IJMBR 1 (2013) 8-14

International Journal of Modern Biological Research
www.bluepenjournals.org/ijmbr

Genetic and biochemical differentiation of pectoral spine variants in *Clarias gariepinus*

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Article History
Received 14 January, 2013
Received in revised form 15 February, 2013
Accepted 19 February, 2013

Key words:
Biochemical differentiation, Phylogeny, Morphotypes, *Clarias gariepinus*.

Full Length Research Article

ABSTRACT

The challenges of morphologic variations in *Clarias gariepinus* without established data on their biochemical and genotypic relationships necessitated the use of molecular tool in establishing their biochemical and phylogenetic relationships. This study investigated the relationship in two (2) pectoral spine variant groups in *C. gariepinus* using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Protein extraction and gel electrophoresis of blood samples from 18 individuals of the species obtained from a dam system were subjected to extraction buffer and SDS PAGE at 12.5%. Variants were biochemically compared based on produced phenogram. Biochemical markers were established by comparing molecular weight of phena-specific bands with standard protein using kDa ladder. Phylogenetic relationship and subsequent test for correctness of phenotypic grouping was established via cluster analysis of scored profiles and Canonical classification, respectively. The produced phenogram revealed that the variants can be separated by a low molecular weight biochemical marker which corresponds with α-amylase inhibitor. Dendrogram generated 8 clusters at 0.63 coefficient of variation. 75% members of one of the groups clustered while canonical classification revealed 100% correctness of original phenotypic grouping. It was concluded that the variants were biochemically and genetically discriminant varieties of *C. gariepinus*. It was also concluded that this information would be of use in taxa discrimination and marker assisted selection for stock improvement in the species while differential biochemical characteristics of the sub-groups would have nutritional and medical importance.

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INTRODUCTION

Clarias gariepinus is widely considered as an important tropical catfish species for aquaculture. It is well known in both culture and artisanal environments in Nigeria where it serves both socio-cultural and research purposes in most regions. Research interest has been on its mass propagation techniques, development of recirculation system, along with quality feed development and genetic improvement of broodstocks (FAO, 2012). *C. gariepinus* and its hybrid, which is reproductively viable (Nukwan et al.,1990), are cultured throughout Nigeria and most especially in the South Western Zone. Knowledge on genetic variation in genus *Clarias* is important as it would facilitate better identification (Teugels et al., 1992; Agnese et al., 1997; Rognon et al., 1998) as well as assist in the detection of introgression and hybridization as reported in other species (Billington et al., 1996). However, there appears dearth of information on this with respect to the species in Nigeria. Na-Nakorn et al. (2002) observed an unreported *Clarias* species which has

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unique genetic profile, possess distinct meristic and morphological traits in Thailand and observed that the application of genetic data (isozymes) appears to provide an excellent method to refine systematic in *Clarias* species. The species was also genetically closest to *C. gariepinus* among the studied species. A similar trend was observed in a recent morphological study of *C. gariepinus* population in a Nigeria dam system. The study revealed morphologic forms which can be differentiated by Anal Fin Length and presence or absence of anterior serration (projection) on the pectoral spines in *C. gariepinus*. However, phenotypically divergent individuals may at the same time reflect genetic differences thus suggesting the presence of sub-species (Mayr, 1969). Such classes of individuals could have differential aquacultural potentials which would be of importance in enhancing productivity and establishing strains of the species. However, this would be possible only when their genetic relationship is established.

Na-Nakorn et al. (2004) reported that levels of hybridization and introgression from introduced catfishes or other genetically improved strains of catfishes used in aquaculture and fisheries development can be more accurately assessed when baseline genetic information is established. There is dearth of such information on the observed phenotypic variants in *C. gariepinus*. Genetically differentiated individuals may reflect bio-chemical differences which would be useful in diagnosing their functional differences. Traditional approach to characterization and evaluation of potential strains is based on morphological features followed by biochemical analysis of total protein and isozyme markers (Lombard et al., 2001; Torkpo et al., 2006). Gottileb (1971) reported that electrophoresis has an advantage that it can directly equate variation in protein banding patterns to genes encoding these proteins. Establishing the biochemical characteristics and genetic differences of the sub-groups will allow an understanding of the role of genotypic composition in the observed morphological differences as proposed by Arbour et al. (2011). Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) could genetically separate taxonomic groups with information on biochemical implication. It has been used in characterization and distinguishing varieties (Rao et al., 1992; Omitogun et al., 1999; Illoh et al., 1993; Torkpo et al., 2006). This study evaluated whether the differences in the pectoral spine sub-groups in *C. gariepinus* had genetic and/ or biochemical basis; and whether this could be referred to as potential strains or varieties.

MATERIALS AND METHODS

Sample collection

Eighteen (18) live samples of *C. gariepinus* were randomly selected from a collection of the species obtained from Asejire dam (South Western, Nigeria) between December, 2009-November, 2011. Number of individuals selected per group was determined based on the relative proportion of the group in the obtained population. Individuals possessing the anterior serration on their pectoral spines were referred to as *peses* and were denoted by C while those without the trait were referred to as smooth/non-*peses* and were denoted by S. About 2 ml of blood was drawn per individual. Blood was drawn from the caudal vein beneath the vertebral column via hypodermal needle into anticoagulant treated (heparinized) vials. Blood collection was carried out at the Department of Aquaculture and Fisheries Management, University of Ibadan, Ibadan and transported in iced container to the Biotechnology Laboratory, Federal University of Agriculture, and Abeokuta where protein extraction and gel electrophoresis was carried out.

Total protein extraction and electrophoresis

Blood samples were homogenized with extraction buffer (800 µl of 0.1 M tris-HCl at pH 7.6), vortexed for 1 min and centrifuged at 10,000 rpm/5 min/4°C. The supernatants were transferred to new Eppendorf tubes and kept in deep freezer until usage. Electrophoresis patterns of the soluble proteins were conducted using gel electrophoresis apparatus (Consort EV 231). Electro-phoresis preparation, electrophoresis conditions, staining and destaining procedures followed Laemmli (1970). The protein extract for all samples was applied to 12.5% polyacrylamide gel. The polyacrylamide resolving and stacking gels for the SDS-PAGE is presented in Table 1, while the composition of loading and running gels are presented in Table 2. Dye stocks were initially stored at 4°C and later boiled for 3 min before gel was loaded. 6 µl protein sample was added to 3 µl of 3x Leammli dye stock.

Gel was polymerized in gel caster, a thin layer of isopropanol added for smoothening the gel surface and poured into the caster after which comb is placed to create wells. Samples were loaded in individual wells made by the comb and were run for 2 h at 150 V and 0.5 mini-amp inside the electrophoresis machine. Nin (9) samples were loaded per time, based on the number of wells made from the comb. Gels were removed from the electrophoresis cells and images were scanned and stored in computer system.

Protein profile scoring

Data were collected from the gels by scoring and viewing the presence (1) or absence (0) of protein bands. The positions of the proteins as enumerated by Gatehouse (1979) and Machuka (2001) were determined using

Table 1. Solution for the 6% stacking gels, 12.5% resolving gel for SDS-PAGE.

	Substance					
	Acrylamide bisacrylamide (ml)	Tris buffer (1.0M Tris-HCl,pH8.8) (ml)	20% (w/v) SDS (µl)	dH_2O (ml)	10%APS (Ammonium persulphate) (µl)	TEMED (Tetramethylenediamine) (µl)
Resolving gel	3.1	3.0	38	1.30	36	10
Stacking gel	1.0	0.63	25.0	3.6	25.0	10.0

Table 2. Composition of solution for loading and running buffers of gels.

Loading buffer (Laemmli Loading dye) (3x stock)	Running buffer (Laemmli buffer) (10x)
1m Tris-HCl pH6.8 (4 ml)	Tris base (30.3 g)
20% SDS (3 ml)	Glycine (144.0 g)
100% Glycerol (3 ml)	SDS (10.0 g)
Bromophenol blue (0.006 g) Make up to 10 ml	dH_2O make to 1 L

standard molecular weight proteo-ladder (Norgen Inc.) and measured in kilodaltons (KDa). Bands were presented in increasing order of alphabet following increasing order of molecular weight of the bands. Similarities and divergence of individual's band scores were carried out via cluster analysis utilizing unweighted pair group method using arithmetic averages (UPGMA) for phenogram grouping (Sneath and Sokal, 1973). Analysis was done with the aid of Computer software NTSYS. The allelic scores for the groups were used for genotypic classification using Canonical Discriminant package of SPSS 15.0 Windows Evaluation Version).

RESULTS

Information on the analyzed individuals is presented in Table 3. Four (4) individuals were in the S group while the rest 14 were in C. The SDS PAGE electrophoretic profile of the populations is shown in Figures 2 and 3. The profile revealed polymorphism of bands across groups. Wide range of molecular weight allele was observed in the population. The band profile revealed 13 bands of molecular weight range of <18.4 to 100 KDa obtained across all genotypes. Most of the bands were within 18.4 and 100 KDa except band A with lower molecular weight (<18.4 KDa). Moreover, band A was distinctively inherited by 75% of the S pectoral spine group (individuals 15, 17 and 18). Analysis of the band scores revealed that 66.67% of the 234 allelic sites inherited protein bands. However, 76.92% of the bands were polymorphic (Table 4) while frequency of occurrence of each of the 13 bands was between the range of 0.17(A) – 1.00(D, L and M).

Figure 1 shows the dendrogram of the UPGMA similarity matrix of the individuals. At 0.63 coefficient of variation, 8 major clusters were identified. A reconciling of the molecular characteristics with morphological sub-groups revealed that 75% of the S group members distinguished themselves by inheriting a common band while 100% of the other group did not show the band. Moreover, canonical discriminant function presented in Table 5 revealed that 100% of the originally grouped phenotypic cases were correctly classified.

DISCUSSION

Biochemical analysis of total protein and isozyme markers has revealed better diagnostic genetic potentials and is usually free from genotype X environment interactions (Lombard et al., 2001; Torkpo et al., 2006). This was also confirmed in this study. Polymorphism of bands across groups as observed in this study showed the usefulness of the SDS PAGE as a biochemical method of genotyping with respect to the studied sub-populations. Although most of the generated bands were within 18.4 and 100 KDa, band A with lower molecular weight was inherited by 75% of

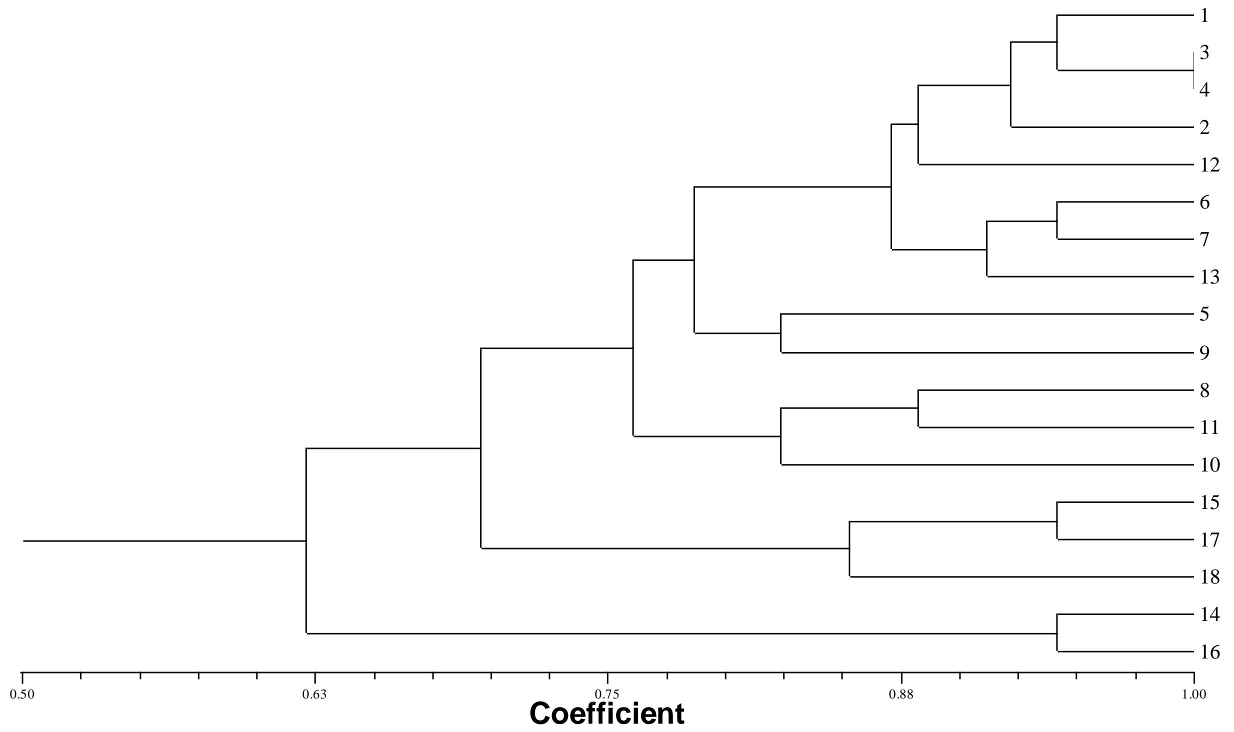

Figure 1. Dendogram of sodium dodecyl sulphate polyacrylamide protein gel of *C. gariepinus* individuals.

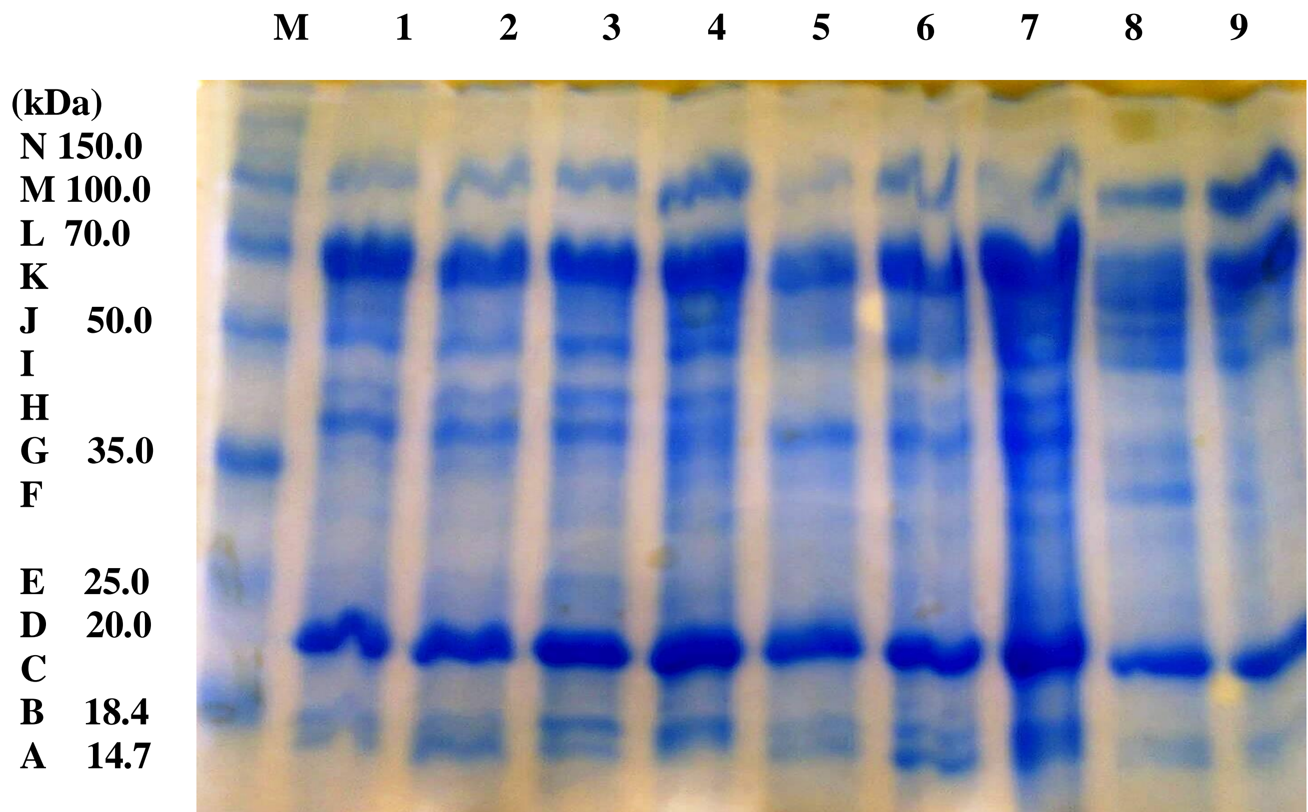

Figure 2. Protein banding pattern of Samples 1 to 9 of *C. gariepinus* in Asejire Dam.

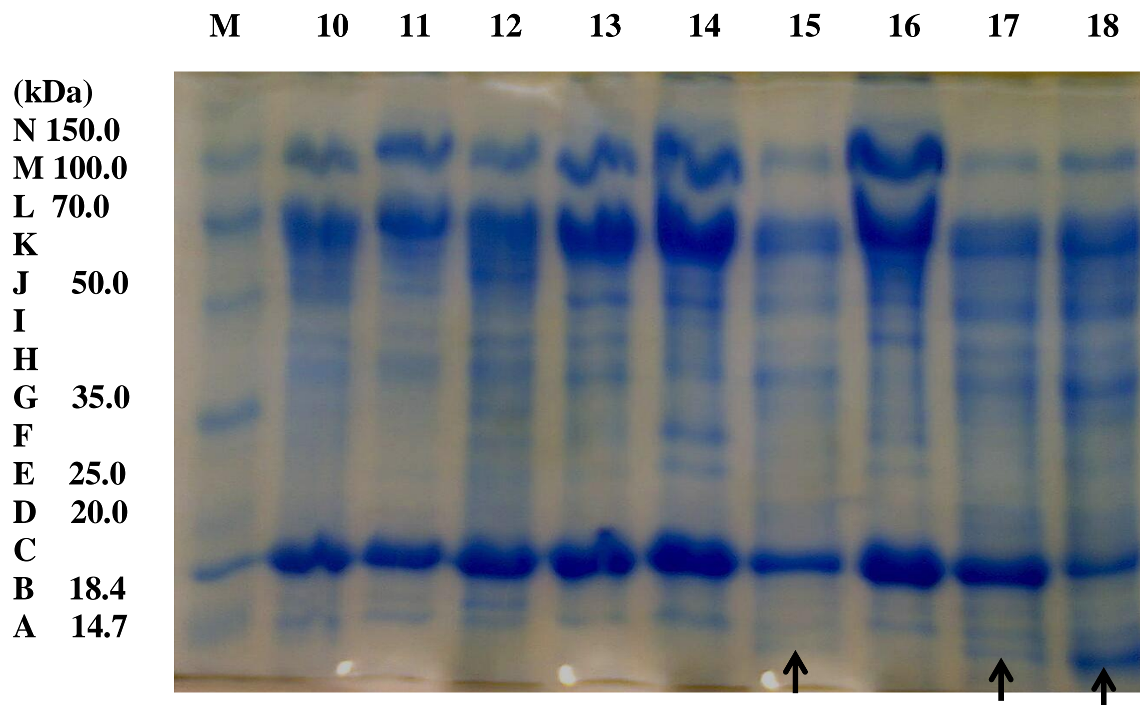

Figure 3. Protein banding pattern of Samples 10 to 18 of *C. gariepinus* in Asejire Dam.

Table3. Information on the analyzed individuals.

Sample identity No.	Score	Group
1	1	c
2	1	c
3	1	c
4	1	c
5	1	c
6	1	c
7	1	c
8	1	c
9	1	c
10	1	c
11	1	c
12	1	c
13	0	s
14	1	c
15	0	s
16	1	c
17	0	s
18	0	s

C, complete anteriorly serrated pectoral spine individual (score-1); S, Smooth anteriorly serrated pectoral spine individuals (Score-0).

members of the S group while the C sub-group did not show the band. This was also supported by the result of the canonical classification which showed that the phenotypic sub-groups were completely different from each other as observed in the phenotypic characterization. Nucleic acid molecules are size-separated with the aid of an electric field, where negatively charged molecules migrate toward anode (positive) pole. The migration flow in electrophoresis is determined by the net charge density (the ratio of charge to molecular weight). Small weight molecules migrate faster than larger ones (Sambrook and Russel, 2001).The S sub-group individuals were also grouped by similarity matrix and canonical classification to be distinctively different from the other sub-group. Analysis of genetic relationship in morphological divergence groups is a kind of characterization that could generate varieties or breeds in fish stocks. It could be of importance in genetic improvement, management and conservation especially when markers for sub-species identity are available. Based on the result of the protein electrophoreses, the sub-groups were observed to be genetically discriminant groups which can be differentiated by allele A. This allele could therefore be observed as a potential marker for deciphering the morphologic sub-groups as the allele A tend to be controlling the absence or presence of the pectoral spine attribute (*peses*). This knowledge could be of use in genetic improvement of the species via marker assisted selection. Omitogun et al. (2001) had earlier observed that genes controlling each character can be mapped and isolated to complement and hasten the work of breeders for genetic improvement. Electrophoresis is a sieving

Table 4. Distribution of identified 13 bands across the studied individuals.

Allelic band	Number of occurrence	Frequency
A	3	0.17
B	15	0.83
C	10	0.56
D	18	1.00
E	9	0.5
F	12	0.67
G	12	0.67
H	10	0.56
I	8	0.44
J	16	0.89
K	8	0.44
L	18	1.00
M	18	1.00

Table 5. Classification results of smooth and completely serrated pectoral spined *C. gariepinus.*

Score		Predicted group	Membership	Total
		0.00	1.00	0.00
Original count	0.00	4	0.0	4
	1.00	0	14	14
	0.00	100.0	0	100.0
	1.00	0	100.0	100.0

process for proteins and this is based on molecular weight of nucleic acids. Allele A had the lowest molecular weight. The low molecular weight allele could be linked with α-amylase inhibitor. Gatehouse (1979) and Machuka (2001) determined position of proteins using standard low and high molecular weight markers in kilodaltons such as: phosphorylase B, 94; bovine serum albumin, 67; ovaaalbumin, 43; carbonic anhydrase, 30; trypsin inhibitor, 20.1 and α-amylase inhibitor, 14.4. All the proteins reported by these authors were within medium range proteo-ladder (14.7-100 kDa). The position of allele A was observed in this study to be the closest to 14.4 which corresponds to the molecular weight of α-amylase inhibitor.

α-Amylases is a family of enzymes that hydrolyse α-D-(1,4)-glucan linkages and plays an important role in the carbohydrate metabolism of many autotrophic and heterotrophic organisms (MacGregor et al., 2001). It is primarily used in heterotrophic organisms to digest starch in their food sources (Silva et al., 2000). However, α-amylase and proteinase inhibitors are attractive candidate for the control of starch dependent organism and have been used in control of seed weevils (Franco et al., 2000). Protein inhibitors of α-amylase are believed to make plants less palatable, even lethal to insects (Sasikiran et al., 2002); they are starch blockers preventing dietary starches from being digested and absorbed by the body (McEwan et al., 2010). Ali et al. (2006) reported that it could be useful in treating obesity and diabetes mellitus resulting from defects in insulin secretion. This information would be of relevance in nutrition and medicine because inhibitory activity of amylases in food source could cause a marked decrease in the availability of digested starch in the consumers and in diabetes patients. Detection of α-amylase inhibitor in almost all the members of the S group could therefore indicate that although the sub-species may not have comparative advantage of been highly palatable and efficient in digesting and absorbing starches when compared with the C sub-group; they would however be of potential use in treating obesity and diabetes mellitus in human. Extensive research has been conducted on the properties and biological effects of these inhibitors in plant physiology, animal and human nutrition because of their possible importance (Garcia-Olmedo et al., 1987; Silano, 1987). Alpha-amylase inhibitors could however be manipulated through genetic engineering (Wang et al., 2006) and could be isolated and purified from specimens (McEwan, 2010). The separation of the species by the presence of a proteinase inhibitor may have evolutionary

basis. Such evolutionary trend could come from pressures of various kinds and may be phylogenetically related. Proteinase inhibitors are a potential model system that is used to study basic evolutionary processes, such as functional diversification (Christeller, 2005). Morphological studies had earlier observed existence of functional disparity in the sub-species.

In conclusion, pectoral spine sub-groups in *C. gariepinus* were biochemically separated by the presence or absence of alpha-amylase inhibitor which is of nutritional and medical importance to consumers. However, the groups could have developed into these sub-species as a result of evolutionary factors.

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