

Integrating morphometric and molecular approaches for snakeheads' phylogeny

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Abstract. Morphometric and molecular phylogenetic study provides interesting perspective for relationship of the snakeheads species. In this study, morphometric method was done by measuring eighteen variables on side view and eleven variables on dorsal view of 53 individuals from four species of snakeheads which were *Channa striata*, *C. micropeltes*, *C. marulioides* and *C. lucius*. Phylogenetics study was carried out on partial mtDNA 16S gene of six *Channa* species which were *C. striata*, *C. micropeltes*, *C. marulioides*, *C. lucius*, *C. melasoma* and *C. gachua*. For the morphometric study, the data were analysed with ANOVA, Principal Component Analysis (PCA), and Discriminant Function Analysis (DFA). All four species were grouped into their respective groups. The variables side and dorsal view showed variability for size among individuals and the species can be grouped into different group based on similarities in the head and caudal peduncle regions. For phylogenetic study, the phylogenetic tree was constructed based on partial mtDNA 16S show a clear grouping among the six species. This study demonstrated that the morphometric and phylogenetic studies were valuable in identification of the species and could be used in determining the relationship of fish species.

Key Words: Channa, mtDNA, truss-network, freshwater fish, species identification.

Introduction. Snakehead is also known as serpent head and is classified into family *Channidae*; genus *Channa* (Mohsin & Ambak 1983). Currently 38 valid species of snakehead are recognized across its natural range from Africa to Asia (Conte-Grand et al 2017) and seven of these species can be found in Peninsular Malaysia which are *Channa striata*, *C. micropeltes*, *C. marulioides*, *C. lucius*, *C. gachua*, *C. melasoma* and *C. bankanensis* (Lee & Ng 1994). Snakeheads represent a very interesting group of fish for research as it has wide geographical range and certain level of endemism in both eastern and western region (Adamson et al 2010). The most common species of snakeheads is *C. striata*, locally known as 'haruan' or common snakehead which has medical properties (Zakaria et al 2004) and is widely sold in wet markets for consumption. It is also widely cultured and subject of numerous researches (Jianguang & Fast 1997; Mat Jais 2007; Hossain et al 2008). The other species are *C. micropeltes* known as giant snakehead, *C. marulioides* known as emperor snakehead, *C. lucius* known as splendid snakehead, *C. gachua* known as dwarf snakehead, *C. melasoma* as black snakehead and *C. bankanensis* as Bangka snakehead. The snakeheads are also very popular with aquarium hobbyist and sport fisher. These seven species of snakeheads are usually identified based on morphological features which are elongated cylindrical body, long and entirely soft-rayed dorsal and anal fins, a large mouth with well-developed teeth on upper and lower jaws, and an accessory air-breathing apparatus known as the suprabranchial organ (Musikasinthorn 1998, 2003; Li et al 2005).

Traditional morphometrics are based on certain features or measurements which do not give actual representation of the fish studied. Truss network analysis is based on certain landmarks that were chosen to represent the important/main shape of the fish that will give a more accurate description of the fish (Strauss & Bookstein 1982). Measurements will go through transformation to eliminate size effects. It was used to differentiate different species and also to discriminate different stocks of fish from the

same species (Bagherian & Rahmani 2009) to facilitate the management and conservation of the species involved.

Snakeheads provide an interesting challenge for phylogenetic study due to potential intra species complexes. Recent works has suggested that *C. gachua* actually consist of two different species: *C. gachua* for the western lineages and *C. limbata* for the eastern lineages (Conte-Grand et al 2017). They also suggested the eastern lineage may also have other sub-units across its natural ranges. These two lineages have limited morphological dissimilarities based on the preserved specimens studied. Further study combining both morphological and genetic approaches may resolve the issue of intra species complexes and help with correct identification of the species. Introduction of snakeheads outside its natural range also has made misidentification of the species was quite common (Courtenay & Williams 2005). Intra species study on *C. striata* show that geographical factor may contribute to genetic structuring of the species (Siti-Balkhis et al 2011).

Morphological studies of the snakeheads species were done using truss-network to obtain the morphometric data of different species of snakeheads while the phylogenetic studies used partial mtDNA 16S in Peninsular Malaysia. Combining these two approaches will enable better identification of snakehead species using quantitative methods and may be able to establish the relationship between *Channa* species in Peninsular Malaysia.

Material and Method

Sampling sites. In this study, 53 specimens were collected which consist of 32 samples of *C. striata*, nine samples of *C. micropeltes*, eight samples of *C. marulioides* and four samples of *C. lucius* for the morphometric study while phylogenetic study used the same sample plus one more sample of *C. melasoma* and *C. gachua*. Only six species were obtained for this study which excluded *C. bankanensis*. Identification was done based on characteristics for each species (Lee & Ng 1994). The samples were obtained from all over Peninsular Malaysia (Table 1) through own sampling or from middle men. Tissues samples of every specimen were obtained from the pectoral and caudal fin and were preserved in 95% ethanol for phylogenetic study while the whole body was kept in -20°C freezer for morphometric study.

Table 1

Sampling site for *Channa* species in Peninsular Malaysia

Species	Location	Number of specimens	
		Morphometric	Phylogenetic
<i>Channa striata</i>	Sungai Sebertak, Pahang	2	2
	Kelantan	11	11
	Terengganu	19	4
<i>Channa micropeltes</i>	Tasik Kenyir, Terengganu	2	2
	Penarik, Terengganu	7	3
	Muar, Johor	5	5
<i>Channa marulioides</i>	Penarik, Terengganu	1	1
	Perak	2	0
<i>Channa lucius</i>	Sungai Sebertak, Pahang	1	1
	Penarik, Terengganu	2	1
	Johor	1	1
<i>Channa melasoma</i>	L. Payong	0	1
<i>Channa gachua</i>	Sungai Pur, Kuala Berang, Terengganu	0	1

Morphometric-truss network measurement. For the morphometric study of *Channa* species in Peninsular Malaysia, the truss-network measurement was used. After sampling, the samples were kept inside sealed plastic bags in -20°C freezer with information about their name, date, place of sampling and number of individuals. For

measurement, the frozen specimens were thawed then measured using vernier callipers according to the morphological landmarks that had been distinguished from the outline of the fish. Total of 18 side views (Figure 1) and 11 dorsal views (Figure 2) were taken on the left side of every specimen (Norainy 2007).

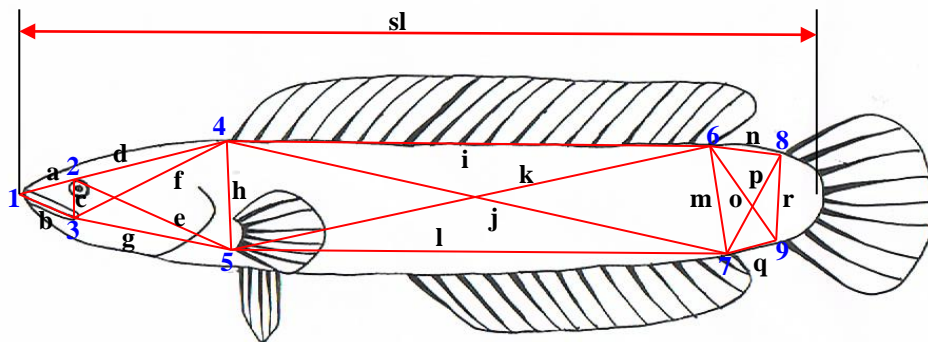


Figure 1. Morphological landmarks of side view. Lines refer to (a) snout to above eye; (b) snout to end of mouth; (c) above eye to end of mouth; (d) above eye to origin of dorsal fin; (e) above eye to origin of pectoral fin; (f) end of mouth to origin of dorsal fin; (g) end of mouth to origin of pectoral fin; (h) origin of dorsal fin to origin of pectoral fin; (i) origin of dorsal fin to end of dorsal fin; (j) origin of dorsal fin to end of anal fin; (k) origin of pectoral fin to end of dorsal fin; (l) origin of pectoral fin to end of anal fin; (m) end of dorsal fin to end of anal fin; (n) end of dorsal fin to upper part origin of caudal fin; (o) end of dorsal fin to lower part of origin of caudal fin; (p) end of anal fin to upper part of origin of caudal fin; (q) end of anal fin to lower part of origin of caudal fin; (r) upper part of origin of caudal fin to lower part origin of caudal fin; and (sl) standard length.

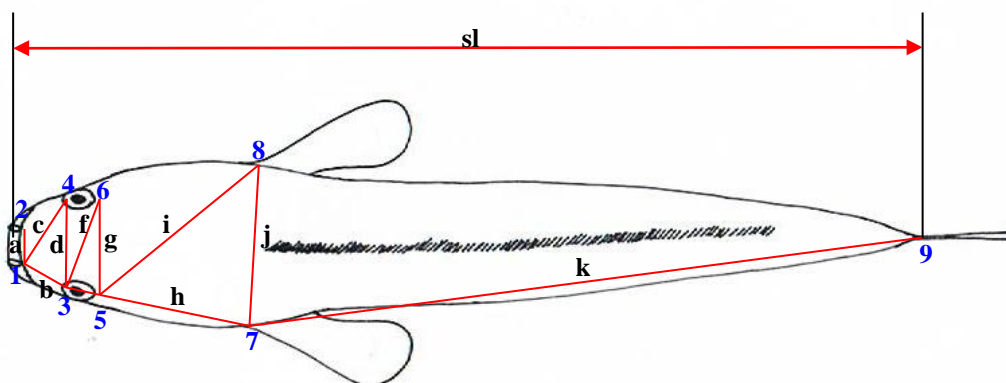


Figure 2. Morphological landmarks of dorsal view. Lines refer to (a) left nostril to right nostril; (b) left nostril to anterior of left eye; (c) left nostril to anterior of right eye; (d) anterior of left eye to anterior of right eye; (e) eye diameter; (f) anterior left eye to posterior right eye; (g) posterior of left eye to posterior of right eye; (h) posterior of left eye to origin of left pectoral fin; (i) posterior of left eye to origin of right pectoral fin; (j) origin of dorsal fin to end of dorsal fin; (k) origin of left pectoral fin to origin of right pectoral fin; and (sl) standard length.

Data analysis for morphological study. The data obtained from morphometric measurement were standardized for size according to Turan et al (2004) using formula:

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

Where M_{adj} was the size-adjusted measurement, M was the original measurement, L_s was the overall mean of standard length for all fish from all samples in each analysis, L_o was the standard length of fish and b was estimated for each character from the observed data as the slope of the regression of $\log M$ on $\log L_o$ which was used on all fish in all groups.

This standardization was done due to different size of the specimens which ranged from 94 mm to 556 mm based on their standard length.

The adjusted data was then analyzed using ANOVA (Analysis of Variance) via SPSS 17.0 software where the comparisons of variation among species for each variable were performed. The ANOVA checked whether there were significance differences within and among species for each variable measured.

Next, the resulted data were analyzed by discriminant function analysis (DFA) and principal component analysis (PCA) also via SPSS 17.0 software. The analysis of DFA described the body shape independently of body size and the PCA determined which variables discriminate between two or more naturally-occurring groups.

DNA extraction and PCR amplification. The extraction of DNA from the 35 specimens was done using Wizard[®] Genomic DNA Purification Kit (Promega, USA) according to manufacturer's instructions.

The DNA extraction product was verified via gel electrophoresis using 1% agarose gel with ethidium bromide in 1x TBE buffer at 70 volt for 40 minutes using B2 EasyCast Mini Gel System (Thermo Scientific Owl, USA). Good genomic DNA was seen as a sharp band while the degraded DNA or contamination was seen as a smear band. The DNA amplification or PCR (Polymerase Chain Reaction) was done using purified DNA obtained from the DNA extraction. The mtDNA 16S gene was amplified using primer 16Sar with sequence 5'-CCGGTCTGAACTCAGATCACGT-3' and 16Sbr with sequence 5'-CGCCTGTTTATCAAAAACAT-3' (Palumbi 1996). All PCR preparation was done in PCR workstation to avoid contamination. The master mix was prepared and aliquots contained 6.2 μ L distilled water, 1.5 μ L 25mM $MgCl_2$, 1.5 μ L 10x Taq Buffer with $(NH_4)_2SO_4$ and 3 μ L 10mM dNTP mix. After that, 1 μ L of each primer was added into the tube. Then, 3 μ L of extracted DNA and 0.5 μ L Taq DNA polymerase were added. PCR was carried out in the MiniOpticon Real-Time PCR System (BioRad, USA) with initial denaturation at 95°C for 2 minutes, 35 cycles of denaturation at 95°C for 30 second, annealing at 38°C for 40 second and extension at 72°C for 1 minute and final extension at 72°C for 5 minutes.

The success of PCR process was confirmed by verification of PCR product via gel electrophoresis using 2% agarose gel with ethidium bromide in 1x TBE buffer at 100 volt for 90 minutes using B2 EasyCast Mini Gel System (Thermo Scientific Owl, USA).

PCR purification. The PCR purification process was done using QIAquick PCR Purification Kit (QIAGEN, Germany) according to manufacturer's protocols. The purified PCR product was confirmed using 2% agarose gel with ethidium bromide in 1x TBE buffer. Then, the PCR product was sent for sequencing.

Data analysis for phylogenetic study. The sequencing results were edited and analysed using MEGA 4.0 (Tamura et al 2007). The phylogenetic tree was constructed based on Maximum-Parsimony method with 1000 bootstrapping.

Results

Descriptive analysis. Morphometric data on side view show that different variables can be used to describe different species of snakeheads. Variables a, b, e, h, m, n, o, p, q and r mainly described *C. micropeltes*. While variables c and d described *C. striata*, variables f and g described *C. lucius* and variables i, j, k, and l was *C. maruloides*. For descriptive analysis of the eleven dorsal view measurements the highest mean obtained

for variables a, c and j was for *C. lucius*, variables b, d, h, and i mainly described *C. micropeltes* and variables e, f, g, and k mainly described *C. maruloides*.

ANOVA. The ANOVA was used to analyze differences among species. The result obtained show that, there were significant 95% confidence levels among the four species except for variables e and h (above eye to origin of pectoral fin and origin of dorsal fin to origin of pectoral fin) for side view measurement and variables d, e, and j (anterior of left eye to anterior of right eye; eye diameter; and origin of left pectoral fin to origin of right pectoral fin) for dorsal view measurement.

The Student Newman Keuls (SNK) test was used to identify the homogenous subsets in the measurement of side and dorsal view. Figure 3 and 4 shows the summary of SNK test of side and dorsal view measurement. The summary of SNK tests on side view measurement show that three of the variables generated discrete subsets (4) for each species which were variables m, o and p. There were four variables, b, n, q and r which generated three subsets and the other were only two subsets and some of them with overlapping among the species. For the summary of SNK test on dorsal view measurement show that only one of the variables generated four discrete subsets for each species which was variable b. The other variables, g and h, generated 2 discrete subsets and variables a and i generated overlapping subsets.

Variables	Species	SNK	Variables	Species	SNK
a	<i>C. striata</i> <i>C. maruloides</i> <i>C. lucius</i> <i>C. micropeltes</i>	1 2	j	<i>C. striata</i> <i>C. lucius</i> <i>C. micropeltes</i> <i>C. maruloides</i>	1 2
b	<i>C. maruloides</i> <i>C. lucius</i> <i>C. striata</i> <i>C. micropeltes</i>	1 2 3	k and l	<i>C. micropeltes</i> <i>C. striata</i> <i>C. lucius</i> <i>C. maruloides</i>	1 2
c	<i>C. lucius</i> <i>C. maruloides</i> <i>C. micropeltes</i> <i>C. striata</i>	1 2	m, o, and p	<i>C. maruloides</i> <i>C. striata</i> <i>C. lucius</i> <i>C. micropeltes</i>	1 2 3 4
d	<i>C. micropeltes</i> <i>C. maruloides</i> <i>C. micropeltes</i> <i>C. striata</i>	1 2 3	n	<i>C. maruloides</i> <i>C. striata</i> <i>C. lucius</i> <i>C. micropeltes</i>	1 2 3
f	<i>C. micropeltes</i> <i>C. maruloides</i> <i>C. striata</i> <i>C. lucius</i>	1 2	q	<i>C. maruloides</i> <i>C. lucius</i> <i>C. striata</i> <i>C. micropeltes</i>	1 2 3
g	<i>C. striata</i> <i>C. maruloides</i> <i>C. micropeltes</i> <i>C. lucius</i>	1 2	r	<i>C. maruloides</i> <i>C. striata</i> <i>C. lucius</i> <i>C. micropeltes</i>	1 2 3

Figure 3. Summary of SNK test of side view measurement.

Variables	Species	SNK
a	<i>C. lucius</i> <i>C. micropeltes</i> <i>C. striata</i> <i>C. marulioides</i>	1 2
b	<i>C. striata</i> <i>C. marulioides</i> <i>C. lucius</i> <i>C. micropeltes</i>	1 2 3 4
g	<i>C. lucius</i> <i>C. striata</i> <i>C. micropeltes</i> <i>C. marulioides</i>	1 2
h	<i>C. marulioides</i> <i>C. lucius</i> <i>C. striata</i> <i>C. micropeltes</i>	1 2
i	<i>C. marulioides</i> <i>C. lucius</i> <i>C. striata</i> <i>C. micropeltes</i>	1 2

Figure 4. Summary of SNK test of dorsal view measurement.

Principal Component Analysis. The Principal Component Analysis (PCA) was used to determine which variables are discriminated between two or more groups. In this analysis, the first five components had total value more than one. Component 1 shows an eigenvalue of 6.752, which explain 37.513% of total variance. Component 2 show an eigenvalue of 3.243 which explain 18.015% of total variance. Component 3 show an eigenvalue of 2.643 which explain 14.683% of total variance, while component 4 show an eigenvalue of 1.313 that explain 7.295% of total variance and component 5 show an eigenvalue of 1.167 which explain 6.483% of total variance. These explained the total variance of 83.989% among the individuals. Then, the rotation matrix using varimax rotation was obtained to review the correlation of variables to all the principal components. The results obtained give the summary of weightings assigned to each of the variables for the extracted components. Component 1 from the table point out that high positive weighting for variables a, m, n, o, p, q, and r, while component 2 points out that the high positive weighting were for variables i, j, and k. For component 3, the high positive weightings were for variables b, c, and e. For component 4, the high positive weighting for variables d, f, and h, and component 5 points out that the high positive weighting for variables e and g.

The eigenvalues, percentage of variance explained and percentage of cumulative values for the dorsal view measurement show that the first four components had a total value more than one. Component 1 showed an eigenvalue of 2.97 that explained 26.96% of the total variance. Component 2 showed an eigenvalue of 2.48 which explained 22.56% of the total variance while component 3 was with value 1.47 which explained 13.34% of total variance, and component 4 with eigenvalue of 1.03 which explain 9.39% of total variance. The four components explained 72.25% of the total variance among the individuals. Then, the rotated matrix was performed to assess the correlation of variables to each principal component.

The rotated component matrix of the dorsal view measurement, the component 1 showed (size variation) the high positive weighting for variables d, f, and g. Component 2 show high positive weighting for variables h and i. Component 3 showed the high positive weighting for variables b and component 4 was variables j and k.

By plotting a scatter plot of individuals' component score between component 1 and component 2 of both side and dorsal view measurement (Figure 5 and 6), the PCA result can be viewed obviously. For side view measurement which was in Figure 5, the four *Channa* species seem to group into their own group which was obvious in *C. striata*. They formed an individual species cluster but were closely related. *C. marulioides* seem to distance away from the major group as well as *C. micropeltes*. For dorsal view measurement, the four *Channa* species seem scattered among them in a cluster group. *C. striata*, *C. micropeltes* and *C. lucius* were clustered in one place while *C. marulioides* seem to form their own group away from the rest of species.

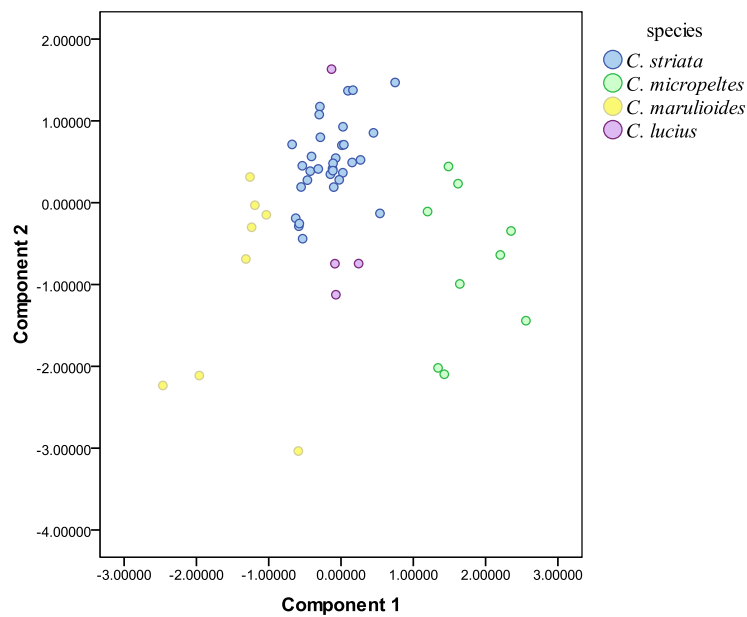


Figure 5. Scatter plot of individuals component score between component 1 and component 2 for side view measurement.

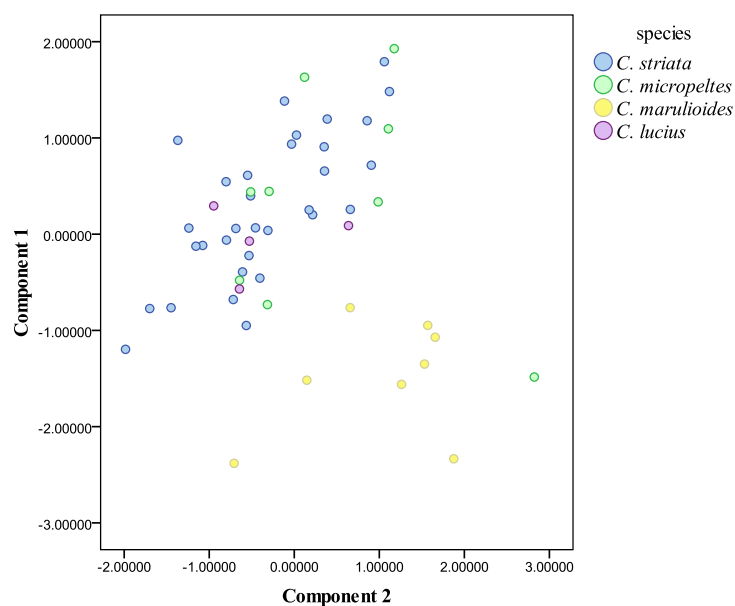


Figure 6. Scatter plot of individuals component score between component 1 and component 2 for dorsal view.

Discriminant Function Analysis. The Discriminant Function Analysis (DFA) was used to summarize the large number of variables into less-derived variables of function. The eigenvalue, percentage of variance, cumulative percentage and canonical correlation for side view measurement are shown in Table 2 and Table 3 for the dorsal view measurement. Table 2 present three functions which were function 1 with 6.21 eigenvalue which explained 46.05% of the total variance, function 2 with 6.07 eigenvalue which explained 45.02% of total variance, and function 3 with 1.20 eigenvalue that explained 8.93% of total variance.

Table 2

Eigen values, percentage of variance, cumulative advantage and canonical correlation for eighteen side view characters

<i>Eigenvalues</i>				
<i>Function</i>	<i>Eigenvalue</i>	<i>% of variance</i>	<i>Cumulative %</i>	<i>Canonical correlation</i>
1	6.21 ^a	46.05	46.05	.93
2	6.07 ^a	45.02	91.07	.93
3	1.20 ^a	8.93	100.00	.74

x^a - first 3 canonical discriminant functions were used in the analysis.

In Table 3, three functions are also present which is function 1 with 20.64 eigenvalue that explained 88.98% of total variance, function 2 with 2.38 eigenvalue which explained 10.26% of total variance and function 3 with 0.18 eigenvalue which explained 0.75% of total variance.

Table 3

Eigen values, percentage of variance, cumulative percentage and canonical correlation for eleven dorsal view characters

<i>Eigenvalues</i>				
<i>Function</i>	<i>Eigenvalue</i>	<i>% of variance</i>	<i>Cumulative %</i>	<i>Canonical correlation</i>
1	20.64 ^a	88.98	88.98	.98
2	2.38 ^a	10.26	99.25	.84
3	.18 ^a	0.75	100.00	.39

x^a - first 3 canonical discriminant functions were used in the analysis.

The results of pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions of side and dorsal view measurement shows that for side view measurement, the discriminatory variables for Function 1 were c, and b which consist of above eye to the mouth. For Function 2, the discriminatory variable was variable which consist from end of dorsal fin to the lower part of origin of caudal fin. Then, for Function 3, the discriminatory variables were d and g which measured the length from above eye to origin of dorsal fin and length of end of mouth to the origin of pectoral fin.

For dorsal view measurement, the discriminatory variable for Function 1 was variable b which was the measurement of left nostril to anterior of left eye. For Function 2, the discriminatory variables were e and i which consist of length of eye diameter and posterior of left eye to the origin of right pectoral fin. Discriminatory variables for Function 3 were g, h, and k. Those variables consist of distance of posterior of left eye to posterior of right eye, posterior of left eye to origin of left pectoral fin and also length of origin of pectoral fin to upper part origin of caudal fin.

The results of discriminant function analysis can be shown more clearly by plotting a distribution graph of individuals' discriminant score within Function 1 and Function 2 as in Figure 7 and 8. In both figures, the four *Channa* species form four distinctive groups. For side view the groups can be differentiated along both functions while for dorsal view the groups can be differentiated more along Function 1.

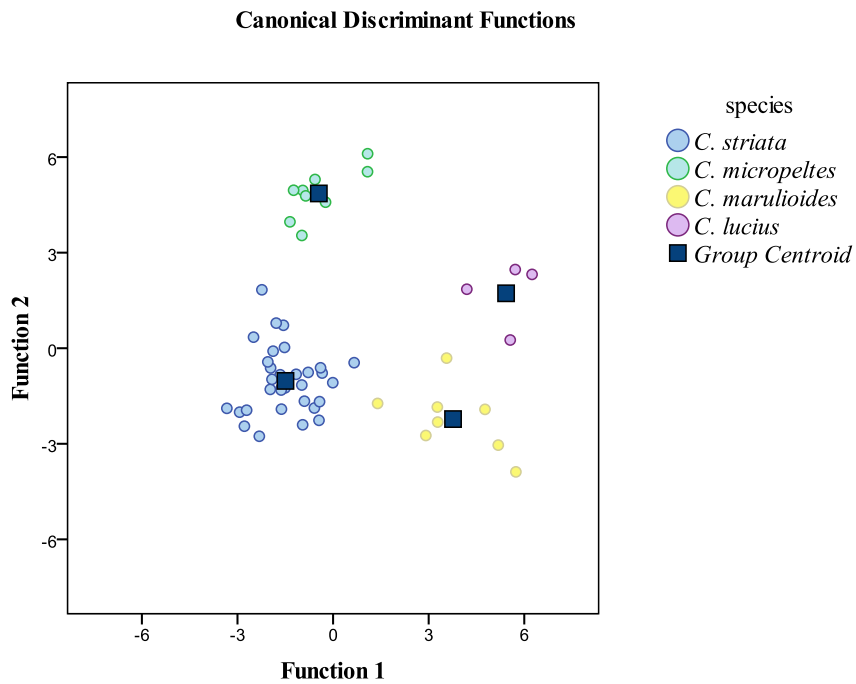


Figure 7. Canonical discriminant functions for side view.

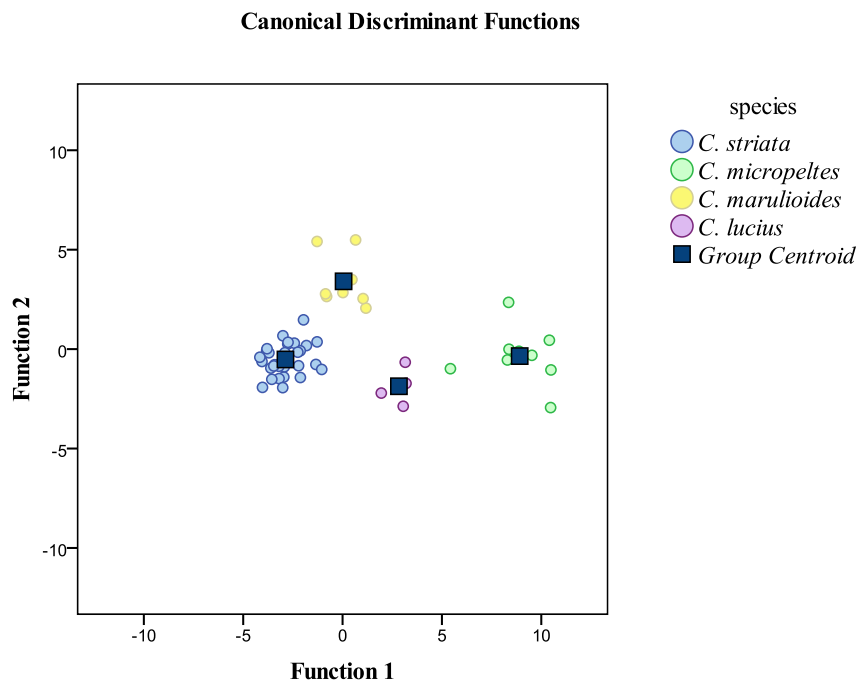


Figure 8. Canonical discriminant function for dorsal view.

The classification results for both side and dorsal view measurement are presented in Table 4 and Table 5. In cross validation, each case is classified by the functions derived from all cases other than that case. In Table 4, 100.0% of original grouped cases were correctly classified and 100.0% of cross-validated grouped cases were correctly classified. These show that all the individuals were grouped successfully in their respective species. In Table 5, 88.9% of original grouped cases were correctly classified

based on dorsal view measurements and even after cross-validation, the same percentage remains. These show that not all the individuals were successfully grouped in their respective species after cross validation.

Table 4

Classification results of predicted group membership for side view

		<i>Species</i>	<i>Predicted group membership</i>				<i>Total</i>
			<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	
Original	Count	1	32	0	0	0	32
		2	0	9	0	0	9
		3	0	0	8	0	8
		4	0	0	0	4	4
	%	1	100.0	.0	.0	.0	100.0
		2	.0	100.0	.0	.0	100.0
		3	.0	.0	100.0	.0	100.0
		4	.0	.0	.0	100.0	100.0
Cross-validated ^a	Count	1	32	0	0	0	32
		2	0	9	0	0	9
		3	0	0	8	0	8
		4	0	0	0	4	4
	%	1	100.0	.0	.0	.0	100.0
		2	.0	100.0	.0	.0	100.0
		3	.0	.0	100.0	.0	100.0
		4	.0	.0	.0	100.0	100.0

Table 5

Classification results of predicted group membership for dorsal view

		<i>Species</i>	<i>Predicted group membership</i>				<i>Total</i>
			<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	
Original	Count	1	32	0	0	0	32
		2	0	8	0	1	9
		3	0	0	8	0	8
		4	0	0	0	4	4
	%	1	100.0	.0	.0	.0	100.0
		2	.0	88.9	.0	11.1	100.0
		3	.0	.0	100.0	.0	100.0
		4	.0	.0	.0	100.0	100.0
Cross-validated ^a	Count	1	32	0	0	0	32
		2	0	8	0	1	9
		3	0	0	8	0	8
		4	0	0	0	4	4
	%	1	100.0	.0	.0	.0	100.0
		2	.0	88.9	.0	11.1	100.0
		3	.0	.0	100.0	.0	100.0
		4	.0	.0	.0	100.0	100.0

The result of pairwise group comparisons on F-statistics for side view measurements are shown in Table 6 and in Table 7 for dorsal view measurement using 45 degrees of freedom. These F-statistics can describe the similarity or differences among the group. All the group results show a significant value which indicate that all groups were significantly different from each other.

Table 6

Pairwise group comparisons for side view measurement

Species		<i>C. striata</i>	<i>C. micropeltes</i>	<i>C. marulioides</i>	<i>C. lucius</i>
<i>C. striata</i>	F		45.697	24.203	35.888
	Sig.		.000	.000	.000
<i>C. micropeltes</i>	F	45.697		49.499	28.221
	Sig.	.000		.000	.000
<i>C. marulioides</i>	F	24.203	49.499		17.610
	Sig.	.000	.000		.000
<i>C. lucius</i>	F	35.888	28.221	17.610	
	Sig.	.000	.000	.000	

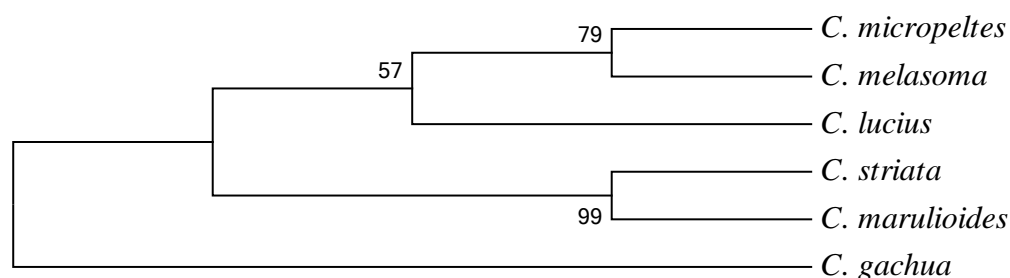
Table 7

Pairwise group comparisons for dorsal view measurement

Species		<i>C. striata</i>	<i>C. micropeltes</i>	<i>C. marulioides</i>	<i>C. lucius</i>
<i>C. striata</i>	F		173.254	21.017	23.785
	Sig.		.000	.000	.000
<i>C. micropeltes</i>	F	173.254		72.254	18.652
	Sig.	.000		.000	.000
<i>C. marulioides</i>	F	21.017	72.254		14.668
	Sig.	.000	.000		.000
<i>C. lucius</i>	F	23.785	18.652	14.668	
	Sig.	.000	.000	.000	

Phylogenetic study

Molecular phylogeny of snakeheads species in Peninsular Malaysia. The phylogenetic results were obtained from analysis using MEGA 4.0 software. There are six species for this phylogenetic study. Figure 9 shows the phylogenetics tree of the six *Channa* species produced from sequence data obtained from phylogenetic project. This tree was produced by Bootstrap test of phylogeny analysis using 1000 replication with maximum-parsimony method in MEGA 4.0. The values were shown only when they were more than 50%. From the phylogenetics tree, it was found that *C. gachua* formed the basal taxon for the *Channa* species. *C. micropeltes*, *C. melasoma* and *C. lucius* and also *C. striata* and *C. marulioides* had formed the sister taxon.

Figure 9. Phylogenetics of six *Channa* species.

Then, the species were compared with sequence data of the same species from the GenBank. The phylogenetics in Figure 10 was produced by Maximum Parsimony method in MEGA 4.0 using sequence data from this study and GenBank. Both sequences of the same species from this study and GenBank were grouped into their respective group.

The species from GenBank were *C. striata* (GenBank) (accession no. EU342186.1), *C. micropeltes* (GenBank) (accession no. DQ532852.1), and *C. gachua* (GenBank) (accession no. EU342179.1) amplified the same gene 16S. The sequence of *Anabas*

testudineus was used as an outgroup which was from GenBank (accession no. EF179145.1) and also used 16S gene which was located isolated from all other species.

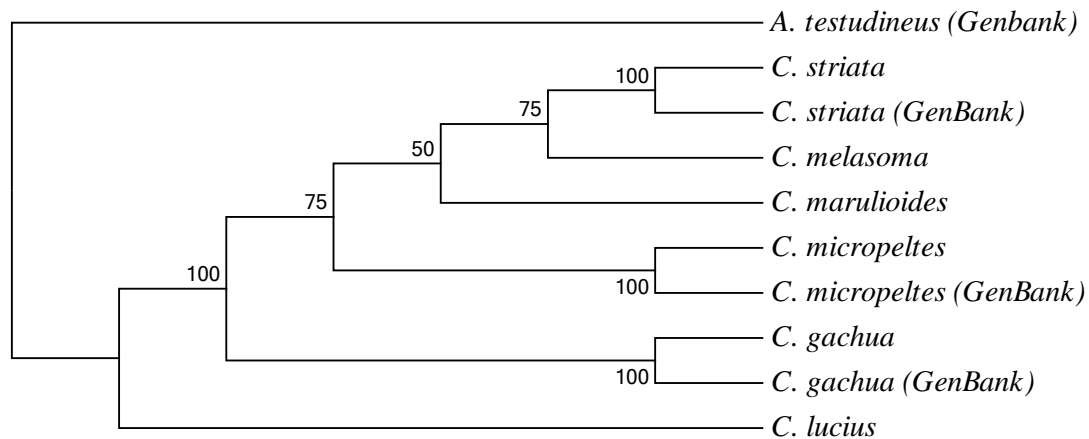


Figure 10. Phylogenetics of six *Channa* species with outgroup and GenBank data using Maximum Parsimony.

Discussion. In the morphometric study, the results obtained from measuring all the eighteen variables of side view and eleven variables of dorsal view of the snakeheads species samples were needed to be standardized to remove the size-dependent variation effects (Turan et al 2004). This was due to the samples were range from 94 mm to 556 mm in standard length.

From the analysis of one-way ANOVA, it was found that both measurements were significant but there were two variables of side view measurement and three variables of dorsal view measurement that was not significant. Variables e, and h of the side view were found not significant. In side view measurement, the variable that was not significant were variable e which was measurement of above eye to origin of pectoral fin, variable h which was measurement of origin of dorsal fin to origin of pectoral fin. In the dorsal view measurement, the three variables are variable d which was anterior of left eye to anterior of right eye, variable e which was anterior to posterior eye or eye diameter, and variable j which was origin of left pectoral fin to origin of right pectoral fin. According to the previous study by Norainy (2007) on morphometric of *Channa* species, the analysis of one-way ANOVA shows that the side and dorsal measurement were significant for all variables which denote that at least one species was significantly different. Even though there were some variable were not significant, the majority of the variable of both side views were significant which can suggest that each species of the four species were different from one another. The not significant result obtained might be due to imprecision during measurement of the samples. The measurement taken was not 0.05 mm precise as was done in previous study (Heras et al 2006).

The principal component analysis (PCA) showed that there are contributions of the side and dorsal view variables for snakeheads size and shape variation. For these four *Channa* species, the side view measurement was scattered fairly along the both component and also for the dorsal view measurement. Compared to previous study by Norainy (2007), there are variability for size among individuals on the entire dorsal side view while on the side view, most of the variables were loaded heavily on the first component. These suggest that in this study, both of the side and dorsal view showed variability for size among individuals. Turan et al (2009) also used principal component analysis to examine the involvements of every variable measured that indicate which variables were crucial for species differentiation. As for this study, variables a, which was in the head region and m, n, o, p, q, and r which were in caudal peduncle region on the side view measurement were crucial in species identification. On the dorsal view, variable d, e, f, g, h, and I, which were all from the head region, were crucial in this *Channa* species identification.

The discriminant functions analysis revealed that the side view and dorsal view measurement of the four *Channa* species can assemble them in different group. According to Norainy (2007), this multivariate analysis was useful in correlating species compared to use of single characteristics which was less efficient. The relationship among individuals of the studied group in Turan et al (2004) was viewed using discriminant function analysis. The comparison of PCA and DFA analysis, can define the quantitative morphological differentiation among the species and also the structures of fish which were different among the species. This indicated that grouping of all four *Channa* species along both Function 1 and 2 were because of the similarities in the head region and also in caudal peduncle region which was the same with the finding by Norainy (2007). Truss network morphometric has been able to discriminate two populations and sexes of *Alburnus chalcoides*, a freshwater fish from Black river systems of Black, Aral and Caspian Seas (Bagherian & Rahmani 2009). Analysis shows that the method able to differentiate sexual dimorphism where the females and males from both populations were grouped together and the females show larger abdomen. Populations from the two populations have different anterior and caudal peduncle due to adaptation to different river systems.

In the phylogenetic study, the phylogram of six *Channa* species reveals that all the species were grouped accordingly to their own group with very high bootstrap value. The *A. testudineus* which is a freshwater fish from another genus was used as an outgroup to root the tree for *Channa* species. *C. gachua* form the basal taxon for the *Channa* species which was the same as previous study on genetic variability of *Channa* spp using RAPD technique (Norainy 2007). The study found that *C. striata* and *C. lucius* formed a sister taxon and also *C. micropeltes* and *C. melasoma*. These were different from this study where *C. striata* formed a sister taxon with *C. marulioides* and *C. micropeltes* formed sister taxon with *C. melasoma* and *C. lucius*. These differences might be due to different type of markers used in both studies. It may be due to different evolutionary histories of the nuclear DNA genes and mtDNA, different rate of variation among either genes, or different number of samples (Mayden et al 2009). Besides that, mtDNA differ from nuclear DNA as it has only a single copy which make it much easier in sequencing, was less correlated with polyploidy and paralogy, have moderately short generation time and also lack of recombination (Mayden et al 2009). In some species studied, variation exists in the individual gene tree of nuclear DNA and mtDNA (Mayden et al 2009). They suggested that addition of large taxon sampling will minimize the variation.

Recent phylogenetic study on existing data of *cox1* from gene bank show that some of the species on the genebank have been wrongly identified and confusion on the correct identification of snakehead species still exist (Conte-Grand et al 2017). There are even mixed up identification of *Channa* sp with *Rasbora* sp. This show the need for further study on snakehead species, particularly study that combine both morphological and genetics approaches. New suggested classification of *C. gachua* to *C. limabata* for eastern lineage shows there is continuous need for research on *Channa* species as there are indications that the existing species may actually consist of more than 38 species (Conte-Grand et al 2017). Combination of morphological and genetic approaches is very useful in managing fisheries resources, especially endangered resources where the planning and exploitation of the species is delicate, such as research on *Tenualosa* sp (Arjunaidi et al 2016). As their habitat decrease due to land development which converting the habitat into plantation and housing area, certain snakehead species may be facing localized extinction. Research on the snakeheads should be done on a larger geographical scale to get a better picture of its morphology and phylogenetic differences and resolve the intra-species complex.

Conclusions. This study has successfully identified and determined the phylogeny of the snakehead species which were *C. striata*, *C. micropeltes*, *C. marulioides*, *C. lucius*, *C. melasoma*, and *C. gachua* that can be found in Peninsular Malaysia using truss-network morphometric and mtDNA techniques. Further research looking at intra-species

complexes using both morphometric and genetic approaches will give useful insight into this interesting air-breathing fish.

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