Article

Morphotypes of cone snails (*Conus mustelinus*), Conidae: a phenetic analysis

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Abstract
This study describes phenotypic differentiation of *Conus mustelinus*, a gastropod species belonging to Family Conidae through phenetic analysis. There exist a number of morphotypes for this species. Thus, it is valuable to look into the nature and variation of this species. In this study, adult shell color polymorphism was observed and six (6) morphotypes were documented and described. Herewith, M5 and M6 among the morphotypes studied, closely resembled each other. A total of 27 characters were used to construct the character matrix. The important characters were spiral ground color, spiral bands color, body whorl color, band between sub-shoulder plus central band and aperture coloration. The generated phenogram revealed possible clusters based on overall phenotypic similarity. Describing variation and relationships based on a combination of discrete shell morphological characters proved to be pertinent in identification process thereby, addressing sibling species and cryptic speciation.

Keywords *Conus mustelinus*; Conidae; phenetic analysis; morphotypes; shell.

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1 Introduction

Species delineation is one of the tremendous challenge and dilemma that confront the field of taxonomy because of the existence of different morphotypes. Kohn, 2005 mentioned that in terms of *Conus* biodiversity descriptions aren't improving. Albeit, he believed that some points remained current and valid while a little progress have been made considering that we have now the ability to apply multivariate statistical techniques to shell morphometric data and to radular tooth characters as well. In addition, in the advent of molecular biology it provided access to more independent characters that are worthy to be considered. Unfortunately, he moreover, expressed the sad fact that descriptions of supposedly new species in recent years were no better than those of Linnaeus and other eighteenth century workers. It is expected that with modern advances, new methods of collection and appreciation of the importance of populations and variation of individuals with the way animals may have evolved thus, modern descriptions should be far superior to those in the eighteenth century. However, people described new species of molluscs in the twentieth century rather in a poor way. Among the most difficult genera to describe is *Conus*. It was noted that typically the reason behind it, is that an author fails to provide enough information about the range of the within-species variation and distinguishing differences between species. Lamarck once wrote that naturalists generally should grant extent to the characters of the species in order to include within its limits the varieties that appear to belong there. It seems that if one doesn't perceive the extent of how much variation exists, one is in jeopardy of either overestimating or underestimating the extent of variation found. It is deemed necessary to re-emphasize these words nowadays. The bad descriptions in the past of *Conus* species by early authors were due to few specimens, little discussions with others, less field study and little knowledge on geographic and morphological variation within species which posed an onerous task on future workers who need the right name for species as a basis for ecological studies. So what is then a good description? It must most importantly distinguished the proposed new species from all previously described species in the genus and distinguished that species to those that are similar to it and thereby, also addressing variation. It is apparently desirable to use as many sets of plausible characters in describing species. In practice, shell characteristics are most important because an objective measure of variation is often present. There are three important sets of shell characters: 1.) color pattern; 2.) size and shape and 3.) sculpture. Significant differences in high proportion with respect to the three characters provide evidence and support for supposedly new species. However, color pattern is the most difficult set of all shell characters to describe objectively and quantitatively and there is a need to develop a method of verbal or mathematical description of color pattern. Apparently, banding patterns of mollusk shells constitute one of the most complex patterns in the animal kingdom and it may contribute to morphotype assignment of species. The production of different shell banding patterns among shelled mollusks is thought to be related to neurophysiological activity of the snail. The nervous stimulations account for the uniqueness in pigmentation patterns i.e. shell bands vary depending on how the pigment excretions are affected by the snail's neural stimulations. Thus, different nerve impulses experienced by a gastropod result into very slight intra-specific variations of its shell's banding pattern (Boettiger et al., 2009). With this, statistical and relatively useful techniques are important and of interest especially, on the morphological aspect that can aide in species delineation. Essentially an approach that allows you to consider various characters on a character matrix and generate possible clusters based on overall phenotypic similarity is pertinent. Hence, describing variation and relationships based on a combination of discrete morphological characters will prove to be useful.

According to Mayr and Ashlock (1991), since organisms can be highly similar in nature and seems that no single character is good enough or may have absolute diagnostic value a combination of characters will allow correct assignment. Hence, a combination of two or more characters is often adequate for diagnosis. To circumvent subjectivity, it is suggested to group species into higher taxa with the aid of computer programs and mathematical process on the basis of their "overall similarity." Herewith, similarity can be determined by recording overall similarities and differences in a large number of variables.

This study considers *Conus mustelinus*, a gastropod species belonging to Family Conidae having venomous sting which can even sting humans. There exist a number of morphotypes for this species thus, it would be valuable to look into the nature and variation of this species. This species is exclusively distributed on the East and West Australia, Japan, Philippines and Fiji. Their size ranges from 40 to 107mm with variety of shell pigmentations (Röckel et al., 1995). Like other cone snail species, *C. mustelinus* can be described through its shell and shell banding patterns (Biggs et al., 2007). Röckel et al. (1995) provided excellent descriptions for identifying the animal. C. *mustelinus* body whorl is mostly yellow-green with white portions and dark chocolate bands. Its shell is encircled by central white band with chocolate marks parallel to each other. The shell's shoulder is emphasized with bands of longitudinal chocolate markings complemented by the spire which is relatively low and striate. Its aperture has a faint chocolate color with pale yellow band. Röckel et al., 1995 also noted that shell variation of *C. mustelinus* specimens are related to the ontogeny of the animal. Though the online malacological community revealed intra-specific morphological variations of *C. mustelinus* (Hardy, 2002; UWBM, 2007; Kersten, 2013), peer-reviewed reports of *C. mustelinus* morphotypes are not available.

Hence, describing morphological variations of this *conus* species is important in identification process thereby, addressing sibling species and cryptic speciation. *C. mustelinus* shells may be classified using its overall physical similarity through Phenetic analysis. It involves clustering and ordination of character traits. This study reports the phenetic analysis results for the shell morphotypes of *C. mustelinus* collected from a reef of Lugait, Misamis Oriental, Philippines.

2 Materials and Methods

2.1 Sampling site, identification and processing of samples

Opportunistic sampling of cone snails (*Conus mustelinus*) was conducted during daytime in Calangahan, Lugait, Iligan City, Philippines [outside the marine protected area (MPA)], location was around N 8º22'07", E 124º15'39" (Fig. 1). Self Contained Underwater Breathing Apparatus (SCUBA) diving was employed. Specimens were carefully handpicked and placed in glass jars with seawater and appropriate labels. The specimens were transported to the laboratory immediately after collection. In the laboratory, the animals were anesthetized using menthol crystals; their head-foot, mantle and visceral mass were removed and their shells were carefully cleaned. Specimens were classified per morphotype and digital images were acquired using Canon Power Shot SX260 HS, 20x optical zoom digital camera. The specimens were examined and identified using the taxonomic descriptions of Walls, 1979; Garcia, 1986; Röckel et al., 1995. Morphological characters of the shell were derived for phenetic analysis. References used for the identification were used to derive the characters for coding.

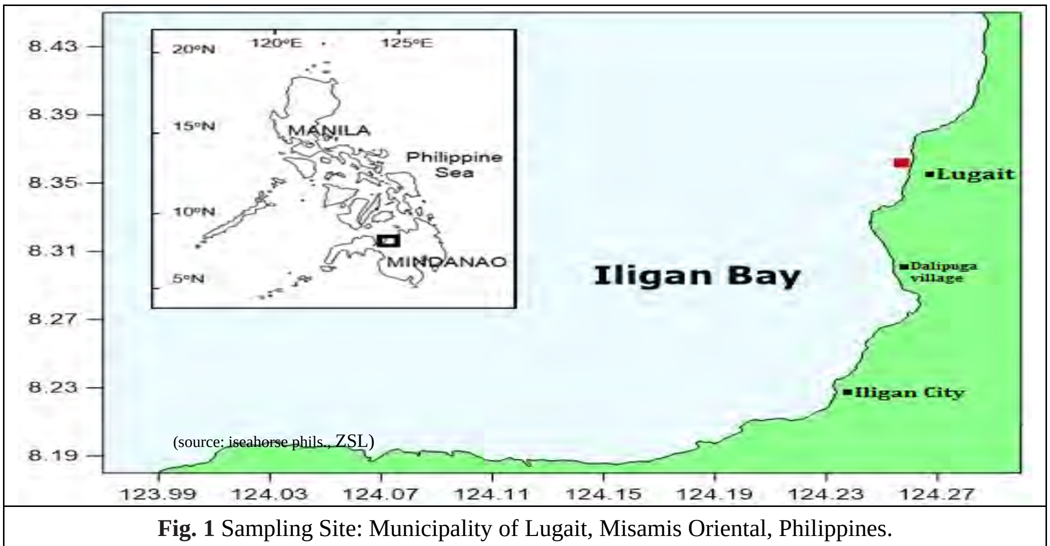

Fig. 1 Sampling Site: Municipality of Lugait, Misamis Oriental, Philippines.

2.2 Phenetic analysis

Shell morphological data were utilized to prepare the character matrix and character state table. A total of 27 characters with character states represented by 0-2 values were used to perform phenetic analysis (Table 1). Hierarchical Cluster Analysis was conducted via average linkage between groups method and Squared Euclidean Distance (Distance (X × Y) = Σ(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 in order to prepare the phenogram. All analysis was done using Statistical Package Social Science (SPSS) for IBM, version 20 (Kim, et al., 2009).

Table 1 Morphological characters and character states (0-2) of *Conus mustelinus* morphotypes.

CHARACTERS
1. Shell ground color: (0) white, (1) cream
2. Spire with (0) six or (1) seven sutures
3. Spiral whorls between sutures: (0) flat, (1) convex
4. Spiral bands almost (0) parallel or (1) scattered to each other
5. Spiral ground color with (0) yellowish, (1) green, (2) cream tint
6. Center of spire: (0) pale green or (1) cream
7. Spire relatively (0) low or (1) high
8. Spiral bands (0) black, (1) chocolate brown, (2) brown with orange tint
9. Shoulder is (0) angulate or (1) subungulate
10. Tip of shoulder bands almost connect to the spire is generally (0) pointed, (1) filamentous
11. Orange tint of chocolate brown shoulder bands (0) almost absent, (1) prominent
12. Area of subshoulder bands: (0) narrow, (1) wide
13. Subshoulder bands generally (0) erect, (1) leaning to diagonal
14. Body whorl color: (0) yellow orange, (1) yellow green, (2) dark green
15. Band between subshoulder and central band: (0) absent, (1) faint, (2) prominent
16. Area of concentric central band: (0) narrow, (1) wide
17. Tan and filamentous bands of the central band: (0) absent, (1) present
18. Chocolate brown bands of midbody (0) erect to (1) irregular or diagonal
19. Chocolate brown bands of midbody: generally (0) parallel or (1) not
20. Lateral growth lines: (0) faint, (1) prominent
21. Basal part of columella: (0) tan to yellow orange or (1) purple
22. Siphonal canal: (0) erect or linear (1) leaning or diagonal relative to columella
23. Plane from the shoulder at the point nearest to aperture to tip of siphon: (0) flat, (1) waving
24. Aperture is (0) translucent or (1) opaque
25. Aperture coloration: (0) purple, (1) brown, (2) cream
26. Curve of the anterior area of peristome (0) levels off with subshoulder bands area or (1) not
27. Peristome width: (0) very thin, (1) relatively thick

3 Results and Discussion

The genus *Conus* remains as taxonomically challenging albeit, the morphological characteristics of the shell is the initial step for identification. *Conus mustelinus* can be described and characterized through its shell and shell banding patterns. Generally, the body whorl is mostly yellowish green with white portions and dark chocolate bands. The shell is encircled by central white band with chocolate marks parallel to each other. Meanwhile, the shell's shoulder is emphasized with bands of longitudinal chocolate markings complemented by the spire which is relatively low and striate. The aperture has a faint chocolate color with pale yellow band (Biggs et al., 2007; Röckel et al., 1995). The shapes of adult shells are alike for both small and large individuals. There are noted variations in color patterns in this species. Variability of colour patterns was documented hence, grouping specimens into six (6) morphotypes (Fig. 2). Observed associated microhabitats vary also according to morphotypes and most often consist of sand or mud into which the cone snail may burrow, but may also include inter-tidal and reef areas. The reef areas are biologically intricate and valuable ecosystem with high diversity and density of life (Tabugo et al., 2016) where *conus* snails often camouflaged itself.

In this study, six morphotypes were identified and were considered for phenetic analysis using 27 characters. Phenetic Analysis revealed the relationships of the 6 morphotypes based on the most useful characters selected on the species level and then were incorporated into a data matrix to allow them to be coded (Table 2).

Between the six morphs of *Conus* species, the lowest dissimilarity indices were found between M5 and M6 because these two morphotypes closely resemble each other based on overall phenotypic similarity. This is followed by M2 and M4 which also had a low dissimilarity value. The highest dissimilarity values were found between M2 and M3, M1 and M4, M1 and M6, M3 and M5 which indicate that they less likely resemble each other based on their overall phenotypic similarity (Table 3).

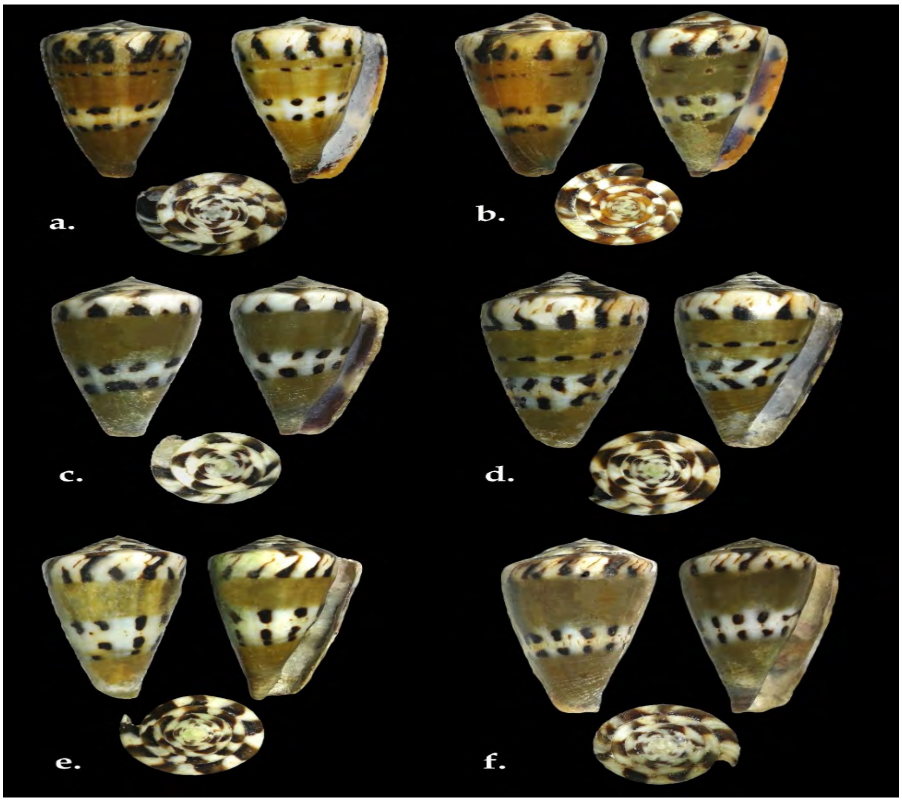

Fig. 2 Dorsal, ventral and spiral views of the six (6) morphotypes of *Conus mustelinus:* a.) M1-morph 1; b.) M2-morph 2; c.) M3-morph 3; d.) M4-morph 4; e.) M5-morph 5; f.) M6-morph 6.

Table 2 Character data matrix for *Conus mustelinus* morphotypes used in phenetic analysis (OTU: operational taxonomic unit).

	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**
M1	0	1	0	1	2	1	0	0	0	0	0	1	0	0	2	0	0	0	0	1	0	1	1	0	1	1	0
M2	1	1	1	0	0	0	1	2	0	1	1	1	0	0	1	0	0	0	1	1	0	1	0	0	1	0	0
M3	0	0	0	1	2	0	1	0	1	0	0	0	0	2	0	0	0	1	1	0	1	0	1	0	0	0	1
M4	1	1	1	0	1	0	1	1	0	1	1	1	1	1	2	1	1	1	1	0	1	0	0	1	0	0	1
M5	1	1	1	1	1	1	0	1	0	1	0	1	1	1	0	1	0	0	0	0	0	1	1	1	2	1	1
M6	1	1	1	1	2	1	1	1	1	1	1	0	1	2	0	0	1	0	0	0	0	1	0	1	2	1	1

Table 3 Dissimilarity matrix in the combined morphological dataset of *Conus mustelinus* morphotypes. The numbers below in the diagonal are the divergence values corrected for multiple substitutions using SPSS for IBM, version 20 (OTU-operational taxonomic unit; M-morphotype).

	Squared Euclidean Distance					
OUT	1	2	3	4	5	6
M1	-					
M2	19.000	-				
M3	21.000	28.000	-			
M4	23.000	14.000	20.000	-		
M5	16.000	17.000	23.000	19.000	-	
M6	23.000	22.000	20.000	20.000	9.000	-

Noteworthy, the important notable characters for delineation were spiral ground color, spiral bands color, body whorl color, band between sub-shoulder plus central band and aperture coloration. The generated phenogram revealed possible clusters based on overall phenotypic similarity (Fig. 3). Results reveal distinct shell banding patterns among the six morphotypes. In terms of similarity relationships, morphotypes M5 and M6 is the most similar and shows lower phenotypic distance. M1 is more similar with morphotypes M5 and M6, although the gap in their distance is quite extensive. M2 closely resembles M4 while, morphotype M3 reveals, however, to be the most distinctly related morphotype based on the low similarity value. Most species differs in appearance during their different life stages which may have contributed to the morphometric variation in M3. Pigmentation patterns may also differ (Meinhart, 1986; Bauchau, 2001). Difference in body length was also observed in M3 having smaller sizes than the rest of the morphotypes.

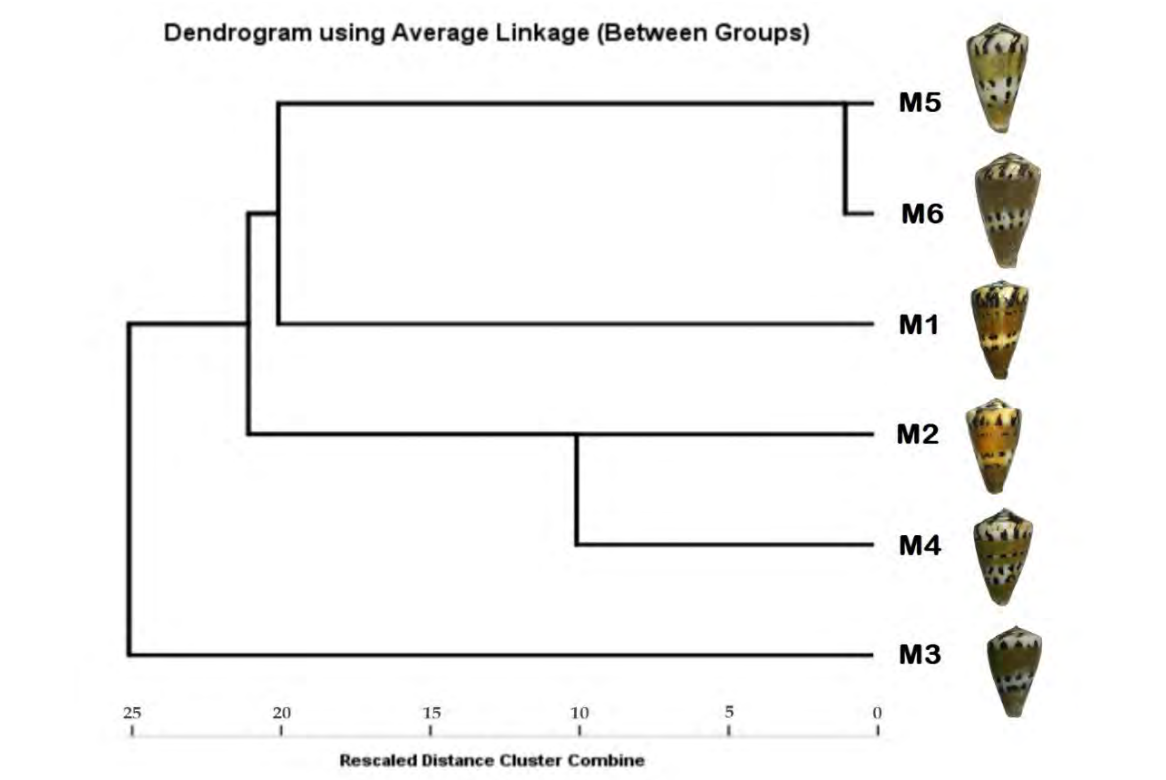

Fig. 3 Phenogram of *Conus mustelinus* morphotypes based on the Squared Euclidean Distance using 27 characters (Average Linkage between groups) (M= morphotype).

In this study, *C. mustelinus* is regarded as a variable species such that in one area alone several morphotypes were observed that may also correspond to respective microhabitats occupied by this species. Highly cryptic adult pigmentation resulting in adult shell color polymorphism was observed. This potentially provides insight in natural selection and population differentiation. This suggests that marine speciation can regularly occur over much finer spatial scales than generally accepted. Herewith, banding patterns of shells constitute one of the most complex patterns and contribute to morphotype assignment of species. Aside from that, different shell banding patterns is thought to be related to neurophysiological activity of the snail (Ermentrout et al., 1986; Boettiger et al., 2009). The nervous stimulations account for the uniqueness in pigmentation patterns i.e. shell bands vary depending on how the pigment excretions are affected by the snail's neural stimulations. Thus, different nerve impulses experienced by a gastropod result into very slight intra-specific variations in shell banding pattern (Boettiger et al., 2009) and can be a respond to stimuli in the environment. In like manner, it was observed in marine gastropod *Littorina mariae* that shell color variation was in response to environment and for mimicry (Saleuddin and Kunigelis, 1984; Reimchen, 1989). Hence, it is hypothesized that the extent of shell coloration could be a function of the substrate in a given environment and foraging positions of predators.

The variability of mollusc shells, and in particular Conoidea shells, is known to be difficult to interpret (Duda et al., 2008, 2009; Puillandre et al., 2010), due to extended polychromatism and rampant homoplasy. The variability of molluscan shells, especially in the case of small samples, is difficult to attribute *a priori* to individual variability, within species geographic variation or differences between species (Meyer et al., 2005; Appleton and Palmer, 1988). Meanwhile, morphologically distinct clusters of specimens may correspond merely to geographical variants, with different environmental conditions acting differentially on the shape and ornamentation of the shell (Puillandre et al., 2010). Several studies on lymnaeid snails also concluded that environmental factors contribute to various shell size and shape (Arthur, 1982; Pip, 1983; Lam and Calow, 1988; Wullschleger and Ward, 1998). Researchers have identified that shifts in growth must occur in several morphometric characters simultaneously and they must be associated with other changes in the ontogeny of the population, e.g. ecological interactions, physiological process and/or behavioral patterns (L'avrincˇı´kova et al., 2005). In this respect, variations observed in *C. mustelinus* could also be attributed by such factors. Herewith, describing morphological variations of *C. mustelinus* is important in identification process especially with the occurrence of sibling species and cryptic speciation.

4 Conclusion

This study gives valuable insights and knowledge on the nature and variability of *Conus mustelinus*. In this study, six morphotypes were documented and described. Herewith, M5 and M6 among the morphotypes studied, closely resembled each other. A total of 27 characters were used to construct the character matrix. The important characters were spiral ground color, spiral bands color, body whorl color, band between sub-shoulder plus central band and aperture coloration. The generated phenogram revealed possible clusters based on overall phenotypic similarity. Thus, *C. mustelinus* is regarded as a variable species such that in one area alone several morphotypes were observed that may also correspond to respective microhabitats occupied by these species. Highly cryptic adult pigmentation resulting in adult shell color polymorphism was observed. Herewith, describing morphological variations of *C. mustelinus* is important in identification process addressing sibling and cryptic species.

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