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Original Article

Molecular Phylogenetics of *Physa acuta* (Pulmonata: Basommatophora): an Invasive species in Central Punjab Pakistan

Molecular Phylogenetics of *Physa acuta* (Pulmonata: Basommatophora): uma espécie invasora no centro de Punjab, Paquistão

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Abstract

Physids belong to Class Gastropoda; belong to Phylum Mollusca and being bioindicators, intermediate hosts of parasites and pests hold a key position in the ecosystem. There are three species of Genus Physa i.e. *P. fontinalis*, *Physa acuta* and *P. gyrina* water bodies of Central Punjab and were characterized on the basis of molecular markers High level of genetic diversity was revealed by polymorphic RAPD, however SSR markers were not amplified. The multivariate analysis revealed polymorphism ranging from 9.09 percent to 50 percent among the three Physid species. Total number of 79 loci were observed for the three species under study and 24 loci were observed to be polymorphic. These RAPD fragment(s) can be developed into co dominant markers (SCAR) by cloning and can be further sequenced for the development of the Physa species specific markers to identify the introduced and native species in Pakistan.

Keywords: mollusca, molecular marker, genetic diversity, polymorphism, physa.

Resumo

Os físidos pertencem à classe Gastropoda; pertencem ao filo Mollusca e, sendo bioindicadores, hospedeiros intermediários de parasitas e pragas, ocupam uma posição-chave no ecossistema. Existem três espécies do gênero Physa, ou seja, *P. fontinalis*, *Physa acuta* e *P. gyrina* em corpos d'água do Punjab Central e foram caracterizadas com base em marcadores moleculares. Alto nível de diversidade genética foi revelado por RAPD polimórfico, no entanto os marcadores SSR não foram amplificados. A análise multivariada revelou polimorfismo variando de 9,09% a 50% entre as três espécies de Physid. Um número total de 79 loci foi observado para as três espécies em estudo e 24 loci foram observados como polimórficos. Esses fragmentos RAPD podem ser desenvolvidos em marcadores codominantes (SCAR) por clonagem e podem ser posteriormente sequenciados para o desenvolvimento de marcadores específicos da espécie Physa para identificar as espécies introduzidas e nativas no Paquistão.

Palavras-chave: mollusca, marcador molecular, diversidade genética, polimorfismo, physa.

1. Introduction

The members of the freshwater family Physidae (Pulmonata: Basommatophora) have invaded the world across all the continents, particularly freshwater lentic habitats except Antarctica. These were native to America (Wethington and Lydeard, 2007; Bousset et al., 2014) yet remained unidentified and as a result ignored which shows lesser interest of scientists (Turner & Montgomery, 2009). The invasion history of the *Physa acuta* showed that it was not reported from oriental region (Ebbs et al., 2018), however Physids have been reported from Rawalpindi

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(Afshan et al., 2013); Baluchistan (Kakar et al., 2017) and central Punjab (Altaf et al., 2017a, b) in Pakistan. This has been recorded as an invasive species in Singapore and Thailand in past few years (Ng et al., 2018). The introduction of these three Physa species is probably much earlier than reported and has well established its population. The distribution of *Physa acuta* might have been facilitated by water birds or mammals (van Leeuwen et al., 2013; Bousset et al., 2013). Therefore the investigation of the pathways along with level of resource competition with other species i.e Lymnea and Planorbis needs to be investigated, which are still poorly known in this part of the world, for better management practices (Kohler et al., 2012). Molecular characterization must be adopted for the confirmation of invasive species (Kongim et al., 2015) as well as native species (Ng et al., 2016) especially showing high plasticity (Gustafson et al., 2014). The study regarding molecular characterization of Physids is the need of time as the cost of management of the invasive species will increase with the time elapse (Simberloff et al., 2013).

Certain snail species are bioindicators (Zeidan et al., 2020) however Physa is cosmopolitan in distribution (Albrecht et al., 2009; Altaf et al., 2017d). These snails are found in large number in the humid season i.e. from July through September as compared to the dry season i.e. December through February, however other abiotic factors also influence the distribution and abundance of these gastropods (Altaf et al., 2016; Qamar et al., 2017).

Shell structure has various characters which provide a primary guide line for identification of snails in taxonomic literature (Kerney and Cameron, 1979). The morphological characters of shell are misleading in this species identification due to phenotypic plasticity and convergent evolution (Albrecht et al., 2009) thereby making these quite unreliable. The molecular markers have been found important to assess the snail diversity (Hershler and Liu, 2004). The molecular markers are very helpful using DNA sequences for the correct and inexpensive identification of species (von Beeren et al., 2015) including 16S RNA and (COI) gene. The RAPD markers revealed genetic diversity more effectively as compared to the COI which are species specific markers (Thaewnon-Ngiw et al., 2004). Secondly species specific markers for molluscs have not been much reported due to which species characterization and genetic diversity revolved around RAPD markers in the last twenty years (Altaf et al., 2017a). The genetic diversity within three species reported in this study has been carried out for the first time in this region which will be useful for management practice of the invasive species of *Physa acuta*.

2. Materials and Methods

2.1. Study area and sampling sites

The sampling has been carried out in suburbs of different districts of Central Punjab 31°N 72 °N (Figure 1). The snails from the different water bodies of the agroecosystem of

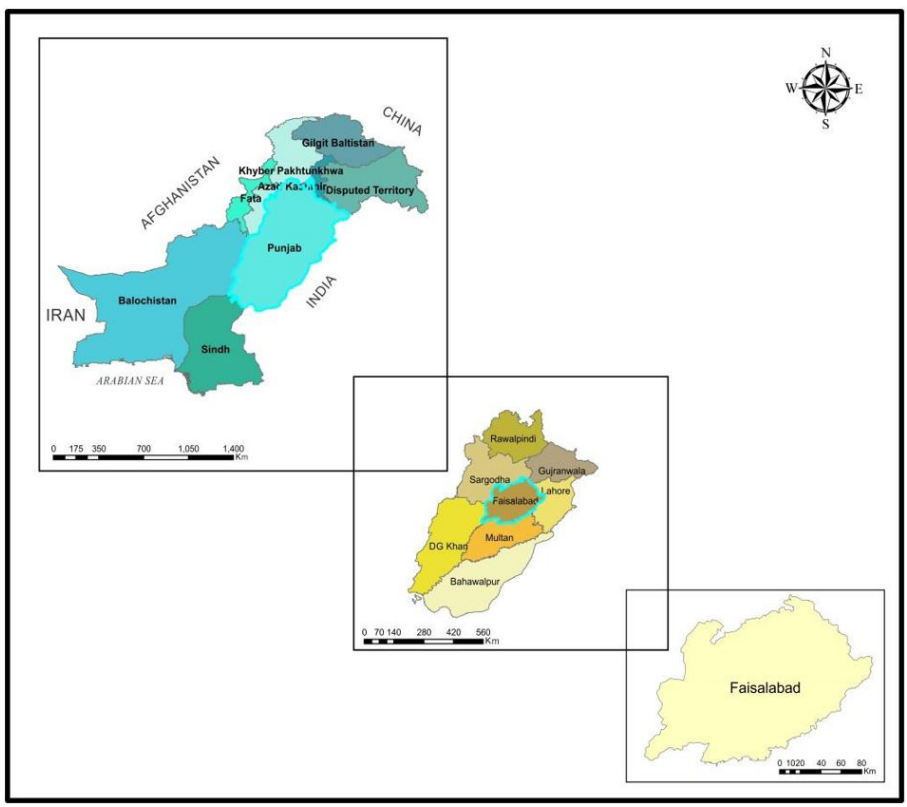

Figure 1. Central Punjab, Pakistan.

the Central Punjab were collected by random sampling during the months of October 2017 to March 2018, by using hand-picking methods from irrigation canals of Faisalabad. Sixty two villages were selected randomly on the using lottery method (Figure 2). Every village was visited once during this period, at the dawn or dusk. The point of irrigation canals with the vegetation/tree cover were keenly observed for an hour to check the presence of species specimens for collection. The Physids species were handpicked for an hour from each village, on site identifications upto genus was carried out followed by the storage of these snail species in small specimen bottles. The stored samples were brought to the lab for preservation in 99.9% ethyl alcohol. All the specimen bottles were labelled with the collectors name, date, ecological information (Table 1).

Morphometric Analysis

The snail samples were identified with the help of keys and the diagrams given by Albrecht et al. (2009), Afshan et al. (2013), Dillon Junior and Jacquemin (2015). The picture of each snail was taken by photocamera following procedures adopted by Altaf et al. (2017b) (Figure 3).

2.2. Genomic DNA extraction

Total genomic DNA extraction from snail samples was done by following the CTAB method as described by Altaf et al. (2017a). The 15ng/ml working dilutions DNA samples were made after measuring its concentration using Nanodrop (Sigma USA).

2.3. Molecular analysis

Total of fifteen decamers (Genelink Co. USA) were selected, based on their polymorphic amplification reported by Altaf et al.2017$_a$. PCR amplification was carried out (PeqLab USA) using optimized concentration. of template DNA, 10X PCR buffer, $MgCl_2$, dNTPs, primer and *Taq* DNA polymerase (MBI, Ferments, Vinius, Lithuania). The PCR amplification profile was used as 94C for 5 min following 40 cycles each of 94°C for 1 minutes, 36°C for 1 min, 72°C for 2 min and final extension at 72°C for 10 min. The PCR products were resolved on 1.2% (w/v) agarose gel stained with Ethidium bromide at 90 V for minutes and visualized

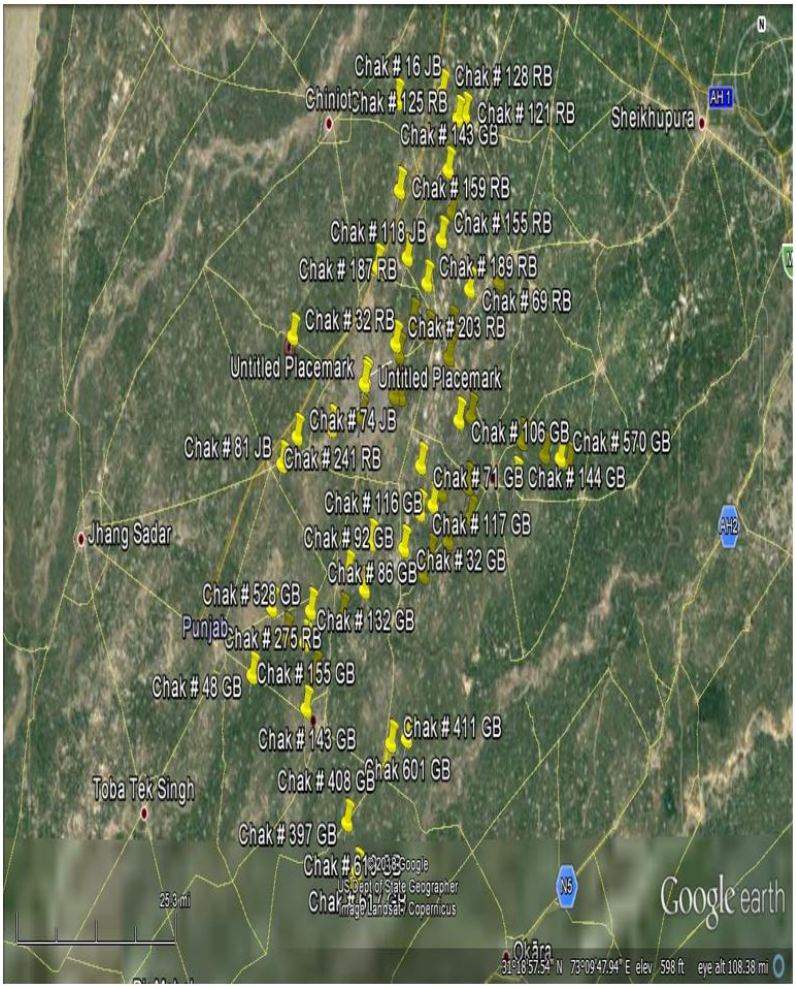

Figure 2. Sampling sites in Punjab.

Table 1. Distributions of species of snails belonging to genus Physa in district Faisalabad.

Sr. no.	Villages	*Physa fontinalis*	*Physa acuta*	*Physa gyrina*
1	23 GB	YES	-	-
2	26 GB	-	YES	-
3	32 GB	YES	YES	YES
4	34 GB	-	-	-
5	35 GB	-	-	-
6	48 GB	-	YES	-
7	55 GB	-	-	-
8	63 GB	-	YES	-
9	68 GB	-	YES	YES
10	71 GB	-	-	-
11	86 GB	-	YES	-
12	92 GB	-	-	-
13	106 GB	-	-	-
14	116 GB	-	-	-
15	117 GB	-	-	-
16	132 GB	YES	-	-
17	137 GB	-	YES	-
18	143 GB	-	-	-
19	146 GB	-	-	-
20	148 GB	-	-	-
21	155 GB	-	-	-
22	397 GB	YES	-	-
23	408 GB	-	YES	-
24	411 GB	YES	-	-
25	528 GB	-	-	-
26	531 GB	-	-	-
27	570 GB	-	-	-
28	601 GB	-	-	-
29	617 GB	-	-	-
30	619 GB	YES	-	-
31	632 GB	-	-	-
32	645 GB	YES	-	-
33	649 GB	YES	-	-
34	659 GB	-	-	-
35	69 RB	-	-	-
36	71 RB	-	YES	-
37	93 RB	-	-	-
38	106 RB	YES	-	-
39	118 RB	-	-	-
40	121 RB	YES	-	-
41	123 RB	-	-	-
42	125 RB	-	YES	-
43	128 RB	YES	-	-

Table 1. Continued...

Sr. no.	Villages	*Physa fontinalis*	*Physa acuta*	*Physa gyrina*
44	143 RB	-	YES	-
45	148 RB	YES	-	-
46	155 RB	-	-	-
47	159 RB	-	-	-
48	187 RB	-	-	-
49	189 RB	YES	-	-
50	198 RB	-	YES	-
51	200 RB	-	-	-
52	203 RB	-	YES	-
53	204 RB	-	-	-
54	217 RB	YES	YES	-
55	222 RB	-	-	-
56	241 RB	-	-	-
57	275 RB	-	-	-
58	16 JB	-	Yes	-
59	32 JB	-	-	-
60	74 JB	-	-	-
61	81 JB	-	-	-
62	118 JB	-	Yes	-

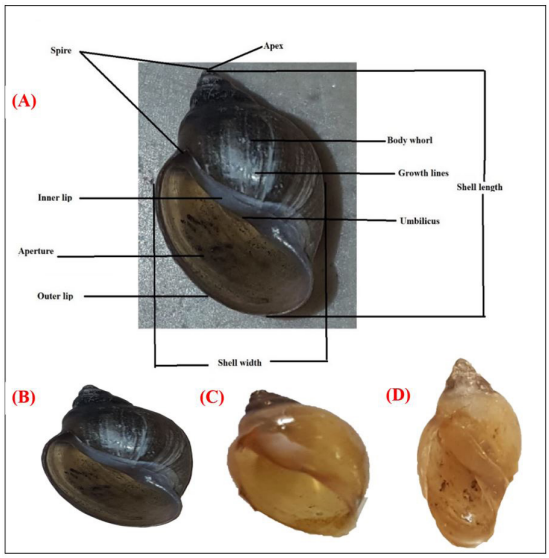

Figure 3. (A) Morphological Characteristics of Snails of Genus Physa (B) *Physa acuta* (C) *Physa fontinalis* (D) *Physa gyrina*.

by the gel doc system (SynGen, UK). Total of four SSR primers were used for genetic diversity estimation of snails. The 20ul SSR (PCR) mixture contained 0.2ul *Taq* DNA polymerase, 2.5ul PCR buffer (10X), 3ul $MgCl_2$ (25mM), 5ul of dNTPs and 3ul template DNA, 2.5 of each forward and reverse primer and final volume of 20 ul of reaction mixture was made by adding d_3H_2O. The amplification profile of PCR comprises 30 cycles 94C for 5 min following 30 cycles each of 94°C for 1 minutes, 60°C for 30 seconds, 72°C for 1min and final extension at 72°C for 10 min.

2.4. Data analysis

The molecular data was analyzed using popgen32 (*Ver.* 1.44) (Yeh et al., 1999). Principal component analysis and analysis of molecular variance (AMOVA) was done using the PAST software Hammer et al.(2001). The PIC value of polymorphic primer was calculated as devised by Anderson et al. (1993). PICi = 1 - ∑P2ij (where pij is frequency of the jth allele for locus i). The genetic similarity between three snail species was calculated by following the methodology adopted by Nei (1973). Based on the genetic similarities the genetic relationship among the snail species was developed by an unweighted pair group method of arithmetic averages (UPGMA).

3. Results

All three species of genus Physa i.e *P. fontinalis, P. acuta* and *P. gyrina* were characterized by employing RAPD.

3.1. RAPD PCR analysis

Total of 15 RAPD primers were amplified out of which eight primers (Table 1) showed polymorphic banding pattern whereas seven RAPD primers showed monomorphic amplification and were excluded from the results. All the

PCR amplification was done in triplicate to ensure the consistency of the RAPD amplification. Good amplification of all the eight RAPD primers was observed. The RAPD bands primers were scored which were generated by the primers and were found polymorphic across the three species. The average NPB (number of polymorphic bands) value was 30.37% of the three Physid species. All three genotypes of genus Physa produced 79 loci out of which 24 loci were found polymorphic. The primer GL-4 showed maximum polymorphism whereas minimum polymorphism was observed in the amplification of GK 03. The primer GL-02 produced maximum number i.e 13 while minimum number of loci were amplified by GL-05 i.e. 5 (Table 2).

Similarity matrix (Table 3) gave the genetic similarities among different Physa species. Maximum genetic similarities was observed between *Physa gyrina* and *Physa acuta* while lowest genetic similarities was observed between *Physa acuta* and *Physa fontinalis*. The polymorphism percentage ranged from 9.09% to 50% among the three genotypes of snail under study. Cluster analysis (Figure 4) shows the genetic relationship among three genotypes. It shows that *Physa gyrina* and *Physa acuta* are genetically close species as compared to *Physa fontinalis.* It was concluded that *Physa gyrina* and *Physa acuta* clustered together showing 77% genetic similarity while the members of this *Physa fontinalis* have the genetic similarity of 70 percent with *Physa acuta* and 74 percent with *Physa gyrina* and *Physa fontinalis.*

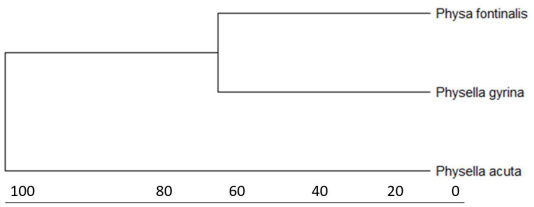

Figure 4. Dendrogram showing relationship among three Physa species.

3.2. SSR analysis

Total of four SSR primers were used to amplify the genomic DNA of three species of genus Physa and there was no amplification observed.

3. Discussion

Physa acuta is an invasive species and found in large numbers in irrigation canals of Central Punjab. All three species of genus Physa i.e *P. fontinalis, P. acuta* and *P. gyrina* were characterized by employing RAPD. It was observed that eight RAPD decamers showed good amplification and useful insight regarding genetic polymorphism as compared to the COI markers (Thaewnon-Ngiw et al., 2004). The Physid species mostly breed by selfing rather than cross fertilization which might be one of the reason of less polymorphism i.e. 30.37%, however polymorphism percentage in Pila snails was 90.91% which have been reported to breed through cross fertilization. RAPD markers have been found to exhibit more genetic polymorphism as compared to the COI in apple snails, which might be due to the female founder effects. The RAPD markers revealed genetic diversity more effectively as compared to the COI which are species specific markers (Thaewnon-Ngiw et al., 2004). Few studies were carried out for genus Pseudamnicola (Delicado et al., 2015) and Hydrobiidae (Delicado et al., 2016) which showed similar polymorphism among species. Moreover, genetic diversity based on RAPD analysis depends on sharing of RAPD-amplified fragments and are scored in electrophoresis. The possibility of comigration of RAPD fragments having similar sizes but different sequences cannot be excluded. Therefore, homology of comigrating fragments should be further verified by sequencing and can further reveal.

Table 2. Genetic polymorphism revealed by RAPD(PCR) analysis.

Sr No.	Primer Name	Sequence(5´-3´)	TNB[1]	NPL[2]	Polymorphism%
1.	GL Decamer L-02	TGGGCGTCAA	13	3	23.07%
2.	GL Decamer L-04	GACTGCACAC	10	7	70%
3.	GL Decamer L-05	ACGCAGGCAC	5	1	20%
4.	GL Decamer L-08	AGCAGGTGGA	16	2	12.5%
5.	GL Decamer A-02	TGCCGAGCTG	8	3	37.5%
6.	GL Decamer A-03	AGTCAGCCAC	10	5	50%
7.	GL Decamer K-01	CATTCGAGCC	6	2	33.33%
8.	GL Decamer K-03	CCAGCTTAGG	11	1	9.09%
Total			**79**	**24**	**30.37**
Mean			**9.88**	**3**	
Variance			**12.88**	**4.28**	

[1]TNB=Total number of bands; [2]NPL=Number of polymorphic loci.

Table 3. Similarity Matrix of genus Physa.

Species	*P. acuta*	*P. fontinalis*	*P. gyrina*
P. acuta	***	0.7419	0.7742
P. fontinalis	0.2985	***	0.7097
P. gyrina	0.2559	0.3429	***

Nei's genetic identity (above diagonal) and genetic distance (below diagonal). *** denotes as 100% similarity as both are the same organisms used in this study.

This study can further lead to reveal the invasive range of *P. acuta* along with its invasion history and phylogenetics structure using other markers i.e. cox1 + 16S, cox1 and 16S which indicated that *P. acuta* genetically related to *P. spelunca* (Ebbs et al., 2018). The *P. acuta* has been held responsible for the decline of the *P. fontinalis* where increase in temperature has been found as the driving force (Früh et al., 2015). In the present study, we used this technique to examine genetic diversity of the three species of the genus Physa in Faisalabad Pakistan. In future these RAPD markers can be converted into sequence characterized amplified regions or SCAR through cloning and can be further sequenced for the development of the Physa species markers. Further the two markers can be used together to identify the introduced and native species in Pakistan. The Physid species have been found to have a negative interaction with other species of the region (Altaf et al., 2017c) which might be due to the phenomenon of antibiosis or ammenalism. The molecular characterization of Physid species is extremely important as we have found potent bioactive substances showing antibacterial activity (Altaf et al., 2018) and antifungal and antiviral activity (unpublished data).

4. Conclusion

The species specific markers are extremely important for the characterization as it will not only be important for the management of the invasive species such as *P. acuta*.

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