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# Genetic diversity analysis of snail species (Mollusca: Gastropoda) using molecular marker technology

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**Abstract**

Members of the Phylum Mollusca can be used as model animals to study the reproductive barriers, for evolution and gamete recognition proteins for which they must be correctly identified and classified. Phenotypic plasticity results in changes in shell morphology, leading to the development of synonyms and homonyms of species, which necessitates molecular characterization. Fifteen species of molluscs collected from different agro-ecosystems of Faisalabad were characterized using polymorphic RAPD dacamers. Total of 114 fragments amplified and only 90 were found polymorphic showing 78.94% polymorphism. The range of amplified RAPD fragments was from 7 to 9 with an average of 7.6 per primer. The overall mean band frequency ranged between 0.27 – 0.68 with an average value of 0.41. Analysis of molecular variance revealed that the variance component among and within populations was 11% and 89% respectively, which is significantly different at probability less than 0.001. Close association was observed among the species of genus *Ariophanta and Oxychilus draparnaudi, Monacha catiana* and these species were found to be closely clustered in one group while the species *Zooctecus insularis* is distantly related with rest of the species in this group showing it to be genetically distant from the same group. The cluster analysis shows that Juvenile *Zooctecus insularis* lies separately in cladogram, as it might be a new species.

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**Introduction**

In marine invertebrates, the members belonging to Phylum Molluscs are considered to be the best that have been studied genetically. There is a great potential for combining genetic and paleontological approaches as molluscs have an excellent fossil record as stated by (Collins *et al*., 1996). As the shells can be readily collected and studied so the attention of alpha taxonomists of gastropods has been attracted to them since long time. There are many descriptions of the shells which are available in the past literature; however, mostly the phenotypic plasticity resulting into differences in shell morphology leads to cases of synonymy or homonymy of species, when compared with the other invertebrate phyla. The differences in the shell morphology (or other characters), which are of taxonomic importance can be understood with the help of genetic data (Johnson and Cumming, 1995). While in the case of allopatric species there arises confusion as the shell variability may be due to the genetic differences and environmental pressures. Besides this, large amount of genetic differentiation or very low amount of genetic difference can be observed in the comparison of members of the species in the same geographic domain and, sometimes, this difference can be found even in same genus as previously stated by (Adamkewicz and Harasewych, 1996).

The use of molecular markers has proved to be a useful tool for complex taxonomic identification where morphological characteristics are ambiguous or cryptic (Douek *et al*., 2002; Westheide *et al*., 2003; Miura *et al*., 2005; Park *et al*., 2005). Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) technique is relatively simple and cheap method capable of differentiating taxa without the need to know their genomes as stated by (Welsh and McClelland, 1990; Williams *et al*., 1990). RAPD markers are dominant and result from the use of short (10 bases long) primers (synthetic oligonucleotides) of random sequence that can amplify multiple segments of genomic DNA by PCR. The number of segments depends on the number of sites of the genome recognized by a particular primer.

The major reason for the success of RAPD analysis is the gain of a large number of genetic markers that require small amounts of DNA without the need of a molecular characterization of the genome of the taxa under study. The species which have been characterized using such markers include oyster genera *Crassostrea*, *Saccostrea*, and *Striostrea* (Klinbunga *et al*., 2000), amphipods (Gammarus: Costa *et al*., 2004) and the tropical abalones *Haliotis asinina*, *Haliotis ovina* and *Haliotis varia* (Klinbunga *et al*., 2004).

In animals like arthropods and spiders, the genitalia show a great deal of structural detail and is an important diagonostic character for species identification. However, at times single species is found to have a great deal of variation among its members or sometimes members of different species may have indistinguishable characters (Mayr, 1991).

Although the genital differences in *Zonitoides nitidus* and *Zonitoides excavatus*, are not clearly found yet there are clear differences in the shell morphology. A great variation has been revealed by using (RAPD) markers between *Zonitoides excavates* and *Zonitoides nitidus*. Sperm transfer has not been observed to be completely absent. The species status of both taxa is supported by the strong genetic differentiation suggested by allozyme and RAPD data (Jordaens *et al*., 2003). Due to this it has become extremely important to use molecular techniques so that the species can be identified with more accuracy. Histone gene primers have been developed for land snails and it has been observed that land snails and bivalves seem to differ in their histone gene organization. Nucleotide polymorphism is of significant importance to understand phylogenetic and systematic relationship among different species and genera. Thus, if adequately used and interpreted, the RAPD method is an informative modern tool for studying genetic diversity of species in nature (Armbruster, 2005).

Most of the studies on the land snails have been carried out approximately a century before in the Indian subcontinent. Hutton (1849) collected and studied the land snails nearby Bolan pass, Suliman range and the hills of south laying west to Indus. According to Blanford and Godwin, (1908) in the areas of Swat, Dir or Chitral no terrestrial snails have been observed except *Petraeus* snails, while in the Kuram valley only very few species were found. They worked chiefly on the conchological side of the families Testacellidae and Zonitidae of Indian subcontinent. Recently 329 specimens of snails were collected by Ali, (2005) from sugarcane fields of village Gutti Faisalabad pertaining to 6 families and 9 genera belonging to order Pulmonata. Pokryszko *et al*., (2009) collected 3500 dry shells and described 22 species out of which 12 were new species from 77 localities. The shell variations in most species were described and nine species were illustrated with figures of detailed reproductive system. In the Northern area of Pakistan there is highest diversity of Pupilliods due to wetter climate and wider altitudinal range. Ten out of twenty two species have been found to be endemic to Pakistan, the distribution of the rest of species was extended to other regions i.e. Asia, Europe and Holarctic. Pupilloid fauna showed great diversity due to Palaearctic/Holarctic influence in general.

So we can say that the studies related to the faunal distribution of snails have been ignored in Pakistan and due to this there is a scanty and scattered knowledge about the snails in this part of the world. The goal of the present study was to obtain species-specific RAPD molecular markers that could distinguish between fifteen species of snails along with their diversity and distribution in different fields of Faisalabad.

## Materials and method

*Collection of molluscs*

Fifteen species of molluscs (Table 1) were used for molecular analysis. The samples were collected from different agricultural fields in Faisalabad region, tagged and immediately stored in 70% ethanol for further analysis.

*Genomic DNA extraction*

Genomic DNA was extracted from all the samples of snails by CTAB (Cetyl Trimethyl Ammonium Bromide) method (Doyle *et al*., 1990). The snail samples of 0.3-0.5 g were ground to a homogeneous paste by using 2 ml of 2×CTAB and transferred into 50 ml falcon tubes. After that, 15 ml of hot (65 ˚C) 2×CTAB was added to the sample tube and mix gently by inverting the tube for several times and incubated at 65 ˚C for half an hour. 15 ml of chloroform isoamylalcohol at the ratio of 24:1 were added and mixed by inverting for 30 sec followed by centrifugation at 10,000 g for 10 min at room temperature. The aqueous phase was transferred to another tube and repeated this step by adding 15 ml chloroform: isoamylalcohol (24:1) in eppendorf tube. To the aqueous phase, 0.6 volume of isopropanol were added, precipitated the genomic DNA and centrifuged at 1000 rounds/minute for 5 minutes and discarded the supernatant solution. Genomic DNA was then washed three times with 70% ethanol, air dried and resuspended in 0.5 ml 0.1×TE buffer. The supernatant was treated with 5 µl of RNAse and incubated for one hour at 37 ˚C followed by addition of equal volume of chloroform: isoamylalcohol (24:1). Thereafter the genomic DNA was centrifuged at 13000 rpm for 10 minutes and transferred aqueous phase in a new eppendorf tube followed by precipitation by adding of 1/10th of 3 M sodium chloride and 2 volume of ethanol and collected by centrifugation at 13,000 rpm for 10 minutes at room temperature. The pellet was air dried and resuspended in 0.1× TE buffer and measured the concentration of the DNA by loading in a 0.8% agarose gel with DNA Quant 200 fluorometer. The DNA quality evaluation was verified on 1% agarose gels stained with ethidium bromide and visualized under ultraviolet light. Genomic DNA concentration of samples of snails was quantified with a IMPLEN Nano Photometer™. The optical density (OD) of each sample was calculated and the working dilutions of all the DNA samples were made by diluting each DNA sample to a uniform concentration of 15 ng $ml^{-1}$ with double distilled deionized water and used for RAPD (PCR) reactions.

*RAPD (PCR) analysis*

Oligonucleotide RAPD primers of different series were custom synthesized from Genelink Co. USA were used in this study. Total of 15 RAPD decamers were selected, which were polymorphic in amplification profile. The PCR thermal cycler (Eppendorf AG No. 533300839, Germany) was used for the RAPD-PCR amplification and with initially optimized concentration of template DNA, 10× PCR buffer, $MgCl_2$, dNTPs, primer and *Taq* DNA polymerase (MBI, Ferments, Vinius, Lithuania). The temperature for DNA amplification was fixed at 94˚C for the period of 5 min as initial denaturation, then 40 cycles with same temperature for 1 minute followed by 36˚C for 1 min, 72˚C for 2 min and 72˚C for 10 min as extension temperature. The PCR product was run on 1.2% (w/v) agarose gel stained with Ethidium bromide at 100 V for 2 hrs, checked under UV trans-illuminator at 300nm and photographed in a gel doc system (Syn Gen, Synoptics Ltd,UK).

*Data analysis*

The RAPD (PCR) products were run on 1.2 % agarose gel and stained with ethidium bromide. The robust and visible RAPD fragments were scored as (1) if present or (0) if absent except those fragments with low amplification and that could not be clearly distinguished. The fingerprints were examined under ultra violet Transilluminator and photographed using SyneGene Gel Documentation System. The data generated from the detection of polymorphic fragments were analyzed using popgen 32 software (Ver. 1.44) as stated by (Yeh *et al.,* 2000) whereas calculation of principal component analysis and analysis of molecular variance (AMOVA) was done using the PAST software. The Polymorphic Information Content value of each RAPD primer was calculated using the equation developed by Anderson *et al.* (1993). $PICi = 1 - \sum P2ij$ (pij is the frequency of the jth allele for locus i). The genetic similarity between 15 snail species was estimated according to the method developed by Nei, (1973). Based on similarity data, an un-weighted pair group method of arithmetic averages (UPGMA) cluster analysis was used to assess genetic diversity among the snail species.

**Results**

*Molecular characterization of snail species*

Optimization of the RAPD-PCR conditions was done due to the sensitivity and reproducibility of the RAPD markers. Different concentrations of genomic DNA, 10X PCR buffer, $MgCl_2$, dNTPs, and *Taq* DNA polymerase were tested for efficient and reliable amplification.

**Table 1.** Accession number and Order of the species.

| Accession Number | Species |
|---|---|
| 1 | *Ariophanta bistrialis ceylanica* |
| 2 | *Ariophanta bistrialis cyix* |
| 3 | *Ariophanta bristrialis taprobanensis* |
| 4 | *Ariophanta bristrialis* |
| 5 | *Ariophanta solata* |
| 6 | *Ariophanta belangeri bombayana* |
| 7 | *Oxychilus draparnaudi* |
| 8 | *Monacha catiana* |
| 9 | *Cernuella virgata* |
| 10 | *Pupoides albilabris* |
| 11 | *Physa fontinalis* |
| 12 | *Zooctecus insularis* |
| 13 | *Juvenile Zooctecus insularis* |
| 14 | *Cecilioides acicula* |
| 15 | *Oxyloma elegans* |

Three different concentrations of template DNA of snails (5, 10, 15, $nguL^{-1}$) were tested and it was observed that 10 ng $\mu L^{-1}$ was found optimum for best amplification. Whereas it was found that 3 mM concentration of $MgCl_2$ and 1.0 unit *Taq* polymerase also found optimum for good amplification in a final volume of 25 µl of reaction mixture. It was also found that higher concentration of *Taq* and $MgCl_2$ gave smear and lower concentrations resulted in light PCR fragments.

Genetic characterization of 15 snail species were done by using 23 RAPD primers and out of which 15 RAPD primers (Table 2) produced polymorphic amplification whereas the remaining 8 primers produced monomorphic banding pattern and thus were excluded from the study.

**Table 2.** RAPD primers with sequence, number of polymorphic bands (NBP), and mean band frequency (MBF).

| Sr. No. | Primer Code | Sequence | TNB | NPB | MBF |
|---|---|---|---|---|---|
| 1 | GL DecamerL-07 | CAGGCCCTTC | 08 | 07 | 0.43 |
| 2 | GL DecamerL-08 | AGTCAGCCAC | 08 | 05 | 0.51 |
| 3 | GL DecamerK-08 | AGGGGTCTTG | 09 | 07 | 0.46 |
| 4 | GL DecamerK-12 | GAAACGGGTG | 07 | 07 | 0.68 |
| 5 | GL DecamerK-19 | TTCCGAACCC | 07 | 05 | 0.36 |
| 6 | GL DecamerK-20 | GGTGACGCAG | 07 | 06 | 0.45 |
| 7 | GL DecamerL-12 | CTGCTGGGAC | 09 | 08 | 0.39 |
| 8 | GL DecamerI-02 | GTGAGGCGTC | 08 | 06 | 0.40 |
| 9 | GL DecamerK-07 | GGGGGTCTTT | 07 | 05 | 0.43 |
| 10 | GL DecamerL-04 | AAAGCTGCGG | 07 | 06 | 0.36 |
| 11 | GL DecamerL-01 | GACGGATCAG | 07 | 04 | 0.31 |
| 12 | GL DecamerK-16 | GTTGCCAGCC | 09 | 07 | 0.41 |
| 13 | GL DecamerB-07 | ACTTCGCCAC | 07 | 06 | 0.33 |
| 14 | GL DecamerB-15 | AGCGCCATTG | 07 | 05 | 0.27 |
| 15 | GL DecamerL-15 | CAGAAGCCCA | 07 | 06 | 0.29 |

Total of 114 fragments were amplified and 90 were found polymorphic that is 78.94% polymorphism. The range of amplified RAPD fragments was found to be 7 to 9 with an average of 7.6 per primer. Three RAPD primers GLK-08, GLL-12 and GLK-09 produced maximum number of bands i.e. 9; The Primer GLD-12 produced maximum number of polymorphic bands (8) and the lowest number of polymorphic RAPD fragments was produced by GLL-1 (4).

**Table 3.** Genetic similarity matrix of 15 snail species based on Nei's genetic identity.

| Pop ID | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | **** | 0.6140 | 0.6842 | 0.6140 | 0.6140 | 0.6842 | 0.5877 | 0.6316 | 0.6140 | 0.6491 | 0.5965 | 0.6491 | 0.6491 | 0.6316 | 0.5614 |
| 2 | | **** | 0.6842 | 0.6316 | 0.6316 | 0.6667 | 0.7281 | 0.7018 | 0.5263 | 0.6140 | 0.6316 | 0.6667 | 0.5789 | 0.6140 | 0.5965 |
| 3 | | | **** | 0.6491 | 0.6491 | 0.7368 | 0.6930 | 0.7368 | 0.6316 | 0.7193 | 0.6491 | 0.6842 | 0.6140 | 0.6667 | 0.6140 |
| 4 | | | | **** | 0.6140 | 0.6491 | 0.6579 | 0.6842 | 0.5439 | 0.6140 | 0.6491 | 0.6667 | 0.6140 | 0.6667 | 0.6140 |
| 5 | | | | | **** | 0.7368 | 0.6053 | 0.6842 | 0.5614 | 0.7193 | 0.6140 | 0.6140 | 0.6316 | 0.6491 | 0.5965 |
| 6 | | | | | | **** | 0.6404 | 0.6667 | 0.6140 | 0.6842 | 0.6491 | 0.6667 | 0.6140 | 0.6842 | 0.6491 |
| 7 | | | | | | | **** | 0.7105 | 0.5877 | 0.6404 | 0.6053 | 0.7281 | 0.6228 | 0.6754 | 0.6228 |
| 8 | | | | | | | | **** | 0.5614 | 0.6842 | 0.6491 | 0.7018 | 0.5965 | 0.6140 | 0.6842 |
| 9 | | | | | | | | | **** | 0.5965 | 0.6491 | 0.6140 | 0.5789 | 0.5614 | 0.5965 |
| 10 | | | | | | | | | | **** | 0.6491 | 0.6667 | 0.5789 | 0.6491 | 0.6667 |
| 11 | | | | | | | | | | | **** | 0.6842 | 0.5965 | 0.6316 | 0.6491 |
| 12 | | | | | | | | | | | | **** | 0.6316 | 0.6491 | 0.6667 |
| 13 | | | | | | | | | | | | | **** | 0.5614 | 0.6316 |
| 14 | | | | | | | | | | | | | | **** | 0.5789 |
| 15 | | | | | | | | | | | | | | | **** |

The overall mean band frequency ranged between 0.27 – 0.68 with an average value of 0.41 (Table 2).

Analysis of molecular variance revealed that the variance component among and within populations was 11% and 89% respectively. Both values are significant at probability less than 0.001 (Table 3, Fig. 1).

*Genetic similarity among snail species*

Genetic similarity estimated by Nei's genetic similarity indices ranged from 0.5 to 0.74. Results depicted that the narrow genetic base was observed among most of the species studies which is more than 50%.

Maximum genetic similarity was found between *Ariophanta belangeri bombayana* and *Ariophanta bristrialis taprobanensis* as well as *Ariophanta belangeri bombayana* and *Ariophanta solata*. Minimum genetic similarity based on Nei's genetic indices was observed among *Cernuella virgata* and *Ariophanta bistrialis cyix* (Table 4).

**Table 4.** Analysis of Molecular Variance Among and Within Populations of Snail Species.

| Source | Df | SS | MS | Est. Var. | % |
|---|---|---|---|---|---|
| Among Pops | 4 | 5.300 | 1.325 | 0.116 | 11% |
| Within Pops | 10 | 9.833 | 0.983 | 0.983 | 89% |
| Total | 14 | 15.133 | | 1.100 | 100% |

*Genetic relationship among snail species*

Genetic relationship among fifteen snail species were estimated using cluster analysis by the unweighted pair group method of arithmetic means (UPGMA) which showed that the two distinct cluster (Fig. 2) First cluster includes the species *Ariophanta bistrialis cyix, Ariophanta bristrialis taprobanensis, Ariophanta bristrialis, Ariophanta solata, Ariophanta belangeri bombayana, Oxychilus draparnaudi, Monacha catiana*.

The most closely related species were *Ariophanta bristrialis taprobanensis, Ariophanta bistrialis ceylanica, Ariophanta bristrialis* and *Ariophanta solata,* followed by species *Ariophanta bistrialis cyix* where as the species *Ariophanta belangeri bombayana, Oxychilus draparnaudi* and *Monacha catiana* also showed close relationship among the members of this group. The most distinct species were found to be *Cernuella virgata* and *Juvenile Zooctecus insularis* which remains unclustered. The other species like *Zooctecus insularis, Pupoides albilabris, Physa fontinalis, Oxyloma elegans* and *Cecilioides acicula* were found to cluster in the second group and linked with each other at some distances. Considering the *Zooctecus insularis* and Juvenile *Zooctecus insularis* they are quite distant from each other in the cluster, it is expected that might belong to some new species, which need further investigations.

The genetic association among the 15 snail species were also detected by principal component analysis and it was found that close association was observed among the species *Ariophanta bistrialis ceylanica, Ariophanta bistrialis cyix, Ariophanta bristrialis taprobanensis, Ariophanta bristrialis, Ariophanta solata, Ariophanta belangeri bombayana, Oxychilus draparnaudi, Monacha catiana* and these species closely clustered in one group while the species *Zooctecus insularis* also present at some distance with rest of the species in this group showing that Juvenile *Zooctecus insularis* might belong to a separate species. The other rest of the species were found scattered into three other groups (Table 4).

**Discussion**

The fresh water gastropods are of extreme conservation concern for example hydrobiid and pleurocerid freshwater gastropods as they are lacking in dispersing capabilities due to which they are found in isolated populations while in general freshwater pulmonate gastropods typically are excellent dispersers.

The major problem which comes in the way of species classification of the gastropods is the correct recognition of the differences between the species at the population level as previously stated by (Edwards and Beerli, 2000; Wiens and Penkrot, 2002; Maddison and Knowles, 2006). The molecular taxonomy is quite effective at this point of time as systematic relationship present in the sister taxa is there even after millions of year and, can be studied with the help of the genetic signatures (Thomaz *et al*., 1996; Arbogast *et al*., 2002).

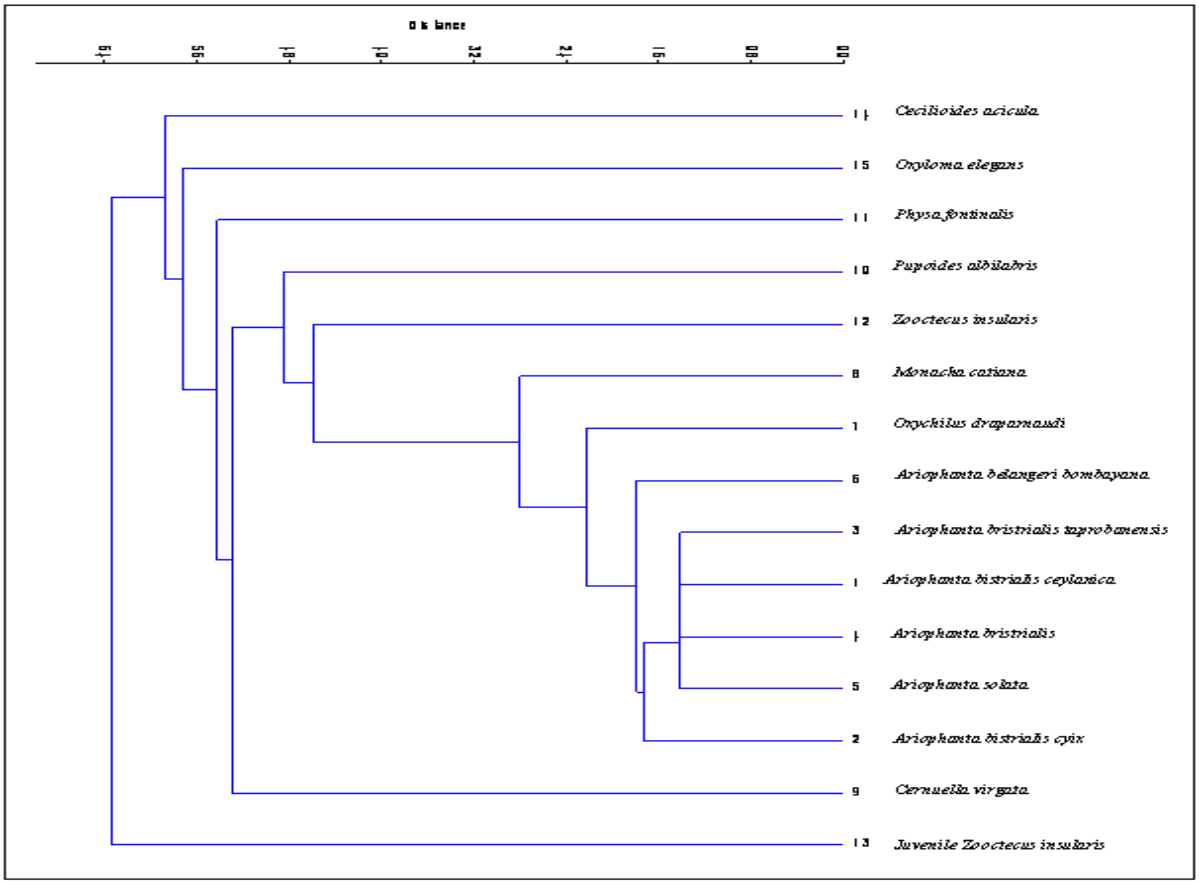


**Fig. 1.** Dendrogram showing relationship among 15 snail species.

The processes occurring at the population level must be considered when taxonomies of recently diverged taxa are being studied. The perception of isolation has been supported by the molecular data to some degree. Considering the distributional patterns there have been very few records of pleurocerid or hydrobiid lineages that are found across unconnected water drainages systems as previously stated by (Minton and Lydeard, 2003; Perez *et al*., 2005; Sides, 2005), and documentation of the level to which there is an exchange in the genes of the upstream and downstream populations. There is an indication of the deep divergence by molecular analyses of intraspecific mitochondrial lineages of freshwater gastropod species as previously stated by (Dillon and Frankis, 2004; Lee *et al*., 2007). The genetic variation due geographical isolation increases the effective population size as previously stated by (Wright, 1943; Wakeley, 2000). The long term retention of ancient mitochondrial alleles, 20 million years, is due to an increase in effective population size as stated by Thomaz *et al*., (1996) much older than the coalescence times expected from mutation rate and population size. In hydrobiid and pleurocerid groups of the gastropods the mutation rates may be elevated up to 8 to 14 base pairs per million years as stated by (Thacker and Hadfield, 2000; Holland and Hadfield, 2002; Rundell *et al*., 2004). There may be an effect of the founder events on the genetic diversity of the closely related species events may include local extinction, and random genetic drift, adaptive genetic change (Goodacre *et al*., 2006). The interpretation of high level of population subdivision that produce a pattern of reciprocal monophyly as differences at the species level due the fact that sampling is done from restricted localities (Arbogast *et al*., 2002). These problems are resolved when dense population-level and geographic sampling are combined with use of the appropriate molecular markers do mitigate but taxa whose ranges are reduced by the human actions, the aforesaid problem might be unavoidable.

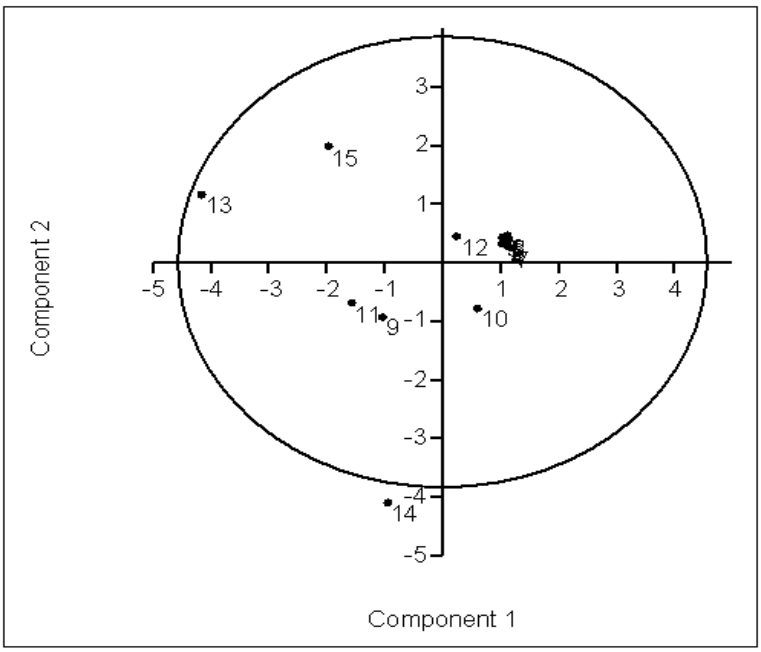


**Fig. 2.** Principal component analysis of 15 snail species based on molecular data.

This misleading pattern is particularly likely when data consist solely of mitochondrial DNA sequences because of the smaller effective population size of the mitochondrial genome. A final complication that must be considered is the status of populations that are in the process of speciation, before or after reproductive isolation has evolved. Historical gene flow in recently diverged species is hard to disentangle from recent contact if introgression is possible as previously stated by (Wakeley, 1996; Rosenberg and Feldman, 2002).

## Conclusion

This study is extremely important in the recognition of the effective markers to identify the gastropod species to further extend our research to find species specific markers.

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