Discrimination analysis of hybrid *Pangasianodon hypophthalmus* (Sauvage, 1983) (♀) × *Pangasius nasutus* (♂) (Bleeker, 1976) and its parental species

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Abstract

Comparative analysis was performed to discriminate a hybrid produced from the crossbreed of *Pangasianodon hypophthalmus* (♀) and *Pangasius nasutus* (♂) and its parental species based on morphology appearances and morphometric characters. Morphological structures of the vomerin and palatal teeth varied between the hybrid and both parents. Results of the univariate analysis revealed 22 morphometric characters were significantly different between the hybrid and its parental species. Under the stepwise discriminate function analysis, the first Function explained 86.10% of total variations and 13.90% in Function 2. Of the 30 characters, only 10 characters which include prepelvic, caudal peduncle length, dorsal fin length, pectoral fin length, anal fin height, anal fin length, adipose fin length, interorbital length, distant to isthmus, and predorsal length can be used to significantly differentiate these species. The predicted fish groups exhibited characters which 100% differentiate and validate them into their respective group. Examination on vomerin and palatal teeth distinct the hybrid and its parental species.

Keywords: *Discriminate function analysis, morphometric, Pangasianodon hypophthalmus, Pangasius nasutus, hybrid*

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Introduction

Fish stock can be differentiating based on their morphological variation (Sokal *et al.*, 2009). This stock identification is crucial to facilitate the management of domestication, and sustainability of aquaculture production (Bailey, 1997; Ibánez *et al.*, 2017). According to Ezeafulukwe *et al.* (2015), determination of phenotypic variation using morphometric characters and meristic counts is the most common method applied in delineating stocks of fishes. Morphometric characters are useful in biological studies as it allows quantitative descriptions of organisms. This quantitative approach allows researchers to differentiate the organisms by comparing the body shape (Gelsvartas, 2005), and combining descriptions such as colour and size (Muchlisin, 2013). Morphometric is important to distinguish the phenotypic of fish with variability in term of growth, development, and maturation (Begg and Waldman, 1999). Identification of fish in different populations using morphometric measurement was applied in previous studies (Tzeng, 2004; Torres e*t al.*, 2014; Chen *et al.*, 2015) because of its simplicity and direct application (Bronte *et al.*, 1999; Hockaday *et al.*, 2000).

In recent decades, advanced techniques through morphometric methods have successfully discriminate fish stocks within fish population (Dwivedi and Dubey, 2013), detecting differences or similarity among groups (To and Ci, 2015). Statistical tools like multivariate techniques such as principal components and discriminant factorial analyses have contributed to the identification of fish stocks efficiently (Kuszniers *et al.*, 2008; Cronin-Fine *et al.*, 2013). Morphometric and multivariate statistical procedures are useful combination for testing and graphically display the differences in fish stock (Baur and Leuenberger, 2011). Selection of characters for morphometric analysis is also important to maximize the effectiveness of stock discrimination (Begg *et al.*, 1999).

Multivariate analysis is applied to reveal the morphometric variables for stock identification (Kusznierz *et al.*, 2008; Specziár *et al.*, 2009; Yakubu and Okunsebor, 2011; Cronin-Fine *et al.*, 2013). Multivariate morphology has been used in population studies of various fishes for stock discrimination (Tzeng *et al.* 2001; Tzeng 2004; Von Cramon-Taubadel *et al.*, 2005; Maynou and Sarda, 1997; Anastasiadou and Leonardos, 2008; Ezeafulukwe *et al.*, 2015; Banerjee *et al.*, 2017). Particularly with the existence of a new hybrid, description of the morphological variation becomes important. Furthermore, establishment of morphological differentiation for identification with the least assumption is possible when using samples of known hybrid (Neff and Smith, 1979). Previous studies (Gustiano *et al.*, 2003; Baharuddin *et al.*, 2014) were able to discriminate the pangasiids species. Lack of adequate information on the discrimination of hybrid in the field has led to the current study for the assessment of phenotypic variations resulting in important characters being derived for rapid discrimination and classification.

Materials and methods

Fish Samplings

Samples of hybrid (n=30) were from a local fish farmer in Temerloh, Pahang cultured to adult stage (14 month old). While samples of *P. hypophthalmus* (n=40) and *P. nasutus* (n=10) were taken from our collections.

Data Collections

Morphometric measurements were taken according to Gustiano (2003). Thirty parts of the fish body were measured (Fig. 1) and character codes and landmarks were employed to represent the description of characters (Table 1). Prior to measurement, fish were anesthetized using MS 222 (35 mg/L) then measured on a measurement board. Measurements of characters were taken using digital vernier calliper to the nearest 0.1 mm.

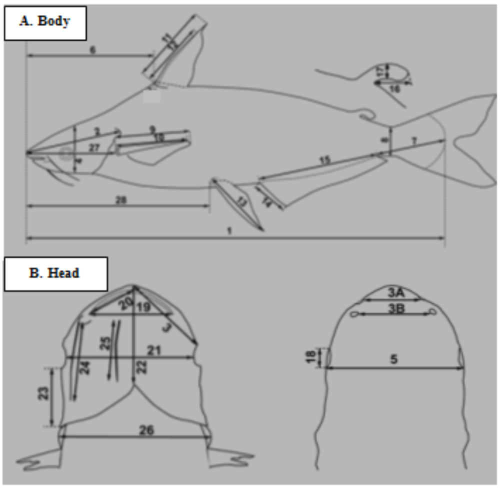

Figure 1: Measurement of the body (A) and head (B) parts of each species. (Source: Gustiano, 2003).

Table 1: Description of the characters used in the study.

Character codes	Landmarks	Description of characters
SL	1	Standard length
HL	2	Head length
SNL	3	Snout length
ASW	3A	Anterior snout width
PSW	3B	Posterior snout width
HD	4	Head depth
HW	5	Head width
PREDL	6	Predorsal length
CPL	7	Caudal peduncle length
CPD	8	Caudal peduncle depth
PFL	9	Pectoral fin length
PSL	10	Pectoral spine length
DFL	11	Dorsal fin length
DSL	12	Dorsal spine length
PEFL	13	Pelvic fin length
AFH	14	Anal fin height
AFL	15	Anal fin length
ADIFH	16	Adipose fin height
ADIFW	17	Adipose fin width
ED	18	Eye diameter
MW	19	Mouth width
LJL	20	Lower jaw length
IL	21	Interorbital length
DSI	22	Distance snout to isthmus
PL	23	Postocular length
MAXBL	24	Maxillary barbell length
MANBL	25	Mandibulary barbell length
BW	26	Body width
PREPL	27	Prepectoral length
PREPEL	28	Prepelvic length

Data Analyses

Data were analysed using univariate and multivariate analyses of variances to test the significance in the morphometric characters. An allometric formula by Elliott *et al.*, (1995) was employed to remove the length effect of the samples due to the morphometric characters (Turan *et al.*, 2005). The formula was calculated as $M_{adj} = M\ (LS/LO)^b$ where, M_{adj}: size adjustment measurement; M: Original measurement; L_O: standard length; $L_{S:}$

overall mean of standard length; b: estimated for each character from the observed data as the slope of the regression of Log_M on Log_{LO} using all samples in all groups (species).

For univariate analysis, the size-adjusted data were submitted to one-way analysis of variance (ANOVA) followed by post hoc multiple tests using Duncan at 5% significant level to compare the variation of each character between species. Multivariate analysis under the discriminate factor analysis (DFA) (Ramsay *et al*., 2009) was carried out to assess the morphological variation. Discriminate analysis was employed to select more important variables as the predictors for the determination of the hybrid and its parental species. The Mahalanobis squared distance was used in a stepwise method. Functions derived are useful in explaining the eigenvalue values that classified the components. Wilk's lambda was projected to test the significant of the combination of the variables in different dimension. It was defined based on value close to 1. Higher discriminator variables indicates fish within the same group, while value close to 0 shows the variability is due to species differences. For visual detection and classification, scatter plots of canonical scores were constructed. All statistical analyses were performed using SPSS software version 20.0

Results and discussion

Morphological Appearances

Body colour of hybrid was observed to be slightly different as compared to parental species (Fig. 2). Live coloration on the head and upper part of body may varies from black to grey for *P. hypophthalmus*, with merely light gold for *P. nasutus* and light green for hybrid. Besides body colour, the vomerin and palatal teeth of hybrid was distinctively different from its parents. The dentition of *P. nasutus* shows large and nearly square vomerin, meanwhile *P. hypophthalmus* with two palatal bands.

Figure 2: Morphological appearance and closer look of on the maxillary and palatal dentition of (A) *Pangasianodon hypophthalmus;* (B) *Pangasius nasutus* (C) hybrid.

Univariate Analysis

Univariate analysis demonstrated all of the characters were significantly different (p<0.05) among all three species except for HL, SNL, ASW, PSW, ED and PPEL (Table 2). Means sizes of every character varied among species and hybrid was closer to *P. hypophthalmus* as most of the characters seem to be insignificant (p>0.05) between these two species.

In general, *P. hypophthalmus* showed longer head parts, in contrast to *P. nasutus* which was longer in fin parts.

Table 2: Mean±SD transformed values of morphometric characters of *Pangasianodon hypophthalmus, Pangasius nasutus* and its hybrid.

Morphometric Characters	*P. hypophthalmus* (n=40)	*P. nasutus* (n=10)	Hybrid (n=30)
Head Length	9.92±0.60	10.02±0.65	9.09±0.74
Snout length	3.94±0.40	4.01±0.39	3.92±0.52
Anterior Snout Width	2.91±0.46	2.98±0.35	2.82±0.70
Posterior snout width	3.86±0.35	3.95±0.27	3.73±0.68
Head depth	4.61±0.66	4.78±0.53	4.62±0.53
Head width	5.66±0.86^{a}	6.73±0.59^{b}	5.64±0.55^{a}
Predorsal length	15.73±0.91^{a}	17.27±0.68^{b}	15.12±0.79^{c}
Caudal peduncle length	6.38±0.62^{a}	6.02±0.53^{a}	5.52±0.87^{b}
Caudal peduncle depth	4.10±0.35^{a}	3.15±0.21^{b}	3.46±0.36^{c}
Pectoral fin length	8.52±0.77^{a}	7.29±0.82^{b}	6.53±0.49^{b}
Pectoral spine length	7.43±0.71^{a}	6.58±0.72^{b}	5.87±0.62^{c}
Dorsal fin length	9.98±0.95^{a}	8.10±0.59^{b}	6.87±0.77^{c}
Dorsal spine length	7.62±0.80^{a}	7.12±0.59^{b}	6.04±0.54^{c}
Pelvic fin length	7.29±0.49^{a}	5.00±0.87^{b}	4.56±0.45^{c}
Anal fin height	6.62±0.94^{a}	4.22±0.98^{b}	4.18±0.4^{b}
Anal fin length	14.75±0.99^{a}	10.68±0.55^{b}	12.02±0.57^{c}
Adipose fin height	1.58±0.22^{a}	1.89±0.22^{b}	2.05±0.45^{b}
Adipose fin width	0.87±0.19^{a}	0.88±0.16^{b}	1.14±0.29^{b}
Eye diameter	1.12±0.10	1.08±0.09	1.07±0.10
Mouth width	3.98±0.48^{a}	4.12±0.36^{a}	3.70±0.34^{b}
Lower jaw length	2.35±0.30^{a}	2.52±0.38^{a}	2.00±0.39^{b}
Interorbital length	5.59±0.53^{a}	6.10±0.64^{b}	5.43±0.88^{a}
Distance snout to isthmus	4.75±0.65^{a}	5.14±0.55^{b}	4.45±0.4^{a}
Postocular length	4.17±0.41^{a}	4.73±0.91^{b}	4.28±0.61^{a}
Maxillary barbell length	2.77±0.92^{a}	4.65±0.50^{b}	3.20±0.53^{a}
Mandibular barbel length	1.22±0.67^{a}	3.54±0.85^{b}	2.04±0.41^{c}
Body width	7.11±0.57^{a}	7.89±0.85^{b}	7.01±0.73^{a}
Prepectoral length	8.73±0.73	9.11±0.98	8.77±0.61
Prepelvic length	18.26±0.94^{a}	20.25±0.89^{b}	18.35±0.98^{a}

Values (mean±SD, mm) in the same row with different superscripts are significantly different ($p<0.05$).

Hybrid has relatively shorter for most characters but longer in ADIFH and ADIFW, and with slender body compared to its parents.

Multivariate Analysis

Discriminate Function analysis has derived two Functions with eigenvalues higher than 1 (Table 3). As illustrated in this table, eigenvalue of 17.71 demonstrated 86.1% of variation among the characters loaded in Function 1. Meanwhile, the eigenvalue in Function 2 was lower than Function 1 (2.854) with the percentage of variance at 13.9%. Eigenvalues demonstrated high correlation of characters derived from Function 1 (0.973) than in Function 2 (0.861) which related to canonical correlation, thus important to describe the discriminating ability a Function.

Function 1 and 2 were loaded by PREL, CPL, DFL, PFL, AFH, AFL, ADIFL, IL, DSI and PREL.

In Function 1, highly loaded with ADIFH, IL, DSI, and PREPEL which positively related to the Function and contribute meaningfully to the species discrimination since most of the variation occurred in these characters. As for Function 2, PREDL, DFL, PFL, ADIFL, DSI and PREPEL contributed most to the species discrimination in the positive direction indicating these characters as good predictors for the hybrid and its parental species.

Table 3: Eigenvalues, percentage of variance, cumulative, canonical correlation and standardized canonical coefficient of DFA loading of characters.

Function	**1**	**2**
Eigenvalues	17.71	2.854
% of variance	86.10	13.90
Cumulative	86.10	100.00
Canonical correlation	0.973	0.861
Standardized canonical coefficient of DFA loading of characters		
Predorsal length	0.124	0.449
Caudal peduncle depth	-0.251	-0.517
Dorsal fin length	-0.002	0.400
Pelvic fin length	-0.451	0.465
Anal fin height	-0.541	0.036
Anal fin length	-0.769	-0.512
Adipose fin height	0.389	-0.207
Interorbital length	0.443	0.363
Distance snout to isthmus	0.684	0.651
Prepelvic length	0.591	0.331

First 2 canonical discriminate Functions were used in the analysis.

The score of species variability of each Function which captured by the value of canonical correlation for Function 1 and 2 are 0.973 and 0.861, respectively, indicated more important correlation with larger canonical correlation. Function 1 exhibited a strong relation between score of its Function as compare to Function 2 and species differences. The significance of the morphometric distinction was also indicated by Wilks' lambda criterion based on the ratio of distinction within-species variability to total variability for the discriminator variables. Based on the result, the first test presented in Table 4 for Function 1 through 2, Wilks' lambda was close to 0, signifying that most of the variables captured from Function 1 attributed to the species differences. In Function 2, Wilks' lambda at 0.259 indicates little variability captured by Function 2 that contributed to between-species differences. Furthermore, chi-square values showed the variability for the species differences prior to extraction was statistically significant at 0.05 level. Even though there was only small amount of group differences observed in Function 2, it is worth consideration due to the proportion of the group was statistically significant.

Table 4: Statistical significance of the derived discriminate Functions for Wilks' Lambda.

Test of Function(s)	**Wilks' Lambda**	**Chi-square**	**df**	**Sig.**
1 through 2	0.014	310.187	20	0.00
2	0.259	97.818	9	0.00

To test the efficacy of a set of Function based on the ability of the Function to accurately classify the species to their respective group is the final determinant in this interpretation. The Function (Table 5) generated in the analysis showed that *Pangasianodon hypophthalmus*, *P. nasutus* and its hybrid were 100% differentiated and validated into their respective group.

Table 5: Predicted group and cross-validated *Pangasianodon hypophthalmus* (PH), *Pangasius nasutus* (PN) and hybrid (HB).

			Predicted Group membership			
		Fish	***P. hypophthalmus* (PH)**	***P. nasutus* (PN)**	**Hybrid (HB)**	**Total**
Original	%	PH	100.0	0.0	0.0	100.0
		PN	0.0	100.0	0.0	100.0
		HB	0.0	0.0	100.0	100.0
Cross-validated	%	PH	97.5	2.5	0.0	100.0
		PN	10.0	90.0	0.0	100.0
		HB	10.0	3.3	90.0	100.0

100.0% of original grouped cases correctly classified.
Cross-validation is done only for those cases in the analysis. In cross validation, each case is classified by the Function derived from all cases other than that case.
95.0% of cross-validated grouped cases correctly classified.

The predicted fish groups exhibited characters which Cross-validate values indicated that misclassification with values lower than the original is common for this classification.

Scatterplot shows there are no overlapping of characters between hybrid and its parents evidently showed that Function 1 discriminated hybrid and its parents into three separate groups (Fig. 3).

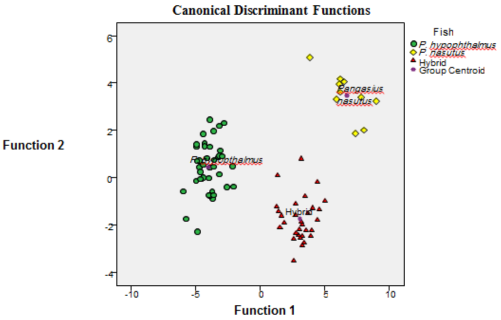

Figure 3: Scatterplot of Function 1 against Function 2 of the morphometric characters of *Pangasianodon hypophthalmus, Pangasius nasutus* and its hybrid.

The present study revealed the variation of hybrid and its parental species based on morphological appearances and morphometric characters. Morphological appearance of body colour, vomerin and palatal teeth band can be used to distinguish the hybrid since rapid diagnostic is needed in identifying fish for stock management. The structure of single vomerin and palatal teeth band of hybrid differentiate it from its parental species. Also, this method has been used in the differentiation of other pangasiids species (Fumihito *et al*., 1989; Roberts and Vidthayanon, 1991; Pouyard *et al*., 2002; Baharuddin *et al*., 2014; Dwivedi *et al*., 2017).

In this study, univariate and multivariate analyses employed were able to discriminate and classify the hybrid based on morphometric characters, where hybrid appeared to resemble *P. hypophthalmus*, evidence with the mean significant values of univariate analysis closer to *P. hypophthalmus*. There is also no overlapping of the characters measured between the hybrid and its parents related to size as observed in scatterplot. Hubb (1955) stated that hybrid is likely to display the characteristics of its parents or immediate traits in the first generation (F1) and backcrossing is considered when hybrid population showed high variability. Several hybrids were found displayed intermediate characters of its parents. For example intraspecific hybridization of native and Thai koi, *Anabas testudineus,* interspecific hybridization between yellow flounder and Winter flounder, *Limanda ferruginea* (♀) × *Pseudopleuronectes americanus* (♂) (Park *et al*., 2003), hybrid between *Gibelion catla* (♀) × *Labeo rohita* (♂) (Bhowmick *et al*., 1981), hybrid between catla and fimbriatus, *Catla* (♀) × *Labeo fimbriatus* (♂) (Basavaraju *et al*., 1995) demonstrated immediate characters of its parents. Similarly, morphological characters of hybrids *Pangasius djambal* and *P. hypophthalmus* and their reciprocal hybrids having intermediate characters except for number of gill rakers which is less than its parental species (Gustiano and Kristanto, 2007).

In fish population studies, the morphology plasticity is commonly occur can be greatly affected by the differences in the environmental conditions such as food availability and variation of water temperature (Wimberger, 1992). For a hybrid, morphology of its parents greatly affected the hybrid characters. It is expected that high phenotypic differences of *P. hypophthalmus* and *P. nasutus* in the present study as these two species belong to different taxa. It was observed dorsal fin length was found larger in *P. hypophthalmus* than *P. nasutus* and its hybrid and the reason for that presumably *P. hypophthalmus* is a benthic species. Therefore it could be part of the adaptation of this species since dorsal fin length is related to the vertical position of the fish in the water column, and claimed posteriorly-placed dorsal fin important for the adaptations to the surface habitat especially for the non-flowing waters (Matthews, 1988). Eyes diameter was larger in *P. hypophthalmus* as compared to *P. nasutus* and its hybrid. While the position of eyes of *P. hypophthalmus* and *P. nasutus* was expected to be different due to species-specific that relate to their vertical habitat preference (Turan, 2005).

This hybrid, however, showed relatively larger ADIFH and ADIFW as compared to its parents, and shorter in most part of the characters which means this hybrid not only performed intermediate shape as anticipated, but their characters demonstrated larger or smaller than its parents, indicating their distinctive morphometric characters. Duong *et al*. (2017) stated that hybrid could express differently than its parents and violated the immediate assumption. In a more distance reported on hybrid between *Clarias macrocephalus* and *Pangasius sutchi* was successfully produced and yielded three morphologically different hybrids (Sittikraiwong 1987) and relatively more on *Pangasius*-like (79.69%), having two dorsal fins, while the second type showed *Clarias*-like appearances (18.27%) and the third group similar to *Clarias* (2.03%). Similarly, Okomoda *et al*. (2018) reported the hybrid of *C.*

gariepinus (CG) × *P. hypophthalmus*(PH) and its reciprocal to be Clarias-like Clariothalmus (CG ♀ × PH ♂) and Pangapinus progenies (PH ♀×CG ♂), which were indistinguishable from their maternal parents and the Panga-like Clariothalmus demonstrated phenotypic intermediary of its pure parentage.

In multivariate analysis interpretation of the results proposed that at least 10 characters which are prepelvic (PREL), caudal peduncle length (CPL), dorsal fin length (DFL), pectoral fin length (PFL), anal fin height (AFH), anal fin length (AFL), adipose fin length (ADIFL), interorbital length (IL), distant to isthmus (DSI) and predorsal length (PREL) were the strongest components as predictors to discriminate these three species which occurred largely at the body, head, and fins parts. The components that derived by Functions 1 and 2 are important to show the characters that exhibit high variation. The total variances shown by the first and second eigenvalues ranged from 86.1 to 13.9% in this study is important to clarify the variation occurred on all of the characters measured. The first eigenvalue showed majority of variances were explained in Function 1 which indicated a good fit of the model derived from the multivariate allometry to the data (Björklund, 1993), and provides information to discriminate this hybrid in the future.

The classification based on the predicted fish groups in the present study was almost perfect with 100.0% of original grouped cases and 95.0% of cross-validated grouped cases. According to Brown and Wicker (2000), investigators need to have some indication of the accuracy of the Functions derived in classifying the groups and it is important to begin with a sample of known group of fish. Most common practice to differentiate the unknown individual which could be considered as hybrid is when morphometric measurement using hybrid indices demonstrated immediate to the value of two parental species (Campton, 1987). Therefore, for a proper data collection of hybrid of known parentage of first filial (F1) generation is necessary to access the accuracy of assumptions of hybrid population in the future (Neff and Smith, 1979). Likewise, result on the cross-validate is crucial if the researches intend to classify other samples into the groups of interests and discrimination based on original or cross-validated are the most commonly used for fish in the wild such as in different geographical areas (Ballesteros-córdova *et al.*, 2016; Banerjee *et al.*, 2017). In cross-validation analysis, the construction of discriminant Function is carried out with multiple repeated analysis by leaving out one individual before categorizing this individual according to their Function which will reduce the possibility of misjudging the efficacy of discriminant Functions to classify the specimens (Ibánez *et al.*, 2017).

In the case of catfish farming in Vietnam (Legendre and Pariselle, 1998) and Thailand (Na-Nakorn and Kamonrat, 2004; Senanan *et al.*, 2004), hybrids detection based on morphology is only applicable for F1 hybrid as misclassification beyond F1 or backcross to its pure parentage occurred quite common (Scribner *et al*., 2001). Mengumphan and Panase (2015) reported that for hybrids analysis beyond F1 generations, the morphometric and meristic divergent of two hybrids beyond F1 generation which were backcross (BC: F1 hybrid, ♀ × *P. gigas* ♂) and reciprocal backcross (RCBC: F1 hybrid ♂ × *P. gigas* ♀) derived from the parental of F1 hybrid (*P. gigas* ♀ × *P. hypophthalmus*♂) and maxillary barbell length and dorsal fin length can be used to separate these two hybrids from *P. gigas* and *P. hypophthalmus*.

It is important to measure the characters with combination of conventional and truss network on different parts of the fish body as it can assist in searching for the most differential characters to differentiate hybrid. Model derived from discriminate Function analysis (DFA) is beneficial to facilitate the search for the strongest components for the discrimination. Most studies on Pangasiid species used as many as 32 components for discrimination (Slembrouck, 2005; Baharuddin *et al*., 2014; Dwivedi *et al*., 2017). Reducing these components to only those contributing most obvious differences will ease the identification of hybrid. In the current study, result derived from the multivariate discriminate analysis after removing the size factor was favourable as compared to previous study when comparison was made based on the ratio of standard length only. This is due to size factor could lead to more variations among the set of variables in morphometric studies (Junquera and Perez-Gandaras, 1993; Tzeng and Yeh, 1999). Removing the size factor is crucial as it will influence the morphometric analysis and lead to invalid result (Tzeng, 2004). In the current study, number of sample for *P. nasutus* n=10, is considerably low. The discrimination could be more efficient if the samples size of *P. nasutus* is larger. However, according to Brown and Wicker (2000), this model of discrimination is still valid as it reflects the real population in the environment since this species is scarce.

Conclusions

In conclusion, morphological variations can be used to differentiate hybrid and its parents. Then characters include PREL, CPL, DFL, PFL, AFH, AFL, ADIFL, IL, DSI and PREL can be used as predictors to discriminate hybrid and its parents. The adipose parts in particular, are larger in hybrid therefore the most favourable characters to be used for the discrimination. In addition, observation on the vomerin and palatal teeth can be used as the most practical method for rapid identification of *P. hypophthalmus*, *P. nasutus* and its hybrid.

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