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# Molecular identification and genetic diversity of apple snail *Pila globosa* by RAPD assay

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## ABSTRACT

*This study was carried out for molecular identification and genetic diversity of freshwater snail Pila globosa in Bangladesh. Traditional taxonomy of different species of the genus Pila has always been obscured and unreliable due to their morphological ambiguity. A rapid and cost effective molecular marker, mitochondrial COI gene was used to establish molecular standards for identification while similar bands were seen in all individuals of Pila globosa at the level of 700 bp of lengths. In addition, genetic diversity of freshwater snail Pila globosa was measured on the basis of PCR-RAPD assay. Three arbitrary primers were screened and 12 polymorphic loci were found in 7 individuals of this experimental species. The percentage of average values of polymorphic loci and polymorphism information content found 21.23 and 0.875 respectively. Highest and lowest Nei genetic similarity was found 0.500 and 0.105 respectively whereas genetic distance was recorded between 0.667 and 0.9444. Furthermore, the highest and lowest linkage distance was observed 17.0 and 9.0 respectively. A distant relationship among individuals was found by studying phylogenetic dendrogram. Though higher genetic diversity was observed in Pila globosa, however, the population of this species in the nature of Bangladesh is not confirm with good genetic status while only seven individuals were investigated.*

**Key words:** Molecular identification, genetic diversity, *Pila globosa*, PCR-RAPD

## INTRODUCTION

The apple snail *Pila globosa* is found in ponds, tanks and rice fields but may also be found in freshwater streams, rivers and even in brackish water of low salinity in Bangladesh (Brown, 1994; Ali and Chakraborty, 1992). Like other snails *Pila globosa* is one of the important mollusks which is environmental friendly agro-economical aquatic organisms, can play a vital role in the development of water quality in aquatic environments. This fauna has not only eco-friendly such as waste treatment but their soft body is also being used in mostly as poultry feed and shell is being used for making fertilizer, lime, etc. Their eggs are generally used as food for fishes, prawns, shrimps, crabs, frogs, turtles and other important aquatic animals. This freshwater snail is also helping the enhancement of prawn and shrimp production by using its flesh as prawn and shrimp feed in their culture of southwest coastal region of Bangladesh (Jahan, 1993). They play a vital role for rice production as natural plough in the rice fields. They have a good medicinal value such as the shell of *Pila* spp. is used in traditional ethnomedicine for weakness of human being (Mahawar

and Jaroli, 2007). Snails including *Pila globosa* are considered as highly digestible and rich in vitamins, fat, proteins and minerals especially calcium. They can play a role for the livelihood of fisherman and fisher women in Bangladesh as well.

Due to several economic demands of this apple snail for the highly priced like 150 to 200 taka/kg led to uncontrolled harvesting and destructive fishing practices by locals and illegal snail poachers, as well as by the overexploitation as shrimp feed, a drastic reduction of snail diversity has been occurred (Jahan *et al.*, 1999). As a result, the populations of apple snails including *Pila globosa* are now a threatened species in the nature of Bangladesh (IUCN, 2000). So, it is now time to save them in nature and that's why their proper identification and genetic variation are needed for future research on breeding, culture and conservation.

Very limited researches have been done on different species of the genus *Pila* in different fields; particularly the molecular identification and genetic study of *Pila* has been done very meagerly while *Pila globosa* is mostly unknown (Thaewnon-ngiw et al. 2003, 2004, Dong et al. 2011). Thus, the objectives of this study are to identify molecular genetic markers capable of facilitating the taxonomic identification of *Pila globosa* and this information will be used in future for developing conservation strategies and management program of this snail in Bangladesh. In this study mitochondrial COI was analyzed for species identification and RAPD assay was studied for genetic diversity of *Pila globosa*. This study will help to develop their adaptation, survival, breeding, culture and stock enhancement, etc.

## MATERIALS AND METHOD

### Sample collection and species identification

The apple snail *Pila globosa* was collected from ponds and canals and transferred them to the Laboratory of the Department of Genetic Engineering and Biotechnology (GEB) at Shahjalal University of Science and Technology (SUST), Sylhet, Bangladesh and kept them into the glass aquariums until tissue isolation. The *Pila globosa* was identified through its morphological characteristics (Ali and Chakraborty, 1992; Jahan, 1993) and seven individuals of this experimental apple snail were considered for this study.

### Tissue isolation and DNA extraction

The snail shell was broken by a small hammer one after another to find out the adductor muscle and visceral mass by using scissors, forceps, and needles. Isolated tissues were kept into the petridish separately and washed them with distilled water. Then the tissues were kept in 1.5 ml eppendorf tubes using 70% ethanol and preserved them at $-20^0$C until DNA extraction.

DNA was extracted from adductor muscles of experimental *Pila globosa* where Krause's (1997) used visceral tissues. DNA quality was checked by electrophoresis on 1% agarose gel with 3µl DNA where 1kb plus ladder was used to compare migration of DNA. The gel was run at 70 volt for 40 minutes. This gel was then placed in gel documentation system and photograph was taken by Semi DSLR camera (Nikon Cool Pix P100 26X Zoom 10.3 MP). Clear bands were found from each individual with quality concentration of DNA.

### PCR amplification

In this study, DNA fragments were analyzed with mitochondrial COI partial (invertebrate universal primer) for species identification which was LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATATTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCAAAA AAT CA-3') (Thaewnon-ngiw et al., 2004). Three RAPD primers, B 03 (5'- CAT CCC CCT G-3') and C 04 (5'- CCG CAT

CTA C-3') by Schwenk *et al*. 1998, and OPB 12 (5'- CCT TGA CGC A-3') by Alam et al (2010) were adopted for studying genetic diversity of *Pila globosa.* PCR reactions were performed each sample in a 15µl reaction mixture containing 8µl of master mix (Promega Hot Start), 2µl of primer, 2µl of template DNA and 3µl deionized distilled water for COI primer. Reactions of COI were amplified through 35 cycles at the following parameters: one minute at 95°C, one minute at 40°C, and one and a half minutes at 72°C, followed by a final extension step at 72°C for seven minutes. PCR reactions of RAPD primers were considered 15µl reaction mixture for each sample with 8µl of master mix (Promega Hot Start), 1µl of primer, 2µl of template DNA and 4µl deionized distilled water. PCR reaction was conducted for pre heating 94˚c 3 minutes, denaturation at 94ºc for 1 minutes; annealing temperature for this PCR was about 34˚c (for B 03)/35ºc (for C 04)/ 34˚c (for OPB 12) in 1 minute and 2 minutes for elongation or extension at 72˚c. A final step of 7 min for 72ºc was added to allow complete extension of the amplified fragments. The PCR was run for 35 cycles. PCR products were checked by electrophoresis on 2% agarose gel with 3µl DNA where 1kb plus ladder was used to compare migration of DNA for mitochondrial COI primers and the gel was run at 70 volt for 40 minutes. All the gels were checked in gel documentation system and photograph was taken by digital camera (Panasonic" DMC-FS20).

**Data Analysis**

Using different software and equations, RAPD data of this experiment was interpreted. The AlphaEaseFC 4.0 software was used for measuring molecular weight of bands. Genetic distance was analyzed by using an equation such as D = 1- Nxy / Nx+Ny- Nxy, where D is the genetic distance between sample x and y, Nxy is number of band shared by sample x and y, Nx is the number of bands in sample x and Ny is the number of bands in sample y. Nei's genetic similarity among individuals were measured by F= 2Nxy/ Nx+Ny, where F is Nei's genetic similarity, Nxy is the number of shared band between X and Y, Nx is the number of band in X, and Ny is the number n of band in Y.

Polymorphism information content (PIC) was calculated by $1- \sum Pij2\ j=1$

where, Pij is the frequency of the jth allele for the ith marker Summed over 'n' alleles. Intra-individual relationship through dandogram was analyzed by using a softwere "Statistica".

## RESULT AND DISCUSSION

**Molecular species identification**

PCR products of an agarose gel with 7 individuals of *Pila globosa* were analyzed by using invertebrate universal mitochondrial COI primers for species identification. All the individuals were banded the sequence at the position of 700bp length based on the 1kb plus ladder (Figure 1) and these seven individuals were considered morphologically as *Pila globosa* as well.

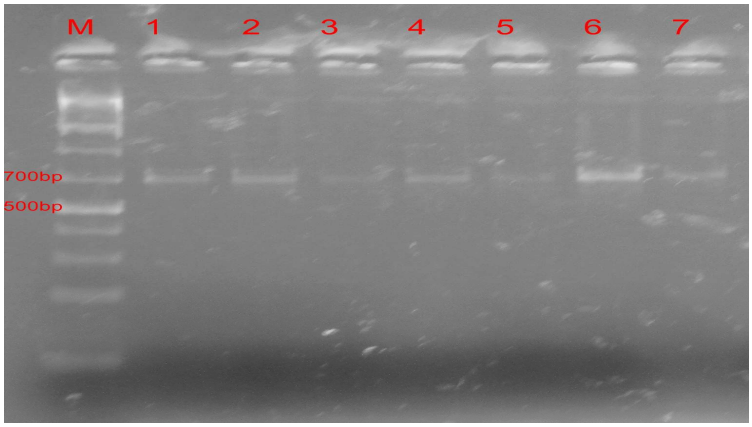


**Figure 1**. Agarose gel electrophoresis showing the specificity of COI DNA at 700bp length in *Pila globosa*

**RAPD based genetic divergence analysis**

**Data profiling and band summary**

In this study three RAPD markers were used to assess genetic diversity among 7 individuals of *P. globosa* genotypes. The bands were compared with 1 kb plus DNA ladder ranged from 75 bp to 20,000 bp (GeneRuler$^{TM}$). Each amplified band profile was defined by the presence or absence of bands at particular positions on the gel. Fragments were scored as 1 if band present or 0 if band absent, separately for each individual and each primer. Very few bands were recorded by B03 primer between the ranges of 270bp-1105bp. Bands were found only 2 to 6 by the primer C04 and the obtained bands were ranged from 85 bp to 485 bp lengths whereas, 1 to 5 bands were ranged from 126 bp to 1670 bp lengths in primer B 12. A total of 56 bands were detected among 7 *P. globosa* individual genotypes out of which 12 were polymorphic bands (Table 1). The highest number of bands (20) amplified by the primer C04 and the lowest number of bands (17) amplified by the primer B03. Primer C04 showed highest polymorphism (25%) and primer B03 showed lowest polymorphism (17.65%) among tested individuals. The highest number of bands (2.85) per individual was amplified from the primer C04 and the lowest number of bands (2.42) per individual was amplified by the primer B03. Primers B03 and OPB12 showed highest Polymorphism Information Content (PIC) (0.9063) while the PIC value was found 0.805 in primer C04.

**Table 1: Summary of the bands revealed from three primers based on RAPD band analysis**

| Primers | SD (bp) | *P. globosa* | | | | |
|---|---|---|---|---|---|---|
| | | TB | PL | % PL | BS | PIC |
| B03 | 143-1105 | 17 | 3 | 17.65 | 2.42 | 0.9063 |
| C04 | 85-485 | 20 | 5 | 25 | 2.85 | 0.805 |
| OPB12 | 126-1670 | 19 | 4 | 21.05 | 2.71 | 0.9063 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Total | | 56 | 12 | - | - | - |
| Average | | 18.66 | 4 | 21.23 | | 0.875 |

SD= Size of DNA (bp), TB= Total Number of DNA Bands, PL= Number of polymorphic Loci, BS= Number of bands per sample, PIC= Polymorphism Information content

**Inter–individual pair wise similarity indices**

Inter individual pair wise similarity of *Pila globosa* was studied and highest similarity was found between individuals 2 and 3 as well as second highest similarity was observed between the individuals 4 and 5, and 5 and 6 whereas lower similarity was recorded rest of the individuals (Table 2).

**Table 2:** Inter-individual pair wise similarity of *Pila globosa*

| samples | Ind. 1 | Ind. 2 | Ind. 3 | Ind. 4 | Ind. 5 | Ind. 6 | Ind. 7 |
|---|---|---|---|---|---|---|---|
| Ind. 1 | --- | 2 | 2 | 1 | 1 | 1 | 1 |
| Ind. 2 | | --- | 5 | 2 | 2 | 1 | 1 |
| Ind. 3 | | | --- | 2 | 1 | 1 | 2 |
| Ind. 4 | | | | -- | 3 | 2 | 1 |
| Ind. 5 | | | | | -- | 3 | 2 |
| Ind. 6 | | | | | | -- | 2 |
| Ind. 7 | | | | | | | --- |

Ind.= Individual

**Genetic distance**

Genetic distance of *Pila globosa* was calculated by using the data from pair wise similarity index (Table 3). Lowest genetic distance was found between the individual 2 and 3 (0.667) and highest distance was found 0.9444 while relatively higher distance was recorded in other individuals.

**Table 3:** Genetic distance among individuals of *Pila globosa*

| Samples | Ind. 1 | Ind. 2 | Ind. 3 | Ind. 4 | Ind. 5 | Ind. 6 | Ind. 7 |
|---|---|---|---|---|---|---|---|
| Ind. 1 | -- | 0.857 | 0.857 | 0.909 | 0.929 | 0.929 | 0.909 |
| Ind. 2 | | -- | 0.667 | 0.857 | 0.882 | 0.944 | 0.933 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ind. 3 | | | -- | 0.857 | 0.944 | 0.944 | 0.857 |
| Ind. 4 | | | | -- | 0.750 | 0.846 | 0.909 |
| Ind. 5 | | | | | -- | 0.813 | 0.846 |
| Ind. 6 | | | | | | -- | 0.846 |
| Ind. 7 | | | | | | | -- |

Ind.= Individual

**Nei's genetic similarity**

Nei genetic similarity (F) was observed among 7 individuals of *P. globosa* (Table 4). Highest genetic similarity was found 0.500 (50%) between samples 2 and 3 followed by the sample 5 and 6 (0.333) and relatively lower similarity was found among other samples.

**Table 4:** Nei's genetic similarity among individuals of *Pila globosa* (Ind.= Individual

| Samples | Ind. 1 | Ind. 2 | Ind. 3 | Ind. 4 | Ind. 5 | Ind. 6 | Ind. 7 |
|---|---|---|---|---|---|---|---|
| Ind. 1 | -- | 0.25 | 0.25 | 0.167 | 0.133 | 0.133 | 0.167 |
| Ind. 2 | | -- | 0.500 | 0.250 | 0.211 | 0.105 | 0.125 |
| Ind. 3 | | | -- | 0.250 | 0.105 | 0.105 | 0.250 |
| Ind. 4 | | | | -- | 0.400 | 0.267 | 0.167 |
| Ind. 5 | | | | | -- | 0.333 | 0.267 |
| Ind. 6 | | | | | | -- | 0.267 |
| Ind. 7 | | | | | | | -- |

**Linkage distances**

The values of pair-wise comparisons of linkage distances analyzed from the combined data for three experimental primers, ranged from 9.0 to 17.0 (Table 5). The highest linkage distance (17.0) was found in 3 and 5, and 3 and 6 individual pairs while the lowest linkage distance (9) was observed in 4 and 5 individual pair.

**Table 5:** Squared Euclidean distances of *Pila globosa*

| Samples | Ind. 1 | Ind. 2 | Ind. 3 | Ind. 4 | Ind. 5 | Ind. 6 | Ind. 7 |
|---|---|---|---|---|---|---|---|

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ind. 1 | 0 | | | | | | |
| Ind. 2 | 12 | 0 | | | | | |
| Ind. 3 | 12 | 10 | 0 | | | | |
| Ind. 4 | 10 | 12 | 12 | 0 | | | |
| Ind. 5 | 13 | 15 | 17 | 9 | 0 | | |
| Ind. 6 | 13 | 17 | 17 | 11 | 12 | 0 | |
| Ind. 7 | 10 | 14 | 12 | 10 | 11 | 11 | 0 |

Ind.= Individual

**Cluster analysis based on RAPD markers**

A cluster analysis using UPGMA based on linkage distance was done to resolve the phylogenetic relationships among 7 individuals of *P. globosa* genotypes (Figure 2). The UPGMA clustering system generated six clusters where for clusters have formed among individual 1 and 7 at linkage distance 10, 4 and 5 at linkage distance 9 and individual 6 forms cluster with cluster 3. Cluster 5 forms between individual 2 and 3 at linkage distance 10. Finally cluster 6 is formed between cluster 4 and 5 at linkage distance 14 which were seen to be most distantly related.

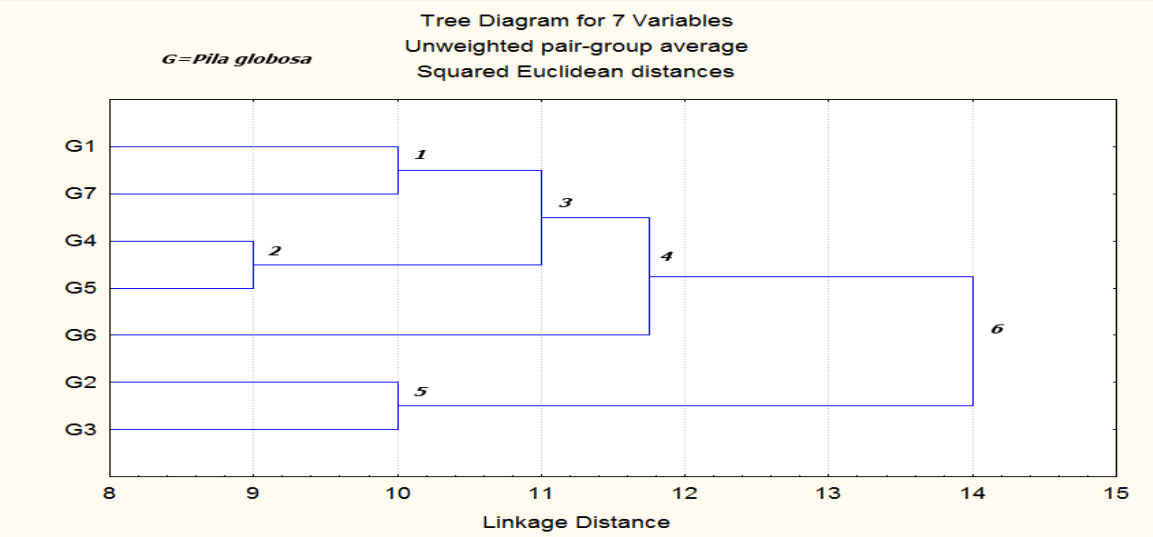


**Figure 2.** Dendrogram of genetic relationships among 7 individuals of *Pila globosa*

**Discussion**

Due to morphological ambiguity of taxonomy, molecular species identification of seven individuals of *Pila globosa* was examined by universal COI primers whereas the sequence was bind at the position of 700bp length in all individuals. Folmer *et al.* (1994) designed this primer as invertebrate

universal primer where they identified 11 invertebrate phyla including molluscs but they did not use the individuals of the *Pila globosa*. On the other hand, this primer was used for identification of the species *Pomacea canaliculata, Pila ampullacea, Pila angelica, Pila pesmei,* and *Pila polita* but only *Pomacea canaliculata* was identified and rest of the species bind at the same length of DNA (Thaewnon-ngiw *et al*. 2004). So, quite similar result was found in the present study.

The freshwater apple snail *Pila globosa* has been considered to be a vulnerable species compared to other snail species in Bangladesh. In this study, genetic diversity of freshwater apple snail *Pila globosa* was studied to know genetic structure of this snail in Bangladesh. The genetic diversity of this species considering seven individuals was studied based on RAPD analysis whereas three arbitrary primers were screened. To our knowledge the present study is the first attempt to determine the genetic variability of this population in Bangladesh. Three selected random primers generated 56 bands ranging from 85 to 1670 bp, corresponding to an average of 18 bands per primer while Thaewnon-ngiw et al (2003) were found two hundred and two polymorphic fragments ranged 180-1500 bp in length from 254 samples of five species by using three other informative primers. The percentages of polymorphic bands were 98.86%, 94.56%, 90.91%, 96.94% and 95.51% for *Pomacea canaliculata, P. ampullacea, P. angelica, P. pesmei* and *P. polita* respectively and found high genetic polymorphism of these taxa whereas very low polymorphic bands (average 21.23%) were found in the present study. High genetic polymorphism detected by RAPD analysis across investigated species were also found in the mud crabs; *Scylla serrata, S. oceanic* and *S. tranquebarica* (Klinbunga et al., 2000) and the cupped oysters of genera *Saccostrea; S. cucullata,* and *S. forskali* and *Striostrea; Striostrea (Parastriostrea) mytiloides* (Klinbunga et al., 2001) which is also related to the present result. Thaewnon-ngiw *et al.* (2004) was also analyzed the genetic diversity and species-diagnostic markers in the introduced apple snail, *Pomacea canaliculata* and in the native Thai apple snails; *Pila ampullacea, P. angelica, P. pesmei,* and *P. polita* by restriction analysis of COI and 16srDNA sequencing and they also found significant level of genetic diversity in different species of apple snails again which is similar to the present results of RAPD analyses.

On the other hand, same primers were used by two different apple snails respectively in *Pila gracilis* (Mahzabin 2014) and in *Pila polita* (Leamon, 2014). But the highest level of polymorphism outcome in their research for B03 primers whereas in the study of *P. globosa* it was C04 primer. In the case of *P. globosa* the highest level of polymorphic outcome was 25% (C04) but for the other two species it was 22.22 (B03) and 35.29 (B03) for *Pila gracilis* and *Pila polita* respectively. From the data analysis of inter-individual pair wise similarity indices the lowest similarity was seen in most of the individual while higher similarity was recorded most of the *Pila polita* (Leamon, 2014) and *Pila gracilis* (Mahzabin, 2014). Depending on these informations, the highest genetic distance was found 0.944 in *Pila globosa* while much higher distance was recorded *Pila polita* (Leamon, 2014) and *Pila gracilis* (Mahzabin, 2014). On the other hand, Nei's genetic similarity among the 7 individuals of *Pila gracilis* also showed the same. Near about most of the individuals showed moderate genetic similarity in between themselves whereas (0.105) and almost similar results were observed in *Pila polita* (Leamon, 2014) whereas *Pila gracilis* (Mahzabin, 2014) showed relatively lower similarity. However, from the study of linkage distance the highest linkage distance (17.0) was found in *Pila globosa* which was similar to the studies on *Pila polita* (Leamon, 2014) and *Pila gracilis* (Mahzabin, 2014).

## CONCLUSION

Due to economic status of *Pila globosa* is recorded in Bangladesh in different point of view and reducing this species day by day, therefore, proper species identification is very important for breeding and culture of this species in Bangladesh and molecular identification of this research will

be helpful for that purposes. And to properly impose the policy to protect snail population it is now so important to assess the genetic diversity of this snail species. Though, the genetic distance value showed higher genetic diversity from this data whereas only seven individuals were studied due to limited laboratory facilities. Thus, this species need to be special attention to assessing genetic diversity for conserving this species in a broad spectrum research.

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