GENETIC STUDIES ON *Rana limnocharis*

ABSTRACT

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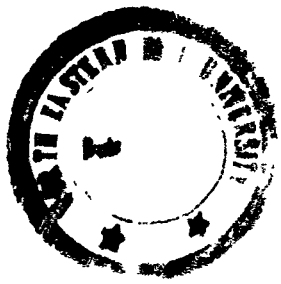
THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENT OF THE DEGREE OF

DOCTOR OF PHILOSOPHY



To

NORTH-EASTERN HILL UNIVERSITY

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Thesis

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In recent years, there has been an increased interest in the study of genetic variation and its application in population genetics to problems of speciation. This interest reflects a variety of ideas but in particular these ideas reflect the potential importance of random and adaptive events in the development of reproductive isolation. The 'strategy of adaptation' in such a changing environment consists in the formation of labile genetic systems capable of responding adaptively to a wide range of variable environmental conditions. Molecular genetics and certain technical developments have made it possible in recent years to answer questions concerning the amount of genetic differentiation in species both at inter and intra population levels, at least to a first approximation. Genetic variation is the basic premise upon which our study has been based. Traditionally, such studies have been based on the geographic classification of the populations. We have discussed here two types of genetic changes, chromosomal and molecular, that have been advocated as important factors in the evolutionary process.

India on the whole and its north eastern part in particular show greatest diversity in form and number in the amphibian fauna. Preliminary survey has revealed that the streaked frog, Rana limnocharis Wiegmann, is the commonest species available in the state of Meghalaya, and its neighbourhood. Moreover, this frog has been also identified

to be Oriental in origin, while most other frogs are palearctic. Subsequently, we have used R. limnocharis as our experimental model because of its abundance and high rate of genetic polymorphism. We have undertaken in our laboratory a detailed study on the molecular and chromosomal genetics of this group and keeping this in view, the present work has been carried out and the findings are presented in two sections (A and B).

SECTION - A : ISOZYMIC STUDIES

The usefulness of an enzyme system as a genetic marker in differentiating between geographically separated populations is determined by its variability, i.e., displaying either the presence of allozymes or difference in the number of bands on the gels. If the enzyme does not show variability, then its isozyme pattern is of little use for comparison among populations. The biochemical techniques of polyacrylamide gel electrophoresis coupled with specific enzyme staining procedures become an effective tool for such analysis.

The whole project has been accomplished in two phases. In phase I six tissues of Rana limnocharis from nine different localities have been electrophoretically surveyed to provide data necessary to make an appropriate selection as to which tissue and enzymes should be analysed for genetic differentiation. In phase II the genetic variation has been studied with emphasis on the tissue and the

and the enzymes indicated from phase I data.

It appears on comparison from all the populations that this frog like most vertebrates possesses a simple pattern of five LDH isozymes. Normal polarity has been observed in the two tetramers A_4 and B_4. Tissue specific LDH isozyme expression is observed due to the inconsistency of LDH B_4, in contrast to the relatively constant A_4.

The tissue MDH isozyme specificity has been placed under two groups - one having the single band and the other with the three banded phenotype. The two loci also express differential activity.

Maximum activity of the ADH isozyme has been observed in the liver tissue while the other tissues failed to show significant activity. The greatest number of ADH isozymes bands resolved is four. This frog carries three separate loci coding for ADH but the product of only two can form heterodimers. The third locus is monomorphic with no identification of a heterozygous combination between the other two ADH loci.

Two different forms of G6PD isozymes in the liver tissue, called A and B have been confirmed. No heterozygous combination is observed between these two forms, thus leaving some doubt on their genetic compatibility.

The preliminary findings from phase I indicated

that the above enzymes in the liver tissue by large warranted extensive investigation because of their more variable phenotypes across the whole population range of Rana limnocharis in Meghalaya.

Under phase II the allozymic variation encoded by nine loci from nine widely separated localities ranging across the whole of Meghalaya has been analysed. These different populations occupy increasingly varying and unpredictable environments. Genetic variation increased in the following order: Tura, Nongstoin, Williamnagar > Cherrapunjee, Mawsynram >Dawki > Nongpoh , Shillong, Jowai.

The mean number of alleles per locus (A), the mean number of loci polymorphic per population (P) and the mean number of loci heterozygous per individual (H) based on the 9 shared loci, were - A = 1.03±0.4, P = 0.48±0.15 and H = 0.08±0.02.

The average number of alleles is low in comparison to th other vertebrates but the mean number of polymorphic loci and heterozygosity is comparable to some of the vertebrates. Low values of alleles may be the characteristics of such populations due to specific environmental conditions. The high levels of polymorphism and heterozygosity are typical for this species when compared with other amphibians.

The expected Hardy-Weinberg proportions have been

observed in majority of the populations with each locus.

The values have been fairly correlated with the environmental - variability model. The genetic variations increased in a definite order, showing that the frogs living in a relatively constant and predictable environment are less genetically variant in contrast to the populations which have distinct spatial and temporal variations. Ecologically two similar populations have the commonest factor that they receive the highest rainfall than any part of the world. But the electrophoretic results do not fit the predicted similarity value. The frogs from one population showed similarity to a ecologically different population. We could not explain this phenomenon on the basis of existing data. Another samples from a population also unequivocally stands out in many respects. However, it is concluded that protein polymorphism is adaptively important and is maintained in the species by natural selection.

Roger's coefficient of genic similarity and Nei's genetic distance formulae were utilized to detect genetic relatedness between the nine populations. The average genetic distance value was 0.11±0.04. The similarity index averaged to 0.96±0.23. The values obtained are difficult to interpret because the approximate or standard values for amphibian species are not available. Our average D value falls approximately in the range of the value obtained in a salamander

species. This could possibly be the confirmation of a standard value in amphibia.

SECTION - B : KARYOLOGICAL STUDIES

The overall karyology is consistent with that of the earlier report and also with the well known homogeneity among the Asiatic Rana. The present report can be considered a satisfactory confirmation of the conclusion shared by several cytogeneticists that the karyotypes of Rana are conservative. This fact attests to the attainment of the analogous level of karyological differentiation by several families of this order, and may be to their common origin as well; nevertheless, it also provides additional evidence of the large number of parallelisms which also at the karyological level have characterized the evolution of Rana.

The sex determining mechanism of amphibia in general is still a controversial issue. Our observations indicate the lack of heteromorphic sex chromosomes and when both the male and female were available the karyotype was identical. Sex determination in this frog could be the task of one pair of chromosomes still morphologically undifferentiated. An accumulation of heteromorphic sex determining genes could be occuring in the chromosome by decreasing the rate of crossing over in a given pair, the morphological differentiation of this chromosome having not begun. It can

also be suggested that the sex in this frog is determined by one or several pairs of male and female determining genes distributed among several chromosomes.

The Nucleolus Organizing Regions (NORs) are located in the homologues of the 10th pair of chromosomes as is true in other Rana. This again points out the fact that the localization of the NOR in Rana karyotype is also very conservative. Additional NOR sites have also been observed in the chromosomes as well as in the nucleoli. Secondary constrictions are acclaimed to be the characteristic feature of the Rana chromosomes but we have not observed secondary constrictions in the karyotypes, which makes this frog to be the third in the list of cytogenetically investigated Rana to lack secondary constrictions.

An overview of the karyological studies shows the limnocharis to possess all the characteristics of Rana except the secondary constrictions on the chromosomes.

Comparative studies made between the other Rana endemic to this region and the Rana limnocharis shows a low similarity value. This could be due to high level of differentiation in this frog. Comparison between the populations of this species shows variations in arm ratios and relative chromosomal lengths. In terms of geographic variations these variations could be considered as 'cytologically diagnostic'.

Some efforts have been made in establishing the evolutionary link of this frog in this region with the data available on other Rana species from the south-east Asian countries. Rana limnocharis has been identified to be Oriental in origin, while most of other frogs are palearctic. Its place of origin in south-east Asia has been fairly established to be Taiwan (Kuramoto, 1985, personal communication). From Taiwan the migration path of Rana limnocharis could be traced easily to the Meghalayan populations. This must have been possible only due to the land and water connections which were established later.

Present evidence relating genetic and environmental variations is still inconclusive and needs critical analytical studies to unveil the potential relationship between the ecological and genetical profiles. Attempts have to be made to compare populations of this species in other localities or with other Rana species in the same localities. As mentioned before there is a relative abundance of Rana limnocharis in whole of south east Asia as compared to other Rana species. It is therefore necessary to study the possible linkage of this frog of this region with other regions. It is evident that time is rapidly approaching when a major effort has to be made towards obtaining complete and reliable estimate of these critical factors to provide a base to build such studies.

We anticipate that exciting development in the next few decades will result from using the new techniques of DNA sequencing and gene cloning. These methods will reveal substitutions and gross variability. This may also permit regulatory gene evolution to be examined directly. Finally, these approaches may provide a high resolution *method for studying evolutionary changes in gene arrangement.*

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DEDICATED

TO

MY GRANDFATHER

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Mayurbhanj Complex, Nongthymmai, Shillong-793014

Prof. K. Chatterjee
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20 Jan., 1988

This is to certify that the thesis entitled "**Genetic Studies on *Rana limnocharis*"**, submitted by Mr. Sant Prakash for the Degree of **Doctor of Philosophy** of the North-Eastern Hill University, Shillong (India), embodies the record of original investigations carried out under my supervision. He has been duly registered and the thesis presented is worthy of being considered for the award of a Ph.D. Degree. This work has not been submitted for any Degree of any other University.

K. Chatterjee

(K. CHATTERJEE)

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Finally, to my parents, brothers and sister who helped in my pursuit with patience, understanding and encouragement. To them I cannot adequately express my indebtedness, gratitude and love.

SANT PRAKASH

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FOREWORD

Polymorphism is a phenomenon which clearly defines the occurrence and significance of variations in animals. According to Kohen (1971), the main cause of polymorphism is the necessity for organism to adapt to the altering environmental conditions. The 'strategy of adaptation' in such changing environment consists in the formation of labile genetic system capable of responding adaptively to a wide range of variable environmental situations. Molecular genetics and certain technical developments have made it possible in recent years to answer questions concerning the amount of genetic differentiation in species both at inter and intra population levels, at least to a first approximation. Such genetically controlled variants provide markers for the investigation of the relationships of populations, races and natural hybrids. Comparisons of gene frequencies for these variants reveal the extent of gene flow and hence the degree of reproductive isolation among the populations comprising a species. Besides protein variations, it is an accepted fact that the chromosomal information of a species are essential to the modern taxonomists since the species are considered to be the objective reality of some particular genetic continuity. However, the importance of chromosomal polymorphism in speciation is still conjectural. Studies of these variants tends to clarify the concepts on the significance of the evolution of chromosomes envisaged not merely as a genotype "containers" but

as organelles in which are integrated the properties of molecules differing in genetic and evolutionary significance. Genetic variations is the basic premise upon which our study has been based.

India on the whole and its north eastern part in particular show greatest diversity in form and number in the amphibian fauna. Preliminary survey has revealed that the streaked frog, Rana limnocharis Wiegmann, is the commonest species available in the state of Meghalaya, and its neighbourhood. Moreover, this frog has been also identified to be Oriental in origin, while most other frogs are palearctic. Subsequently, we have used R. limnocharis as our experimental model because of its abundance and high rate of genetic polymorphism. We have undertaken in our laboratory a detailed study on the biology and genetics of this group and keeping this in view, the present work has been carried out and the findings have been presented in two sections (A and B).

Section A includes the isozymic investigations and has been accomplished in two phases. In phase I electropho/-retic survey were made to provide data necessary to make an appropriate selection of samples for the analysis of genetic differentiation. In phase II the genetic variation has been studied. Section B deals with the cytogenetical status of this frog and its relationships with the other Ranas.

The final results of the investigations carried out under section A and B have been concluded under section C.

PLATE I The frog, Rana limnocharis

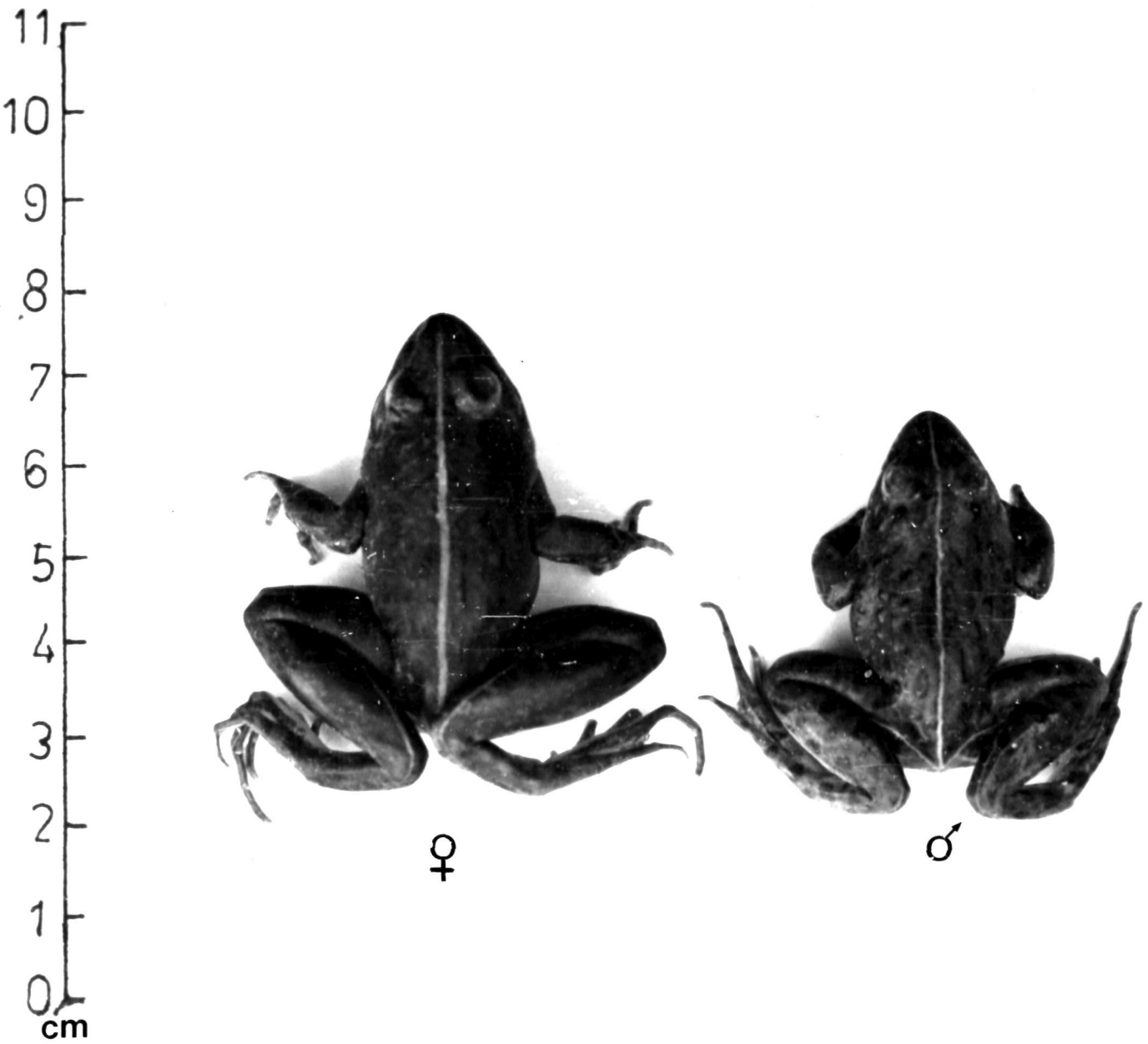
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PLATE II Habitat Variation

I Pond

II Weed Choked ditch


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PLATE III Habitat Variation

I Sluggish Creek

II Paddy field


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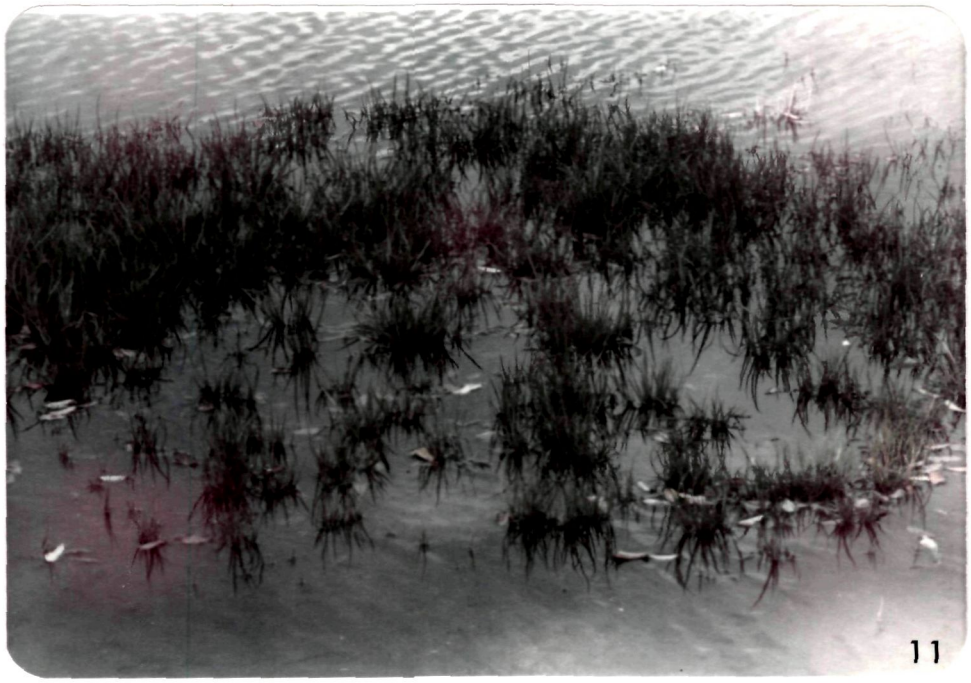
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SECTION - A

ISOZYMIC STUDIES

1. INTRODUCTION

The exploitation of Biochemical events in terms of genetics mainly depends upon the presence of enormous specificity of macromolecules and metabolic processes at every genetic level which are basically same in the lowest and highest organisms. Numerous fundamental questions concerning the structure of species and processes of speciation seem to be capable of being answered by studies concentrated at the molecular level. Studies of multimolecular forms of proteins are of importance not only in determining interspecific relationships but also in revealing genetically controlled variants among populations of the same species. Such genetic variants provide markers for the investigations of the relationships of populations, races and natural hybrids. Differing selection pressures may result in unique bands of a protein having arisen in populations which are geographically isolated but more often result in differences in frequency of specific genes since the amount of divervence brought about by selective advantage depends on the function of the concerned gene. Comparison of gene frequencies for these variant proteins reveal the extent of gene flow and hence the degree of reproductive isolation among the populations comprising a species. Thus the degree of genetic differentiation suggested by the gene frequencies may not be a simple reflection of the relationship between populations. This means that individuals do not necessarily

belong to the same population even if no differences in genetic pattern are found where formerly isolated species have been brought into secondary contact by habitat changes; the effects of introgression may be revealed by the patterns of gene frequencies throughout the range of the species concerned. According to Kohen (1971), the main cause of polymorphism is the necessity for organisms to adapt to the altering environmental conditions. The 'strategy of adaptation' in such changing environment consists in the formation of labile genetic system capable of responding adaptively to a wide range of variable environmental conditions.

Molecular genetics and certain technical developments have made it possible in recent years to answer questions concerning the amount of genetic differentiation between and within populations, at least to a first approximation. The basic conceptional break-through was the discovery of the relationship between genes and the proteins coded by them. Enzymes and other proteins substantiate allelic variations in the genes coding for them. The existence of a gene can now be ascertained even when the gene is not variable. The important thing is that the genes can be selected for study without a prior knowledge of variations, both within and between the populations. It can also be chosen for studies that represent a random sample of the

genome with respect to their variations in the populations to be compared. The results obtained from these moderate loci can, therefore, be extrapolated to the whole genome.

It is well established that the metabolic pathways are multistep processes, each step is catalysed by a particular functional protein or enzyme. It is also known that each cell type and tissue, contributing to a specific organ, contains a specific set of enzymes. The presence of a specific enzyme (i.e. functional protein) in a particular organ of an organism enables the organ to perform a special function. The morphological adaptations are expected to be closely related to environmental fluctuations. It has been observed that the relationship between certain enzymes and morphological differentiation is so close that often the appearance or disappearance of a specific enzyme is considered as a 'biochemical marker' (Scholl and Anders, 1973, Brody et al, 1976). The variation in the primary structure of proteins can be detected by electrophoresis and this technique has been found to be the most reliable as well as a practical tool for detecting genetic variation. It makes it possible to obtain a random sample of the genome and to detect allelic variation at individual loci, allowing one to calculate the frequency of the specific alleles in different populations. Although, the number of sampled loci is relatively small compared to the total genome,

sensitive data can be obtained by analysing the molecular changes by differences in migration (Case et al., 1975).

The vertebrates are well suited for such a study and some vertebrate lineages have experienced faster rates of phenotypic evolution than others. Placental mammals, for instance, have experienced rapid organismal evolution compared with lower vertebrates. Although, there are thousands of frog species living today, they are so uniform phenotypically that they are grouped under a single order (Anura). In contrast, the placental mammals are divided into at least 16 orders. Also, the present day frogs are not easily distinguishable morphologically from those that lived 90 million years ago (Estes, 1975). It is clear that the organismal evolution in frogs has been slow in comparison to mammals yet at the molecular level, evolution has been just as rapid.

When one attempts to look at the overall picture of vertebrate evolution, it soon becomes evident that the genetic formation which assured the eventual emergence of mammals and of man from the fish was laid at the very beginning, that is, as soon as the amphibians emerged. This is the very reason that amphibians as a group constitute the most fascinating subject for the study of vertebrate evolution.

There is burgeoning literature available for research and study related to the biochemical and genetical studies of amphibians. A very comprehensive review is presented here which could be taken as a representative one for the isozyme distribution pattern in individual anurans and their population significance.

The first attempt to review the molecular basis of isozymes and the suggestion for the term 'Isozyme' as a useful designation for the multiple molecular forms of an enzyme found within a single organism (or a member of a single species) was made by Markert et al. (1959). Later, Shaw et al. (1969) reviewed the variations found in different enzyme systems through electrophoretic techniques and commented on their significance in biological sciences and research.

Amphibians exhibit a wide spectrum of variations in proteins along with other evidence of diversity at the biochemical level. Wells (1964) developed a key to five named subspecies based upon the electrophoretic patterns of their plasma proteins. Wilson et al. (1964) found differences in molecular expression of LDH Isozymes amongst various amphibian species. Salthe (1965) studied the catalytic properties on the heart and muscle extracts for the LDH isozymes obtained from eighty five species of frogs from various populations and detected only the heart LDH to

be highly polymorphic. Chen (1966) explained that metamorphosis in amphibians involve differential gene action as the basis for at least some of the changing proteins. Wright et al. (1966) have put forward a simple, direct method for changes in specific enzymes during embryonic development in both normal and hybrid frogs. Balek et al. (1967) found variable Creatine kinase activity with an electrophoretic mobility different from that of mammalian or avian enzymes. Population and species structure of cricket frogs, Acris crepitans and A. gryllus were examined in terms of protein variation throughout the southeastern part of United States by Dessaur et al. (1969). Salthe et al. (1969) has reviewed the genetic variations found in relation to species specificity, phylogeny and environmental conditions.

The problem in anuran evolution was analysed by a large number of workers through various techniques (Guttman, 1973; Nevo, 1974; Johnson, 1979; Vonwyl, 1983). Inger et al. (1974) were the first to investigate genetic variation and population ecology of some South East Asian frogs of the genus Bufo and Rana. Case (1974) investigated protein variation in several species of Hyla from seventeen populations spread all over United States. Nevo (1974) correlated strategies of genetic systems in constant and varying environment of four anuran species. Schmid et al. (1974) made electrophoretic analysis of the lens and muscle proteins

of selected anurans. Vogel & Chen (1975) have investigated the lactate dehydrogenase isozyme patterns in Rana esculenta complex and observed that it has a genetic control mechanism. Dessauer et al (1975) have detected high genetic variability in an ecologically variant Bufo viridis and suggested its usefulness in population studies. Ling et al. (1976) have found acid phosphatase activity in the development of the cement gland in Xenopus laevis. Tabachnick (1977) has analysed allelic variation at five polymorphic loci coding for four proteins among nine populations of Notophthalamus viridescens and concluded that all the populations showed non random pattern of variation. Case (1978) has worked on the evolutionary relationship of the members of the genus Rana, native to the western part of North America. Ushakov et al. (1978) have found selective advantage or 'neutrality' of genotypes on the loci of esterases and the functional homeostasis in the frog population. Nevo (1979) surveyed the spatial patterns on environmental correlates and predictors of genic variation in Hyla in Israel and suggested that protein polymorphisms are largely adaptive and are moulded primarily by climatic selection rather than by stochastic processes or neutrality and the environmental variation model seems to be the best predictor of genic variation in tree frogs. Weslowski et al. (1979) investigated the hexokinase and alcohol dehydrogenase isozymes in Xenopus

and found it suitable for the study of gene action in development. Gerhardt et al. (1980) made a detailed survey on the morphology, vocalization and electrophoretic analysis of natural hybrids between Hyla cinerea and H. gratiosa. Pierce et al. (1980) reported significant genotype heterogeneity over very short distances in terms of allozymic studies in Ambystoma species. Vonwyl Fischberg (1980) compared isoelectric focussing (IEF) and electrophoresis as separation methods for LDH isozymes and investigated the expression of LDH isozymes in Xenopus species hybrids. Webb 1980) has analysed malate dehydrogenase (MDH) isozymes from anuran ovary with the help of isoelectric focussing.

Daugherty et al. (1981) surveyed the variation in the New Zealand frogs and discussed their implications as taxonomical tools. Dunlop et al. (1981) examined the geographic variations of proteins and mating call in Rana pipiens from north central United States. Kubiez et al. (1981) have compared the multiple molecular forms of acid phosphatase of Rana esculenta with those of Cyprinus carpio. Tilley (1981) detected electrophoretic variation in the Applachian population of the Desmognathus fuscus complex.

The studies on the linkage of enzymes to the chromosomes started very late during 1983 and most of the enzymes studied have been sex-linked. Cayrol et al. (1983) demon-

strated the relationship between sex-linked Peptidase-I, its expression and gene-dose effect in Pleurodeles wallii. Ferrier et al. (1983) also demonstrated the sex-linked expression of the enzyme peptidase in several Pleurodeles species. Elinson (1983) detected the inheritance pattern and expression of a sex-linked enzyme in the frog Rana clamitans.

Since 1983 work on the isozyme pattern has diversed so much that its implications have been observed in just about anything in variation, evolutionary or speciation studies as well as in clinical genetics. Formas et al. (1983) studied the allozymic and morphological differentiation in the South American frogs of the genus Eupsophus. Gunther et al. (1983) surveyed the LDH-B isozyme in water frogs from different parts of Europe and central Asia and commented on its polymorphic nature among geographically widely separated populations. Weslowski et al. (1983) discussed the advantage and methods for application of LDH isozymes in the clawed frog Xenopus, its utility in studying the evolutionary patterns and speciation. Cooper et al. (1985) have established allelic isozyme variants in the Mexican Axolotl as a potential marker for developmental genetics. Easteal (1986) has found that in New Zealand the introduced populations of giant toad have been established through introgression of other populations that previously had been isolated.

Review of literature reveals that works on the Biochemical genetics of Indian frogs are extremely scanty in comparison to the work done in the rest of the world. India on the whole and its northeastern part in particular is very rich and diverse in amphibian fauna. As many as 40 species belonging to 11 genera are known from this area out of which Meghalaya gets the highest representation of 30 species (Pillai et al. 1976). High rainfall is undoubtedly an important factor responsible for the abundance of frogs (Inger et al. 1974). Preliminary survey has revealed that the streaked frog Rana limnocharis Wiegmann is the commonest species available in the state of Meghalaya and its neighbourhood (Roy, 1980). It is used as a food item among certain tribal communities in this region. It has also been reported as a delicacy in Korea (Heusser, 1974).

We have used Rana limnocharis as our experimental model because of it abundance and high rate of genetic polymorphism. The main purpose of our study is to determine the amount of genetic variation within and between the different populations to unravel their relationship. Subsequently we have used electrophoretic estimates of various isozymes as the tool for determining the genetic characteristics of various individuals from selected populations falling under this group from the state of Meghalaya (India).

2. MATERIALS AND METHODS

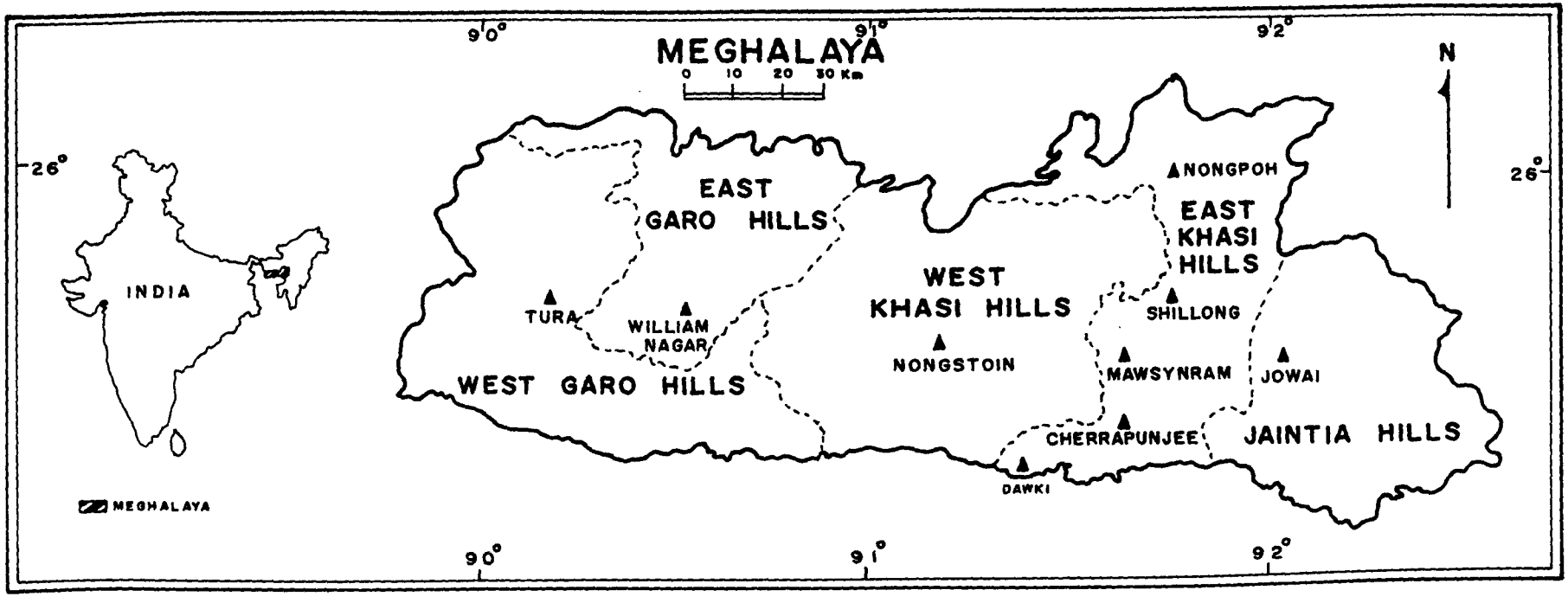

Fig. 1. Geographical distribution of sampling localities of R. limnocharis

TABLE 1: Geographical and climatological data of Rana limnocharis populations of Meghalaya.

Population No	Locality	Isolation distance (Km)	Elevation (ASL) (m)	Latitude-Longitude	Annual rainfall (mm)	Humidity %	Mean temperature (°C) Jan.	August
1	Tura	323	1418	25°31'S-90°12'W	2.7	97.1	14	32
2	Williamnagar	258	520	25°15'S-90°40'W	2.4	93.1	24	28
3	Nongstoin	180	1101	25°15'S-91°18'W	4.6	96.2	22	34
4	Cherrapunjee	55	1337	25°18'S-91°46'N	14.2	82.0	18	32.5
5	Mawsynram	51	1320	25°10'S-91°40'N	12.9	80.0	10	28
6	Dawki	98	1221	25°14'S-91°52'W	3.7	91.2	20	28
7	Nongpoh	65	310	25°58'S-91°48'E	2.7	78.5	11	29
8	Shillong	0	1450	25°30'N-91°52'E	22	76.5	4	24
9	Jowai	60	1350	26°70'N-92°51'E	5.1	92.1	10	29
Range		0-323	310-1450		2.2-14.2	76.5-97.1	4	34

2.1. Experimental animal

The streaked frog, Rana limnocharis Wiegmann constitutes the research specimen for the present study. The size of the males ranges from 3.1-4.1 cm and that of females from 3.7-5.6 cm. The body weight shows differences between males and females. The males range between 2.5-4.5 g and females from 8.1-13.8 g. The males can further be identified by their small slender body, presence of vocal sacs and thumb pads during the active breeding period; while the females lack these qualities but are relatively larger and have a swollen abdomen. Both the sexes possess imperfectly webbed toes.

2.1.1 Habitat

Rana limnocharis invariably breeds during the months of April-September, at times in temporary, but preferably in permanent bodies of water, rich in vegetation, such as in marshes, ponds, pools and rivers. It has also been reported to breed in the paddy fields.

2.1.2 Distribution

It is one of the widely distributed frog species in India and has also been reported from the whole of south east Asia (Satyamurti, 1967; Inger, 1974 and Kuramato, 1980). In the Himalayas, it has been recorded from Kangra

and Kulu valley and also from Nepal, Kumaon and Simla Hills (Acharji and Kripalani, 1951). It has been reported from the Khasi and Garo Hills first by Boulenger (1920) and a detailed survey was made by Pillai and Chanda (1976) from North East India.

2.1.3 Sampling

A total of 2416 adult frogs representing nine populations of R. limnocharis were sampled during the breeding seasons. Complete data on localities, geography and climate are given in Table 1.

The populations selected range across the whole state of Meghalaya, India, from a relatively constant to a highly varying environment (Table 1). The sampled populations included ponds, small streams, lakes, paddy fields, weed-choked-ditches and sluggish-creeks, Fig 1; Pl II&III

Shillong was considered the central point as the laboratory where the analyses were made is located there. It was designated as 0 in terms of Km. (Table 2). The maximum distance covered (North-South) was 323 kms, and (East-West) distance was 80 kms. The distances [illegible] though short [illegible] are accompanied by steep gradient in elevation ranging between 310 m to 1450 m. The place which receives world's largest rainfall, Cherrapunjee, also falls under this range.

The climatological variables in this estimate (Table 2) provide major comparative data with the genic diversity found. All the climatological values for the localities are from the Meteorological Service and the Statistical handbook of Meghalaya, India (1982-'83-'84-'85).

We did not attempt to measure ecological data, either physical (climatic, hydrological, etc.) or biotic (vegetation predators, competitors, etc.) locally, but used long term climatological data (humidity and temperature variables) and geographical data (latitude, longitude and altitude) which directly or indirectly affect the biology of R. limnocharis.

2.2 Experimental methodology

Live specimens were processed in the laboratory for different tissue samples (brain, heart, liver, kidney, muscles and eye). Tissue samples were stored in a -20°C freezer.

2.2.1 Homogenization

Tissues were blotted and weighed accurately and homogenized in a glass homogenizer containing measured volume of ice cold 0.25 M sucrose solution. The percentage of homogenates varied depending upon the maximum weight of the tissue taken.

2.2.2 Extraction

Tissue extracts were obtained by centrifuging the homogenates at 15,000 x g for 15 minutes at 4°C. The resulting clear supernatant contained the soluble protein. The residue was discarded.

2.2.3 Electrophoresis

When a particle of effective charge (Q) is forced to migrate in a viscous medium (liquid or gel) by the action of an electric field (potential gradient, E), the phenomenon is generally called electrophoresis (Maurer, 1971). The driving force which acts upon the particle migrating with a constant velocity is equal to the frictional resistance (F) which the particle must overcome in the medium, i.e.

$$QE = F$$

The electrophoretic mobility of a particle is defined as

$$m = \frac{d}{tE} = \frac{V}{E} = \frac{Q}{F} = \left(\frac{Cm^2}{Volt \times Sec}\right)$$

Where 'd' is the migration distance of the particle in time 't', 'V' is the velocity and 'F' is the frictional resistance.

The vertical disc electrophoresis system of Davis (1964) was employed. It is a discontinuous separating system with regard to pH value, buffer composition and gel pore size in which polyacrylamide gel serves as the matrix. Disc electrophoresis is carried out with small columns

of polyacrylamide gel consisting of three layers in a suitable container like cylindrical tubes. The three layers are: (1) a large pore spacer or gel, (2) a small pore separation or running gel in which the sample constituents are separated, and (3) contains the sample solution. Electrophoresis is performed with a vertical column of gels attached to two different reservoirs, sample gel uppermost, attached to an upper reservoir and the lower end submerged in the buffer solution of the lower reservoir. Electrodes are placed in each reservoir and polarity is set so that the sample ions migrate towards the small pore gel. A constant voltage is applied for a specific time. The gel is then removed from the container and placed for a specific period of time in an enzyme staining solution. Unbound dye is removed from the gel slowly by washing in 7% acetic acid and then the gel is preserved in a suitable solution.

2.2.3.1 Reagents

These solutions are stored in dark bottles in a refrigerator and their shelf life are upto two months.

Reagent A : pH 8.9

1N HCl	48 ml.
Tris (hydroxymethyl) methylamine	36.6 g.
N,N,N,N-Tetramethyl ethylenediamine (TEMED)	0.23 ml.

The volume is made upto 100 ml with distilled water.

Reagent B : pH 6.7

1N HCl	48 ml
Tris	5.98 g
TEMED	0.46 ml
Distilled water	51.54 ml

Reagent C

Acrylamide	10 g
Bis	0.8 g
Distilled water	100 ml

Reagent D

Acrylamide	10 g
Bis	2.5 g
Distilled water	100 ml

Reagent E

Riboflavin	4.0 g
Distilled water	100 ml

Reagent F

Sucrose	40 g
Distilled water	100 ml

Reagent G

Ammonium persulphate	0.14 g
Distilled water	100 ml

Stock buffer solution : pH 8.3

Tris	6.0 g
Glycine	28.8 g
Distilled water	1000 ml

For reservoir, 10% strength of this stock solution is used.

Destaining solution

Acetic acid	70 ml
Distilled water	1000 ml

2.2.3.2 Equipment used

The size of the cylindrical gel tubes used was about 10 cm in length with an inner diameter of 5 mm. These tubes were fixed vertically in the electrophoresis running chamber, consisting primarily of the upper and lower chambers with platinum electrodes. The upper and lower chambers were the buffer reservoirs. Arrangements were there to hold the gel tubes vertically in the upper reservoir and to make a link with the lower reservoir. The additional equipment comprise of electrodes, cables and a power supply (Systronic 604), gel tubes rack for loading, rubber caps for closing one end of the gel tube and polymerizing lamps, needle and syringe, test tubes etc.

2.2.3.3 Gel systems and their composition

Separation gel was prepared just before use by mixing the reagents in the following proportion (mixing ration V/V)

Reagent A	1 part
Reagent C	2 parts
Distilled water	1 part
Reagent G	4 parts

Spacer gel system was prepared by mixing the reagents in the following proportion:

Reagent B	1 part
Reagent D	2 parts
Reagent E	1 part
Reagent F	4 parts

2.2.3.4 Procedure

About 1 ml to 1.5 ml (upto 7 cm in length of the gel tubes) of the separation gel system was poured into the gel tubes which were capped at the bottom. Care was taken not to allow any air bubble in the gel column. A few drops of distilled water were carefully layered above the separation gel system. After polymerization, the water from the top of the gel column was removed carefully and about 0.2 ml of the spacer gel system was poured carefully into the gel tubes. A few drops of water were again layered above this gel system and the gel tubes were left under fluorescence light for 30-45 minutes to polymerize.

The gel tubes were then fixed vertically as described by Davis (1964) in the disc electrophoresis chamber. The upper and the lower chambers of the apparatus were filled with 1M Tris-Glycine (pH 8.3). About 0.1 ml of the test solution was directly poured over the spacer gel system after removing the water layer. The remaining vacant part of the gel-tube was filled with the buffer used for the chambers. Then both the upper and lower chambers were filled with buffer completely. A drop of 1% Bromophenol blue indicator solution was mixed in the upper buffer chamber. After

all these preparations, the apparatus was placed at 4-5°C and the two electrodes were connected with the electrophoresis power supply. The current strength was raised upto 3 mA/gel tube. After the requisite time of running, the current supply was stopped and the gel tubes were removed from the upper chamber. The gels were taken out carefully with the help of a long needle and water force from a syringe and immediately subjected to specific treatment to obtain different isozyme patterns.

2.3 Separation of isozymes

Isozymes of an enzyme system are proteins of the same configuration but with a slight difference in molecular weight and electrophoretic mobility, hence the above mentioned separation technique can be utilized for their detection.

The gels were incubated with specific stains for a specific period. Gels incubated without the substrate served as controls. After the reaction the gels were washed and preserved in 7% acetic acid (v/v).

The following enzymes were investigated:

Lactate dehydrogenase (LDH; E.C. 1.1.1.27.)

Electrophoresis : Tris-Glycine buffer pH 8.3

Stain : 1M Tris-HCl (pH 8.0) - 2.5 ml
1N Lithium Lactate - 0.5 ml
standard solution
NAD (B-Nicotinamide - 80 mg
adenine dinucleotide)
PMS (Phenazine metho- - 1.2 mg
sulphate)
NBT (P-Nitroblue tetra- - 0.8 mg
zolium chloride)
Distilled water - 47 ml

The gels were incubated in the above solution at 37°C for 15 minutes. LDH bands were violet in colour.

Malate Dehydrogenase (MDH; E.C. 1.1.1.37)

Electrophoresis : Tris-Glycine pH 8.3
Stain : Only 0.5 ml of 1N Malic acid is used as the substrate instead of Lactic acid rest of the solution remains as above.

Alcohol Dehydrogenase (ADH; E.C. 1.1.1.1.)

Electrophoresis : Tris-Glycine buffer pH 8.3
Stain : The solution remains as in LDH except that the substrate has been substituted for 1 ml Ethyl alcohol

Glucose-6-Phosphate Dehydrogenase (G6PD; E.C. 1.1.1.49)

Electrophoresis : Tris-Glycine buffer pH 8.3

Stain : 0.05 M Tris pH 8.0 - 50 ml
Glucose 6 Phosphate - 40 mg
NADP - 20 mg
PMS - 0.6 mg
NBT - 15 mg

The gels are incubated in the above mixture at 37°C for 55 minutes. The G6PD bands appear deep blue in colour.

2.4 Heat inactivation

All the four dehydrogenases were subjected to heat inactivation studies in the following manner:

LDH - Heating upto 55°-65°C
MDH - Heating upto 40°-45°C
ADH - Heating upto 25°-30°C
G6PD - Heating upto 50°-70°C

The extracts were placed in test tubes and subjected to heating as mentioned for an hour or so. Then they were cooled, filtered and subjected to electrophoresis and finally stained as before. These gels were subjected to densitometric analysis with a spectrophotometer (Beckman) for exact correlation of the bands.

2.5 Quantitative estimation of Protein

Estimation of protein is necessary so that a known amount of it can be loaded from the sample of different tissues for electrophoresis. Protein content was determined according to the procedure of Lowry et al. (1951).

Principle: Proteins when react with Folid-Ciocalteau (Phenol-reagent), form a blue colour, the intensity of which at 750 nm is proportional to the amount of proteins

present in a sample. The final colour reactions are the bi-uret reaction of phosphomolybdic phosphotungstic reagent by tyrosine and tryptophan present in the treated protein.

Reagents: Stock A : 1% $CuSO_4$ solution
Stock B : 2% K-Na-tartarate
Stock C : 2% Na_2CO_3 in 0.1 N NaoH.

0.5 ml of the Stock A was mixed with 0.5 ml of Stock B and the volume was made upto 50 ml with Stock C, and was treated as the alkaline solution. Folin-Ciocalteau reagent was prepared by diluting this with equal amount of distilled water just before use.

Procedure: 5 ml of alkaline solution was added to 1 ml of test solution. They were mixed thoroughly and allowed to stand at room temperature for 10 minutes. 0.5ml of diluted F-C reagent was added rapidly with immediate mixing. After 30 minutes, the extinction was read against an appropriate blank at 750 nm. in a spectrophotometer (Beckman). Protein concentration was determined after preparing a standard curve using Bovine serum albumen.

2.6 Statistical analyses

All the data obtained were statistically analysed according to Nei & Roychoudhury (1972), Roger (1972) and Ayala (1980).

Nei's Genetic distance (D) and Roger's coefficient of similarity (S) formulae were utilized.

We used the system of locus nomenclature as suggested by Ayala (1980) where the allelic products, were designated by their mobility from the origin. One allele, usually the most common in Rana limnocharis was designated as 100; the number assigned to their alleles is the distance moved from the origin towards the anode expressed as a percentage of the distance moved by the allele 100.

Allele frequency, degree of polymorphism (P) and heterozygosity (H) were analysed from each population. A locus was considered polymorphic when the frequency of the most common allele was 0.95 of less. Average individual heterozygosity (H) was determined by calculating the percentage of heterozygotes per locus and averaging over all loci. The chi-square test was used to test the significant differences in gene frequencies between populations. Hardy-Weinberg proportions were worked out from the genotype frequencies.

The variance between the population in allele frequencies of loci were tested by analysis of variance (ANOVA). The significant value used were 0.05 and 0.01.

3. RESULTS

3.1 Lactate dehydrogenase isozyme pattern

Lactate dehydrogenase is ubiquitous in vertebrates and is in most composed of two genetically and biochemically distinct polypeptide subunits which, upon random association, generate five tetrameric isozymes. These isozymes differ in net charge and thus can be separated by electrophoresis. The molecular forms of lactate dehydrogenase are designated as LDH 1=B_4; LDH 2=A_1B_3; LDH 3=A_2B_2; LDH 4=A_3B_1; and LDH 5=A_4. In normal cases, LDH A_4 shows comparatively high activity in the skeletal muscle than LDH B_4 whereas the latter is more active in heart tissue than the former. LDH B_4 bears more net negative charges than LDH A_4 and, therefore, migrates towards the anode while A_4 remains relatively confined to the cathodal end. Reversal of this pattern has also been observed in some amphibians.

3.1.1 General profile

Observations have been made from the various tissues of Rana limnocharis and a general LDH isozyme profile is described below:

Heart: A five banded LDH isozyme phenotype has been observed (Fig.2) indicating that prominent binomial degree of heterotetramer A-B hybridisation occurs in this tissue. There is a characteristic anodal band of high activity, confirmed by heat inactivation studies, to be the B_4 LDH

homotetramer (please refer to section 2.4). The A_4 homotetramer exists with lesser activity (Fig.6). The existence of normal polarity of A and B subunits is confirmed by the presence of highly active LDH B_4 at the anodal end.

Muscle: This tissue exhibits a three banded LDH phenotype (Fig.2). There is a distinct A_4 activity zone where the bands are thick and have a complete disc like appearance. The A_4 bands are confined to the cathodal end (Fig.2). This tissue does not show any B_4 activity (Fig.6). The characteristic of this tissue is the high A_3B_1 activity.

Liver: This tissue has a striking similarity with the muscle pattern with respect to A_4 and B_4 (Fig.2). The A_4 shows very high activity. LDH B_4 is usually absent (Fig.6). The total number of bands resolved is four. The space between each band is more than in other tissues (Fig.2). The A_1B_3 band is prominent.

Kidney: The kidney tissue shows a five banded pattern (Fig.2). Both A_4 and B_4 show equal activity. However, A_3B_1 is less active when compared to A_2B_2 and A_1B_3 (Fig.6). The characteristic feature of this tissue is the closeness between these five bands (Fig.2).

Brain: The total number of LDH isozymes observed is five. Similar to the kidney tissue, the brain tissue

also exhibits high A_2B_2 and A_1B_3 activity in comparison to A_3B_1 (Fig.6). The homotetramers A_4 and B_4 show equal activity. The A_4 activity matches with that of the other tissues but B_4 differs (Fig.2) from them.

Eye: This tissue exhibits the classic pattern of LDH isozyme phenotypes as found in other vertebrates. Total bands resolved are five. Unlike the other tissue the predominance of one subunit is very common. This tissue shows the LDH homotetramers A_4 and B_4 and their heteropolymer A_1B_3, A_2B_2 and A_3B_1 to be in the appropriate proportion (Fig.2).

3.1.2 Heat stability

This experiment has been performed to detect the LDH A_4 and B_4 activity zones. LDH B_4 is more heat labile than A_4. Similar observations have been made in this frog. Crude extracts incubated at 55°-60°C for 5 minutes show no activity at the anodal end, and the activity at the cathodal end also disappears at 65°C at 25 minutes. The latter is, therefore, the A_4 and the former the B_4 LDH subunit respectively.

3.1.3 Variation

Heart tissue of all the adult individuals of Rana limnocharis examined is characterized by strongly stained

anodal bands. The pattern of liver and skeletal muscle differs strikingly from that of the heart (Fig.2). The more basic bands are clearly visible, whereas the more acidic bands are absent. Exception of this rule is the population from Tura, where some of the individuals displayed asymmetric pattern of strongly stained bands.

Several individuals have only four bands in their heart tissue. A view of the band spacing reveals that the second fastest moving band is missing. It must be noted that in almost all the tissues of many individuals subbandings or parallel bands are expressed. The activities of these subbandings vary with individuals. The kidney pattern in many individuals are difficult to interpret. However, on comparison with the other tissues it becomes clear that there are principally five bands. The difficulty in interpretation resides in the occurrence of subbandings and the existence of quantitative differences in staining of bands and subbands between the individuals. Since normal polarity was observed in Rana limnocharis, it can be assumed that the most positively charged band, at least in cases where only five bands exist, is identical with the A_4 homotetramer. The electrophoretic mobility of A_4 and B_4 is the same in all the tissues. In contrast to the A subunit, the activity of B subunit differs from tissue to tissue. The characteristic predominance of A_1B_3 band in muscle

and liver which is not observed in other tissues is a clear indication of its specific nature. Consequently, tissue specific LDH isozyme pattern exists in this frog as described above.

3.2 Malate dehydrogenase isozyme pattern

Malate dehydrogenase exists as a dimer in frog species with a molecular weight ranging from 35,000 to 60,000 (Voelker et al. 1979). The isozyme pattern is less complex as might be expected from the dimeric nature of enzymatically active MDH molecules (Scholl & Anders, 1973).

The random association of the duplicated MDH loci, MDH A and MDH B, as found in many frog species and higher vertebrates, results in the formation of three dimeric MDH isozymes, A_2, A_1B_1, and B_2. The electrophoretic mobilities of these two MDH homodimers in general show the A_2 homodimer to be very active at the anodal region whereas the B_2 occupies the cathodal zone. The B_2 isozyme is present with usually low activity and with restricted tissue expression predominating in skeletal muscle and liver.

3.2.1 General profile

Tissues from heart, kidney, brain, liver, eye and skeletal muscle have been subjected to the MDH isozyme pattern analysis. A general pattern has been established

after analysing all the nine populations of Rana limnocharis (see Fig. 3 and 6).

Extracts from heart, kidney, brain and eye tissues show a monomorphic band (Fig.3). The liver and the muscle extracts show the dimeric nature by resolving three equally spaced bands, A_2, A_1B_1, and B_2. However, the muscle and the liver tissuesdiffer in their band intensities and electrophoretic mobilities (Fig.3). The isozymes of A subunit composition shows higher activity in all the tissues analysed as compared to the B_2 homodimer (Fig.6). The monomorphic band found in many tissues has been confirmed to be the A_2 dimer by the heat inactivation studies.

3.2.2 Heat stability

The MDH A subunit starts disappearing when the extract is incubated at 45°C for about 10 minutes. After 25 minutes the total activity of A_2 stops. This confirms the position of the two subunits as well as their random association.

3.2.3 Variation

The greatest number of MDH isozyme bands observed from any tissue has been three though it varied amongst individuals and also between populations. The allelic variations are dealt in a latter section (3.5). Individuals

from many populations showed a single banded phenotype in their kidney or muscle extracts where normally three banded phenotypes should be expressed. The number of such cases was very small. It may be assumed that one of the two genes coding for the MDH subunits is not being expressed in these individuals (Fig.3).

None of these bands stains if NAD in the staining solution is replaced by NADP thus ruling out the possibility that any of these bands might be MOD rather than MDH.

3.3 Alcohol dehydrogenase isozyme pattern

This is primarily a liver specific protein with a substrate specificity, mediating the conversion of primary alcohols to aldehydes and secondary alcohols to ketones utilizing NAD^+ as the electron acceptor (Pietruszko et al.,1973).

In frogs, ADH is almost confined to the liver tissue. The Electrophoretic study resolves three to four isozymes which could be explained genetically by assuming that there are atleast three structural genes coding for ADH monomers and provide evidence for the dimeric nature of most of the isozymes.

3.2.1 General Profile

Alcohol dehydrogenase activity has been maximum in the liver tissue, although minor activities are observed

in other tissues including heart and kidney (Fig.6). The liver tissue has been resolved into four distinct ADH isozyme bands. Heart and kidney tissue have been found to exhibit a monomorphic band with minor activity. Fig.6 depicts the array of ADH isozyme patterns in these tissues. The tissues which failed to exhibit significant activity are brain, eyes and muscle. Hence, these latter tissues are not scored in our analysis.

The greatest number of ADH isozymes resolved was four. Out of these four banded phenotype, three equally spaced bands (A_2, A_1B_1 and B_2) migrate anodally to the fourth band (C_2) (Fig.4). The total possible sites of ADH activity is six if it is assumed that from the most anodal to the least anodal, the isozymes would be ADH A_2, A_1B_1, A_1C_1, B_2, B_2C_2, and C_2. But no heterodimers were observed between A_2C_2 or B_2C_2.

3.4 Glucose 6 phosphate dehydrogenase isozyme pattern

In higher vertebrates, the G6PD isozyme is dimeric in nature (Shaw, 1966; Yamanchi et al., 1974) and in most of the animals it exists in just two forms without exhibiting the intermediate hybrid zone (Scholl, 1973). In mammals, G6PD isozymes are encoded in two separate loci, one of it being sex-chromosomal linked (Kirkman, et al., 1963) while the other is autosomal in origin (Shaw et al., 1965).

The autosomally inherited mammalian isozyme reacts more actively with galactose 6 phosphate and is also referred to as "Hexose 6 phosphate dehydrogenase, H6PD". Two G6PD isozymes with similar substrate specificities as the mammalian G6PD have been also observed in the teleosts (Scholl, 1973). However, there is no definite report of the molecular mechanism governing the expression of G6PD genes in amphibians.

3.4.1 General profile

Six different tissues have been analysed for the G6PD isozyme pattern. Out of these six, the liver tissue exhibits two forms of highly active G6PD isozyme (Fig.5). Two other tissues show a single banded phenotype. The muscle tissues exhibits a highly active anodal band and a faint activity in the cathodal region. The electrophoretic mobility of the anodal band is relatively similar in all the tissues except the heart where it has a faster mobility (Fig.5). The liver tissue also shows high activity in the cathodal zone. Tissues of heart and eye show a monomorphic phenotype whereas brain and kidney are G6PD negative (Fig.6). Minor bands appear occasionally running adjacent to the anodal band. They could be interpreted as subbands or secondary isozymes. The intensities of the bands also varied with individuals but in general, the G6PD isozyme pattern in this frog is in agreement with the pattern found in other vertebrates.

Genetic variant of the B enzyme was detected in which homozygotes presented a single zone of activity at one of the two different positions, while the heterozygotes express bands at both the positions. No third pattern intermediate between these two was observed.

3.5 Allozymic Variation

Nine loci from nine populations of Rana limnocharis ranging across the whole of Meghalaya have been scored for allozyme variation. The electrophoretic pattern in general has been to some extent consistent with patterns and interpretation of the respective enzymes as reported in other species (Case et al 1975; Nevo, 1980).

Two guidelines have been useful in formulating our interpretations: 1) banding pattern has to be consistent with known molecular structures of similar proteins in other frog species; 2) wherever the same locus was expressed in two or more tissues the banding pattern of variants had to be consistent among tissues.

3.5.1 Lactate dehydrogenase

Gene products of both the LDH loci, LDH A and B, have been scored from the heart and the liver extracts. The allelic distribution at these loci partly correspond to that documented by Nevo (1978) in the marsh frog of

Israel. Majority of populations resolve into two alleles of LDH A, LDH A^{65} and LDH A^{100}; two alleles have also been resolved at the LDH B locus, LDH B^{190}, and LDH B^{196} (please see section 2.6 for nomenclature). Most populations have been fixed at either LDH^{190} or LDH^{196} except for the population from Nongpoh which showed LDH B^{182} in a low frequency (Fig.8). Additional LDH alleles observed could not be scored because of extremely low frequencies. LDH B gene has been more variant than ~~the~~ A in majority of the populations (Table 3).

3.5.2 Malate dehydrogenase

Typical dimeric variants have been observed at two of these loci, MDH A and B. MDH A resolves into two alleles, 80 and 100. MDH B resolves into the alleles, 154 and 172. MDH A_2 showed variants having mobility of about 30 per cent relative to the most common allele (Fig.6). Three banded heterozygotes, resulting from the interaction of dimeric subunits have also been resolved (Table 3). The variants of MDH B type could be classed under the faint types. Overlapping of electrophoretic mobilities renders it impossible to devise a genetic model to explain the observed effect.

3.5.3 Alcohol dehydrogenase

The four banded phenotype resolved in the liver tissue is arranged in the manner that the first three are the result of interaction of dimeric ADH A and B and the highly anodal is the ADH C. Heterodimers between B and C or A and C have not been observed. MDH C has been relatively invariant while A and B are highly polymorphic. MDH A resolves into alleles, 77 and 100, MDH B into 146 and 152, and MDH C into 160 and 180, respectively (Fig. 7; Table 3).

3.5.4 Glucose 6 phosphate dehydrogenase

It expresses both narrow and broad banded phenotypes, and depending upon their relative position on the gel, are interpreted to reflect homozygotes or heterozygotes of the two allelic products of presumably dimeric nature. The fast broad band variant G6PD, seen at a very low frequency in the majority of the populations, resolves into alleles 184 and 198. G6PD A locus is also fairly variant with the two alleles, 100 and 140 (Fig. 7; Table 3). From overall comparison with the other enzymes scored this enzyme is relatively less variant.

3.5.5 Genetic variation

Table 2 and Fig. 7 show levels of genetic variation at nine loci in nine natural populations of Rana limnocharis

Table 1 shows the geographic variations. The observed patterns of variation are consistent with a model of codominant alleles following Mendelian inheritance. The expected Hardy-Weinberg proportions have been observed in most populations (Table 6,7,8,9).

Three measurements have been used to assess levels of variability: mean number of alleles per locus (A), proportion of polymorphic loci (P), and mean number of heterozygotes per locus per individual (H). For each population and locus, heterozygosity has been calculated from the genotypic frequencies. A locus or population is considered polymorphic when the frequency of the commonest allele is equal or less than 0.95 (Table 2).

Allele frequencies are given in Table (3). Frequencies for Rana limnocharis is pooled across localities after obtaining the individual population data.

The mean number of alleles per locus (A) is 1.03±0.40, ranging from 0.44 in Dawki (population 6), to 1.66 in Shillong (population 8) and the mean of polymorphic loci(P) is 48 per cent ranging from 20 per cent in Dawki (population 6) and 70 per cent in Shillong (population 8, Table 2). The mean heterozygosity (H) is 7 per cent ranging again between Dawki and Shillong with 4 per cent and 10 per cent, respectively (Table 2).

Intrapopulation patterns of genetic variation are also observed in many population. Polymorphic isozyme phenotypes are shown in the Fig. 7.

Genetic relatedness

The electrophoretic data obtained were extrapolated to measure the overall genetic similarity and the genetic distance among different populations of this frog. The two methods of comparison used are Roger's coefficient of genic similarity (I) and Nei's genetic distance (D). The coefficient of genetic similarity and genetic distance are calculated for paired combinations of all the nine populations based on the normalized identity of the genes between each pair of populations (Please see section 2.6). Table 4 presents a matrix of both measures from the nine populations.

The most striking feature of the data obtained by the estimation of the genetic distance are the large number of comparisons that yield a D value of 0.01 while the greatest value obtained out of all the populations is between Dawki (6) and Shillong (8) showing 0.19. Overall comparison of samples with all populations reveals that the average genetic distance is extremely low 0.111±0.001 (Table 4).

The similarity value between the samples ranged between 88-99 per cent, the greatest being between Cherrapunjee (4) and Mawsynram (5) (Table 4).

Anova

Analysis of variance for a two way classified data with one replicate per cell was made with mean allele frequency from all nine loci scored over the nine populations

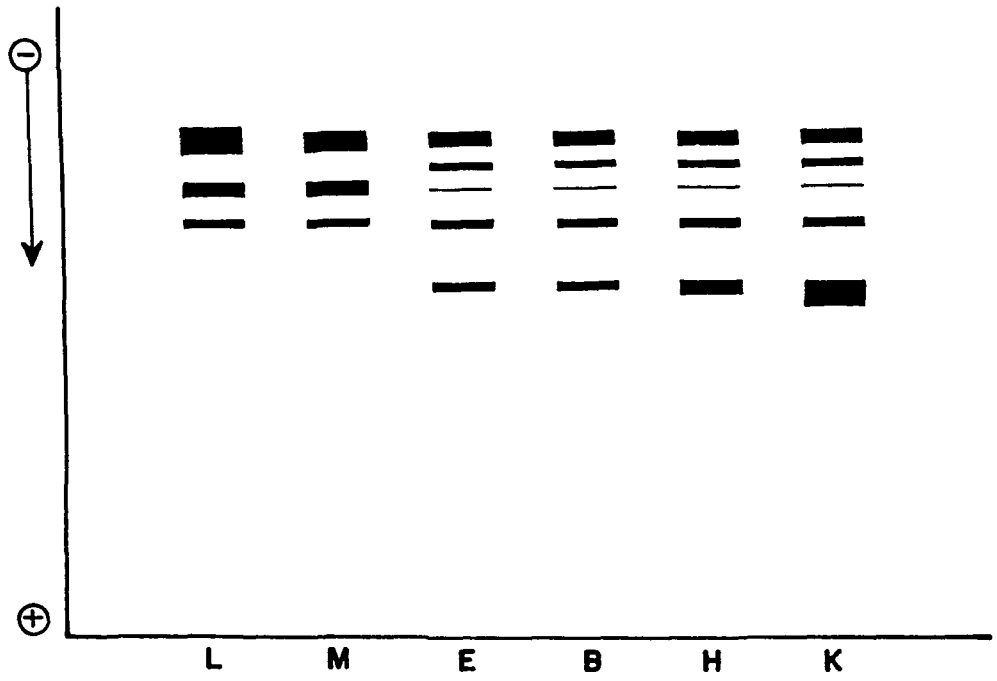

Fig. 2. Tissue LDH isozyme pattern

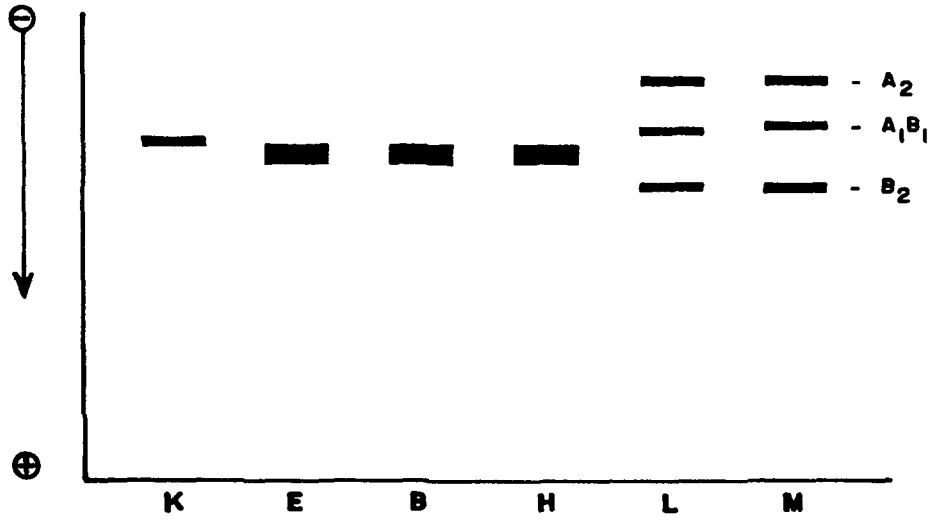

Fig. 3. Tissue MDH isozyme pattern

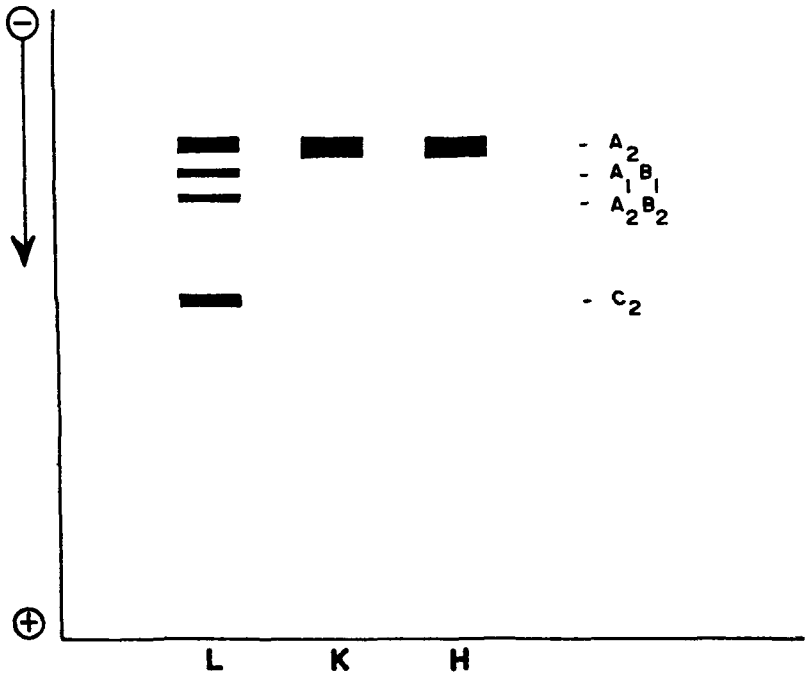

Fig. 4. Tissue ADH isozyme pattern

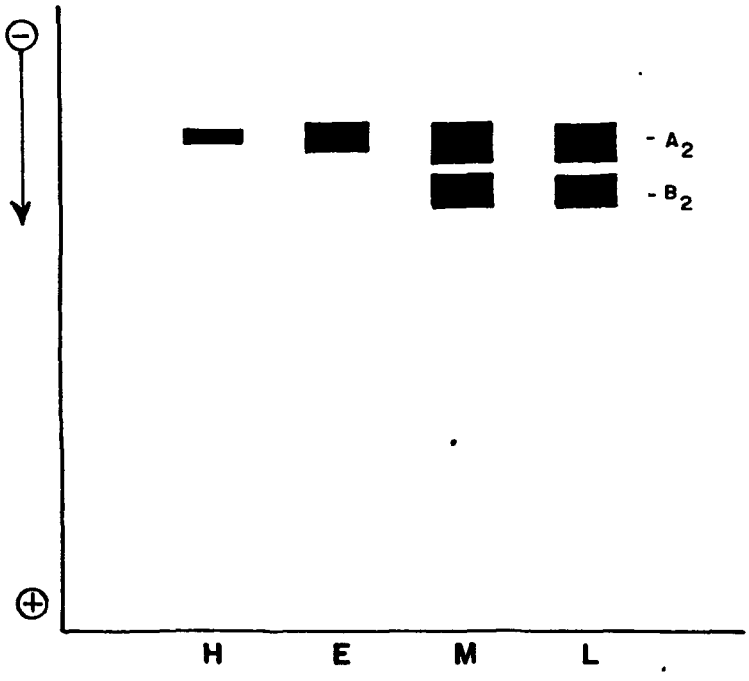

Fig. 5. Tissue G6PD isozyme patterm

FIG. 6. ENZYME TISSUE SPECEFICITY IN RANA LIMNOCHARIS

ENZYMES	BANDS	TISSUES					
		EYE	BRAIN	HEART	KIDNEY	LIVER	MUSCLE
LDH	A_4	+ +	+	+	+	+ +	+ +
	$A_1 B_3$	+	+	±	±	+ +	+
	$A_2 B_2$	+	±	+	±	+	+ +
	$A B_3$	+	±	±	±	±	+
	B_4	+ +	+	+ +	+	−	−
MDH	A_2	+ +	+ +	+	+	+	+
	$A_1 B_1$	−	−	−	−	+	+
	B_2	−	−	−	−	+	+
ADH	A_2	−	−	−	−	+	−
	$A_1 B_1$	−	−	+	−	+	−
	B_2	−	−	+	+	+	−
	C_2	−	−	−	−	+	−
G6PD	A_2	−	−	−	−	+ +	±
	B_2	+	−	+	−	+ +	+

+ + = VERY HIGH ACTIVITY
+ = HIGH ACTIVITY
± = LOW ACTIVITY
− = ACTIVITY NOT OBSERVED

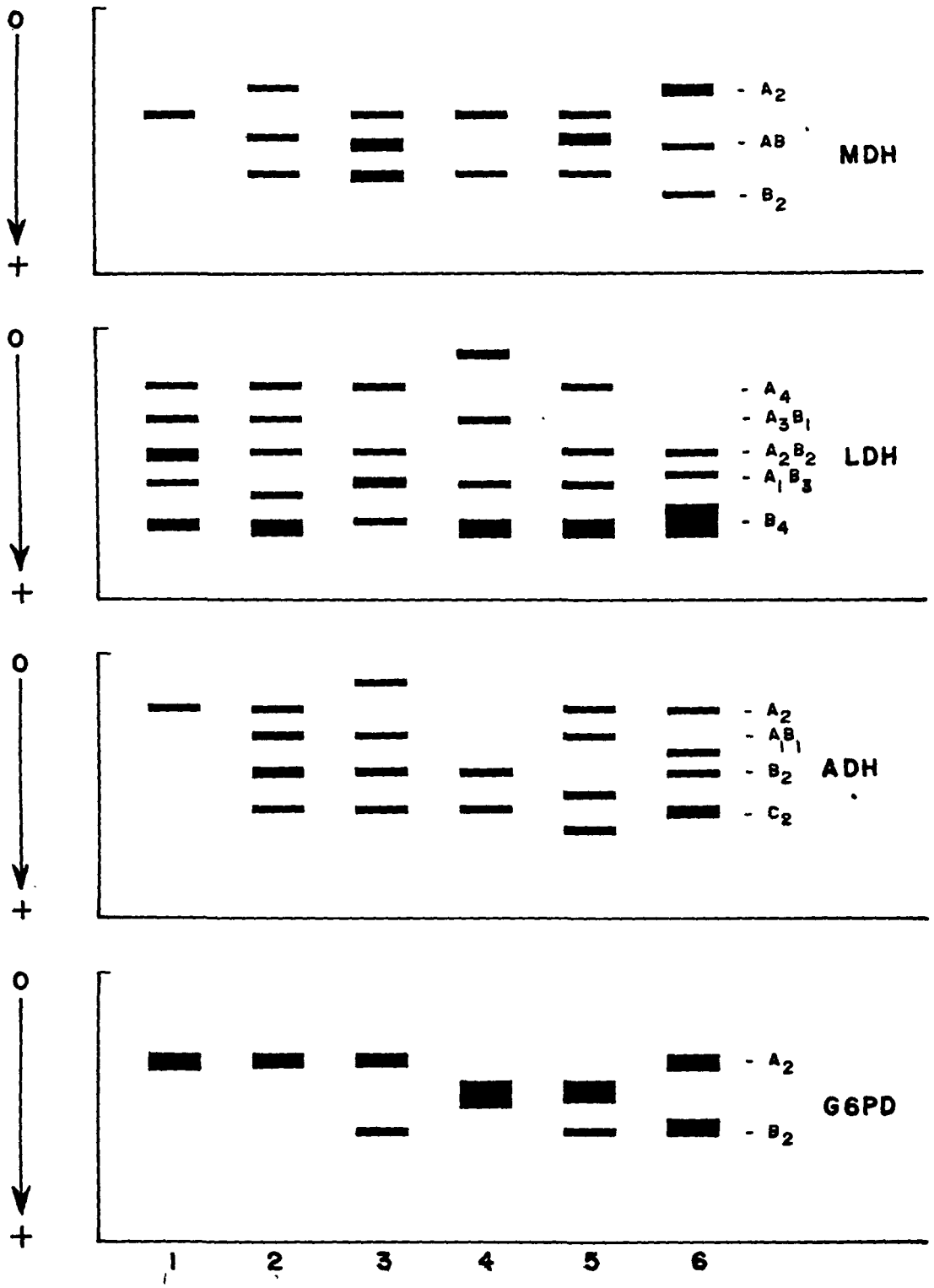

Fig. 7. Phenotypic variations among the four isozymes.

TABLE 2: Genetic variations in populations of *Rana limnocharis*

Locality	Sample size N	Mean No. of Allele A±SE	Polymorphism P*	Heterozygosity H±SE
1	101	1.33±0.98	0.60	0.08±0.03
2	140	1.00±0.84	0.51	0.07±0.02
3	110	0.66±0.32	0.37	0.05±0.03
4	125	0.77±0.22	0.40	0.06±0.02
5	85	0.88±0.24	0.44	0.06±0.03
6	96	0.44±0.02	0.20	0.04±0.02
7	152	1.11±0.86	0.53	0.08±0.02
8	146	1.66±1.01	0.70	0.10±0.03
9	125	1.44±0.96	0.62	0.09±0.02
Total	1080			
Mean		1.03±0.40	0.48±0.15	0.07±0.02
Range		0.44-1.66	0.20-0.70	0.04-0.10

*Criterion of polymorphism 0.95

TABLE 3: Allele frequencies at nine loci in Rana limnocharis

Enzyme	Allele	Populations								
		1	2	3	4	5	6	7	8	9
LDH A_4	65	0.15	0.18	0.06	0.12	0.07	0.00	1.00	0.12	0.16
	100	0.85	0.82	0.94	0.88	0.93	1.00	0.00	0.88	0.84
LDH B_4	190	1.00	0.98	1.00	1.00	1.00	1.00	0.95	1.00	0.86
	196	0.00	0.02	0.00	0.00	0.00	0.00	0.05	0.00	0.14
MDH A_2	80	0.00	0.01	0.26	0.00	0.00	0.00	0.04	0.16	0.06
	100	1.00	0.81	0.74	1.00	1.00	1.00	0.86	0.84	0.94
MDH B_2	154	0.22	0.19	0.26	0.00	0.00	1.00	0.10	0.13	0.80
	177	0.78	0.81	0.74	1.00	1.00	0.00	0.90	0.87	0.20
ADH A_2	77	0.02	1.00	1.00	0.00	0.95	0.06	1.00	0.34	0.72
	100	0.98	0.00	0.00	1.00	0.05	0.94	0.00	0.56	0.28
ADH B_2	146	0.92	0.73	0.83	0.87	0.86	1.00	1.00	0.89	1.00
	152	0.08	0.27	0.17	0.13	0.14	0.00	0.00	0.11	0.00
ADH C_2	160	0.08	0.00	0.00	0.04	0.02	0.16	0.08	0.13	0.14
	180	0.92	1.00	1.00	0.96	0.98	0.84	0.92	0.87	0.86
G6PD A_2	100	0.97	0.98	1.00	1.00	1.00	1.00	0.95	0.92	1.00
	140	0.03	0.02	0.00	0.00	0.00	0.00	0.05	0.08	0.00
G6PD B_2	184	1.00	1.00	1.00	0.84	0.88	1.00	0.84	0.95	0.86
	198	0.00	0.00	0.00	0.16	0.22	0.00	0.16	0.05	0.14

TABLE 4: Genetic relatedness among nine populations of Rana limnocharis

Locality	1	2	3	4	5	6	7	8	9
1	-	0.86	0.97	0.92	0.95	0.88	0.96	0.95	0.84
2	0.15	-	0.96	0.98	0.87	0.84	0.86	0.96	0.92
3	0.03	0.04	-	0.88	0.96	0.98	0.97	0.87	0.86
4	0.08	0.03	0.12	-	0.99	0.96	0.92	0.95	0.87
5	0.05	0.18	0.04	0.17	-	0.98	0.95	0.96	0.84
6	0.12	0.17	0.03	0.04	0.03	-	0.97	0.84	0.82
7	0.04	0.15	0.02	0.08	0.05	0.03	-	0.86	0.87
8	0.05	0.04	0.18	0.05	0.04	0.17	0.15	-	0.92
9	0.17	0.08	0.15	0.17	0.01	0.19	0.18	0.08	-

Genetic similarity	Mean	0.96±0.23	Range	0.82-0.99
Genetic distance	Mean	0.11±0.04	Range	0.01-0.19

4. DISCUSSIONS

4.1 Isozymes

4.1.1 Lactate dehydrogenase

The enzyme lactate dehydrogenase (LDH E.C. 1.1.1.27.) is one of the most extensively studied enzyme systems and occupies an important position in cell metabolism off the glycolytic pathway. During anaerobic periods when the Kreb's cycle is inoperative, pyruvate is converted to lactate by LDH with a concomitant production of NAD^+. This oxidation of NADH to NAD^+ allows glycolysis to continue, which in turn permits the continued production of ATP for energy (Whitt et al., 1973).

LDH is reported to be present in almost all groups of vertebrates and invertebrates (Markert and Ursprung, 1974). In vertebrates, the polypeptide subunits of LDH are encoded by two genetic loci whose alleles are codominantly expressed (Shaw and Berto, 1963). Some of the Agnatha possess only the A locus. In the more evolved chondricthyes, the two loci A and B are expressed. In some fishes, three LDH loci have been discovered (Whitt et al., 1975; Chatterjee et al., 1984). In most other fishes, the occurrence of an additional locus, which probably arose by duplication of the B locus, could be demonstrated (Schott, 1974). Recently another tissue specific locus in the kidney of air breathing fish, Clarias batrachus has also been reported (Joseph et al., 1985). In many species of fish, fewer than five isozymes are readily separable by electrophoresis because of restric-

TABLE 10: No. of LDH bands observed in Rana species

Species	No. of bands	Authors
Rana gryllio	4	Wright et al., 1966
R. catesbiana	8	Moyer et al., 1968
R. clamitans	4	"
R. aurora	3	"
R. palustris	4	"
R. pipiens	3	"
R. septentrionalis	6	"
R. virgatipes	25	"
R. sylvatica	18	"
R. boylei	5	Case, 1975.
R. esculenta	5	"
R. muscosa	5	Case, 1978
R. pretiosa	4	"
R. tarahumarae	4	"
R. cascadae	3	"
R. limnocharis	5	Present study

tions upon subunit association as tetrameric instability (Markert et al., 1965; Whitt et al., 1970). Mammals and birds possess an additional LDH locus, the C locus (LDH-X; Bianco et al., 1963; Goldberg, 1977), restricted to mature testes. However, in many tissues in general there are five isozymes formed by the random association of the A and B subunits into all possible tetramers (A_4,A_3,B_1,A_2 B_2,A_1B_3 and B_4).

Among the vertebrates, the situation in the living amphibians seems to be more complicated. Various number of bands ranging from 2 to 25 have originally been reported from the three orders - Anura (or Salientia): frogs and toads; Urodela (or Caudata): Salamanders and newts; and Apoda (or Gymnophiona): the limbless amphibians (Haupt et al., 1958; Wieland et al., 1959; Wright 1964; Adams et al., 1965; Grainger et al., 1966; Balek et al., 1967; Moyer et al., 1968; Chen, 1968; Gallien et al., 1973; Vonwyl et al., 1980). Among these the classical pattern of five isozymes, as found in the diploid and tetraploid species of the family Ceratophrydidae (Schwantes et al., 1969), seems to be the exception. Later it became clear that in amphibians, only the two common genes, coding for A and B subunits, are present, as claimed by Maxson et al., (1974) in Hyla. Bands additional to the expected five could be

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explained as the result of allelic polymorphism (Wright et al., 1968; Salthe, 1969; Maxson et al., 1974; Vogel et al., 1977; and Graff et al., 1977). The occurrence of only two bands is now understood in atleast one case; Triturus vulgaris exhibits just two bands with no hybridisation between these bands. These bands were identified as the A and B subunits (Faulhaber et al., 1978). In Xenopus laevis a number of investigators have reported more than five bands (Kunz et al., 1967; Claycomb et al., 1971; Kunz, 1973; Wall et al., 1974; and Faulhaber et al., 1975). Inspite of the more number of bands the Xenopus possesses only two different subunits and the excess bands are due to allelic polymorphism (Lyra et al., 1966).

Studies on the Rana species started from the work of Moyer et al., (1968). They also provided considerable evidences on the pattern changes of LDH isozymes during ontogeny. Their comparative LDH isozyme pattern from nine Rana species showed either more or less than the expected five isozymes. The highest number ranged to twentysix. There after for the next twelve years no substantial progress were made. A rethrust to this studies came from the work on the biochemical systematics of the members of the genus Rana (Case, 1978). There after such studies picked momentum elsewhere but in India no attempt has been made so far on these aspects. Table 10 represents the list of Rana

species analysed so far for the LDH isozyme pattern.

In our course of investigation with Rana limnocharis a simple pattern of five LDH isozymes was found with the activity similar to the standard pattern. This is rather a surprise finding, since, as mentioned above the situation in Rana species is more complicated. When the LDH data are considered together, they strongly suggest that the usual LDH isozymes of frogs in the genus Rana are tetramers consisting of two different kinds of subunits (Moyer et al., 1968). It is likely that the enzyme is similarly constructed in our species. The existence of two loci A and B are confirmed. This assignment is supported to a certain extent, by the tissue specific concentrations of the isozyme. The LDH A_4 is most concentrated in muscle and liver tissue and LDH B_4 is mostly in the heart extract, a condition similar to that found throughout the genus Rana (Vonwyl et al., 1980). Out of these two loci the A_4 homotetramer is constant in most of the tissue but the activity of B_4 gives a distinct pattern to the tissues. The muscle and liver tissue show A_3B_1 band which is lacking in the other tissues. Hence, it is possible to infer that in Rana limnocharis tissue specific pattern of LDH isozymes exists. A group of relatively rapid migrating isozymes or the occurrence of some characteristic bands in some tissues have been also used as a criteria for determining tissue specificity

in Rana pipiens and R. palusris (Moyer et al., 1968). There was no characteristic band in our frog but the variation in electrophoretic mobility of B_4 resulted in a characteristic patterns for each tissue.

The occurrence of more than five bands has also been observed in some of our frogs, as has been demonstrated in other amphibians. The absence of some LDH isozymes in heterozygotes and even in homozygotes has also been noted in amphibians (Balek et al., 1967; Wright and Moyer, 1968, 1973; Vogel and Chen 1967, 1977; Faulhaber and Lange-Iyra, 1978). In our case, it could not be ascertained whether missing or extra bands were the result of the resolution of the electrophoretic system, restrictions on random aggregation, the absence of activity in formed polymers, or more rapid degradation of polymers. Theoretically, postranslational modifications (epigenetic) at the level of subunits of polymers, could also give rise to additional bands as in Xenopus (Vonwyl et al., 1980). Postranslational modifications, acting directly on polymers seem unlikely atleast in one case, where five closely spaced bands can be seen in the cathodal region. It is unlikely that exactly five bands are produced by modified A_4 tetramers, while it is much more probable that these bands are due to random aggregation of two different A subunits. The idea of post translational modifications, acting directly on the A subunits,

is however, difficult to reject in case of possible duplication without a partial analyses of the primary sequence of the two subunits.

4.1.2 Malate dehydrogenase

Malate dehydrogenase (MDH. E.C. 1.1.1.37) catalyses the reversible oxidation of L-Malate to oxalo acetate with the concomitant reduction of NAD and as mentioned earlier is an essential component of the aerobic metabolism. The tissues, both muscle and liver, play active part in these activities hence, the total expression of the MDH isozymes is specifically restricted to these tissues.

Malate dehydrogenase is known to be dimeric in nature being encoded in the majority of vertebrates at two gene loci, A and B (Banaszak et al., 1975). The random association of A and B polypeptides results in the formation of these dimeric MDH isozyme A_2; A_1B_1, and B_2 as observed in many teleosts (Bailey et al., 1970; Wilson et al., 1973; Whitt et al., 1977; and Frankel et al., 1984). Codominancy in the teleosts have also been reported (Teniguchi et al., 1980).

Less complex patterns of MDH isozymes has been observed in the amphibian group (Danzmann, 1982). The earliest work is traced back to the Urodele Pleurodeles (Gallien

et al., 1973). Substantial work, particularly, in the Rana species is lacking. In our species, Rana limnocharis, the MDH isozyme system can be attributed to the codominant action of the two MDH loci resulting in a three banded phenotype. Out of all the six tissues examined only liver and muscle tissues show the dimorphic nature by resolving three equally spaced bands. In the other tissues monomorphic band of A_2 dimer was confirmed. The isozymes of A subunit composition shows higher activity in all the tissues analysed as compared to the B_2. The MDH isozyme tissue-specificity could be placed under two groups - one having the single band and the other with the three banded phenotype. Furthermore, relative electrophoretic mobilities of the MDH isozymes are in general agreement with electrophoretic distribution for this system in other amphibians, with the B_2 homodimer being the most anodal.

The greatest number of MDH isozyme bands from any tissue has been three though it varied between individuals and also between populations. Although these populations could be identified on the basis of the differential activities of MDH A_2 or B_2 isozymes but in general the activities of these subunits are in random proportion.

Since we couldn't separate the mitochondrial and supernatant MDH fractions due to our limited laboratory working condition it is difficult to conclude that the

bands stained are for which fraction. However, from the general review of literature it could be assumed to be the supernatant MDH isozyme fraction.

4.1.3 Alcohol dehydrogenase

Alcohol dehydrogenase (E.C. 1.1.1.1.) has been observed predominantly in the liver tissue as it is primarily a liver specific protein found in parenchymal cells (Raiha et al., 1967). It mediates the conversion of primary alcohols to aldehydes and secondary alcohols to ketones utilizing NAD^+ as the electron acceptor (Pietruszko, et al., 1973).

Alcohol dehydrogenase is known to be dimeric in nature, being encoded in the majority of vertebrates at a single gene locus (Hitzeroth et al., 1968; Shaklee et al., 1974; Levine et al., 1975; and Frankel, 1978, 1980, 1981 and 1983). ADHJ occurs at its highest concentrations in liver, but has also been reported to be present to a lesser extent in the stomach and kidney of several species (Hitzeroth et al., 1968 and Shaklee et al., 1974).

Work on amphibian species are scanty. Weslowski (1983) had reported in Xenopus species that altogether four ADH isozyme phenotypes has been observed from any tissue. The group Rana has not yet been subjected to such investigations.

Rana limnocharis resolved the greatest number of ADH isozymes to four which is similar to the Xenopus pattern (Weslowski, 1983). Maximum activity has been observed only in the liver tissue, although minor activities are observed in other tissues including heart and kidney. The tissues which failed to exhibit significant activity are brain, eyes and muscle which may probably be due to the less physiological requirement of ADH in these tissues. Between the four banded phenotypes, three equally spaced bands (A_2,A_1B_1, and B_2) migrate anodally to the fourth band (C_2) (Fig.4).

The various ADH phenotypes can be explained genetically on the basis of the assumption made by (Weslowski et al., 1979) in Xenopus. It is assumed that (1) R. limnocharis carries at least three genes that code for ADH isozymes separable electrophoretically, (2) only two of these loci code for monomers that can form heterodimers and (3) one of these loci has two alleles, one coding for an electrophoretically fast (1^f) and the other for a comparatively slow (1^s) monomer. Thus, the consistant triple banded pattern would be due to homo and heterodimers coded by two separate loci. The fast migrating array would result from individuals homozygous for the fast allelic variant of one of these loci, the slow array from homozygotes for the slow allele, and the fast plus slow pattern form heterozygotes for this gene. The product of the third locus is assumed to comigrate

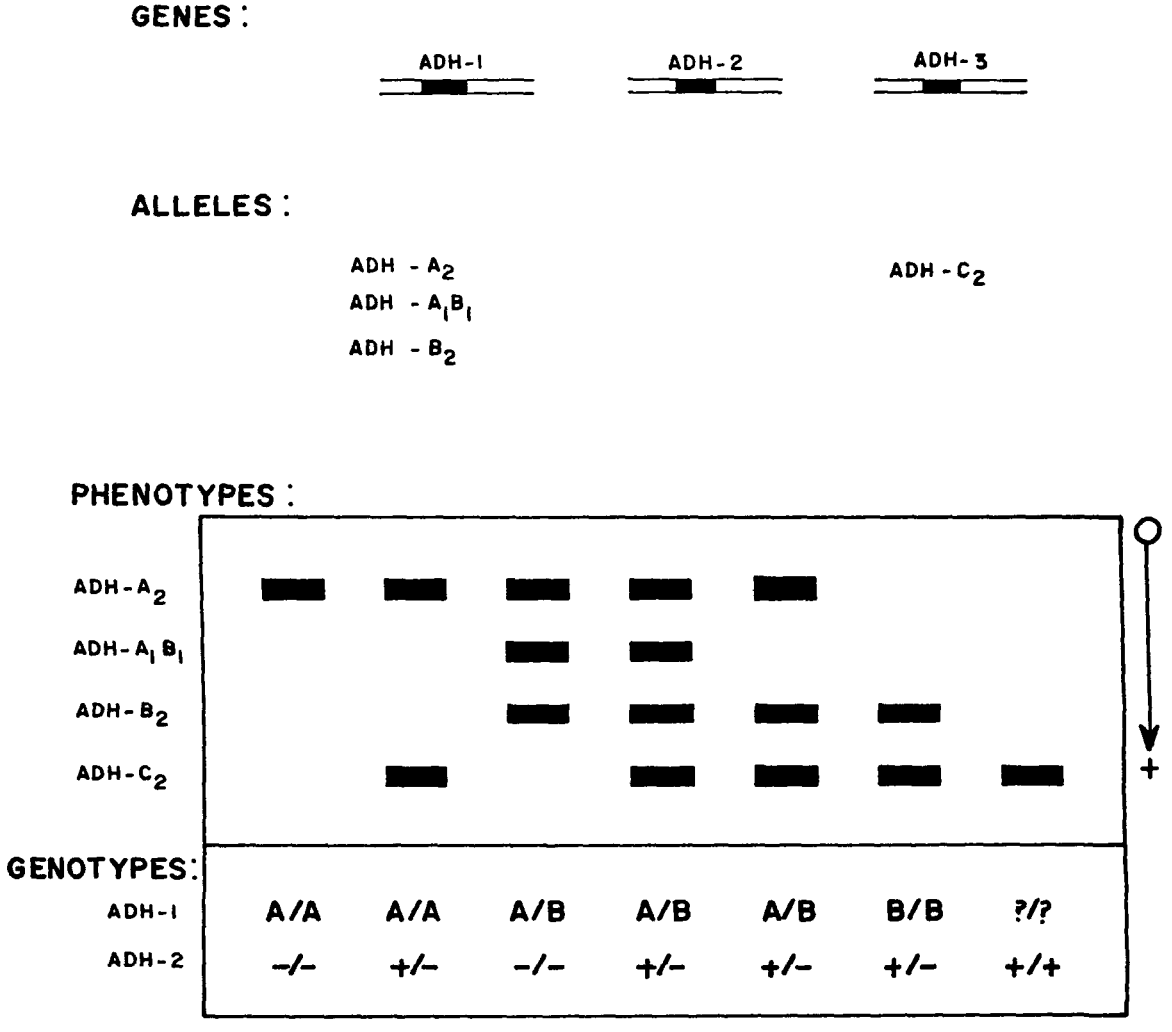

Fig. 8. Genetic interpretation of ADH isozyme in R. limnocharis

with that of the slow allelic variant for one of the two loci whose products form heterodimers and results in a dense cathodal-most band when both are present. Fig.8 depicts the possible phenotypes and genotypes arising due to the three genes. There are two groups of Alleles. ADH 1 and 2 includes the homo and heterodimers which form the consistent triple banded pattern (A_2B_2).

The ADH 3 or C_2 gene has been observed as monomorphic with no indication of a heterozygous combinations between A_2C_2 or B_2C_2 has been observed. This probably leaves some doubt on the molecular structure. Although, the possibility of its dimeric nature is more as ADH C_3 isozymes from most of the other vertebrates are dimeric (Rossman et al.,1975). Our report is in agreement with that of Xenopus laevis (Weslowski, 1979) in assuming that Rana limnocharis carries at least three separate loci coding for ADH monomers, but the products of only two can form heterodimers. The tissue specificity and multiple isozymic forms warrant future investigations concerning their mode of activation.

4.1.4. Glucose - 6 - phosphate dehydrogenase

In higher vertebrates, the occurrence of two different forms of Glucose-6-phosphate dehydrogenase (G6PD, E.C.1.1.1.49) have been observed (Shaw et al., 1965; and Ohno et al.,1966). Its dimeric nature has also been reported (Shaw, 1966;

and Yamanchi et al., 1974) and in most of the animals they do not exhibit the intermediate hybrid zone (Scholl, 1973). In mammals, G6PD isozymes are encoded in two separate loci, one of it being sex-chromosomally linked (Kirkman, et al., 1963) while the other is autosomaly in origin (Shaw et al.,1965). The autosomally inherited mammalian isozyme reacts more actively with galactose-6-phosphate and is also referred as Hexose 6 phosphate dehydrogenase, H6PD. Two G6PD isozyme with similar substrate specificities as the mammalian G6pD have also been observed in the teleost (Scholl, 1973). Similar observation has been made in insects (Rao, 1980). However, there is no definite report of the molecular mechanism governing the expression of the G6PD genes in amphibians.

The occurrence of two different forms of G6PD isozyme in the liver tissue, called A and B has been confirmed in Rana limnocharis, as in other vertebrates. We can then assume that the two isozymes observed are homologous to the G6PD and H6PD isozymes. Both the isozymes are consistently scorable inspite of the fact that considerable variation is observed with respect to the amount of stain deposit at the sites of both the isozymes. No heterozygous combinations were observed between these two isozymes and this leaves some doubt on their genetic compatibility. No sex differential activity was observed in this frog.

4.2 Genetic Variations

Reliable estimates of genetic variation in natural populations require examination of polymorphic loci (Lewontin, 1974; Nei and Roy Choudhary, 1974). Studies of such genetic variability between and within populations is based on a random sample of the genome. It requires a sample which is both diverse with regard to biological function of loci and unbiased with regard to monomorphism versus polymorphism. This is probably the only reason why the knowledge of variation in the genome of a species in a natural population is poor. Our investigation with the natural populations of the streaked frog, R. limnocharis, has shown that the observed allozymic variation is under genetic control since the phenotypic frequencies are in close agreement with those expected on the basis of Hardy-Weinberg value and the electrophoretic patterns were consistent with the previously described enzyme subunit structures in other species. If possible, the interpretation of electrophoretic data should, of course, be based on inheritance experiments. In many wild species, however, this is very difficult or even impossible. On the contrary there is strong need for genetic studies of population in many species which are not easily stocked (Allendorf and Utter, 1977). Further, for a correct understanding, a general picture of genetic variation in natural population cannot be obtained from

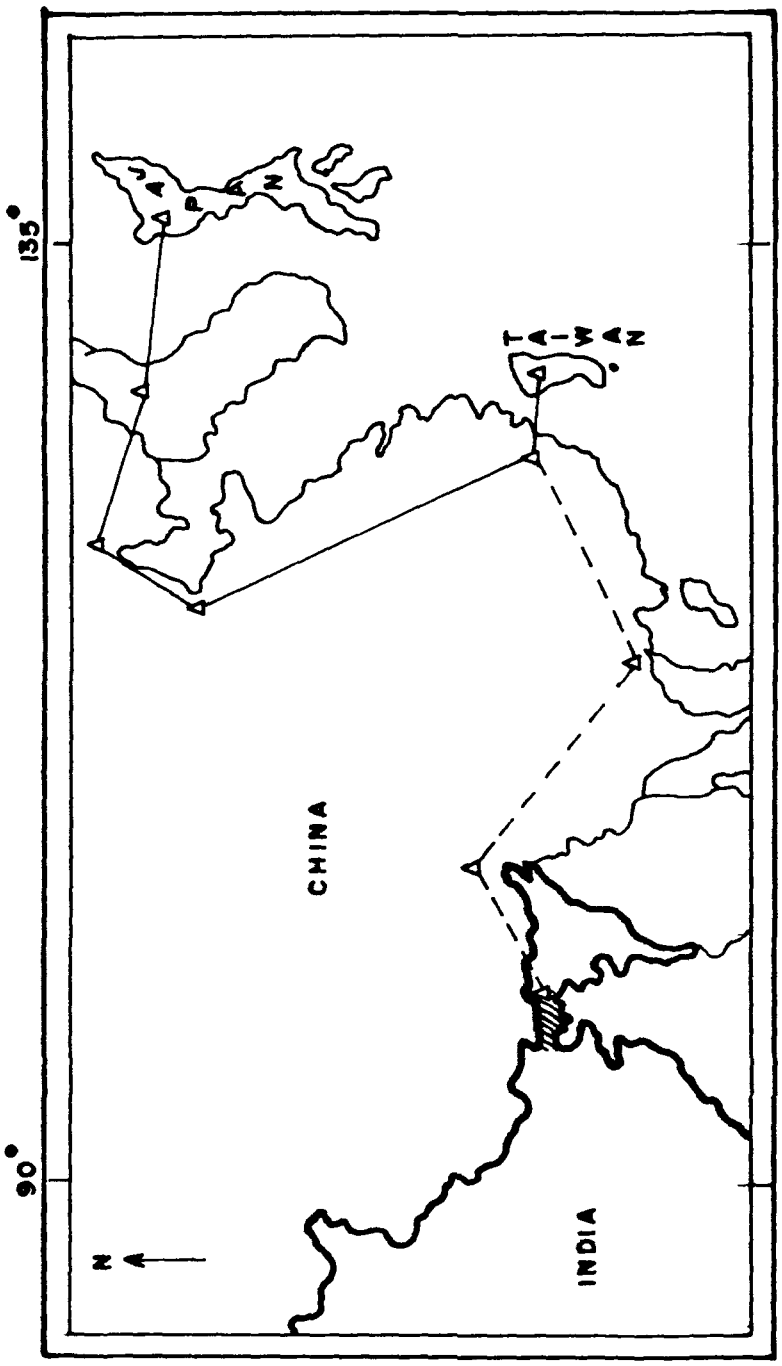

Fig.9. Probable origin and extention route map of R. limnocharis (modified after Kuromoto, 1967).

studies which are restricted to organisms only bred in the laboratory.

The species Rana limnocharis is one of the most widely distributed species among the Asian frogs. Its range covers most of the countries in south-east Asia. The unique features of this species is that it is apparently Oriental in origin (Inger, 1954), while the other Rana species are palaearctic. Its probable place of origin in this region has been found to be in Formosa (now Taiwan) and this frog must have extended its distribution area because of its adaptability to watery lowlands and invaded Japan, China, Burma and subsequently to the north-eastern part of India (Kuramoto, 1967; Fig.9). It is primarily a riparian species. In Meghalaya despite its riparian habit, it is also found near human habitation in cultivated or disturbed areas. However, it never moves far from standing or flowing water. These bodies of water serve as buffer to climatic perturbations of both humidity and temperature. Though breeding populations are large, they do not form specialised breeding aggregation in the sense of semi-isolated clusters of adults that have moved from normal feeding areas to breeding sites, as reported in many anurans (Inger, 1979). Breeding takes place from April to September, varying geographically.

The general character of the habitat has an effect

on gene flow among our frog populations. Those living in open man-modified environments have irregularly shaped but more or less continuous areas available in the surroundings of Shillong and more so over large sections of Meghalaya. Forest environments of Tura, on the other hand, are becoming increasingly constructed into islands, thus isolating populations of forest species. Inger (1973) encountered similar ecological conditions with Bufo species in Malaya and Kualalumpur. He observed that if all the factors remain constant the gene flow among those populations are greatest in which the habitat is open and man-modified than for those confined to forests. Similar observations were made in our course of study. The populations from Shillong, Jowai, Nongpoh and to some extent Cherrapunjee, where the disturbance is more, showed relatively increased genetic variability, while the isolated and stable populations from Tura and Nongstoin were relatively invariable. Similar reports have also come from Hyla species (Case, 1975). The characteristics of any population that are most likely to affect the rate and amount of local differentiation and the extent of local heterogeneity are the relationship of the non breeding range to the breeding site, and the tendency to form breeding aggregations. The absence of breeding aggregation and no separate cut area for feeding in the various populations of this frog, tends to decrease the effective population

size, also the amount of heterogeneity locally. Inger (1974) found that the degree to which these effects are felt is probably directly related to the size of the aggregation. No breeding aggregation was observed in this frog and hence the effect on the population size must be minimun.

If the nine populations of this frog are considered in the light of distributional characteristics (Table 1) and their potential effects on genetic variation, we would expect the Tura population to show more homogeneity locally than in relations to other wider areas. The restriction of Tura population to forests, which now exists only in isolated patches in and around Tura, certainly limits gene flow among populations. It differs from the other populations in all the distributional features (Table 1). The electrophoretic data substantiate this prediction.

We would not expect populations from Cherrapunjee and Mawsynram to differ greatly in the amount of genetic variation since they share many important characteristics (Table 1). Both these populations receive the highest rainfall than in any part of the world (See Section 2.1.4). However, the electrophoretic results do not fit into the predicted value. The population of Mawsynram shows more closeness to Dawki in terms of variation than to Cherrapunjee (Table-2). This is demonstrated by the fact both populations from

Mawsynram and Dawki were found to be statistically less variable than the Cherrapunjee populations in terms of allelic variation. Here the lack of more detailed measures of the population structure of Rana limnocharis is likely the reason for the "discrepencies" between prediction and findings.

The alitudinal habitat variation between Shillong and Nongpoh and between Shillong and Jowai was clearly reflected on the electrophoretic pattern (Table 1 & 2). Nongstoin also appeared to be isolated but the analysis of variance showed similar value to all the lower elevated population. Two important conclusions emerge from these data: First with limited knowledge of the ecological background of the populations we have difficulties to predict a fairly realistic outlines of the pattern of genetic variation. In population about which we know less, our prediction becomes less useful, partly because some factors remain unknown in a quantitative sense and partly because our meausres of the relative intensity of various factors may be too crude to allow an estimate of net effect. For example, the high variation in some of the populations (Shillong, Cherrapunjee and Jowai) was not anticipated.

For each population, the percentage of polymorphic loci and the average individual heterozygosity Lakhean

calculated (Table 2). The percentage of polymorphic loci ranged from 20% in Dawki to 70% in Nongpoh. Heterozygosity ranged from 0.04 in Dawki to 0.1 in Shillong population. There is no cline in either the percentage of polymorphic loci or average heterozygosity. In general, population with a high percentage of polymorphic loci also has high $\bar{H}$ value (Table 2). Throughout the species range in Shillong, Jowai, Nongpoh, Tura, and Williamnagar, the percentage of polymorphic loci is very high (51-70%). These values are similar to those reported in Hyla (Case, 1975), Bufo (Rogers, 1973) and Taricha (Hedgecock et al., 1974) and higher than those found in local populations of Acris (Dessaner and Nevo, 1969). Average heterozygosities, which range between approximately 4-10% are similar to values for "typical" vertebrates which have an $\bar{H}$ value of 5-6% (Salender et al., 1973, and Case 1978). There were four exceptions within this range with regards to percentage polymorphic loci (Cherrapunjee, Nongstoin, Mawsynram and Dawki). Possible explanation for the low polymorphic values observed include the relative isolation of these populations relatively isolated from other, the amount of environmental variability in these areas particularly the habitat, and the relative age of the populations. We have no data which could aid in distinguishing among these three possibilities, so until detailed ecological studies are conducted in these four

areas, the true reason for the high polymorphic values observed will not be properly understood.

The four low polymorphic populations (Mawsynram, Dawki, Cherrapunjee and Nongstoin) show lower percentage of average individual heterozygosity levels, ranging from 0.4 to 0.5 within this region. Such values are comparable to those found in some species of Dipodomys (Johnson et al., 1971) and some fossorial rodents (Nevo, et al., 1974). Since detailed ecological and microhabitat data are not available for any of these populations, the significance of the observed differences is not clear. It has been assumed that high levels of polymorphism and $\bar{H}$ are "typical" for this species, since other amphibians which have been studied elsewhere generally seem to have a high percentage of polymorphic loci and average individual heterozygosities which are similar to those of other vertebrates. However, the possibility that the converse is true, that low levels of polymorphism and low $\bar{H}$ values are typical for this species, should not be dismissed automatically.

The average number of alleles per locus population show that their frequencies significantly change between several population (Table 2). This difference also indicates the extent of biochemical differentiation at the population level due to various aforesaid factors particularly the

habitat. The average number of alleles per locus per population was observed to be 1.03±0.40. The value is low in comparison to the other vertebrates studied where it ranges between 1.11-2.41 (Inger et al., 1977; Culthart et al.,1984). The range of values was between 0.44 to 1.66. Possibility of this low value due to a small sample size could be rejected because some populations scoring high values had more reduced population size. The reduced nature of all the values may be the characteristic of such population due to a specific environmental condition.

Allele frequencies are gien in Table 3 for each locus from all the nine populations. The analysis of variance (ANOVA) of these date indicates that statistically significant amount of variation (P 0.001) was present between localities for all the nine loci (Table 4). One of the possible reason for the inconsistency in allele frequency and variation could be that the uniform, invariant alleles or genes 'co-adapt' the species for high average fitness in a slowly but inevitably changing environment (Wright, 1974), whereas the more variant allele provides fine adjustment to the immediate environments. This fine adjustment could be due to the physiological stress, viz., temperature increase/decrease, adaptive response to the water conditions and so on. Possibility exists to confirm the allelic variance

to be of adaptive nature if breeding studies are made. Although, no breeding tests were conducted in our frog due to limited laboratory working conditions to confirm Mendelian inheritance at the loci analysed, we assume that the electrophoretic variation represented allelic variation. Similar assumption has also been made by several workers (Hadfield, et al., 1979; Fujio et al., 1979; Nevo et al., 1982).

Most of the loci studied have a major allele in high frequencies in all the populations with a few less common alleles occurring in various populations (see section 3.5). Most populations are fixed at certain allele. The expected Hardy-Weinberg proportions have been observed in majority of the population in each locus.

4.3 Environment Variation Model

The idea that genetic variation is related to environmental variation dates back to Dobzhansky (1951). It was framed theoretically by Levins (1968) and tested experimentally by Powell (1971) and Ayala (1974). Increased enmvironmental heterogeneity and unpredictability leads to heterozygosity (Nevo, 1980). The frogs, in our study, living in a relatively constant and predictable environment (Tura, Nongstoin, William Nagar) and thus insulated from short term environmental changes, have significant lower genetic variation than

the habitat intermediate (Jowai, Shillong and Nongpoh). On the contrary, populations which are spatially and temporally distinctly varying (Cherrapunjee and Mawsynram) and unpredictable has a much larger range of genetic variation. The genetic variation increases in the order - Tura, Nongstoin, Williamnagar >Cherrapunjee, Mawsynram>Dawki>Nongpoh>Shillong, Jowai.

The genetic profiles of this frog can also be correlated with the apparent difference between semi aquatic and semi terrestrial habitats (see section 2.1.4). The environmental model of genetic variation predicting a moderate variation (Tura, Nongstoin and Williamnagar), as compared to the extremely unpredictable (Cherrapunjee, Mawsynram and Dawki) and disturbed (Shillong, Jowai and Nongpoh) is reasonably substantiated.

The environmental variation model predicting lower genetic variation in relatively more predictable environments was confirmed also in a variety of unrelated subterranean taxa such as fussorial mammals, amphisbaenians and lizards, skinks, frogs, subterranean mole crickets, Gryllotalpa, Land snail species of Bulminus and Sphincterochila, and habitat specialist species in general (Nevo, 1978, 1979). Inger et al. (1974) made an attempt to correlate genetic variation with ecology and population structure of amphibians

but his predictability was lrss satisfactory among the Ranid species in relation to other species.

4.4. Genetic Relatedness

Roger's coefficient of genic similarity (S) and Nei's genetic distance (D) between all pairs are shown in Table (4). The genetic distance values between the nien populations vary widely (0.01 to 0.19) and could be averaged to 0.11±0.04. The similarity index ranges between 0.82 to 0.99 and its average value is 0.96±0.23. Although, the distance between Dawki and Shillong is not much (Table-1) but the greatest genetic distance values are obtained between them (0.19). The greatest similarity index value is obtained between Cherrapunjee and Mawsynram (0.99). Incidently, these two populations also fall under similar microhabitat. The values obtained are difficult to interpret in taxonomic terms because approximate S values for amphibian species or sub species are not available. The only available estimate is that of Hedgcock et al., (1974) in the salamander, Taricha, there the average genetic distance was D = 0.168±0.042. Our average D value falls approximately in the same range and could perhaps be a possible confirmation of a standard D value for amphibians.

Reports of high genetical similarity between populations of a species are unusual if there is no overlapping

parameters. Genetic similarities therefore, appear to reflect properties of biological and evolutionary significance. The pattern of allozymic variation (mentioned above) and the consideration of genic diversity, support natural selection as an important determinant of population genetic structure in R. limnocharis and make random factors and neutrality alone unlikely explanatory model. Large number of frogs have been found in all surveys, also during the breeding season. Consequently, effects of small population size are excluded as major determinants of differentiation. Likewise, the expected outcome of genetic drift, i.e., fixation of many alleles in small isolated populations, has not been found. In contrast to this expectation, the same alleles in polymorphic loci predominates in all the population. In particular, population of Tura and Williamnagar are separated from the Shillong population by several dozen kilometers uncrossable by frogs. If these isolates date back to geological times then their genetic pattern involving uniformity of alleles and the continuation of pattern in levels of genic diversity, make the drift and/or neutrality hypotheses unlikely, and suggests selection as the major factor of genetic differentiation of populations. It therefore appears plausible that allelic variation of protein polymorphisms is atleast partly adaptive and is regulated in natural populations by selection in accord

with an index of environmental diversity.

Despite of some evidence to the contrary (Gooch, et al., 1972) a growing body of evidence reinforces the idea that genetic polymorphism, including protein polymorphism, is adaptively important rather than neutral. Environmental heterozygosity probably selects for higher heterozygoxity and thereby for higher fitness. The latter may not only depend on individual polymorphic loci but also on greater overall enzyme heterozygosity per individual as suggested by Wills (1973).

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PLATES

PLATE 1.1 Comparison of LDH isozyme tissue pattern

PLATE 1.2 Comparison of MDH isozyme tissue pattern

M L E B K H

1.1

H K L

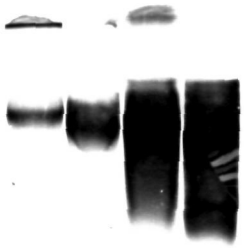
1.2

PLATE 2.1 Comparison of ADH isozyme tissue pattern

PLATE 2.2 Comparison of G6PD isozyme tissue pattern

2.1

K E B H L M



2.2

H E M L

PLATE 3 Allelic polymorphisms at MDH loci

3

SECTION - B

KARYOLOGICAL STUDIES

1. INTRODUCTION

It is an accepted fact that the chromosomal information of a species are essential to the modern taxonomists since the species are considered to be the objective reality of some particular genetic continuity (Manna 1969). Karyological studies of frogs have lagged for behind than that of other vertabrates inspite of their abundance and easy availability. Out of about 400 living species of frogs only about forty two are chromosomally known and karyotyped (Morescalchi, 1973; Bogart and Tandy, 1981; Wiley, 1982; Kuramoto, 1985). This is probably due to the inconsistency in number and forms of chromosome and also because the traditional techniques have not been useful in studying frog chromosomes.

In the last few decades there has been renewed vigour in the study of chromosome complements and karyotypes of amphibians due to the technological advances which facilitated preparations and resolution of individual chromosomes within a complement. The initial thrust of such studies came from Morescalchi (1963) who gave a comparative account of chromosomes of various groups of amphibians. It was also during this decade the great controversy over the nomenclature of chromosome types arose, which was, however, clarified later by Levan et al. (1964) when they proposed that the chromosomes should be categorised according to their centromeric position. This proposal was universally

adopted in 1966 at the Paris conference.

Members of the families of Hylidae, Centrolemidae, Ranidae, Leptodactylidae came under thorough investigation for their cytological identity (Goin and Goin, 1962; Kobyashi, 1962; Duellman et al., 1965). The most popular method for obtaining somatic chromosomes was from spleen (Manna and Bhuya, 1966). The meiotic behavior and the chromosome number of the genus Acris was established (Cole, 1966). Reports of Bufo came from India and abroad (Volpe et al., 1968; Bogart, 1968; Cole et al., 1968; Manna and Bhunya, 1968; Chatterjee & Barik, 1970). Unlike other families, Leptodactylidae, presented serious problems on the taxonomic and the systematic identity of its members from the karyomorphological data which differed to that of the conventional parameters (Bogart, 1970). A monograph on the Brazilian anuran chromosomes helped in solving some of the problems relating to the chromosomal evolution and specification (Rabello, 1970). The karyotypic similarities between the various hylids and their evolutionary significance have been dealt by Stephenson and Stephenson (1970).

It was only in the seventies that the cytogenetical studies centered on the genus Rana. Interests were also directed on the study of the utility of the giant chromosomes in cytotaxonomy (Giorgi and Gallieni, 1972). A systematic Karyomorphological survey was made to evolutionary link

Table 1. Chromosome number in the genus Rana.

Species	Chromosome no. (2n)	References
1. Rana limnocharis	26	Sato, 1934
2. R. plancyi	26	Ting, 1939.
3. R. hexadactyla	26	Natarajan, 1958.
4. R. cyanophlyctis	26	-do-
5. R. narina	26	Kobayashi, 1962.
6. R. chinsinensis	24	-do-, 1962.
7. R. arvalis	24	-do-; Ullerich, 1967.
8. R. ornativentris	24	-do-; Seto, 1965.
9. R. esculenta	26	Morescalchi, 1962; Gunther, 1970.
10.R. palustris	26	Goin et al., 1962.
11.R. pipiens	26	Di Berardino, 1962; Volpe et al, 1970.
12.R. okinavana	26	Nakamura et al., 1963.
13.R. kuhlii	26	Nakamura et al., 1963.
14.R. sylvatica	26	Hennen, 1964; Rafferty, 1969.
15.R. ishikawae	26	Seto, 1965.
16.R. catesbiana	26	-do-
17.R. rugosa	26	-do-
18.R. nigromaculata	26	-do-
19.R. japonica	26	-do-
20.R. holsti	26	Okada, 1966.
21.R. temporaria	26	Guillmin, 1967; Morescalchi, 1967.
22.R. papua	26	Duellman, 1967.
23.R. grisea	26	-do-
24.R. graeca	26	Morescalchi, 1967.

contd....

Table-1 contd..

Species	Chromosome no. (2n)	References
25. R. ridibunda	26	Morescalchi, 1967; Gunther, 1970.
26. R. dalmatina	26	Guillemin, 1967.
27. R. tigrina	26	Singh et al., 1970.
28. R. subaspera	26	Kuramoto, 1970.
29. R. dybowskii	24	-do-; 1972.
30. R. namiyei	22	-do-, 1972.
31. R. boylii	26	-do-
32. R. cascadae	26	-do-
33. R. brevipoda	26	Nishioka, 1972.
34. R. longicrus	26	Kuramoto et al., 1973.
35. R. adspera	26	Morescalchi, 1973.
36. R. preteiosa	26	Haertel et al., 1974.
37. R. muscosa	26	Ward, 1977.
38. R. angolensis	26	Bogart et al., 1981.
39 R. occipitilis	26	-do-
40 R. lessonae	26	Kuramoto, 1983.
41. R. macronemis	26	Birstein, 1984.
42. R. latestii	26	-do-

the widespread Rana species in the American continent (Cole, 1974). A similar study was also tried on the Cuban populations and also a monograph on the Malagasy Rana was put forward in which the general mechanisms of chromosomal evolution, ecological and genetic significance of chromosomal variation were explained (Bogart et al 1981). Chromosomal conservation has been exemplified between the African populations (Morescalchi, 1981).

The Rana species with established dipoid (2n) chromosome numbers are listed in Table I. It is evident that most of the species have a diploid number of 26. Those for which the karyotypes have been studied in greater detail reveal pairs of chromosomes that differ only in few aspects from one species to another. The chromosome number fluctuated between 22-26. The monograph presented by Morescalchi (1979) confirmed the diploid number of 26 chromosomes in fifteen Rana species. The earlier investigations on few south east Asian groups of Rana also confirmed the same number (Kuromoto, 1972).

Apart f rom the karyological investigations, work on various other aspects of chromosomes like aneuoploidy, sex chromosomes morphology and behaviour, secondary constrictions - Nucleolus organizing regions (NORs) and level of heterochromatisation were also considered.

The question whether there are morphologically differentiated and recognizable sex chromosomes in the Rana species still remains unresolved. Rebello (1970) stated, "when both sexes were available, male and female presented identical karyotypes". Raychoudhary and Singh (1975) have made generalisation for the whole of amphibia and stated, "there is no unequivocal evidence of heteromorphic pairs which can be designated as sex chromosomes". Similar observations have also come from various other workers (Hennen, 1964; Seto, 1964; Ullerich, 1966; Chatterjee and Barik, 1970; Herrero, 1982; Wiley, 1982; Kuramoto, 1985). However, several authors have reported the presence of heteromorphic pairs which they have claimed as sex chromosomes. Manna and Bhunya (1966) suggested female heterogameity for Bufonidae. Kawamura (1977) established the existence of male heterogameity in some Asiatic Rana by sex reversal experiments. Yadav and Pillai (1976) observed female heterogameity in two species of Rana. Cytochemical bandings have revealed heteromorphism in male frogs (Session, 1980; Schmid, 1980; Chakravorty et al., 1983).

The presence and position of secondary constrictions which are potentially the Nucleoulus organizer regions (NORs, the sites of 18s + 28s rNDA) are important characteristics in karyosystematics. With regards to amphibians karyotypes, especially those of the Rana species, secondary

constrictions were discussed by Hennen (1964); Seto (1965); Guellemin (1967); Ullerich (1967); Gunther (1970); Nishioka (1972); Kuramoto (1972, 1980); Kuromoto et al., (1973); Ivanov and Madyanov (1973); Belcheva and Genova (1974); Haertel et al. (1974); Popov (1974); Orlova et al. (1977); Koref-Santibanez and Gunther (1980); Green et al. (1981); Green (1983); Birstein (1984).

The silver stained Rana chromosomes revealed dimorphic NORs in the chromosome sets (Ward, 1977; Schmid, 1978; Belcheva et al., 1981; Birstein, 1981, 1984). 14 to 16 NORs in the diploid chromosome compliments have been reported (Schmid, 1978) which incidently is the highest number recorded till date.

Constituitive heterochromatin bandings revealed the structural heterochromatic as thin blocks in the centromere and the adjacent regions of the centromere (Grafadatsky et al., 1970). A cytochemical classification of the various chromatin types in 26 species of Rana on the basis of differential labelling on the constituitive heterochromatin with fluorochrome dyes was made by Schmid (1980). All these and the other advanced techniques are now showing tremendous potential for providing valuable cytogenetical informations on amphibia.

The amphibian fauna of the Indian subcontingent

show greatest diversity in form and number. The Rana species are relatively more abundant in the North eastern regions of India. Cytogenetically they still remain an enigma. We, therefore, have chosen one of the common frog Rana limnocharis for our present study with a view to determine the diploid (2n) number, morphology, centromeric index of somatic and germinal chromosomes of both the sexes, behaviour of chromosomes during meiosis, sex chromosome constitution and sex determining mechanism, structural and numerical change polymorphism and banding patterns, which are essential to obtain for getting a complete knowledge of the cytogenetics of any organisms (Manna et al., 1968; Chatterjee and Majhi 1974; Bogart, 1974; Kawamura 1977; Schmid, 1980; Morescalchi 1981; Kuramoto, 1983, 1985). These informations would add to the reportoir of the Cytogenetics of Rana on the whole.

Efforts have also been made to infer cytogenetical changes in the karyotypes of geographically spread populations. Feasibility of correlating the chromosomal and the biochemical data have also been tested.

2. MATERIALS AND METHODS

2.1 The Frog

The characteristics and the collection sites of Rana limnocharis have been described in chapter I.

2.2 Chromosome Preparation

Adult individuals were injected intra peritoneally with 0.1% Colchicin solution (1 ml/100g body weight). The injected frogs were sacrificed after 6 hr.

Cytological preparations were made from spleen and gonads following the technique of Dhar and Chatterjee (1984) with some necessary modifications. Spleen and testes were removed and was either subjected to squash or air dried technique. For squash preparations the tissues were finely minced in a hypotonic solution of Sodium citrate for at least 45 minutes and then fixed in (1:3 v:v) Glacial acetic : Absolute alcohol solution, for at least 30 minutes at room temperature and subsequently thumb squashed on an albuminised slide and stained in Heidenhein's haemotoxylin.

For the air-dried preparation the tissues were flushed vigorously in tri-sodium citrate solution (0.09%) to make a homogenuous cell suspension and then allowed to stand for about 45 minutes. The homogenous suspension was centrifuged at 1500-2000 rpm, for 15 minutes. The super-natant was removed and the residue was fixed in 4 ml. of

freshly prepared fixative and subjected to vigourous aspiration. The suspension was allowed to stand for 30 minutes at room temperature and again centrifuged at 1500-2000 rpm for 10 minutes. Two to three drops of the suspension were then placed on a clean slide (chilled in absolute alcohol overnight) and ignited by passing the slide over the flame, dried, and stained in Giemsa solution.

2.2.1 Haematoxylin staining

The procedure described by Smith (1949) was followed with slight modification.

i) Mordant solution : 3g Ammonium ferric sulphate
100 ml dist.water.

ii)Stain : 1g Haematoxylin powder
5 ml. absolute alcohol
95 ml. dist. water
Time : 45 minutes.

iii)Differentiating solution : Saturated aqueous solution of Picric acid.

The slides were processed through ascending alcohol grades before mounting.

2.2.2 Giemsa staining

The procedure as described by Chatterjee et al., (1981) were followed with slight modifications:

i) Buffer A : m/15 di-sodium hydrogen orthro phosphate.

freshly prepared fixative and subjected to vigourous aspiration. The suspension was allowed to stand for 30 minutes at room temperature and again centrifuged at 1500-2000 rpm for 10 minutes. Two to three drops of the suspension were then placed on a clean slide (chilled in absolute alcohol overnight) and ignited by passing the slide over the flame, dried, and stained in Giemsa solution.

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2.2.2 Giemsa staining

The procedure as described by Chatterjee et al., (1981) were followed with slight modifications:

i) Buffer A : m/15 di-sodium hydrogen orthro phosphate.

ii) Buffer B : m/15 Potassium dihydrogen orthro phosphate

iii) Working solution: Buffer A and B (1:1) pH 6.8-7.2

iv) Stain : 10 ml. Giemsa solution (BDH)
90 ml. Working buffer solution
Time : 10 minutes.

2.3 Chromosome analysis

All measurements were taken in the best metaphase plates that could be obtained (the numbers of metaphases analysed are shown in Table 3). The following parameters were considered while studying the chromosome morphology.

(i) Length of the Long arm of the Chromosome.

(ii) Length of the Short arm of the Chromosome.

(iii) Arm-ratio or 'r' = $\frac{\text{Length of the Long Arm}}{\text{Length of the Short arm}}$

(iv) Centromeric indices = $\frac{\text{Length of the short arm}}{\text{Length of the long arm}} \times 100$

(v) Total length of the Chromosome.

(vi) Relative length of the Chromosome.

$$RL = \frac{\text{Total length of a chromosome}}{\text{Total length of the whole chromosome set}}$$

(vii) Number of arms or NF value : total number of arms in a complete chromosome set.

(viii) Centromeric position and chromosome type.

The position of the centromere was determined according to Levan et al. (1964) by dividing the long arm by the short arm to provide an arm ratio (r). If 'r' is 1.0 to 1.7, the centromere has been considered as median and the chromosome has been designated as 'm' type (metacentric);

a 'r' or 1.7 to 3.0 reflects a submedian centromere and the chromosome has been designated as 'st' type (subtelocentric). A 'r' of 7.0 to ∞ indicates a terminal centromere and the chromosome has been referred as 't' type terminal. Idiograms were constructed using the average measurements of all homologous arms or chromosomes analysed.

2.4 NOR staining

The one stage method of silver staining was used (Howell and Black, 1980). One drop of gelatin solution (2% solution in water acidified with formic acid) and two drops of 50% $AgNO_3$ solution were placed on the preparation, then the drops were mixed, and the mixture was covered with a cover glass. The preparation was heated at 70°C for 2.0-2.5 min, the cover glass taken off and the preparation washed with water, dried, analyzed and photographed.

2.5 Banding studies

We failed to obtain banding karyotypes although various methods were tried.

3. RESULTS

3.1 Karyology

A total of 240 metaphase plates were photographed and analysed (126 ♂ and 114 ♀, Table 2). The chromosomes were counted, measured, and the idiograms established. As no difference were found between males and females, the measurements of both the sexes were pooled.

Like other species of genus Rana, R. limnocharis have also a diploid chromosome number (2n) of 26. Minor deviations from this number arise occasionally (Table 2). The chromosomes are grouped into two series : 5 large and 8 smaller pairs (Plate 1, 2, 3; Fig.1). They can be arranged according to size, coinciding in general with the scheme established by Morescalchi (1973, 1979). The quantitative data of the measurements is shown in table 3, and the idiograms in Fig. 1. The variations in size of the chromosomes obtained in individuals are attributed to difference in the degree of spiralizaton of the chromosomes in various cells.

All the five large pairs of chromosomes are metacentric. In eight shorter chromosomes, pair numbers 6, 10, 12 and 13 are submetacentric. Although, pair 8 and 9 occasionally overlap within the limits of variations of submetacentric value. Otherwise, they remain metacentric. The chromosomes in general are biarmed and have been classi-

fied as per Levan et al. (1961) formula. The fundamental number (NF) is 52.

No chromosomes is characterised by any secondary constriction in its long areas as has been generalized for all Rana species. Nor was there any evidence of heteromorphic pair of sex chromosomes in either female or male karyotypes. The karyotypes in both the sexes are identical.

The relative chromosomal lengths of most of this frog in the population is similar and is always around the average values found in other Rana. The point of interest is the first pair of chromosomes whose mean relative length is extremely large when compared to the corresponding pairs. The situation in the eight small chromosome pairs is different as there are often considerable overlapping of the chromosomal lengths between corresponding pairs from individuals collected from various populations.

An overview confirms that Rana limnocharis has all the karyotypic characteristics found in the other Rana (Table 1) except the presence of secondary constrictions on the chromosomes.

3.2 Inter populations karyological comparison

An effort was made to compare the karyometrical data from three major populations of R. limnocharis so as to express more precisely the degree of similarity and dissimilarity. Each chromosome pair is compared on the point whether the differences in mean value of relative length and arm ratio is statistically significant or not (Table 4,5). When the values of mean ± 2 SE overlap each other the difference is not regarded as statistically significant. Therefore, in our comparison of karyometrical values from Jowai, Shillong and Tura populations following symbols are used (> < =) e.g., T>S = J<T (Table 5) which indicates that the difference between Shillong and Jowai is minimum, but the value of Tura is greater to both Shillong and Jowai. For the sake of visual comparison, schematic representations of three idiograms and tables have been shown (Table 4, Fig. 1).

The significant differences obtained in the Karyometrical survey from different populations of the species are in general on the arm ratio (r) or the relative chromosomal length (r.l.). The differences are not in the magnitude of being interpreted in terms of sub-specificity.

3.3 Meiotic Studies

In the meiotic stages a consistent 13 bivalents are observed (Plate 4). A large number of diplotene, diakinesis and metaphase I nuclei are analysed to study the chiasma and the subsequent terminalisation.

Diplotene shows maximum elongation of chromosome (Plate 4) and the greatest number of chiasmata (Table 3). The highest number of chiasmata per chromosome is 2 and the minimum 1. Crossing over manifestations have been studied from the number of chiasmata. In the Diakinesis plates the chromosome appear comparatively condensed and chiasma shows tendency towards terminalisation (Plate 4). The metaphase I chmosomes are slender but condensed (Plate 4).

The mean frequency of chiasma per nucleus between diplotene, diakinesis and metaphase I do not reveal much variations. Between Diplotene and metaphase I most of the chiasmata gets terminalized.

No heteromorphic structures were observed and all the chromosome pairs form bivalents. The first meiotic division is reductional (Plate 4) and the heploid number (=n) is 13. The centromeric position of all the chromosomes are clear.

The spermatocytic prophase nuclei have a well-defined structure (Plate 4). The chromatin strands can be seen

clearly throughout the stages of prophase. the morphological detaiul of the chromosomes in the second meiotic division is also very clear.

3.4 NOR staining

Table 9 shows karyotypes of the investigated Rana species after silver-staining of the chromosomes.

In all these frogs NORs are located in the long arms of the hamologues of the 10th chromosome pairs (Plate 5). Apart from the NOR's other silver positive sites are also observed. In no case there was any variation observed in the position of the NORs between the populations.

No secondary constrictions in the chromosomes have been observed either after silver-staining removing the silver and staining the same preparations with Giemsa.

Table 2. Chromosome countings at metaphase.

Sex	Frequency of Chromosome Counts (2n)				Number of metaphases analysed
	24	25	26	27	
Male	16	08	198	04	126
Female	09	07	92	08	116

Table 3. Karyomorphological studies of the chromosome compliment of R. limnocharis

Chromosome pair	Length of short arm (μ)	Length of long arm (μ)	Total length (μ)	Relative length (%)	Arm ratio (r)	Centromeric indices (ci)	Chromosome type (t)
1	7.0	7.0	14.0	14.61	1.00	100	m
2	4.0	8.5	12.5	13.03	2.12	47.05	sm
3	5.5	5.5	11.0	11.47	1.00	100	m
4	4.5	6.5	11.0	11.47	1.44	69.23	m
5	4.8	5.2	10.0	10.42	1.08	92.31	m
6	2.8	3.2	6.0	6.25	1.14	87.56	m
7	2.0	3.5	5.5	5.73	1.75	57.14	sm
8	2.6	2.6	5.2	5.42	1.00	100	m
9	1.6	3.6	5.0	5.21	2.25	44.44	sm
10	2.5	2.5	5.0	5.21	1.00	100	m
11	1.5	2.7	4.2	4.37	1.81	55.55	sm
12	1.8	1.8	3.6	3.75	1.00	100	m
13	1.5	1.5	3.0	3.12	1.00	100	m

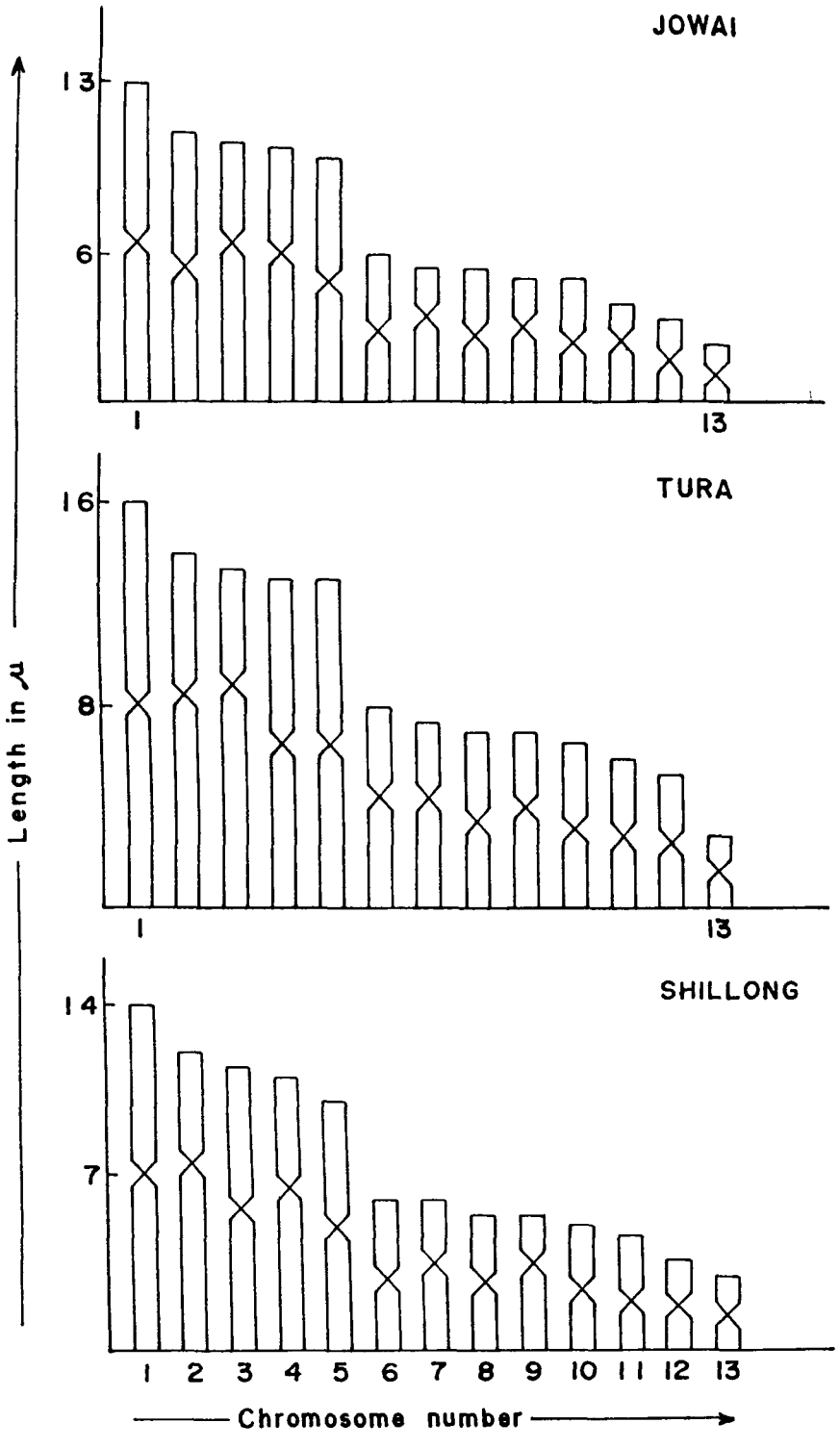

Fig.1. Idiograms of different populations of Rana limnocharis

Table 4. Quantitative characteristic of the population of R. limnocharis.

Population		Pair number												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Jowai	r.l.*	14.30	12.10	11.66	11.88	11.0	6.60	5.83	5.83	5.50	5.28	5.06	3.63	2.53
	A.r.*	1.0	1.0	1.76	1.36	1.0	1.14	1.94	1.12	1.71	1.0	2.06	1.21	1.42
Tura	r.l.	13.46	11.78	11.11	10.94	10.94	6.73	6.39	5.9	5.9	5.21	5.05	4.04	2.52
	A.r.	1.0	1.54	2.14	1.0	1.0	1.28	1.71	1.0	1.74	1.06	1.0	1.0	1.0
Shillong	r.l.	14.61	13.03	11.47	11.47	10.42	6.25	5.73	5.42	5.21	5.21	4.37	3.75	3.12
	A.r.	1.0	2.12	1.0	1.44	1.08	1.14	1.75	1.0	2.25	1.0	1.8	1.0	1.0

*r.l. = % realtive length; A.r. = arm ratio.

Table 5. Comparison of chromosome pairs of three population of R. limnocharis.

Chromosome pair	Relative length	Arm ratio
1	T < S = J > T	T = S ≠ J ≠ T
2	T < S = J > T	T > S = J < T
3	T < S = J > T	T > S < J < T
4	T > S = J < T	T < S = J > T
5	T > S = J = T	T = S = J = T
6	T > S = J < T	T = S = J = T
7	T > S = J < T	T > S < J = T
8	T = S = J = T	T = S = J > T
9	T > S = J < T	T < S > J = T
10	T = S + J + T	T = S = J = T
11	T > S = J < T	T = S < J > T
12	T = S = J > T	T = S = T = T
13	T = S = J > T	T = S = J > T

S = Shillong; J = Jowai; and T = Tura.

Table 6. Interpopulation differences in total chromosomal length of R. limnocharis.

Populations	Total length ± 2SE	Range
Jowai	90.9 ± 0.80	92.4 - 88.5
Tura	118.8 ± 0.66	120.2 - 116.8
Shillong	96.1 ± 0.70	98.3 - 95.2

Table 7. Chiasma frequency and terminalisation coefficient.

No. of Nuclei studied	Stages of meiosis	Total chiasmata (m X)	Total terminalised chiasmata (TmX)	Chiasma Frequency per nucleus	Terminalisation coefficient
62	Diplotene	1302	640	21.0	0.491
62	Diakinesis	906	807	14.6	0.890
62	Metaphase I	1416	748	22.8	0.528

4. DISCUSSIONS

4.1 Karyotypic status of Rana

Forty two species of the genus Rana (including the present work) have been, so far, extended to chromosomal investigations, all having 26 chromosomes except R. namiyei whose diploid complement is reduced to 22. (Nishioka, 1972; Kuramoto, 1972; Haertel et al., 1974; Singh, 1974; Ward, 1977; Morescalchi, 1979; Table 1). Investigation on the six Rana endemic to south east Asia has also revealed similar diploid number (Kuromoto, 1982). Our frog, Rana limnocharis, confirms similar pattern of chromosome numbers. Many of the earlier results reassert the hypothesis according to which the 'higher' families of Anurans have a basic karyotype of 26 biarmed chromosomes (Bogart, 1973; Morescalchi, 1973, 1979). This facts attests to the attainment of an analogous level of karyological differentiation by several authors of this order, and may be to their common origin as well; nevertheless, it also provides additional evidence of the large number of parallelismes which also at the karyological level have characterized the evolution of the genus Rana.

The 26 chromosomes of Rana limnocharis can be grouped under two series : 5 large and 8 smaller pairs (Plate 1,2,3). All the large chromosomes (pairs 1-5) are metacentric while the smaller ones (pairs 6-13) are meta- or sub metacentric. In most of the cells the larger chromosomes are morphologically

distinguishable, the smaller ones, except for the conspicuously submetacentric pair, are too similar to be identified and paire with certainity.

The data obtained from Rana limnocharis are consistent with the well known karyological homogeneity among Asiatic Rana (Herrero, 1982), of which all those studied have 26 biarmed chromosomes of regularly decreasing length with a discontinuity in size between the 8th and the 9th pair and their formula differ very little even at the intergeneic level. The diploid number of this frog is also in agreement with the earlier number given by Sato (1934) but we differ in the subdivision of the five pairs of large chromosomes into two groups of different lengths. We are in agreement with Kuramoto's (1970) grouping of chromosomes in 5 large and 8 small.

On comparing the chromosome morphology of Rana limnocharis with other amphibians, one is struck by an apparent uniformity of chromosome number, relative chromosome size, and centromeric position (Morscalchi, 1973; Mancino et al. 1977). This feature has been responsible for the widely expressed atitude that Ranas are chromosomally conservative. Wilson et al. (1974) have gone as far as to argue that amphibians have approximately twenty times fewer chromosomal rearrangements than mammalian species. Bogart and Nelson (1976) have argued against this generalisation as they

have encountered variability in the genus Rana and their analysis shows that there appears to be two major lineage of Rana which are chromosomally discrete. One line reffereable toa 26- chromosome ancestral karyotype and the other line related to a 24- chromosome ancestral type. Surveying the anuran chromosome members (Kuramoto, 1985), it is evident that the number tends to be reduced from generalized to derived forms, as well as from primitive to advanced groups. The 26- chromosome lineage is considered to be of greatest antiquity as karyotypic similarities have been used to link most of the higher families of frogs which have a putative ancestral 26- chromosome karyotype (Bogart, 1970, 1973; Morescalchi, 1973). Since R. limnocharis has this basic karyotype, it is highly probable that it belongs to the primitive stock in this genus (also see sec. 4.1.3).

It is true that many species of amphibians are chromosomally conservative in terms of lacking of gross chromosome change. If the karyotypic endpoint in an evolutionary series consists of completely biarmed chromosomes, than the probability that new chromosomal structural changes translocation, fission/fusion, inversions, became fixed in a given population is lower than in undifferentiated karyotypes, where many acro- and telocentric chromosomes still occur (Macgregor, 1982). Although, we have not encountered any gross chromosomal changes but variations in karyo-

metrical data have been obtained for R. limnocharis from various geographically separated populations (Table 4,5,6). These variations are not enough to declare the variant groups as different species or subspecies. It has also been found that the first and second chromosome pairs in a few individuals is metacentric and submetacentric in respect to standard karyotype. This can be due to two reasons - (i) a translocation of a segment from the terminal end of chromosome one to chromosome two, (ii) pericentric inversion may also be the causative factor in bringing about a change in the centromeric position and subsequently the arm ratio and type of the chromosome. Kuramoto (1970) had reported the presence of two subspecies of R. limnocharis based on such chromosomal variations. We do not consider our data to be sufficient enough for subdividing into two subspecies but asa matter of understanding we consider the variations are due to the geographical location of the populations (see sec. 4.1.2.). Experimental hybridisation between the two subspecies have produced viable and fertile tadpoles rejecting the theory of subspecificity (Kuramoto, 1983). The NF values was 52 in all the cases which is in agreement with the generalised NF values of Rana.

No secondary constrictions were evident on any of the larger or smaller chromosomes of this frog. Thus the generalisation that secondary constrictions in the Rana

is characteristic does not hold true for atleast this species. Schmid (1978) had emphasized that all Rana species (excepting R. arvalis and R. dalmatina) have a large secondary constriction in a near-centric position of the long arm of chromosome pair 10. It is, therefore, evident that this Rana limnocharis becomes, todate, the third frog in lacking the characteristic secondary constrictions.

4.1.1 Karyotypic interrelationship

As emphasised earlier, species of Rana endemic to the south east Asia have apparently originated and derived from Taiwan, erstwhile Formosa (Inger, 1950). It is obvious that R. limnocharis must have differentiated quite recently before entering the Indian territory. Kuramoto (1972) had made a preliminary study through karyotypic characteristic to understand the phylogenetic relationship in six species of Rana endemic to south east Asia. They are R. okinavane, R. narina, R. ishikawae, R. subaspera, R. holsti and R. hamiyei. On the comparison with the chromosome compliment and the data obtained by the 't' test in our frog (Table 3,4), it is obvious that R. limnocharis differ from other members as can be observed from Table 7.

Comparison of the karyotypes indicates that the large chromosomes were similar in size and shape but the small chromosomes differed significantly. 4 pairs agreed

Table 8. Number of chromosome pairs that agree both relative length and arm ratio (above diagonal) and those that differ in both (lower diagonal).

	R. narina	R. ishikawae	R. subaspera	R. holsti	R. okinavana	R. limnocharis	R. namyei
R. narina		7	3	5	4	4	3
R. ishikawae	0		8	8	4	6	4
R. subaspera	2	0		9	6	4	2
R. holsti	1	1	0		5	6	3
R. okinavana	2	1	3	1		3	2
R. limnocharis	2	2	2	1	1		5
R. namyei	5	6	8	4	5	6	

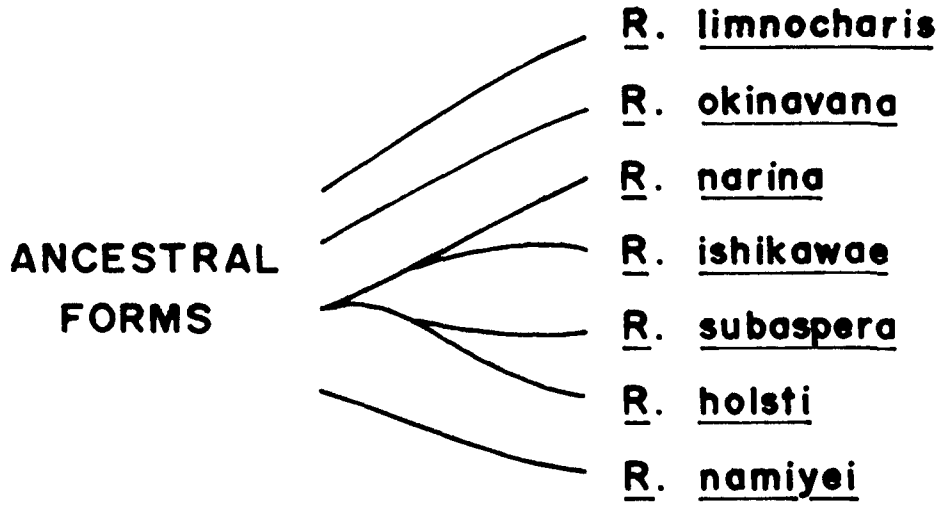

Fig.2 Hypothetical phylogeny of R. limnocharis on Karyotypic similarity with six Rana species endemic to south east Asia.

both in relative length and in arm ratio with R. narina and R. sub spera: 6 pairs resembled between R. ishikawae, R. holstei and R. namyei: and only 3 pairs agreed with R. okinavana. The remarkable similarity between R. narina, R. ishikawae, R. subaspera and R. holstei is because they have differentiated locally in Japan as a result of long geographical isolation (Kuramoto, 1972). R. limnocharis showed some resemblance to R. ishikawae and R. holsti may be because of the common origin from the continental China. R. namiyei could not be related with limnocharis as it has 2n = 22 suggesting that it is the most diverged stock in the genus and may well be separated from the other Rana by giving it a subgeneric rank (Kuramoto, 1972).

R. okivavana stands apart from the other species although our data indicates resemblance of 3 pairs while only one pair differed both in arm ratio and relative lengths. These data indicates that R. limnocharis in mere close to R. okinavana than to any other species. One can, therefore, assure and understand that these two species must have derived from the common stock and then had differentiated independently in terms of karyotypic (Fig. 2) relationship.

Further detailed studies on all aspects of cytogenetics is required to understand the phylogenetic origin and differentiation of this frog.

4.1.2 Intra-populations karyotypical comparison

Many authors have revealed introspecific differences in the karyotypes of anuran amphibians (Wright, 1978; Gale, 1980; Falconer, 1981). It is expected, therefore, that the populations of intermediate phase in speciation, subspecies or geographically isolated populations, differ from one another to some degree in their karyotypes. Kuramoto (1970) had made a karyotypic comparison of Rana limnocharis from Fakuoka and Amami populations of Japan. These two populations of this species differed considerably in chromosomal length and to a lesser extent in arm ratio of the chromosomes as well as in the position of the secondary constrictions. Considering this as the first step of karyotypic differentiation they had devided the two populations into two subspecies - R. limnocharis limnocharis and R. limnocharis vitigera. With this background the present study was carried to detect such variations between the geographically widely seperated populations of R. limnocharis in Meghalaya.

Although the data obtained are from rather a limited populations (Table 4 and 5), it is apparent that karyotypes differ between populations. In our case the differences obtained in the karyometrical survey are also on the relative chromosomal length and the arm ratio of the chromosome. No secondary constrictions were observed. In relative length,

only four pairs (Nos. 3,6,10 and 11) are similar in length. The populations from Shillong and Jowai do not show much variations but the populations from Tura has value greater than the other two (Table 4) and can be indicated by the symbol T>S = J<T (Table 5). It is noticed that the total length of the chromosome of the Tura group of Rana are larger than the other two populations while between Shillong and Jowai species the TCL do not significantly differ (table 6).

Differences in the arm ratio are smaller than the relative length. Chromosome pairs of Tura again differs from both the other populations. Three pairs (4, 9 and 11) have values greater than the Tura groups while two pairs (2 and 7) differ either between Jowai and Tura or between Shillong and Tura. No. 10 is the only pair which does not differ in both chromosomal length and arm ratio in all the three populations (Table 4,5). These differences observed between the Karyotypes of Tura species with the other populations could be regarded as 'Cytologically diagnostic' feature in terms variations but lack of reproductive isolation between the populations (Roy, personal communication, 1986) removes all doubts on its specificity. This a matter of course, since, according to the biological species concept (Dobzhansky, et al., 1977; Mayr, 1963), two populations are rarded to belong to two different species

when the hybrids between them are inviable. No genetic incompatibility was observed in our populations. Therefore, we are in no position to disagree/agree with Kuramoto (1970) designation of subspecificity. Nevertheless, these variation for our understanding is considered as some sign of geographic speciation if viewd from a cytlogical point. Some morphological variations have also been observed among the populations of limnocharis (see appendix). It can be concluded, therefore, that the populations of R. limnocharis from the geogrpahically spread Shillong, Jowai and Tura may be differentiating marginally both morphologically and chromosomally but the degree of genetic divergence has not reached the point where each population is blocked to exchange respective genome with one another by hybrid inviability.

4.2 Sex determining mechanism

The problems of sex-chromosome in amphibians in recent years have become of great interest. A diversity of opinion exists among the investigators. Heteromorphic sex chromosome have been demonstrated in only five species of the anura, to date (Schmid et al., 1982). In majority of the species, the sex chromosomes are still in an initial stage of morphological differentiation and cannot be distinguished micropically. It has not been possible to generalize heteromorphism even with the new improved techniques of chromosome banding (Singh, 1974; Schmid, 1980; King, 1987).

The history of the sex determining controversy can be traced from Witschi (1929) who claimed for the first time that the males of some north American toads were heterogenetic. This observation was challenged by Saez, Rojas and De Robertis (1936) as well as by Wickbom (1945). White (1954) found similarities in the sex determining mechanism in amphibians to those of the fish Xiphophorus. Galien (1956) established the existence of female heterogenety in Xenopus leaevis by sex reversal experiments where a small acrocentevic. X and a large metacentric Y were demonstrated in female metaphases by Wieler and Ohno (1962) whose results have been confirmed by Morescalchi (1963). Manna and Bhunya (1966) found female heterogenety in Bufo melanostictus and they also commented on the failure of detecting the same phenomena by other workers by the use of orthodox cytological techniques on the germinal tissues. Robello (1970) stated, "when both sexes are available, male and female presented identical karyotype in Ranidae". Stephenson and Stephenson (1970) did not find any proven case of chromosomal heteromorphism among the cytologically known species of the genus Hyla. Brum-zorilla and Saez (1971) casted doubts on the reports on heteromorphism, they concluded that the presumed sex chromosomes found in different species of amphibia were only bivalents that had different behaviour because the bivalent elements are the only chromosomes

which can have different shapes and sizes. Raychoudhury and Singh (1975) have generalized for the whole group of amphibians and stated, "there is no unequivocal evidence of heteromorphic pair which can be designated as sex chromosomes". Kawamura (1977) found male heterogenty by sex reversal experiments cytochemical bandings were tried effectively in some toads (Session, 1980; Schmid, 1980; Chakravarty et al., 1983).

Our observation with Rana limnocharis indicate the lack of heteromorphic sex chromosomes which is in accordance with the earlier reports of Sato (1934) and Kuramoto (1970). When both sexes were available both male and female presented identical karyotypes cytochemical bandings studies were also found uneffective.

The lack of morphological differentiation at the chromosomal level does not imply, however, an absence of gentical digamety. Sex determination in our frog could be the task of one pair of chromosomes still morphologically indifferentiated, status found in Boidae (Serpentes) by Becak (1968). An accumulation of heteromorphic sex determining genes could be occuring in one chromosome by decreasing the rate of crossing over in a given pair, the morphological differentiation of this chromosome having not yet begun.

Another hypothesis can be suggested is the sex in this frog is determined by one or several pairs of male and female determining genes distributed among several chromosomes.

4.3 Meiotic studies

Large, distinguishable somatic chromosomes and analyzable meiotic stages are features which contributed to the cytological usefulness of an organism, this seems true specially as regards the male line where these processes can be studied with greater care because of the abundance of spermotocytes (John and Levis, 1965). The meiotic study in R. limnocharis indicates orthodox type (Plate 4), beginning with short diplotene to a long diakinesis. The general clarity of the early meiotic prophase stages is quite remarkable. Bivalents appear highly fuzzy (spiralized) with one to two chiasmata in both large and smaller bivalents. However, most of the bivalents assume the characteristic ring like appearance in Metaphase I. This is the genral pattern found in Ranids and Rhacophorids. Some Ranids like R. adspera frequently reveals diakinetic bivalents provided with pro-terminal chiasmata. The present study shows 13 perfectly paired bivalents which also indicates that there is no heteromorphic sex chromosome pair. The lack of heteromorphic ompliments in the chromosomes in meiosis is consis-

tent with the concept of homogenety in amphibians (see sec. 4.2).

A good amount of chiasma movement to terminalisation is observed from Diakinesis to Metaphase I. The chromosome outlines becomes smooth and clear due to continued condensation. In no case, however, has a secondary constriction near the distal end of the long arm, a structure so clearly seen in the meiotic bivalent in Rana been noted at any stage.

During the different substage of prophase I all the chromosomes exhibit uniform stainability in other words no heteropycnotic behavior was shown by any chromosome. The generalised presence of bivalent with terminal chiasmata in the spermatocyte meiosis of the Anura suggests that the aquision of this type of meiosis may have had a certain importance in the evolution of this order.

4.4 Nucleolus Organizing Regions

In most of the Rana species the NORs are located in th long arms of the homologues of the 10th chromosome pair (Table 8). Only in the African species of Pyxicephalus which are closely related to Rana, NOR's are located in the 6th or 13th pairs (Schmid, 1980). This presence of additional NORs in the arms of a homologue of the heteromorphic pair no. 8 was also reported by Schmid (1980). It

Table 9. The localization of Nucleolar organizer regions in Karyotypes of Rana.

Species	Origin	2n	Position of Ag-NOR*	References
Rana esculenta	Central Europe	26	10	Schmid, 1978; Birstein, 1981.
R. graeca	Western Europe	26	10	Vitelli et al., 1982.
R. latestei	Western Europe	26	10	Birstein, 1984
R. lessonae	Central Europe	26	10	Birstein, 1981.
R. ridibunda	Central Europe	26	10	Schmid, 1978; Belcheva et al., 1981; Birstein, 1981.
R. temporaria	Central Eurape	26	10	Schmid, 1978; Birstein, 1981.
R. macrocnemis	The Caucusus	26	10	Birstein, 1984.
R. erythrea	south east Asia	26	10 (6,8,11,13)	Schmid, 1978; 1980.
R. limnocharis	south east Asia	26	10 (1,2,5,12)	Present work.
R. nigromaculata	Central Asia	26	10	Birstein, 1984.
R. chensinensis	Far East	24	10	Birstein, 1984.
R. macrodon	South east Asia	24	10	Schmid, 1980.
R. blairi	North America	26	10	Ward, 1977.
R. catesbiana	North America	26	10 (8,12)	Schmid, 1978; Ward, 1977.
R. palustris	North America	26	10 (6,7,12,13)	Schmid, 1978, 1980.
R. magnaocularis	North America	26	10	Schmid, 1978, 1980.
R. sphenocephala	North America	26	10 (9,13)	Schmid, 1978.
Pyxicephalus adspersus	South Africa	26	6 (8)	Schmid, 1978.
P. delanlandii	South Africa	26	13	Schmid, 1980.

*Number in the parenthesis represent additional NORs sites on the chromosome compliment.

seems possible that this change in the karyotype, in comparison to Rana, is probable due to some chromosome arrangements.

In our frog, Rana limnocharis the NOR's are on the 10th pair (sometimes on the 11th). As these chromosomes are submetacentric the NOR lies somewhere in the end of the long arm (Plate 5). Although we consistently found only one pair of chromosomes with Ag - NORs, more than two nucleoli were observed frequently in the interphase. The latter exceptions are attributed probable to the incidental occurrance of polyploid cells in the diploid species. However, the existence of a second nucleolus organizing chromosome pair cannot be excluded with absolute certainity because additional sites were found to be positively stained with silver. Similar results were observed by Schmid (1978, 1980). It is not yet clear if rDNA is localized in these additional sites, or if these silver positive regions are the sites of 5S DNA and other genes, as in the case of triturus vulgaris (Nardi et al., 1978).

Unfortunately, the silver (Ag-As) method has not beenused in many Rana species. Table 9 and our data point out the fast that the localization of NOR in Rana, in general, is very conservative. Apparently less conservativeness is found in Bufo (Schmid, 1978; Beck and Mohan, 1979; Bürstein, 1981) and this conservativeness may be the characteristics

of only some of the higher anurans because even in the karyotypes of closely related species of Urodeles, the NORs are located in different chromosome pairs (Mancino et al., 1977; Macgreger and Sherwood, 1979).

The presence and position of secondary constrictions which are potentially the nucleolus organizer regions (NORs, the sites of 18S + 28S rDNA) are important characteristics of karyo ystematics. Schmid (1982) and Birstein (1984) have detected and localized NOR in the 10th pair to the secondary constrictions. Some authors have found in other pairs and that is why there are different numbers assigned to the chromosome having secondary constrictions (Kuramoto, 1973). We have found no secondary constrictions in our frog. Both with or without silver staining. Ivanov and Madyanov (1973) had also reported the absence of secondary constrictions in some elected Rana species. Therefore, the general characteristic of Rana does not hold true for our frog.

Size differences between the homologous silver stained NORs are frequently encountered in amphibians (Ward, 1977; Ruiz et al., 1981; Schmid, 1982). Such unequal - sizes were particularly noted in fishes (Foresti et al., 1981) and mammals, mainly man (Goodpasture and Bloom, 1975; Miller et al., 1977; Varley, 1977). These NOR - heteromor-

phisms are simple size differences between the Ag-stained blocks; no tandem duplication or triplication of the NOR was ever found in these studies. In our study with R. limnocharis about 40% of the animal with NOR - heteromorphisms had these simple size differences. Since these have been observed in both mitotic and meiotic I metaphase, it must be constitutional, intra individual characteristic of the NORs.

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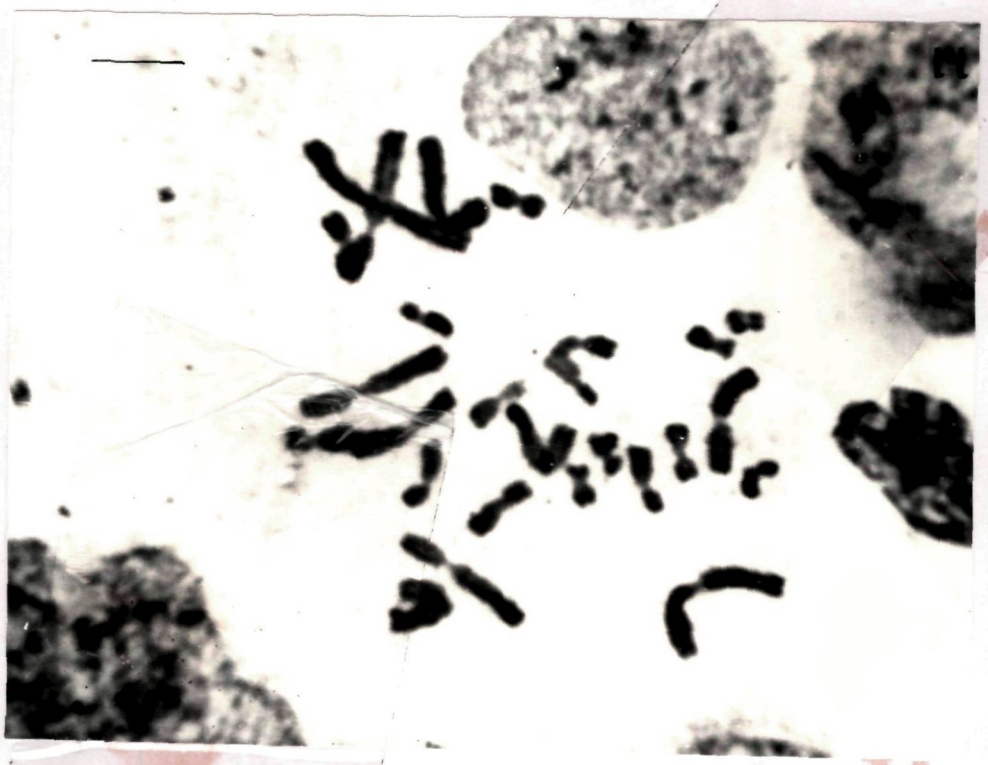
PLATE 1 Metaphase spread from two populations of Rana limnocharis (Bar 10μ)

1. Shillong
2. Jowai



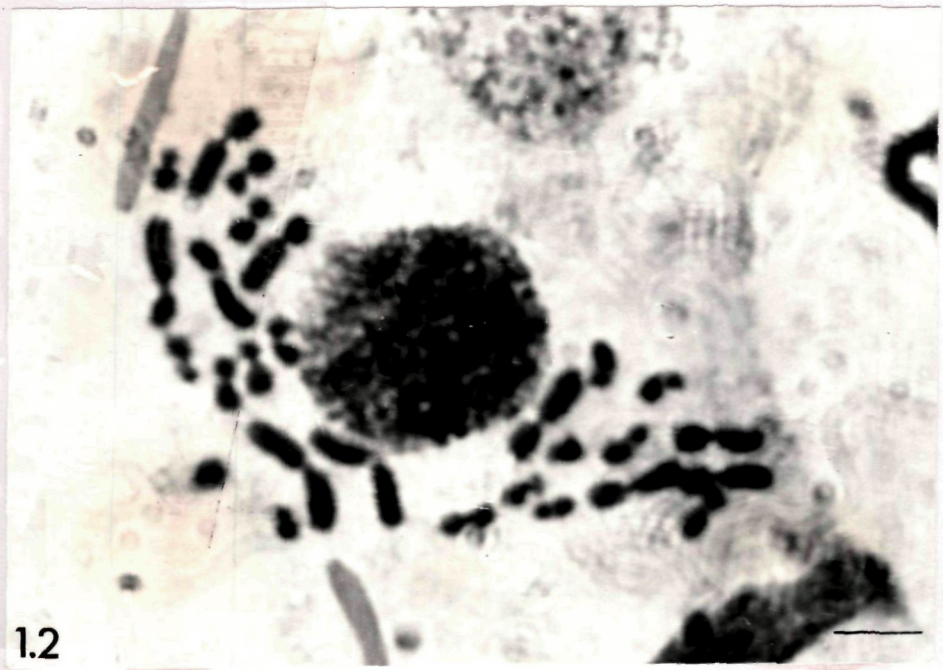


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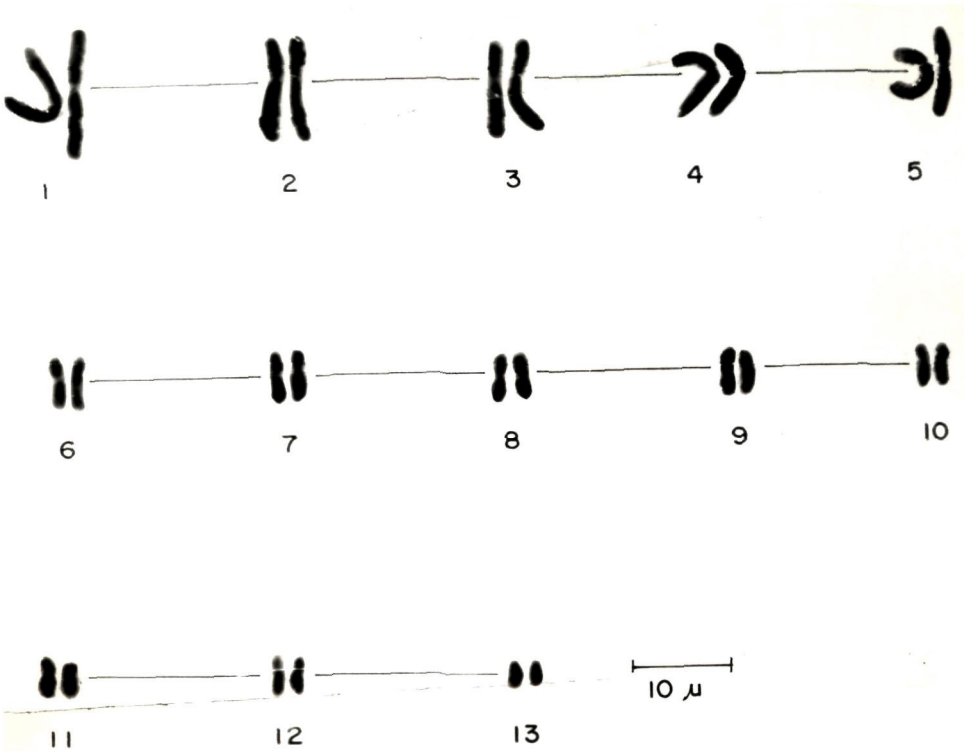
PLATE 2 Karotypes of two populations of R. limnocharis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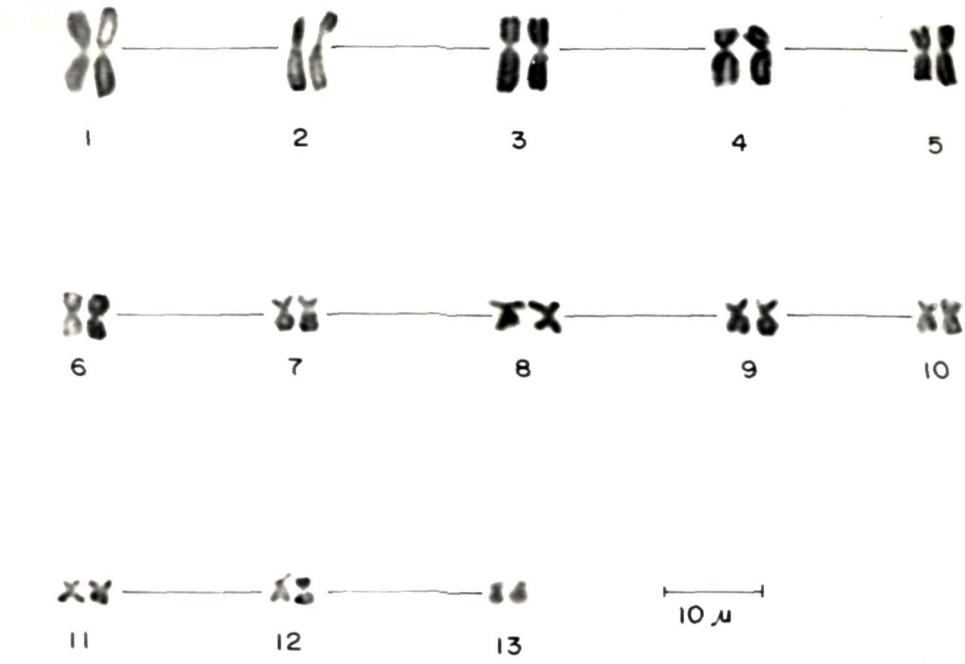
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PLATE 3 **Metaphase spread of R. limnocharis** and **Karyotypes** (Bar 10μ)

3

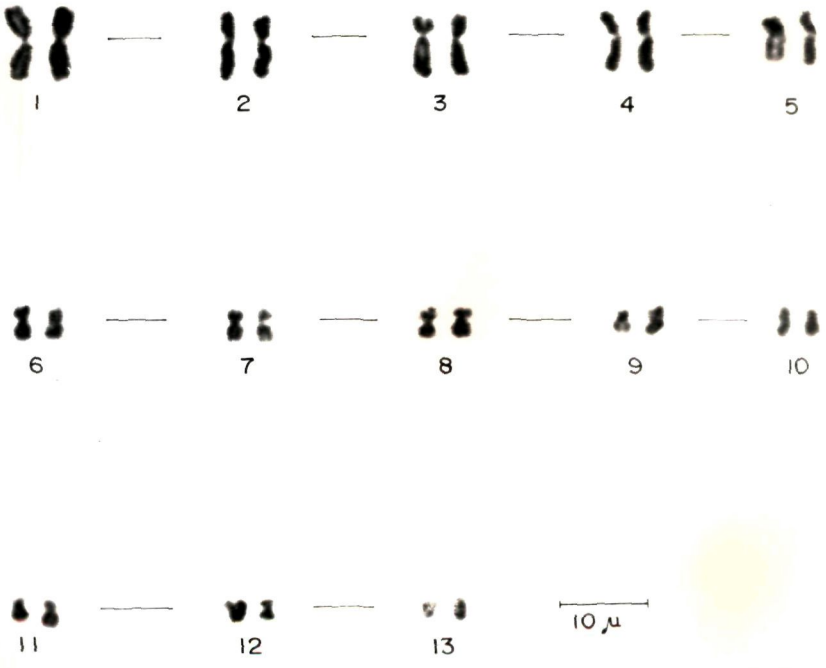

PLATE 5 Nucleolus Organizer Regions
(Bar 10μ)

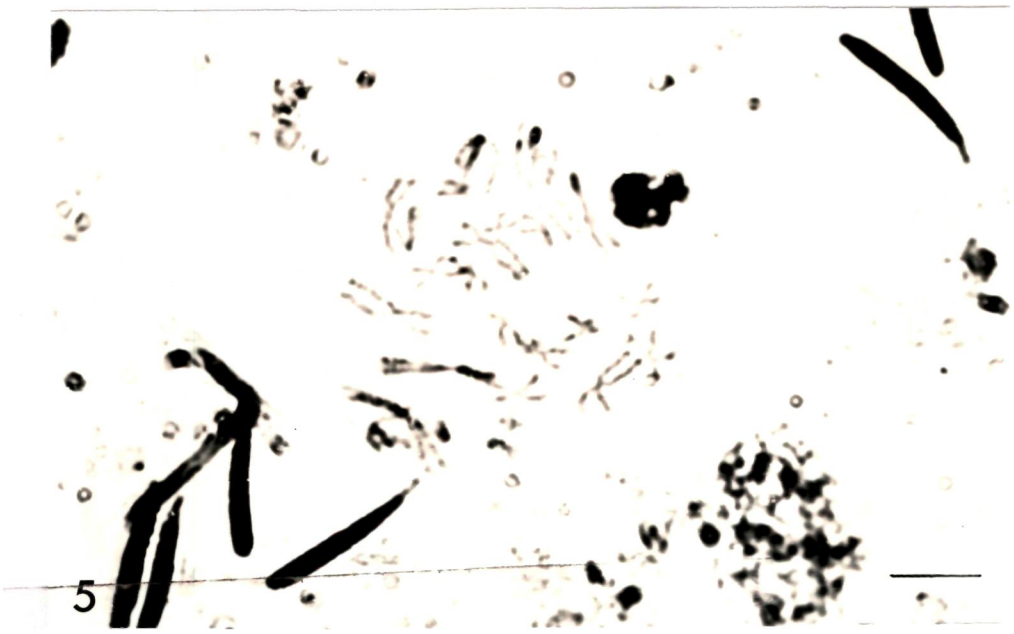
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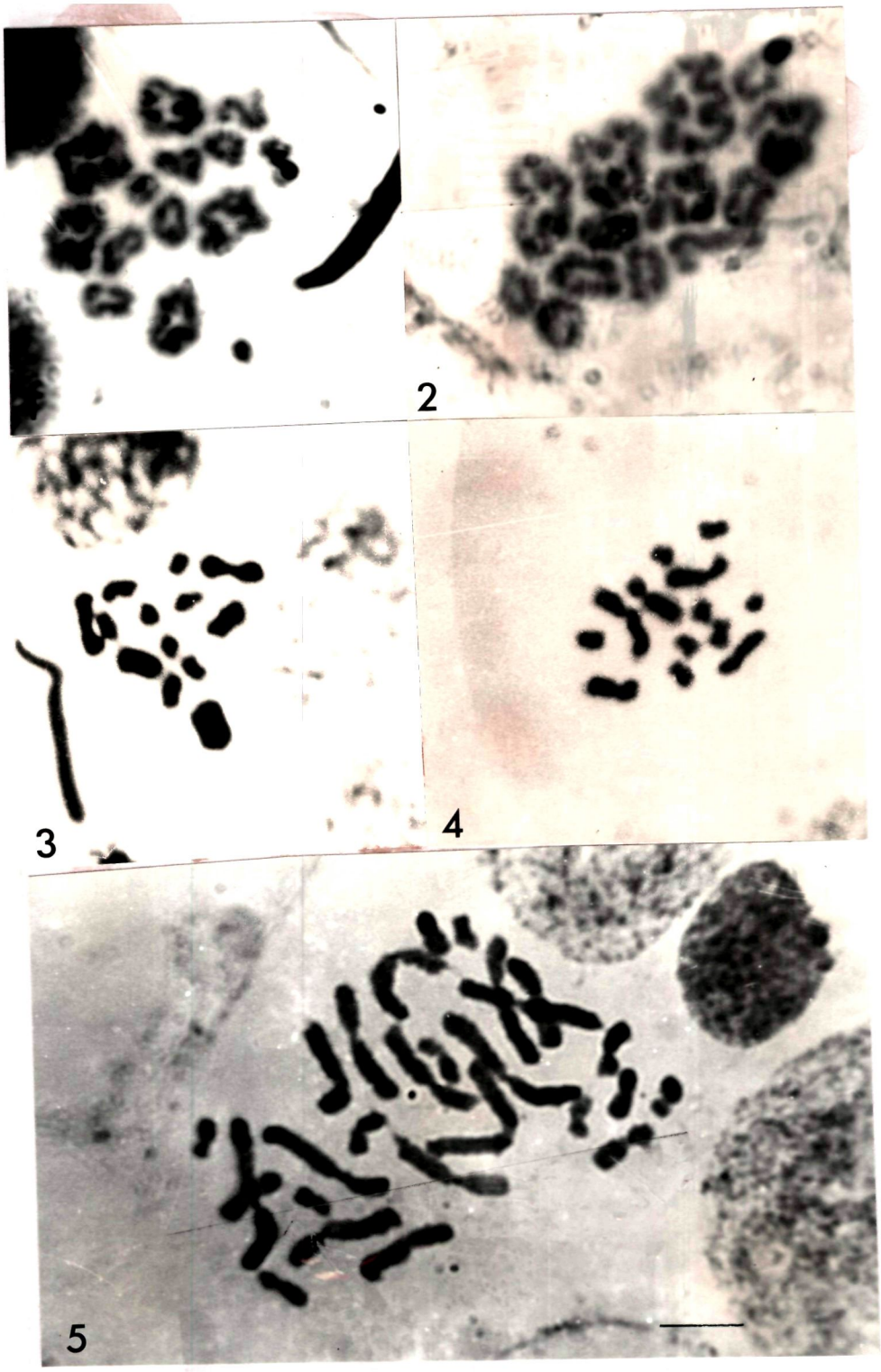
PLATE 4 Meiotic studies

(Bar 10μ)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SECTION - C

ONCLUDING REMARKS

The usefulness of an enzyme system as a genetic marker in differentiating between geographically separated populations is determined by its variability, i.e., displaying either the presence of allozyme or difference in the number of bands on the gels. If the enzyme does not show variability, then its isozyme pattern is of little use for comparison among populations. The biochemical techniques of polyacrylamide gel electrophoresis coupled with specific enzyme staining procedures become an effective tool for such analysis. Genetic variation is the basic premise upon which our study has been based.

The whole project has been accomplished in two phases. In phase I six tissues of Rana limnocharis from nine different localities have been electrophoretically surveyed to provide data necessary to make an appropriate selection as to which tissue and enzymes should be analysed for genetic differentiation. In phase II the genetic variation has been studied with emphasis on the tissue and the enzymes indicated from phase I data.

It appears on comparison from all the populations that this frog like most vertebrates possesses a simple pattern of five LDH isozymes. Normal polarity has been observed in the two tetramers A_4 and B_4. Tissue specific LDH isozyme expression is observed due to the inconsistency of LDH B_4, in contrast to the relatively constant A_4.

The tissue MDH isozyme specificity has been placed under two groups - one having the single band and the other with the three banded phenotype. The two loci also express differential activity.

Maximum activity of the ADH isozyme has been observed in the liver tissue while the other tissues failed to show significant activity. The greatest number of ADH isozymes bands resolved is four. This frog carries three separate loci coding for ADH but the product of only two can from heterodimers. The third locus is monomorphic with no identification of a heterozygous combination between the other two ADH loci.

Two different forms of G6PD isozymes in the liver tissue, called A and B has been confirmed. No heterozygous combination is observed between these two forms, thus leaving some doubt on their genetic compatibility.

The preliminary findings from phase I indicated that the above enzymes in the liver tissue by large warranted extensive investigation because of their more variable phenotypes across the whole population range of Rana limnocharis in Meghalaya.

Under phase II the allozymic variation encoded by nine loci from nine widely separated localities ranging across the whole of Meghalaya has been analysed. These

different populations occupy increasingly varrying and unpredictable environments.

The average number of alleles is low in comparison to the other vertebrates but the mean number of polymorphic loci and heterozygosity is comparable to some of the vertebrates. Low values of alleles may be the characteristic of such populations due to specific environmental conditions. The high levels of polymorphism and heterozygosity are typical for this species when compared with other amphibians.

The expected Hardy-Weinberg proportions have been observed in majority of the populations with each locus.

The values have been fairly correlated with the environmental - variability model. The genetic variations increased in a definite order, showing that the frogs living in a relatively constant and predictable environment are less genetically variant in contrast to the populations which have distinct spatial and temporal variations. Ecologically two similar populations have the commonest factor that they receive the highest rainfall than any part of the world. But the electrophoretic results do not fit the predicted similarity value. The frog from one population showed similarity to a ecologically different population. We could not explain this phenomenon on the basis of existing data. Another samples from a population also unequivocally

stands out in many respects. However, it is concluded that protein polymorphism is adaptively important and is maintained in the species by natural selection.

Roger's coefficient of genic similarity and Nei's genetic distance formulae were utilized to detect genetic relatedness between the nine populations. The average genetic distance value was 0.11 ± 0.04. The similarity index averaged to 0.96 ± 0.23. The values obtained are difficult to interpret because the approximate or standard values for amphibian species are not available. Our average D value fall approximately in the range of the value obtained in a salamandar species. This could possibly be the confirmation of a standard value in amphibia.

Some efforts have been made in establishing the evolutionary link of this frog in this region with the data available on other Rana species from the south-east Asianountries. Rana limnocharis has been identified to be Oriental in origin, while most of other frogs are Palearctic. Its place of origin in south-east Asia have been fairly established to be Taiwan (Kuramoto, 1985, personal communication). From Taiwan the migration path of Rana limnocharis could be traced easily to the Meghalayan populations. Thks must have been possible only due to the land and water connections which were established later.

These data draw out two general conclusions. Firstly it is evident that our prediction becomes less useful when our knowledge of the ecology and population structure of the frog species is not enough. This is partly because some factors remain unknown in a qualitative sense and partly because some measures of the relative intensity of various factors may be too crude to allow an estimate of net effect. Secondly, the 'prediction' approach, we have taken to explore the relationship between genetic variation and the ecology of the species has an important inherent difficulty. We constantly run the risk of making the correct prediction for wrong reason(s).

The overall karyology is consistent with that of the earlier report and also with the well known homogeneity among the Asiatic Rana. The present report can be considered a satisfactgory confirmation of the conclusion shared by several cytogeneticists that the karyotype of Rana are conservative. This fact attests to the attainment of the analogous level of karyological differentiation by several families of this order, and may be to their common origin as well; nevertheless, it also provides additional evidence of the large number of parallelism which also at the karyological level have characterized the evolution of Rana.

The sex determining mechanism of amphibia in general

is still a controversial issue. Our observation indicate the lack of heteromorphic sex chromosomes and when both the male and female were available the karyotype was identical. Sex determination in this frog could be the task of one pair of chromosomes still morphologically indifferentiated. An accumulation of heteromorphic sex determining genes could be occuring in one chromosome by decreasing the rate of crossing over in a given pair, the morphological differentiation of this chromosome having not begun. It can also be suggested that the sex in this frog is determined by one or several pairs of male and female determining genes distributed among several chromosomes.

The Nucleolus Organizing Regions (NORs) are located in the homologues of the 10th pair of chromosomes as is true in other Rana. This again points out the fact that the localizationf the NOR in Rana karyotype is also very conservative. Additional NOR sites have also been observed in the chromosomes as well as in the nucloli. Secondary constrictions are acclaimed to be the characteristic feature of the Rana chromosomes but we have not observed secondary constrictions in the karyotypes, which makes this frog to be the third in the list of cytogenetically investigated Rana to lack secondary constrictions.

An overview of the karyological studies shows the limnocharis to possess all the characteristics of Rana except

the secondary constrictions on the chromosomes.

Comparative studies made between the other Rana endemic to this region and the Rana limnocharis shows a low similarity value. This could be due to high level of differentiation in this frog. Comparison between the populations of this species shows variations in arm ratios and relative chromosomal lengths. In terms of geographic variations these variations could be considered as 'cytologically diagnostic'.

Present evidence relating genetic and environmental variations is still inconclusive and needs critical analytical studies to unveil the potential relationship between the ecological and genetical profiles. Attempts have to be made to compare populations of this species in other localities or with other Rana species in the same localities. As mentioned before their is a relative abundance of Rana limnocharis in whole of south east Asia as compared to other Rana species. It is therefore necessary to study the possible linkage of this frog of this region with other regions. It is evident that time is rapidly approaching when a major effort has to be made toward obtaining complete and reliable estimates of these critical factors to provide a base to build such studies.

We anticipate that exciting development in the next

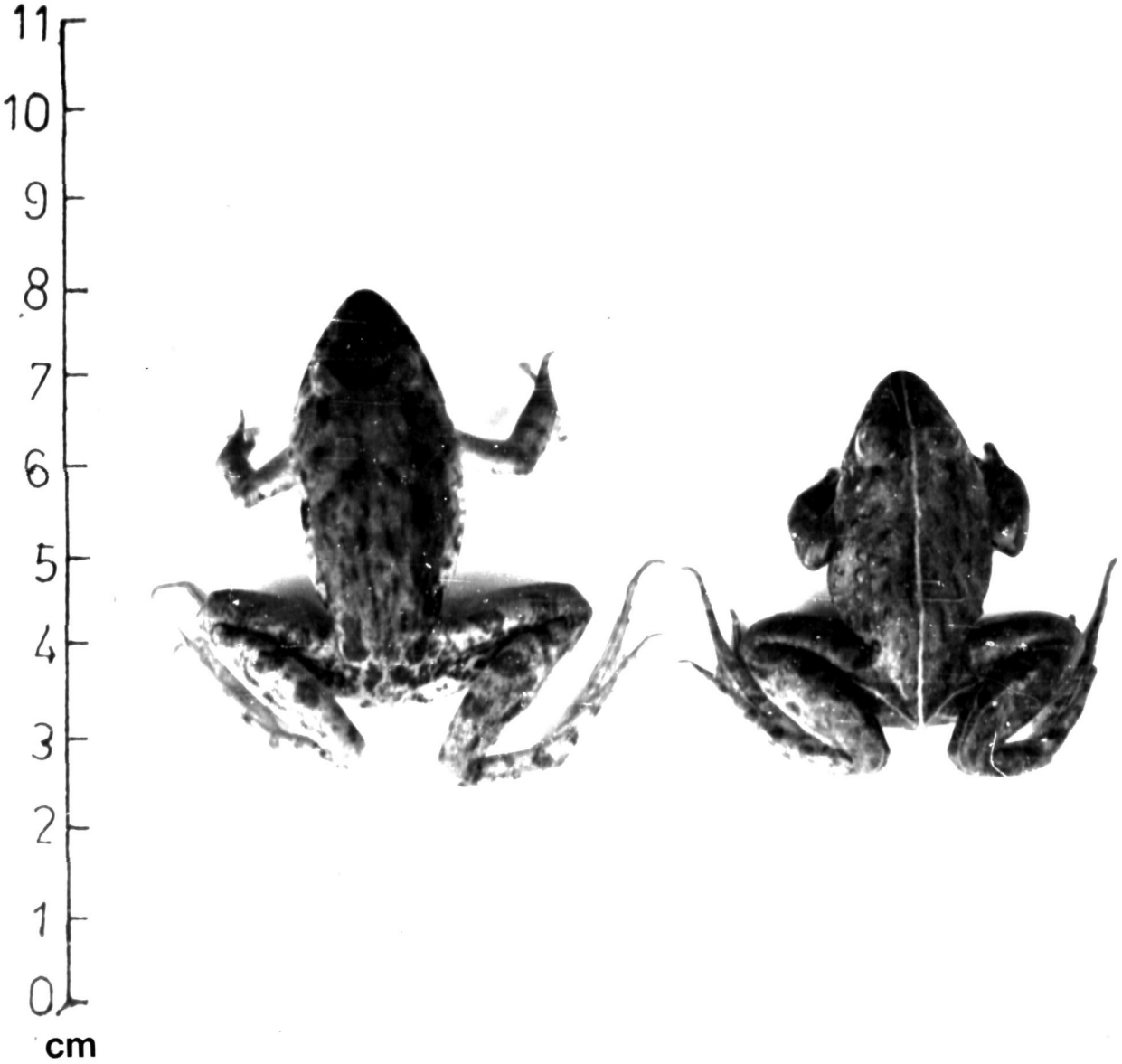
few decade will result from using the new techniques of DNA sequencing and gene cloning. These methods will reveal substitutions and gross variability. This may also permit regulatory gene evolution to be examined directly. Finally, these approaches may provide a high resolutionmethod for studying evolutionary changes in gene arrangement.

APPENDIX

MID-DORSAL STRIPE PATTERN VARIATION IN R. LIMNOCHARIS

PLATE 1 Mid-dorsal stripe pattern variation in R. limnocharis

PLATE 1 Mid-dorsal stripe pattern variation in R. limnocharis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Mid dorsal stripe pattern variations in Rana limnocharis

Studies made on the South East Asian populations of Rana limnocharis indicated morphological differences (Kuramoto, 1968). The unique differences in the external characters, mid-dorsal stripes, were selected for the purpose of clarifying the pattern of variation and casting additional light on the evolutionary history of this species in the north east part of India.

Two types of this frog, one with mid-dorsal stripe and the other without it, occur in this part in various populations. Similar observation had been made on Japanese populations (Inger, 1947; Moriwaki, 1953). As the genetic basis of this character has been established by Moriwaki (1953), the genetic constitution of a population could be inferred from the phenotypic constitution.

Our study concerning this pattern of variation, reveal the genetic differences among the populations that did not manifest in the biochemical analysis.

Geographic variation of mid-dorsal stripe

Number and populations of striped and non-striped frogs in each population are given in table 1 and Plate 1. All populations of Meghalaya had some proportions of both striped and non-striped frogs. Apparently, there is no clinal change in the proportion of striped types and the differences among the localities are not significant statistically. For instance, the result of chi-square test between Jowai and Shillong is as follows:

$\chi^2 = 1.95$ $\qquad\qquad$ $0.1 < P < 0.25$

Moriwaki (1953) had reported that the mid dorsal stripe of R. limnocharis is a dominant character. According to Hardy Weinberg's

Table 1. Frequencies of two phenotypes and gene S in various populations of *R. limnocharis*.

Locality	No. of Frogs	No. of striped frogs (%)	No. of non-striped frogs (%)	Frequency of gene S
Tura	24	14 (58.3)	10 (42.7)	0.31
Nongstoin	95	46 (48.4)	49 (51.6)	0.28
Cherrapunjee	251	130 (51.8)	121 (48.2)	0.31
Mawsynram	236	124 (52.2)	112 (47.8)	0.31
Nongpoh	446	228 (51.1)	218 (48.9)	0.30
Dawki	123	66 (53.7)	57 (46.3)	0.33
William Nagar	18	8 (44.4)	10 (55.5)	0.29
Shillong	244	135 (55.3)	109 (44.7)	0.33
Jowai	112	53 (47.3)	59 (52.7)	0.27

rule, freqauency of striped or non-striped genes in a population is given by the followi ng formula:

$$P = 1 - R$$

Where P is the frequency of striped gene and R is the frequency of the recessive non-striped gene. The value of P during our study is given in table 1. The striped gene is about 30% while the non-striped being 70% on an average in the various populations.

Genetic differentiation of Population

It is obvious from the above result that R. limnocharis in the populations of Meghalaya also differs in this external character, more so with the mid-dorsal stripe pattern. Overall, each population apparently exhibits a mixed group of striped and non-striped frogs. Proportion of striped type pattern has been reported by various authors for a fairly large number localities scattered all over Asia (Merlevis, 1930; Fang and Chang, 1931; Inger, 1966; Moriwaki, 1953; Lice and Hu, 1961). No report has been made from India and this is probably the first report from this region. When closely observed the proportion of the striped gene tends to change randomly.

Evidently the populations of Meghalaya are similar phenotypically and undoubtedly in genic constitutions to many other populations of Asia. Selective ignificance of the mid-dorsal stripe has not been investigated. It is probable that the dominant and recessive genes for the stripe have been presumed by some selective advantage of the heterozygote, namely, by balanced polymorphism. Otherwise, one of the genes would have a low frequency to form a balance between selection and mutation.