproductive isolation from other species are only considered after a rigorous search for discontinuities in characters between species using a set of criteria (Appendix IX) to give a robust and clear species description. The South African species of *Syngnathus* were evaluated using information from a historical perspective via morphological data, phylogenetic trees (genetic analyses), and biological and ecological data provided in the previous chapters. This was done to present a hypothesis regarding the speciation process and the phylogenetic relationships among various species of *Syngnathus*.

6.2 Are the species units phylogenetically distinct?

6.2.1 Morphological divergence

Significant differences among the analysed 11 morphological characters using both univariate and multivariate analyses were identified between the three species examined in this study. The results therefore supported the hypothesis that the three species groups (*S. temminckii*, *S. watermeyeri* and *S. acus*) have diverged from each other in their morphology. The extent of differentiation was illustrated by the scatterplots of the PCA and DA analyses of all the species in which there was no overlap between individuals of *S. watermeyeri* and *S. temminckii* (Figure 3.11). There was congruence between the unadjusted and adjusted data sets resulting in the same species group segregations for both procedures. Size was observed to be an important defining character as it was significant in separating *S. watermeyeri* from the other two species. Therefore, size may thus be significant in separating adult specimens of species of *Syngnathus*. It has been

suggested that size may bias statistical analysis of morphological data if not corrected for. *Syngnathus watermeyeri* had a much shorter snout (as % of SL), lower number of fin rays (pectoral and dorsal) and body rings (trunk and tail) than the other two species. Specimens of the South African *S. temminckii* were more morphologically similar to the geographically distant population of *S. acus* than the more proximal *S. watermeyeri*. It was thus a combination of different characters that was significant in separating the three species groups. Phenotypic diversification through either genetic differentiation or phenotypic plasticity can drive microevolutionary change within a species and consequently lead to speciation (Langerhans *et al.*, 2003). The different environments on the South African coastline and their effects on dispersal and differential adaptation as well as the spatial distance between the species groups may explain some of the morphological differences observed. The two South African species and *S. acus* are geographically separated from each other such that they are exposed to different environments and hence selection pressures. The environmental factors or local habitats are critical in promoting morphological variation as they may promote or limit gene flow and therefore population mixing (Turan, 2004). The morphological differences observed were sufficient to support the recognition of *S. temminckii* as a distinct species.

6.2.2 Genetic divergence – Molecular Phylogenetic analysis

The major aim of this study was to determine the taxonomic status of the disjunctly distributed South African species of *Syngnathus* by reconstructing their evolutionary history using a phylogenetic tree. The phylogenetic tree of cytochrome *b* gene sequences of 12 species of *Syngnathus* resulted in a well-supported monophyly of the genus and featured various distinct mitochondrial lineages that were separated from each other by about 3 to 21 % nucleotide divergence. Individuals from different localities across the range of all the three species as has been suggested by various studies (Monaghan *et al.*, 2005; Goldstein *et al.*, 2000; Walsh, 2000; Davies & Nixon, 1992) were used to distinguish variation within a species from variation between species in order to identify those characters that are uniquely shared among all members of a species. The diversity indices and sequence divergence values obtained showed significant differences in mtDNA diversity among the three species (*S. temminckii, S. watermeyeri* and *S. acus*)

with no shared mutations while the number of fixed differences among them ranging from 45–76. These specific fixed differences and the genetic divergence were also in the range that is considered sufficient for species designations (Monaghan *et al.,* 2005; Edwards & Beerli, 2000; Kocher & Carleton, 1997; Davies & Nixon, 1992). The fact that these taxa showed great divergence in genetic distance and together with the evidence obtained from the morphological analyses is suggestive that there may be unrecognized taxonomic diversity present. The molecular identification of the two South African species has implications for the systematics and the taxonomy of the genus. First of all, the high sequence divergence values observed between *S. acus* and *S. temminckii* (11%) specimens indicate that these two taxa are distinct species. Although *S. acus* and *S. temminckii* are descended from a recent common ancestor, the two species have been genetically isolated with no gene flow between them. Secondly, the cytochrome *b* results showed that *S. watermeyeri* is nested within *Syngnathus* as a sister taxon of *S. temminckii*.

The phylogeny further suggests that the two African species are closely related to the north-eastern Atlantic species and may thus be a result of dispersal from this region. The tree showed a clear divergence between the eastern Pacific (*S. leptorhynchus*) and the Atlantic and Mediterranean species. However, there was little support for the node joining the two Atlantic populations of *Syngnathus* (West and East), such that it therefore appears that these two groups may have diverged rapidly over a short evolutionary period. The basal position of the Pacific American *S. leptorhynchus* and the other American (North Western Atlantic) species is consistent with the idea that the ancestor of the genus *Syngnathus* may have originated from the Americas or that this region reflects ancestral polymorphisms (Fritzsche, 1980). The high diversity of *Syngnathus* species within the Atlantic may be evidence that they are actively evolving. To date 20 valid species have been recorded from the Atlantic region, most of which are separated by small morphological differences. Therefore, the observed levels of sequence divergence and the phylogenetic tree suggest that the data of the 12 species represent long separated distinct species and diverse evolutionary histories within *Syngnathus.*

The genetic and morphologic data reveal the existence of two groups of "*S. acus*", South African and north-eastern Atlantic, and some evidence of genetic divergence between Mediterranean and Atlantics *S. acus* samples (Figure 4.9). Further understanding of these inter-basin relationships must await broader sampling throughout the distribution range of this genus. Collections of potentially more molecular genetic data such as microsatellites with higher mutation rates (Meyer, 1993) and physical tagging programs designed to measure long distance movements of pipefishes would be useful in understanding these species. Mitochondrial DNA is an ideal marker for lineage diagnosis because isolated populations should achieve reciprocal monophyly more rapidly for mtDNA than nDNA due to the smaller effective population size of mtDNA under ideal conditions (Glor *et al.*, 2004; Wiens & Penkrot, 2002). Since this genus is known for its morphological conservatism (Kuiter, 2000; Dawson, 1985), it is hypothesized that although morphological characters are useful they should be used together with genetic methods for full systematic resolution among pipefishes. Morphological stasis (i.e., the lack of significant morphological change in a species over long periods of time) has been suggested as a common phenomenon among pipefishes (Lourie *et al.*, 2005). Furthermore, the morphological and meristic characters of pipefishes have not been studied in an evolutionary context, such that the influence of selection on phenotypic expression is still unknown. Exploring the genetic and environmental basis of the observed morphological patterns is important and should be the next step in the investigation of the divergence observed in the three species, as it would define whether the selected morphological characters are independent of non-heritable variation. Although molecular data sets could likewise have this flaw, discovering the flaw and finding appropriate alternatives are likely to be much easier than for morphological data sets (Hillis & Wiens, 2000).

6.3 Do the species show any degree of geographic overlap?

This is an important question to consider when distinguishing between species because of the influence of geography on speciation. Populations that are continuous and widespread and overlap geographically have a greater chance of dispersing and of mixing where they come into contact (Turan, 2004). Dispersal and population mixing are important for

speciation as they encourage individuals to transfer genetic material between gene pools and therefore constrain adaptive diversification and the process of speciation. The genetic distinctiveness and morphological divergence the North-eastern and Mediterranean populations of *S. acus* from the two South African species was supported by the data, as there is no geographical overlap between these populations. Furthermore, the disjunct distribution and spatial isolation of the two South African species from all the other species of the genus was also significant. The estimated average N_m and F_{ST} values of 0.03 and 0.89, respectively were very high and suggest that there is incomplete homogenization of populations with little or no gene flow between these species. These high values were also indicative of genetic divergence among the South African species (Slatkin, 1985).

The results also showed that South African pipefishes are highly restricted in their dispersal by geographical distance, as there was a positive correlation between F_{ST} and distance between localities. The level of morphological divergence between different localities also tended to increase with increasing spatial distance. It has been suggested that dispersal and consequently gene flow among pipefish populations occurs primarily by drift via currents or moving seaweeds, as these fish are poor swimmers (Teske *et al.*, 2004). Although *S. temminckii* is distributed throughout the South African coastline, dispersal of pipefish may be restricted due to the peculiarities of complex oceanographic circulation patterns of the Agulhas Current (Lutjeharms *et al.*, 2003; Olivar & Beckley, 1994). The high dispersal parameters were also evident between the two South African species and also imply low levels of migration and therefore reduced gene flow between these species. As the two South African species are not isolated from each other by geography or physical barriers to gene flow, other isolating factors must be involved in the genetic differentiation between these species. Populations of *S. temminckii* were estimated to exchange one individual migrant per three generations, and therefore are expected to diverge mainly by neutral processes such as genetic drift. The major selective forces that may have contributed to the divergence of these species may be similar to those that have been identified for other fish species like sticklebacks and cichlids such as selection on ecological traits, sexual selection and genetic conflicts (Johnson & Taylor,

2004; McKinnon & Rundle, 2002; McPhail, 1994Kocher, 2004). Another interesting result was the genetic divergence between the two population groups of *S. acus* that are geographically isolated from each other by the Strait of Gibraltar. It is not clear how distinct these two populations are from each other, however, geographic groups or allopatric populations are generally considered to be at various degrees or stages of speciation (Turan, 2004). Allopatry views?

6.4 Are the species reproductively isolated from each other?

The significance of reproductive isolation as a mechanism for defining species is well supported (Coyne & Orr, 2004; Brooks & McLennan, 2002; Mayr, 1963). Reproductive isolation is the process that promotes the maintenance of the integrity of well-balanced and harmonious gene pools (Via, 2002). It prevents the homogenizing effect of gene flow and therefore the mixing of different gene pools of different populations. Although reproductive-isolating mechanisms will not always prevent the occasional interbreeding of non-conspecific individuals, they nevertheless affect the complete fusion of such populations (McPhail, 1994). Various factors such as differences in the time of reproduction, and mate recognition and fertilization patterns have been observed to be significant in discouraging mating between pipefish species (Vincent, 1995b; Howard & Koehn, 1985). The extent of the male brood pouch development among syngnathids has been linked to the evolutionary history of this family (Wilson *et al.*, 2001; Helfman *et al.*, 1997). Furthermore, these fishes represent an interesting evolutionary model as they have evolved sex roles that are reversed, with females competing for males. This may therefore imply that sexual selection and reproductive isolating mechanisms are significant and may have played an important role in the speciation of syngnathids. Therefore using reproductive isolation as a mechanism for defining species of *Syngnathus* should be useful in defining the mechanisms of speciation among these species.

The results of the haplotype analysis among the three species (chapter five) suggest that these species are reproductively isolated from each other. The data revealed no evidence of hybridization between individuals of the three species with very little or no gene flow

reflected by very low N_m values (0.03). The species were genetically distinct with more than 30 mutations for comparisons between any species pair. There was no overlap in the morphological analysis between the two South African species, suggesting that there is no hybridization or that if there is, it is not reflected in the morphology. Although there was an overlap in morphological characters between *S. acus* and *S. temminckii*, reproduction isolation can still be proposed as due to the geographical separation and genetic divergence between these two species populations.

The results of this study (chapter two) showed that a large number of newly hatched young, gravid females and pregnant males of the two South African species were abundant in spring and summer with reduced numbers in the winter months. It therefore appears that the timing of reproduction may not be a cause for reproductive isolation among the species, as the two species breed at the same time and thus would be able to interbreed if they come into contact. However, little is known and could be deduced from the biological data about the nature of the reproduction isolating mechanisms between these species. Detailed fieldwork and laboratory work of organisms in their environment is required to determine which characters really influence reproductive isolation. Most of the work that has been done on examining premating isolation mechanisms among pipefishes of *Syngnathus* has depended on field survey observations and laboratory experiments of mate choice (Vincent, 1995b; Berglund, 1993; Ryer & Orth, 1987; Gronell, 1984). Experimental tests of these assumptions are difficult to carry out and male preference functions are difficult to measure, as it is hard to predict which of the several female characteristics, are attractive to the males. Assembling a group of such studies in an array of taxa, would allow researchers to make generalizations about which characters most impede gene flow in particular ecological situations (Via, 2002). The highly structured population of *S. temminckii* should make it much easier to map the major factors that have led to reproductive isolation and consequently genetic and morphological diversification between these species.

6.5 How did the species come to be reproductively isolated from each other?

There are two speciation models that can be used to explain the evolution of the South African species of *Syngnathus*: the sympatric and allopatric models (Kocher, 2005; Coyne & Orr, 2004; Turner, 1999; Templeton, 1989). Although the sympatric model is very difficult to describe and has been widely debated (Kocher, 2004; Jones *et al.*, 2003; Stauffer *et al.*, 2002; McPhail, 1994), it could possibly be responsible for the divergence between *S. temminckii* and *S. watermeyeri*. The basic requirement of models of sympatric speciation is that there is either a genetic association between mate recognition systems and traits under disruptive selection, or that the mating of the two species is in separate habitats (McPhail, 1994). These two South African species occur together and have been captured in the same habitats in estuarine systems of South Africa (Whitfield, 1998). Whether these estuarine habitats provide two discrete or segregated microhabitats that may have promoted specialization (e.g. different foraging abilities) and therefore encouraged mate choice selection to occur is unknown. Of the two species, *S. watermeyeri* has a restricted distribution and has not been recorded in the marine environment. It should be tested whether this species is more specialized and requires specific environmental conditions in order to survive. On the other hand, *S. temminckii* has a wide distribution and has adapted to various environments being present in estuaries, coastal bays and reef areas. Therefore, it is possible that adaptation linked to the availability of alternative habitats may have been an important factor in the origin and diversification of these two sister species. There is also strong evidence of genetic association among the members of each species as there was high genetic divergence based on cyt *b* sequences between these species. The results also showed no evidence of population mixing or hybridization. However, the reproduction of two sister species in the same environments is one requirement of a sympatric model and needs to be verified with laboratory experiments or field surveys.

The allopatric model of speciation was considered for explaining the observed genetic and morphological divergence between the other species of the genus and *S. acus* from the two South African species. Under this model, divergence is regarded as either a consequence of the splitting of a species' range into two or more isolated fragments or

the dispersal of a small group of individuals into a new area (Brooks & McLennan, 2002). These isolated or separated populations may then diverge genetically in response to different local selection regimes and eventually evolve into two distinct species (McPhail, 1994). Although there is a wealth of evidence to support the theory that classic allopatric speciation has been important in the radiation of many fishes (Casey *et al.*, 2004; McKinnon & Mori, 2003; Hewitt, 2001; Avise & Walker, 1999; Turner, 1999; Randall, 1998; Templeton, 1989), this mechanism does not sufficiently explain the origin of all new species (Kocher, 2004; Jones *et al.*, 2003; Albertson *et al.*, 1999). For example, the adaptive radiations of cichlid species in African lakes have occurred in such a short period of time (and with significant levels of gene flow) that classic allopatric models of speciation do not easily explain their evolution (Kocher, 2005; Kocher, 2004). However, the genetic differences observed between the disjunctly distributed South African species and *S. acus* are consistent with populations that have been reproductively isolated due to vicariance for a long period of time by geographical barriers to gene flow.

The distributional concordance observed across fish taxa suggests that the geographic ranges of extant lineages have generally gone through a pronounced shift through time in response to common vicariant events (Bermingham *et al.*, 1997). The phylogenetic and biogeographic patterns suggest that diversification in taxa of *Syngnathus* has been a basin-wide phenomenon, occurring over tens of millions of years in the Atlantic Ocean. Based on the hypothesis of a molecular clock of 1.4% per million years and the rise of the strait of Gibraltar, these *Syngnathus* lineages may have diverged about 12–18 Mya. That the genus appears to be monophyletic suggests that these lineages have been sufficiently isolated to account for different species. The basal division in the phylogeny was between clades endemic to the Pacific and Atlantic oceans but suggests a diversification of these populations before the Pliocene rise of the Panamanian land bridge. The Atlantic species richness may therefore be a consequence of local and regional processes. Phylogenetic data and divergence time estimates of the two South African species suggest that diversification begun by the late Miocene (5-8 Mya). Hence, the geographical disjunction of the South African species of *Syngnathus* corresponds to historical boundaries established by geological events and climate changes. The boundaries separated these

populations, led to the reproductive isolation of these species and therefore promoted genetic and morphological diversification in the Indian Ocean.

6.6 Final conclusions

Pipefishes of the genus *Syngnathus* offer many advantages as a model system for studying the genetic basis of evolutionary change and how it relates to biogeographical regions. The species in this group are diverse in ecology and behavior, and many of the species are common enough to be ideal subjects for biological study. The occurrence of the species of the genus in the Atlantic, Pacific and Indian Oceans poses various questions, on the historical lineages, dispersal and historical events that led to this distribution. It has been observed by paleontologists working on the geological record of the entire history of life, that evolution occurs in coordinated patterns in many different species lineages based on their regional ecological setting and the physical environment, via ecological systems to influence the processes of speciation (Eldredge, 2000). For examples it has been observed that global cooling triggered new ecosystems and new species about 2.5 million years ago (Beerli *et al.,* 1996; Hewitt, 1996; Tsigenopoulos *et al.,* 2003; Ravelo *et al.,* 2004; Bremer *et al,* 2005). Therefore, the radiations or selective forces that have created and shaped these recently diverged South African species of *Syngnathus* are therefore due to a remarkable combination of various factors that have also been significant in the speciation of other groups of fishes (Kocher, 2004; Bermingham *et al.*, 1997). The aim of this work was to unify biological data, morphometric analysis and molecular analysis (quantitative and phylogeography) to make inferences about the evolution and speciation within *Syngnathus*.

This study has also demonstrated that the knowledge about South African pipefishes that has accumulated over considerable time in museum collections is very useful in understanding life history and population dynamics. This knowledge would be essential to understand and conserve these species, particularly as they are part of the large endemic component of the South African ichthyofauna. Although these species may live in a variety of habitats with a moderately large geographical range, the study shows that they are highly vulnerable due to the unstable environments they reside in and their

complex reproductive strategies (Vincent, 1996). The taxonomic affinities among the two South African pipefishes and the similarity of habitats that they occupy should stimulate interest in their comparative physiology and ecology. This integration or fusion of different ideas and methodology is needed in the field of systematics, which has for too long relied on a single method based on comparative studies of morphometric and biological data to explain the diversity of fishes. The use of different systematic procedures offers a wider scope of understanding the nature of speciation and allows for the first time a comprehensive understanding of pipefish diversity and life history in an evolutionary context. Species-level understanding of these geographically widespread and diverse taxa can advance the use of pipefishes as a model group for studies on the origins and maintenance of species diversity.

APPENDIX I

List of the 32 valid pipefish species of *Syngnathus* and their geographical distribution (Myers *et al.*, 2005; Wilson *et al.*, 2003; Kuiter, 2000; Dawson, 1986; Dawson, 1985; Fritzsche, 1980; Herald, 1940)

Species	Author**	Distribution
S. schlegeli	Kaup, 1856	North-western Pacific
S. auliscus	Swain, 1882	Eastern Pacific
S. californiensis	Storer, 1845	Eastern Pacific
S. carinatus	Gilbert, 1892	Eastern Central Pacific
S. euchrous	Fritzsche, 1980	Eastern Pacific
S. exilis	Osburn & Nichols, 1916	Eastern Pacific
S. insulae	Fritzsche, 1980	Eastern Central Pacific
S. leptorhynchus	Girard, 1854	Eastern Pacific
S. macrobrachium	Fritzsche, 1980	South-eastern Pacific
S. affinis	Günther, 1870	Western Atlantic
S. caribbaeus	Dawson, 1979	Western Atlantic
S. dawsoni	Herald, 1969	Western Central Atlantic
S. floridae	Jordan & Gilbert, 1882	Western Atlantic
S. folleti	Herald, 1942	Southwest Atlantic
S. fuscus	Storer, 1839	Western Atlantic
S. louisianae	Günther, 1870	Western Atlantic
S. makaxi	Herald & Dawson, 1972	Western Central Atlantic
*S. pelagicus**	Linnaeus, 1758	Western Atlantic (sub-tropical)
S. scovelli	Evermann & Kendall, 1896	Western Atlantic
S. springeri	Herald, 1942	Western Atlantic
S. rostellatus	Nilsson, 1855	North-eastern Atlantic
S. abaster	Risso, 1827	Eastern Atlantic, Mediterranean & Black sea
S. phlegon	Risso, 1827	Eastern Atlantic & Mediterranean Sea
S. typhle	Linnaeus, 1758	Eastern Atlantic, Baltic, Mediterranean, Black & Azov Sea
S. schmidti	Popov, 1927	Europe: Black Sea & Sea of Azov
S. taenionotus	Canestrini, 1871	Mediterranean Sea, northwestern Adriatic Sea.
S. tenuirostris	Rathke, 1837	Mediterranean, South Adriatic, Tyrrhenian, Black & Azov Sea
S. variegatus	Pallas, 1814	Eurasia: Black Sea & Sea of Azov.
S. acus	Linnaeus, 1758	Western Indian Ocean, South western Atlantic, Eastern Atlantic, North Sea, Mediterranean & Black Sea
S. macrophthalmus	Duncker, 1915	Western Indian Ocean: Suez & northwestern Red Sea
*S. safina**	Paulus, 1992	Western Indian Ocean: Gulf of Aqaba, Red Sea
S. watermeyeri	Smith, 1963	Africa: Known from tidal areas within the Kariega, Kasouga and Bushmans Rivers, South Africa

Note: * species provisionally placed in genus. **The authors listed in this appendix are not included in the reference section

APPENDIX II

Extracts from Kaup's 1856 descriptions of South African species of *Syngnathus* from the Cape of Good Hope (pages 36-37 and 45-46).

"**7**. ***Syngnathus temminckii***, Kp.

Diagnosis - Snout as long as the distance between the hinder border of the orbits and the extremity of the gill cover. Head and snout roughly shagreened and measuring one-ninth of the whole length. Seventeen body-rings before the dorsal fin. Tail-rings 37. Dorsal fin standing on nine rings, of which two belong to the body.

Description - This species is distinguished by its combined characters, from the others which inhabit the seas around the Cape of Good Hope. The prominent line on the snout is not toothed and the lateral line ridge is interrupted. Thirty-one rays exist in the dorsal fin, which is somewhat longer than the head. Colour a smutty yellowish-brown, irregularly speckled. A female 6.26 inches long, has a snout 0.28 inch long, the tail 3.66 inches, the dorsal fin 0.73 inch. In a smaller male 5.08 inches, the egg-pouch measures 1.58 inch. Its predominating colour is brownish with smutty yellowish specks.

A male and a female specimen, procured by M. Horstock at the Cape, exist in the Leyden Museum; and I have named the species after the Director of that Museum out of gratitude and esteem."

Table II. 1: Summary of morphological data from the two specimens of *S. temminckii* used for Kaup's description. Note this table is not in Kaup's text.

Morphological characters	Female		Male	
Morphometrics	Inches	Millimeters	Inches	Millimeters
Total length	6.26	159.0	5.08	129.0
Snout length	0.28	7.1		
Tail length	3.66	93.0		
Doral fin base	0.73	18.5		
Egg pouch			1.58	40.0
Meristics				
Dorsal fin rays	31		31	
Trunk rings	17		17	
Tail rings	37		37	
Sub-dorsal rings	9		9	

Appendix II: continued…

Extracts from Kaup's 1856 descriptions of South African species of *Syngnathus* from the Cape of Good Hope (pages 36-37 and 45-46).

21. ***Syngnathus delalandii***

Diagnosis – Length of the slender snout equal to the distance between the fore border of the orbit and the root end of the pectoral fin. Head and snout together either a little shorter or a little longer than the space in which the dorsal fin stands. The dorsal is based on 10 rings, and there are 18-20 rings before it.

Description – In the Paris museum, there is a Cape of Good Hope specimen procured by Delalande. Its snout is more slender than that of *rufescens*, and the body longer, but the tail again shorter. The colour is a uniform brown, with a bronze luster. Its length is 7.12 inches. Length of its head 0.91 inches; of its snout 0.51 inch; of the body measured from the edge of the pectoral ring to the vent, 2.17 inches; of the dorsal fin 0.95 inch; of the tail 4.25 inches. In the Berlin museum there are two male specimens sent by M. Krebs, also from the Cape seas. Length 5.91 inches; of head 0.82 inch; of snout 0.47 inch; of the body 1.85 inch; of dorsal fin 0.78 inch; of the tail pouch 1.85 inch. These have 18 rings before the dorsal fin, whose length rather exceeds that if the snout and head together; their colour is reddish-brown with lighter and darker specs.

APPENDIX III

Names of museums and voucher specimen numbers of material that was used in the biological and morphological analysis

Museum	Voucher Numbers			
South African Institute for Aquatic Biodiversity	SAIAB (RUSI)			
	2760	9026	17395	38610
	4308	9027	17398	38617
	7551	9034	17404	38618
	7552	10849	17405	39504
	9014	10868	17417	41113
	9015	11375	21557	41114
	9016	13229	21562	41456
	9017	13591	28338	44672
	9018	14193	30438	47275
	9019	14213	31806	49136
	9020	14218	32087	49238
	9021	14236	32131	49395
	9022	17214	34021	60052
	9023	17230	36271	60703
	9024	17234	37529	61830
	9025	17342	37567	
South African Museum	SAM			
	12800	12803	22027	35197
	12801	12804	24690	35388
	12802	13494	26408	
Museum National d'Histoire Naturelle, Paris	MNHN			
	1995-0043	1974-0270	1975-0665	1989-0111
	1959-0214	1975-0664	1977-0162	1989-0112
	1961-0858			

Appendix III: continued…
Names of museums and voucher specimen numbers of material that was used in the biological and morphological analysis

Museum	Voucher Numbers			
British Museum (Natural History), London	BMNH			
	53 1851.4.1.37 1889.8.14.39-41 1893.2.24.4-9 1907.6.27.1 1910.4.25.1 1922.11.17.1 1926.12.21.9 1928.7.16.1-2 1928.9.19.1	1929.10.3.1 1930.9.30.13 1931.1.28.5 1933.5.1.1 1933.5.24.1 1934.10.8.6 1938.9.30.1-2 1951.2.19.9 1961.12.12.5 1962.12.20.130	1962.6.1.1 192.7.30.72 1969.7.24.27 1971.2.16.319 1971.2.16.320 1971.2.16.321 1971.2.16.322-323 1971.2.16.324 1971.2.16.325-327 1971.2.16.328	1981.6.16.16 1981.9.22.5 1982.9.16.4 1982.9.17.61 1983.8.3.11 1989.3.13.1 68.8.13.40 76.9.12.2 81.10.29.3 89.9.14.5
Zoologisches Institut und Zoologisches Museum, Hamburg	ZMH			
	5 7	28 129	1201 1346	2468 6068

APPENDIX IV

A summary of the main characteristics of r and K selected species (FAO, 1984).

Characteristics	r-selected	K-selected
Climate	Usually variable and/or unpredictable	Fairly constant and/or predictable (or species shows migratory behaviour)
Risk of natural death	Often high or catastrophic; largely independent of population size	Death rate is more scheduled and dependent on population size
Population size	Variable in time, non-equilibrium conditions prevail; occupies ecological vacuums but rarely reaches the carrying capacity of the environment	Fairly constant in time, at or near carrying capacity
Competition between and within species	Generally lax	Usually keen
Length of life Natural selection in favour of	Short (1) Rapid development (2) High rate of population increase (3) High rate of egg production (4) Small body size (5) Single reproduction (6) Less emphasis on behavioural and morphological characteristics to increase individual survival habits	Longer (1) Slow development (2) Low rate of population increase (3) Low rate of egg production (4) Large body size (5) Multiple reproduction (6) Behaviour and morphology assures good individual survival, e.g., territorial behaviour, spines, special dentition and special feeding habits
All above lead to:	Productivity	Efficiency

APPENDIX V

Holotype and syntype* material for the two South African species of *Syngnathus* and morphological data of the two syntypes of *S. temminckii*.

Table V.1: Holotype and syntype* material for the two South African species of *Syngnathus* that were examined and included in the morphological analysis.

Museum	Species	Author	Type	Museum	Voucher number
South African Institute for Aquatic Biodiversity	*S. watermeyeri*	Smith, 1963	Holotype	SAIAB (RUSI)	124
Museum National d'Histoire Naturelle, Paris	*S. brachyrhynchus*	Kaup, 1856		MNHN	0000-6133
	*S. delalandii**	Kaup, 1856			0000-6139
	S. leptorhynchus	Girard, 1854	Holotype		1917-3273
British Museum (Natural History), London	*S. acus*	Linnaeus, 1758	Holotype	BMNH	1853.11.12.184
	*S. alternans**	Günther, 1870	Syntype		1855.9.19.1395
	S. rubescens	Risso, 1810	Syntype		64.12.12.29
National Museum of Natural History, Leiden	*S. temminckii*	Kaup, 1856	Syntype	RMNH	3876

Table V.2: Morphometric and meristic measurements of the holotypes of *Syngnathus acus* and the two South African syntypes *S. temminckii* and *S. delalandii* from the Cape of Good Hope (RMNH 3876). All morphometrics measurements are in mm.

Fish No.	Sex	SL	HL	SnL	SnD	OD	IOW	TD	DFB	CFL	DFR	PFR	Trunk	Tail	SDR1	SDR2	SDR
S. acus	-	434	56.80	30.40	4.62	6.30	-	21.70	49.90	10.30	39	-	20	44	1.00	8.50	9.50
S. temminckii	Female	155	16.72	7.20	1.90	2.82	1.56	6.56	18.34	4.68	31	11	19	37	1.25	7.25	9.00
S. temminckii	Male	126	13.68	5.60	1.30	2.16	1.10	3.80	16.00	4.66	31	12	19	37	1.50	6.75	8.25
S. delalandii		179	21.10	13.47		3.40	1.60	6.00	25.40	5.30	40	13	20	41	0.75	9.25	10.00

APPENDIX VI

The list of locations and biogeographical regions of the European (non-South African) museum specimens of *S. acus* used in the morphological analysis.

Location	Country/region	Biogeographical region
Famagusta	Cyprus	Mediterranean sea
near Moralaix	France	
Gulf of Thermaitos (North)	Greece	
Gulf of Evoikos (South)	Greece	
Herm	Channel islands	North-eastern Atlantic Ocean
Alderney	Channel islands	
Bay of Arcachon,	France	
Galway bay	Ireland	
Port Erin bay	Isle of Mann, Irish sea	
West of Bradda head	Isle of Mann	
Mogadore	Morocco	
Rabat	Morocco	
Madeira	Spain	
North Minch, UK	UK	
Roscoff, English Channel	UK	
Teignmouth, English channel	UK	
Yealm mouth, Devon	UK	
Weymouth, English channel	UK	
Fareham creek, English channel	UK	
English channel	UK	
Essex	UK	
River wear	UK	
Pembrokeshire, Saint George's channel	UK	
Cardigan bay, Saint George's channel	UK	
off Poole	UK	
Falmouth	UK	
South of river Humber	UK	North Sea
Leig-on-sea	UK	
South-end	UK	
South of Gomer, Norfolk	UK	
Great Yarmouth	UK	

APPENDIX VII

Discriminant function analysis summaries.

Table VII.1: Discriminant function analysis summary for size-adjusted data for the biogeographical regions of *S. acus* (SA). Variables in model: 7; n = 246; Wilk's Lambda: 0.38; F (7,238) = 55.97; and $P < 0.05$.

Character	Wilk's λ	Partial λ	F-remove	*P*-level	Tolerance	Root 1
SnL	0.54	**0.70**	100.48	0.00	0.90	-0.73
IOW	0.44	**0.87**	36.14	0.00	0.90	0.49
OD	0.44	**0.86**	40.09	0.00	0.92	-0.50
TD	0.40	0.95	12.42	0.00	0.83	0.31
SDR	0.38	0.99	2.85	0.09	0.79	0.16
Trunk	0.38	0.99	2.28	0.13	0.85	-0.13
DFR	0.38	0.99	1.90	0.17	0.80	0.13
Eigenvalue						1.65
Cum. Prop.						1.00
Canonical means						
E. Cape						-1.53
W. Cape						1.07

Note: Values in bold indicate variables that are responsible for the discrimination.

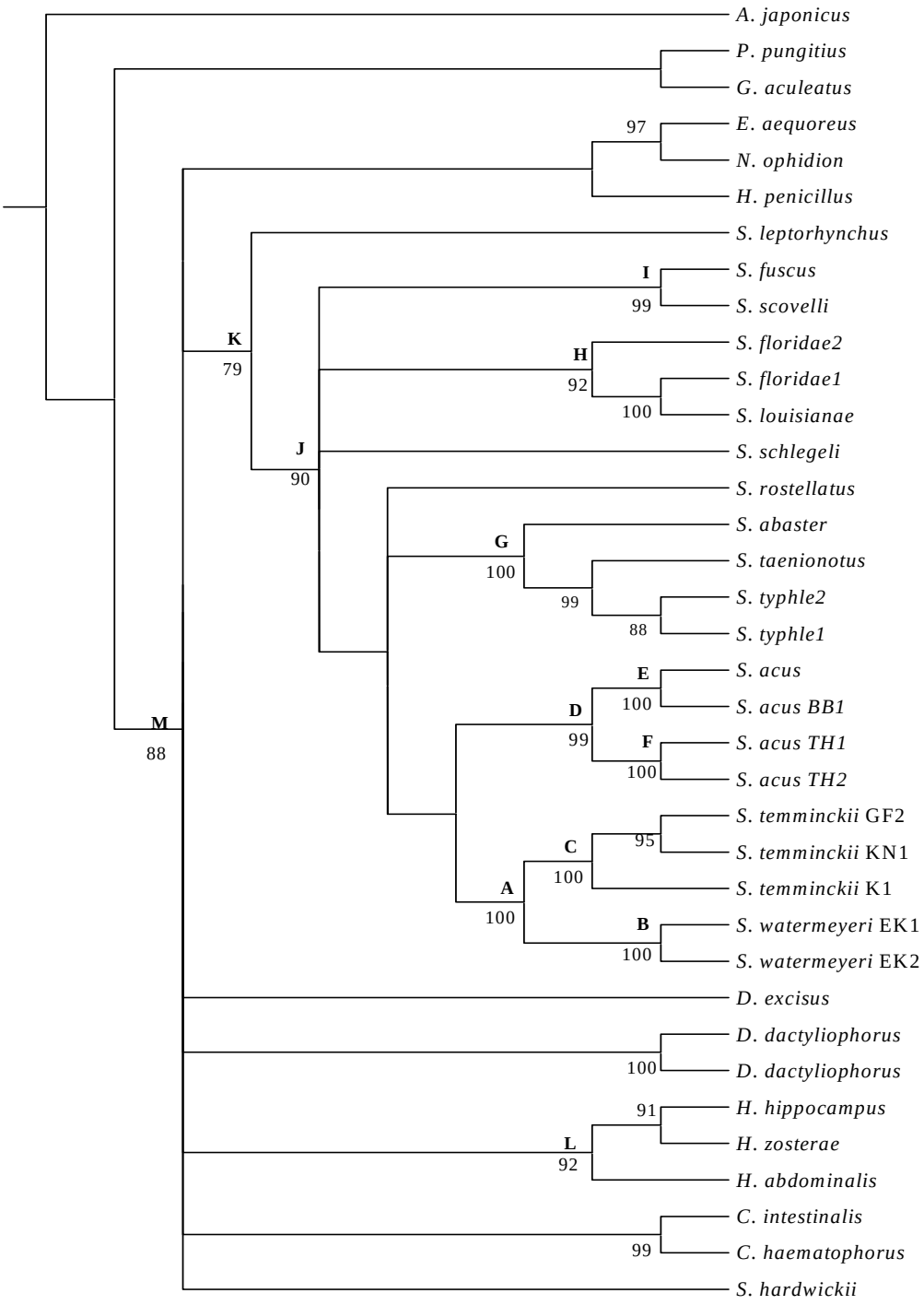
Table VII.2: Discriminant function analysis summary for morphological differences between male and female pipefishes using size-adjusted data: Variables in model 10; n = 186; Wilk's λ: 0.77; F (10,175) = 5.13; and $P < 0.05$.

Character	Wilk's λ	Partial λ	F-remove	P-level	Tolerance	Root 1
HL	0.78	0.99	1.85	0.18	0.33	-0.38
TD	0.87	**0.89**	21.97	0.00	0.62	-0.89
SnL	0.78	0.98	2.59	0.11	0.46	-0.37
CFL	0.79	0.98	3.44	0.06	0.82	0.32
PFR1	0.78	0.99	1.22	0.27	0.22	-0.37
SDR1	0.79	**0.97**	4.61	0.03	0.54	0.46
OD	0.78	0.99	1.68	0.20	0.60	0.26
Trunk	0.78	0.99	2.38	0.12	0.31	-0.44
Tail	0.78	0.99	2.33	0.12	0.48	0.35
DFR	0.78	0.99	1.56	0.21	0.24	-0.40
Eigenvalue						0.29
Cum. Prop.						1.00
Canonical means						
Females						-0.49
Males						0.59

Note: Values in bold indicate variables that are responsible for the discrimination.

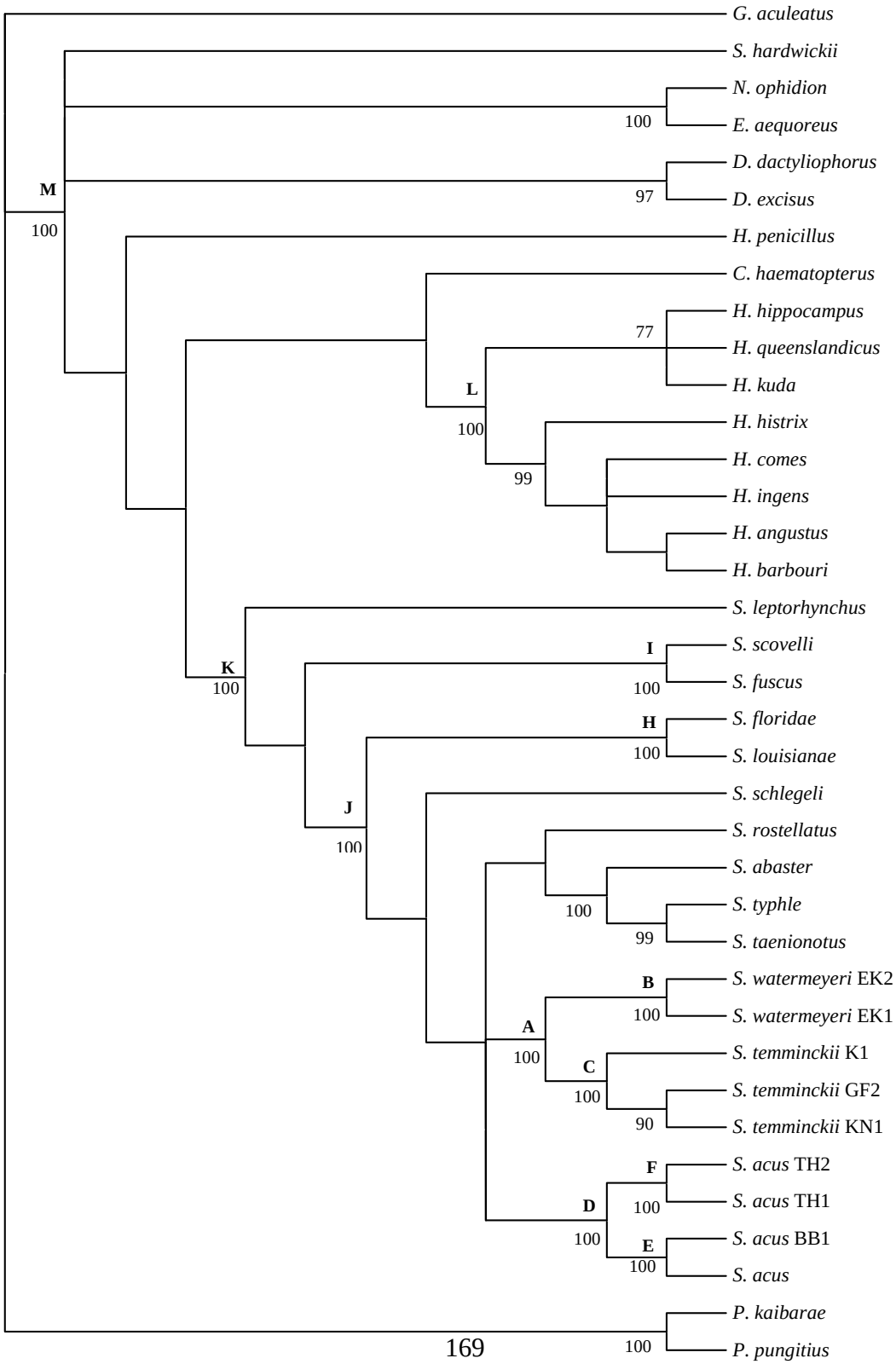
APPENDIX VIII

Figure 1: Phylogenetic relationships among species of *Syngnathus* as reflected by the consensus of the three recovered trees from the parsimony analysis of cytochrome *b* sequence data (420 aligned base pairs) of 36 sequences.



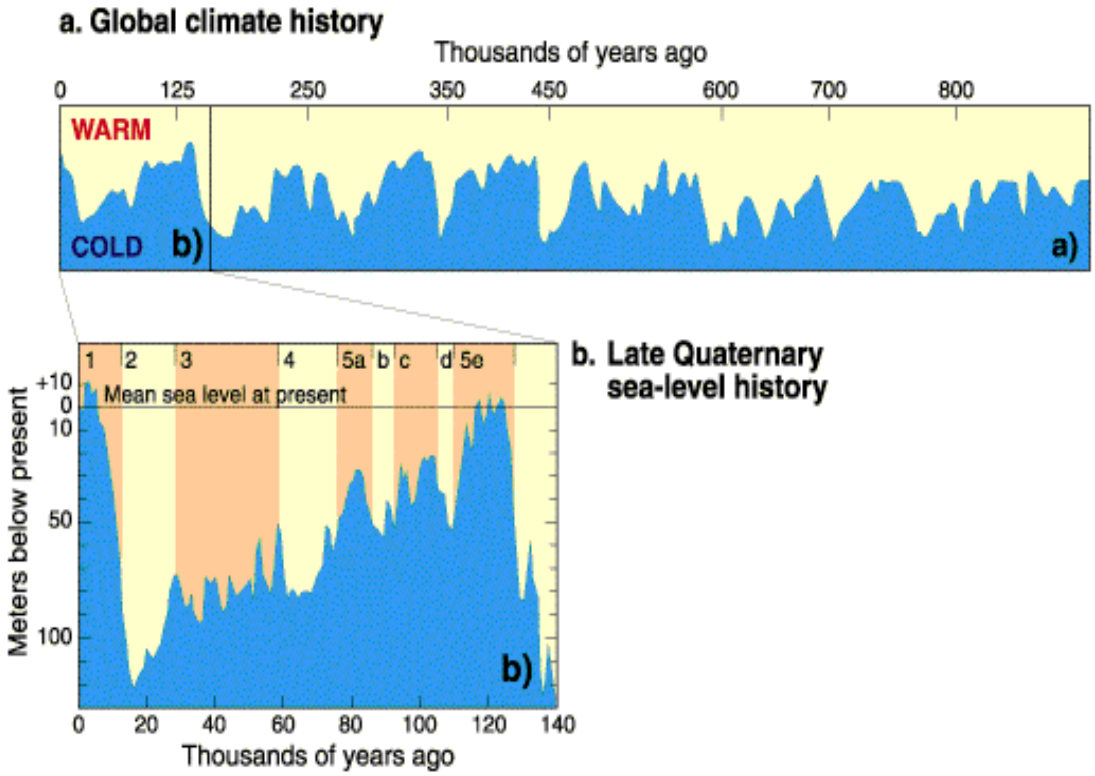
Appendix VIII

Figure 2: Phylogenetic relationships among species of *Syngnathus* as reflected the parsimony analysis of cytochrome *b* sequence data (420 aligned base pairs) of 37 sequences.



APPENDIX IX

The relative temperature over the past million years and sea level changes over the past 140,000 years (NOAA, 2004).



APPENDIX X

Flow chart showing the different steps needed to move from a description of a species to a robust explanation of the way in which a species is produced (reproduced from Brooks & McLennan, 2002)

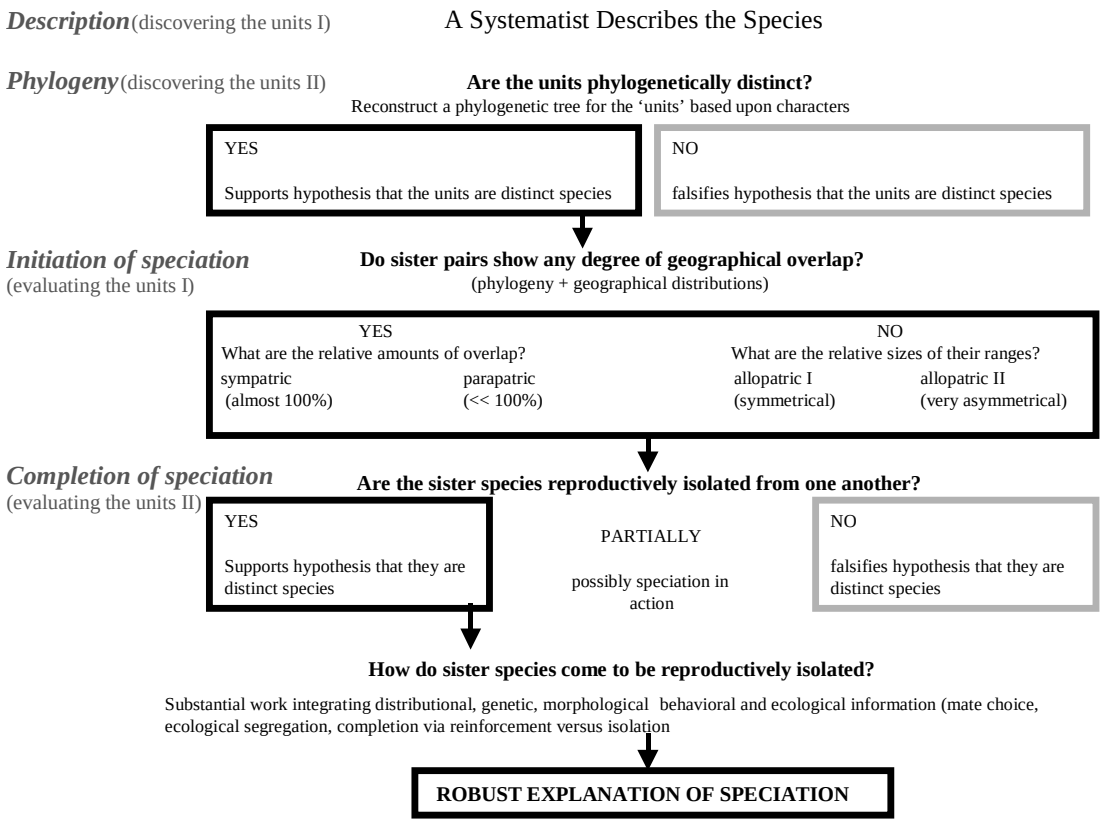


PLATE I

Plate I.1: *Syngnathus temminckii* (Photograph by P. C. Heemstra).

Plate I.2: *Syngnathus watermeyeri* (Photograph by P. C. Heemstra).

Plate I.3: *Syngnathus temminckii* male with eggs in a fully developed brood pouch (Photograph by P. C. Heemstra).

Plate I.4: *Syngnathus temminckii* Kaup, 1856 holotype (Photograph by J. van Egmond).

Plate I.5: *Syngnathus delalandii* (Kaup, 1856) holotype - MNHN 0000-6139.

Plate I.6: *Syngnathus brachyrhynchus* (Kaup, 1856) holotype - MNHN 0000-6133.

Plate I.7: *Syngnathus leptorhynchus* holotype (eastern Pacific) - MNHN 1917-3273.

Plate I.8: *Syngnathus acus* specimens (North eastern Atlantic) - MNHN 1974-0270 and 1961-0858.

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