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First record of body colour polymorphism in giant African snail *Achatina fulica* (Bowdich, 1822) - a comparative study using mitochondrial cytochrome oxidase subunit I (COI) gene

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ABSTRACT: The presence of the body colour polymorphism in the tropical invasive pest giant African snail is reported for the first time from South India. Three different body colour polymorphs were recognised *viz*. grey, black and white. The grey body colour is the most common polymorph. The black and white colour polymorphs are found to be in almost equal proportions in the reported localities with the grey counterparts. The cytochrome oxidase subunit I (COI) sequences of the three colour polymorphs are found to be identical. The presence of the body colour polymorphism in south India may be attributed to the avian predation and other selection pressures.

KEYWORDS: Polymorphism, giant African snail, COI gene

Colour polymorphism is the co-occurrence of two or more different colour patterns within a population. The physical structures which reflects light or the pigments which are present in the body of an organism is the major cause of different colours in nature. Both these are extremely sensitive to genetic variation (White and Kemp, 2016). Generally, colour polymorphism patterns in animal groups influence the predator-prey relationships. That is while considering the predators which consumes polymorphic preys, suffers from a lesser performance as compared with the others who consumes only monomorphic preys (Karpestam *et al*., 2016). Colour polymorphism also hinders the competence or efficiency of the predators which focuses on specific coloured preys and this increases the survival of the prey species (Karpestam *et al*., 2014). This is demonstrated by apostatic selection in which the common colour polymorph of a species is memorised by the predator and the rare colour polymorph is maintained in the population. The typical case of this is the polymorphic grove snail *Cepaea nemoralis*. Here the rare colour morph is rarely recognised by the avian predators and they become a common variety. Functionally, body colour polymorphism is used for phenomena such as sexual signalling, crypsis, thermoregulation, mutualism, aposematism and sometimes in Batesian and Mullerian mimicry (White and Kemp, 2016). Another aspect of colour polymorphism is the melanin induced body colour change due to climate change in which pale body coloured organisms were

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present in those areas where more desertification occurred and black body coloured ones in those areas with high humidity (Roulin, 2014).

Shell colour polymorphism has been studied intensely in many species of land snails like *Thais* (or *Nucella*) *emarginata* of North America (Palmer, 1985). In *Cepaea nemoralis* snails, those snails living in shaded habitats are having dark coloured shells and those living in open habitats are having light coloured shells (Schilthuizen, 2013). Shell colour is also associated with the substrate colour in the species *Littorina saxatilis* which varies from grey to brown. The grey coloured shells were found on the grey coloured rocks and brown coloured shells on red coloured rocks (Byers, 1990). Dark to light shell colours were exhibited in the snail *Bradybaena similaris* along with presence and absence of bands. The colours of the shell and banding pattern was controlled by two different alleles B and C which are linked (Asami and Asami, 2008). Homozygous recessive alleles make the shells of the African Achatinidae un-streaked and the dominant alleles provide streaked shells (Allen, 1985). Shell banding pattern and chirality of the shells of the Giant African snails revealed dextral and considerable variation in the banding pattern in the Bengaluru region of India (Jayasankar *et al*., 2014). Very few studies have described body colour polymorphism in land snails. The body colour polymorphism in the snail *Pomaceae canaliculata* exhibited two different body colours- yellow and brown- and their inheritance followed simple Mendelian inheritance pattern with yellow recessive to Brown (Yusa, 2004). The black and white skinned snails of *Archachatina marginata* has shown significant difference in the genetic level when examined using RAPD markers (Etukudo *et al*., 2016). The Giant African land snails *Achatina achatina* and *Archachatina marginata,* showed marked body colour polymorphism with black and white snails, while white in urban and black in the forest zones (Owen and Reid, 1986). Fresh water snails also exhibit body colour polymorphism (Tiecher *et al*., 2017). No reports are available regarding the body colour polymorphism in the Giant African Snail *Achatina fulica* (Bowdich, 1822), an invasive alien pest in the tropical region.

The Giant African Snail *Achatina fulica* (Bowdich, 1822) native of East Africa is one of the worst of 100 invasive species in the world. The snail was introduced to India from Mauritius in 1847 and spread to many parts. In South India, the snails were first reported around 1900s (Mead, 1961). In this study we present three different body colour polymorphs of the Giant African Snail from south India and have used a partial sequence of cytochrome oxidase subunit I (COI) gene to identify the extent of genetic variation in the colour polymorphic snails.

Field surveys were conducted in the states of Kerala and Tamil Nadu during the years 2016 to 2018 and collected samples of giant African snails from the infested areas. The geo-coordinates of all the collected localities were recorded in a hand-held Global Positioning System (GPS). We also recorded the body colour in the field and the collected snails were reared in a glass house. The dead snails were preserved in 70% ethanol for molecular analysis.

DNA isolation

DNA isolation was achieved from the foot muscle tissue of ethanol preserved snail samples. The tissue parts were soaked in sterile water for an hour or two to remove ethanol. The general Cetyl Trimethyl Ammonium Bromide CTAB method modified from Winnepenninckx *et al*., (1993) was used. The washing step using Phenol: Chloroform: Isoamyl alcohol (25:24:1) solution was repeated several times to remove the mucopolysaccharides present in the snail tissues. The DNA was precipitated by adding iso-propyl alcohol and overnight incubation. The isolated DNA was stored at -20°C for further use.

PCR Amplification and Sequencing

The isolated DNA was subjected to PCR amplification using the Cytochrome Oxidase Subunit I (COI) gene. The universal COI primers developed by Folmer *et al*., (1994) LCO-1490: 5' GGTCAACAAATCATAAAGATATTGG 3' HCO-2198: 5' AAACTTCAGGGTGACCAAAAAA TCA 3' was used for gene amplification. PCR amplification was standardised with the following

Fig 1. Body colour polymorphism in Giant African Snail *Achatina fulica*

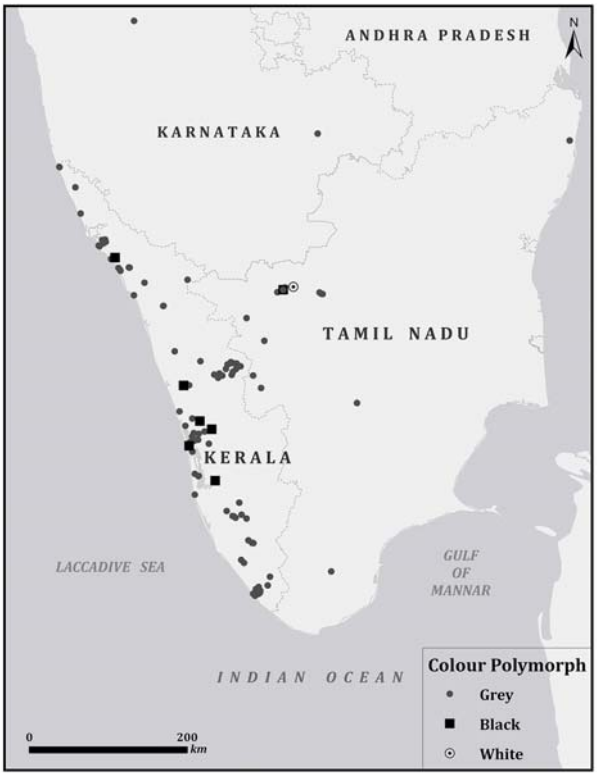

Fig 2. Localities of the three different colour polymorphs of *Achatina fulica*

conditions: initial denaturation at 94°C for 2'; 38 cycles of 94°C for 30", 48°C for 30" and 65°C for 1'; followed by final elongation of 65°C for 10'. The PCR reaction yielded 406 base pair sequence of the COI gene of the snails. The PCR products were visualised on a 1.2% agarose gel and the sequencing of the PCR products was done through service providers.

Matching of the sequences was done using NCBI BLAST search tool. The sequences obtained from the study have shown similarity with the complete mitochondrial sequence and the Cytochrome Oxidase Subunit I gene sequences of *Achatina fulica* available in the NCBI. The raw sequences were trimmed using Bioedit and aligned using ClustalW and assembled manually in Molecular Evolutionary Genetic Analysis (MEGA) version 7.0.26 (Kumar *et al*., 2016).

A total of 263 Giant African Snail infested localities were surveyed in south India of which 246 were from Kerala and 17 were from Tamil Nadu. Three different colour polymorphs, grey, black and white were distinguished from the surveys and the samples collected (Figure 1). Grey is the common body colour and are thoroughly distributed all along the snail distribution localities. The black body colour polymorph was reported from seven localities in Kerala and Tamil Nadu. They were from Willington Island, Angamaly, Kottayam, Perumbavoor, Poonkunnam and Pinarayi in Kerala, and in Sathyamangalam from Tamil Nadu. The white bodied snails were seen in two localities *viz*., Konamoolai and Kenjanur near Sathyamangalam in the Erode district of Tamil Nadu. The three body colour polymorphs were seen together in a single locality from Kenjanur near Sathyamangalam in Tamil Nadu. In all other places either grey and black or grey and white (from one locality Konamoolai in Tamil Nadu) were seen together. In the populations from two localities in Tamil Nadu, the white and black polymorphs were seen almost in equal proportion with the grey polymorphs. The black snails in Kerala where it was reported from six localities, they are found to be in almost equal numbers with that of the grey polymorphs (Figure 2). The young ones of the white bodied snails and black bodied snails were checked to know whether the snails are leached out due to the manure applied in the fields or due to some other natural phenomenon. But the young ones of these snails which were collected from the field are also having the same body colour like their adults. But the eggs laid by the white bodied snails and the black bodied snails failed to hatch in the laboratory conditions. The grey, black and white polymorphs of three snails from Kenjanur near Sathyamangalam in Tamil Nadu, where the three exists together were subjected to molecular analysis using the partial sequence of the 406 base pair long COI. The sequence was deposited in the National Centre for Biotechnology Information (NCBI) GenBank database and the accession number is LC440023.1.

The alignment and assembly of the sequences obtained from the colour polymorphic snails showed no genetic variation in their sequences. The sequences were found to be completely similar. This shows that in the Giant African snails are exhibiting colour polymorphism. All the colour polymorphic

snails are of the same species and not even a different variety. The complete similarity in the genetic makeup of the organism confirms this. Also, the presence of the colour polymorphism in the snail is not due to any external factors like application of manure, extremely harsh climates or some other environmental factors. Because, the colour polymorphic snails were found in equal proportion with that of its grey counter parts.

Along with high reproductive potential, ability to withstand harsh climatic conditions and adaptability in many environments of the snail in South India and the presence of colour polymorphism makes *Achatina fulica* an excellent invasive species. The black snails of Kerala are very useful ecotype to study the movement of snails within the state. Generally the snails which were captive bred are found to show a phenomenon of white body colour and this occurs due to continuous inbreeding. The white polymorph is available from only one report outside India from a single source in Venezuela (http://caracol.cbm.usb.ve/). The Giant African snails *Archachatina marginata* and *Achatina achatina* are found to be showing remarkable body colour polymorphism from black to white in Africa. The local people are consuming the snails in that region but due to cultural and medicinal beliefs they avoid collection of the white bodied snails. This helps in maintaining the polymorphism and species (Owen and Reid, 1986). Avian predators also play very important in maintaining the colour polymorphism in snails. The cryptic behaviour of the snails based on the background is helpful for the snails to maintain its population (Surmacki *et al*., 2013). The black and the white skinned snails are showing genetic differences in Africa also. This proves high heterogeneity in the Giant African land snails in Africa (Etukudo *et al*., 2016). In one sampled locality in Tamil Nadu, the snails are seen to be predated in large numbers by the open billed stork *Anastomus oscitans*, which could be a reason for the presence of white polymorphs in an equal proportion. Similarly, in Kerala the black polymorphs are seen in equal proportions which might supports the phenomenon of dark colour which is adapted to live in the high rainfall regions. This is the first report of the body colour polymorphism in giant African snails. The presence of body colour polymorphism in *A. fulica* and its huge adaptability to different environments makes the species a very successful invasive species. This adaptation makes the snail to escape from different avian and other predators. More molecular studies have to be attempted in this regard using suitable nuclear markers to understand the hereditary mechanism behind the adaptation and the movement of the body colour polymorphism in the species.

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