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Arpita Rakshit
(a) Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.
(b) Ramsaday College, Amta, Howrah – 711401, West Bengal, India.

Aditya Paul
(a) Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.
(b) Ramsaday College, Amta, Howrah – 711401, West Bengal, India.

Somnath Bhattacharjee
Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.

Tanmoy Banik
Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.

Rita Saran
Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.

Banasree Mandal
Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.

Deep Poddar
Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.

Krishna Gangopadhyay
Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.

Correspondence
Krishna Gangopadhyay
Rishi Bankim Chandra College [P.G. Studies], Kanthalpara, Naihati, North 24 Parganas – 743165, West Bengal, India.

Cytogenetic and molecular profiling of spotted snake head fish *Channa punctatus* (Bloch, 1793) from three districts (Nadia, Hooghly and north 24 Parganas) of west Bengal, India

Arpita Rakshit, Aditya Paul, Somnath Bhattacharjee, Tanmoy Banik, Rita Saran, Banasree Mandal, Deep Poddar, Krishna Gangopadhyay

Abstract
Cytogenetic and genomic studies in recent years play a vital role in clarifying the considerable importance regarding species characterization, evolution and systemic studies. Spotted snakehead fish [*Channa punctatus*], belonging to family Channidae, collected from Nadia, Hooghly and North 24 Parganas, the three districts of West Bengal, [two sites from each district], India, and was characterized by karyotype analysis with Nucleolus Organizer Regions [NORs] and RAPD markers. Nucleolus Organizer Regions [NORs] are usually detected on the metaphase and interphase fish chromosomes through controlled silver nitrate staining technique. RAPD profiles were generated using eight oligonucleotide primers which produced repeatable and consistent results. Karyotypic study with NOR and the RAPD study of the *C. punctatus* species, from three districts of West Bengal, India, shows emphasis on intraspecies diversity in *C. punctatus* species. Therefore, the differences in results might not be only due to geographical differentiation, but also due to genetic differences of initial founder individual [s] between geographical populations which were widely migrated as a result of area effects like flood of river, founder events, genetic drift, reduced gene flow, climatic and other local adaptations, and also by transposable genetic elements.

Keywords: Karyotype, AgNOR, Genetic diversity, RAPD.

1. Introduction
Channa punctatus (Bloch 1793), a freshwater common green snake-headed spotted murrel, is belonging to the family Channidae of the order Channiformes with accessory respiratory organs that help the fish to survive in shallow muddy water. They are freshwater predatory fishes found throughout South, East and Southeast Asia and are very important in fishery, aquaculture, food fish species, pharmaceutical products , traditional medicine and as well as food fish species. The studied *Channa sp* in Indian subcontinent have been studied karyotypically so far, each having a different diploid number in family Channidae: *C. punctatus* 2n=32 [1], *C. striatus*, 2n=40 [2], *C. gachua*, 2n=78 [1], *C. orientalis*, 2n=52 [4]. Variations on chromosome number and morphology can be found between different populations of the same species, as reported in *Channa gachua* [3], [4] or within different individuals of the same population [5].
The Detailed cytological analysis of fish chromosome can throw light on the field of aquaculture, conservation process of a particular species, taxonomic relationship in between the species and among the species and their phylogenetic relationship. Cytogenetic studies have been proved successful in higher vertebrates but due to large number and relatively small size of chromosomes in fish species, the study is not sufficient [6]. The basic information on the number, size and morphology of chromosomes [7] is important to study chromosomal differentiation. For evolutionary study of indigenous species like *Channa,* the knowledge of genetic divergence of population and their local adaptations can provide a potential resource.
The nucleolus organizer regions [NORs] are the chromosomal sites of genes, which transcribe for 18s and 28s ribosomal RNA, that were presumably transcribed at preceding interphase and are important in view of their intimate relationship with protein synthesis [8]. The development of silver staining technique, which actually involves staining of the acidic proteins located in

and associated with the fibrillar structure of NORs [8]. to detect metaphase chromosome sites of NORs has greatly facilitated comparative studies of NOR variation within and in between species and in studying phylogenetics and systematics [9].

In recent years, Random Amplified Polymorphic DNA [RAPD] analyses through the Polymerase Chain Reaction (PCR) has been widely used in molecular characterization and tracing the phylogeny of diverse plant and animal species. This technique has contributed widely in the studies of genetic diversity and phylogenetic analysis [10, 11]. These markers are used as versatile tools to provide the correct estimate of genetic diversity. The main advantages of RAPD analysis over other molecular methods are low sample DNA requirements, the high frequency of detectable polymorphic bands [12] and independent from the effects of environmental factors [13, 14]. Recently, molecular markers have been used for genetic structure analyses in different fish species [15], but the studies are still insufficient in the case of indigenous fish like *Channa punctatus* of the studied region.

Due to different anthropogenic activities like construction of thermal power station (Bandel), increased use of pesticides in the agricultural fields, establishment of new industrial units, the most serious environmental changes occur for species inhabiting either land or water. The reservoirs can cause modifications in the pattern of dispersion of various species of fish migration [16] and, under certain conditions, may lead to the extinction of species that cannot adapt to this kind of change [17]. Reduction of dispersion and gene flow may in time alter the relative proportions of the intra- and inter-species diversity [18]. The study of genetic variability is of prime importance for genetic approaches to fish conservation or breeding, which depend on knowledge of the amount of variation existing in a local reproductive unit [19]. According to Haig, 1998 [20], the most important contribution that conservation geneticists can make to the assessment of the viability of populations is to determine the relative amounts of genetic diversity within and among the populations.

The present study is therefore designed to determine the karyotype of the fish specimen of the six populations from three districts of West Bengal, India, to ascertain the taxonomic and evolutionary status of this particular fish species using silver staining as well as RAPD analysis. The objective of the present study is to increase the knowledge of cytology and the effects of genetic variability on distribution of *Channa* sp to better understand the role of diversity, within and among the populations. The study also aims to gain more insight into the relationship between the chromosomal differentiation and evolution of *Channa punctatus* as and to understand evolution of fishes in general.

2. Materials and methods

2.1 Location of the study Area

Thirty live specimens of *Channa punctatus* (Five each from a particular area) were collected from water bodies of Bartir Bill, Birati, Bandel, Simurali, Singur and Kalyani, West Bengal, India. The initial identification was made on the basis of morphological characters [21]. These studied areas, as shown in Table 1, are not only differing in their microclimatic conditions but also in ecological conditions.

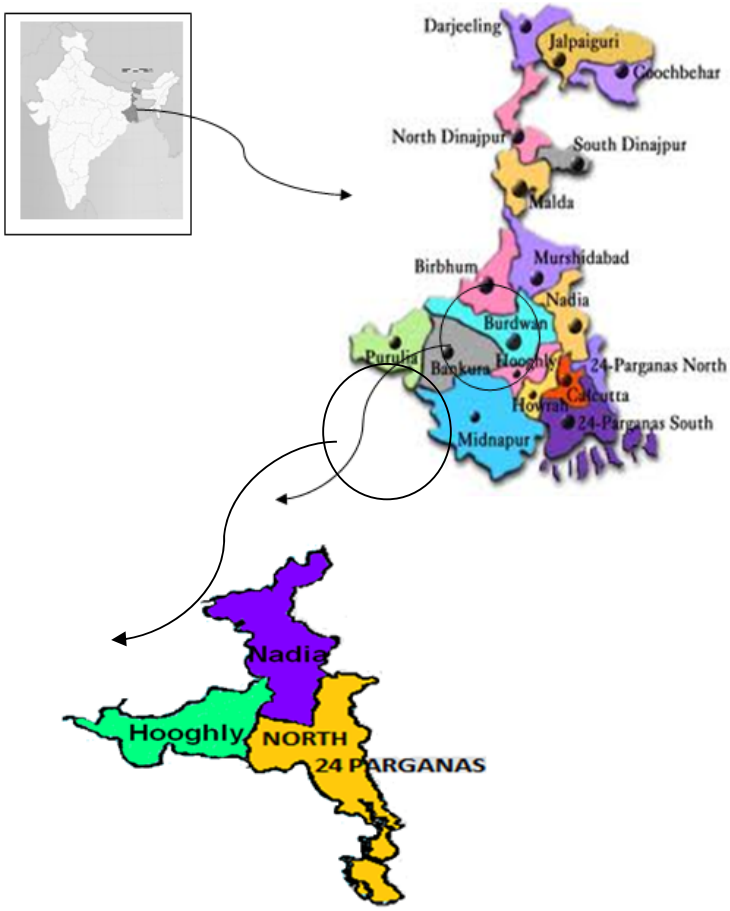

Fig 1: [a] Map of West Bengal, India showing study area, [b] Detailed Map of study area of West Bengal, India

Table 1: Study Area Details of the Collected *Channa punctatus* species

Districts	Study sites	Latitude	Longitude
North 24 Parganas	Birati	22.6592°N	88.4384°E
	Bartir Bill	22.83°N	88.37°E
Hooghly	Bandel	22°55′22″N	88°22′46″ E
	Singur	22.8100° N	88.2300°E
Nadia	Kalyani	22.9800° N	88.4800° E
	Simurali	22.9400° N	88.4200° E

2.2 Karyotype Analysis and Ag NOR Staining

Live specimens of *Channa punctatus* were collected from the fish farm at different studied locations and kept them for seven days in the laboratory for acclimatization. Chromosome preparation was obtained from gill and head kidney tissues using the technique described by Khuda-Bukhsh and Barat, 1987 [22]. Silver staining of the nucleolar organizer region was performed according to Ploton *et al*., 1986 [23].

Chromosome morphology was determined on the basis of arm ratios as proposed by Levan *et al*., 1964 [24]. Metacentric [M], submetacentric [SM], and subtelocentric [ST] chromosomes were considered as bi-armed.

Leica DM LS2 trinoccular microscope fitted with a camera and 100x×10x oil immersion lens combination was used to scan the cells and take the photographs.

2.3 DNA isolation and RAPD- PCR amplification of DNA

Genomic DNA was isolated from fish scales using the protocol of Wasko *et al*, 2003 [25]. After ensuring complete solubility of DNA, the purity factor [A_{260} / A_{280} nm] was measured spectrophotometrically and its integrity was checked.

Polymerase chain reaction [PCR] was conducted in a thermo-cycler [Applied Biosystems] in 50 µl of the reaction medium containing10x PCR Buffer [Genei, Bangalore]; 50 mM KCl; 2 mM $MgCl_2$ [Genei, Bangalore]; 0.2 mM of each dNTPs [Genei, Bangalore]; 1 mM primer; 20–50 ng DNA template; and 1 U of *Taq [Thermophilus aquaticus]* polymerase. The amplification cycles were repeated 30 times and comprised denaturation [94 °C, 1 min], annealing [55 °C, 1 min], and elongation [72 °C, 2 min]. Upon synthesis of amplified DNA, the reaction was terminated by decreasing the temperature at 4 °C. The amplicons were thereafter, fractionated in 1% agarose gel in 1×TAE buffer supplemented with ethidium bromide at 70-80 volt. The DNA Ladder [2000 bp- 100 bp in size] [Gene Ruler®, Fermentas] was used as molecular weight markers. Eight random primers with a length of 10 - 16 bp were used for our experiment. The primers, as shown in Table 2, were commercially synthesized by IDT integrated DNA technologies [Genei™, Bangalore].

Only distinctly visible fragments reproducible in repeated experiments were taken into account when analyzing the electrophoretic patterns. The differences in staining intensity of the bands were not considered.

Table 2: Primer and their code used for PCR amplification

Sl. No.	Name	Primer sequenced	GC [%]
1	OPA1	5'-TGC CGA GCT G-3'	70
2	OPA2	5'-AGG GGT CTT G-3'	60
3	OPA 3	5'-GAA ACG GGT G-3'	60
4	OPA 4	5'-GTG ACG TAG G-3'	60
5	OPA 5	5'-GGG TAA CGC C-3'	70
6	OPA 6	5′ - AGCTCACTGA - 3′	50
7	OPA 8	5'-AGT CGG GCT G-3'	70
8	OPA 9	5'-AAT CGG GCT G-3'	60

2.4 Matrix by Jaccard's similarity coefficient method and Cluster Analysis

The calculation of Jaccard's Coefficient was made with numbers of total bands and with numbers of primers used in the study [27] and the Jaccard Similarity Coefficient, J was defined as

$$J(A,B) = \frac{|A \cap B|}{|A \cup B|}.$$

Where A = First species, B = Second species whose relatedness to the first species needs to be ascertained. In simpler cases, where only relatedness or similarities between two species were to be determined, the mean of all the J values for different arbitrary primers were calculated [27].

The Jaccard Similarity Co-efficient values were between 1, for Operational Taxonomic Unit [OTU] with total similarity and 0 for OTUs with no similarity at all. For an average analysis with two primers, if the coefficient falls between any values between 0.75 ~ 1, one arbitrary assumes the species to belong same species complex [Tulsa: Stat Soft, 1996].

A classification assay of the studied *Channa* species was performed by cluster analysis and multidimensional scaling using the program package STATISTICA. The cluster analysis was performed using bootstrap and the phylogram was constructed based on the data [28, 29].

For statistical analysis of the fragments detected, a consolidated binary matrix was constructed, where the presence or absence of an amplified fragment in the pattern of the corresponding sample was denoted by 1 or 0, respectively.

3. Results and Discussions

All the studied species of *C. punctatus*, presented a diploid chromosome number 2n= 32, from all locations. But the species has different numbers of metacentric, sub metacentric, telocentric, acrocentric chromosomes, as shown in Table 3.

All the fish from different locations showed 3 pairs of AgNOR region, i.e., 3rd, 9th and 11th pair of chromosome, as shown in Table 3.

Table 3: Karyotype Pattern of *Channa punctatus* in studied locations of West Bengal, India

Districts	Study sites	Species	Karyotype	Fundamental Arm Number [FN]	AgNOR regions
North 24 Parganas	Birati	*Channa punctatus*	16 m + 16 sm	64	3 pairs, i.e., 4th, 9th and 11th pair of chromosomes
	Bartir Bill	*Channa punctatus*	18 m + 14 sm	64	3 pairs, i.e., 4th, 9th and 11th pair of chromosomes
Hooghly	Bandel	*Channa punctatus*	16 m + 12 sm+ 4a	60	3 pairs, i.e., 4th, 9th and 11th pair of chromosomes
	Singur	*Channa punctatus*	16 m + 14 sm+ 2a	62	3 pairs, i.e., 4th, 9th and 11th pair of chromosomes
Nadia	Kalyani	*Channa punctatus*	16 m + 16 sm	64	3 pairs, i.e., 4th, 9th and 11th pair of chromosomes
	Simurali	*Channa punctatus*	20 m + 12 sm	64	3 pairs, i.e., 4th, 9th and 11th pair of chromosomes

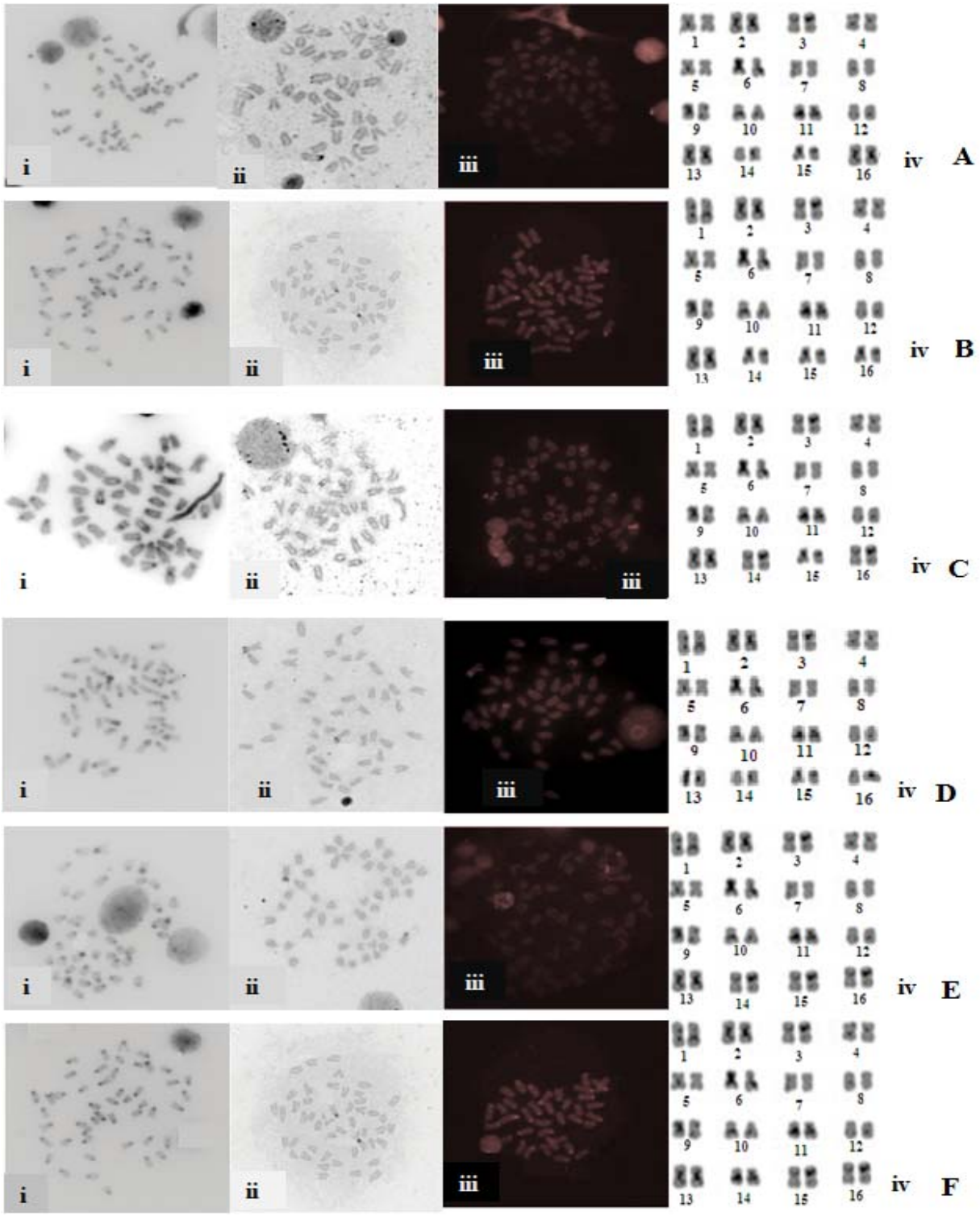

Fig 2: Mitotic metaphase and karyotype from kidney cells of Channa punctatus from six population in three districts of West Bengal. A. Singur, Hooghly, B. Kalyani, Nadia, C. Simurali, Nadia , D. Bandel, Hooghly , E. Birati, North 24 Parganas, F. Bartir bill, Shyamnagar.

A reference to the karyotypes of five *Channa* species so far analyzed indicates that species with large number of chromosomes have higher number of acrocentric chromosomes [1, 3]. This observation supports the hypothesis of origin of biarmed chromosomes from acrocentric counterparts in *Channa* species. Chromosome polymorphism in *Channa* species due to subtelocentric and acrocentric members evidences that pericentric inversion operated in the karyotype evolution of channid fishes. Current knowledge of molecular genetics can explain the molecular basis of speciation through pericentric inversion.

Chromosomal differentiations help with the storage of genetic variability, with the establishment of isolating mechanisms and with protecting the gene pool against the influx of alien genes. Molecular basis of these evolution related functions of chromosomal changes can now be examined in the context of changes in gene expression brought by chromosomal changes since the effects of various genetic and epigenetic changes on

gene expression are fairly understood. Robertsonian rearrangements, pericentric inversion and polyploidy have been implicated for evolution of the Channid fishes. These chromosomal changes are, however, inadequate to explain differentiation of these species because some of these species have similar chromosome number and closely similar chromosome morphology [2]. Moreover, karyotype evolution in fishes through pericentric inversion would require long-drawn cytological adaptations operated for long many years and through Robersonian rearrangements would lead to too many complicated steps. Total number of chromosome is same in all populations of *Channa punctatus* fishes but there is difference in the number of metacentric, submetacentric, telocentric, acrocentric chromosomes. Similarity in number but dissimilarity in morphology of chromosome complements within a *Channa* species cannot be fully explained by pericentric inversion alone. These observations can be rather attributed to centromere repositioning via neocentromere emergence as demonstrated for primates.

The Nucleolar Organizer Regions [NOR] are described as highly repetitive genome sites related to the rRNA synthesis. These repetitive regions present small, active transcription sites and non-transcribed spacing segments with their own structural dynamics, in which the presence of transposons located close to the genome regions has been identified. The NOR location can describe the chromosome evolution. Interstitial NOR probably was originated by tandem fusion between NOR-bearing chromosomes and other chromosomes but *C. punctatus* might have been produced NOR-bearing chromosomes through pericentric inversion or even through centric fusion of two telocentric chromosomes, one of which had telomeric NOR [23].

After silver-staining, the NORs can be easily identified as black dots exclusively localised throughout the nucleolar area, and are called "AgNORs". The NORs' argyrophilia is due to a group of nucleolar proteins, which have a high affinity for silver [AgNOR proteins]. One pair of NOR in *C. orientalis* and three pairs of NOR in *C. striatus* and *C. punctatus* indicate NOR division by chromosome rearrangements or DNA magnification. These mechanisms can adjust rDNA redundancy under selection pressure for a changed phenotype [22]. This effect may be due to disturbance of transcribing sequence by the inserted NOR similar to the effect of transposon on gene expression [23]. The ribosomal genes are organised into tandem arrays in the heterochromatin in multicellular organisms. Some regions of heterochromatin contain actively transcribed gene which require proximity to heterochromatin for their normal expression. Chromosome rearrangements that separate these genes from large blocks of heterochromatin result in their variegated expression. NOR differentiation might be an effective process in the evolution of *Channa* species.

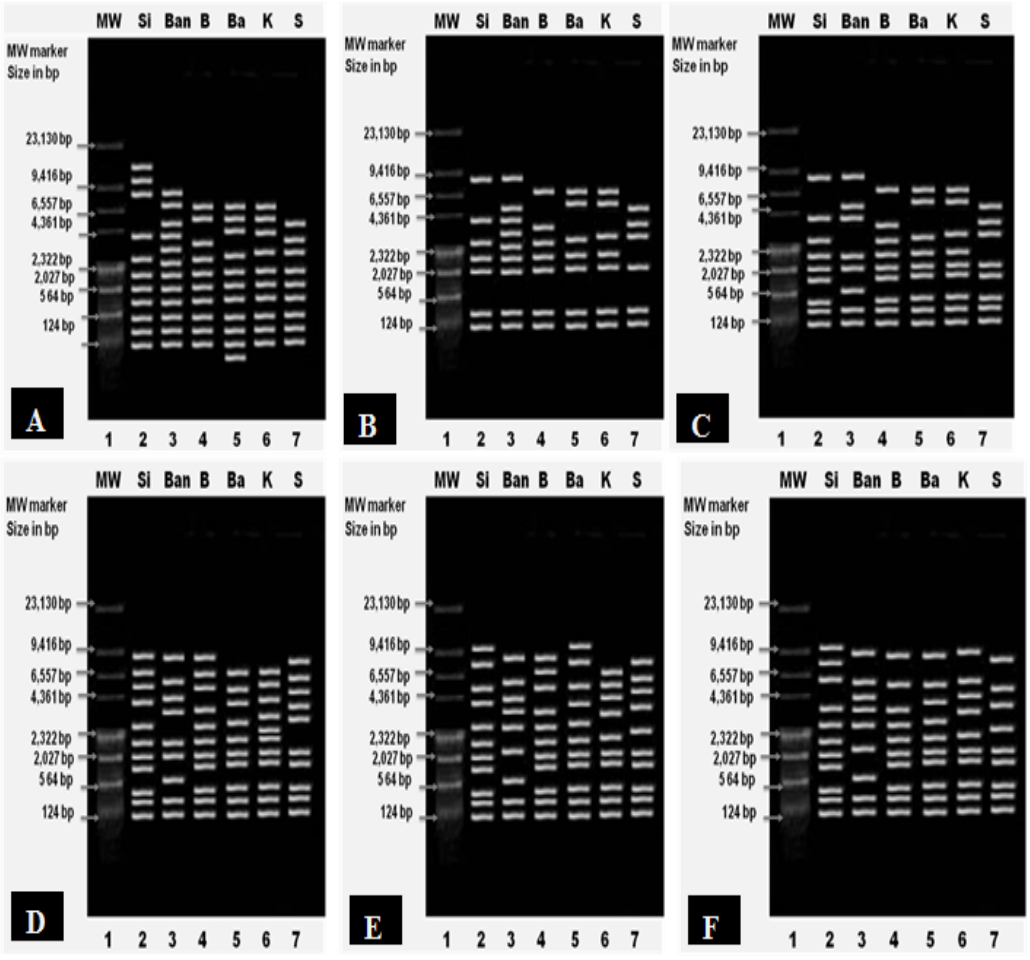

Fig 3: RAPD – PCR amplification of 50 ng of total nucleic acid of *Channa punctatus* from three districts of West Bengal. [A] by Primer OPA 1, [B] by Primer OPA 2, [C] by Primer OPA 3, [D] by Primer OPA 4, [E] by Primer OPA 5, [F] by Primer OPA 6, [G] by Primer OPA 8, [H] by Primer OPA 9. Si – Singur, Hooghly; Ban – Bandel, Hoogly; B—Birati, North 24 Parganas; Ba – Bartir Bill, Shyamnagar, North 24 Parganas; K—Kalyani, Nadia; S – Simurali, Nadia.

Results presented in Figure 3 show the amplified banding patterns of the six populations of *C. punctatus* species from the six locations of three districts of West Bengal, Inida, studied using the RAPD primers.

Figure 3A, 3B, 3C, 3D, 3E, 3F show the agarose gel electrophoresis micrograph showing amplified banding patterns of by OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 6, OPA 8 and OPA 9 respectively. In figure 3A, seven monomorphic bands are produced in each lane and polymorphic bands are varied in each lane. Figure 3B, 3C, 3D, 3E, 3F, 3G and 3H show seven, six, eight, nine, seven, six and nine polymorphic bands are produced in each lane of the figures respectively, as shown in Table 4.

Table 4: Primer sequence and their Polymorphism

Primers	Sequence	No. of markers	No. of Polymorphic markers	% of Polymorphism
OPA 1	5'-TGC CGA GCT G-3'	19	11	57.89
OPA 2	5'-AGG GGT CTT G-3'	15	8	53.33
OPA 3	5'-GAA ACG GGT G-3'	16	7	43.75
OPA 4	5'-GTG ACG TAG G-3'	16	8	50.00
OPA 5	5'-GGG TAA CGC C-3'	15	6	40.00
OPA 6	5´ - AGCTCACTGA - 3´	17	8	77.06
OPA 8	5'-AGT CGG GCT G-3'	18	7	38.89
OPA 9	5'-AAT CGG GCT G-3'	16	6	37.5

3.1 Matrix for Jaccard's similarity coefficient method and Cluster Analysis: Using the RAPD data set, when we calculated the relatedness among collected species studied by Jaccard's Similarity Coefficient method [26], we noted that there were marked differences in the values of coefficient between the species, as shown in Table 5.

Table 5: Jaccard similarity co-efficient of *Channa punctatus* from six populations of three districts of West Bengal, India, calculated from RAPD-PCR analysis

Districts	Study sites	Singur	Bandel	Birati	Bartir Bill, Shyamnagar	Kalyani	Simurali
Hooghly	Singur	1	0.76	0.65	0.68	0.69	0.61
	Bandel		1	0.67	0.72	0.60	0.62
North 24 Parganas	Birati			1	0.63	0.66	0.64
	Bartir Bill, Shyamnagar				1	0.62	0.60
Nadia	Kalyani					1	0.78
	Simurali						1

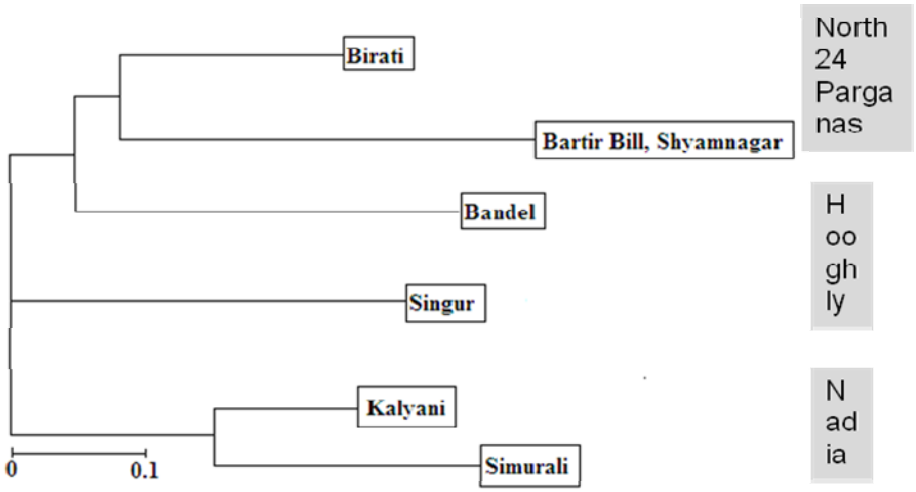

Fig 4: Dendogram generated using UPGMA cluster analysis showing the relationship and diversity among the *Channa punctatus*

Our molecular phylogenetic tree constructed by UPGMA method showed that 6 populations are apparently classified into different cluster, where Simurali, Nadia and Birati, North 24 Parganas can be considered as the boundary. Fish populations collected from different distinct hydographic basins lacking connectivity with the other sub basins, formed distinct clusters. Such geographic distances lead to isolation in relation to the chromosome of species from Southern part of West Bengal and it might have been responsible for present grouping pattern.

4. Conclusions

The presence of the transposable element might be contributing to genetic variation, acting as a natural mutant in its host organism. Hence, the development and performance of the transposable elements in the vertebrate genome evolution seems to be more significant than it has usually been postulated. Then, it can be established that the transposable elements participate and possibly even interfere in the genome evolutionary processes, either for their transposable activity, causing structural mutations within the chromosomes, or for

their repetitive nature, which is related to the increase in chromosome rearrangement rates. Moreover, RAPD clearly indicated genetic variation and similarity in *Channa punctatus* species depending upon isolation of sampling sites by distance i.e. neighbouring localities were usually genetically similar to each other, while genetic heterogeneity increased with the distance in localities. Therefore, the locations of species collection which were nearer, [Kalyani, Simurali and Birati, Bartir Bill] were grouped in one cluster by dendrograms, as shown in Figure 4. Present investigation also confirms the genetic variability of *Channa punctatus* species of six populations from three districts of West Bengal, India.

Genetic diversity or variation and its measurement have vital importance in interpretation, understanding and management of populations and individuals. These molecular markers combined with new statistical developments enable the determination of differences and similarities between stocks and individuals, and the population of origin of single fish species, resulting in numerous new research possibilities and applications in practical fisheries and aquaculture stock management. Various molecular markers, proteins or DNA [mitochondrial DNA or nuclear DNA such as minisatellites, microsatellites, transcribed sequences, anonymous cDNA or RAPDs] [12] are now being used in fisheries and aquaculture.

Data obtained with chromosomal analysis were co-related with RAPD PCR study, species sharing the same Fundamental Arm Number [FN] = 64, are clustered together, i.e., Birati and Bartur Bill; Kalyani and Simurali. The populations from Kalyani and Simurali are more related to each other due to the fact that they belong to the sub basins located geographically closer, revealing a close relationship among them in accordance with geological history and their original Basins.

The populations in different cluster can be further grouped into sub-clusters. Therefore, the results above might not be due to geographical differentiation, but it may be due to genetic differences of initial founder individual [s] between geographical populations which are widely distributed as a result of area effects like flood of river, founder events, genetic drift, reduced gene flow, climatic and other local adaptations, and also by transposable genetic elements. The evolutionary significance of polyploidy and the role of chromosomal rearrangements was also play an important role in this intra species diversity.

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