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 Archana¹*, Singh Anjali² and Majumdar Soham³
¹High Altitude Regional Center, Zoological Survey of India, Saproon, Solan, Himachal Pradesh, India
²DBS PG College (Srib Dev Suman University), Karanpur Road, Chironwali, Dehradun, Uttarakhand, India
³Forest 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,
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 *Hoplobatractus tigerinus*) were included in CITES Appendix II due to overexploitation and the decline in their populations (Abdulahi, 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 *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 &
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 MgCl2, 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 martensis. 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 cancrivorastudy 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%.
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 Fejervarya 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. cancivora 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. multiistriata 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

<table>
<thead>
<tr>
<th>No. of Species</th>
<th>N</th>
<th>C &amp; CT</th>
<th>V</th>
<th>S</th>
<th>H</th>
<th>Hd</th>
<th>PI</th>
<th>D</th>
<th>K</th>
<th>InDel</th>
<th>Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>21</td>
<td>36</td>
<td>203 &amp; 0.57</td>
<td>225</td>
<td>34</td>
<td>30</td>
<td>0.981</td>
<td>0.18176</td>
<td>0.58632</td>
<td>71.2</td>
<td>551</td>
<td>4.595</td>
</tr>
</tbody>
</table>

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 cancivora, Fejervarya syhadrensens

<table>
<thead>
<tr>
<th>Species</th>
<th>N</th>
<th>C</th>
<th>V</th>
<th>S</th>
<th>Pi</th>
</tr>
</thead>
<tbody>
<tr>
<td>F. limnocharis</td>
<td>6</td>
<td>403</td>
<td>6</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>F. cancivora</td>
<td>4</td>
<td>367</td>
<td>53</td>
<td>2</td>
<td>50</td>
</tr>
<tr>
<td>Fejervarya syhadrensens from</td>
<td>5</td>
<td>412</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DehraDun (Phulsane village)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fejervarya syhadrensens vs</td>
<td>1</td>
<td>322</td>
<td>87</td>
<td>84</td>
<td>3</td>
</tr>
<tr>
<td>Fejervarya limnocharis</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fejervarya cancivora vs</td>
<td>4</td>
<td>290</td>
<td>130,</td>
<td>64</td>
<td>66</td>
</tr>
<tr>
<td>Fejervarya syhadrensens</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fejervarya cancivora vs</td>
<td>4</td>
<td>329</td>
<td>93</td>
<td>4</td>
<td>89</td>
</tr>
<tr>
<td>Fejervarya limnocharis</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fejervarya syhadrensens vs</td>
<td>5</td>
<td>300</td>
<td>116</td>
<td>2</td>
<td>114</td>
</tr>
<tr>
<td>DehraDun vs Fejervarya limnocharis</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N Number of sequences, C conserved sites, V variable sites, S singleton sites, Pi Parsimony informative sites.
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
Table 3. Haplotypes of species of *Fejervarya* with accession numbers from NCBI

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

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
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 (Zhiang 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 Cambodia 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, RAG1, RAG2, 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
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 parameter 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 limbs 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* 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. multistriata* 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.
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 Cambodia. 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 phylogeony 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
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:**


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


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


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.


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


https://www.amphibiaweb.org
