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ZYMMOGRAMMATIC STUDY OF DIFFERENT FORMS OF *CLARIAS* SPECIES

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Abstract

In present study 0,1 matrix was prepared by using electropherogram. Neighbor Joining tree based on Nei and Li distances and Neighbor Joining tree - Jaccard's similarity coefficient was erected. Neighbor Joining tree based on Nei and Li distances clearly indicated that intermediate form is hybrid of C.b and C.g so it can not be given a status of independent taxon.

INTRODUCTION

It is well established that certain proteins usually the water soluble ones like muscle myogen can be separated by electrophoresis and used to identify species and correlate the species using computer softwares. The separation patterns are species specific and found to be independent of physiological state or environment. The methods described are of immense value to the taxonomists in ascertaining the identity of closely related species and sub-species showing very similar morphological characters or if the fish show any deformity in the external morphology due to injury at embryonic or post embryonic development stages. Usual morophological techniques supplemented with biochemical studies can relieve the taxonomists from many anxieties of proper identification.

Therefore, zymogrammatic study of all the three forms were also carried out using SDS-PAGE and the results were analysed statistically to derive correlation its intermediate form among the two species and relate them taxonomically.

Sample Collection

The acclimatized fishes viz., *Clarias batrachus, Clarias gariepinus* and Intermediate form from the aquarium were taken and sacrificed to

collect muscle samples. The muscle samples were taken from just below the dorsal fin, care was taken to isolate the muscle from other tissue. The separated tissue was immediately aliquoted and preserved at -20°C in a freezer. The process of collection was repeated at least five times with all the three forms.

Myogen extraction preparation

White muscles from the freezer was taken and washed thoroughly with distilled water and soaked dry on blotting paper.Care was taken not to include pieces of skin,blood etc,since their presence might alter the separation pattern.

About 10 gm of muscles was homogenized with an equal weight of distilled water and then centrifuged at 3000 rpm for 20 minutes

Clear supernatant was decanted and diluted with an equal volume of 40% sucrose solution and stored at 0°C until required.

Preparation of loading buffer

The gel loading buffer was prepared according to – Sambrook, et al (1989). 50 mM Tris HCl (PH 6.8), 100 mM ditiothreitol (DTT), 2% SDS (electrophoresis grade), 0.1% bromophenol blue,10% glycerol,1X SDS gel loading buffer lacking ditiothreitol was stored at room temperature. DTT was then added just before the buffer was used from 1M stock solution.

Prepration of different chemicals

Acrylamide and Bis acrylamide

Electrophoresis grade acrylamide and bisacrylamide was arranged and stock solution of 29 % (w/v) acrylamide and 1 % (w/v) N, N'–methylene bisacrylamide was prepared in deionized water. The resultant solution was stored in dark coloured bottles. Extreme care was taken in handling these chemicals owing to their neurotoxic nature

Sodium dodecyl Sulfate (SDS) also called sodium lauryl sulfate

Special electrophoresis grade sodium dodecyl sulfate was used to make 10 % (w/v) stock solution with deionized water. The solution was stored at room temperature. 100 gm SDS and 900 ml H_2O were mixed and heated to about 60 °C. The pH was adjusted to 7.2 by adding a few drops of concentrated HCl. The volume was later adjusted to 1 It. The solution was aliquoted and stored.

Staining Solution

0.25 g Coomassie Brilliant Blue R 250 in 90 ml of methanol: (1:1 w/v) and 10 ml of glacial acetic acid.

TABLE – A

TABLE - B

Tris buffers for the preparation of resolving gel:

Tris base was dissolved in deionized water and pH was adjusted to pH 8.8 using conc HCl.

Tris buffer for the preparation of stacking gel:

Tris base was dissolved in deionized water and pH was adjusted to pH 6.8 using conc HCl.

Ammonium persulfate:

10 % (w/v) solution was prepared fresh every time the gel was run.

Tris–glycine electrophoresis buffer:

This buffer contains 25 mM Tris base, 250 mM glycine (electrophoresis grade, pH 8.3), 0.1% SDS. A 5X stock

TABLE – A

For preparing resolving gels (15%) for tris-glycine SDS – Polyacrylamide Gel Electrophoresis Component Volume (ml) per gel mould volume of

Solution Components	5	10	15	20	25	30	40	50
H_2O	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
30% acrylamide mix	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5M Tris (pH 8.8)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% SDS	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
10% ammonium Persulfate	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED (fresh)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

TABLE – B

For preparing resolving gels (5%) for tris-glycine SDS – Polyacrylamide Gel Electrophoresis Solution Component Volume (ml) per gel mould volume of Component

Solution Components	1	2	3	4	5	6	8	10
H2O	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
30% acrylamide mix	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0M Tris (pH 6.8)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
10% SDS	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
10% ammonium Persulfate	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

was prepared by dissolving 15 .1 g of Tris base and 94 g of glycine in 900 ml of deionizedO. Then 50 ml of 10% (w/v) stock solution of electrophoresis grade SDS was added and the volume was adjusted to 1000 ml with deionizedO.

Pouring SDS – Polyacrylamide Gel:

The glass plates of electrophoresis unit were assembled according to manufacturer's instructions. After determining the volume of the gel mould (from instruction manual provided by manufacturers) the solution for resolving gel was prepared according to table A. Special care was taken to proceed to next step after addition of TEMED as it begins the polymerization process.

The solution was poured into the gap of the glass plates (which were arranged using 1.5 mm spacers). Care was taken to leave sufficient space for stacking gel (the length of the teeth of the comb plus 1 cm); using pipette overlay butanol was carefully layered. The gel was then carefully placed in a vertical position at room temperature (The overlay prevents oxygen from diffusing into the gel and inhibiting polymerization). After polymerization is complete (30 minutes to 1 hr.) the overlay was poured off and the top of the gel was washed several times with deionized water to remove any unpolymerized acrylamide. As much fluid as possible was drained and the remaining water was carefully removed with the edge of the blotting paper. In a disposable plastic tube appropriate volume of stacking gel solution was made according to Table B.

The stacking gel solution was immediately poured directly on to the surface of the polymerized resolving gel. Teflon comb was immediately inserted. Care was taken to avoid air bubbles. More stacking gel solution was filled in spaces of the comb completely. The gel was placed in a vertical position at room temperature. Overlay of butanol was added in drops over the spaces between the combs. For preparation of sample equal amount (1/2 a gm) of muscle was homogenized with tissue homogeniser after adding 1X running buffer (1 ml) after thorough homogenisation the samples were subjected to centrifugation at 5000 rpm for 15-20 minutes. The supernatant was taken and mixed with loading gel buffer in the ratio of 9:1. While the stacking gel polymerized, the samples were prepared by heating them to 90-100 °C for three minutes with 1X SDS gel loading buffer in a waterbath to denature the proteins. After polymerization was complete the comb was removed carefully. The wells were washed immediately with deionized water; the gel was mounted in the electrophoresis apparatus. Tris-glycine electrophoresis

buffer was added to the top and the bottom reservoirs.

5X Tris glycine electrophoresis buffer was prepared by dissolving 15.1g of Tris base and 94 gm of glycine is 900 ml of deionized water, afterwards 5 ml of a 10% (w/v) stock solution of SDS was added and the volume adjusted to 1000 ml with deionized water.

The gel was loaded with samples 10-15 µl of each sample in the predetermined order were deposited at the bottom of the gel, any unused well was filled with 1X SDS gel loading buffer. The electrophoresis unit was attached to electric power supply 150 to 230 volts, 35 m amp. was applied. After the dye front moved into the resolving gel the voltage was increased. After some time when bromophenol blue reached the bottom of the gel. Then the power supply was turned off.

Care was taken to ensure uninterrupted power supply. Care was also taken so that there is no change in room temperature during the run. The glass plates were removed from the electrophoresis apparatus. The gel was removed from the plates and stained with coomassie brilliant blue solution. The staining continued for 4 hrs. and immediately put in destaining solution for at least 2 hrs., destaining solution was changed three or four times .After destaining the gels were stored in sealed plastic bags in 20% glycerol to avoid fading of protein bands.

The stained gels were photographed for permanent record and analysed by making these graphs on graph paper; 0, 1 matrix was prepared and NT-SYS software was used to generate Neighbor Joining Tree based on Nei and Li distances and Nj tree based on jaccard's similarity coefficient to find out phylogenetic relationship among all three forms.

§ For each aspect, approximately 40 experiments were carried out for each species and 30 readings were randomly picked up and statistically analyzed. The data were analyzed by Analysis of Variance (ANOVA) followed by Post HOC multiple comparison test (Tukey HSD).

§ **ZYMOGRAMMATIC STUDY**

§ For zymogrammatic study electrophoresis was carried out on all three forms of *Clarias* (Pl V, VI, and VII) to establish phylogenetic relationship. Photographs were taken and 0, 1 matrix (Table 22, Pl VIII) was prepared to make Neighbor Joining tree based on Nei and Li distances and Nj tree-Jaccard's similarity coefficient.

TABLE -22

Marker	Number	*Clarias batrachus*	*Clarias gariepinus*	Intermediate form
I	1	1	0	0
	2	1	1	1
	3	1	1	1
II	4	0	0	1
	5	0	1	1
III	6	1	1	1
	7	0	1	0
IV	8	1	1	1
V	9	1	1	1
	10	0	1	1
	11	0	1	1
VI	12	0	1	1
	13	1	1	1
VII	14	1	0	1
	15	1	1	1
	16	1	1	1
	17	1	1	1
VIII	18	1	1	1

Electropherogram of muscle myogens were compared among three forms of genus *Clarias* of Meerut region. The pattern was studied in order to estimate the degree of biochemical (muscle protein) difference among them. Neighbor joining Tree based on Nei and Li distances (PL IXA) and Njtree – Jaccard's similarity coefficient (PL IXB) were erected using NT–SYS software. The three forms of Genus *Clarias* viz. *Clarias batrachus, Clarias gariepinus* and Intermediate form were compared to bring about interspecific relationship. Neighbor Joining tree based on Nei and Li distances (PLATE IX-A) clearly indicates that intermediate form is hybrid of C.b and C.g so it cannot be given a status of independent taxon.Neighbor Joining tree - Jaccard's similarity coefficient (PLATE IX-B) showed that intermediate form is closer to C.g as compared to C.b as is also supported from morphological characterstics and other biochemical parameters. Clearly indicating the fact that there is an interbreeding between C.b and C.g.

§ **Zymogrammatic Sudy**

§ In zymogrammatic study *Clarias batrachus, Clarias gariepinus*

DISTANCE AND SIMILARITY COEFFICIENTS

Dice Coef	C. b	C. g	I. f
C. b		0.74074	0.78571
C. g	0.74074		0.90323
I. f	0.78571	0.90323	
Dice dist	C. b	I. f	C. g
C. b		0.33333	0.47826
I. f	0.33333		0.16129
C. g	0.47826	0.16129	
Jaccard	C. b	I. f	C. g
C. b		0.5	0.64706
I. f	0.5		0.27778
C. g	0.64706	0.27778	

and Intermediate form were studied to identify the species specific myogen bands. A catalogue of the characteristic band pattern for identification of fish species of Genus *Clarias* can be prepared as suggested by Mackie (1969). Although this technique cannot be used as a substitute for general morphological technique but can be used as a supplement to it.

§ Electrophoretic analysis of muscle myogen is a better means for identify closely related species because in morphological identification we may need to examine large number of fishes due to interspecific and intraspecfic overlapping in characters whereas in this analysis single specimen is sufficient to identify a species. Another very important aspect of this technique is identifying a fish species which has lost its identifiable morphological characters. Many a times, the young population of a particular species looks entirely different morphologically from its adult population and have been in the past described as new species, similarly sexually dimorphic forms have also been described as different species of the same Genus. These confusions can very easily be clarified and verified by doing muscle myogen analysis of these fishes if the band patterns are similar in these species they belong to same group and if they differ they belong to different species.

§ In present study 0,1 matrix was prepared by using electropherogram.

Neighbor Joining tree based on Nei and Li distances and Neighbor Joining tree - Jaccard's similarity coefficient was erected. Neighbor Joining tree based on Nei and Li distances clearly indicated that intermediate form is hybrid of C.b and C.g so it can not be given a status of independent taxon. Neighbor joining tree - Jaccard's similarity coefficient showed that Intermediate form is closer to C.g as compared to C.b as is also supported from morphological characterstics and other biochemical parameters. Clearly indicating the fact there is inbreeding between C.b and C.g.

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