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Geometric Morphometrics Analysis of Inter-Population Wing Shape Variations in Bats

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Competing interests
The authors have declared that no competing interests exist.

Abstract

Background: *The cryptic diversity of bat fauna in Pakistan demands to incorporate an efficient and reliable approach for morphological species identification. The traditional taxonomic approaches are effective in exploring variations of characters but have proved to be less efficient in quantifying the interspecific and intraspecific differences. Geometric morphometric method has recently act as an efficient tool to analyze the overall changes in shape and size of biological features. The present study is therefore conducted to exploit the use of geometric morphometric methods along with traditional morphological measurements to examine the size and shape differences among four geographically isolated population groups of insectivorous bat species* (Pipistrellus coromandra).

Methods: *Specimens were collected from different locations of Punjab, Pakistan. Twelve well-defined landmarks to quantify the variation in right wing of bats were analyzed using geometric morphometric tools and wing measurements of 5 selected parameters were also taken using traditional morphological measurements.*

Results: *The results of external measurements for wing overlapped for most part among the different studied population groups. Fur colour photographs displayed in the inter-population had shown visible change from dark brown to light brown giving an indication of more morphological differences. Regarding the geometric morphometric results, wing-shape differences were found to dominate in inter-population as compared to intra-population for bats species (*Pipistrellus coromandra*) which clearly reflects the effects of habitat factors on different populations phenotypically. The wireframe for the first two PCs indicated an overall shape change trend with the displacement of landmark points representing the expansion along the upper wing margins in PC1 compared to PC2.*

Conclusion: *The current study has successfully explored the power of geometric morphometric in reflecting the variations in wing shape among different populations of bats species (*Pipistrellus coromandra*).*

Key words: *Bats, Geometric Morphometry, Inter-population, Pakistan, Wing shape*

Introduction

Size and shape variations in organisms have long been studied for species differentiation (Cordeiro-Estrela et al., 2008) and to infer the ecological relationships (Aldridge & Rautenbach, 1987; Norberg, 1981) among different population groups (dos Reis et al., 2002). The use of geometric morphometrics (GM) approaches has gained the tremendous popularity since the late 1980s and has been used across many taxa (Adams et al., 2013; Adams et al., 2004). The increasing fame of this approach is related to its ability to offer visualization of results in terms of shape changes (Zelditch et al., 2012) and quantifying variations in the anatomical structures position relative to one another which is difficult to analyze in linear morphometric techniques. Wide application of geometric morphometrics approach comes from insect taxonomy and systematics where minute morphological variation has been detected (Baracchi et al., 2011; Francuski et al., 2009; Neto et al., 2013; Pepinelli et al., 2013). Among bats wing morphology has been extensively studied to determine foraging behaviour, flight capabilities (Adams, 1996;

Aldridge, 1986; Findley & Black, 1983) along with habitat use (Kalcounis & Brigham, 1995). Traditional taxonomic approaches explore morphological variations of characters (McPeek, 1990; Michaux, 1989) but it is sometimes difficult to quantify intraspecific or interspecific variations (Barao et al., 2014; Nedeljković et al., 2013; Riedel et al., 2013). Geometric morphometrics based on two- or three-dimensional Cartesian coordinates of landmarks act as an efficient tool to analyze and quantify the overall changes in shape of biological structures (Adams et al., 2013; Bookstein, 1991). Loss of habitat and urbanization are seriously causing threats to bat populations in their natural habitats and has led many species to change their distribution range or become extinct (Meyer et al., 2010). Therefore, studies employing species distribution and effects of habitat factors on population are extremely important for the conservation of species.

According to reported data the Chiropteran diversity of Pakistan consists of 8 families, 26 genera and 50 species (Roberts, 1997). Bat fauna in the country has not been explored extensively with some reported studies focusing on generating taxonomic data based on traditional morphological measurements only (Perveen & Faiz-ur-Rahman, 2015; Javid et al., 2012; Javid et al., 2014; Hamidullah et al., 2018; Mahmood-ul-Hassan & Salim, 2014; Salim et al., 2016). Geometric morphometric methods have been employed by many researchers for examining the wing morphology of bats (Hedrick & Dumont, 2018; Nogueira et al., 2009; Ospina-Garcés et al., 2016; Richards et al., 2012; Schmieder et al., 2015) but these methods have been explored relatively more among other taxa (Birch, 1997; Camargo & de Oliveira, 2012). Bats wing morphology is mostly studied based on five standard measurements (Dietz et al., 2006) including forearm length with wrist representing bat size, length of 5th finger excluding wrist determines wing width (Fenton, 1990), 3rd finger length excluding wrist reflects hand wing length(Blood & McFarlane, 1988; Fenton, 1990) length of 4th digit; 1st phalanx and 2nd phalanx are important for the identification of species (Paunović & Stamenković, 1998). Such efforts have stimulated the other workers to carry out further research in the taxa, but efforts to examine the diversity based on recent techniques is severely lacking in Pakistan.

Therefore, the present study has been aimed to analyze the efficiency of geometric morphometric method over traditional morphological techniques in examining the size and shape variations among four isolated population groups of insectivorous bat species (*Pipistrellus coromandra*). Specimens were captured from locations including urban (Kahna, District Lahore); semi-urban (Head Baloki, District Kasur) and rural areas (Chak 107, District Sargodha & Piru Chak, District Gujranwala) to visualize the effects of habitat factors which might lead to difference in external morphological features.

Materials and Methods

Study area

The study was conducted across different locations in Punjab, Pakistan. Sampling areas included Kahna District, Lahore (KA) and Head Baloki District, Kasur (HB) in the East of Punjab, District, Sargodha (SA) in the Northwest of Punjab and District, Gujranwala (GJ) in the North of Punjab.

Animal Sampling & Processing

From each population six specimens (*Pipistrellus coromandra*) were collected with hands/hand nets from their roosting sites during the daytime. Field work was carried out under the permission from institutional bioethics committee (D/503/UZ) & from the Wildlife & Parks Department, Punjab (No. 2839). Only adult bats were captured, and adult status was confirmed by transilluminating the wing and checking for epiphyseal fusion of long phalanges (Brunet-Rossinni, & Wilkinson, 2009). The specimens were euthanized by cervical dislocation for further taking morphometric measurements. Sample ID along with other details is given in (Table 1).

Traditional Morphological Analysis

Wing measurements of 5 parameters were taken using Whitworth Electronic Digital Caliper following Dietz et al., (2006) (Table 2). Digital photographs (16 megapixels) of randomly selected specimens from each population were taken under standard lightening conditions to represent fur colour variation from the dorsal side.

Geometric Morphometric Analysis

Geometric morphometric (GM) analysis was conducted to quantify the size and shape variations in right wing of bats used in the current study in order to assess the relationship of habitat factors with morphometric shape changes.

Landmark Positioning & Digitization

Bat specimens were fully stretched on a graph sheet using an adhesive fixed on a soft board so as to open their wings at their maximum length. Each specimen was kept with its dorsal side held towards the board. Twelve well defined landmarks were chosen from the literature (Schmieder et al., 2015; Von Busse et al., 2012) and defined in (Table 3).

Each landmark position was marked on the stretched wings using a sharp needle and resulting points were then numbered on graph sheet followed by multiple photos (16 megapixels) of each specimen. The coordinates of each selected landmarks were digitized using Morphometric tools at SUNY Stony Brook; tpsDIG2w32 (Rohlf, 2010a) after converting the image file into tps file format using the application tpsUtil 1.76 (Rohlf, 2010b). To reduce measurement error, the best picture was selected based on clarity of landmarks positions marked, digitized three times and landmark positioning error was measured by calculating standard deviation between Procrustes coordinates along each dimension and repeat data of landmark coordinates on each digitized image to ensure the landmarks repeatability.
To examine the wing size and shape variations among species collected from different locations analysis was performed including: Procrustes Superimposition and Principal Component Analysis (PCA). The results were computed using the Morpho J software (Klingenberg, 2011). (http://www.flywings.org.uk/morphoj_guide/index.htm).

Procrustes Superimposition (PS)

Dataset was subjected to procrustes superimposition on Morpho J; the method performs the minimization and centralization of distances between landmarks to obtain landmarks standardization from the centroid size (CS). CS reflects the me-

Table 1: Locality and methods of collection for all specimens used in the study.

ID	Location	District	GPS coordinates		Roosting site	Method of capturing
			Latitude	Longitude		
BI to BVI (KA)	Kahna	Lahore	31.3738	74.3675	Wall of old building	Hand
BI to BVI (HB)	Head Baloki	Kasur	31.2222	73.8589	Old Bridge	Hand net
BI to BVI (SA)	Chak 107	Sargodha	31.9669	72.5724	Trees	Hand
BI to BVI (GJ)	Piru Chak	Gujranwala	32.1821	74.2692	Old House ceiling	Hand net

Table 2: Wing Morphological features of captured bat specimen used in the study (adapted from Dietz et al., 2006).

Wing	Abbreviations
Forearm length	FA
Length of 5th finger	D5
Length of 3rd finger	D3
Length of 1st phalanx of 4th digit	P4.1
Length of 2nd phalanx of 4th digit	P4.2

Table 3: Landmarks with allotted numbers and their respective position on right wing of bat for digitization.

Wing Region	Landmark Number
The wing tips	LM-1
3rd digit; joint between the first and second phalanges	LM-2
2nd digit tip	LM-3
4th digit tip	LM-4
4th digit joint; between the first and second phalanges	LM-5
4th digit joint; between the first phalanx and metacarpal	LM-6
Point between leading edge of the wing and thumb	LM-7
5th digit tip; foot	LM-8
Wrist; tip of the elbow	LM-9
The connection of the elbow to the body	LM-10
The connection of the wing membrane to the body; elbow	LM-11
The connection of the wing membrane to the foot; and hip	LM-12

-asurement of the variation of the shape analyzed and is calculated by the "Square root of the sum of the square distance of each anatomical landmark to the mass center of each configuration (centroid)" (Bookstein, 1991).

Principal Component Analysis (PCA)

The detailed analysis of shape changes among datasets was investigated by principal component analysis (PCA) from the covariance matrix. PCA depicts the major features of shape variation in a dataset. PCA results of the current study are presented in the form of wire frame graphs (scale 0.1), transformation grids and scatter plots.

Statistical Analysis

Morphological variations were statistically tested in each population group using the mean and Standard Deviation (SD) for all wing parameters. The data was also subjected to ANOVA (one way analysis of variance) with the Tukey's Multiple Comparison test at 95% confidence interval. All statistical analyses were performed using GraphPad prism 5.

Results

Traditional Morphological Analysis

The means of means ($\bar{x}$) of measured wing parameters had shown overlapped values among the different population groups (KA, HB, SA and GJ). Analysis of variance results had shown no significant difference ($P < 0.05$) for most of the external wing measurements except for KA population showing significant difference ($P > 0.05$) for P4.1 with the rest of the populations and for SA and GJ populations showing significant difference ($P > 0.05$) for P4.2 (Figure 1; Table 4).

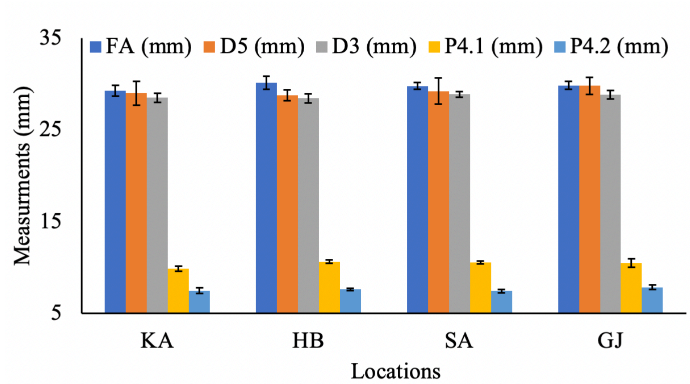

Figure 1: Comparison of mean of means ($\bar{x}$) of wing parameters measurements for different populations.

Fur colour photographs displayed in (Figure 2) had shown change in fur colour among different population groups from dark brown to light brown i.e., GJ Population > HB Population > SA Population > KA Population. These variations in fur colour are indicative that there could be more morphological differences across the population groups and must be further analyzed.

Geometric Morphometric Analysis

Landmark positioning error

Standard deviation (SD) around each centroid (X & Y coordinates) for each landmark was calculated for all specimens in a population which had shown the mean and standard deviation values within the range of 0 to 4.

Procrustes superimposition

Superimposition of right wing among different population had shown the following inter population landmarks (LM) observations. Regarding Kahna (KA) population LM-2 (3rd digit; joint between the first and second phalanges), 3 (2nd digit

*Table 4: Mean of means (x̄) and Minimum-Maximum Range (R) of External wing measurements of adult bats (*Pipistrellus coromandra*) collected from different sites of Punjab (Pakistan). Abbreviations of wing parameters are given in Table 2.*

Sample Sites	FA (mm)		D5 (mm)		D3 (mm)		P4.1 (mm)		P4.2 (mm)	
	x̄ ± SD	R	x̄ ± SD	R	x̄ ± SD	R	x̄ ± SD	R	x̄ ± SD	R
KA	29.28 ± 0.58	28.62-29.77	28.98 ± 1.31	27.36-30.59	28.51± 0.50	27.92-29.21	9.88 ± 0.28	9.72-10.37	7.48 ± 0.30	7.31-7.91
HB	30.12 ± 0.71	28.86-30.48	28.78± 0.58	28.26-29.74	28.43± 0.48	27.70-28.94	10.64 ± 0.17	10.45-10.90	7.62 ± 0.11	7.49-7.74
SA	29.77± 0.36	29.39-30.26	29.22± 1.41	27.07-30.44	28.87 ± 0.32	28.49-29.26	10.56 ± 0.13	10.37-10.72	7.43 ± 0.17	7.18-7.65
GJ	29.83± 0.43	29.39-30.52	29.79 ± 0.93	28.67-30.90	28.85 ± 0.47	28.31-29.29	10.50± 0.48	9.67-10.84	7.86 ± 0.23	7.56-8.19

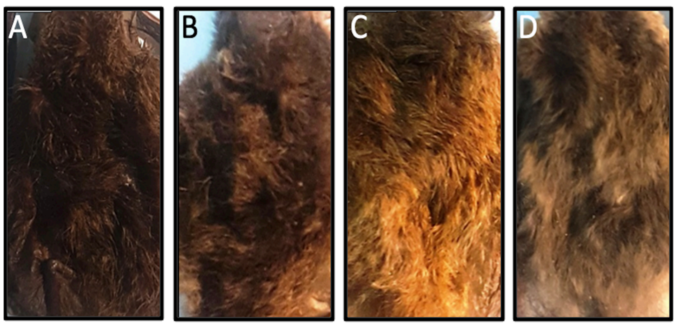

Figure 2: Variation of fur colour (dorsal side) for four individuals of Pipistrellus coromandra *collected from different locations. (A) GJ (B) HB (C) SA (D) KA.*

tip), 7 (point between leading edge of the wing and thumb) & 9 (wrist; tip of the elbow; wrist) marking the upper boundary of the right wing had shown a change from the centroid as compared to its lower boundary of the same wing. Whereas on LM- 4 (4th digit tip) & 5 (4th digit joint; between the first and second phalanges) a sharp change has been noted from its centroid (Figure 3A).

Specimen collected from Head Baloki (HB) had shown very close pattern with the centroid, while in the same population there is a change at LM-4 (4th digit tip), 5 (4th digit joint; between the first and second phalanges) & and from landmark 5 to 6 (4th digit joint; between the first phalanx and metacarpal) when compared to the KA and GJ population (Figure 3B).

Specimen collected from Sargodha (SA) had shown narrowing of wings along upper margins at landmarks 2, 3, 7, 9, 10 & 11 (Figure 3C).

When Gujranwala (GJ) population is considered, a clear change has been noted at landmarks 9 & 10 i.e., wrist & connection of the elbow to the body, which had shown a downward change at its tip from the centroid. Concurrently, narrowing of the wing pattern along upper margins has been noted in this population when compared with KA population (Figure 3D).

Principal component analysis (PCA)

Already described landmarks yielded 20 shape variables, and accordingly 20 principal components (Table 5). Not all PCs generated are included in the study and only those PCs are retained having high percentage of total shape variation so only PC1 and PC2 were analyzed further. The 20 PCs generated each had progressively less variance, with PC1 (62.272%) and PC2 (21.373%) representing more than 83% of the total variance (Figure 4). Scatter plots of PCA illustrate the individual specimens separated by color category of different locations. It depicts that individuals which share common shape are clustered together while distant population groups are clustered far apart.

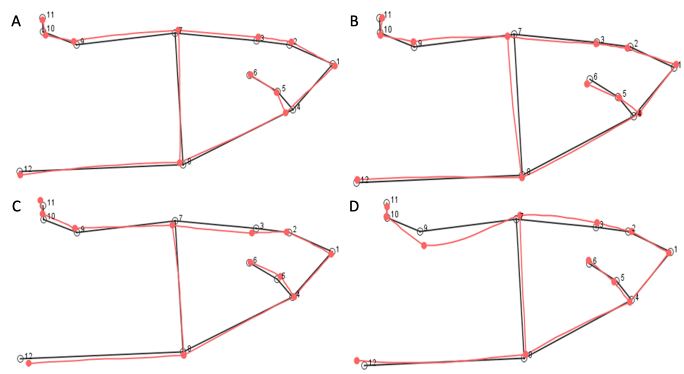

Figure 3: Procrustes superimposition results for right wing of bats for different populations A: KA Population: B: HB Population C: SA Population D: GJ Population. Shape change is given by the red line while; black line represents the mean shape.

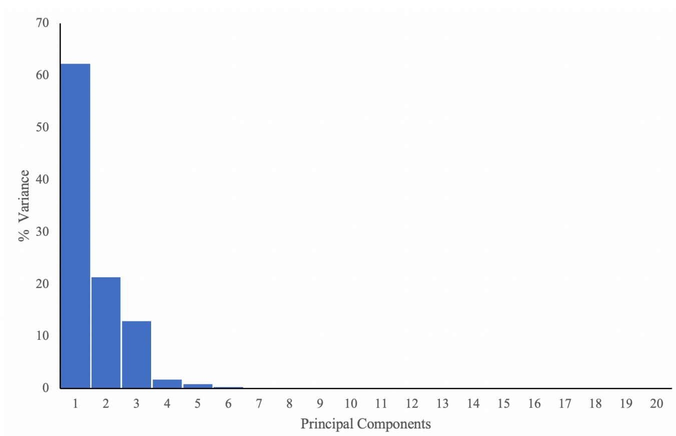

Figure 4: Total percentage variance depicted by all Principal Components (PCs) generated from analysis complete dataset of all studied Bats population groups from Punjab, Pakistan.

The scatter plot of different populations along PC1 and PC2 is presented in (Figure 5A-C); The GJ population (Red) are clustered together at PC1 (the positive end axis) whereas KA population (Cyan) came at the positive end along the PC2 axis. The remaining two population groups, SA (purple) and HB

(Neon Green) have clustered close relative to each other with HB population along negative axis of PC1 and SA population along positive axis of PC2 (Figure 5C).

PCA represented by wireframes depicted overall shape changes from the centroid and indicate which landmark points contribute most to shape differences. Wireframe graph depicted by PC1 & PC2 (Figure 5D & E) had shown that only slight variation occurred on LM-1 (wing tip), 2 (3rd digit; joint between the first and second phalanges), 10 (elbow connection point to the body) and 11 (wing membrane connection to the body) in PC1 whereas in PC2 slight change was found on LM-3 (2nd digit tip), 5 (4th digit joint; between the first and second phalanges), 6 (4th digit joint; between the first phalanx and metacarpal) and 11 (wing membrane connection to the body).

Table 5: Principal Components Analysis (PCA) of complete dataset for all population groups studied from Punjab, Pakistan.

PC	Eigenvalues	%Variance	Total%
1	0.00078909	62.272	62.272
2	0.00027083	21.373	83.645
3	0.00016357	12.908	96.553
4	0.00002200	1.736	98.289
5	0.00001042	0.823	99.111
6	0.00000386	0.305	99.416
7	0.00000142	0.112	99.529
8	0.00000130	0.103	99.632
9	0.00000107	0.084	99.716
10	0.00000088	0.069	99.785
11.	0.00000081	0.064	99.849
12.	0.00000055	0.043	99.892
13.	0.00000037	0.029	99.921
14.	0.00000031	0.025	99.946
15.	0.00000026	0.020	99.966
16.	0.00000019	0.015	99.981
17.	0.00000015	0.012	99.992
18.	0.00000006	0.005	99.997
19.	0.00000002	0.002	99.999
20.	0.00000001	0.001	100.00

Discussion

This work has highlighted the importance of geometric morphometrics in considering the non-isotropic variations among studied population groups of bats from selected areas of Punjab, Pakistan. Geometric morphometrics as a powerful tool enables to detect shape changes visually among different groups, which is not possible when only employing traditional morphometric approaches. Measurements of external wing morphology of bats have long been used for the identification of species and forearm length (FA) is considered as most important predictor. In the present study mean FA lengths of the captured specimens were compared with reference measurements for spe-

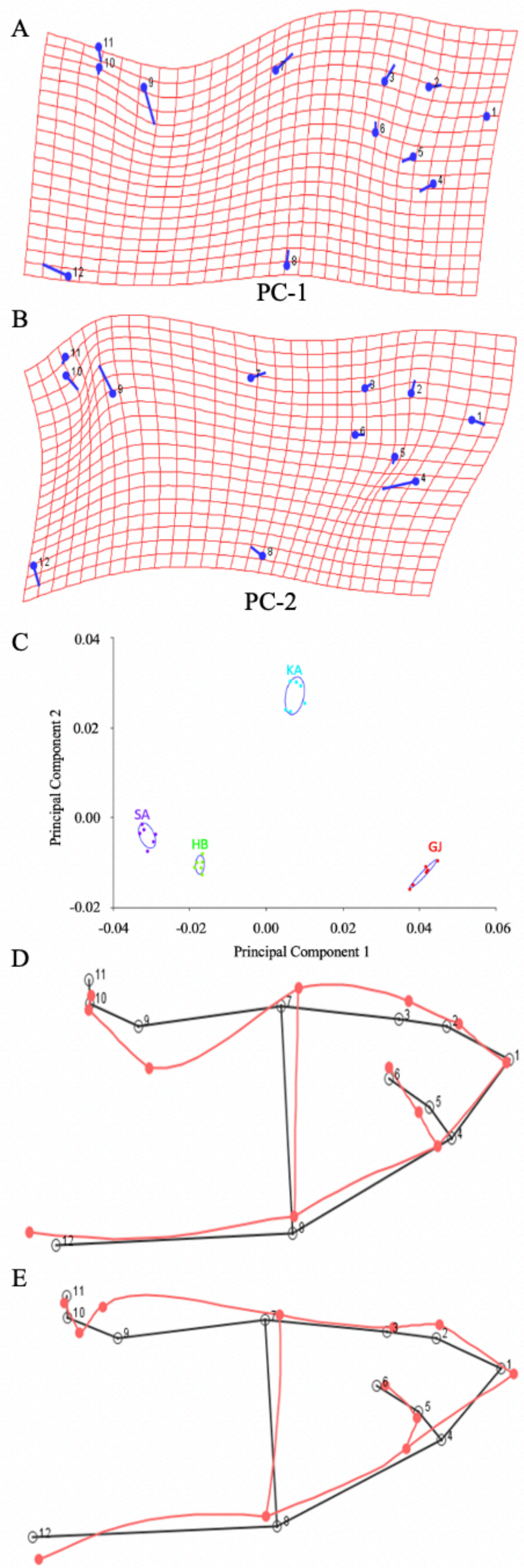

Figure 5: Distribution of specimens from the whole dataset categorized by Location along axis of PC1 (A) and PC2 (B). The specimens from different locations are coded with different colors: Red (Gujranwala); Purple (Sargodha); Neon Green (Head Baloki); Cyan (Kahna) (C). Wireframe graphs illustrating the shape changes on the PC1 (D) & PC2 (E). Red line represents shape change; black line represents the mean shape.

cies *Pipistrellus coromandra*. According to (Roberts, 1997) the average length of forearm for the species was 32 mm long (range 31-33 mm) while the findings of (Bates & Harrison, 1998) had shown the forearm length of 30.0 mm (25.5 -34.3 mm). These lengths are comparable to the findings of the current study. These wing measurements provide an excellent predictor to determine the habitat use and habitat structure of bats. Shorthand wing in relation to forearm and wing width along with small body size is associated with dense vegetation cover whereas the species with longer hand wings will prefer the open spaces (Dietz et al., 2006).

In this study the wing shape was analyzed in different populations of insectivorous bats (*Pipistrellus coromandra*). Regarding the results wing-shape differences were found to dominate in inter-population as compared to intra-population which clearly reflects the effects of habitat factors on the bat's populations phenotypically. (SEVCIK, 2003) analyzed the wing shape variability of the bat genus Plecotus in Central Europe and found interspecific differences which clearly reflected among the species especially in the relative length of phalanges of digit III, IV and V. The minor variations among the species reflect the differences in habitat use and foraging adaptations (Kalcounis & Brigham, 1995) and provide excellent examples in the context of functional ecology.

All studied population groups in the present study had shown variation at landmark points from the centroid with population from HB showing less variation from the mean shape. There was a clear trend of widening of wing from upper margins in KA population as compared with other populations. In GJ population from leading edge of the wing (LM-7) to wrist (LM-9) and connection of the elbow to the body (LM-10) there was sharp narrowing of the wing. Wing tip (LM-1) has contributed least to the shape variations among the populations. Hence, it is justified to analyze morphological variations in intraspecific geographically distant population groups exposed to different environmental conditions. Two species of bats Genus *Pipistrellus* i.e., *Pipistrellus pipistrellus* & *Pipistrellus pygmaeus* were successfully discriminated morphologically based on variations in baculum shape using geometric morphometrics methods (Herdina et al., 2014). The two species although have been recognized as separate species genetically since 1997 but no reliable morphological species discriminating trait was validated. It is extremely important to determine morphological details quantitatively so that cryptic species diversity could be understood and their phylogeographic distributions can be explained.

The wireframe for the first two PCs indicated overall shape change trend with the displacement of landmark points representing the expansion along the upper wing margins in PC1 compared to PC2. (Schmieder et al., 2015) analyzed interspecies differences in European horseshoe bats (Rhinolophidae, Chiroptera) using geometric morphometrics based on wing morphology wherein between group principal components highlighted the difference between species lies in the extent to which wing reaches in the direction of the head. Regarding the scatter plot along PC1 and PC2 of the current work had shown clear integration among individuals from one population forming clusters which is further supporting the inter-population shape differences.

Conclusion

The current study clearly shows the power of geometric morphometric methods over traditional morphological approaches for determining the variations among different population groups of insectivorous bats species (*Pipistrellus coromandra*). Moreover, it is further concluded that analyzing the bat wing morphology with procrustes methods and multivariate statistical analyses has demonstrated efficiently even slight changes in wing shape in populations from different locations; hence indicating the effects of habitat factors on morphology of species.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: ZA, MHA, NS; data collection: ZA, MHA; analysis and interpretation of results: ZA, MHA, SRA, NS; draft manuscript preparation: ZA, MHA. All authors reviewed the results and approved the final version of the manuscript.

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