**International Journal of Global Science Research** **ISSN: 2348-8344 (Online)**
**Vol. 8, Issue. 2, October 2021, pp. 1518-1530** **DOI: 10.26540/ijgsr.v8.i2.2021.178**
***Available Online* at** www.ijgsr.com


**Research Paper**

# Molecular characterization of species of *Fejervarya* (Amphibia: Anura: Dicroglossidae), known to be a culinary delicacy, used as novelties and curious: A study to focus their conservation.

Bahuguna Archana[1*], Singh Anjali[2] and Majumdar Soham[3]
[1]High Altitude Regional Center, Zoological Survey of India, Saproon, Solan, Himachal Pradesh, India
[2]DBS PG College (Srib Dev Suman University), Karanpur Road, Chironwali, Dehradun, Uttarakhand, India
[3]Forest Research Institute, Deemed to be University, Kaulagarh Road, Dehradun, Uttarakhand, India
*Corresponding author email: archana.bahuguna65@gmail.com

Received: 08/06/2021
Revised: 13/06/2021
Accepted: 24/06/2021

**Abstract:** Frogs are traded not only for meat, but they are also being caught and killed for use in making novelties and curios for the tourist industry such as purses and key chains. The skin of frog is also used in the leather and glue trade. In China thirty two species are recognised as components of traditional Chinese medicine. The selling of *Fejervarya limnocharis* is common as food in Southeast Asia, including Thailand, Laos, and Cambodia but is widely regarded as culinary delicacies in most region of the world including Europe, USA and Australia. The present study describes the use of 12SrRNA as marker for identification of species of *Fejervarya* based on molecular data like haplotypes, haplotype diversity, conservation sites, variable sites, parsimony informative sites, singleton sites, nuclear diversity, Tajima's d, average number of nucleotide difference, In Del polymorphism. Road killed and dead 15 specimens of species of *Fejervarya* from DehraDun, Uttarakhand, India were collected in the present study and processed for molecular analysis using 12SrRNA as marker and other genetic data including 21 taxa of *Fejervarya*. The work is useful in identification of species and the origin of wildlife parts and products, so that conservation strategies can be adopted for the control of overexploitation of the species and also to develop strategies by regulating the food consumption.
**Keywords:** 12srRNA, *Fejervarya*, Molecular characterization, Wildlife parts and products

## Introduction:

The selling of *Fejervarya limnocharis* is common as food in Southeast Asia. It is also known as culinary delicacies in most region of the world including Europe, USA and Australia (Jening and Hayes, 1985; Martin, 2000; Patel, 1993; Torok,

**International Journal of Global Science Research** **ISSN: 2348-8344 (Online)**
**Vol. 8, Issue. 2, October 2021, pp. 1518-1530** **DOI: 10.26540/ijgsr.v8.i2.2021.178**
***Available Online* at** www.ijgsr.com


2003). In Cambodia, *Fejervarya limnocharis* is frequently collected for human consumption, along with *Hoplobatrachus rugulosus*, *Glyphoglossus molossus*, *Kaloula pulchra*, *Duttaphrynus melanostictus* and *Pelophylax laterali* (Neang, 2010). Frogs are traded not only for meat, but they are also being caught and killed for use in making novelties and curios for the tourist industry such as purses, key chains, for leather and glue trade (Pough *et. al*. 1998). In China thirty two species are recognised as components of traditional Chinese medicine (Carpenter *et al.*, 2007). The depletion of a giant *Limnonectes* species in Sumatra is due to overexploitation of the species to make stuffed ornaments. *Fejervarya cancrivora* is the source of around three fourth of Indonesia's exported frog legs, to consume as food (Kusirini and Alford 2006).It is estimated that between 180 million to a billion frogs are collected from the wild in Asia alone each year (http://www.amphibiaweb.org/declines/exploitation.html). Thus the frog trade has raised concern related to the decline of population of certain frog species around the world; moreover the industry has not been properly monitored. Frog farming is also introduced in some countries in order to meet the demand for frogs, an initiative to reduce pressure on wild populations, but can cause risks of the introduction of non-native, potentially harmful exotic species and the spread of chytrid fungus and other diseases (Jennings and Hayes, 1985). It was noted that harvesters in west Java are exporting skinless leg mass of *Limnonectes macrodon* (IUCN status Vulnerable 2004) *Fejervarya cancrivora*. In Indonesia *Limnonectes macrodon* is now considered uncommon, which was known to be common earlier. The price of *Limnonectes macrodon* was usually higher than that for rice field frogs. 83.2% of the total exported frog legs to Europe is contributed by Indonesia (Ohler and Nicolas, 2017). In 1985, two edible frogs species from India and Bangladesh (*Euphlyctis hexadactylus* and *Hoplobatracus tigerinus*) were included in CITES Appendix II due to overexploitation and the decline in their populations (Abdulali, 1985; Dash and Mahanta, 1993; Pandian and Marian, 1986). It was also noted by Schumuck, in 2000 that Indonesian exports of frog legs rose markedly and exports from Bangladesh and India decreased.

It is difficult to identify the species in trade and also the numbers as the frog's legs are usually exported without their skins thus difficult to identify. Even if they were identified the documentation in export papers may be incorrect (Veith *et al.*, 2000). In the present study we collected the specimens of species of *Fejervarya* from Dehradun, Uttarakhand, India and processed for molecular analysis using 12SrRNA as marker.The present study describes the use of 12SrRNA as marker for identification of species of *Fejervarya* based on haplotypes and other molecular data like haplotypes, haplotype diversity, conservation sites, variable sites, parsimony informative sites, singleton sites, nuclear diversity, Tajima's d, average number of nucleotide difference,In Del polymorphism, phylogenetic trees and haplotype network of species (21 taxa) of *Fejervarya* by using 12SrRNA for their identification and origin. The work is useful in identification of species and the origin of wildlife parts and products, so that conservation strategies can be adopted for the control of overexploitation of the species and also the strategies for conservation of the species of the genus by regulating the food consumption.

## Materials and Methods:

Road killed and dead 15 specimens of *Fejervarya* species from Dehradun, Uttarakhand, India were collected and washed with sterile Milli-Q water &

International Journal of Global Science Research
Vol. 8, Issue. 2, October 2021, pp. 1518-1530
*Available Online* at www.ijgsr.com

ISSN: 2348-8344 (Online)
DOI: 10.26540/ijgsr.v8.i2.2021.178

ethanol 70% (v/v) respectively. DNA was isolated from the leg muscles using HiPur A™ Forensic Sample Genomic DNA Purification Kit (HIMEDIA) following manufacturer protocol.

12s rRNA sequences were generated using a set of primer pair, L1091 and H1478 (Kocher *et al*., 1989). PCR reaction was performed in Q-cycler, Quanta Biotech, in a total volume of 25µl of reaction mixture (10X PCR-with $MgCl_2$, 2.5µl; 10mM dNTP's, 2.5µl; 5 pmol primer, 0.45µl each; 15ng of DNA template; 1.5U Taq enzyme). Polymerase chain reaction consisted of initial denaturation of 94°C for 4 minutes and each cycle of denaturation for 1 min at 94°C, hybridization for 1 min at 55°C and extension for 1 min at 72°C followed by final elongation for 10 min at 72°C was done in EppendorfmastercyclerX50. The cycle was set for 35 times. We sequenced the PCR products using ABI's AmpliTaq FS dye terminator cycle sequencing chemistry on an automated ABI 3100 Genetic Analyzer. Negative controls were used in all DNA extraction and PCR amplification to control for potential contamination.12SrRNA gene. The partial sequences i.e. 488 bp were submitted to NCBI after conducting sequence alignment by Bioedit and by checking their similarity with species of genus *Fejervarya*. Accession numbers were obtained of five partial gene sequences of the samples, submitted to NCBI i.e. MT768054-MT768058.

## Mitochondrial DNA analysis

Partial Sequences (488bp) of 12SrRNA thus generated, edited using Chromas 1.6 (Technelysium Pty Ltd., South Brisbane Australia). Quarry sequences were crosschecked, and compared using GenBank BLAST (http://www.ncbi.nlm.nih.gov/BLAST).
CLUSTAL W was used to compare DNA sequence data implemented in BioEdit v 7.0.9.0 software (Hall, 1999) with outgroups *Limnonectes fujianensis, Sphaerotheca breviceps, Occidozygma lima* and *Occidozygma martensii*. All sequences were proof read and analyzed by using MEGA- X (Kumar *et. al*., 2018) and were aligned by using ClustalW (Thompson *et. al*., 2003). MEGA- X and DNA sp were used for finding the haplotypes, haplotype diversity, conservation sites, variable sites, parsimony informative sites. Network ver 10 was used to generate haplotype network.

## Results:

Genetic data for all sequences examined (number of sequences 36 belonging to 21 taxa) indicated 203 conserved sites with 0.57 conserved threshold, 225 number polymorphic sites, 34 number of Singleton sites, 30 haplotypes, 0.9810 haplotype diversity, 0.18176 nucleotide diversity, 0.58632 Tajima's d and 71.25average number of nucleotide difference (k), 4.595 Insertion deletion polymorphism InDel polymorphism, 191 Pi Parsimony informative sites (Table 1).
*Fejervarya syhadrensis* vs *Fejervarya limnocharis* indicated 412 conserved sites, 0 variable sites, 0 singleton and 0 parsimony informative sites. *Fejervarya cancrivora* vs *Fejervarya limnocharis* reported conserved sites 329, variable 93, parsimo informative sites 89 and singleton sites 4; *Fejervarya syhadrensis* DehraDun vs *Fejervarya cancrivora*study indicated conserved sites 278, variable sites 146, parsimo iformative sites 144, singleton sites 2. *Fejervarya syhadrensis* DehraDun vs *Fejervarya limnocharis* study for genetic data indicated conserved sites 300, variable sites 116, parsimo-informative 114 and singleton sites 2; (Table 2). The nucleotide frequencies were A = 30.40%, T/U = 24.00%, C = 25.8%, and G = 19.8%.

International Journal of Global Science Research
Vol. 8, Issue. 2, October 2021, pp. 1518-1530
*Available Online* at www.ijgsr.com

ISSN: 2348-8344 (Online)
DOI: 10.26540/ijgsr.v8.i2.2021.178

The phylogeny analysis resulted in formation of 10 clades in the phylogeny using Maximum Likelihood with Kimura 2 para-meter model as test model (Figure.1). *F. kudremukhensis* formed a subclade with *F. nilgarica*. *Fejervarya* species of Nepal Chitwan formed a clade with *F. granosa* and *Fejervaryan* species of Assam. Close to them is the clade of *F. greenei* and *F. kirtinghei* of Sri Lanka Hakgola. *F. rufescens* of India, Mangalore formed a clade with *Fejervarya* species from Andaman island. *F. mudduraja* India Madiken and *F. kalinga* formed the separate clade. *F. cancrivora* formed a separate clade. *F. triora* formed the clade with *F. sakishimensis* Japan, a species from Iriomote island. *F multistrata* of Taiwan Green Island formed a clade with *Fejervarya* species of Japan, Hiroshima. *F. limnocharis* formed the separate clade with *F. multistriata* of China. *F. iskandari* noted to be close to *F. orissaensis*, Orissa, India (Figure 1).

**Table 1 Molecular characteristic data for species of *Fejervarya* examined**

| No. of Species | N | C &CT | V | S | H | Hd | Π | D | K | InDel | Pi |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 21 | 36 | 203 & 0.57 | 225 | 34 | 30 | 0.981 | 0.18176 | 0.58632 | 71.2 551 | 4.595 | 191 |

N Number of sequences, C Conserved sites, CT Conservation Threshold, V Number polymorphic sites, S number of Singleton sites, H total number of haplotypes, h haplotype diversity, π nucleotide diversity, D Tajima's d and average number of nucleotide difference (k) , Insertion deletion polymorphism InDel p, Pi Parsimony informative sites.

**Table 2 Comparative molecular data for *Fejervarya limnocharis*, *Fejervarya cancrivora*, *Fejervarya syhadrensis***

| Species | N | C | V | S | Pi |
|---|---|---|---|---|---|
| *F.limnocharis* | 6 | 403 | 6 | 4 | 2 |
| *F.cancrivora* | 4 | 367 | 53 | 3 | 50 |
| *Fejervarya syhadrensis* from DehraDun (Phulsanee village) | 5 | 412 | 0 | 0 | 0 |
| *Fejervarya syhadrensis* vs *Fejervarya limnocharis* | 1 vs 6 | 322 | 87 | 84 | 3 |
| *Fejervarya cancrivora* vs *Fejervarya syhadrensis* | 4 vs 1 | 290 | 130, | 64 | 66 |
| *Fejervarya cancrivora* vs *Fejervarya limnocharis* | 4 vs 6 | 329 | 93 | 4 | 89 |
| *Fejervarya syhadrensis* DehraDun *vs Fejervarya limnocharis* | 5 vs 6 | 300 | 116 | 2 | 114 |

N Number of sequences, C conserved sites, V variable sites, S singleton sites, Pi Parsimony informative sites

**International Journal of Global Science Research** **ISSN: 2348-8344 (Online)**
**Vol. 8, Issue. 2, October 2021, pp. 1518-1530** **DOI: 10.26540/ijgsr.v8.i2.2021.178**
***Available Online* at** [www.ijgsr.com](http://www.ijgsr.com)


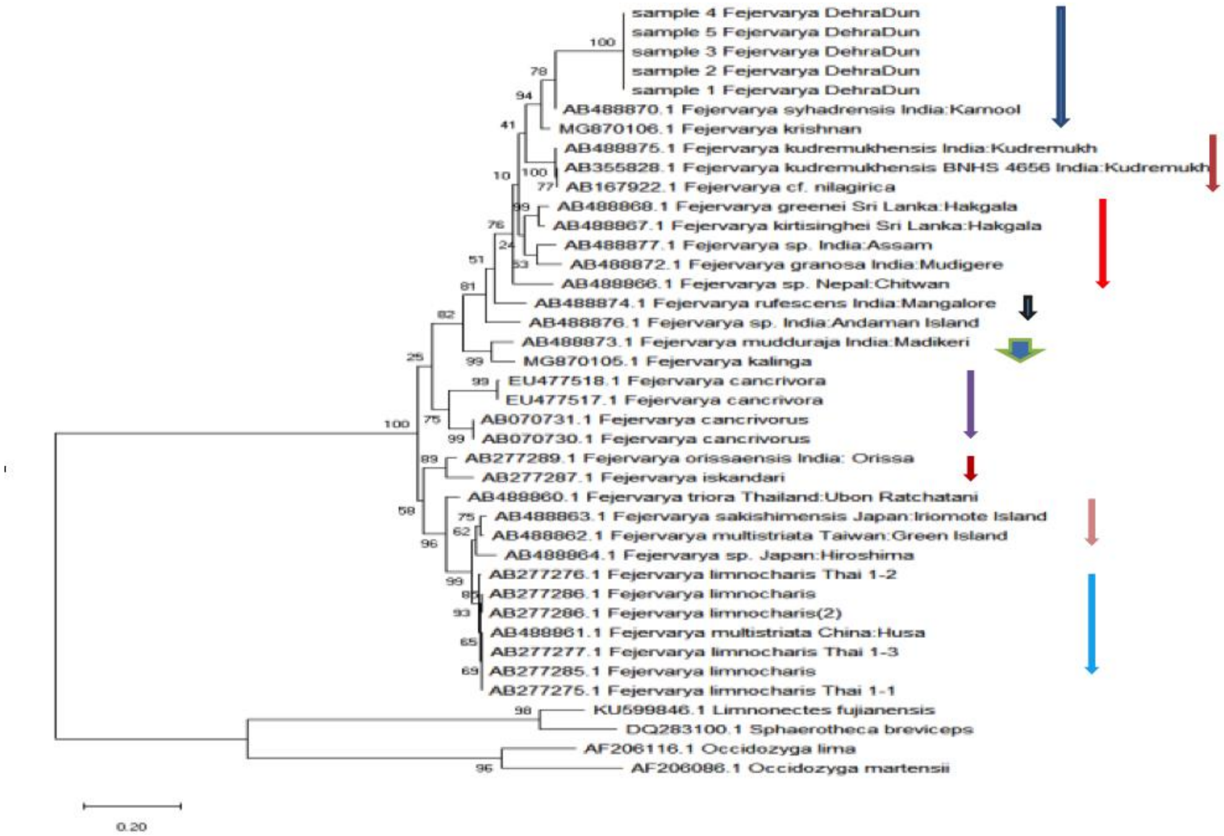


**Figure 1: Maximum Likelihood tree based on 12S rRNA for 36 *Fejervarya* taxa with *Limnonectes fujianensis*, *Sphaerotheca breviceps*, *Occidozygma lima* and *Occidozygma martensii* as outgroup with boot-strap values.**

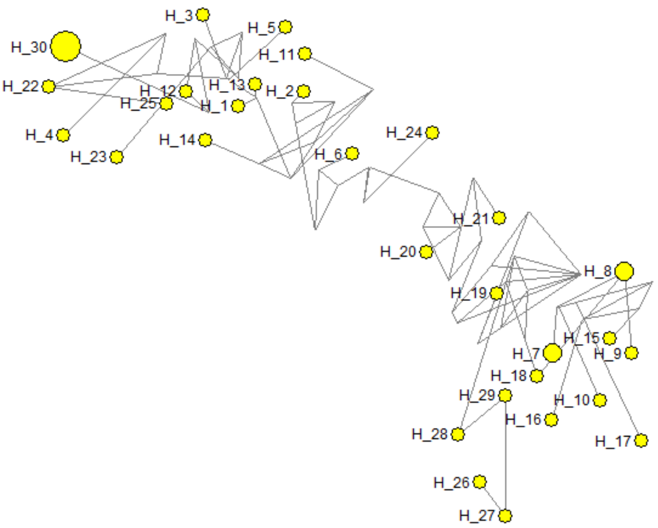


**Figure 2: Haplotype Network of *Fejervarya* species using Network10**

**International Journal of Global Science Research** **ISSN: 2348-8344 (Online)**
**Vol. 8, Issue. 2, October 2021, pp. 1518-1530** **DOI: 10.26540/ijgsr.v8.i2.2021.178**
***Available Online* at** www.ijgsr.com


**Table 3. Haplotypes of species of *Fejervarya* with accession numbers from NCBI**

| Haplotypes | Species | Haplotypes | Species |
|---|---|---|---|
| H1 | *F.greenei* Sri Lanka Hgkgala AB488868.1 | H16 | *F.sakishmemsis* Japan AB488863.1 |
| H2 | *Fejervarya* sp India Assam AB488877.1 | H17 | *F. multistrata*Taiwan AB488862.1 |
| H3 | *Fejervarya* sp India, Andaman island AB488876.1 | H18 | *F.multistrata* China Husa AB488861.1 |
| H4 | *F.kudremukhensis* AB488875.1 | H19 | *F. trio* Thailand AB488860.1 |
| H5 | *F.rufescens* India, Mangalore AB488874.1 | H20 | *F.orissaensis* [AB277289.1] |
| H6 | *F.muduraja* India Madikeri AB488873.1 | H21 | *F.iskandari* AB277287.1 |
| H7 | *F.limnocharis* AB277286.1 AB277286.1 | H22 | *F.kudremukhensis*India AB355828.1 |
| H8 | *F.limnocharis*, AB277285.1 *F.limnocharis* Thai AB277275.1 | H23 | *F.krishnan* [MG870106.1 |
| H9 | *F.limnocharis* Thai AB277277.1 | H24 | *F.kalinga* MG870105.1] |
| H10 | *Fejervarya limnocharis* Thai AB277276.1 | H25 | *Fejervarya nilgarica* AB167922.1 |
| H11 | *F granosa* India Mudigera AB488872.1 | H26 | *F.cancrivora* EU477518.1 |
| H12 | *F.syhadrensis* Karnool AB488870.1 | H27 | *F.cancrivora* EU477517.1 |
| H13 | *Fejervary akirtisinghi* Sri Lanka Hakgala AB488867.1 | H28 | *F.cancrivora* AB070731.1 |
| H14 | *Fejervarya*sp Nepal Chitwan [AB488866.1] | H29 | *F.cancrivora* AB070730.1 |
| H15 | Hap_15: *Fejervarya*sp Japan, Hiroshima AB488864.1 | H30 | *Fejervarya syhadrensis* From Phulsanee, DehraDun, Uttarakhand, India |

**Discussion:**

First proposed in 1915 by István József Bolkay, a Hungarian naturalist, *Fejervarya* is one of the Asian genera of frogs in the Dicroglossidae family. Biochemical and molecular phylogenetic analyses (Toda *et. al.*, 1998; Kurabayashi *et. al.*, 2005; Djong *et. al.*, 2007) indicated the existence of several cryptic species in *Fejervarya* from its wide distribution range. Dubois and Ohler, 2000; Veith *et. al.*, 2001 partially revised the taxonomy of the *Fejervarya* species from southeastern Asia. However the status of southern Asian species is still in confusion, except for those of Nepal and

**International Journal of Global Science Research** **ISSN: 2348-8344 (Online)**
**Vol. 8, Issue. 2, October 2021, pp. 1518-1530** **DOI: 10.26540/ijgsr.v8.i2.2021.178**
***Available Online* at** www.ijgsr.com


Sri Lanka (Dutta and Manamendra, 1996; Manamendra and Gabadage 1996). Dubois and Ohler (2000) summed up some phonetic differences that exist between *Fejervarya* Bolkay, 1915 and *Limnonectes* Fitzinger, 1843, such as shape of the digits of adults (Dubois and Ohler, 2000), their male secondary characters (Boulenger,1920) and the differences in the mouthparts of their tadpoles (Fei *et. al.*, 1991). Dubois and Ohler, 2000 pointed out the existence of a dark ventro-lateral line from ampit to groin, a unique common derived character only to be found in *Fejervarya.* Frogs of the genus *Fejervarya* are distributed throughout South and Southeast Asia, from India, Sri Lanka, and Nepal eastwards to Indonesia, China, and Japan (Frost, 2007). *Fejervarya*, is known to be distributed from eastern India (Orissa) eastwards through Myanmar to southern China to the islands of the Sunda Shelf as well as Japan. The widespread Cricket Frog (*F. limnocharis*) have also been suspected to be cryptic species complexes. The call characteristics of the males were known to be the main criteria along with the morphological features for differentiating the species (Frost, 2007, Dubois and Ohler 2000). Moreover identification of species of the genus from food products like legs, curios and novelties etc. is not possible unless and until molecular tools and techniques are applied.

In India 35 species has been listed under genus *Fejervarya* by Dinesh *et. al*., 2015 and all of them are data deficient or least concern except two species *F. nicobariensis* (Stolicka, 1870) and *F. nilagirica* (Jerdon, 1853) which has been listed as endangered under IUCN. There are around 38 species of *Fejervarya* present in Asian countries, and some of them are about to extinct due to environmental conditions, pollution , overexploitation as food, overharvesting, habitat destruction, overexploitation as novelties, curios and climate change (Zhigang *et. al.*, 2004).

As much as 75% of Indonesia's exported frog legs for food consumption consists of *Fejervarya cancrivora* (Kusirini and Alford, 2006). Frog consumption among local people in Cambodia is widespread, and many communities depend on collecting frogs to supplement protein intake and also to generate additional income (Allen *et. al*., 2008). In Combodia the focus on herpetological studies is on taxonomic and systematic work (Grismer *et. al*., 2007a, 2007b, 2008, Ohler *et. al.*, 2009; Stuart *et al.* 2006a, 2006b) and very few studies have been done on ecological aspects and impact on species of frogs due to over exploitation. It was reported officially in Cambodia that 15 tons of frogs were exported in the past few years, but without any information on where these frogs were going, or what species were being collected (Allen *et. al*., 2008).

Manabu *et. al*., in 2010 carried out molecular study for species identification and solve phylogenetic problems related to this genus by using 67 *Fejervarya* specimens from 12 Asian countries and sequenced part of the mitochondrial (mt) Cytochrome b gene, 12S and 16S rRNA genes and seven nuclear genes (BDNF, CXCR4, NCX1, RAG-1, RAG-2, Rhod, and Tyr) for 25 *Fejervarya* taxa. These molecular markers appear to be adequate for the identification of species. They subjected the molecular data to phylogenetic analyses and their study indicated that *F. limnocharis* and "*F. multistriata*" (from China) formed a clade. On the other hand, neither "*F. limnocharis*" from the Japan mainland nor "*F. limnocharis*" from eastern Taiwan formed a clade with *F. limnocharis,* similar results were also obtained in the present study (Figure 1). Their results also suggest that several cryptic species may be included among the widely distributed *Fejervarya* species. Their datasets support

**International Journal of Global Science Research** **ISSN: 2348-8344 (Online)**
**Vol. 8, Issue. 2, October 2021, pp. 1518-1530** **DOI: 10.26540/ijgsr.v8.i2.2021.178**
***Available Online* at** www.ijgsr.com


paraphyly for the genus *Fejervarya*. In present study we produced the phylogeny by using 12SrRNA gene sequences of 21 species (with total sequences 36) with *Limnonectes fujianensis*, *Sphaerotheca breviceps*, *Occidozyga lima* and *Occidozyga martensii* as outgroups. Maximum Likelihood was applied using Kimura 2 para-meter model. Five gene sequences of *Fejervarya* (accession numbers MT768054, MT768055, MT768056, MT768057 and MT768058 from NCBI) from Dehradun, Uttarakhand formed a clade with *Fejervarya syhadrensis* from India, Karnool and also with *Fejervarya krishnan*. *Fejervarya syhadrensis* has morphological features like the presence of distinct circular spots on hind limbs (both in male and female) with pale colour center, pointed snout, basic dorsal colour olive green with distinct mid dorsal line, ventrum smooth and yellow. In male the ventrum on throat is with black spots & the hind limbis with intense yellow colour on ventrum. The digital formula (fingers) is 3>1>2>4. Subarticular tubercles are rounded with oval inner metatarsal tubercles and two additional oval palmer tubercles. The subarticular tubercles smaller than those of the fingers with oblong laterally flattened inner metatarsal tubercles and smaller outer tubercles. All these features are noted to be present in all adult specimens and subadult specimens of the intact samples collected from DehraDun, Uttarakhand. Further molecular analysis revealed that the samples collected belong to *Fejervarya syhadrensis* (Annandale). BLAST search also showed 99.9% similarity with *Fejervarya syhadrensis* (Annandale).Total of 10 clades were formed in the phylogeny using Maximum Likelihood with Kimura 2 para-meter model as test model (Figure.1). Among these clades *F. kudremukhensis* formed a subclade with *F. nilgarica*. i.e. Kudremukh Cricket frog, is endemic to the central Western Ghats of Karnataka State, India. The name *kudremukhensis* refers to the type locality, Kundremukh (Frost et al. 2013). *F. nilgarica* is a species of frog endemic to Western Ghats, India. It is known from Nilgiri mountains in Tamil Nadu and district in Karnataka (IUCN threatened) (Frost et al. 2013, Biju *et al.* 2016). *Fejervarya* species of Nepal Chitwan formed a clade with *F. granosa* and Fejervaryan species of Assam. Close to them is the clade of *F. greenei* and *F. kirtinghei* of Sri Lanka Hakgola. *F. rufescens* of India, Mangalore formed a clade with *Fejervarya* species from Andaman island. *F. rufescens* has type locality Malabar. *F. mudduraja* India Madiken and *F. kalinga* formed the separate clade. *F. cancrivora* formed separate clade. *F. triora* formed the clade with *F. sakishimensis* Japan, a species from Iriomote island. *F multistrata* of Taiwan Green Island formed a clade with *Fejervarya* species of Japan, Hiroshima. *F. limnocharis* formed the separate clade with *F. multistriata* of China Husa. *F. iskandari* is close to *F. orissaensis*, Orissa, India. Among all species of genus *Fejervarya* present in India, only few i.e. *F. nepalensis* (Dubois), *F. pierrie* (Dubois), *F. teraiensis* (Dubois), *F. syhadrensis* (Annandale), *F. sengupti* (Purkayastha and Matusi) have been reported from Northern and Northeastern India, including recently described species by Archana Bahuguna i.e. *Fejervarya jhilmilensis* in 2017 from JhilmilJheel, Haridwar, Uttarakhand. The specimens thus analyzed from Dehradun belong to *F. syhadrensis*, India Kurnool with bootstrap value 94. Moreover genetic data obtained in the present study (Tables 1, 2, 3) and haplotype network (Figure 2) produced by using NETWORK 10 are also useful to know the origin of the species as well as for identification of the species.

Molecular tool thus is effective in identification of species of *Fejervarya* to know their origin by generating haplotypes

**International Journal of Global Science Research** **ISSN: 2348-8344 (Online)**
**Vol. 8, Issue. 2, October 2021, pp. 1518-1530** **DOI: 10.26540/ijgsr.v8.i2.2021.178**
***Available Online* at www.ijgsr.com**


for effective conservation plans as presented in the present study (Figures 1, 2). Amphibians globally are facing a growing crisis, with between a third and one half of all known species threatened with extinction (Stuart *et. al*., 2004). Although new amphibian species are being discovered and described every year, with 6433 species currently recognized worldwide (Frost *et. al.,* 2006) but recent studies have shown that amphibian populations are drastically declining across the planet due to various threats (Stuart *et. al*., 2004; Rowley *et. al*., 2009). The International Union for Conservation of Nature declared 2008 the "Year of the Frog"; while the World Association of Zoos and Aquariums established the "Amphibian Ark" an initiative to start a captive breeding programme for the most threatened species (Attenborough 2008). Over-collection of *Rana draytonii* during the Californian gold rush of 1849 caused a significant depletion in the abundance of this species within 20 years (Jennings and Hayes, 1985). Similar situation was noted in case of the Indian bullfrog *Hoplobatrachus tigrina.* There is need to find out the exact status of *Hoplobatrachus tigrina* and species of *Fejervarya* and also to keep an eye on its trade. In China a ban on harvesting the frogs was imposed after over-collecting resulted falling frog numbers but a reciprocal increase in the agricultural insect pests on which this frog feeds (Fugler, 1985). *Fejervarya limnocharis* is commonly sold as food in Southeast Asia, including Thailand, Laos, and Cambodia. It is frequently collected for human consumption, along with *Hoplobatrachus rugulosus, Glyphoglossus molossus, Kaloula pulchra, Duttaphrynus melanostictus* and *Pelophylax lateralis* in Combodia. In Southeast Asia, the crab-eating frog is locally hunted for food and is often farmed for its edible legs (Neang, 2010). In the Southeast Asian region, Indonesia is known historically been the largest exporter of frog's legs (Warkentin *et al*., 2009) with 5,600 tons exported in 1992 (Kusirini and Alford, 2006); while in 1981 alone India exported an estimated total of 4,368 tons (http://www.american.edu/ted/frogs.htm). Harvesting of amphibians is often associated with the rural poor supplementing their diet but the trade in frogs is a worldwide business. Legs of bullfrogs from Asia, are exported to Europe as a delicacy. It is reported that 6,000 tons of frog legs were imported to Europe each year during the 1990s (Jensen and Camp, 2003) and in 1999, the quantity reported to rise to 9700 tons. The chief importers are Belgium, Luxembourg and France (Warkentin *et. al*., 2009; Patel, 1993). Currently United States is reported to import more than 3000 tons of frog meat a year from abroad (https://www.amphibiaweb.org) and harvested 5200 tons of wild caught frogs between 1998 and 2002 (Martin *et. al*., 2005). Between 1981-1984, over 6 million rugulose frogs *Hoplobatrachus rugulosus*, caught from the wild, were exported from Thailand to Hong Kong (Wai-Neng Lau *et al.* 1999). All these reports indicates that such a huge numbers of export of frog legs and its use as curious and novelties is likely to be causing an adverse effect on the frog populations and the ecosystems and is a serious issue to take care.

**Conclusion:**

Genetic data with haplotype network and phylogeny produced by using 12srRNA partial gene sequences for 21 taxa of *Fejervarya* is useful for identification of the species of *Fejervarya* as well as for getting the information of the origin of species of the genus which is a source of food, novelties, curious and other ornamental products. From our study it was concluded that 12S rRNA is a feasible marker for differentiating the species of

International Journal of Global Science Research
Vol. 8, Issue. 2, October 2021, pp. 1518-1530
*Available Online* at www.ijgsr.com

ISSN: 2348-8344 (Online)
DOI: 10.26540/ijgsr.v8.i2.2021.178

genus *Fejervarya* and can be used for identification of species of *Fejervarya*. We also recommend the following strategies to be adopted to enhance the conservation of amphibians.

## Recommendations:

In India, there is need to do the status survey of the species of *Fejervarya* and also in other Asian countries.

1. More studies need to be done on ecological and molecular aspects of the species of the genus as genetic data are lacking for delimiting the species of the genus.
2. Permits to be given by authorized Administration of Ministry of Forestry and Environment of the concerned country for export/ import of frog legs and other products.
3. Authorized officials at all border checkpoints should check the regulations of export and import. In case valid permits are not with exporters and importers then fines must be imposed.
4. Introduction of harmful exotic species should be avoided, and also there is need to take measures to prevent the spread of chytrid fungus.
5. The import of non-native frog species for frog farms must be banned.
6. All harvesting persons in the country where amphibian harvesting is going on should be registered by concerned Ministry. Awareness programmes need to be conducted to educate villagers and farmers about maintaining a healthy frog population in agriculture fields and in village. Conduct various programme highlighting the beneficial effects on the careful use of pesticides to avoid frog and other crop pest predator mortality.

## Acknowledgements:

We are grateful to the Director, Zoological Survey of India, Dr. Kailash Chandra for providing necessary facility for the work and encouragement. The practical work for molecular study was done at Molecular Systematic Laboratory of Northern Regional Center, Zoological Survey of India. We are thankful to Officer in Charge Dr. Avtar Kaur Sidhu for the support to carry out this work. The work was accomplished with the fund support by Ministry of Forest, Environment and Climate Change, New Delhi, India and for this the authors are highly obliged to MoEFCC.

## References:

Jennings M. R. and Hayes M. P. (1985) Pre-1900 overharvest of California red-legged frogs (Rana aurora draytonii): The inducement for bullfrog (*Rana catesbeiana*) introduction. Herpetologica, 94-103.

Martin R. E. (2000) Other aquatic life of economic significance: Frogs and frog legs. Marine and Freshwater Products Handbook, Technomic Pub. Co. Inc., Lancaster, 279-287.

Patel T. (1993) French may eat Indonesia out of frogs. New Scientist 1868, 7

Torok Z. (2003) Action plan for sustainable exploitation of *Ranaridibunda* stocks from the Danube delta biosphere reserve (Romania) Frog leg, (60).

Neang T. (2010) An investigation into frog consumption and trade in Cambodia. Flora and Fauna International, Cambodia Programme, Phnom Penh, Cambodia.

Pough F. H., Andrews R. M., Cadle J. E., Crump M. L., Savitsky A. H, Wells K. D. (2004) Herpetology, 3rd edn. Prentice Hall, New Jersey.

Carpenter A. I., Dublin H., Lau M., Syed G., Mckay J. E. and Moore R. D. (2007). Chapter 5: Over-harvesting. In: Gascon, C., Collins, J.P., Moore, R.D., Church, D.R., McKay J.E.and Mendelson J.R. III (eds.), Amphibian Conservation Action Plan, Gland, International Union for Conservation of Nature and Natural Resources: 26-31.

Kusrini M. D. and Alford R. A. (2006). Indonesia's exports of frogs' legs. Traffic Bulletin, 21, 13-24.

http://www.ncbi.nlm.nih.gov/BLAST

https://www.amphibiaweb.org/declines/exploitation.html

Ohler A., and Nicolas V. (2017) Which frog's legs do froggies eat? The use of DNA barcoding for identification of deep frozen frog legs (Dicroglossidae, Amphibia) commercialized in France. European Journal of Taxonomy, 271: 1-19.http://dx.doi.org/10.5852/ejt.2017.271

Abdulali H. (1985) On the export of frog legs from India. Journal of the Bombay Natural History Society. Bombay, 82(2), 347-375.

Dash M. C. and Mahanta J. K. (1993) Quantitative analysis of the community structure of tropical amphibian assemblages and its significance to conservation. Journal of biosciences. 18(1), 121-139.

Pandian T. J. and Marian M. P. (1986). Production and utilization of frogs: an ecological view. Proceedings: Animal Sciences, 95(3), 289-301.

Schumuck J. (2000). Trade and species conservation. In: R. Hofrichter (ed) The encyclopedia of amphibians. (Ontario, Key Porter Books Lim)., 228-241

Veith M., Kosuch J., Feldmann R., Martens, H. and Seitz A. (2000). A test for correct species declaration of frog legs imports from Indonesia into the European Union. Biodiversity & Conservation, 9(3), 333-341.

Kocher T. D., Thomas W. K., Meyer A., Edwards S. V., Pääbo S., Villablanca F. X., and Wilson A. C. (1989). Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. Proceedings of the National Academy of Sciences, 86(16), 6196-6200.

http www ncbi nih.gov/BLAST

Hall T. A. (1999, January). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In: Nucleic acids symposium series (Vol. 41, No. 41, pp. 95-98). [London]: Information Retrieval Ltd., c1979-c2000.

Kumar S., Stretcher G., Li, M., Kyan C. and Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution **35**: 1547-1549.

Thompson J. D., Gibson T. J. and Higgins D. G. (2003). Multiple sequence alignment using Clustal W and Clustal X. Current protocols in bioinformatics. (1), 2-3.

Toda M., Nishida M., Matusi M. and Ota H. (1998). Genetic divergence among Southeast and East Asian population of Ranalimnocharis (Amphibia: Anura), with species reference to sympatric cryptic species in Java. Zoological Science. 14, 607–613.

Kurabayashi A, Kuramoto M., Joshy H. and Sumida M. (2005) Molecular Phylogeny of the Ranid Frogs from Southwest India Based on the Mitochondrial Ribosomal RNA Gene Sequences, Zoological Science. 22, 525-534.

Djong T. H., Islam M. M., Nishioka M., Matsui M., Ota H., Kuramoto M., Khan M. R., Alam M. S., Khonsue W. and Sumida M. (2007). Genetic relationships and reproductive-isolation mechanisms among the *Fejervarya limnocharis* complex from Indonesia (Java) and other Asian countries. Zoological Science, 24(4), 360-375.

Dubois A., and Ohler A. (2000). Systematics of *Fejervarya limnocharis* (Gravenhorst, 1829)(Amphibia, Anura, Ranidae) and related species. 1. Nomenclatural status and type-specimens of the nominal species Ranalimnocharis Gravenhorst, 1829. Alytes, 18 (1-2), 15-50.

Veith M., Kosuch J., Ohler A and Dubois A. (2001). Systematics of *Fejervarya* *limnocharis* (Gravenhorst, 1829) (Amphibia, Anura, Ranidae) and related species. 2. Morphological and molecular variation in frogs from the Greater Sunda Islands (Sumatra, Java, Borneo) with the definition of two species. Alytes, 19(1), 5-28.

Dutta S. K. and Manamendra-Arachchi K. (1996). The amphibian fauna of Sri Lanka. Wildlife Heritage Trust of Sri Lanka.

Manamendra-Arachchi K., and Gabadage D. (1996). *Limnonectes kirtisinghei*, a new species of ranid frog from Sri Lanka. Journal of South Asian Natural History, 2(1), 31-42.

Boulenger G. A. (1920). A monograph of the South Asian, Papuan, Melanesian, and Australian frogs of the genus *Rana*. Rec Ind Mus, 20, 1-126.

Fei L., Ye C. Y. and Huang Y. Z. (1991). Key to Chinese Amphibia. Chongqing Branch Sci. Technol.

Frost D. R. (2007). Amphibian species of the world: an online reference. Version 5.0 (1 February, 2007). Electronic Database accessible at http://research.amnh. org/herpetology/amphibia/index. php.

Dinesh K. P., Vijayakumar S. P., Channakeshavamurthy B. H., Toreskar V. R., Kulkarni N. U. and Shanker K. (2015) Systematic status of *Fejervarya* (Amphibia, Anura, Dicroglossidae) from South and SE Asia with the description of a new species from the Western Ghats of Peninsular India. Zootaxa, 3999(1), 79-94.

Zhigang Y., Ermizhao Haitaoshi, Diesmos A., Alcala A., R., Brown L., Afuang G., Gee J. Sukumaran, Yaakob N., Leong T. M., Yodchaiy C., Kumthorn T., Das I., Iakandarmumpuni D. and Robert I. (2004) *Fejervarya cancrivora*. IUCN Red List of Threatened Species.

Allen D., Darwall W., Dubois M., Sreng K. K., Lopez A., McIvor A, Springate-Baginski O. and Try T. (2008) Integrating people in conservation planning. An integrated assessment of the biodiversity, livelihood and economic implications of the proposed special management zones in the Stung Treng Ramsar Site. https://portals.iucn.org/library/sites/library/files/documents/2008-018.pdf

Grismer, L. L., Chav, T., Neang, T., Wood, P. L., Grismer, J. L., Youmans, T. M., Ponce A., Daltry J.C. and Kaiser H. (2007a) The herpetofauna of the Phnom Aural Wildlife Sanctuary and checklist of the herpetofauna of the Cardamom Mountains, Cambodia. Hamadryad-Madras-, 31(2), 216.

Grismer L., Thy N., Chav T., and Holden J. (2007b) A new species of *Chiromantis* Peters 1854 (Anura: Rhacophoridae) from Phnom Samkos in the northwestern Cardamom Mountains, Cambodia. Herpetologica, 63(3), 392-400.

Grismer L. L., Neang T., Chav T., Wood Jr P. L., Oaks J. R., Holden J., Grismer J. L., Szutz T. R. and Youmans, T. M. (2008). Additional amphibians and reptiles from the Phnom Samkos Wildlife Sanctuary in northwestern Cardamom Mountains, Cambodia, with comments on their taxonomy and the discovery of three new species. The Raffles Bulletin of Zoology, 56(1), 161-175.

Ohler A., Deuti K., Grosjean S., Paul S., Ayyaswamy A. K., Ahmed M. F. and Dutta S. K. (2009). Small-sized dicroglossids from India, with the description of a new species from West Bengal, India. Zootaxa, 2209 (1), 43-56.

Stuart B. L., Sok K. and T. Neang, (2006)a. A collection of the amphibians and reptiles from hilly eastern Cambodia.The Raffles Bulletin of Zoology, 54,129-155.

Stuart B. L. and Emmett D. A. (2006)b. A collection of amphibians and reptiles from the Elephant and Cardamom Mountains, southwestern Cambodia. Fieldiana Zoology, (New series) 109:1–27.

Manabu Kotaki, Atsushi Kurabayashi, Masafumi Matusui, Mitsuru Kuramoto, Tjong Hon Djong and Masayuki, Sumida (2010). Molecular phylogeny of the diversified frogs of genus *Fejervarya* (Anura: Dicrolossidae). Zoological science

International Journal of Global Science Research
Vol. 8, Issue. 2, October 2021, pp. 1518-1530
*Available Online* at www.ijgsr.com

ISSN: 2348-8344 (Online)
DOI: 10.26540/ijgsr.v8.i2.2021.178

27 (5):386-395. http://doi.org/10.2108/zsj.27.386

Frost D. R. (2013) Amphibian Species of the World: an online reference. Version 6.0 – http://research.amnh.org/vz/ herpetology/amphibia/ (22 Sep. 2013). – (American Museum of Natural History) .

Biju S. D., Senevirathne G., Garg S., Mahony S., Kamei R. G., Thomas A Shouche Y., Raxworthy C. J., Meegaskumbura M. and Bocxlaer, I. V. (2016) Frankixalus, a new rhacophorid genus of tree hole breeding frogs with oophagous tadpoles. PloS one, 11(1), e0145727.https://doi.org/10.1371/journal.pone.0145727https://www.mapress.com/j/zt/article/view/zootaxa.4674.1.5

Stuart S. N., Chanson J. S., Cox N. A., Young B. E., Rodrigues A. S. L., Fischman D. L. and Waller R. W. (2004) Status and trends of amphibian declines and extinctions worldwide.Science 306: 1783- 1786.

Frost D. R., Grant T., Faivovich J. N, Bain R.H., Haas A., Haddad C. F. B., De Sa R.O., Channing A., Wilkinson M., Donnellan S.C., Raxworthy C. J., Campbell J.A., Blotto B. L.,Moler,P., Drewes, R.C., Nussbaum R.A., Lynch J. D., Green D. M. and Wheeler W. C. (2006). The Amphibian tree of life, Bulletin of the Ammerican Museum of Natural History Central Park West, New York, 297, 370 .

Rowley J., Brown R., Raoul B., Kursrini M., Inger R., Stuart B., Wogan G., Neang T., Chanard T., Cao, T. T., Diesmos A., Iskandar D. T., Lau M., Ming L. Tzi, Makchai S., Nguyen Q. T. and Phimmahimmachak S. (2009) Impending Conservation Crisis for Southeast Asian Amphibians. Biol. Lett. published online 9 December 2009, 1-3, (doi: 10.1098/rsbl.2009.0793)

Attenborough, S. D., (2008) Amphibian Ark 2008 YEAR OF THE FROG. Teach The Amphibian Crisis! http://www.amphibianark.org/pdf/Teach%20the%20Amphibian%20Crisis.pdf

Fugler C. M. (1985) A proposed management programme for the Indian bullfrog, *Rana tigrina*, in Bangladesh, comments pertaining to its intensive cultivation with observations on the status of the exploited chelonians.

https://www.american.edu/ted/frogs.html

Jensen J. B. and Camp C. D. (2003) Human exploitation of amphibians: direct and indirect impacts. Amphibian conservation, 199-213.

Warkentin I. G., Bickford D., Sodhi N. S., & Bradshaw, C. J. (2009) Eating frogs to extinction. *Conservation Biology*, *23*(4), 1056-1059.

https://www.amphibiaweb.org

Martín J., Luque-Larena J. J. and López, P. (2005) Factors affecting escape behavior of Iberian green frogs (Ranaperezi). Canadian Journal of Zoology, 83(9), 1189-1194.

Wai-Neng Lau M. Ades G., Goodyer N. and Zou F. S. (1999) Wildlife Trade in Southern China including Hong Kong and Macau. The Biodiversity Working Group: China Council for International Cooperation on Environment and Development, Beijing.