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RESEARCH ARTICLE

Deciphering species-group taxonomic complexity of common, *Barbodes binotatus* and saddle barbs, *B. banksi* in Peninsular Malaysia

Muhammad Fahmi Ahmad [a], Evia Chan Mei Theng [a], Siti Azizah Mohd Nor [b], Amirrudin Ahmad [a,c,*]

[a]*Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu*
[b]*Marine Biotechnology Institute, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu*
[c]*Institute of Tropical Biodiversity and Sustainable Development, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu*

* *Corresponding author: amirrudin@umt.edu.my*

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Abstract

Correct assessment of freshwater fishes depends on the most recent taxonomic nomenclature and accurate identification. The existing of the complex between closely related species is one of the prominent issues that sometimes poorly addressed that leads to misidentification of the species and inaccurate diversity measurement. This study addressed the issue through morphometrics methods, to correctly evaluate the diversity of freshwater fish within species group of genus *Barbodes*, i.e., *B. binotatus* and *B. banksi*. In examining species complex of *B. binotatus* and *B. banksi*, traditional and landmark-based geometric morphometrics methods were applied. Traditional morphometric displayed characters that distinguished both species were generally located at the anterior part of the body, and specifically in the cephalic area of the fish. Landmark-based geometric morphometric revealed a highly similar body shape on both species. Variations within both species were subtle and could not be significantly distinguished by both methods. The overall outcomes of this study suggest that in order to achieve a proper assessment to freshwater fishes, one need to solve the identification problems in species complex. This effort could consequently lead to a better understanding of freshwater fish status, for a better conservation plan and management.

Keywords: Geometric morphometric, shape variation, species complex, species diversity, traditional morphometric

INTRODUCTION

The dynamism of life involved wide variety of taxa with species complex such as Amazonian tree frogs of genus *Hypsiboas* (Caminer and Ron, 2014) and Midas cichlid fish (Barluenga and Meyer, 2010). Species complex can be defined as a close but distinct group of species (Couldridge and Alexander, 2002). The grouping is considered complex when the boundaries in defining and discerning a species to closely related species could not be confidently established. Recognizing species complex is a prelude to delimit a species before dealing with the complexities of morphological and morphometrics differences plus molecular data as the vital supportive materials which will change the preceding view on the species (Davis *et al*., 2016; Muñoz *et al*., 2013).

Peninsular Malaysia has two freshwater fish species commonly known as common barb and saddle barb, scientifically known as *Barbodes binotatus* (Valenciennes, in Cuvier and Valenciennes) and *Barbodes banksi* (Herre), respectively. Previously, these two species were placed under genus *Puntius* Hamilton (see Kottelat *et al*., 1993) and *Systomus* McClelland (see Rainboth, 1996). In fish, distinct markings are often used as taxonomic characteristics to diagnose the fish species (Kottelat, 2013; Pethiyagoda *et al*., 2012; Stevens, 2005). Genus *Barbodes* Bleeker has a prominent character that the body color pattern changes ontogenically (Kottelat, 2013). This character was used to distinguish them from other genera formerly assigned in *Puntius* in Southeast Asia (Kottelat, 2013).

Previously, Herre (1940) listed *B. binotatus* as *Puntius binotatus* and *B. banksi* as its subspecies named as *Puntius binotatus banksi* from Kuching, Sarawak, Malaysia. Later, both *B. binotatus* and *B. banksi* were considered as two distinct species by Kottelat and Lim (1996). Both species can be distinguished by the marking found just under the dorsal fin. *B. binotatus* has a round spot under its dorsal fin while *B. banksi* has a dark wedge-shaped marking under its dorsal fin; as a key to differentiate the two species (Ng and Tan, 1999). *B. binotatus* was originally described from Java, Indonesia and *B. banksi* was from Kuching, Sarawak, Malaysia.

However, it is also found that there are some variations of both *Barbodes* spp. populations. Variation in the two species has created individuals with markings that look similar to the other species or markings that difficult to be positively assigned to any species that leads to the uncertainties in recognizing both species. This has caused a great confusion and misidentification of the two species and perhaps it forms a species-complex of more species than it was previously expected. This led to Ng and Tan (1999) suggesting that both *B. binotatus* and *B. banksi* are likely to be two color forms of a single species because of their similarities.

There is also an issue on the status of *B. binotatus* that occurred in the Peninsular Malaysia. According to Kottelat (2000), the actual and valid *B. binotatus* (*Puntius binotatus*) is restricted to Java, Bali, Lombok, and highlands of Sumatera in Indonesia. The specimens that were referred to as *B. binotatus* (*P. binotatus*) from other parts of Southeast Asia were tentatively belong to different species and generally known as "*B. binotatus* (*P. binotatus*) group" (Kottelat, 2000). *B. banksi* is one of the members of *B. binotatus* group which previously thought as a synonym of *B. binotatus* (Roberts, 1989). This nominal species has been considered as valid species despite the lack of a complete taxonomic revision to confirm this (Ng and Tan, 1999). Besides the distinguish pattern on its body, it is understood to have clear geographical distribution in comparison to other species in the group (Kottelat and Lim, 1996).

Shape variation has been quantitatively analyzed using morphometrics method to disentangle queries in biology that are caused by evolutionary, pathology, ontogenetic development, phenotypic plasticity, and interspecific hybridization in many organisms (Monti *et al*., 2001; Zelditch *et al*., 2004). Morphometrics is the study of shape variation and its covariation with other variables (Webster and Sheets, 2010). This analysis is divided into two approaches – traditional morphometrics and geometric morphometrics. Traditional morphometrics uses the relationships between the distance of the body parts in the form of length measurements, ratios, or angles, to describe the shape variation among and within groups (Webster and Sheets, 2010). Some of the limitations in traditional morphometric is it did not contain information on spatial distribution of shape changes across organism and it is not possible to depicted graphical presentation because geometric relationships among the variables were not retained (Bo *et al*., 2014). Geometric morphometrics, on the other hand, are the quantitative representation and analysis of morphological shape using geometrical coordinates, either in two or three dimensions, instead of measurement of distances (Kerschbaumer and Sturmbauer, 2011). Landmark-based geometric morphometrics uses a set of biologically important and homogenously distributed landmarks on the organism's body as points to create an image of the organism virtually (Rohlf and Marcus, 1993; Zelditch *et al*., 2004).

Geometric morphometric is considered to be more efficient compared to traditional morphometrics in explaining variations in shape between and within population. This advantage is from its ability to enhance interpretation by increasing the statistical power and allowed direct visualization of shape transformation of the organisms (Franssen *et al*., 2013; Nacua *et al*., 2012; Rohlf and Marcus, 1993). Biologist had demonstrated that geometric morphometrics was the best tools in extricating them from taxonomic problems such as in species complex in fish (Kerschbaumer and Strumbauer, 2011), interspecific hybridization (Conte-Grand *et al*., 2015), relationship between fish ecomorphology and body shape (Bower and Piller, 2015), sexual dimorphism in body shapes of fish (Dorado *et al*., 2012), or colonization and evolution of cryptic fish (Bichuette *et al*., 2015). Gunawickrama and Damayanthi (2012) uses traditional morphometric to confirm the species level divergence between *Puntius dorsalis* (Pisces: Cyprinidae) from its presumed red-fin variety in Sri Lanka. Manimegalai *et al*. (2010) used traditional morphometric as a tool to identify different variants in *Etroplus maculatus*, an endemic fish species in India. Some studies combined both methods such as in Maderbacher *et al*. (2008), to discriminate population of *Tropheus moorii* fish species complex in southern part of Lake Tanganyika in Africa. Maderbacher *et al*. (2008) found that traditional morphometric was less flexible and less powerful statistically in discriminating the closely related species compared to geometric morphometrics method.

Confusion and misidentification between species of *B. binotatus* and *B. banksi* have led to many taxonomic perplexities. Therefore, the usage of additional methods to distinguish between the two species is important for taxonomic identification of species and for conservation purposes. Thus, this research focus on the application of traditional and geometric morphometrics, to distinguish body shape variation between *B. binotatus* and *B. banksi* plus any intermediate forms of the two species occurred in Peninsular Malaysia as an effort to elucidate the status of the fish in question.

EXPERIMENTAL

Sample and data collection

Both preserved specimens and freshly collected samples of *B. binotatus* and *B. banksi* were used in this study. Preserved specimens deposited in the ichthyological collection at Universiti Malaysia Terengganu (UMT), together with fresh samples caught from freshwater streams in a few localities throughout Peninsular Malaysia (Figure 1) were closely examined. Specimens were identified according to the currently understood morphological differences to separate them into two species of which is *B. binotatus* and *B. banksi* with the help of various taxonomic keys. Within the two *Barbodes* species, we further grouped them into three species-groups to identify the intra-species variants that account for their morphological differences and the variants identified were then examined separately. The descriptions of each variant and the total number of individuals in each variant were also noted.

Traditional morphometric

Measurements were taken using hand-held electronic digital calliper for each individual to the nearest 0.1 mm. Eleven outline measurements were made on each specimen (Figure 2) plus the other four morphometric measurements (not shown in the figure). The four measurements were standard length (SL), head length (HL), pectoral fin base length (PBL), and horizontal eye diameter (ED). Errors in observation and instruments were avoided by appointing the same person to conduct the measurement and using the same calliper throughout the measurements process (Hayek et al., 2001).

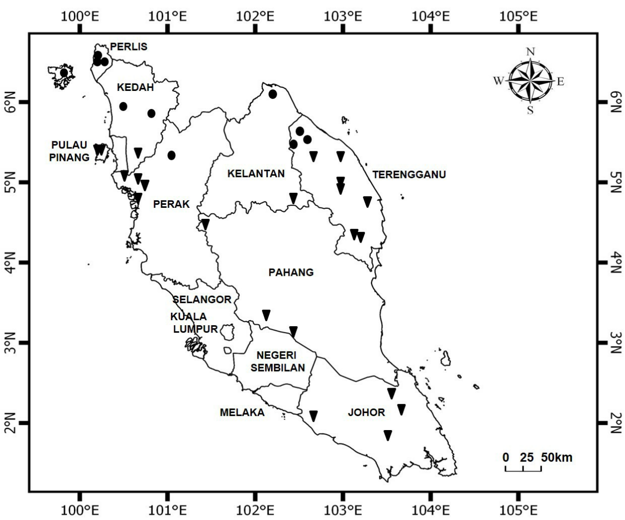


Fig. 1 Map showing the sources of the specimens in Peninsular Malaysia. Filled circles indicated location for *B. binotatus*, inverted filled triangles indicated location for *B. banksi*.

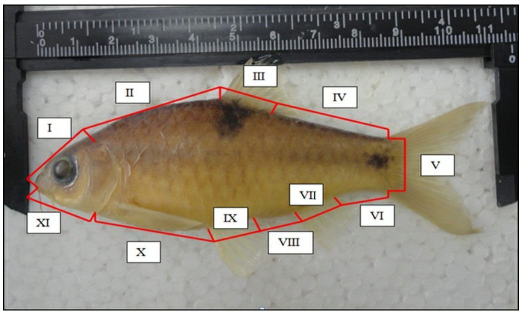


Fig. 2 Outline measurements of the *Barbodes* specimen. (I) from the tip of the snout to the upper end of the operculum; (II) from end of the operculum to the origin of the dorsal fin; (III) dorsal fin base length; (IV) from the end of dorsal fin to the origin of the caudal fin; (V) upper to lower of caudal fin origin; (VI) origin of caudal fin to the end of anal fin; (VII) anal fin base length; (VIII) from the end of pelvic fin to the origin of anal fin; (IX) pelvic fin base length; (X) from the end of operculum to the origin of pelvic fin and (XI) from the tip of the snout to the lower end of the operculum.

Prior to the analysis, the variation caused by the allometric growth were first remove by transforming each of the characters measured (Reist, 1985). All morphometric measurements were standardized by using an allometric formula as suggested by Elliot *et al*. (1995): $M_{adj} = M(L_s/L_o)^b$, where M_{adj} is the size adjusted measurement; M is the original measurement; L_s is the overall mean of standard component (standard length or head length); L_o is the standard length or head length of the fish. For each morphometric character, parameter b was estimated as the slope of the regression of log M on log L_o using all fish from every group. Prior to further analysis, the data were transformed accordingly. The transformed data were subjected to IBM SPSS Statistics for Windows, version 23 for Independent Samples t-Test and one-way analysis of variance (ANOVA). Independent Samples t-Test was used to examine the difference for each character between populations of *B. binotatus* and *B. banksi*. The one-way ANOVA and subsequent post-hoc test (Tukey test) were applied to identifying the significant variables between variations within each population. After that, Principal Component Analysis (PCA) was done on the size adjusted measurements to assess inter- and intraspecies variation between *B. binotatus* and *B. banksi*, using the Paleontological Statistics (PAST) software version 1.27 (Hammer et al., 2001).

Geometric morphometric

Landmark-based geometric morphometric was applied in this study to represent the morphology of the fish in term of coordinates of a set of landmark points (Webster and Sheets, 2010). Landmark points were selected and marked with pins on the outline of the specimens. Digital images of the left side of each specimen was taken and loaded to computer for landmark digitization. Twenty landmarks as in Figure 3 were digitized using tpsDig version 2.0 (Rohlf, 2006) into geometric coordinates of x and y.

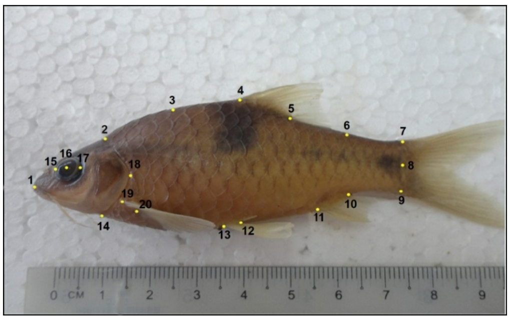


Fig. 3 Landmarks point on the left side of the lateral view of the fish.

Before further analyses, non-shape variations from position, orientation, and scale between specimens were removed using General Procrustes Analysis (GPA) landmark superimposition method. This method also generated a consensus that had the average body shape of the group of specimens. Variables left after the removal were then used to generate weight matrix comprised of partial warp scores and uniform component. Both GPA and the obtained partial warp scores were carried out using tpsRelw version 1.36 (Rohlf, 2003).

The partial warp scores plus uniform component generated from tpsRelw were exported to Microsoft Office Excel 2013 before further analyses of inter- and intraspecific variation of the fish in PAST software. The partial warp scores were used to discriminate shape differences interspecies by using Discriminant Analysis (DA). Hotelling's T-square test was used to compare the equality of the means between the two species. For comparing variation within species, Multivariate Analysis of Variance (MANOVA) was applied on partial warp scores plus uniform. Canonical Variate Analysis (CVA) was executed to examine the difference between pre-defined groups or variation within species based on multivariate shape data.

RESULTS

Variation in *Barbodes binotatus* and *B. banksi* population

A total of 86 individuals of *B. binotatus* and 178 individuals of *B. banksi* were examined in this study. Both species were firstly distinguished by their marking beneath dorsal fin. Individuals of *B. binotatus* has markings that does not extend further than two scales vertically and/or horizontally with markings that does not reach to lateral line. While individuals of *B. banksi* has marking that were extended to three to four scales vertically below dorsal fin base (covering partially or fully the base of dorsal fin) and/or horizontally with markings often reaching the lateral line of the fish.

Three a priori groups or variations were identified from 86 individuals of *B. binotatus* population (Table 1). There were 40 individuals grouped into variation A which was the most common variant, 17 individuals into variation B, and 29 individuals grouped into variation C. *Barbodes banksi* also have three a priori group identified from a total of 178 individuals in (Table 2). Variation A was the most common one with 114 individuals, 23 individuals grouped into variation B, and 41 individuals were in variation C. Variants were however not sympatric.

Table 1 Description of variation of *B. binotatus* (N – Number of individuals).

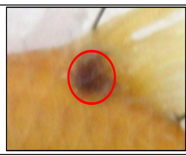
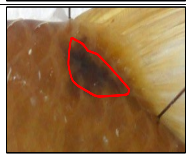
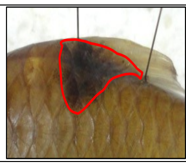
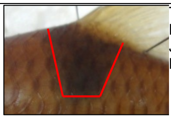
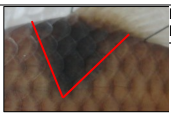
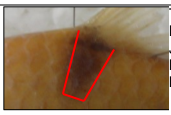
Variation	Blotch shape	Locality	Marking description	N
A		Kedah, Perlis, Perak, Kelantan, Terengganu	Round, typical blotch shape for *B. binotatus*, 1 and a half scales vertically and horizontally	40
B		Perlis, Kedah	Elongated, longer than the typical round blotch, 1 and a half scales vertically and 2 to 3 scales horizontally	17
C		Terengganu, Kelantan	Irregular shape, bigger blotch than the other variations, 2 to 3 scales vertically and horizontally	29
			TOTAL	**86**

Table 2 Description of variation of *B. banksi* (N – Number of individuals).

Variation	Blotch Shape	Locality	Marking description	N
A		Terengganu, Perak, Johor, Pulau Pinang	Wedged-shaped blotch with tapering end, typical marking for *B. banksi*, 3 to 4 scales vertically and 4 to 5 scales horizontally	114
B		Perak, Pahang, Terengganu	Similar to typical blotch except it has sharp end towards lateral line, 3 to 4 scales vertically and 4 to 5 scales horizontally	23
C		Terengganu, Perak, Johor, Pahang, Kedah	More to square than wedge shape, 3 to 4 scales vertically and 1 to 2 and a half scales horizontally, does not extend further than the dorsal fin base	41
			TOTAL	**178**

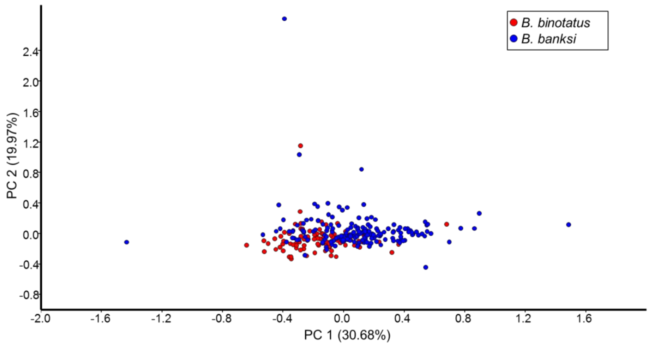


Fig. 4 The scatter plot of principal component analysis (PCA) results from 12 morphometric measurements performed on *B. binotatus* and *B. banksi*.

Traditional morphometric

Of 14 morphometric measurements analyzed (excluding SL), 12 variables showed significant differences between *B. binotatus* and *B. banksi* ($p < 0.05$) (Table 3). The 12 variables were HL (head length), ED (eye diameter), PBL (pectoral fin base length), II (end of the operculum to the origin of the dorsal fin), III (dorsal fin base length), V (upper to lower of caudal fin origin), VI (origin of caudal fin to the end of anal fin), VII (anal fin base length), VIII (end of pelvic fin to the origin of anal fin), IX (pelvic fin base length), X (end of operculum to the origin of pelvic fin) and XI (tip of the snout to the lower end of the operculum). *B. banksi* also reported higher mean for all measurements compared to *B. binotatus*.

Table 3 Mean and standard error (s.e.) of transformed value of morphometric measurements (cm) of *B. binotatus* and *B. banksi* (different superscript letters indicate significant differences).

Morphometric measurements	**Mean (cm) ± s.e.**	
	***Barbodes binotatus* (n=86)**	***Barbodes banksi* (n=178)**
SL Range (cm)	4.0-9.6	3.8-10.4
SL	6.34 ± 0.125	6.72 ± 0.098
HL	1.79 ± 0.021^a	1.92 ± 0.016^b
PBL	0.37 ± 0.006^a	0.40 ± 0.004^b
ED	0.48 ± 0.004^a	0.53 ± 0.004^b
I	1.57 ± 0.008^a	1.76 ± 0.010^a
II	2.37 ± 0.018^a	2.49 ± 0.017^b
III	1.06 ± 0.015^a	1.14 ± 0.011^b
IV	2.13 ± 0.023^a	2.15 ± 0.019^a
V	1.02 ± 0.010^a	1.07 ± 0.007^b
VI	1.03 ± 0.016^a	1.07 ± 0.011^b
VII	0.75 ± 0.009^a	0.82 ± 0.012^b
VIII	1.66 ± 0.021^a	1.72 ± 0.011^b
IX	0.31 ± 0.005^a	0.34 ± 0.004^b
X	2.04 ± 0.016^a	2.20 ± 0.014^b
XI	1.47 ± 0.019^a	1.56 ± 0.020^b

The 12 significant morphometric measurements were further analyzed in PCA to investigate the individual variance that affected by the morphometric measurements. Individuals of *B. binotatus* and *B. banksi* were scarcely discriminated by the transformed morphometric measurements in the first two components of PCA (PC 1 and PC 2). This was indicated by the major overlapping between the two species groups in the PCA (Figure 4). Despite the great overlap, individuals of *B. binotatus* still showed a slight separation from *B. banksi* at the negative side of PC 1 which signified the differences between species based on morphometric characters.

Three components were extracted from the PCA performed on size-adjusted morphometrics measurements that explained about 64.39% of the variation. The highest loadings in each component indicated the importance of the variables to the component. PC 1 and PC 2 accounted for 30.68% and 19.97% of the total variance respectively (Table 4). The first component (PC 1) was composed primarily by II (end of the operculum to the origin of the dorsal fin), X (end of operculum to the origin of pelvic fin), and HL (head length), with loading value of 0.595, 0.481, and 0.411 respectively. In PC 2, the highest loading (0.967) was from variable XI (tip of the snout to the lower end of the operculum). The third component (PC 3) explained 13.73% of the total variation. This component was dominated by HL (0.805), II (-0.393), and VIII (end of pelvic fin to the origin of anal fin) (-0.331).

Table 4 Principal component analysis (PCA) loadings in the first three components for morphometric measurements of *B. binotatus* and *B. banksi*. The highest component loadings are indicated by the bold font.

Variables	**PC 1 (30.68%)**	**PC 2 (19.97%)**	**PC 3 (13.73%)**
HL	**0.411**	0.161	**0.805**
PBL	0.063	0.025	0.002
ED	0.027	0.020	-0.009
II	**0.595**	-0.002	**-0.393**
III	0.165	0.094	0.108
V	0.215	0.054	0.115
VI	0.190	0.067	0.116
VII	0.156	0.081	0.010
VIII	0.304	0.060	**-0.331**
IX	0.076	0.008	-0.015
X	**0.481**	-0.104	-0.158
XI	-0.092	**0.967**	-0.157

The three components that explained the most variance in PCA were associated mainly with several measurements of the anterior part of the fish body. HL and II particularly gave high loadings in both PC 1 and PC 3. This underlined the importance of the anterior part of fish, to distinguish *B. binotatus* from *B. banksi* by using morphometric measurements.

One-way ANOVA test on the three variations of *B. binotatus*, variation B showed significantly higher mean value of HL compared to the other two variations. Other characters measured in *B. binotatus* did not revealed any differences between three variations (Table 5).

Table 5 Mean and standard error (s.e.) of morphometric measurements (cm) for three variations of *B. binotatus* (different superscript letters indicate significant differences).

Morphometric measurements	Mean (cm) ± s.e.		
	A (n=40)	B (n=17)	C (n=29)
SL	6.26 ± 0.209	6.64 ± 0.209	6.28 ± 0.198
HL	1.78 ± 0.028[a]	1.91 ± 0.062[b]	1.73 ± 0.026[a]
PBL	0.36 ± 0.009[a]	0.35 ± 0.012[a]	0.38 ± 0.010[a]
ED	0.47 ± 0.005[a]	0.47 ± 0.006[a]	0.49 ± 0.007[a]
I	1.58 ± 0.011[a]	1.55 ± 0.016[a]	1.56 ± 0.016[a]
II	2.34 ± 0.021[a]	2.34 ± 0.034[a]	2.42 ± 0.041[a]
III	1.05 ± 0.021[a]	1.09 ± 0.040[a]	1.06 ± 0.024[a]
IV	2.13 ± 0.038[a]	2.10 ± 0.044[a]	2.16 ± 0.034[a]
V	1.02 ± 0.016[a]	1.01 ± 0.017[a]	1.01 ± 0.018[a]
VI	1.04 ± 0.022[a]	0.97 ± 0.036[a]	1.04 ± 0.030[a]
VII	0.74 ± 0.012[a]	0.75 ± 0.020[a]	0.74 ± 0.019[a]
VIII	1.65 ± 0.037[a]	1.62 ± 0.026[a]	1.68 ± 0.030[a]
IX	0.30 ± 0.007[a]	0.30 ± 0.009[a]	0.33 ± 0.009[a]
X	2.04 ± 0.021[a]	2.07 ± 0.035[a]	2.03 ± 0.031[a]
XI	1.48 ± 0.038[a]	1.46 ± 0.022[a]	1.47 ± 0.013[a]

While in *B. banksi*, only one significant difference in morphometric characters that was XI (A-B and B-C) (Table 6). Variation B of *B. banksi* had higher mean value of XI compared to variation A and C. PCA was not conducted on the three variations in both species because there was no difference identified in all characters measured except one. All morphometric measurements were expected to cause vast overlap in individuals between variations A, B, and C in both species.

Table 6 Mean and standard error (s.e.) of morphometric measurements (cm) for three variations of *B. banksi* (different superscript letters indicate significant differences).

Morphometric measurements	Mean (cm) ± s.e.		
	A (n=114)	B (n=23)	C (n=41)
SL	6.68 ± 0.112	7.41 ± 0.259	6.45 ± 0.243
HL	1.93 ± 0.021[a]	1.92 ± 0.049[a]	1.89 ± 0.026[a]
PBL	0.40 ± 0.005[a]	0.41 ± 0.012[a]	0.41 ± 0.008[a]
ED	0.53 ± 0.004[a]	0.52 ± 0.013[a]	0.53 ± 0.009[a]
I	1.76 ± 0.013[a]	1.78 ± 0.027[a]	1.74 ± 0.018[a]
II	2.48 ± 0.023[a]	2.46 ± 0.051[a]	2.53 ± 0.026[a]
III	1.14 ± 0.014[a]	1.11 ± 0.035[a]	1.15 ± 0.021[a]
IV	2.14 ± 0.024[a]	2.21 ± 0.044[a]	2.16 ± 0.043[a]
V	1.07 ± 0.009[a]	1.08 ± 0.022[a]	1.09 ± 0.014[a]
VI	1.07 ± 0.014[a]	1.04 ± 0.036[a]	1.07 ± 0.022[a]
VII	0.81 ± 0.014[a]	0.80 ± 0.016[a]	0.85 ± 0.033[a]
VIII	1.71 ± 0.013[a]	1.75 ± 0.049[a]	1.72 ± 0.018[a]
IX	0.34 ± 0.005[a]	0.35 ± 0.014[a]	0.34 ± 0.007[a]
X	2.20 ± 0.019[a]	2.18 ± 0.043[a]	2.18 ± 0.025[a]
XI	1.53 ± 0.013[a]	1.73 ± 0.126[b]	1.56 ± 0.030[a]

Geometric morphometric

Multivariate Hotelling's T-square test mean shape showed no difference in mean body shape ($p > 0.05$) between *B. binotatus* and *B. banksi*. Large overlaps in histogram bar of discriminant analysis explained the high similarity in body shape of both species' groups (Figure 5). Discriminant analysis gave a value of 48.86% of correctly classified group which was not enough to discriminate *B. binotatus* from *B. banksi* in terms of body shape.

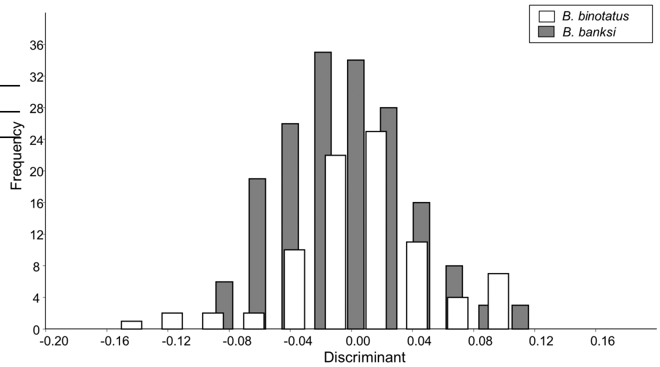


Fig. 5 Histogram of frequency distribution of discriminant analysis score on geometric shape variables of *B. binotatus* and *B. banksi*.

Barbodes binotatus and *B. banksi* exhibited no significant difference ($p > 0.05$) in mean body shape between intraspecies variation groups (A-B-C). MANOVA provided a high p value for comparison between variation groups of *B. binotatus* (Wilks' Λ = 1, p = 0.9978) and *B. banksi* (Wilks' Λ = 0.9983, p = 0.9384). Canonical variate analysis (CVA) scatterplot of non-affine and affine component of landmark-based geometric morphometric, depicted a large overlap between the convex hulls of variation A, B, and C of *B. binotatus* (Figure 6A). The same is observed in a CVA scatterplot for *B. banksi* variation groups, which signified the huge similarity in overall body shape of variation A, B, and C (Figure 6B).

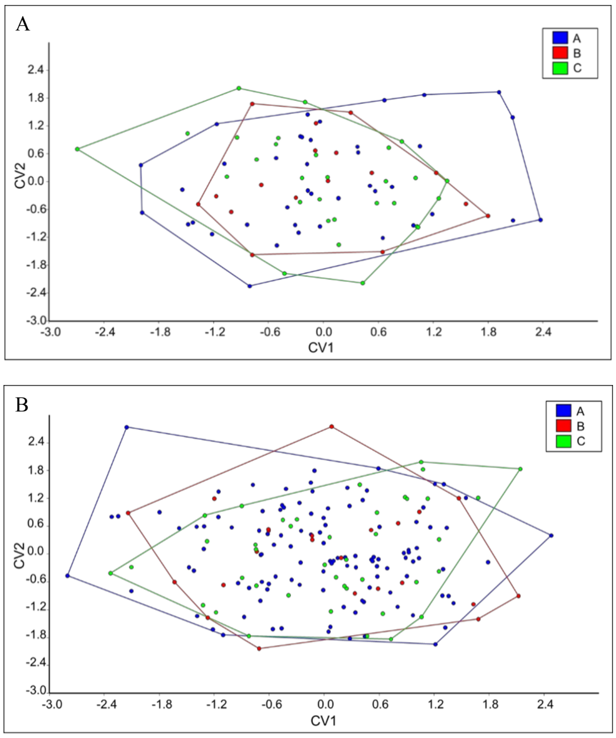


Fig. 6 Canonical variate analysis (CVA) scatterplot based on partial warps plus the uniform component of variation A, B, and C of (A) *B. binotatus* (B) *B. banksi*. Specimens with same body color pattern are connected by the convex hull line

DISCUSSION

Closely related species or species complex frequently caused taxonomic inaccuracy, i.e., misidentification due to the confusion of similar looking congener species. Morphometrics analyses has been widely used to assess shape variation in a wide variety of taxa especially fish (Bower and Piller, 2015; Santos and Quilang, 2012). These methods had perceived to be useful tools in species delimitation and accurate identification. In the previous study, the two species were deemed distinctively different and traditional morphometrics used in this study revealed that there were substantial disparities between *B. binotatus* and *B. banksi*. However, only one character could be used to distinguish between three variations within each species. Alternatively, landmark based geometric morphometric displayed deviations in mean shape between species and variations within species but could not be significantly discriminated statistically.

Interspecies shape variations

This study presumptively distinguishes *B. binotatus* from *B. banksi* as two different species *sensu* Kottelat and Lim (1996) and Kottelat (2013). From the traditional morphometric analysis, *B. binotatus* was differentiated from *B. banksi* in 12 morphometric characters (from 14 characters analysed) used in this study. This confirmed the disparity in body shape of the two species. *B. banksi* exhibited higher mean value of morphometric measurements for each character which showed that *B. banksi* had a bigger body size and shape compared to *B. binotatus* (Table 3). Earlier, the character, i.e., head length of *B. binotatus*, was shown to be larger compared to *B. banksi* (3.4 to 3.8 vs 3.2 to 3.3 times in the standard length) (see Herre, 1940). In contrast, this study showed that *B. banksi* had higher head length, 4.2 to 3.6 times in standard length (HL: 1.92 ± 0.016 cm) compared to *B. binotatus*, 3.4 to 3.6 times in standard length (HL: 1.79 ± 0.021 cm). Since the published data on morphometric measurements of *B. banksi* were limited and its restricted known distribution, the true size of this species was still unknown. Herre (1940) reported the largest size of *B. banksi* was 9.4 cm in Kota Tinggi, Johor. In this study, the biggest specimen examined was 10.4 cm, showed that this species may grow bigger in their distribution range. For that, variations in morphological measurement could be size-dependent (Ezeafulukwe *et al*., 2015). *Barbodes binotatus* was thought to be widely distributed species in Southeast Asia compared to *B. banksi*. This species has been reported from Phillipines, Borneo, Java, Sumatra, Peninsular Malaysia to the middle of Mekong of Thailand (Rainboth, 1996; Pethiyagoda *et al*., 2012), however, it may involve several distinct species (Kottelat, 2000; Pethiyagoda *et al*., 2012; Kottelat, 2013). Rainboth (1996) in his study in Cambodian Mekong river system, stated that *B. binotatus* was caught with approximately 10 cm length in size but it can reach up to 20 cm length. For both species, specimen size examined in this study were considered appropriate (Table 3) thus the results obtained in the current study were considered highly acceptable.

PCA has been regarded as a reliable method in emphasizing the most significant morphological variation in discriminating species (Grismer et al., 2016). Data dimension reduction of the 12 significant character using PCA revealed the importance of the anterior parts of the body in discriminating both species. Predorsal distance and head length particularly, gave the highest loading on PC 1 (30.68%) of PCA which highlighted the importance of the characters in discriminating body shape of both fish species. Head length was a prevalent character in discriminating fish inter- or intraspecies (Atobatele, 2013; Maderbacher *et al*., 2008). Head measurements had become one of the most important characters in detecting three morphological differences in the population of Bluefish (*Pomatomus saltatrix*) in Turkish territorial seas (Turan *et al*., 2006). Other species that exhibited the significance of head measurement in species discrimination includes, gobiid fish (*Glossogobius giuris*), orange-fin labeo (*Labeo calbasu*), and snow trout (*Schizothorax richardsonii*) (Mollah *et al*., 2012; Ramasamy and Rajangam, 2016; Wagle *et al*., 2015).

Among vertebrates, fish have the highest morphological diversity, which mainly associated with their environment (Cunico and Agostinho, 2006; Wimberger, 1992). The variations in fish's head morphology could be essential in discriminating species. Conventional morphometric method alone was inadequate to explain the variation within the two species. Landmark-based geometric morphometric also spotted a few differences between the two species especially in head region and body depth (*B. banksi* had a slightly larger head shape and deeper body depth compared to *B. binotatus*). This corroborates the traditional morphometric result which emphasized the head part to differentiate both species. The head section of fish usually associated with trophic niche and foraging habit (Aguilar-Medrano *et al*., 2011; Ingram, 2015). Head size for example could enhance the capabilities to capture prey, i.e., relatively large head size could heighten the capture of small prey (Wagle *et al*., 2015). According to trophic level model in Froese and Pauly (2016), the estimated feeding type for *B. binotatus* and *B. banksi* were omnivores and carnivores respectively. Besides this information, there are no other studies reported on the feeding habit and its relation to cephalic structure of both species.

Despite the slight differences on head shape and body depth of *B. binotatus* and *B. banksi*, as shown by the consensus in geometric morphometric, the multivariate analysis run using discriminant analysis and Hotelling's T-square showed no difference in mean body shape of both species. This result is congruent to the Ng and Tan (1999) whose inferred that *B. binotatus* and *B. banksi* might be a single species with two extreme color forms. However, this study is still tentatively recognized both species as two different species based on two reasons. The first one is, both species are conspicuously separated by geographical barrier (Figure 1), though there is a little overlap between the geographical distributions of the two populations. For example, in Terengganu, both species occurred in Sungai Setiu drainage but in different streams (pers. obs.). *Barbodes banksi* distributed from Sungai Setiu to the southern part of Terengganu and south while *B. binotatus* was recorded from Sungai Setiu to the north part of Terengganu and northern region (Figure 1). Based on the present knowledge, *B. banksi* could be well distributed all over Peninsular Malaysia while *B. binotatus* were restricted to the northern part of the Malay peninsula.

There is also no report that both species to occur sympatrically. This vicariance instigated event could promote allopatric speciation which was conjectured to be the main mode of speciation for freshwater fish (Seehausen and Wagner, 2014). Allopatric speciation could promote different kind of selection pressures on isolated population hence allowing adaptation and evolution into distinct biological entities (Luceño *et al*., 2013). The second reason is because of the clearly distinguishable marking or color morph located immediately below the dorsal fin on both species. *Barbodes binotatus* has a round spot marking while *B. banksi* has wedge-shaped marking (Ng and Tan, 1999). Hitherto, this marking character has been frequently used to effectively differentiate both species in the field. The problem with these species color morph is that, there are so many variations within each species which will confused taxonomist whether the variation is due to reproductive isolation or phenotypic plasticity (Grady and Quattro, 1999; Schultz *et al*., 2007).

Intraspecies shape variations

Three variation of body markings were identified for each species of *B. binotatus* and *B. banksi* respectively. Apparently, there were lack of significant morphological variation between color morph A, B and C in both species. Morphometric measurement HL (head length) and XI (tip of the snout to the lower end of the operculum) were the only significant characters that showed disparity between variations (i.e., A-B and B-C) within *B. binotatus* and *B. banksi* respectively. Both measurements (HL and XI) are placed on cephalic area of the fish which highlighted the important of head area in distinguishing morphological variation between and within both species. Multivariate analysis on landmark-based geometric morphometric also gave the same result as conventional morphometric with lack of dissimilarity ($p > 0.05$) in overall body shape between variations within both species as shown by the MANOVA and CVA.

The effort to discriminate between variations in *B. binotatus* and *B. banksi* maybe inconclusive since the variations were not well divided in multivariate analysis. Additional biological information such as habitat use, trophic niche, and predator-prey adaptation is required in order to define the variation groups (Bolnick *et al*., 2011; Mattson and Belk, 2013). Intraspecific variation of organism typically related to the environment they thrive in (Benítez, 2013; Parker *et al*., 2009). The paucity of information on *B. binotatus* and *B. banksi* functional traits and feeding behavior could conceal the importance of the morphometrics differences in each species, which is instigated by the ecological process. A study on intraspecific variation in *Poecilia reticulata* had detected several ecomorphotypes within the same species that triggered by the different environments, i.e., males of *Poecilia reticulata* in stream had wider mouth while population in lake had more protractible mouth (Mise *et al*., 2015). The development of several intraspecific ecomorphotypes in a same population could facilitated the resource partitioning and habitat utilization of the fish thus reduce competition (Bolnick *et al*., 2011; Sampaio *et al*., 2013).

Variations within *B. binotatus* and *B. banksi* were only conspicuously differ in body marking, whereas in the body shape they were almost similar. Intraspecific variations in terms of body marking within *B. binotatus* and *B. banksi* were also not localized. There was a possibility that we could find the two body markings of *B. banksi* in the same stream, e.g., Sungai Setiu drainages. The occurrence of two body marking of the same species sympatrically may be because of the phenotypic plasticity, in response to the selective pressure in the environment (Maan and Sefc, 2013). Generally, phenotypic plasticity in organism is attributed to the environmental gradients (Hampton *et al*., 2014). In response to the stream impoundments, stream fishes adapted improved their locomotion with decreased body depths, larger caudal areas, and changes in pectoral fin positioning (Franssen *et al*., 2013). Phenotypic plasticity also impeded the development of conspicuous color morphs in haplochromine cichlid fish for anti-predation adaptation (Maan *et al*., 2008). The body marking polymorphism could be prompted by the sexual dimorphism but in *B. binotatus* this was not the case (Lim *et al*., 2014).

Intraspecific variations of both species examined in this study can be further explained by examination of larger number of specimens as well as size distribution of each collection collected over the wider geographical areas of its distribution limits that may be able to discriminate the intraspecific disparity that exists between the two species. Others studies had shown the needs to employ additional methods such as molecular analysis to discriminate cryptic species where taxonomy is not an issue (Karanovic *et al*., 2016). We strongly belief genetic information might hold more information about these two remarkable species.

CONCLUSION

Combining conventional morphometric and the more advanced landmark-based geometric morphometric, proved to be useful in elucidating species complex in *B. binotatus* and *B. banksi*. Traditional morphometric specialized in detecting characters that varies whereas geometric morphometric specialized in depicting the intermediate form and subtle disparity in body shape of inter- and intraspecies. Despite the morphometric results, the color or spot polymorphism was still the most reliable character to be used in the field to distinguish inter- and intraspecific variations of the fish.

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