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Identification of a uniquely southern African clade of coastal pipefishes *Syngnathus* spp.

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The taxonomic status of two southern African coastal pipefish species, *Syngnathus temminckii* and *Syngnathus watermeyeri*, was investigated using a combination of morphological and genetic data. Morphological data showed that *S. temminckii* is distinct from the broadly distributed European pipefish *Syngnathus acus*, and a molecular phylogeny reconstructed using mitochondrial DNA recovered *S. temminckii* and *S. watermeyeri* as sister taxa. The southern African species share an evolutionary origin with north-eastern Atlantic Ocean and Mediterranean Sea species, including *S. acus*. These data support the existence of a distinct southern African clade of *Syngnathus* pipefishes that has diverged *in situ* to form the two species present in the region today.

Key words: mitochondrial DNA; morphometrics; phylogeny; *Syngnathus acus*; *Syngnathus temminckii*; *Syngnathus watermeyeri*.

INTRODUCTION

Syngnathus L. 1758 is the most species-rich pipefish genus in the family Syngnathidae, with 32 recognized species (Dawson, 1985). The genus has a wide geographical distribution and is particularly common in the Atlantic Ocean, with additional species in the Pacific and Indian Oceans (Dawson, 1985; Kuiter, 2001). Despite considerable work on the phylogenetics and phylogeography of this genus (Wilson & Orr, 2011), phylogenetic relationships of species of *Syngnathus* are not fully resolved and some species have only been provisionally included within the genus due to considerable morphological variability in characteristics, such as the configuration of

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the lateral body ridge (Dawson, 1985, 1986; Kuiter, 2000). In addition, intraspecific variation in morphology has been documented in widely distributed species such as the bay pipefish *Syngnathus leptorhynchus* Girard 1854 and the greater pipefish *Syngnathus acus* L. 1758 (Fritzsche, 1980; Kuiter, 2000; Wilson, 2006). Localized colour forms of these pipefishes are lumped as single species with a high degree of ecotypic variation. This uncertainty has contributed to the confusion concerning the geographic ranges of many species in the genus. On the other hand, several species (*e.g.* the chocolate pipefish *Syngnathus euchrous* Fritzsche 1980 and the Pacific seaweed pipefish *Syngnathus schlegeli* Kaup 1856) are very similar in general morphology, so that identifying specimens without locality data can be very difficult (Dawson, 1985).

Designations of species of *Syngnathus* have therefore been a confusion of synonyms, and this is also true for the two isolated southern African species. The long snout pipefish *Syngnathus temminckii* Kaup 1856 is the most common pipefish in estuaries and coastal areas of southern Africa, ranging from Walvis Bay (Namibia) to the Tugela system (South Africa). This species and its synonym *Syngnathus delalandi* Kaup 1856 (Dawson, 1985) were described from the Cape of Good Hope, South Africa, and were previously synonymized with *S. acus* from the north-eastern Atlantic Ocean and Mediterranean Sea (Heemstra & Heemstra, 2004). The river pipefish *Syngnathus watermeyeri* Smith 1963, in contrast, is poorly known and is restricted to the estuarine environments of the East and West Kleinemonde, Bushmans and Kariega Rivers of South Africa (Whitfield, 1995, 1998; Vorwerk *et al*., 2007). *Syngnathus watermeyeri* has a shorter snout and lower pectoral-fin ray count than its congeners, and its taxonomic status in *Syngnathus* remains provisional (Dawson, 1986; Kuiter, 2000).

Prior analyses of intrageneric diversity and evolution of pipefishes (Herald, 1959; Helfman *et al*., 1997; Wilson *et al*., 2001, 2003) have neglected southern African species. Wilson *et al*. (2001, 2003) studied European and American pipefishes to reconstruct the evolution of the brood pouch in the family Syngnathidae, with some focus on relationships within the genus *Syngnathus*. They suggested that interoceanic migration of an ancestor from a centre of origin in the Pacific Ocean may have been responsible for seeding marine and freshwater habitats in the Atlantic Ocean (Wilson *et al*., 2001). North-eastern Atlantic Ocean species were separated from western Atlantic Ocean species, but had a close relationship with the eastern Pacific Ocean *S. leptorhynchus* and the western Pacific Ocean *S. schlegeli*. Intrageneric relationships and the placement of southern African species remain unresolved due to incomplete species and population sampling. The primary goals of this study were to evaluate the taxonomic status of the two southern African species, *S. temminckii* and *S. watermeyeri*, and to determine their phylogenetic placement among other species of the genus *Syngnathus* by applying both morphological and molecular approaches.

MATERIALS AND METHODS

VOUCHER SPECIMEN MATERIAL EXAMINED

Voucher specimens of species of *Syngnathus* for morphological analyses representing several localities (Fig. 1) were sourced from various museum collections (Table I). Additional

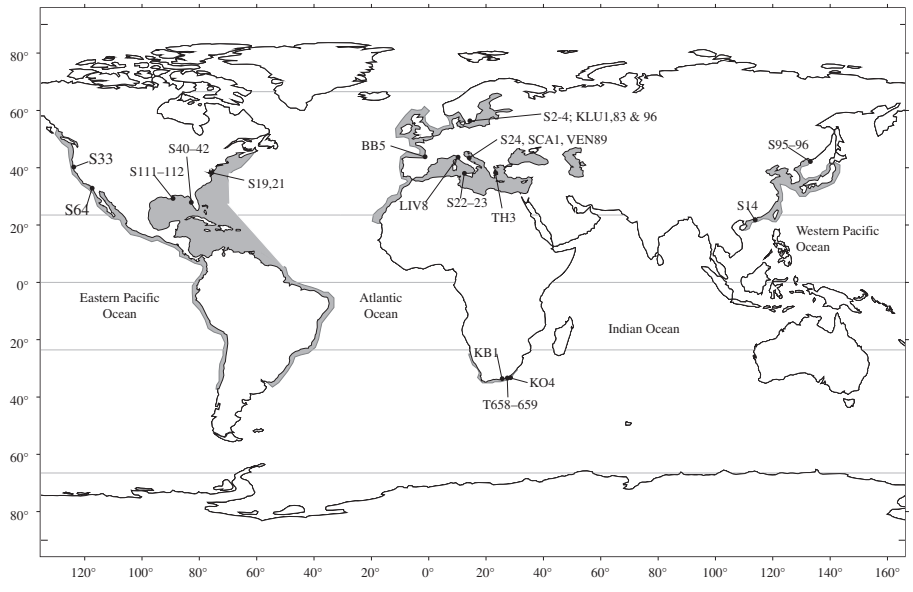

FIG. 1. The global distribution of species of *Syngnathus* (Dawson, 1985) and collection localities of DNA samples used in the phylogenetic analyses (see Tables I and III for details of collection information of all samples).

material was collected from estuaries and coastal areas using a 2 m seine (Table I) and as dead specimens from breaching events in three estuaries (*S. temminckii*: Swartvlei; *S. watermeyeri*: East and West Kleinemonde). The live fishes were euthanized with an overdose of anaesthetic clove oil before dissection. The following type material was examined: *S. watermeyeri*, Bushmans River near Port Alfred, 33° 50′ S, South Africa. Holotype (unique), South African Institute for Aquatic Biodiversity(SAIAB; formerly RUSI) 124; *S. acus*, Europe. Syntype, BMNH 1853.11.12.184 (Gronovius coll.) (1, skin); *S. temminckii*, Cape of Good Hope, South Africa. Syntypes: RMNH 3876 (2) and *S. delalandii*, Cape of Good Hope, South Africa. Holotype, MNHN 0000-6139.

MORPHOLOGICAL ANALYSES

Counts and measurements follow the standard methods given by Herald (1941) and Dawson (1985). Sixteen meristic and morphometric characters were examined for all specimens (Table II and Fig. 2). Linear measurement characters were standardized prior to statistical analysis using two procedures. The first procedure calculated proportional data by dividing all morphometric characters of a particular individual by its standard length (L_S) in mm, with the exception of snout length (L_{SN}), which was standardized as a ratio of the head length (L_H). The second procedure adjusted size-dependent morphological variation using the formula $M_{adj} = M\ (L_{SM}\ L_S^{-1})^b$ (Elliott *et al*., 1995), where M_{adj} is the size-adjusted measurement for the character, M is the original morphometric measurement and L_{SM} is the mean L_S. The slope of the regression model of the $\log_{10}$ of M and L_S using all specimens is represented by b. For the analysis, specimens were also grouped according to sex. All analyses were performed on unadjusted, proportional data and size-adjusted data (M_{adj}).

Comparative analyses were performed using the STATISTICA software package (StatSoft; www.statsoft.com). Exploratory analysis was done as an initial step to test for homogeneity of variance. The normality of residual values was tested using a Shapiro–Wilk's W test. One-way ANOVAs were used to check for phenotypic variation in morphological characters between species and their holotypes or syntypes. Statistical tests employed Tukey's *post hoc*

HSD test for unequal sample size to assess the relative importance of each character for species discrimination. Separate analyses were initially done for morphometric and meristic characters based on the assumption that these two classes of variables respond differently to environmental conditions and genetic composition. As there were no significant differences in species discrimination ($P > 0{\cdot}05$), all morphological characters were analysed in combined analyses. L_S and all other measurements were used for estimation of length relationship correlations among the three species. The slopes of least squares regression models of L_S *v*. other morphometric characters were compared using ANCOVA to determine whether these measurements were significantly different between species. Principal component analysis (PCA) and discriminate function analysis (DFA), both of which are useful in analysing intraspecific variation, were also implemented. PCA was performed to detect morphological differences between species, to determine the contribution of each character to these differences and to choose the appropriate sub-set of variables to be used in DFA. DFA was then used to obtain a function for discriminating groups previously defined by the PCA using stepwise and forward multiple regression analysis.

TABLE I. List of species, locations and number of specimens (*n*) used for morphological analyses

Location	Latitude; longitude	Biogeographic region	*n*
Syngnathus temminckii (Southern Africa)*,†,‡			
Walvis Bay (Namibia)	22° 56′ S; 14° 30′ E	West coast, Atlantic Ocean	3
Lüderitz Bay (Namibia)	26° 65′ S; 15° 15′ E	West coast, Atlantic Ocean	3
False Bay–Cape of Good Hope	34° 09′ S; 22° 07′ E	South-west coast, Atlantic Ocean	2
Klein	34° 25′ 25·0″ S; 19° 18′ 13·4″ E	South-west coast, Atlantic Ocean	10
Swartvlei	34° 01′ 51·2″ S; 22° 47′ 49·3″ E	South coast, Indian Ocean	111
Knysna	34° 04′ 38·9″ S; 23° 03′ 33·4″ E	South coast, Indian Ocean	34
Kromme	34° 08′ 27·9″ S; 24° 50′ 36·7″ E	South coast, Indian Ocean	5
Kariega	33° 40′ 55·9″ S; 26° 41′ 15·6″ E	South coast, Indian Ocean	86
Kowie	33° 36′ 11·2″ S; 26° 54′ 10·2″ E	South coast, Indian Ocean	7
East London	33° 01′ 42·9″ S; 27° 54′ 57·3″ E	South coast, Indian Ocean	15
*Syngnathus watermeyeri**			
Bushmans	33° 41′ 41·0″ S; 26° 39′ 48·6″ E	South coast, Indian Ocean	10
Kariega	33° 40′ 55·9″ S; 26° 41′ 15·6″ E	South coast, Indian Ocean	9
West Kleinemonde	33° 32′ 28·2″ S; 27° 02′ 51·7″ E	South coast, Indian Ocean	3
East Kleinemonde	33° 32′ 21·8″ S; 27° 02′ 55·2″ E	South coast, Indian Ocean	33

TABLE I. Continued

Location	Latitude; longitude	Biogeographic region	*n*
Syngnathus acus§,‖,¶			
U.K., France, Spain and Morocco	Not available	North eastern Atlantic Ocean	84
U.K.	Not available	North Sea	6
Cyprus, Greece and France	Not available	Mediterranean Sea	5

The following voucher specimen materials were examined.

*South African Institute of Aquatic Biodiversity, Grahamstown (SAIAB/RUSI) 920, 2760, 4308, 5731, 7551, 7552, 9014, 9015–9027, 9034, 10849, 10868, 11375, 13229, 13591, 14193, 14213, 14218, 14236, 17214, 17230, 17234, 17342, 17395, 17398, 17404, 17405, 17417, 21557, 21562, 28338, 30438, 31806, 32087, 32131, 34021, 36271, 37529, 37567, 38610, 38617, 38618, 39504, 41113, 41114, 41456, 44672, 47275, 49136, 49238, 49395, 59803, 60052, 60703 and 61830 (underlined numbers are *S. watermeyeri*).

†South African Museum (SAM) 12800–12804, 13494, 22027, 24690, 26408, 35197 and 35388;

‡National Museum of Natural History, Leiden (RMNH) syntypes.

§Museum National d'Histoire Naturelle, Paris (MNHN) 1995-0043, 1959-0214, 1961-0858, 1974-0270, 1975-0664, 1975-0665, 1977-0162, 1989-0111 and 1989-0112.

‖Natural History Museum, 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 and 89.9.14.5.

¶Zoologisches Institut und Zoologisches Museum, Hamburg (ZMH) 5, 7, 28, 129, 1201, 1346, 2468 and 6068. Institutional abbreviations follow Dawson (1985).

DNA TISSUE COLLECTION

Syngnathus temminckii was collected from the Kowie (KO, 33° 36′ 11·2″ S; 26° 54′ 10·2″ E) and Kabeljous (KB, 34° 00′ 17·4″ S; 24° 56′ 15·5″ E) Estuaries. The two specimens of *S. watermeyeri* originated from the Kariega Estuary (Vorwerk *et al*., 2007), one of only four locations from which this species has been recovered (Table III). Specimens of the European population of *S. acus* were obtained from the Thracian Sea (TH3), Mediterranean and from the Bay of Biscay (BB5), Atlantic Ocean. Tissue samples were preserved in 99% ethanol. Additional European samples were collected as part of a broad-scale phylogeographic investigation of European pipefishes (Wilson & Eigenmann Veraguth, 2010).

DNA EXTRACTION, AMPLIFICATION AND SEQUENCING

DNA was extracted using a DNeasy QIAGEN tissue extraction kit (Qiagen; www.qiagen.com). Phylogenetic relationships among the southern African species of *Syngnathus* and congeners for which published sequence data are available were reconstructed by amplifying portions of mitochondrial DNA cytochrome b (*cytb*), *12S* and *16S* rRNA (Table III). Primers used for amplification of these markers are listed in Table IV. The *cytb* gene was amplified using the specifically designed primer combination of SynL and SynH, while the reverse primer HI16091 (Roos, 2005) was used for sequencing.

Polymerase chain reactions (PCR) were performed using 1–2 µl of DNA in a 50 µl reaction, containing 1 U of Taq DNA polymerase (BIOTAQ), with 5 µl of 10× buffer, 0·2 mM of each deoxynucleotide triphosphate (dNTP) and of 5 mM of $MgCl_2$. The amplification procedure

TABLE II. Description of the morphological characters following Dawson (1986)

Number	Character	Abbreviation	Description
1	Standard length	L_S	Distance from tip of lower jaw to base of median caudal-fin rays
2	Head length	L_H	Tip of lower jaw to posterior margin of operculum
3	Snout length	L_{SN}	Anterior tip of snout to anterior margin of bony orbit
4	Snout depth	D_{Sn}	Smallest vertical dimension of snout
5	Caudal fin length	L_{CF}	Length of caudal fin
6	Orbit diameter	O_D	Bony orbit diameter
7	Inter-orbital width	W_{IO}	Smallest bony width measured above centres of eyes
8	Trunk depth	D_T	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	R_{PF}	Number of pectoral-fin rays (average of both fins)
11	Dorsal-fin rays	R_{DF}	Number of dorsal-fin rays
12	Tail rings	TaR	From first ring behind anus to penultimate ring excluding terminal element bearing caudal fin
13	Trunk rings	TrR	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	Total subdorsal rings (SDR1 + SDR2)

included a denaturation step of 2 min at 94° C, followed by 35 cycles of 1 min denaturation at 94° C, 1 min annealing at a primer-specific annealing temperature (60° C for *cytb* and 55° C for both *12S* and *16S* rRNA) and 1 min of extension at 72° C, followed by a final extension step at 72° C for 10 min. Thermal cycling was performed in either a Thermo Hybaid PCR Sprint Temperature Cycling system machine (www.thermoscientific.com) or a Corbett Research PC-960G Micro-plate Gradient thermal cycler (www.corbettlifescience.com). The PCR product was purified using the QIAquick (Qiagen) PCR purification kit. Cycle sequencing was conducted using the ABI Prism BigDye V3.1 terminator cycle sequencing kit (Applied Biosystems; www.appliedbiosystems.com) according to the manufacturer's instructions. The cycle sequencing reaction comprised 25 cycles at 96° C for 10 s, 50° C for 5 s and 60° C for 4 min.

Sequences generated for this study were assembled using Sequencher 3.1.1 (Gene Codes Corporation; http://genecodes.com/), and other sequences from congeners were downloaded from GenBank and included in the analyses (Table III). The sequences were aligned using the ClustalX multiple sequence alignment package (Thompson *et al*., 1994; Chenna *et al*., 2003) using default parameters, and verified by eye using BioEdit (Hall, 1999).

PHYLOGENY RECONSTRUCTION

Maximum likelihood, parsimony and Bayesian inference were all used to reconstruct phylogenetic relationships among the species of *Syngnathus*. A maximum likelihood search was conducted using default settings at the RAxML Black Box Server (Stamatakis *et al*., 2008;

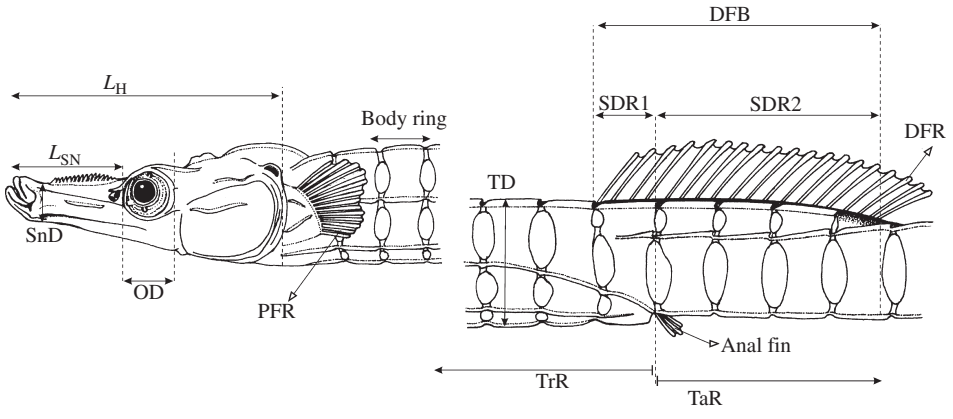

FIG. 2. An illustration of some of the morphometric and meristic characters described in Table II (after Dawson, 1986). Pipefish drawings have been accessed from the South African Institute for Aquatic Biodiversity (SAIAB) illustration collection database with accession numbers SAIAB 94925 (body) and SAIAB 94926 (head).

http://phylobench.vital-it.ch/raxml-bb/). Six partitions were specified: codon positions 1, 2 and 3 of *cytb*, *12S* rRNA, *16S* rRNA (including a larger fragment of the marker that was generated using primers for *12S* rRNA for some individuals, see Table III) and tRNA-*val*. Per-gene branch-length optimization and a maximum likelihood search were specified to find the best-scoring tree following 100 bootstrap replications (Felsenstein, 1985). A parsimony tree using combined data was reconstructed using default settings in MEGA5 (Tamura *et al*., 2011), and support for nodes was assessed using 1000 bootstrap replications. Finally, Bayesian inference was conducted using MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The same partitions as those used for the maximum likelihood analysis were specified, with an additional seventh partition represented by a data set comprising single-nucleotide indels that were shared among at least two sequences. For all six nucleotide partitions, a standard model of DNA substitution was specified using the general time reversible (GTR) model (Rodríguez *et al*., 1990), a gamma distribution parameter and a proportion of invariable sites. Two runs with four Markov chain Monte-Carlo (MCMC) chains each (one cold chain and three heated chains) were run simultaneously for 3×10^6 generations, storing trees every 100 generations. Following examination of likelihood scores, the first 10% of stored trees were discarded as burn-in, and a 50% majority rule consensus tree was constructed from all the remaining trees. Posterior probabilities and likelihood scores during stationarity were compared between the two runs, and two additional runs with the same settings were performed for 5×10^6 generations.

RESULTS

MORPHOLOGICAL VARIATION

Summary statistics for morphometric and meristic characters for all 430 specimens (including types) are summarized according to species in Table V. Significant differences in most characters (ANOVA, $P < 0{\cdot}05$) were found among the three species *S. temminckii*, *S. watermeyeri* and *S. acus*. Despite evidence of sexual dimorphism in morphometric characters, only three characters [L_H, orbit diameter (OD) and trunk depth (TD)] were found to be significantly different among the sexes. Variation in

TABLE III. Collection numbers and GenBank accession numbers of specimens of species used for phylogenetic analyses

Species	Collection number	GenBank accession		
		Cytochrome b	*12S rRNA*	*16S* rRNA
Syngnathus temminckii	KO4	JX228137	JX228149*	JX228161
	KB1	JX228138	JX228150*	JX228162
Syngnathus watermeyeri	T658	JX228139	JX228151*	JX228163
	T659	JX228140	JX228152*	JX228164
Syngnathus abaster	S23	AF356060[a]	AF354959[a]	AF355010[a]
	SCA1	JX228141	JX228153*	JX228165
Syngnathus acus	BB5	JX228142	JX228154*	JX228166
	KLU83	JX228143	JX228155*	JX228167
	S2	AF356040[a]	AF354940[a]	AF354991[a]
	TH3	JX228144	JX228156*	JX228168
Syngnathus exilis	S64	JF273424[b]	JF273442[b]	JF273406[b]
Syngnathus floridae	S21	AF356058[a]	AF354957[a]	AF355008[a]
	S41	AF356069[a]	AF354971[a]	AF355018[a]
Syngnathus fuscus	S19	AF356056[a]	AF354955[a]	AF355006[a]
Syngnathus leptorhynchus	S33 (except 16S)	AF356064[a]	AF354964[a]	DQ309797[c]
Syngnathus louisianae	S42	AF356070[a]	AF354972[a]	AF355019[a]
Syngnathus pelagicus	S111	JF273436[b]	JF273454[b]	JF273418[b]
	S112	JF273437[b]	JF273455[b]	JF273419[b]
Syngnathus rostellatus	S3	AF356041[a]	AF354941[a]	AF354992[a]
	KLU96	JX228145	JX228157*	JX228169
Syngnathus schlegeli	S14	AF356051[a]	AF354951[a]	AF355002[a]
	S95	JF273434[b]	JF273452[b]	JF273416[b]
	S96	JF273433[b]	JF273451[b]	JF273415[b]
Syngnathus scovelli	S40	AF356068[a]	AF354970[a]	AF355017[a]
Syngnathus taenionotus	S24	AF356061[a]	AF354960[a]	AF355011[a]
	VEN89	JX228146	JX228158*	JX228170
Syngnathus tenuirostris	LIV8	JX228147	JX228159*	JX228171
Syngnathus typhle	S4	AF356042[a]	AF354942[a]	AF354993[a]
	S22	AF356059[a]	AF354958[a]	AF355009[a]
	KLU1	JX228148	JX228160*	JX228172

Previously published sequence data and locality information: [a]Wilson *et al*. (2001); [b]Wilson & Orr (2011); [c]Wilson (2006).

**12S* rRNA sequences also contained sequence data of tRNA-*val* and a short portion of 16S rRNA.

L_{SN}, and dorsal and pectoral-fin ray counts, is particularly useful in distinguishing *S. watermeyeri* and its holotype from *S. temminckii* and *S. acus*. The pectoral-fin ray count discriminates *S. watermeyeri* (6–8 rays) from the other species (10–14). *Syngnathus watermeyeri* also has a shorter snout and a lower number of both dorsal-fin rays and body rings (Table V). Other morphological differences among species were observed in the measurements of head characters. The measurements of the holotypes of the three species were segregated according to locality groupings. Although some characters [inter-orbital width (IOW) and pectoral-fin rays (PFR)] were unavailable for the type specimen of *S. acus*, which was preserved as a skin, characters associated with the head (L_{SN} and L_H) separate *S. acus* from southern

TABLE IV. Primers used to amplify three mitochondrial DNA markers of *Syngnathus*

Molecular marker	Primer name	Sequence (5′–3′)	Reference
Cytochrome b	SynL	ATG ACC AAT TTA CGA AAA AC	This study
	SynH	GGC TTT ATT TTC CGT TCA GC	This study
	HI16091	GTA TCA TTC TGG TTT GAT GTG	Roos (2005)
12S rRNA	L1091	AAA CTG GGA TTA GAT ACC CCA CTA	Hrbek & Larson (1999)
	H2001	AAC CAG CTA TCA CCA GGC TCG	Hrbek & Larson (1999)
16S rRNA	16SarL	CGC CTG TTT ATC AAA AAC AT	Palumbi *et al*. (2002)
	16SbrH	CGG GTC TGA ACT CAG ATC ACG T	Palumbi *et al*. (2002)

African specimens. Other morphological measurements of the types and synonyms of *S. acus* and *S. temminckii* are similar and within the range of these two taxa. In general, morphometric characters, other than the inter-orbital width, show very high variation among the three species (Table V).

Most morphometric characters were significantly correlated with L_S (ANCOVA, $P < 0{\cdot}05$), but relationships differ among species groups for unadjusted data. Snout depth, orbit diameter and dorsal fin base were significantly correlated with L_S. The relationship of the snout to L_H among specimens of *S. acus* was significantly different from the two southern African species. These two characters (L_{SN} and L_H) were significantly different with regard to all comparisons with *S. watermeyeri*, while trunk depth and caudal-fin length were only different between *S. acus* and *S. temminckii*. The slopes of the majority of meristic characters were different among the species, except for trunk and subdorsal rings covered by the dorsal fin base (SDR1) ring counts between *S. acus* and *S. temminckii*.

MULTIVARIATE ANALYSIS

Variation in the morphometric characters, L_H, L_{SN} and dorsal-fin base (DFB), explained a significant proportion of phenotypic variation among species using PCA (Table VI). The PCA classified 90% of the specimens designated as *S. temminckii*, 70% of *S. acus* and 100% of *S. watermeyeri* into independent groups. Inter *v*. intra-group distances were large enough to confidently conclude that the three groups represented by the specimens were distinct. About 70% of the total variation (associated with L_{SN}, L_H, DFB and IOW) was accounted for by the first three PCA factors, with factors 1 and 2 accounting for 63% of the total variation. DFB had the highest loading in factor 1, while L_H and PFR had the highest loadings in factor 2. Factor 2 had a much smaller eigenvalue (1·36) and only explained 10% of the variance, while factor 3 explained 7% of the variance. Factor 3 was therefore not included in the scatter plots, as it contributed relatively little to the observed variance, with only one variable (IOW) having a high factor loading (0·78). A plot of the two first PCA axes revealed an overlap between *S. acus* and *S. temminckii*, with complete distinction

TABLE V. Variation in morphometric (1–9) and meristic (10–16) characters for three putative pipefish species and their type specimens

Character	Species	*n*	Mean	S.D.	S.E.	Minimum	Maximum	Type specimens	*P*
L_S	1	277	133·8	37·7	2·3	32·0	261·0	126–179	*
	2	84	244·1	90·9	9·9	69·0	412·0	434	
	3	53	111·5	14·7	2·0	85·0	144·0	110	
L_H	1	277	16·7	5·3	0·3	4·5	34·2	13·7–21·1	*
	2	84	34·0	12·2	1·3	10·1	58·0	56·8	
	3	53	10·5	1·1	0·2	7·9	12·7	9·96	
L_{SN}	1	277	8·6	3·7	0·2	1·8	21·2	5·6–13·5	*
	2	84	19·6	7·7	0·8	4·7	32·9	30·4	
	3	53	3·6	0·6	0·1	2·4	4·8	2·9	
SnD	1	80	1·6	0·4	0·0	0·8	2·7	1·3–1·9	a, c
	2	81	2·9	0·9	0·1	0·8	4·9	4·62	
	3	41	1·3	0·2	0·0	0·4	1·7	1·26	
OD	1	272	2·5	0·6	0·0	0·9	3·9	2·2–3·4	*
	2	84	3·9	1·2	0·1	1·3	6·4	6·3	
	3	53	1·8	0·2	0·0	1·3	2·6	1·6	
IOW	1	272	1·2	0·2	0·0	0·4	2·3	1·1–1·6	a, c
	2	84	2·1	0·7	0·1	0·8	3·8	–	
	3	53	1·0	0·2	0·0	0·7	2·1	0·7	
TD	1	277	3·9	1·4	0·1	1·0	10·9	3·8–6·6	a, c
	2	84	8·4	3·6	0·4	1·7	18·6	21·7	
	3	53	4·1	0·8	0·1	2·7	6·0	5·2	
DFB	1	277	16·8	4·7	0·3	4·5	35·1	16·0–25·4	*
	2	84	29·3	11·8	1·3	8·1	58·9	49·9	
	3	53	12·4	1·7	0·2	8·0	15·5	11·4	
L_{CF}	1	271	4·6	1·2	0·1	1·50	8·1	4·7–5·3	*
	2	81	8·1	2·9	0·3	2·79	13·5	1·03	
	3	53	3·7	0·6	0·1	2·34	4·9	3·9	
DFR	1	275	37	2	0	33	42	31–40	*
	2	83	39	3	0	33	44	39	
	3	53	30	1	0	28	33	25	
PFR	1	202	12	1	0	11	14	11–13	b, c
	2	45	12	1	0	10	14	–	
	3	21	7	1	0	6	8	6	
TrR	1	277	20	1	0	17	20	19–20	b, c
	2	84	20	1	0	15	21	20	
	3	53	17	0	0	16	18	17	
TaR	1	277	39	1	0	36	43	37–41	*
	2	84	43	2	0	37	45	44	
	3	53	38	1	0	36	41	36	
SDR1	1	277	1·17	0·35	0·02	0·25	2·00	0·75–1·5	b, c
	2	83	1·26	0·38	0·04	0·50	2·75	1	
	3	42	0·50	0·21	0·03	0·25	1·00	0	

TABLE V. Continued

Character	Species	*n*	Mean	S.D.	S.E.	Minimum	Maximum	Type specimens	*P*
SDR2	1	277	8·18	0·55	0·03	6·75	9·50	6·75–9·25	*
	2	83	7·93	0·62	0·07	6·50	9·50	8·50	
	3	53	6·82	0·34	0·05	6·00	7·50	7	
SDR	1	277	9·36	0·55	0·03	7·75	10·75	8·25–10·00	
	2	83	9·19	0·71	0·08	7·50	11·25	9·50	
	3	53	7·22	0·35	0·05	6·25	8·00	7·00	

1, *Syngnathus temminckii*; 2, *Syngnathus acus* (Europe); 3, *Syngnathus watermeyeri*; *n*, number of specimens; L_H, head length; L_{SN}, snout length; SnD, snout depth; L_{CF}, caudal-fin length; OD, orbit diameter; IOW, inter-orbital width; TD, trunk depth; DFB, dorsal-fin base; PFR, pectoral-fin rays; DFR, dorsal-fin rays; TaR, tail rings; TrR, trunk rings; SDR, subdorsal rings. *, Significant results ($P < 0{\cdot}05$) for differences between species groups: all species groups different from each other; a = 1 and 2, b = 1 and 3 and c = 2 and 3 are significantly different from each other. Type museum specimens were analysed from the South African Institute of Aquatic Biodiversity, Grahamstown (SAIAB), Museum National d'Histoire Naturelle, Paris (MNHN) and Natural History Museum, London (BMNH), except for *S. temminckii* where the type specimen information was taken from Kaup (1856) and from the National Museum of Natural History, Leiden (RMNH). The measurements of *S. temminckii* also reflect the two syntypes of this description and *S. delalandi* its synonym. Morphometrics were measured in mm while meristic characters are counts.

between these species and *S. watermeyeri* using morphometric variables (Fig. 3). PCA results for proportional data were slightly different from size-adjusted data, particularly with regard to the distribution of significant characters among factors. The proportion of specimens correctly classified was 97% overall. The two principal components, however, explained only 54% of the variation among specimens. All meristic characters (except for SDR1) were responsible for explaining variation. The PCA for both data sets did not indicate significant phenotypic variation due to sex among the southern African specimens of *Syngnathus*.

Discriminant function analysis of size-adjusted variables indicated that species and sex contributed significantly ($P < 0{\cdot}05$) to discriminating specimens into distinct groups (Table VII). Two characters, L_S and SDR, were redundant (highly size correlated) and were therefore not included in the model. There were significant differences in characters among the three species ($F = 142{\cdot}6$, Wilk's $\lambda = 0{\cdot}025$, $P < 0{\cdot}05$). Partial Wilk's λ indicated that meristic characters, PFR, tail rings (TaR), subdorsal rings covered by the tail (SDR2), trunk rings (TrR) and dorsal-fin rays (DFR) (ranked from first to fifth, respectively), contribute most to the overall discrimination among the three taxa (Table VIII). The first function separated *S. watermeyeri* from *S. acus* and *S. temminckii*, and the canonical mean for this species was found to differ significantly from that of the other two species (Fig. 3). The second function chiefly distinguished specimens of *S. acus* from the two southern African species. The magnitude of this discrimination, however, was much smaller than for the first function and accounted for less of the morphological variation. Sex significantly contributed to only two characters (TD and SDR1) that are associated with the trunk and brood pouch area. There were no differences among meristic characters with regard to sex across species.

TABLE VI. Factor loadings of the principal component analysis of all specimens using proportional and size-adjusted (M_{adj}) data. Values in bold contributed >70% to the observed variation and are significant at $P < 0{\cdot}05$

	Proportional data		Size-adjusted data		
Character	Factor 1	Factor 2	Factor 1	Factor 2	Factor 3
L_{SN}:L_H*	**0·86***	0·16*			
L_H	**0·89**	−0·07	0·17	**0·84**	0·21
L_{SN}	**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
L_{CF}	−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 that * is for raw and proportional data sets only and refers to the ratio of snout length (L_{SN}) to head length (L_H). All other characters in this data set are the ratios of morphometric characters with standard length (L_S). SnD, snout depth; L_{CF}, caudal-fin length; OD, orbit diameter; IOW, inter-orbital width; TD, trunk depth; DFB, dorsal-fin base; PFR, pectoral-fin rays; DFR, dorsal-fin rays; TaR, tail rings; TrR, trunk rings; SDR, subdorsal rings.

PHYLOGENETIC ANALYSES

All three methods of phylogeny reconstruction recovered the southern African species *S. temminckii* and *S. watermeyeri* in a strongly supported sister group relationship (Fig. 4). There is also a strong support for the monophyly of a clade that

TABLE VII. The classification matrix for the observed classifications according to discriminate analysis for species and the effects of sex on morphological variation using size-adjusted data

Effect	Group	*n*	Per cent correct in cluster (size adjusted)
Species group	*Syngnathus temminckii*	250	97·30
	Syngnathus acus	77	97·40
	Syngnathus watermeyeri	42	100·00
Sex	Female	95	75·26
	Male	79	71·43

n, sample size.

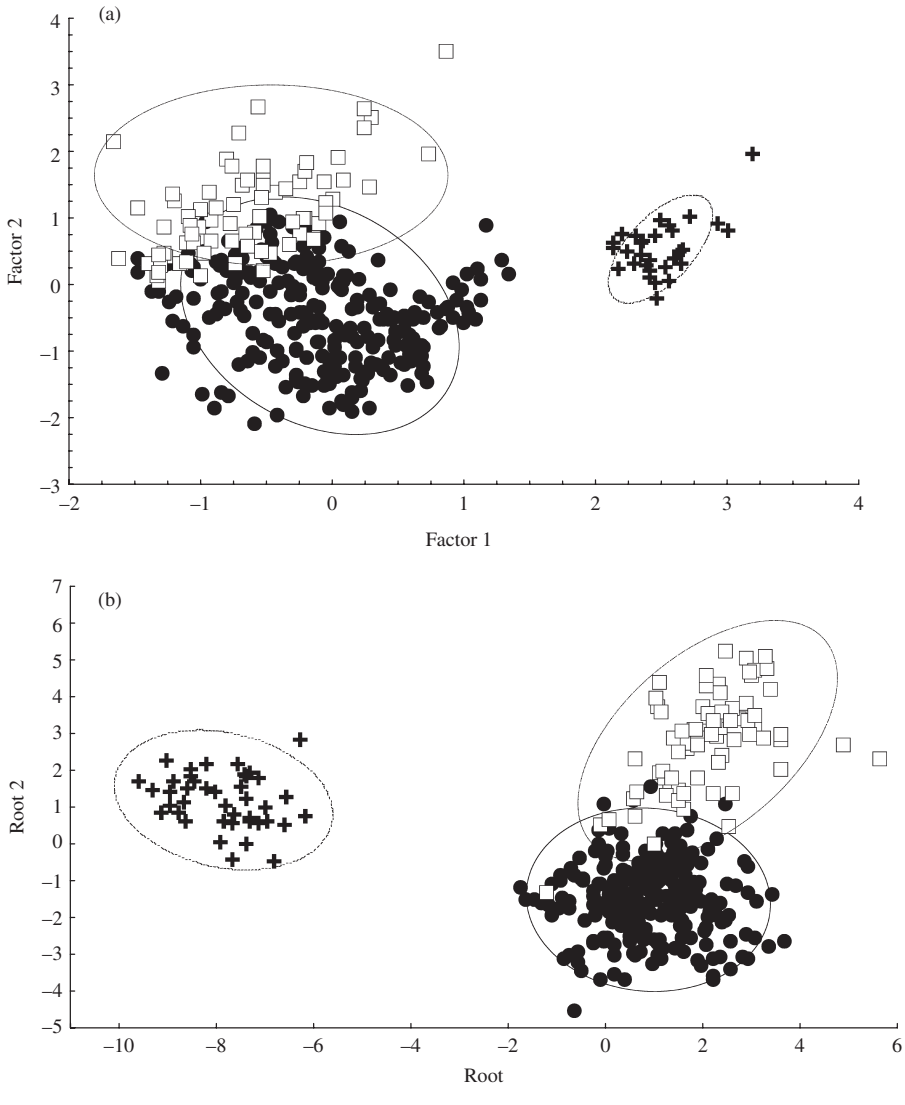

FIG. 3. (a) Principal component analysis (PCA) factor scores and (b) discriminate function analysis (DFA) canonical scores with 95% confidence ellipsoids of the three species, *Syngnathus temminckii* (●), *Syngnathus acus* (□) and *Syngnathus watermeyeri* (+), and size-adjusted morphometric characters.

includes the southern African species and six species from the north-eastern Atlantic Ocean and Mediterranean Sea: *S. acus*, the black-striped pipefish *Syngnathus abaster* Risso 1827, Nilsson's pipefish *Syngnathus rostellatus* Nilsson 1855, the darkflank pipefish *Syngnathus taenionotus* Canestrini 1871, the narrow snouted pipefish *Syngnathus tenuirostris* Rathke 1837 and the broadnosed pipefish *Syngnathus typhle* L. 1758. In the phylogenetic tree with the highest likelihood score, *S. acus* was recovered in a sister group relationship with the other five species from the northern hemisphere rather than the southern African species. Although bootstrap support for this placement was low, it was also supported by the other methods of phylogeny

TABLE VIII. Discriminant function analysis summary for morphological differences between the three species groups using size-adjusted data. Variables in model: 13; $n = 366$, Wilk's λ: 0·025, F (26 702) = 142·60 and $P < 0·05$. Values in bold indicate species groups that are responsible for the discrimination.

Character	Wilk's λ	Partial λ	F-remove	P-value	Tolerance	Root 1	Root 2
PFR1	0·054	**0·47**	201·14	<0·001	0·98	**−0·77**	0·14
Tail	0·049	**0·51**	166·33	<0·001	0·61	0·14	**−1·03**
SDR2	0·029	0·88	23·64	<0·001	0·53	−0·20	**0·50**
Trunk	0·029	0·86	28·05	<0·001	0·71	**−0·34**	0·34
DFR	0·030	**0·84**	32·45	<0·001	0·68	−0·26	**−0·48**
L_H	0·030	**0·85**	30·28	<0·001	0·55	−0·31	**−0·49**
TD	0·027	0·92	14·17	<0·001	0·72	−0·02	−0·37
IOW	0·026	0·96	7·05	<0·01	0·83	−0·05	−0·24
SDR1	0·026	0·97	6·20	<0·01	0·82	−0·21	−0·05
DFB	0·026	0·97	6·11	<0·01	0·63	0·05	0·26
L_{CF}	0·026	0·98	4·31	<0·05	0·80	−0·18	−0·04
OD	0·026	0·98	3·07	<0·05	0·82	−0·04	0·16
L_{SN}	0·026	0·99	2·12	>0·05	0·57	0·02	0·17
Eigenvalue						9·14	2·89
Cumulative proportion						0·76	1·00
Canonical means							
Syngnathus temminckii						−1·07	1·02
Syngnathus acus						−1·12	**−3·19**
Syngnathus watermeyeri						**8·36**	−0·03

L_H, head length; L_{SN}, snout length; SnD, *s*nout depth; L_{CF}, caudal-fin length; OD, orbit diameter; IOW, inter-orbital width; TD, trunk depth; DFB, dorsal-fin base; PFR, pectoral-fin rays; DFR, dorsal-fin rays; TaR, tail rings; TrR, trunk rings; SDR, subdorsal rings.

reconstruction (parsimony bootstrap support: 78%, Bayesian posterior probability: 97%). *Syngnathus acus* was paraphyletic, as one individual (TH3) was recovered as a sister taxon of *S. tenuirostris*. It would be worthwhile to investigate the relationship between these two species using a larger sample size and both mtDNA and nDNA markers to study the evolutionary history of this taxon.

DISCUSSION

This study is the first to investigate genetic and morphological variation of southern African species of *Syngnathus*. The non-monophyly of *S. acus* and *S. temminckii* supports the hypothesis that the southern African long snout pipefish previously treated as *S. acus* (Dawson, 1986) is in fact distinct from the species found in the northern hemisphere, and should thus be referred to as *S. temminckii*. Differences in body size seem to be an important source of morphological variation, with *S. acus* growing to a significantly larger size than *S. temminckii*. Meristic characters (*e.g.* dorsal-fin ray counts as well as trunk tail and subdorsal rings) are more effective than morphometric characters in separating *S. acus* and *S. temminckii* from the smaller *S. watermeyeri*. Although there is a considerable overlap in meristic and

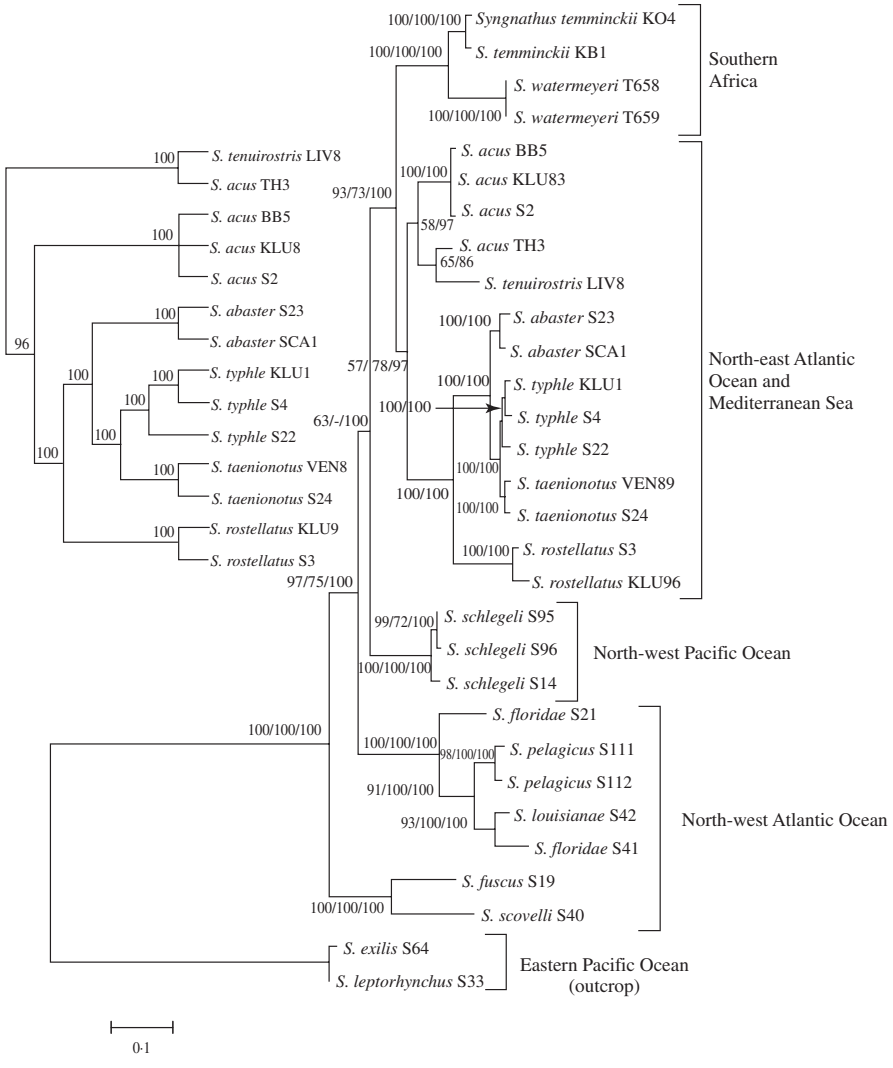

FIG. 4. Phylogenetic placement of the southern African coastal pipefish species *Syngnathus temminckii* and *S. watermeyeri* among congeners. The phylogenetic tree depicted was reconstructed from combined mtDNA *cytochrome b*, *12S* rRNA, tRNA-*val* and *16S* rRNA sequences using the maximum likelihood method. Support for nodes based on three alternative methods of phylogenetic reconstruction is indicated by three numbers (bootstrap support ≥50% from maximum likelihood and parsimony, as well as posterior probabilities ≥95% from Bayesian inference; the latter is shown separately for north-east Atlantic Ocean and Mediterranean Sea species in the insert, as the tree topology reconstructed using Bayesian inference was not identical to that of the other methods).

morphometric characters between *S. acus* and *S. temminckii*, such overlap appears to be common among syngnathids that are either closely related or live in similar habitats (Herald, 1965; Fritzsche, 1980; Kuiter, 2000).

Although the generic status of *S. watermeyeri* was previously considered uncertain (Dawson, 1985), the results presented here show that this species is not only

firmly placed within *Syngnathus*, but is also the sister taxon of *S. temminckii*. Morphological characters indicating distinctness of *S. watermeyeri* from other members of the genus, including a shorter snout, a lower number of pectoral and dorsal-fin rays, a lower number of trunk and tail rings and a lower number of sub-dorsal rings, are clearly not taxonomically informative and seem to be correlated with this species' short and broad body shape. Reductions in the counts for meristic characters, such as fin rays and trunk rings, are also evident in closely related species of seahorses (Syngnathidae: *Hippocampus*) that differ in size, such as the dwarf seahorse *Hippocampus zosterae* Jordan & Gilbert 1882 and its sister species, the lined seahorse *Hippocampus erectus* Perry 1810 (Lourie *et al*., 1999). Unlike all other species of the genus *Syngnathus*, *S. watermeyeri* is an estuarine endemic that does not occur in the marine habitat (Dawson, 1986; Whitfield, 1998). Adaptation to this habitat may also explain the considerable morphological differences between *S. watermeyeri* and its congeners.

The sister group relationship of the two southern African species *S. temminckii* and *S. watermeyeri* is strongly supported in all phylogenetic analyses, and there is also support for a sister group relationship between the two southern African species and all species from the north-eastern Atlantic Ocean and the Mediterranean Sea, including *S. acus*. Although morphological similarities between *S. acus* and *S. temminckii* indicate that *S. acus* could be basal to the southern African lineage, phylogenetic reconstructions do not support this hypothesis. Sequence data from the nuclear genome may be required to unambiguously resolve the phylogeny of the group (Teske & Beheregaray, 2009).

The sister group relationship of *S. temminckii* and *S. watermeyeri* indicates that *S. temminckii* is not simply a southern African population of *S. acus*, rejecting the hypothesis of an anti-tropical distribution of this species. The finding that the temperate southern African species share an evolutionary origin with a group of temperate pipefishes from the northern hemisphere highlights the importance of inter-hemispheric migration in the evolutionary history of this group. Genetic analyses of evolutionary lineages with congruent geographic structure indicate that such colonization events may have occurred in either direction (Teske *et al*., 2011). Evolutionary lineages basal to the *Syngnathus* clade of interest all comprise pipefishes from the northern hemisphere, which supports the derived status of the southern African species. This result, however, may be strongly affected by both sampling bias and limited resolution of mtDNA data at deeper phylogenetic levels. In addition to sequencing nuclear loci, more sampling will be necessary to resolve phylogenetic relationships within *Syngnathus* and to test biogeographic hypotheses related to their evolutionary origin.

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