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Genetic variations among Ecologically diverse species of Anurans at the level of Genus based on ISSR Marker

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Abstract

Anurans (frogs and toads) collected from South Kanara, Udupi and North Kanara districts, Karnataka viz: Euphlyctis cyanophlyctis (an aquatic), Fejervarya rufescens (a burrowing), Duttaphrynus melanostictus (a terrestrial), Fejervarya caperata (a semi aquatic), Ramanella montana (a semi arboreal) and Polypedates maculatus (an arboreal) belonging to five genera placed under four families were compared using Inter Simple Sequence Repeat (ISSR) marker to analyze genetic variability and relationship between the investigated taxa. ISSR profile obtained exhibited 100% presence of polymorphic bands. The ISSR bands could also be categorized as high (H), moderate (M) and light (L) based on the intensity of amplification. Dendrograms were generated using similarity coefficient and relationship coefficient values based on the count of only high or high and moderate or high, moderate and light bands in each case. Analysis of the data suggests that the genetic relatedness between the taxa as exhibited in the generated dendrograms varies based on the type of amplified bands considered. The clustering pattern obtained in the case of high and moderate bands was more close to the conventional taxonomical pattern of grouping based on the phenotypic characters. The specific unique bands present in each case may be of importance as molecular markers. Observed genetic variability (presence/absence, total number, intensity of amplification and size of polymorphic bands) among taxa probably provide factors contributing partially to diverse adaptive fitness supporting varied ecological demands. However, an intensive study incorporating more representive species from each of the habitat is needed to pinpoint the importance of the unique bands as the genus / species / ecological molecular markers.

Key words: Anurans, genetic variability, India, inter simple sequence repeat (ISSR) marker, Karnataka.

Introduction

Inter Simple Sequence Repeat (ISSR) a genome region of about 100-3000bp is located between adjacent oppositely oriented microsatellite (sequence of 2-6 base pairs of DNA) loci. In ISSR technology the complementary sequences to two neighboring microsatellites are used as PCR primers resulting in the amplification of the variable region between them. The generated fragments from multiple loci are separated by electrophoresis and scored for the presence or absence of fragments of particular size. Sequences amplified by ISSR-PCR can be used to characterize genetic relatedness among population, genetic fingerprinting, gene tagging, phylogenetic analysis, detection of genomic instability and assessment of hybridization[1,2].

Earlier studies on anurans have demonstrated genetic differences among anuran populations[3-7] using random amplified polymorphic DNA (RAPD) technique and microsatellite markers developed recently for anurans have been suggested to be useful for resolving taxonomic uncertainties within the genus and for conservation studies[8-11]. ISSR technology unlike RAPD is cheaper and easy to handle.

A study on phylogeographical and population demographic analysis detected by ISSR markers has suggested that the current distribution of Chinese black-spotted frog, *Pelophylax nigromaculata* population is the result of its range expansion from two independent refugia[12].

However, there are no reports documenting genetic variations in ecologically diverse anurans based on ISSR markers. The present study therefore, aims to compare the Inter Simple Sequence Repeat (ISSR) profiles of the anuran (frogs and toads) viz: *Euphlyctis cyanophlyctis* (an aquatic), *Fejervarya caperata* (a semi aquatic), *Fejervarya rufescens* (a burrowing), *Duttaphrynus melanostictus* (a terrestrial), *Ramanella montana* (a semi arboreal) and *Polypedates maculatus* (an arboreal) species belonging to five genera and record the degree of genetic relatedness.

Material and Methods

Anurans collected from certain localities as shown in figure-1 viz: **1**: Mirjan (14°30'48"N 74°25'09"E; 78ft) and **2:** Haldipur (14°20'17"N 74°25'46"E; 34ft) in North Kannara district; **3**: Thekkatte (13°33'08"N 74°41'58"E; 49ft) in Udupi district and **4**: Konaje (12°48'55"N 74°55'54"E; 364ft) in South Kanara

district in Karnataka includes *Ramanella montana* from Mirjan; *Polypedates maculatus* from Haldipur; *Fejervarya caperata* from Thekkatte and *Euphlyctis cyanophlyctis*, *Fejervarya rufescens* and *Duttaphrynus melanostictus* from Konaje. The Sartorius muscle piece was used to isolate genomic DNA.

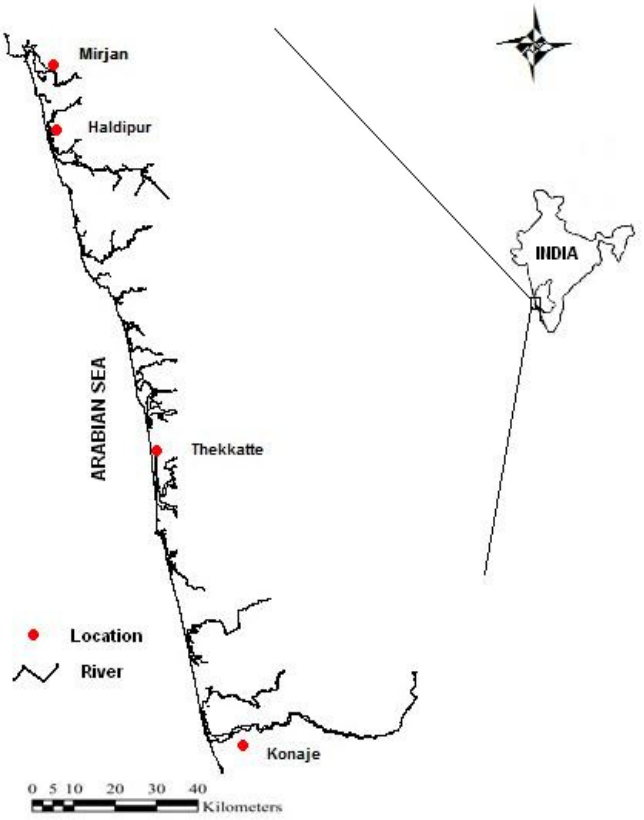

Figure – 1
The map showing the sites (●) of anuran collection from Mirjan and Haldipur in North Kannara, Thekkatte in Udupi and Konaje in South Kannara districts, Karnataka, India

Isolation of Genomic DNA: The tissues were homogenized in 5ml extraction buffer (100 mM Tris HCl, pH 8; 50mM EDTA, pH 8; 150mM NaCl; 1% SDS) and incubated at 60˚C for 30 minutes. Further 5ml Tris equilibrated phenol and 1/10 volume of 3M sodium acetate was added and mixed gently. This was kept at room temperature for 5 min and centrifuged at 2000 rpm for 8 min and further transferred the aqueous phase into another centrifuge tube and added equal volume of chloroform:isoamylalcohol (24:1) to it and centrifuged at 2000 rpm for 15 min. The aqueous phase was further transferred to a new test tube and 2-3 volume of chilled absolute alcohol was added. White fibrous DNA precipitated. This was further washed with 70% alcohol and centrifuged at 7000 rpm for 8 min. The precipitated DNA is dried and dissolved in TE buffer (20mM Tris and 10mM EDTA) and store at 4˚C until further use.

Electrophoresis of genomic DNA: The isolated genomic DNA was further processed by centrifuging at 8000 rpm for 5 min and the supernatant is removed. 1ml of 70% alcohol was added to it and centrifuged for 5 min at 8000 rpm. Further the supernatant was removed and 1 ml of absolute alcohol was added. Again centrifuged for 5 min at 8000 rpm, removed the supernatant and the pellet was dried (30 min). The dried pellet was dissolved in 100ul of TE buffer. Boil the contents in the tube in water bath for 5 min and placed the tubes on ice immediately for 5 min. Shear the DNA well with a syringe and 10 ul of DNA was loaded on the 0.8% agarose gel and run for 20-30 min at 50-100 V and observed under UV transilluminator.

PCR amplification: PCR amplification was performed according to the procedure[13] with slight modifications. The annealing temperature of the primer $(ACAG)_4$ was optimized through gradient PCR and 54ºC temperature (annealing) was found to be optimum. Each reaction mixture of 25 µl contained 1 µl of genomic DNA (20 ng), 2 µl of primer (10 pM / µl), 2.5 µl of 10x buffer (100 mM Tris at pH 9.0; 500 mM KCl and 1% Triton X-100), 1.5 µl of 1.5 mM $MgCl_2$, 2 µl of 200 µM dNTPs and 1.0 U of *Taq* DNA polymerase (Bangalore Genei, India). PCR amplification were performed in a Karger thermal cycler with an initial denaturation of 94ºC for 5 min, followed by 40 cycles of denaturing at 94ºC for 1 min, annealing for 1 min at 54ºC, extension at 72ºC for 2 min, and final extension at 72ºC for 15 min and then 4ºC soak.

Separation and detection of PCR products: PCR amplified products were separated on 1.5% agarose gel electrophoresis. The entire 25µl reaction volume was mixed with 1x loading buffer (Bangalore Genei, India) and loaded onto gel and electrophoresed for 3 hrs at 60 V. A 100 bp molecular weight marker was used for band sizing. Gel images were recorded and the band sizes were quantified by Mega bioprint 1000 system (Vilber Lourmat, France).

Data Analysis: DNA fragments separated by electrophoresis as exhibited in figure-2 were scored present (1) or absent (0) based on their sizes to generate binary matrix as shown in table-1. The ISSR bands could also be categorized as high (H), moderate (M) and light (L) based on the intensity of amplification as listed in table-2. Percent polymorphism (% P) values were calculated as number of polymorphic bands × 100/ total number of bands as in table-3.

The scored ISSR band pattern was used to obtain Nei and Li (NL) similarity coefficient values[14], Jaccard's similarity coefficient values[15] and relationship coefficient values[16.] The obtained values in each case were subjected to generate UPGMA (unweighted pair group method with arithmetic mean) based dendrograms using PAST software[15]. Nei's genetic distance[17] and gene diversity parameters viz: polymorphic loci, Nei's gene diversity (h), Shannon's Information Index (I) and Gene flow (Nm) values were obtained as shown in table -3, table -4, figure -3. using software[18]. Polymorphism information

content (PIC) was calculated using PIC calculator extra software[19] which assumes Hardy-Weinberg equilibrium.

Results and Discussion

Genomic variability is an attribute of all biological populations. ISSR markers have been shown to be useful to trace phylogeography and demographic history of Chinese black-spotted frog, *Pelophylax nigromaculata* populations[12] and genetic diversity among tarantulas based on amplified DNA fragments[20].

DNA fragments generated in the present study using ISSR -PCR method are as shown in figure 2. The presence (1) or absence (0) of ISSR fragments (bands) of different sizes were scored as shown in table-1.

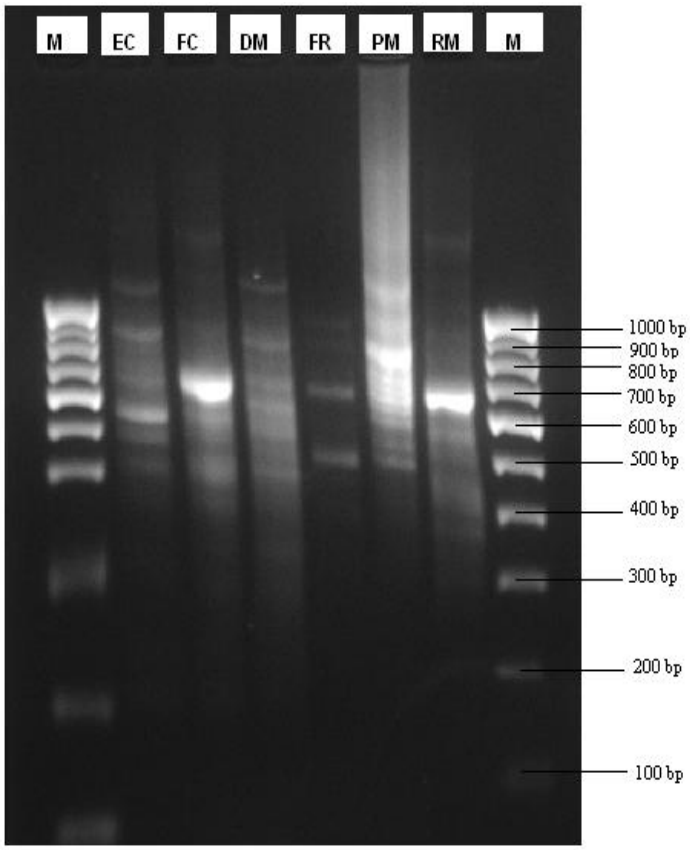

Figure – 2
ISSR profile of anurans viz *Euphlyctis cyanophlyctis* (EC), *Fejervarya caperata* (FC), *Duttaphrynus melanostictus* (DM), *Fejervarya rufescens* (FR), *Polypedates maculatus* (PM) and *Ramanella montana* (RM) from aquatic, semi aquatic, terrestrial, burrowing, arboreal and semi arboreal habitats respectively. The marker lane (base pairs ladder) is represented by letter 'M'

A total of 28 polymorphic bands were generated by the primer used. The shared bands between the investigated taxa were counted once only. No monomorphic (present in every taxa) band was observed and 100% presence of polymorphic (present in some taxa only) bands points to a demarcate variability in the genome of the studied genera. The total number of polymorphic bands counts to six, eight, five, eleven, nine and ten in *Euphlyctis cyanophlyctis* (an aquatic), *Fejervarya caperata* (a semi aquatic), *Fejervarya rufescens* (a burrowing), *Duttaphrynus melanostictus* (a terrestrial), *Ramanella montana* (a semi arboreal) and *Polypedates maculatus* (an arboreal) in the studied anuran species respectively as listed in table-1.

The bands categorized as high (**H**), moderate (**M**) and light (**L**) based on the intensity of amplification, exhibited are shown in figure-2 and listed in table-2. The proportion of polymorphic loci across the genome referred as gene diversity is one of the measure to assess genetic diversity of a population. The percent polymorphic values obtained based on the count of either only H or H and M or H, M and L bands as shown in table-3 were found to vary among the anuran taxa, the highest polymorphism being 22.45% in the case of *Duttaphrynus melanostictus*, a toad (family: Bufonidae) and the lowest being 10.2 % in the case of *Fejervarya rufescens*, a burrowing anuran.

Genetic variability refers to the tendency of the genetic characters to vary. The ISSR data (number, size, intensity of amplified bands and percent polymorphic values) variations in the genome of anurans recorded in the present study, pinpoints to the concurrent diversity of the nucleotides in the primer complementary sites which may be due to mutation, insertion, deletion and near neutrality of alleles[21,22]. The high genetic diversity further support greater differentiation[23, 24]. Variations in the gene characteristics are necessary to produce changes that enable the species to adapt and survive. Varied genetic characters probably form the basis to confer varied adaptive fitness/survival ability in the diverse ecological habitats[25].

The number of unique/diagnostic bands (not shared between the taxa) demarcated in table 2 counts as two in *Euphlyctis cyanophlyctis* and *Fejervarya rufescens*, four in *Fejervarya caperata*, five in *Ramanella montana* and seven in *Duttaphrynus melanostictus* and *Polypedates maculatus*. The presence/ absence of unique bands may be of importance as inter genera/inter specific markers**.**

Using Nei and Li and Jaccard's similarity coefficient values and Gao's *et al.* relationship coefficient values based on the count of only H or H and M and or H, M and L bands as shown in table 4 were used to generate UPGMA based dendrograms are shown in figure 3. The cophenetic correlation coefficient values in each of these generated dendrogram are more than ≈ 0.8 as desired[26].

Table -1
The ISSR profile of six anuran species viz: *Euphlyctis cyanophlyctis* (EC), *Fejervarya caperata* (FC), *Fejervarya rufescens* (FR), *Duttaphrynus melanostictus* (DM), *Ramanella montana* (RM) and *Polypedates maculatus* (PM) showing presence (1) or absence (0) of amplified ISSR fragment

Molecular Marker (bp)	EC	FC	FR	DM	RM	PM
-	0	1	0	0	1	0
-	0	1	0	0	1	0
-	1	0	0	1	0	1
-	0	0	0	1	0	0
1000	0	1	1	0	0	1
-	1	0	0	0	1	0
900	0	0	1	1	0	0
-	0	0	0	0	0	1
-	0	0	0	1	1	0
800	0	0	1	0	0	0
-	1	1	1	1	0	1
-	0	0	0	0	0	1
700	-	-	-	-	-	-
-	0	0	0	0	1	1
-	0	0	0	1	0	0
-	0	0	0	0	0	1
-	1	1	0	0	0	0
-	0	0	0	0	0	1
600	0	0	0	1	0	0
-	1	1	0	0	1	0
-	0	0	0	0	0	1
-	0	0	1	1	1	1
500	1	1	0	0	0	0
-	0	0	0	1	0	0
-	0	0	0	0	1	0
-	0	1	0	0	0	0
-	0	0	0	1	0	0
400	-	-	-	-	-	-
-	0	0	0	0	1	0
-	0	0	0	1	0	0
300	-	-	-	-	-	-
Total number of amplified ISSR fragments	**6**	**8**	**5**	**11**	**9**	**10**

Table – 2

The ISSR banding profile of six anurans viz: *Euphlyctis cyanophlyctis* (EC), *Fejervarya caperata* (FC), *Fejervarya rufescens* (FR), *Duttaphrynus melanostictus* (DM), *Ramanella montana* (RM) and *Polypedates maculatus* (PM) based on intensity of amplification of bands categorized as high (H), moderate (M) and light (L). The unique bands are shown in bold letters. Only clear bands were counted

Molecular Marker (bp)		EC	FC	FR	DM	RM	PM
-		-	**M**	-	-	**L**	-
-		-	L	-	-	L	-
-		M	-	-	M	-	**L**
-		-	-	-	**L**	-	-
1000		-	L	L	-	-	L
-		**M**	-	-	-	**L**	-
900		-	-	**L**	**M**	-	-
-		-	-	-	-	-	**H**
-		-	-	-	L	L	-
800		-	-	**L**	-	-	-
-		M	**H**	M	M	-	**L**
-		-	-	-	-	-	**L**
700		-	-	-	-	-	-
-		-	-	-	-	H	H
-		-	-	-	**M**	-	-
-		-	-	-	-	-	**M**
-		**H**	**M**	-	-	-	-
-		-	-	-	-	-	**M**
600		-	-	-	**L**	-	-
-		L	L	-	-	**M**	-
-		-	-	-	-	-	**M**
-		-	-	M	L	L	M
500		M	M	-	-	-	-
-		-	-	-	**M**	-	-
-		-	-	-	-	**L**	-
-		-	**L**	-	-	-	-
-		-	-	-	**L**	-	-
400		-	-	-	-	-	-
-		-	-	-	-	**L**	-
-		-	-	-	**L**	-	-
300		-	-	-	-	-	-
Number of bands based on intensity of amplification	H	1	1	0	0	1	2
	M	4	3	2	5	1	4
	L	1	4	3	6	7	4
Number of unique bands	H	1	1	0	0	0	1
	M	1	2	0	3	1	3
	L	0	1	2	4	4	3

Table – 3

Genetic diversity among six anuran species based on % polymorphism, polymorphic loci, polymorphic information content, Nei's gene diversity, Shannon's Information index and gene flow based on categories of amplified bands

Anuran species		Intensity of amplified bands		
		High	High and Moderate	High, Moderate and Light
Percent polymorphism	*Euphlyctis cyanophlyctis*	20	21	12.24
	Fejervarya caperata	20	17	16.33
	Fejervarya rufescens	0	8	10.2
	Duttaphrynus melanostictus	0	21	22.45
	Ramanella montana	20	8	18.37
	Polypedates maculatus	40	25	20.41
Number of scored loci		4	16	28
Number of polymorphic loci		4	16	28
Total percent of polymorphic loci		100	100	100
Polymorphism information content		0.152	0.778	0.8124
Nei's gene diversity ± standard deviation		0.3194 ± 0.0833	0.3403 ± 0.0833	0.3552 ± 0.0924
Shannon's information index ± standard deviation		0.4970 ± 0.0930	0.5203 ± 0.0930	0.5363 ± 0.1022
Gene flow		0	0	0

Table – 4

Nei and Li and Jaccard's similarity coefficient, Gao et al's relationship coefficient and Nei's genetic distance values based on the count of high (H) or high and moderate (HandM) or high, moderate and light (H, M and L) amplified bands for six anurans namely *Euphlyctis cyanophlyctis* (EC), *Fejervarya caperata* (FC), *Fejervarya rufescens* (FR), *Duttaphrynus melanostictus* (DM), *Ramanella montana* (RM) and *Polypedates maculatus* (PM).

		H band						H and M band						H, M and L band					
		EC	FC	FR	DM	RM	PM	EC	FC	FR	DM	RM	PM	EC	FC	FR	DM	RM	PM
Nei and Li similarity coefficient	EC	1						1						1					
	FC	0	1					0.67	1					0.57	1				
	FR	0	0	1				0.29	0.33	1				0	0.15	1			
	DM	0	0	0	1			0.4	0.22	0.29	1			0.36	0.11	0.25	1		
	RM	0	0	0	0	1		0	0	0	0	1		0.27	0.35	0.14	0.2	1	
	PM	0	0	0	0	0.67	1	0	0	0.25	0	0.25	1	0.25	0.22	0.27	0.29	0.21	1
Jaccard similarity coefficient	EC	1						1						1					
	FC	0	1					0.5	1					0.4	1				
	FR	0	0	1				0.17	0.2	1				0.1	0.18	1			
	DM	0	0	1	1			0.25	0.13	0.17	1			0.13	0.06	0.23	1		
	RM	0	0	0	0	1		0	0	0	0	1		0.16	0.21	0.08	0.11	1	
	PM	0	0	0	0	0.5	1	0	0	0.14	0	0.14	1	0.14	0.13	0.25	0.17	0.12	1
Gao *et al.* relationship coefficient	EC	1						1						1					
	FC	0	1					0.45	1					0.33	1				
	FR	0	0	1				0.1	0.13	1				0	0.03	1			
	DM	0	0	0	1			0.16	0.05	0.1	1			0.06	0.01	0.07	1		
	RM	0	0	0	0	1		0	0	0	0	1		0.07	0.13	0.02	0.04	1	
	PM	0	0	0	0	0.5	1	0	0	0.08	0	0.08	1	0.07	0.05	0.08	0.08	0.04	1
Nei's genetic distance	EC	0						0						0					
	FC	0.69	0					0.21	0					0.24	0				
	FR	0.29	0.29	0				0.38	0.29	0				0.39	0.39	0			
	DM	0.29	0.29	0	0			0.47	0.58	0.38	0			0.62	0.93	0.44	0		
	RM	0.69	0.69	0.29	0.29	0		0.58	0.47	0.29	0.58	0		0.5	0.5	0.56	0.85	0	
	PM	1.39	1.39	0.69	0.69	0.29	0	1.16	0.98	0.47	1.16	0.47	0	0.56	0.69	0.39	0.77	0.77	0

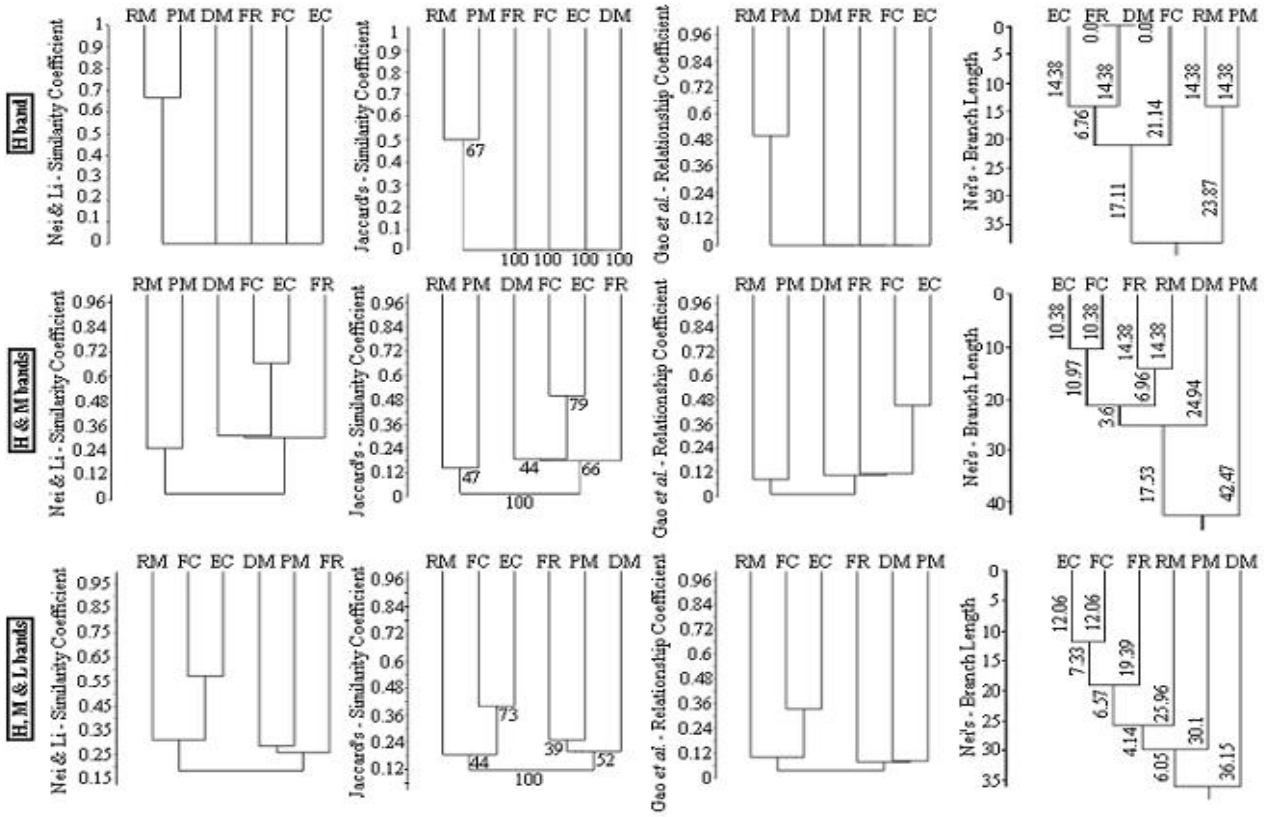

Figure – 3

Dendrograms generated using Nei and Li and Jaccard's similarity coefficient, Gao *et al*. relationship coefficient and Nei's genetic distance values using ISSR data based on high or high and moderate or high, moderate and light amplified bands showing genetic relatedness among anuran species viz: *Euphlyctis cyanophlyctis* (EC), *Fejervarya caperata* (FC), *Fejervarya rufescens* (FR), *Duttaphrynus melanostictus* (DM), *Ramanella montana* (RM) and *Polypedates maculatus* (PM). The cophenetic correlation values lies between 0.8 and 1.0. The bootstrap values at nodes (Jaccard's) support phylogenetic inference for grouping. The Nei's branch length denotes the amount of character change

The generated dendrograms using Nei and Li (NL) and Jaccard's similarity coefficient values and Gao *et al.* relationship coefficient values based on the count of only H bands as shown in figure-3 indicates the presence of a single cluster which groups *Ramanella montana* (a semi-arboreal frog) and *Polypedates maculatus* (an arboreal frog). The rest of the taxa namely *Euphlyctis cyanophlyctis* (an aquatic frog), *Fejervarya caperata* (semi aquatic frog), *Duttaphrynus melanostictus* (terrestrial toad) and *Fejervarya rufescens* (a burrowing frog) exhibits a case of polytomy (with more than one branches coming off from a single node) suggesting an unclear genetic relationship between them.

While the dendrograms generated based on the count of only H and M bands using NL and Jaccard's similarity coefficient and Gao *et al.* relationship coefficient values exhibits the presence of two clusters in each of these generated dendrograms as shown in figure 3. One of the cluster groups *Ramanella montana* and *Polypedates maculatus*, while the second cluster primarily groups *Euphlyctis cyanophlyctis* and *Fejervarya caperata.* The terrestrial toad, *Duttaphrynus melanostictus* and the burrowing frog, *Fejervarya rufescens* joins this second cluster showing varying degree of relatedness in each case. The *Fejervarya rufescens* (burrowing frog) joins the second cluster at the level of 0.11 while the terrestrial toad, *Duttaphrynus melanostictus* joins this cluster at the level of 0.10 in the case of the dendrogram generated using Gao *et al.* relationship coefficient values, probably suggesting that *Fejervarya rufescens* is more closer to aquatic frog, *Euphlyctis cyanophlyctis* and semi-aquatic frog, *Fejervarya caperata* than to the terrestrial toad, *Duttaphrynus melanostictus* unlike the relatedness depicted in the case of the dendrograms generated using NL and Jaccard's similarity coefficient values, where *Duttaphrynus melanostictus* is more closer to *Euphlyctis cyanophlyctis* and *Fejervarya caperata.* The overall topology of the dendrogram generated is same based on NL and Jaccard's similarity coefficient values but differs from that of the dendrogram generated based on the relationship coefficient values.

On the other hand, the dendrogram generated counting all the H, M and L bands indicates the presence of two clusters using NL and Jaccard's similarity coefficient and Gao's *et al.* relationship coefficient values as depicted in table 4 but with a different clustering pattern. The first cluster as shown in figure 3 is similar which groups *Euphlyctis cyanophlyctis*, *Fejervarya caperata* and *Ramanella montana* but exhibits varying degree of relatedness in each of these generated dendrograms. However, the second cluster which groups *Polypedates maculatus*, *Duttaphrynus melanostictus* and *Fejervarya rufescens,* depicts similar topology in NL and Gao's *et al.* based dendrograms unlike in the case of the Jaccard's dendrogram. The terrestrial toad, *Duttaphrynus melanostictus* joins at the level of 0.19 to the group which includes the burrowing frog, *Fejervarya rufescens* and the arboreal frog*, Polypedates maculatus* in the case of the Jaccard's based dendrogram, while it is the burrowing frog, *Fejervarya rufescens* which joins at the level of 0.26 and 0.07 respectively to the group which includes the terrestrial toad*, Duttaphrynus melanostictus* and the arboreal frog*, Polypedates maculatus* in the case of NL and Gao's *et al.* based dendrograms.

The clustering pattern of taxa was found to exhibit variations based on either the number of shared (present and absent) bands and or the total number of amplified bands. The present data as detailed in figure 3 and table 4 suggest that the genetic relatedness between the taxa varies based on the count of amplified bands viz., H or H and M or H, M and L bands. The clustering pattern generated using H and M bands (unlike the pattern obtained using H, M and L bands) relates closely to conventional taxonomical grouping[27] based on the phenotypic characters as shown in figure 4 suggesting that light bands may not be of much significance to support the level of genetic relatedness between the taxa.

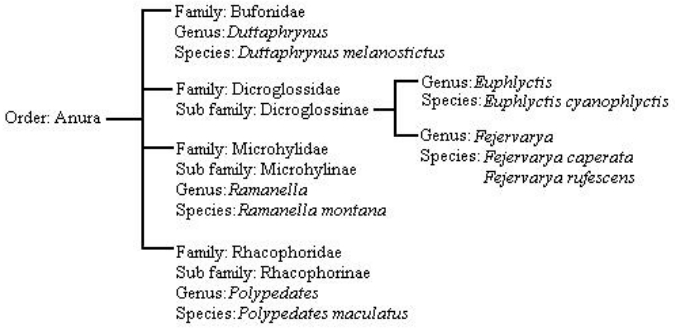

Figure – 4
The conventional taxonomic grouping of the studied anurans as documented by Frost, 2011

Nei's genetic distance values varied as exhibited in table 4 and figure 3 based on the count of H bands or H and M bands or H, M and L bands. The toad, *Duttaphrynus melanostictus*, (family Bufonidae) is placed at the farthest end compared to the other anurans based on H, M and L bands, whereas *Polypedates maculatus* (an arboreal frog) is located at the farthest end compared to the other anurans based on H and M bands.

Polymorphism information content (PIC), Nei's gene diversity (which is equivalent to the average heterozygosity), Shanon's information index (I) values did not differ much based on the count of H and M or H, M and L bands and also the Gene flow (Nm) value being zero among ecologically diverse anurans studied in the present study as shown in table 3 suggest a relatively low dispersal ability known in amphibians[3, 28-30] which probably relates to their respective reproductive strategies.

The present data thus indicates the presence of a varied genotype among the studied anurans suggesting that ISSR is a useful tool to differentiate and exhibit genetic relatedness among taxa at the genus level. However, an intensive study on more representive species from each of the habitat is needed to pinpoint the importance of the unique bands as the genus / species / ecological molecular markers.

Conclusion

Inter Simple Sequence Repeat (ISSR) is a useful tool to differentiate the taxa at the genus level. The Unique bands are of importance as molecular marker. Absence of monomorphic and the presence of 100% polymorphic bands among anurans (frogs and toads) belonging to five genera placed under four families suggest a high level of genetic variability pointing to the diverse adaptive fitness among anurans supporting to their ecological demands. The UPGMA based dendrograms based on the count of high and moderate amplified bands is more close to the conventional grouping pattern of the taxa based on the phenotypic characters, as the species with more similar morphological characters shared higher similarity on ISSR profiles. Gene flow value suggests low dispersal ability among amphibians. However, an intensive study on more representive species from each of the habitat is needed to pinpoint the importance of the unique bands as the genus / species / ecological molecular markers.

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