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Integrative approach in describing *Neurothemis* species using correlation analysis based on distances

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ABSTRACT

Describing species variation and delineation are fundamental to biology and much debate exists surrounding on what applied approach is appropriate. Species delineation now used separate elaborate datasets to quantify independently and test species criteria. However, the complexity of the speciation process has ushered the need to infuse studies with new tools and techniques capable of aiding in species delineation. Herewith, an integrative approach using Correlation Analysis based on Distances was used to circumvent the traditional morphological analysis and provide a novel means of describing closely related complex species (sibling species) diversity using the genus Neurothemis as a case study. Correlation Analysis based on distances proved to be useful by looking into the relative contribution of each trait considered to species/group divergence and distinctiveness. Results demonstrate noted differences between female and male morphs. On one hand, females exhibited female-limited polymorphism which was suggested to have possibly evolved throughout sexual selection. On the other hand, polymorphism being limited to males mostly plays a role in male-male competition for access to females. Differences were attributed mainly by external morphological wing characters such as the fore- wing triangle, hind wing triangle, the radial planate, fore- wing subtriangle, number of anal loop 'sole cells', hind wing supertriangle, number of cross veins in the cubital space of the hind wing (behind the median space), wing pigmentation, shape of synthorax, shape of epiproct and shape of left and right cerci. Here, the utilization of a number of characters for species delineation proved to be effective in understanding variation and the nature of the Neurothemis species found in Iligan City.

INTRODUCTION

It has been a subject of tremendous debates about the applied approach to describing diversity. Wherein, one must vividly distinguish the nature of these two scientific tasks: delineating and classifying species and the most fundamental question of taxonomy is not how to identify species, but rather how to delineate them. Species delineation is said to be fundamental to the discovery of life's diversity because through it one can determine and recognize whether or not different specimens are members of the same cohesive lineage. Careful consideration of species delimitation is required to support crucial decisions based on accurate species identification [1,2]. Erroneous species boundaries or diversity estimates may often lead to incorrect answers to such questions as to how phylogenetic trees must be reconstructed rigorously and analyzed critically because these are first steps toward discussing broader questions. Proponents from different disciplines raised issues as to what characters should be used to delineate organisms. It is evident that no single individual is "typical" of the characters of the population and

the continuous variation among the members of a population manifests itself most conspicuously in linear measurements and proportions. Mean values, variances and coefficients for each trait are characteristic of each population and species [3,4,5,1]. Hence, each character may show a different degree of variability within a single population. Similarly, there are different degrees of variability among related species. Thus, the question on what characters to use or what characters would best delineate species.

Apparently, the uses of morphological characters of adult specimens among others have become frequent, but they are supplemented already to an increasing extent by other characters [6,7]. This is particularly true for "difficult" species, genera, and families in which the evidence from morphology has been equivocal or contradictory. The increasing utilization of new characters is justified because (1) morphology reflects only part of the genotype and may not reflect genetic relationship accurately, (2) morphology in certain taxa does not supply sufficient characters, and (3) any character may be misleading because of special adaptations. Thus, the introduction of new kinds of systematic/taxonomic characters has been one feature of the so called new systematics. However, these characters do not displace the use of traditional morphological characters [5]. Meanwhile, the emergence of Geometric Morphometrics (GM), also known as the statistical analysis of shapes, somehow, became a breakthrough because it is a collaboration of different fields (Image Analysis, Biology, Geometry, Statistics) based on Cartesian landmark coordinates. The method works by separating shape from overall size, position, and orientation of the landmark configurations, thus the resulting Procrustes shape coordinates can be used for statistical analysis. The powerful visualization tools and the typically large amount of shape variables gave rise to a specific exploratory style of analysis, allowing the identification and quantification of previously unknown shape features [8,9]. Herewith, these systems of identification and delineation should not be seen as competing against each other or exclusive, but rather as approaches to the same goal which simply differ in the characters they consider. One system or the other may be favoured for particular cases depending on the results it provides, but they also can be used concurrently. It is very likely that what will work best for identification will depend on the particular species being considered, even among closely related species.

Thereby, an integrative approach to taxonomy should now become general. Wherein, it became necessary because the complexity of species biology requires that species boundaries be studied from multiple, complementary perspectives [10]. Also, the level of confidence in species supported by different kinds of data is much higher than for species supported by only one kind. A way to collate all available characters is important. Such approach could help solve problematic species complexes also addressing cryptic speciation occurring in some odonate species. But, it is inevitable that discordant characters may create a problem. To circumvent the issue, the utilization of a technical approach is necessary which has the capacity to collate all available datasets thus, demonstrating the process of integrative taxonomy for studies on species delineation and variations.

In Odonata an enormous amount of variation is prevalent both in their life histories and their activities [11]. Odonates as model organisms are of particular interest due to the number of characters that can relatively be considered. The wings alone are a rich source of taxonomic characters. Here, a clear understanding of the relationships among the Odonata can have far reaching implications in Odonate biology and over the past 45 years, there have been many studies attempting to resolve the relationships within the Odonata [12,13,14,15,16,17] based on morphological characters. In particular, the genus *Neurothemis* is said to be a difficult genus having exhibited notable variations even within sibling species. This had befuddled many taxonomists where species can be very similar in terms of appearance, behaviour and other notable characteristics [18,19]. There are numerous species of Libellulid dragonflies under the genus *Neurothemis* where most species are red in color and among these are the two (2) most popular species found in Iligan City: *Neurothemis terminata*, Ris 1911 and *Neurothemis ramburii*, Kaup and Brauer 1866. These two species were considered problematic because they showed similarities in terms of color and patterns which often lead to confusion and difficulty in identification process. Males of the species are very similar except for a few traits and the female usually exhibits female-limited polymorphism [20,21,22,19]. Although there are many morphological studies that had attempted to use different characters to resolve the relationships of the Odonates based on wing venation [12,14,16], morphology of the flight apparatus and copulatory structures [15], none have been able to come to robust conclusions. Recently there were also several studies conducted on Odonates (Libellulidae) using cytotaxonomical method [23], evaluation based on morphological and physiochemical characters [24], characters based on external genitalia [25], and a study on the relationships of Anisoptera based on mitochondrial 16S rRNA gene sequences [26]. However, there is a dearth of studies demonstrating the process of integrative taxonomy for studies on species delineation and variations. In this study, a new procedure which is a combination of already available analytical methods: Squared Euclidean distance, geometric morphometics:

landmark and outline based approaches and correlation analysis based on distances were utilized to look into relationships of species belonging to one controversial and difficult genus. Independent of any form there be should a dependable and fast method for species delineation and incorporating characters from other disciplines will increase reliability. Herewith, morphological inference is enhanced by providing sets of data directly applicable to a taxonomic problem [10,27].

A major hurdle would be a corroboration of species boundaries via independent lines of evidence for diagnosing species [28], and it is in this perspective that motivates the investigation of a integrative approach for species delimitation. This study is a simulation that was used to evaluate the accuracy of an integrative approach for delineation and understanding species variation on the genus *Neurothemis*. Linnaeus's dictum: "It is the genus that gives the characters, and not the characters that make the genus." The soundest genera are based on an overall appreciation and weighing of various considerations [29,30]. The species included in a genus usually have many features in common, and recognition of a higher taxon is generally based on the presence of a correlated character complex, but as Darwin (1859) said "The importance, of classification of characters, mainly depends on their being correlated with other several characters of more or less importance, the value indeed of an aggregate of characters is very evident in natural history," [31, 5].

MATERIALS AND METHODS

1. Specimen Collection, Preservation and Identification

The specimens were collected from different areas in Iligan City. A total of 12 morphotypes (8 female morphs and 4 male morphs) were collected belonging to two *Neurothemis* species. This was done by using sweep nets for catching samples and appropriate preservation techniques were applied. The specimens were then placed in envelopes for a while to void any intestinal contents then, were immersed briefly in 95% acetone. Acetone extracts fat and water from specimens, and suited for better drying and color preservation. The abdomens were straightened and the legs were arranged in place for each specimen, such that the genitalia were not obscured on the second abdominal segments of males. Specimens were then placed in labeled envelopes and submerged in acetone (in a tightly closed plastic container) for 16 – 24 hours inside air-tight specimen containers. Afterwards, the specimens were removed from the acetone and placed in the open for a few days, by then the acetone had completely evaporated. Specimens were then taken out of the glassine envelopes and stored permanently in clear envelopes made of cellophane, mylar or polypropylene. The identification and collection data were typed on 3" x 5" cards, which were inserted in the envelopes behind the specimen. The envelopes were then stored like a card file in cabinets [32]. Identification process was done using available taxonomic keys, field guides, monographs and consultation with odonate experts under International Dragonfly Fund [19].

2. Morphological Analysis and Phenetic Analysis

Thirty (30) individuals per morphotype (morph) were examined based on their morphology for a set of characters. Adult morphological and biological characters were selected for coding. A character is the 'presence' versus 'absence' of something (taxon, species with a particular morphological structure) or it could be a plain description of the external morphology of organisms [33,34,35]. External morphological data were utilized in order to prepare the character state table and the character matrix. A total of 57 characters were used. All of the observed characters were assigned values of 0, 1,2,3 or 4… and these values were used to conduct the phenetic analysis using Statistical Package Social Science (SPSS) for IBM, version 19 (Table 1 and 2). The characteristics of the species were then equally weighted and treated unordered for quantification, objectification and efficient classification. The names of the species were substituted with the Operational Taxonomic Unit (OTU) code numbers in order to be suitable for computer processing. The raw data obtained were then organized in order to measure the similarity between the OTUs. The Squared Euclidean Distance (Distance $(X \times Y) = \Sigma(Xi - Yi)^2$) algorithm was employed to calculate the dissimilarity coefficient and then categorized the ones with the lowest coefficient and connected them to the one with the higher coefficient. Resulting squared distances values were later loaded to CORIANDIS software for data analysis. A total of 57 characters were used inclusive of qualitative and quantitative characters [36,37].

Table 1. Morphological Character Selection and Coding

CHARACTERS
1. Compound eye: (0) contact; (1) apart
2. Triangle direction of forewing and hindwing: (0) different; (1) the same
3. Corpora incerta of compound eye: (0) curved; (1) smooth
4. Lobe on abdomen's second node: (0) absent; (1) present
5. Metallic luster on thorax: (0) absent; (1) present
6. Anal angle of hindwing: (0) projected; (1) round
7. Abdomen length: (0) over 40 mm; (1) under 40mm
8. Hindwing length: (0) over 40 mm; (1) under 40mm
9. Abdomen's third node: (0) not; (1) slender
10. Shape of abdomen's first, second, and third nodes: (0) not; (1) club–shaped
11. Superior appendage, compared with inferior appendage:(0) not; (1) longer
12. Back side of superior appendage: (0) bending; (1) straight
13. Pterostigma: (0) not; (1) black
14. Yellow line on front head: (0) none; (1) 1; (2) 2
15. Two black spot patterns on frons: (0) absent; (1) present
16. A band on the brim of the upper frons: (0) absent; (1) straight; (2) T–shaped
17. A pattern on the whole abdomen: (0) absent; (1) present
18. Membranule: (0) small; (1) big
19. Brindle on wing: (0) absent; (1) present
20. Basal part of hindwing: (0) not; (1) transparent
21. Macrotrichium on prothorax anal margin: (0) dense; (1) thin
22. White powder on abdomen: (0) absent; (1) present
23. The end of the wing: (0) not; (1) colored
24. Lobe on the abdomen's tenth node: (0) absent; (1) present
25. Abdomen: (0) not; (1) wide and flat
26. Wide yellow band on the abdomen's second, third, and fourth nodes: (0) absent; (1) present
27. Both sides of the abdomen's node: (0) not; (1) saw–toothed
28. Two yellow bands on the medithorax: (0) absent; (1) present
29. Vein: (0) not; (1) black
30. Costa: (0) not; (1) black
31. Leg: (0) not; (1) black
32. Cercus: (0) black; (1) brown; (2) white
33. Cercus length: (0) long; (1) short
34. Brace vein: (0) absent; (1) present
35. Antenodal crossvein: (0) not; (1) in line
36. Right Fore- wing triangle: (0) one cell; (1) two cells; (2) three cells; (3) four cells; (4) five cells; (5) six cells; (6) seven cells; (7) eight cells; (nine cells; (9) ten cells; (10) eleven cells; (11) twelve cells; (12) thirteen cells
37. Left Fore- wing triangle: (0) one cell; (1) two cells; (2) three cells; (3) four cells; (4) five cells; (5) six cells; (6) seven cells; (7) eight cells; (nine cells; (9) ten cells; (10) eleven cells; (11) twelve cells; (12) thirteen cells
38. Right hindwing triangle:(0) one cell; (1)two cells; (2) three cells; (3) four cells
39. Left hindwing triangle:(0) one cell; (1)two cells; (2) three cells; (3) four cells
40. Right wing Radial planate: (0) one cell wide; (1) combination of one and two cells wide; (2) two cells wide;(3) combination of two and three ce wide. The radial planate is outlined by two longitudinal veins. This character has been used as a key character at the generic level.
41. Left wing Radial planate: (0) one cell wide; (1) combination of one and two cells wide; (2) two cells wide;(3) combination of two and three ce wide.
42. Right fore- wing subtriangle: (0) one cell; (1) two cells; (2) three cells; (3) four cells; (4) five cells; (5) six cells; (6) seven cells; (7) eight cells; twelve to twenty cells.
43. Left fore- wing subtriangle: (0) one cell; (1) two cells; (2) three cells; (3) four cells; (4) five cells; (5) six cells; (6) seven cells; (7) eight cells; twelve to twenty cells.
44. Anal loop shape: (0) indistinct or absent ('boot' shape not present); (1) boot-shaped with straight midrib; (2) boot-shaped with midrib ankled "ankle." Fraser (1957) viewed an indistinct anal loop as an ancestral state for Libellulidae.
45. Right wing Anal loop 'sole' cells: (0) three cells; (1) four cells; (2) five cells; (3) six cells; (4) seven cells; (5) eight cells; (6) nine cells; (7) elev cells; (8) twelve to twenty
46. Left wing Anal loop 'sole' cells: (0) three cells; (1) four cells; (2) five cells; (3) six cells; (4) seven cells; (5) eight cells; (6) nine cells; (7) elev cells; (8) twelve to twenty
47. Radial planate: (0) absent or weakly defined; (1) strongly defined
48. Medial planate: (0) absent or weakly defined; (1) strongly defined. Most libellulids have obvious medial planates, but some species do not have.
49. Hind wing subtriangle: (0) absent; (1) present
50. Right hind wing supertriangle: (0) one cell; (1) two cells; (2) three cells
51. Left hind wing supertriangle: (0) one cell; (1) two cells; (2) three cells
52. Dominant body and wing color or pigmentation: (0) deep red; (1) light golden brown; (2) darker shade of brown; (3) brownish but w transparent hues
53. Fore- wing antenodal cross veins (ACVs): (0) continuous through subcosta; (1) some skewed below subcosta making crossveins app discontinuous
54. Hind wing ACVs: (0) continuous through subcosta; (1) some skewed below subcosta making crossveins appear discontinuous. The skewed state of the ACVs in either the fore- wing or hind wing is rare in libellulids, but when present, is quite visible as crossveins that are not collinear above and below the subcosta.

55. Hind wing nodus position: (0) equidistant between wing base and pterostigma; (1) closer to pterostigma than wing base; (2) closer to wing base than pterostigma. This character appears to be influenced by the wing size and lifestyle of the taxon.
56. Basal area of the hindwing: (0) unexpanded with wing width nearly equal throughout; (1) wing base expanded and widest part of wing giving hindwing a triangular shape.
57. Number of cross vein in the cubital space of the hind wing (behind the median space): (0) one; (1) more than one

** Adapted from Kim, et al., 2009; Pilgrim and Von Dohlen, 2008*

Table 2. The Character data matrix for phenetic analysis

	CHARACTERS																																																							
OTU	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
N. ter(f)	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	1	1	1	1	1	0	0	5	5	2	5	3	1	1	0	1	1	2	0	0	1	0	1
N. ramM1(f)	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	1	1	0	1	1	0	1	2	1	2	1	0	0	5	4	2	4	3	1	1	0	1	1	2	0	0	1	0	0
N. ramM2(f)	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	1	0	1	1	0	1	3	4	1	1	0	0	4	4	2	3	2	1	1	0	2	1	2	0	0	1	0	1
N. ramM3(f)	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	1	6	6	2	2	1	1	6	5	2	4	4	1	1	0	1	1	2	0	0	1	0	0
N. ramM4(f)	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	1	0	1	2	0	1	1	1	1	4	4	2	3	5	1	1	0	0	0	2	0	0	1	0	0
N. ramM5(f)	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	2	5	2	2	1	1	5	6	2	5	4	1	1	0	2	2	2	0	0	1	0	0
N. ramM6(f)	1	0	0	1	0	0	1	1	0	0	1	1	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	1	0	1	6	5	1	2	1	1	7	7	2	3	3	1	1	0	1	1	2	0	0	1	0	0
N. ramM7(f)	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	1	0	1	5	4	2	2	1	1	7	7	2	4	4	1	1	0	2	2	1	0	0	1	0	0
N. terM1(m)	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	8	8	1	1	1	1	8	8	2	7	7	1	1	0	1	1	0	0	0	1	0	1
N. terM2(m)	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	1	8	8	1	1	3	3	8	8	2	8	8	1	1	0	1	1	1	0	0	1	0	1
N. ramM1(m)	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	10	12	3	4	3	3	8	8	2	7	7	1	1	0	2	2	0	0	0	1	0	0
N. ramM2(m)	1	0	0	1	0	0	1	1	0	0	1	0	0	0	0	0	1	0	1	0	1	0	0	0	0	0	0	0	0	1	0	1	0	0	1	9	10	2	4	1	1	8	8	2	7	7	1	1	0	2	2	2	0	0	1	0	0

3. Geometric Morphometrics (GM)

a. General Procrustes Analysis (GPA)

Geometric morphometrics (GM), as a statistical analysis of shape has been employed to clarify the relationships of closely related taxa [38,8,39,40,41,42,43,44,45,46,47]. This technique makes use of x, y coordinates ('landmarks') to quantify shape and allows both comparison of shape and investigation as to what landmarks caused such variations [48]. The coordinates obtained were compared between specimens after removing the effects of size, position, and orientation, allowing evaluation of differences in shape [49]. The specific body parts used in the study were carefully removed with the aid of forceps and digital images were obtained using HP2400 flatbed scanner at 1200 resolution (dpi) for the wings and a digital microscope 20-400x magnification or stereomicroscope for the other body parts (synthorax) then landmarks (Figure 1 and 2; Table 3) were established on the digitized images. Landmarks are defined as specific points on a biological structure that can be located according to some rules and can be considered homologous across a sample of the same kind of structure [50,51]. The first order landmarks (type I), being located at fixed points while the other landmarks (type II) were located at points of inflexion. In the original sense of the term "landmarks," are supposed to be located at homologous sites on the organism, however, it has been found useful to expand this concept so as to encompass "pseudolandmarks" which are located at mid-points of rounding of the outline of the organism [52]. Landmarks were captured using the computer program tpsDig, version 2.10 (copyright© 2006, F. James Rohlf, Ecology & Evolution, SUNY at Stony Brook; available from the State University of New York [SUNY] Stony Brook. Generalized orthogonal least squares, Procrustes average configuration of landmarks were computed using the Generalized Procrustes Analysis (GPA) superimposition method [53,54]. This method was used because of low bias [55,56] as such it eliminated non-shape variation in configurations of landmarks by superimposing landmark configurations using least squares estimates for translation and rotation parameters. Unit centroid size was used as the alignment-scaling method. This procedure yields a consensus configuration, the central trend of an observed sample of landmarks, which is similar to a multidimensional average [57,8]. This procedure was used to extract the shape of the left fore-wing (LFW), right fore-wing (RFW), left hind wing (LHW), right hind wing (RHW) and synthorax (SYN). Procrustes fitted x and y coordinates defining the shape for each anatomical structure under study were then loaded to CORIANDIS software for further analysis.

Table 3A. Description of assigned landmarks on both left and right fore- wing of *Neurothemis* dragonflies respectively

Landmark #	Descriptive location	Landmark #	Descriptive location
1	Proximal End of the Costa (C)	**16**	Distal End of the Radius (R)
2	Proximal End of the Subcosta (Sc)	**17**	Origin of the Radial Branches (R2 and R3)
3	Proximal End of the Radius + Media (R+M)	**18**	Anterior End of the 2nd Crossvein between Radial Branches (R2 and R3)
4	Proximal End of the Cubitus (Cu)	**19**	Posterior End of the 2nd Crossvein between Radial Branches (R2 and R3); Origin of Radial Supplement (Rspl)
5	Proximal End of the 1st Anal Vein (A/IA)	**20**	Proximal End of Radial Supplement (Rspl)
6	Basal End of the Arculus (Arc)	**21**	Distal End of Radial Supplement
7	Proximal End of the Anterior Margin of the Triangle (T)	**22**	Distal End of Anterior Media (MA)
8	Distal End of the Anterior Margin of the Triangle (T)	**23**	Distal End of Radial Branch (R4)
9	Midpoint of the Triangle (T)	**24**	Distal End of Intercalary Radial Vein (IR2)
10	Midpoint of the Triangle (T)	**25**	Distal End of Radial Branch (R2)
11	Posterior End of the Triangle (T)	**26**	Antero-lateral and Distal End of the Pterostigma
12	Origin of Radial Branches (R2 and R4)	**27**	Postero-lateral and Distal End of the Pterostigma
13	Origin of Intercalary Vein (IR3)	**28**	Antero-lateral and Proximal End of the Pterostigma
14	Nodus (N)	**29**	Postero-lateral and Proximal End of the Pterostigma
15	Distal End of the Subcosta (Sc)		

Table 3B. Description of assigned landmarks on both left and right hindwing of *Neurothemis* dragonflies respectively

Landmark #	Descriptive location	Landmark #	Descriptive location
1	Proximal End of the Costa (C)	**19**	Origin of the Intercalary Radial Vein (IR3)
2	Proximal End of the Subcosta (Sc)	**20**	Nodus (n)
3	Proximal End of the Media (m)	**21**	Distal End of the Subcosta (Sc)
4	Proximal End of the Cubitus (Cu)	**22**	Distal End of the Radius (R)
5	Posterior End of the Anal Crossing (Ac)	**23**	Origin of the Radial Branches (R2 and R3)
6	Basal End of the Arculus (Arc)	**24**	Distal End of Radial Supplement
7	Posterior and Proximal Vertex of the Hypertrigone (ht)	**25**	Posterior End of the 2nd Crossvein between Radial Branches (R2 and R3); Origin of Radial Supplement (Rspl)
8	Anterior and Proximal Vertex of the Subtrigone (t)	**26**	Distal End of the Anterior Media (AM)
9	Anterior and Proximal Vertex of the Hypertrigone (ht)	**27**	Distal End of the Radial Branch (R4)
10	Posterior and Proximal Vertex of the Subtrigone (t)	**28**	Distal End of the Intercalary Radial Vein (IR3)
11	(Cu2 + A2)	**29**	Distal End of the Radial Branch (R3)
12	Distal Vertex of the Subtrigone (t)	**30**	Distal End of Intercalary Radial Vein (IR2)
13	Anal Supplement (Aspl)	**31**	Distal End of Radial Branch (R2)
14	Basal end of the Anal Vein (A3)	**32**	Antero-lateral and Distal end of the Pterostigma
15	Second Branch of Cubital Vein (Cu2)	**33**	Postero- lateral and Distal end of the Pterostigma
16	Distal End of the Cubito-anal Vein (Cu2)	**34**	Antero-lateral and Proximal end of the Pterostigma
17	Distal End of the Posterior Cubital Vein	**35**	Postero-lateral and Proximal End of the Pterostigma
18	Origin of the Radial Branch (R4)		

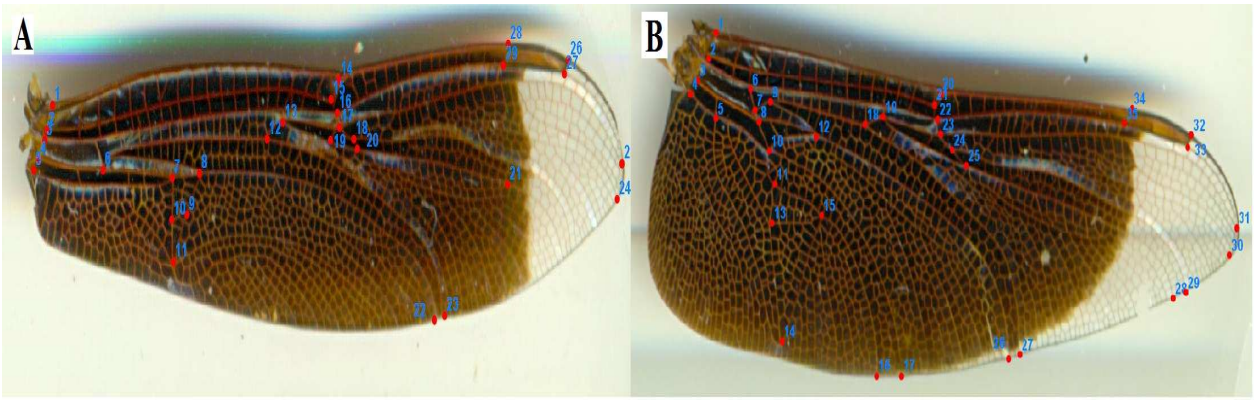

Figure 1. Landmarks for left and right fore- and hind wing of *Neurothemis* Dragonflies (Anisoptera)

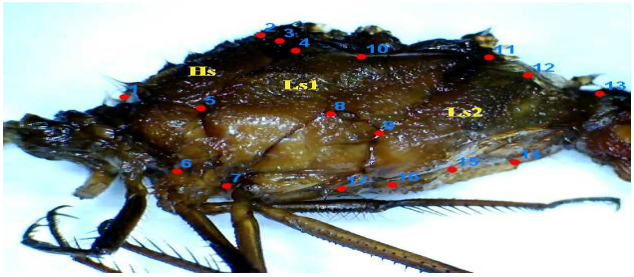

Figure 2. Landmarks on lateral view of the synthorax of Odonata :Anisoptera, *Neurothemis* dragonfly ;Hs-humeral suture, Ls1-1st lateral suture, and Ls2-2nd lateral suture

Table 4. Description of assigned landmarks on the synthorax of *Neurothemis*

Landmark #	Descriptive location
1	Anterior proximal end of the humeral suture
2	Posterior distal end of the humeral suture
3	Postero-lateral distal end of the humeral suture
4	Posterior distal end of the first lateral suture
5	Anterior proximal end of the first lateral suture
6	Antero-lateral proximal end of the 1st lateral suture
7	Sub antero-lateral proximal end of the 1st lateral suture
8	Sub anterior proximal end of the 1st lateral suture
9	Anterior proximal end of the 2nd lateral suture
10	Posterior distal end of the 1st lateral suture
11	Posterior distal end of the 2nd lateral suture
12	Sub posterior distal end of the 2nd lateral suture
13	Sub postero-lateral distal end of the 2nd lateral suture
14	Postero-lateral distal end of the 2nd lateral suture
15	Sub posterior distal end of the 2nd lateral suture
16	Sub postero-lateral proximal end of the 2nd lateral suture
17	Sub posterior proximal end of the 2nd lateral suture

b. Elliptic Fourier Analysis (EFA) - Shape Analysis

The upper appendages (cerci) and lower appendages (epiproct) were removed and placed under a Leica Dissecting Microscope for careful examination (figure 3). Image acquisition was done using a Canon Digital Camera Powershot A810 5x, 16 megapixels. The full colored images of male appendages were converted to 24-bitmap type, binary (black & white color) images. Then outlines of male appendages were digitized using the software package SHAPE version 1.3 [58] for examination of shape variation and were recorded as chain codes [59]. Herewith, the objects of interests were distinguished via segmentation technique through a "threshold procedure" where a parameter called the brightness threshold is manually chosen from brightness histogram and applied. Undesirable marks also termed as "noise" were consequently eliminated by erosion-dilation filter process. After noise reduction, the closed contour shape of the appendages was extracted via edge detection and the contours were stored in the form of chain codes [60]. Chain coding technique was used which relied on a contour representation to code shape

information. This method tracks the shape of the appendages and represents each movement by a chain code symbol ranging from 0-7. The set of possible movement depends on the type of contour representation, a pixel based contour representation were used in this study. Normalized Elliptic Fourier Descriptors (EFD) obtained from the chaincodes were calculated using Elliptic Fourier transformation as suggested by Kuhl and Giardina (1982) [61]. Normalization of data obtained from chain codes used the first harmonic ellipse as a basis which corresponds to the first Fourier approximation and utilized the 20 harmonics number to be calculated as suggested by Iwata and Ukai (2002) [58]. Principal component scores defining the shape of the left and right upper appendages (cerci) and lower appendage (epiproct) were obtained and loaded to CORIANDIS software for further analysis [62].

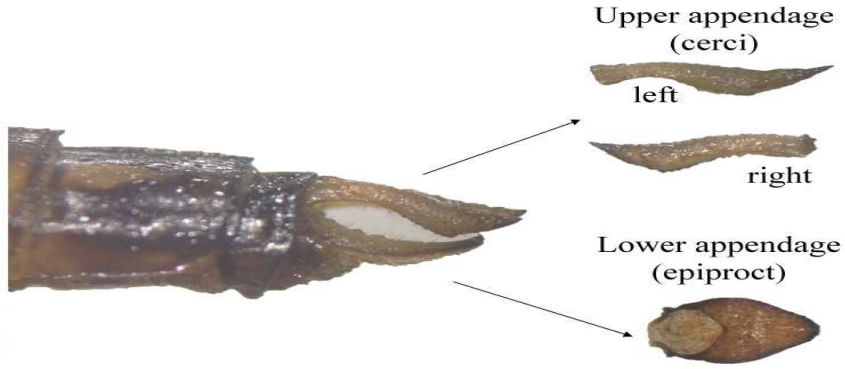

Figure 3. *Neurothemis* male appendages: upper (cerci) and lower (epiproct)

4. Correlation Analysis based on Distances.

Correlation based on distances implements a method for a broad spectrum of data types, including 2-D landmark and distance data. It allows a study of associations among multivariate datasets. The main core of this method is that it allows comparison via correlation analyses of matrices of distances among specimens or groups. In this case, landmark and non-landmark data were loaded to the software and utilized. This provides a modern approach and vividly look into associations among groups respectively, based on the relative contribution and distribution of each trait (each dataset) to species diversity, distinctiveness and similarities in terms of congruence among traits [63,64] by considering multiple set of characters. In other words, it is a measure of how much of the information in the datasets/squared distances was used in defining the weights used to compute the compromise [65]. The resulting graph contained plots or clusters (stars), each corresponding to a different species group. Congruence and multivariate covariance measure how similar the interspecific locations of traits/datasets (represented as colored points) in space. If two traits tend to be consistently different or similar between pairs of species, they were said to be (positively) congruent, and show in the plot as a general tendency to cluster together within species. If two traits were inconsistent in how similar they were between species, results show low congruence and covariance values. Trait variance or disparity was proportional to the area occupied by datasets.

RESULTS AND DISCUSSION

This study utilized a technical approach which has the capacity to collate all available datasets thus, demonstrating the process of integrative taxonomy for studies on species delineation and variations on two cosmopolitan *Neurothemis* species having different morphotypes through Correlation Analysis based on Distances (CORIANDIS). *Neurothemis* species, also known as “red dragonflies,” are often seen in drains, ditches, ponds, shallow streams and not so densely shaded areas in Iligan City. The two cosmopolitan species often seen and confused are: *Neurothemis terminata*, Ris 1911 and *Neurothemis ramburii*, Kaup and Brauer 1866. This study shed light on the nature and variation of *Neurothemis* species after much dilemma. Correlation Analysis based on Distances (CORIANDIS) has the capacity to integrate all available character data sets, investigate, visualize underlying relationships and sources of variability among the groups in terms of relative contribution of traits and congruence among characters [63,64]. Figure 4a shows the resulting clusters that represent the location of the female morphs (*m1, m2, m3*…) on the ‘compromise’ space reflecting overall similarity based on six (6) combined character sets. This procedure allows both looking into similarities among specimens/groups, and interpreting such similarities in terms of congruence among traits where, N. ram m4 and N. ter m1; N. ram m3, N. ram m5, N. ram m6 and N. ram m7 were clustered together. While, N.ram m1 and N.ram m2 departs considerably from other populations,

although this seems to be largely a function of the external morphological wing characters such as fore- wing triangle, hind wing triangle, the radial planate, fore- wing subtriangle, number of anal loop 'sole cells', hind wing supertriangle and number of cross veins in the cubital space of the hind wing (behind the median space), wing pigmentation and the shape of the synthorax. This is expressed as the magnitude of the vectors emanating from the centroid in the compromise space. Figure 4b is a disparity plot for female *Neurothemis* morphs showing vividly the relative contribution of the combined character sets to species' divergence. Here, the total height of the stacked-bar chart equals the total or standardized sum of squared distances for each character set considered to the origin (a measure of variance or disparity). This shows how much each population differs from the rest by interpreting such differences in terms of individual character [65]. Trait variance or disparity was proportional to the area occupied by datasets. Herewith, noted differences have been observed between female morphs. It was apparent that wing characters and shape of synthorax play a great role in differentiating one morphotype to the other. This is further evidence to the homoplasious nature of the wing. Also, the life habits of these odonates may be selecting for convergent changes such that the nature of the wings and shape of synthorax may be reflective of their lifestyle and their habitats [66,67]. In addition, it was also evident that females of these species exhibit female-limited polymorphism (wing and body coloration) [20,21,22,19]. This is suggested to have evolved throughout sexual selection. On one hand, when polymorphism is limited to males, it mostly plays a role in male-male competition for access to females [68,69,70]. On the other hand, female-limited polymorphisms are important for regulating costly intersexual interactions [71,68,72]. Moreover, figure 5a shows the resulting clusters and respective location on the 'compromise' space for male *Neurothemis* morphs based on nine (9) combined character sets and figure 5b (disparity plot) shows vividly the relative contribution of the combined character sets to species' divergence. Additional 3 characters (shape of epiproct, left and right cerci) were included for the male populations since these characters are of importance especially to investigation of possible species divergence and differentiation. The terminal abdominal appendages on the males are called claspers. The claspers are formed by a pair of upper appendages, called cerci, and a single lower appendage, an epiproct. These two are designed to lock into species-specific grooves and notches on the female in order to secure the two together for mating. With this, Inter-specific sexual activity is normally discouraged by the lock-and-key aspect of the attachment process. For the females, the terminal appendages consist of a pair of cerci, which have little or no function [73]. Herewith, noted differences have been observed between the male morphs which seems to be a function of the external morphological wing characters such as the fore- wing triangle, hind wing triangle, the radial planate, fore- wing subtriangle, number of anal loop 'sole cells', hind wing supertriangle, number of cross veins in the cubital space of the hind wing (behind the median space), shape of synthorax, shape of epiproct, shape of left and right cerci. It is noted though, that significant difference was observed between groups especially in terms of the lower appendage (epiproct). With this, it is hypothesized that this feature proved to be an important factor in the lock-and-key aspect and specificity of the attachment process dealing with copulation in *Neurothemis* species. Thus, draws the line between groups leading to possible reproductive isolation. For the upper appendages (cerci), which function somewhat as hooks to hold the female in tandem prior to mating, significant difference was notable between species and not between morphs belonging to same species. However, by looking on the overall contribution of all character sets, close association was notable between N. ram morph 1 and 2 and between N. ter morph 1 and 2.

Hence, it is hypothesized that differences between groups may be reflective of their lifestyle and their habitats [66,67]. However, current and strong sexual selection may promote adaptive population divergence in these species and that premating sexual isolation may have arisen as a correlated response to divergent sexual selection. The results may highlight the importance of sexual selection, rather than natural selection in the adaptive radiation of odonates, and supports previous suggestions that divergent sexual selection promotes speciation in this group. Accordingly, several meta-analysis on selection studies especially in natural populations have been published and one conclusion from these studies indicate that the strength of selection on phenotypic traits found to be strong in natural populations although the definition of weak and strong is still subject to discussion [74,75,76]. Here, the utilization of a number of character data sets and an integrative approach for species delineation proved to be effective in understanding variation and the nature of the *Neurothemis* species found in Iligan City.

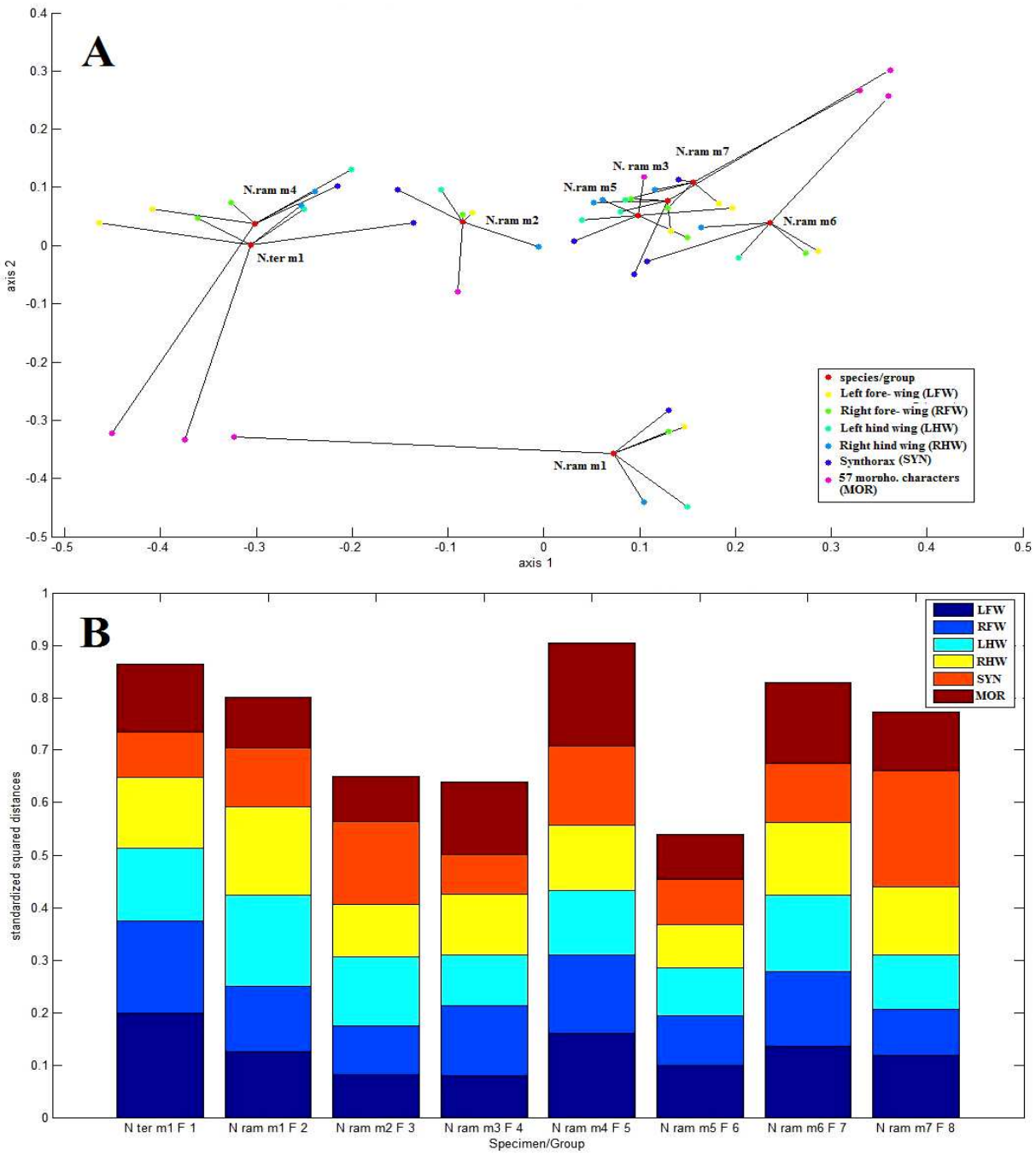

Figure 4. Species/ groups original distance matrices and their compromise projected onto compromise space (a); Disparity plot showing squared distances to centroid for each character set (b) for female *Neurothemis* morphs

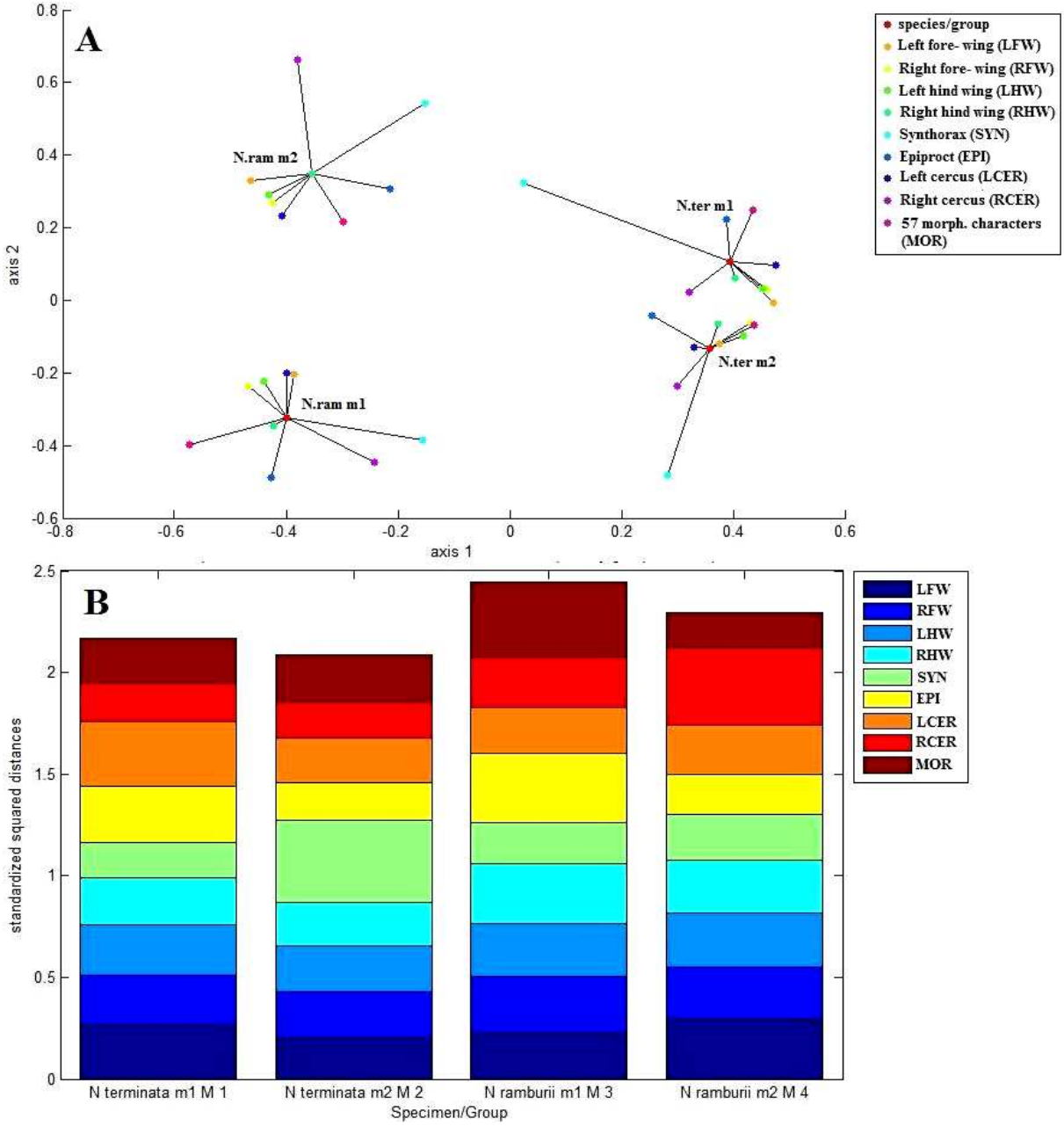

Figure 5. Species/ groups original distance matrices and their compromise projected onto compromise space (a); Disparity plot showing squared distances to centroid for each character set (b) for male *Neurothemis* morphs

CONCLUSION

This study is a simulation that was used to evaluate the accuracy of an integrative approach for species delineation and understanding variation on the genus *Neurothemis*. Among the characters that best contribute to species divergence/ distinctiveness were external morphological wing characters such as the fore- wing triangle, hind wing triangle, the radial planate, fore- wing subtriangle, number of anal loop 'sole cells', hind wing supertriangle, number of cross veins in the cubital space of the hind wing (behind the median space), wing pigmentation, shape of synthorax, shape of epiproct and shape of left and right cerci. In this study, the utilization of a number of characters for species delineation proved to be effective in understanding variation and the nature of the *Neurothemis* species found in Iligan City. The results of this study indicate that indeed the value and utilization of an aggregate of

characters is important and evident in natural history. Herewith, morphological inference is enhanced by providing sets of data directly applicable to the taxonomic problem.

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REFERENCES

[1] DWA Noble; Y Qi; J Fu. *BMC Evolutionary Biology*,**2010**,10 (197),1471-2148.
[2] LM Boykin; KF Armstrong; L Kubatko; P De Barro. *EvolBionform Online*, **2012**,8-37.
[3] RK Selander; RF Johnson; TH Hamilton. *Condor*, **1965**, 66,491-495.
[4] MJ Bishop. *Zool. J. Linn. Soc*. **1982**, 74, 197-206.
[5] E Mayr; P Ashlock. Principles of Systematic Zoology, 2nd ed., McGraw-Hill, Singapore, 1991; pp. 158-188.
[6] WF Blair (ed.). Vertebrate Speciation, University of Texas Press, Austin, 1961.
[7] E Munroe. *Rev. Canad. Biol*., **1960**,19, 293-319.
[8] DC Adams; FJ Rohlf; DE Slice. *J. Zool*., **2004**, 71, 5-10.
[9] P Mitteroecker; G. Gunz. *Evol. Biol*., **2009**, 36:235-247.
[10] B Dayrat. *Biological Journal of the Linnaean Society*, **2005**, 85, 407-415.
[11] R Lyons, Accessed 02 January,**1999** http://casswww.ucsd.edu/archive/personal/ron/CVNC/odonata/ipsodonata.html.
[12] F C Fraser. A reclassification of the order Odonata, Royal Zoological Society of New South Wales, Sydney, Australia, 1957.
[13] W Hennig. Insect phylogeny, Wiley, New York, 1981.
[14] FL Carle. *Soc. Int. Odonatol. (Rapid Commun.)*, **1982**, 4, 66.
[15] HK Pfau. *Adv. Odonatol*., **1991**, 5, 109-141.
[16] JWH Trueman. *Odonatologica*, **1996**, 25, 59-72.
[17] G Bechley. Accessed 05 January, **2002** http://www.bechly.de/phylosys.htm.
[18] RA Dow; V Clausnitzer. The IUCN red list of threatened species, **2012.**
[19] SRM Tabugo; CG Demayo; MAJ Torres. *Natura Journal*, **2013**, 17 (10), 2-14.
[20] M Schorr; M Lindeboom; D Paulson. World Odonata List, University of Puget Sound, 2010.
[21] V Kalkman. Accessed 19 February, **2009** www.iucnredlist.org.
[22] CG Demayo; SA Harun; MAJ Torres. *Australian Journal of Basic and Applied Sciences*, **2011**, 5(6), 748-759.
[23] WH Park; JW Lee; DH Kim; YJ Shin. *J. Nat. Sci. Yeungnam Univ*., **1988**, 8,175–184.
[24] JH Yoon. Ph.D. thesis, Kyungpook National Univ., (Daegu, Korea, 1997).
[25] SM Lee. Dragonflies of Korean Peninsula (Odonata), Junghaeng–sa, Seoul, 2001; pp. 228.
[26] SB Kim; HS Jeon; HS Oh; YH Jung; WT Kim. *Korean J. Genetics*, **2008**, 30, 53–61.
[27] M Pola; Y Valles; JL Cervera; M Medina; TM Gosliner. *Ann. Zool. Fennici.*, **2006**, 43, 52-64.
[28] RB Payne; MD Sorenson. *Bull. B.O.C.*, **2007**, 127(1), 4-26.
[29] CD Michener. *Syst. Zool*., **1957**, 6, 160-173.
[30] RF Inger. *Evolution*, **1958,** 12, 370-384.
[31] C Darwin. On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggled for Life, [Facsimile ed. Mayr, E. (ed.)], John Murray, Cambridge, MA, Harvard University Press, London, 1964.
[32] RA Munawwirah; H Rahman. ODONATA *(*DRAGONFLIES AND DAMSELFLIES), Intoductory Course to Entomology, 2012.
[33] S Damm; B Schierwater; H Hardrys. *Molecular Ecology*, **2010**,19, 3881-3893.
[34] A Nel; P Nel; JF Petrulevicius; V Perrichot; J Prokop; D Azar. *Ann. Soc. Entomol. Fr.(n.s.)*, **2010**, 46(1-2),276-292.
[35] AMA Garcia; TR Gregory. *Journal of Zoology*, **2009**, 278, 163-173.
[36] PHA Sneath; RR Sokal. Numerical taxonomy: The principles and practice of numerical classification, W. H. Freeman and company, San Francisco, 1973.
[37] SB Kim; HS Oh; WT Kim; O Tadauchi. *J. Fac.Agr., Kyushu Univ.*, **2009**, 54(1), 123-132.
[38] JT Richtsmeier; VB Deleon; SR Lele. *Yearbook of Physical Anthropology*, **2002**, 45, 63-91.

[39] LF Marcus; M Corti; A Loy; GJP Naylor; DE Slice. Advances in Morphometrics, Plenum Press New York, 1996; pp. 284: 587.
[40] FJ Rohlf; LP Marcus. *Trends Ecol. Evol.*, **1993**, 8, 129-132.
[41] AM Aytekin; M Terzo; P Rasmont; N Çagatay. *Ann. Soc. Entomol. Fr.*, **2007**, 43, 95-102.
[42] S Schröder; W Drescher; V Steinhage; B Kastenholz. *Proc. Intern. Symp. on Conserving Europe's Bees. Int. Bee Research Ass. & Linnean Society*, **1995**, 6-7.
[43] V Steinhage; T Arbuckle; S Schröder; AB Cremers et al. *German Programme on Biodiversity and Global Change Status Report, Bonn*, **2001**, 194-195.
[44] M Drauschke; V Steinhage; A Pogoda; S Müller et al. Proceedings of the 7th International Workshop on Pattern Recognition in Information Systems (Fred Aand Jain AK, eds.), INSTICC (Institute for Systems and Technologies of Information, Control and Communication) Press, Funchal, 2007; pp. 25-36.
[45] MFM Mendes; TM Francoy; P Nunes-silva; C Menezes et al. *Biosci. J.*, **2007**, 23(Suppl 1), 147-152.
[46] TM Francoy; D Wittmann; M Drauschke; S Müller et al. *Apidologie*, **2008**, 39, 488-494.
[47] TM Francoy; PPR Prado; LS Goncalves; LD Costa et al. *Apidologie*, **2006**, 37, 91-97.
[48] E Pretorius. *Australian Journal of Entomology*, **2005**, 44, 113-121.
[49] FJ Rohlf. 1996. Introduction to outlines *in* Marcus, L. F., M. Corti, A. Loy, G. Naylor, and D. E. Slice (eds.) Advances in Morphometrics, Plenum Publishing Corp., New York, **1996**; pp. 209-210.
[50] DE Slice. Three dimentional generalized resistant fitting and the comparison of least squares and resistant-fit residuals, In L.F. Marcus, M. Corti, A. Loy, E. Naylor & D. Slice (eds) Advances in Morphometrics, Plenium Press, New York, **1996**; pp. 179-199.
[51] A Dickinson. Morphometric method and data collection, University of Toronto, Canada, **2001**.
[52] AM Elewa. Two application of geometric morphometrics to the study of shape polymorphism in Eocene ostracodes from Egypt and Spain, **2002**.
[53] FJ Rohlf; D Slice. *Systematic Zool.*, **1990**, 39, 40-59.
[54] DE Slice. *Syst. Biol.*, **2001**, 50, 141-149.
[55] FJ Rohlf. *Amer. J. Phys. Anthropol.*, **2000**, 111,463-478.
[56] FJ Rohlf. *Journal of Human Evolution*, **2003**, 44, 665-683.
[57] JA Walker. *Sytematic biology*, **2000**, 49, 686-696.
[58] H Iwata; Y Ukai. *J. Heredity*, **2002**, 93, 384-385.
[59] H Freeman. *Comp. Surv.*, **1974**, 6, 57-97.
[60] RM Dalayap; MAJ Torres; CG Demayo. *Int. J.Agric., Biol.*, **2011**, 13, 652-658.
[61] FP Kuhl; CR Giardina. *Comp. Graphics Image Proc.*, **1982**, 18, 236-258.
[62] SRM Tabugo; MAJ Torres; LF Olowa; MAB Sabaduquia; RM Macapil; AM Acevedo; CG Demayo. *International Research Journal of Biological Sciences*, **2012**, 1(8), 19-26.
[63] H Abdi; D Valentin; A. J. O'Toole; B Edelman. DISTATIS. The analysis of multiple distance matrices. In : Proceedings of the IEEE Computer Society: International Conference on Computer Vision and Pattern Recognition, San Diego, 2005; pp. 42-47.
[64] H Abdi, D Valentin, S Chollet, C Chrea. *Food Quality and Preference*, **2007**,18, 627-640.
[65] EJ Márquez; LL Knowles. *Journal of Evolutionary Biology, in press*. doi: 10.1111/j.1420-9101.2007.01415.x, **2007**.
[66] EM Pilgrim; CD Von Dohlen. *Systematic Entomology*, **2008**, 33, 159-174.
[67] PS Corbet. Dragonflies: Behavior and Ecology of Odonata, Comstock Publishing Associates, Ithaca, NY, 1999.
[68] EI Svensson; F Eroukhmanoff; F Magne. *Evolution*, **2006**, 60(6), 1242-1253.
[69] Y Tsubaki; R Hooper; M Siva-Jothy. *Res. Popul. Ecol.*, **1997**, 39,149–155.
[70] SJ Plaistow; Y Tsubaki. *Proc. Biol. Sci.*, **2000**, 267, 969–975.
[71] S Cook; J Vernon; M Bateson; T Guilford. *Anim. Behav.*, **1994**, 47, 389–397.
[72] Y Takahashi; J Yoshimura; S Morita; M Watanabe. *Evolution*, **2010**, 64,3620–3628.
[73] K Mead. Dragonflies of the North Woods (North Woods Naturalists Guides), Kollath-Stensaas Publishing, Duluth M.N., 2003.
[74] HE Hoekstra; JM Hoekstra; D Berrigan; SN Vignieri; A Hoang; CE Hill; P Beerli; JG Kingsolver. *Proc. Natl. Acad. Sci. USA*, **2001**,98,9157–9160.
[75] JG Kingsolver; HE Hoekstra; JM Hoekstra; D Berrigan; SN Vignieri; CE Hill; A Hoang; P Gibert; P Beerli. *Am. Nat.*, **2001**,157, 245–261.
[76] JT Hereford; F Hansen; D Houle. *Evolution*, **2004**, 58, 2133–2143.