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RESEARCH ARTICLE

KARYOLOGICAL ANALYSIS OF INDIAN SKITTERING FROG, *EUPHLYCTIS CYANOPHLYCTIS* FROM JAMMU AND KASHMIR (INDIA)

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ABSTRACT

Euphlyctis cyanophlyctis is a common dicroglossid frog found throughout the Indian subcontinent and is the native frog of Kashmir. This frog is a highly aquatic and littoral species found in marshes, pools and various other wetlands. Chromosomal complements of both male and female specimens were analyzed using conventional Giemsa staining, C-banding and NOR-banding. The karyotypes prepared formed of 13 pairs biarmed homologous chromosomes (2n=26) largely forming a graded series and thereby the fundamental arm number was NF=52. The chromosomes of this species got divided into two groups; the first group comprised of the pairs of large chromosomes 1-5 and second group had the pairs 6-13 of smaller chromosomes. Of these, chromosomes of pairs 2, 3, 4 (from first group), 9, 10 and 11 (from second group) were submetacentric; the remainder were metacentric and the chromosomal formula for the species is n=7M+6SM. C-banding analysis revealed the presence of centromeric C-bands in all the chromosomes of the karyotype while secondary constrictions were evident on the long arm of pair 10. Results of the present study have scientific and practical significance complementary to biochemical and molecular studies in animal taxonomy.

INTRODUCTION

Euphlyctis cyanophlyctis is known under numerous common names, including Indian skipper frog or skittering frog or green frog and is claimed to be the native frog of Kashmir. It is the mostly widely distributed oriental frog and is highly aquatic and littoral frog belonging to family Dicroglossidae (Anura, Amphibia). Dicroglossidae comprises of 186 species in 13 genera (Frost, 2013). Presently genus *Euphlyctis cyanophlyctis* is represented by six species worldwide. Taxonomic revision of ranoid frogs renamed the species *Rana cyanophlyctis* as *Euphlyctis cyanophlyctis.* Its distribution extends from Thailand to Nepal throughout India, Sri Lanka, almost throughout Pakistan, westward to Iran and Afghanistan. This study was confined to Jammu division of Jammu and Kashmir State, India. It remains permanently resident in most fresh water bodies that sustain submerged aquatic plants, paddy fields, canals and rainwater ditches. The adults, largely aquatic,floating amongst plants with the lower half of the body and hind limbs submerged in water.This green frog dives under water when disturbed often staying beneath for long durations. Female lay eggs after the first rains and throughout the rainy season and a female may lay 2000eggs in ponds or paddy fields. The frog can tolerate a wide range of oxygen, temperature and pH variations, from fresh water to considerably brakish and polluted refuse water; it thrives equally well in sewer systems of towns and cities. It is reported to be declining at an alarming rate especially in the urban and semi-urban areas of Kashmir valley, warning environmentalists (Kashmir frogs croaking towards extinction; Well, 2007). It is therefore, widespread and abundant but is sensitive to environmental pollutions and thus can serve as an indicator species of water conditions. It is believed that cytogenetic studies serve as a significant tool providing relevant data that are used in Taxonomy, in identification of species and in understanding the mechanism of speciation and evolution (Burch, 1968). However, it may also contribute valuable systematic characters and the knowledge of chromosomal morphology which may be used to resolve any cytotoxic and genotoxic threats to the group.

MATERIAL AND METHODS

Cytogenetic analysis of male and female specimens of model species was carried out following the conventional colchicine-hypotonic-acetic-alcohol air-drying Giemsa staining technique (Tijo and Whang, 1965). *In-vivo* injection of colchicine treatment was used. An intramuscular and intraperitoneal injection of 0.1% colchicine solution was given to the frog at the rate of 1ml/100gm body weight. After 4 hour, frogs were dissected from the ventral side so as to remove the kidneys, intestine and bone marrow (Haertel *et al*, 1974; MacGregor and Varley, 1986). The tissue was chopped into very fine pieces

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and subjected to hypotonic treatment at room temperature for an hour. Tissue was fixed in freshly prepared Carnoys fixative for 45minutes and after every 15 minutes fixative was changed. Air-drying dabbing method was used for preparing slides. Tissue was dabbed on clean and dry slides and eventually air-dried. Slides were stained in 2% Giemsa stain for about 40-50 minutes and differentiated in distilled water and then again air-dried. Some slides were subjected to differential staining or banding. C-banding was done using Sumner (1972) technique with some modifications. Ag-NOR banding was done using Howell and Black (1980) protocol with slight modifications. The stained slides were scanned thoroughly for well spread metaphase plates which were then observed under 10X eyepiece and 100X objective using oil immersion and the best complements were selected for photomicrography. Selected suitable metaphases conventionally stained with Giemsa and those showing NOR and C-banding were photomicrographed at 1000X magnification showing clear images by using Nikon YS100 binocular research microscope and Samsung SDC-313camera. Morphometric analysis of chromosomes was done using stage micrometer and oculometer.

RESULTS

During the present study, screening of mitotic metaphase plate of both male and female revealed that chromosomal complement of *E. cyanophlyctis* (Fig. 1) consist of 26 chromosomes i.e. diploid number=26. (Fig.2, 3, 6 and 7). No heteromorphic sex chromosome was observed in the karyotype and general chromosomal form and type was found to be same in both the sexes. For karyotyping, chromosomes of well-spread metaphase plates were cut out from photomicrographs and placed according to the size and centromere position. Sixteen pair of chromosomes was categorized into two groups:

Group A: five pairs of large chromosomes
Group B: eight pairs of smaller chromosomes.

The karyotype comprised of all biarmed chromosomes with NF=52. Chromosomal formula for the complement was calculated as 2n=14M+12SM (Fig.3 and 7). The chromosomes of both the groups were of two types that are, metacentric and submetacentric (Levan *et al*, 1964). Six pairs of chromosomes were submetacentric type. Three pairs, i.e. pair 2, 3 and 4 in group A, were found to be submetacentric while rest all the pairs in this group were submetacentric type. Similarly in group B, three pairs, 9, 10 and 11 were found to be submetacentric while other chromosomes were found to be metacentric type (Fig. 3 and 7). Morphometric measurement of the chromosomes in male karyotype showed mean total haploid length was 9.7 and total diploid length of chromosome was 19.4. Similarly for female karyotype, mean total haploid length and total diploid length of chromosomes were 9.8 and 19.6 respectively. Detailed morphometric data of chromosome complement for male and female karyotypes are given in the Table 1 and 2 respectively. Histogram (Fig. 4) and Idiogram (Fig. 5) and for male and Histogram (Fig. 8) and Idiogram (Fig. 9) and for female were prepared using morphological data. C-banding showed the presence of heterochromatin and centromeric C-bands were found in all the chromosomes of karyotype (Fig. 10 and 11). According to the literature, C-heterochromatin is concentrated in the centromere region in many species of the family Ranidae (Miura, 1995; Schmid, 1978). Ag-NOR banding showed a well-defined and conspicuous pair of nuclear organizer regions was found on pair no. 10 on long arm (10q) (Fig. 12 and 13).

Fig1 An adult male and female *Euphlyctis cyanophlyctis*

Fig. 2 A conventional stained somatic metaphase complement of male *Euphlyctis cyanophlyctis* (2n=26)

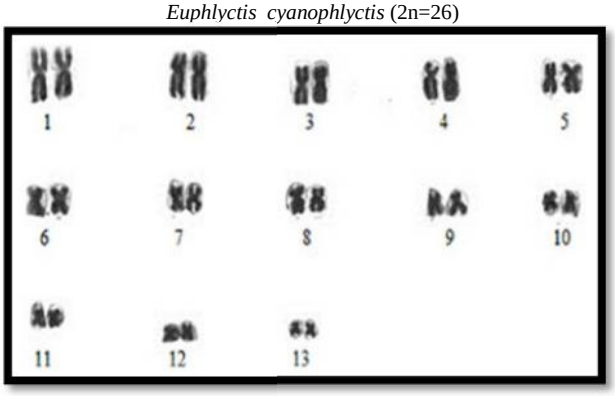

Fig. 3 Karyotype of male *Euphlyctis cyanophlyctis* (2n=26) Chromosomal formula=7M+6SM

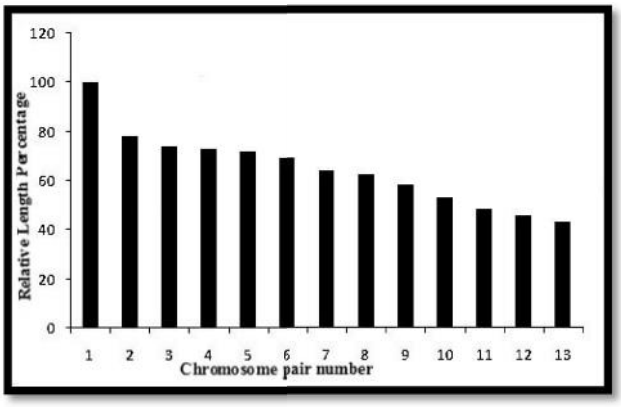

Fig. 4 Histogram of male specimen of *E. cyanophlyctis*

Table 1 Morphometric data of karyotype of male *Euphlyctis cyanophlyctis* showing 2n=26 (14m+12sm)

Chromosome pair no.	Mean length of the short arm in µm (p)	Mean length of the long arm in µm (q)	Absolute length (p+q) of the chromosome in µm	Arm ratio (q/p)	Relative length percentage	Total complement length percentage	Centromeric index	Nomenclature
1	0.55	0.60	1.15	1.09	100.0	5.92	47.82	Metacentric
2	0.33	0.57	0.90	1.73	78.26	4.63	36.66	Submetacentric
3	0.31	0.54	0.85	1.74	73.91	4.38	36.47	Submetacentric
4	0.31	0.53	0.84	1.71	73.04	4.32	36.90	Submetacentric
5	0.32	0.51	0.83	1.59	72.17	4.27	38.55	Metacentric
6	0.31	0.49	0.80	1.58	69.56	4.12	38.75	Metacentric
7	0.28	0.46	0.74	1.64	64.34	3.81	37.83	Metacentric
8	0.27	0.45	0.72	1.66	62.60	3.71	37.50	Metacentric
9	0.24	0.43	0.67	1.79	58.26	3.45	35.82	Submetacentric
10	0.21	0.40	0.61	1.90	53.04	3.14	34.42	Submetacentric
11	0.19	0.37	0.56	1.94	48.69	2.88	33.92	Submetacentric
12	0.23	0.30	0.53	1.30	46.08	2.73	43.39	Metacentric
13	0.22	0.28	0.50	1.27	43.47	2.57	44.00	Metacentric

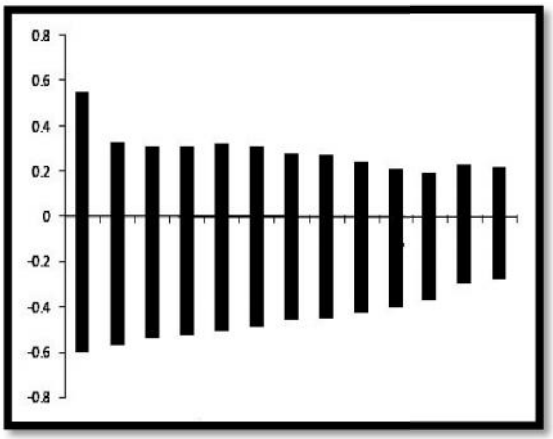

Fig. 5 Idiogram of male specimen of *E. cyanophlyctis*

Fig. 6 A conventional stained somatic metaphase complement of female *Euphlyctis cyanophlyctis* (2n=26)

Fig. 7 Karyotype of female *Euphlyctis cyanophlyctis* (2n=26)Chromosomal formula=7M+6SM

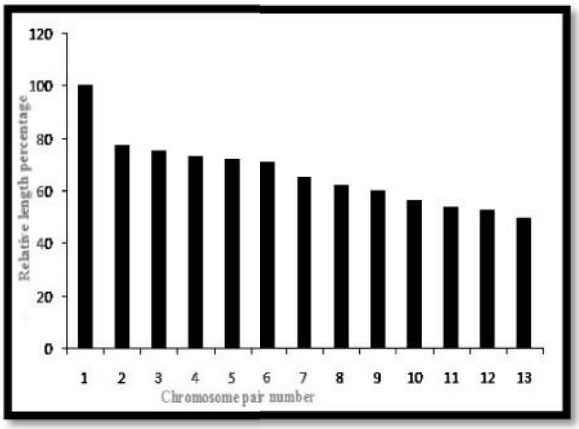

Fig. 8 Histogram of female specimen of *E. cyanophlyctis*

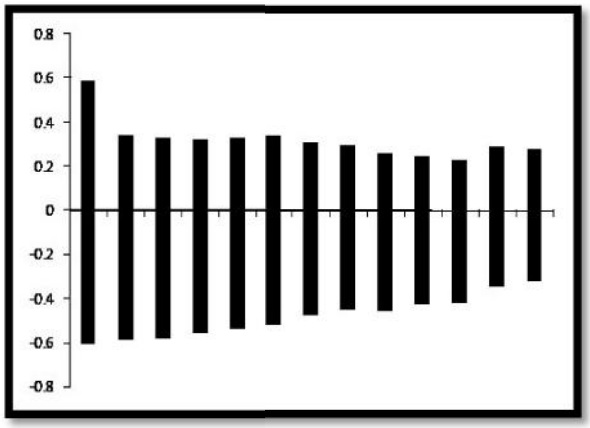

Fig. 9 Idiogram of female specimen of *E. cyanophlyctis*

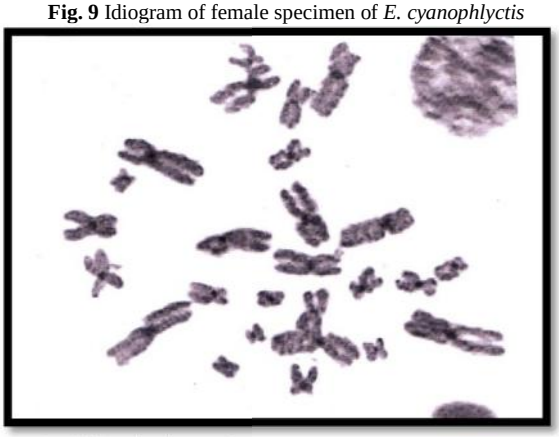

Fig. 10 C-stained metaphase complement of *E. cyanophlyctis*

Table 2 Morphometric data of karyotype of female *Euphlyctis cyanophlyctis* showing 2n=26 (14m+12sm)

Chromosome pair no.	Mean length of the short arm in µm (p)	Mean length of the long arm in µm (q)	Absolute length (p+q) of the chromosome in µm	Arm ratio (q/p)	Relative length percentage	Total complement length percentage	Centromeric index	Nomenclature
1	0.59	0.61	1.20	1.03	100.00	5.72	49.17	Metacentric
2	0.34	0.59	0.93	1.73	77.50	4.43	36.56	Submetacentric
3	0.33	0.58	0.91	1.75	75.83	4.34	36.26	Submetacentric
4	0.32	0.56	0.88	1.75	73.33	4.19	36.36	Submetacentric
5	0.33	0.54	0.87	1.63	72.50	4.15	37.93	Metacentric
6	0.34	0.52	0.86	1.52	71.67	4.10	39.53	Metacentric
7	0.31	0.48	0.79	1.54	65.83	3.77	39.24	Metacentric
8	0.30	0.45	0.75	1.50	62.50	3.58	40.00	Metacentric
9	0.26	0.46	0.72	1.76	60.00	3.44	36.11	Submetacentric
10	0.25	0.43	0.68	1.72	56.67	3.24	36.76	Submetacentric
11	0.23	0.42	0.65	1.82	54.17	3.10	35.38	Submetacentric
12	0.29	0.35	0.64	1.20	53.33	3.05	45.31	Metacentric
13	0.28	0.32	0.60	1.14	50.00	2.86	46.66	Metacentric

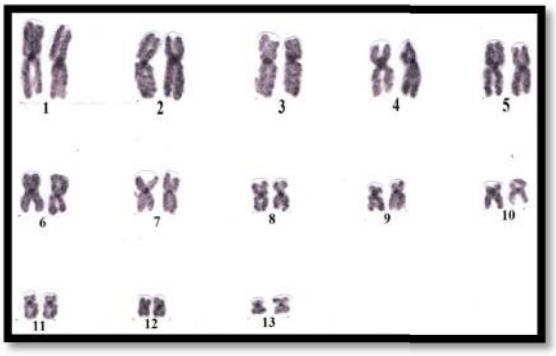

Fig. 11 C-banded karyotype of *Euphlyctis cyanophlyctis* showing the presence of centromeric C-bands in all the chromosomes

Fig. 12 NOR-stained metaphase complement of *E. cyanophlyctis*

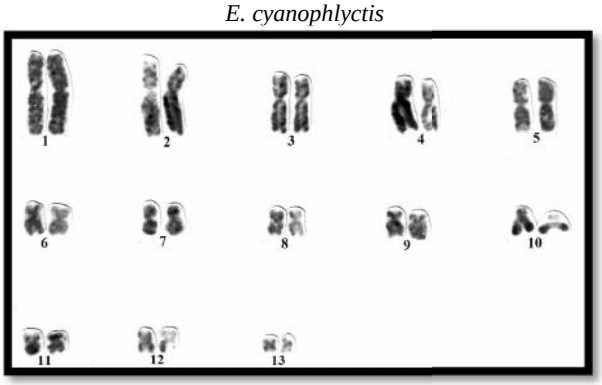

Fig. 13 NOR-stained karyotype of *Euphlyctis cyanophlyctis* showing the location of NORs on the 10th pair of submetacentric chromosomes

DISCUSSION

The present species of frog, *Euphlyctis cyanophlyctis* shows the presence of twenty six chromosomes in its somatic karyotype which reveal that the karyotypic characters of this species are conserved like other members of ranoid stock since the past studies has confirmed the basic diploid number 2n=26 in almost all the ranoids studied till date (Kawamura, 1939a and b; Wickbom, 1945 and 1949; Mathey, 1951; Seto, 1965; Nishioka, 1972; Duda and Koul, 1973; Ivanov and Madiyanov, 1973; Morescalchi, 1973; Haertel *et al*, 1974; Bardhan *et al*, 1978; Schmid, 1978; Chakrabarti *et al*, 1983; Nishioka *et al*, 1987; King, 1990; Kuramoto, 1992; Mohammad *et al*, 1997; Vences *et al*, 2000; Joshy *et al*, 2006; Arslan *et al*, 2010; Shanthi *et al*, 2010; Jazayeri *et al*, 2012). Family Dicroglossidae basically belongs to ranoid anuran stock. Almost all the ranoid have symmetrical karyotypes with all biarmed chromosomes. Karyotypic details are in confirmation with the general cytogenetic trends in ranoid anurans. The information displayed in literature points out that the most frogs of the *Rana* genus have the karyotype formed of 26 chromosomes (metacentric and submetacentric) and in the present study, the chromosomes within the karyotype can be distinguished into two groups on the basis of their sizes, first group of five pairs of large sized chromosomes and second group of eight pairs of smaller chromosomes. All the chromosomes of both the groups are either metacentric or submetacentric according to chromosomal classification of Levan *et al*, 1964. However, no telocentric chromosome and also no sex chromosome could be designated even on the basis of differential staining (Fig.11 and 13).

In the present study, cytogenetic analysis of *Euphlyctis cyanophlyctis* are in partial agreement with the earlier studies (Bardhan *et al*, 1978; Shanthi *et al*, 2010) but the retention of the diploid chromosome number 26 is a characteristic of the ranoid anurans. Duda and Koul (1973) reported metacentric, submetacentric and subtelocentric chromosomes in *Rana cyanophlyctis* (presently known as *E. cyanophlyctis*) however, the present work reported the categorization of chromosomal complement of *E. cyanophlyctis* into metacentric and submetacentric only. Amphibians throughout the world are disappearing at an alarming rate (Blaustein and Wake, 1990) and this frog claimed to be native of Kashmir is declining there (Kashmir frogs croaking towards extinction; Well, 2007).

Although the present work is a preliminary chromosomal investigation of the species with the morphology and morphometric characteristics of the chromosomal complement but any change or damage to the chromosomal morphology due to any genotoxic or cytotoxic agent can be evaluated with the application of various genotoxicity assays.

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