Article

Elliptic Fourier Analysis of body shape variation of *Hippocampus* spp. (seahorse) in Danajon Bank, Philippines

S. R. M. Tabugo-Rico[1], L.A. Tumanda[1], C.K. Cabrido[1], C.M. Apale[2], E. Dumalagan[2]

[1]Department of Biological Sciences, College of Science and Mathematics, Mindanao State University-Iligan Institute of Technology, Philippines

[2]Zoological Society of London iSeahorse, Philippines

E-mail: sharonrose0297@gmail.com

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Abstract

Seahorses inhabit various ecosystems hence, had become a flagship species of the marine environment. The Philippines as a hot spot of biodiversity in Asia holds a number of species of seahorses. This serve as an exploratory study to describe body shape variation of selected common seahorse species: *Hippocampus comes*, *Hippocampus histrix, Hippocampus spinosissimus* and *Hippocampus kuda* from Danajon bank using Elliptic Fourier Analysis. The method was done to test whether significant yet subtle differences in body shape variation can be species-specific, habitat-influenced and provide evidence of sexual dimorphism. It is hypothesized that phenotypic divergence may provide evidence for genetic differentiation or mere adaptations to habitat variation. Results show significant considerable differences in the body shapes of the five populations based on the canonical variate analysis (CVA) and multivariate analysis of variance (MANOVA) with significant p values. Populations were found to be distinct from each other suggesting that body shape variation is species-specific, habitat-influenced and provided evidence for sexual dimorphism. Results of discriminant analysis show further support for species specific traits and sexual dimorphism. This study shows the application of the method of geometric morphometrics specifically elliptic fourier analysis in describing subtle body shape variation of selected *Hippocampus* species.

Keywords elliptic fourier analysis; *Hippocampus*; seahorse; Danajon bank.

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1 Introduction

In the marine environment, *Hippocampus* spp. (seahorses), are associated with varied habitats such as sea grass beds, mangroves, sandy silt bottoms, sponge gardens, gorgonians corals, sea whips, sea fans, estuaries and coral reefs, which harbors the highest biodiversity in the marine environment (Foster and Vincent, 2004; Loh et al., 2014; Wu and Zhang, 2012). Thus, they are considered as flagship species of various ecosystems. They are among the many genera whose life histories render them vulnerable to overfishing and habitat destruction. Protecting seahorses would mean protecting diverse habitats including all marine life (Foster and Vincent, 2004; Loh et al., 2014). They are generally characterized by their sparse distribution, low mobility, small home ranges, low fecundity, lengthy parental care and mate fidelity and listed as 'vulnerable' or 'endangered' on the 2003 IUCN Red list of Threatened Species (IUCN, 2003). Inferred life spans ranged from 1 to 5 years (Foster and Vincent, 2004). As such, they are not only good and suitable for relatively long term monitoring and ecological studies but could also be fascinating subject for phenotypic studies, owing to complex shapes and forms. Phenotypes exhibit the interplay of genes and the environment. Several studies show that different species are associated with specific or various habitats. For instance, *H. comes* typically found as juveniles on floating sargassum, moving to corals and sponges when older. The *H. kuda*, often seen in shallow waters in coastal bays and lagoons, seagrass beds, floating seaweeds, sandy sediments in rocky littoral zone, muddy bottoms, mangroves, estuaries and lower reaches of rivers. *H. histrix* occur in sea grass beds, sandy sediments, weedy rocky reefs, hard & soft corals, sea whips & sea fans, and sponges in deep waters. Other species spotted in deep waters are *H. kelloggi* and *H. spinosissimus*. The *H. kelloggi* are found in gorgonian corals and sea whips. Whereas, *H. spinosissimus* are associated with octocorals and sandy bottoms but not mud. *H. bargibanti* and *H. denise* live only on gorgonian sea fans and *H. pontohi* were found among hydroid crops, bryozoans and coralline algae (Lourie et al., 2003; Lourie and Kuiter, 2008). Majority of seahorse populations in the wild are inadequately studied. Albeit, easily recognized as a group, many seahorse species superficially similar in appearance and species delineation is often a dilemma. Hence, the problem arises in seahorse identification and delineation. Thus, is body shape variation species-specific? Or habitat influenced? With this, it is pertinent to employ reliable and unambiguous method to distinguish populations. Along this line, is the incessant search for tools to characterize subtle body shape variation in populations as evidence for phenotypic differentiation in the genus *Hippocampus*. The emergence of the Geometric morphometrics or Statistical analysis of shapes for biological forms revolutionized our method and understanding of biological shape variations at the population level (Adams et al., 2004). Meanwhile, Danajon Bank located off northern Bohol Island, is the only double barrier reef in the Philippines and is 1 of only 3 such sites in the Indo-Pacific. This double barrier reef consists of 3 large reefs and spread across almost 130 kms. Based on dive surveys and local information from fisher folks, four species of seahorse out of seven that thrive in the Philippines are found in Danajon (Ready, 2013; Pichon, 1977; Armada, White, and Christie, 2009).

Accordingly, this study serve as an exploratory study to describe body shape variation of selected common seahorse *Hippocampus* species: *H. comes*, *H. histrix, H. spinosissimus* and *H. kuda* from Danajon bank using Elliptic Fourier Analysis.

Moreover, considerable research is needed to advance seahorse conservation and management and current available data confirm that seahorses are likely vulnerable to high levels of exploitation. Following seahorse populations through time will enable researchers to track population status, reveal potential impacts from anthropogenic activities and discover new aspects of seahorse biology. Such information can be compiled and released to relevant authorities to directly influence conservation efforts.

2 Materials and Methods

2.1 Sampling site, specimen collection and identification

The sampling site was in Danajon bank, a double barrier, in the Pacific, spanning 97 miles along the islands of Cebu, Bohol, mainland Leyte and Southern Leyte (Fig. 1). This is the only well-documented seahorse sanctuary in the Philippines and was considered as an area where marine animals thought to have first evolved. It has a rare geologic formation and considered as one of the six double barrier reefs in the world with the richest areas in Biodiversity. It is considered as a home of over 200 threatened species including species of *Hippocampus* (seahorse) (Ready, 2013; Pichon, 1977; Armada, White, and Christie, 2009; White and Cruz-Trinidad, 1998). Adult seahorse specimens were in courtesy of iseahorse, phils., ZSL. Specimens were bycatch samples of fishermen in the area. Identification of samples was done through illustrated keys, Guide to the identification of Seahorses (Lourie et al., 2004) and consultation of experts. Photographs were taken for all the samples then processed for image analysis. Microhabitats associated with species of seahorses were noted based on iseahorse underwater surveys (through SCUBA diving) undertaken in the area.

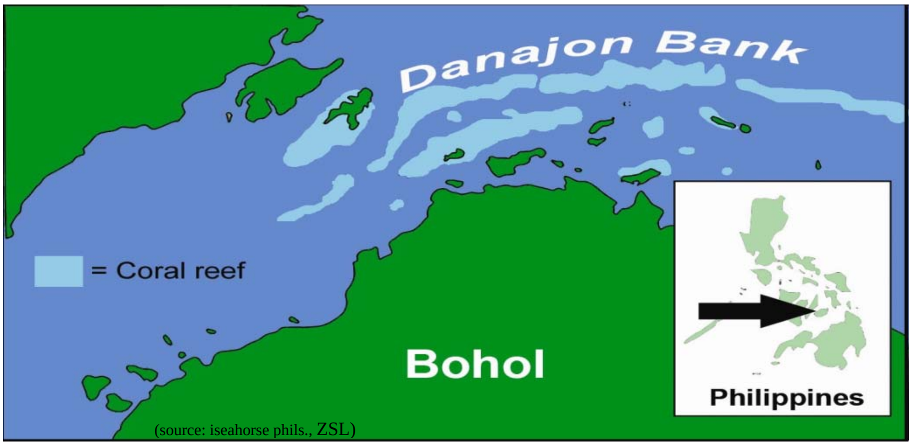

Fig. 1 Study site: map of Danajon bank, a double barrier reef.

2.2 Image acquisition, processing, shape analysis and statistical analysis

Image acquisition was done using Canon DSLR 550D. Images were processed in triplicates. The full colored images of seahorses were pre-processed in Photoshop and converted to 24- bitmap type, binary (black & white color) images for noise reduction. Moreover, images were pre-processed with respect to the position of the trunk to exclude the part of the tail demarcated by the first tail ring in females and the 5^{th} tail ring in males (to include the pouch), such that only the body shape with respect to curved trunk length was considered for the analysis. This is to avoid bias on the distal part of the tail due to orientation. Then outlines of body shape were digitized using the software package SHAPE version 1.3 (Iwata and Ukai, 2002) for examination of shape variation and chain codes were recorded (Freeman, 1974). Herewith, the objects of interests were distinguished via segmentation techniques through a “threshold procedure” where a parameter called the brightness threshold is manually chosen from brightness histogram and applied. Undesirable marks also termed as “noise” were consequently eliminated by erosion-dilation filter process. After noise reduction, the closed contour body shapes of seahorses were extracted via edge detection and the contours were stored in the form of chain codes (Dalayap et al., 2011). Chain coding technique was employed, which relied on a contour representation to code shape information. This method tracks the body shapes of seahorses and represents each movement by a chain code symbol ranging from 0-7. The set of possible movement depends on the type of contour representation, a pixel based contour representation were used in this study. Normalized Elliptic Fourier Descriptors (EFD) obtained from the chaincodes were calculated using Elliptic Fourier transformation as suggested by Kuhl and Giardina (1982). Normalization of data obtained from chain codes used the first harmonic ellipse as a basis which corresponds to the first Fourier approximation and utilized the 20 harmonics number to be calculated as suggested by Iwata and Ukai (2002). Principal component analysis was used to summarize independent shape characteristics. It can efficiently extract and summarized information obtained from the coefficients generated.

Moreover, to test the level of significance of principal component scores extracted from the procedure, the differences in body shape among seahorses were determined and subjected to Multivariate Analysis of Variance and Canonical Variate Analysis (MANOVA/CVA). Wilks' lambda and Pillai trace values and p values were obtained. Box- and whiskers and scatter plots showing the variations were generated, this is to visualize the distribution of shape variation using the principal component scores (Tabugo et al., 2014, 2012) (Fig. 2). Discriminant analysis show further support for species specific traits and sexual dimorphism.

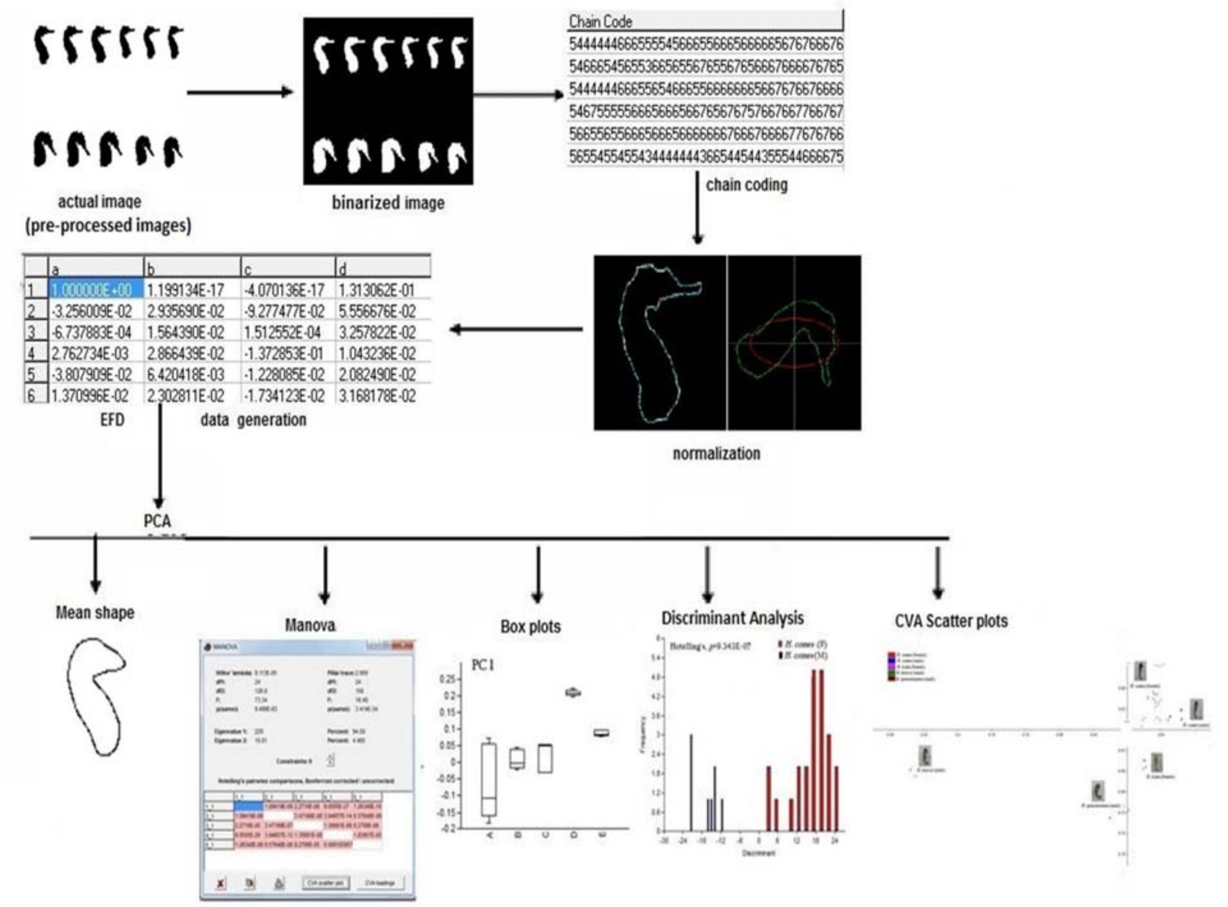

Fig. 2 Outline of Elliptic Fourier Analysis of *Hippocampus* spp. (seahorse) found in Danajon Bank.

3 Results and Discussion

In the Philippines, there were recorded seahorse sightings throughout the country however, Danajon Bank, serves as an important sanctuary for many species. Important marine habitats like coral reefs, sea grass beds, mangrove forests, sponge gardens and gorgonians serve as a haven for such species. Owing to their presence in various habitats, they are considered as valuable and flagship species of the marine ecosystem. The sad part, is that seahorses are often targeted by fisher folks supplying traders for medicinal and aquarium use (Lourie et al., 2003; Lourie et al., 1999; Pajaro and Vincent, 2015). Moreover, they are considered as vulnerable to bycatch fishing and habitat degradation. Albeit, seahorse research had made advances, there is still a dearth of data when it comes to populations. There is still much to learn also about the nature of populations, key life history parameters such as growth rates, dispersal and natural mortality. Data on body shape variation in this group remains lacking.

In this study, statistical analysis of shape has been employed to look into relationships of species populations under the genus *Hippocampus* based on body shape variation. In the past, this method had paved way for fast and reliable approach of studying biological forms (Adams et al., 2004; Marcus, et al., 1996; Rohlf and Marcus, 1993). Previous studies suggested that generally, it is a good tool in elucidating variations in organisms especially between population variations (Hassall, Thompson and Harvey, 2008). The Elliptic Fourier Analysis (EFA) using Elliptic Fourier descriptors (EFDs), originally proposed by Kuhl and Giardina (1982), can delineate any type of shape with a closed two dimensional contour. EFDs have been effectively applied to the analysis of various biological shapes in animals (Rohlf and Archie, 1984; Ferson et al., 1985; Bierbaum and Ferson, 1986; Diaz et al., 1989; Liu et al., 1996; Laurie et al., 1997) and plants (White et al., 1988; McLellan, 1993; Furuta et al., 1995; Ohsawa et al., 1998; Iwata et al., 1998; Iwata et al., 2000; Toyohara et al., 2000; Uga et al., 2003; Yoshioka et al., 2005a, 2005b, 2006a, 2006b). Herewith, the principal component scores obtained were used as observed values of morphological features in subsequent analysis, such as analysis of the body shapes of seahorse populations.

Results yield significant differences in the body shape of the seahorses for the five populations examined based on the distribution of the individuals along the first two canonical variate axes (Wilk’s lambda: 3.08E-04; p-value: 1.096E-55; Pillai trace: 2.873; p-value: 3.27E-34). Here, CV1 accounts for 80.2% and CV2 explains 14.27% of the overall variation respectively. Individuals in the CV1 axis described variations were based on body shape and depth with respect to curved trunk length while those individuals in the CV2 axis vary in the length-width aspect ratio, shape of head and snout (Fig. 3).

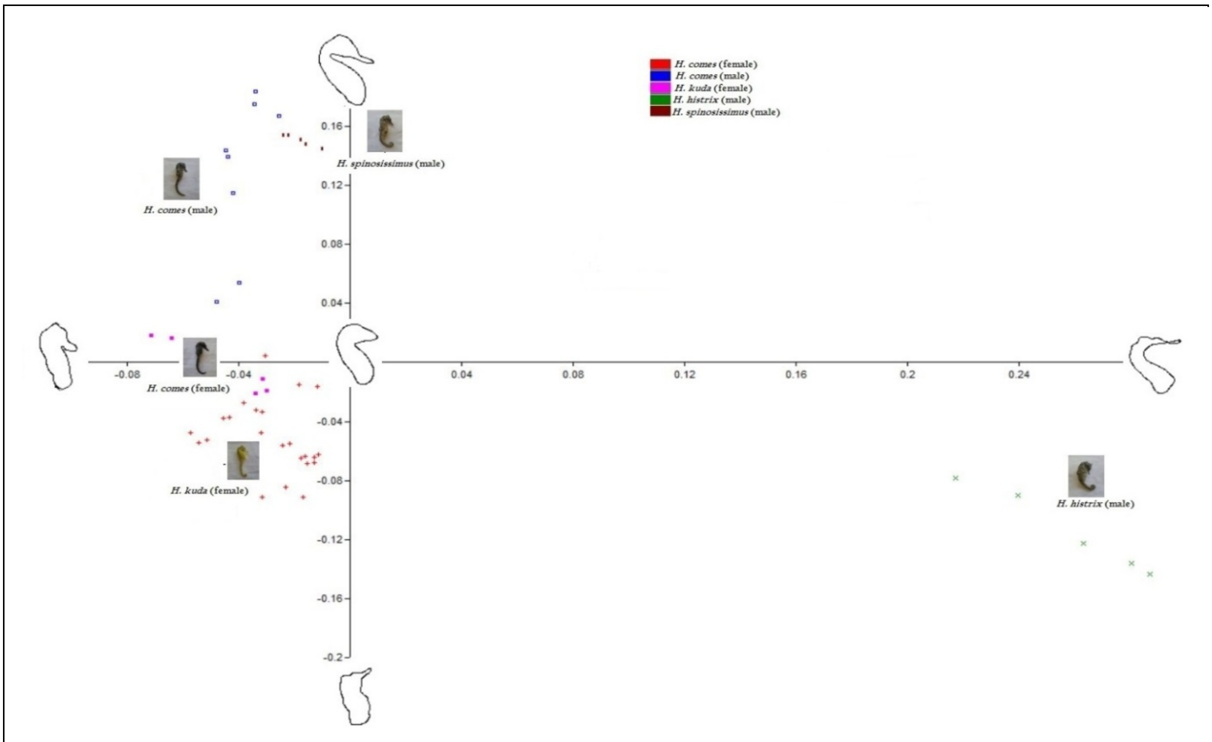

Fig. 3 Plots of the first two canonical axes showing significant difference in body shapes among species of seahorses (Wilk’s lambda: 3.08E-04; p-value: 1.096E-55; Pillai trace: 2.873; p-value: 3.27E-34) found in Danajon Bank, including positive and negative deviations from consensus shape.

Noteworthy, based on the distribution of points on the compromise space of the CVA plot (Fig. 3) both *H. comes* male and female species appeared close to each other but remained relatively distinct populations perhaps, due to shared habitats. *H. comes* typically found as juveniles on floating sargassum, moving to corals and sponges when older. The male species have smaller home ranges than females because they need to conserve their energy for pregnancy and brooding. One of the fascinating characteristics of seahorses is that the males bear the young. Females produce eggs and the males produce the sperm. However, when the eggs become ripe the females deposit it to the male brooding pouch (Lourie, 2016). Thus, males have deep ventro-lateral medial portion, broader and fuller shape because of the presence of the brooding pouch drawing a line between male and female seahorse species. This implies the role of sexual dimorphism in the significant difference between male and female *H. comes* species in the CVA plot. Meanwhile, *H. kuda* female occupies the lower left portion of the plot clustered near *H. comes* female. Thus, it is also significantly different as such it occupies different habitats. They are often seen in shallow waters in coastal bays and lagoons, sea grass beds, floating seaweeds, sandy sediments in rocky littoral zone, muddy bottoms, mangroves, estuaries and lower reaches of rivers. *H. kuda* have deep body, thick snout and deep head. But, generally females have more narrow body then males. *H. spinosissimus* resembles that of *H. comes* in some aspect though, it is classified as spiny seahorse, and their body shape is similar. Both have slender snout but *H. spinosissimus* has quite short snout compared to *H. comes*. Perhaps, this explains why they are quite close together in the plot yet remained relatively distinct. *H. spinosissimus* are associated with octocorals and sandy bottoms but not mud. *H. histrix,* can be found in sandy sediments, weedy rocky reefs, hard & soft corals, sea whips & sea fans, and sponges. *H. histrix* has long snout and average to narrow body. Male species highlight the deep ventro-lateral medial portion with conspicuous bellies (Lourie, 2016).

Table 1 shows the Multivariate Analysis of Variance (MANOVA), p-values are Hotelling's pairwise comparisons (Bonferroni corrected), showing significant difference in body shape variation of seahorses between groups. Notably, significant observed variation suggest that body shape variation can be species-specific, sex-influenced and habitat-influenced.

Table 1 Multivariate Analysis of Variance (MANOVA), p-values are Hotelling's pairwise comparisons (Bonferroni corrected), showing significant difference in body shape variation of seahorses between groups.

	H. comes (F)	*H. comes* (M)	*H. kuda* (F)	*H. histrix* (M)
H. comes (F)				
H. comes (M)	**7.16E-09**			
H. kuda (F)	**2.56E-09**	**7.39E-07**		
H. histrix (M)	**1.36E-20**	**3.15E-06**	**7.47E-05**	
H. spinosissimus (M)	**3.12E-17**	**3.30E-05**	**6.04E-05**	**1.13E-05**

**p*-values<0.05 is significant.

Accordingly, form and function said to have evolved hand-in-hand such that the unique curved shape bodies in seahorses provided them increased feeding efficiency as they adapt to the environment. Studies have pointed out that the evolution of the upright posture of seahorses was linked to Oligocene expansion of seagrass habitats. In a study by Wassenbergh (2011), the seahorse's S-curved body was a recent evolutionary innovation. Studies also showed that aside from expansion of habitats, seahorse shape play a pertinent role in capturing prey. This is to support that form and function could evolve hand-in-hand. Hence, the unique curved shape in seahorses increased their efficiency in feeding (Wassenbergh et al., 2011; Teske and Beheregaray, 2009). Such mechanism is dependent on food availability in a given environment. Varied food corresponds to varied habitats. Thus, different seahorse species are often associated with their preferred habitats thus, seahorse' body shape evolve in respond to its important role in capturing prey in a given habitat. In addition, Wassenbergh (2011) stated that it was believed that ancestral seahorses took advantage of expanded shallow water seagrass habitats by simply adopting a more cryptic lifestyle that is by changing their hunting lifestyle to 'sit and wait' ambush feeders. They simply attached their prehensile tail to certain holdfasts then wait for the food to pass by striking distance. This is to compensate for being weak swimmers. The S-shaped bodies in seahorses allowed them to tense their muscles and also designed to snap forward to gulp their prey (Bergert and Wainwright, 1997). Feeding behaviour and microhabitat take its role in body shape variation.

Fig. 4 shows the significant difference in body shapes among species of seahorses: *H. comes* (F), *H. comes* (M), *H. kuda* (F), *H. histrix* (M) and *H. spinosissimus* (M)) found in Danajon Bank. Herewith, species tend to have a consensus shape which is common for all populations examined. However, notable differences in body shape between species and sex were also evident. But, generally variations observed were based on shape of head, snout and body depth with respect to curved trunk length. The males have deeper body due to brood pouch and females have more narrow body with small to medium sized head relative to body.

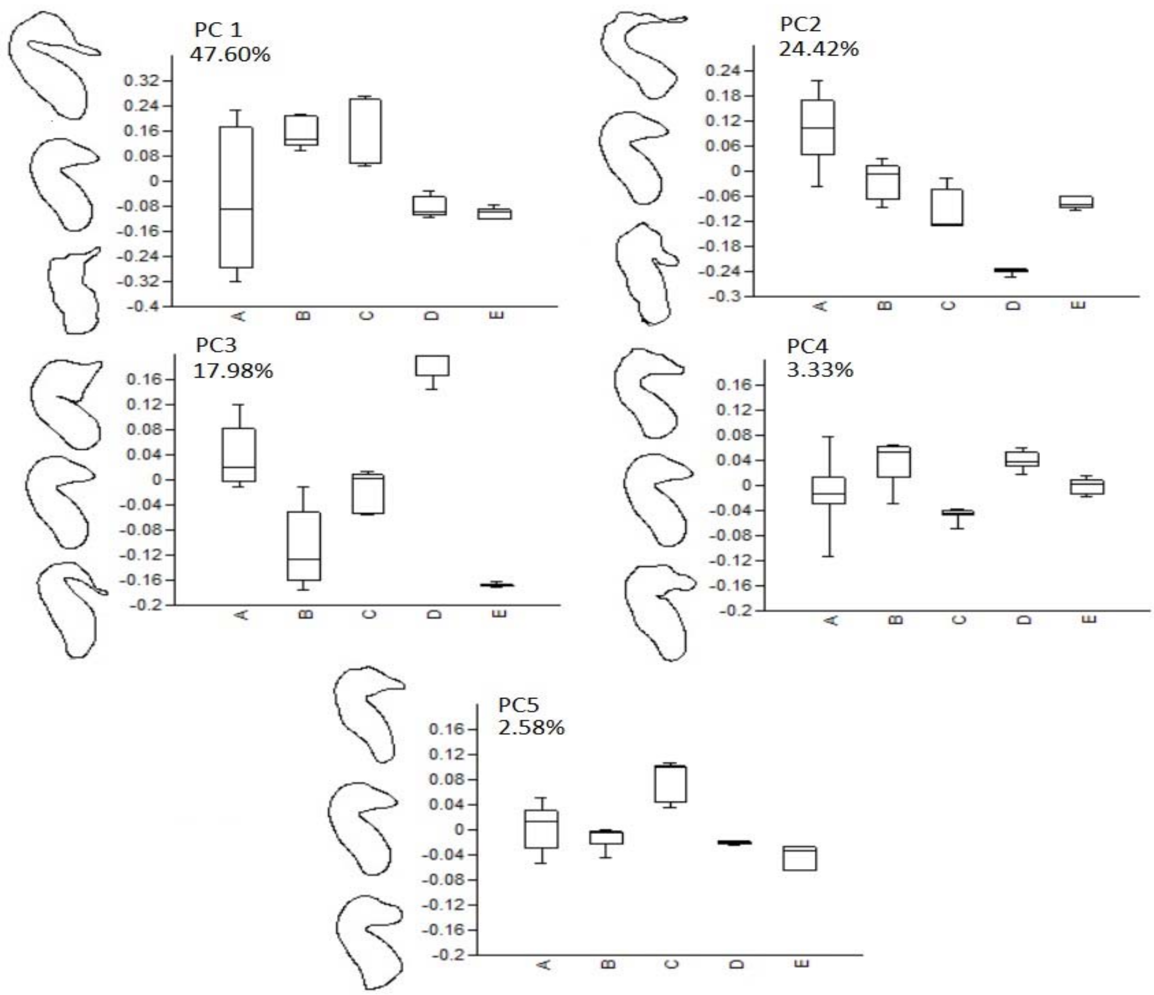

Fig. 4 Boxplots showing significant difference in body shapes among species of seahorses: A. *H. comes* (F); B. *H. comes* (M); C. *H. kuda* (F); D. *H. histrix* (M); E. *H. spinosissimus* (M) found in Danajon Bank.

Principal component analysis was performed to summarize independent body shape characteristics. The method efficiently extracts and summarized information obtained from the coefficients generated. Standardized Elliptic Fourier Coefficients were calculated and used to reconstruct the consensus morphology including the positive (+) and negative (-) deviations from the mean body shape of seahorses. This is an exploratory process in order to determine comparison between shapes and elucidate possible biological significance. Considerable differences in the body shape of seahorses were noted. Table 2 shows the detailed descriptions of body shape variation accounted by each principal component for seahorses (*Hippocampus* spp.) found in Danajon Bank.

Table 2 Descriptions of body shape variation accounted by each principal component for seahorses (*Hippocampus* spp.) found in Danajon Bank.

PC s	Descriptions
PC 1 (47.60%)	Variations observed were based on shape of head, snout and body depth with respect to curved trunk length; *H. comes* have narrow head, slender snout; the males have deeper body due to brood pouch and females have more narrow body with small head relative to body; For *H. kuda*, deep head, thick snout and deep body; *H. histrix*, have average to narrow body; *H. spinosissimus* have short snout and mean towards narrow body; Observed variations were notable between species and between sex;
PC 2 (24.42%)	Variations ranged from mean shape towards either narrow or broad body; *H. spinosissimus*, short and slender snout; *H. histrix* has narrow body;
PC 3 (17.98%)	Individuals have mean shape except *H. kuda* (F) that has short, thick snout and quite broad on the ventro-medial part of body; *H. spinosissimus* and *H. histrix* have average to narrow body but conspicuous belly for males; head is relatively narrow;
PC 4 (3.33%)	Individuals tend to approach a common body shape ranging from narrow to deep ventro-lateral medial portion; head can be deep and narrow on the antero-lateral proximal end;
PC 5 (2.58%)	Observed variations were notable between species and between sex; Individuals tend to approach a common shape ranging from body narrow to deep ventro-lateral medial portion; head can be deep and narrow on the antero-lateral proximal end; snout shape ranged from average to relatively narrow to thick and short; females generally have narrower body shape on the ventro-lateral medial part; while males have broader and fuller shape; this is conspicuous for *H. comes*, *H. spinosissimus* and *H histrix*;

Moreover, PC scores were subjected to Discriminant Function Analysis (DFA) to test for sexual dimorphism and species distinctiveness. Percentage of correct classification higher than the cut-off value of 75 indicates clear separation of the independent datasets examined. This is shown as no overlapping of bins in the generated histogram (Fig. 5). Herewith, this analysis is used as a standard method to visually confirm or reject the hypothesis that two groups are morphologically distinct. The equality of means of the two groups were determined, tested and further reclassified to previously defined groups (Hammer et al., 2001). Results yield significant result for DFA thus, confirming the hypothesis that groups are morphologically distinct. Hence, implies sexual dimorphism and species distinctiveness based on body shapes.

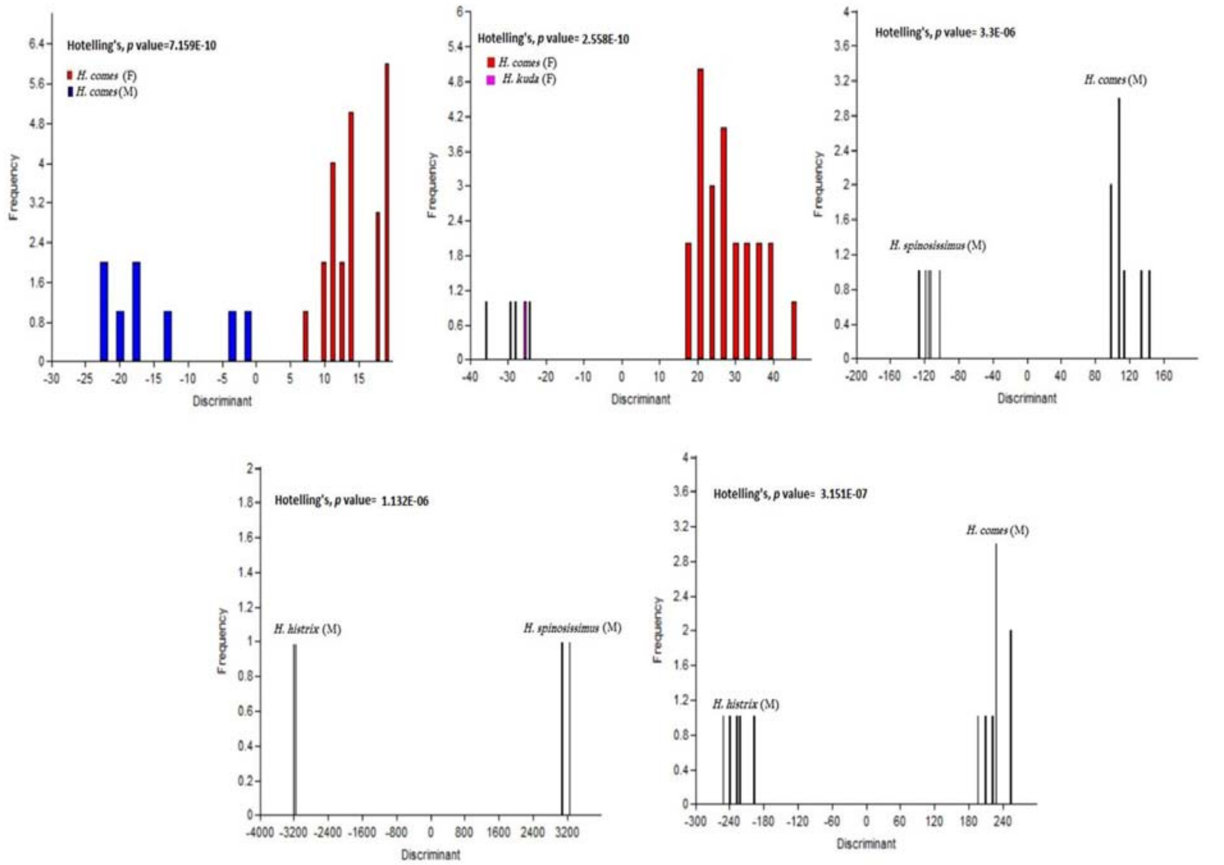

Fig. 5 Discriminant Analysis showing significant difference in body shapes among species of seahorses found in Danajon Bank ($p<0.05$ is significant).

4 Conclusion

This study provided a baseline data on body shape variation of species of seahorses. In this study, elliptic fourier analysis provided a means to increase efficiency in understanding the nature of body shape variation in seahorses from Danajon bank. Results show significant considerable differences in the body shapes of the five populations based on the canonical variate analysis (CVA) and multivariate analysis of variance (MANOVA) with significant *p* values. Herewith, populations were found to be distinct from each other suggesting that body shape variation is species-specific, habitat-influenced and provided evidence for sexual dimorphism. Results of discriminant analysis show further support for sexual dimorphism and species specific traits. This study shows the application of the method of elliptic fourier analysis in describing subtle body shape variation of selected *Hippocampus* species. Moreover, seahorse' body shape evolve in respond to its important role in capturing prey in a given habitat. Hence, conservation of microhabitats associated with species of seahorses in the marine environment is important

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