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ORIGINAL ARTICLE

Evaluation of haplotype diversity of *Achatina fulica* (Lissachatina) [Bowdich] from Indian sub-continent by means of 16S rDNA sequence and its phylogenetic relationships with other global populations

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Abstract *Achatina fulica* (*Lissachatina fulica*) is one of the most invasive species found across the globe causing a significant damage to crops, vegetables, and horticultural plants. This terrestrial snail is native to east Africa and spread to different parts of the world by introductions. India, a hot spot for biodiversity of several endemic gastropods, has witnessed an outburst of this snail population in several parts of the country posing a serious threat to crop loss and also to human health. With an objective to evaluate the genetic diversity of this snail, we have sampled this snail from different parts of India and analyzed its haplotype diversity by means of 16S rDNA sequence information. Apart from this, we have studied the phylogenetic relationships of the isolates sequenced in the present study in relation with other global populations by Bayesian and Maximum-likelihood approaches. Of the isolates sequenced, haplotype 'C' is the predominant one. A new haplotype 'S' from the state of Odisha was observed. The isolates sequenced in the present study clustered with its conspecifics from the Indian sub-continent. Haplotype network analyses were also carried out for studying the evolution of different haplotypes. It was observed that haplotype 'S' was associated with a Mauritius haplotype 'H', indicating the possibility of multiple introductions of *A. fulica* to India.

Keywords *Achatina fulica* · Haplotype · Indian sub-continent · 16S rDNA · Network analysis · Phylogeny

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Introduction

Achatina fulica (Bowdich, 1882) is a pulmonate land snail found in different parts of the world. It has its origin from East Africa and has spread to different parts of the world by introductions (Albuquerque et al. 2008). It is distributed to all the continents except Antarctic. Madagascar is the first place of introduction outside the African continent from Kenya prior to the year 1800. From Madagascar, it was introduced to Mauritius. From Mauritius, it has spread to different parts of the world (Raut and Baker 2002). It is a pestiferous snail and is recorded as one among the 100 most invasive species found in the world by IUCN (Lowe et al. 2000). It is a vector for rat lung worm *Angiostrongylus cantonensis* that causes eosinophilic meningitis in humans, *Angiostrongylus costaricensis* that causes abdominal angiostrongyliasis, and another worm *Angiostrongylus abstrusus*, whose effects are yet to be known (Silvana et al. 2007; Jayashankar et al. 2014; Joseph 1966; Fernanda et al. 2010). Apart from causing damage to the human health, *A. fulica* is also a carrier of several pathogens that infests different crops (Rudra et al. 2009), thus damaging both the human health as well as agricultural crops.

Indian sub-continent harbours rich biodiversity of several endemic gastropods (Ramakrishna et al. 2010). In the recent past, the country has witnessed an outbreak of *A. fulica* in different parts of India causing a significant damage to agro-horticultural plants. As per the historical records, this snail was first introduced into the country at Kolkata (erst while Calcutta) in April 1847 by a famous malacologist, Sir. Henry Benson. He had collected two individuals of *A. fulica* from Mauritius in February, 1847. These were handed over to his friend, who had released them into his garden at Chowringhee, Kolkata (Naggs

1997). Later, it was spread to different parts of the country mostly by introductions. From West Bengal, it was introduced to Odisha and Bihar (Sheela 1999). From Odisha, these were introduced at Araku Valley in the Visakhapatnam district in the state of Andhra Pradesh (Rekha et al. 2015). This snail was introduced to North Bihar from Kolkata by a black smith (Sheela 1999). In 1959, a few snails were introduced to Nagaland from West Bengal by R.S. Bedi. He had brought these snails out of curiosity and released them into his garden at Dimapur. Later, it was spread to in and around areas of Dimapur and caused significant damages to the crop plants. It was introduced for the first time at Chennai (erst while Madras) in the south Indian state of Tamil Nadu at 'My lady Garden' during British period. From there, it was spread to different parts of Tamil Nadu (Raut and Ghose 1984). It was introduced to Andaman and Nicobar Islands around 1940s by Japanese soldiers during World War II. This has resulted in its present day dispersal in this region (Paras Nath 2007). This snail was also introduced at Mussoorie, a hill station in India by Captain Hutton. However, all the introduced specimens were dead due to severe winter (Mead 1961). Deliberate transport of this snail from one region to another region is one of the main reasons for its wide spread in India and to different parts of the world (Mead 1961; Budha and Naggs 2008). *A. fulica* has spread to different states in India, viz., Karnataka, Andhra Pradesh, Uttar Pradesh, Bihar, Tamil Nadu, Maharashtra, Kerala, Gujarat, Nagaland, Assam, Jharkhand, Daman and Diu, Tripura, Mizoram, Goa, Andaman and Nicobar Islands, and Lakshadweep Islands.

In India, Agriculture sustains the livelihood of 70% of its population and is a significant contributor to the nation's economy. Agricultural yield in the country is dependent on several factors like onset of monsoon, availability of water resources, etc (Khan et al. 2009). Any change in the aforementioned parameters adversely affects the agricultural yield and thus affects the livelihood of farmers. Apart from these abiotic factors, yield is also significantly affected by several diseases and infestations by different pests. In such a scenario, the prevalence of *A. fulica* has become a great menace causing severe damage to crops, vegetables, and horticultural plants, thereby adversely affecting the economies of the families dependent on agriculture and agro-based industries.

The outburst of this snail was first reported in the country in Odisha as an epidemic form in 1928 (Nath 2007). It is a voracious feeder with diverse food habits and feeds on different horticultural, ornamental, and crop plants viz., banana (Mathai 2014), Vanilla (Vanitha et al. 2011), cucumber, brinjal, lady's finger, cabbage, cauliflower, turmeric (Sheela 1999), mulberry, ground nut, French beans, marigold (Jadhav et al. 2016), etc. This has made its survival in the newly introduced areas a success and a great loss to the crop plants. It is overwhelming to see the list of wide varieties of crops, vegetables, horticultural, and ornamental plants that it feeds on (Albuquerque et al. 2008; Jadhav et al. 2016).

In the recent past, there are several unpublished reports that are growingly available with respect to the severity of its infestations in new areas and its damage to the agro-horticultural plants. In a recently published study carried out by Jadhav et al. (2016) in the Kolhapur district from the state of Maharashtra, this snail was found to cause severe damage to the foliage of Mulberry plants. Furthermore, the leaves were found unsuitable for feeding the silkworm because of the presence of mucus and excreta of this snail. As a result, the local sericulture industry was severely damaged and several farmers were choosing to opt out sericulture.

Introduced out of its native range, its outburst in different geographical regions is attributed mainly due to its high reproductive capacity, thus out competing the endemic snail populations which lead to their extinction, thereby posing a serious threat to the local biodiversity (Silvana et al. 2007). There are significant costs involved in managing this pestiferous snail, which has become a burden to the states. This includes the cost towards the purchase of various chemicals, expenses related to manpower, etc (Civeyrel and Simberloff 1996). For example, it costed an amount of 700,000 dollars to the United States for the complete eradication of *A. fulica* from Florida (Poucher 1975). Brazil has incurred agricultural losses up to US 42.6 billion dollars (Silvana et al. 2007). Most importantly, these expenses do not cover the losses to human health. Apart from this, its presence is a nuisance on daily basis in the backyards of homes, offices, universities (Mathai 2014), and roads (Rekha et al. 2015).

Given the severity of damage caused to the agricultural crops, an immediate step is required to prevent its further spread. Hence, there are several approaches which includes the intervention at human level by clearing the areas where these snails resides (Budha and Naggs 2008; Albuquerque et al. 2008); chemical control by spraying different molluscicides; and also by biological control mechanisms (Mathai 2014).

Apart from its negative impact on the agriculture and human health, it possesses medicinal properties and is also edible in different parts of the world. These are one of the main reasons for its introduction to Madagascar from its native range (Raut and Baker 2002; Fontanilla et al. 2014). However, people have deliberately introduced from one place to another place out of wonder and curiosity after they encountered this snail (Budha and Naggs 2008).

Achatina fulica has long historical association to India. However, genetic diversity of this snail is not explored in

entirety. The present study is carried out with an objective to look for the presence of any new haplotypes in the Indian sub-continent by increasing the extent of sampling than that carried out by Fontanilla et al. (2014). The first step towards studying the haplotype diversity of this snail by 16S rDNA was carried out by Fontanilla et al. (2014). They have sampled this snail from different parts of the world. With respect to India, they have confined their sampling only to four localities from the state of Maharashtra. Hence, in the present study, we have sampled this snail from new areas. Because of the spread of this snail over wide geographical regions, and due to logistic constraints and limited man power, *A. fulica* from all the regions of India was not sampled. Especially, we were unable to sample this snail from Kolkata, which is considered to be the first place of introduction to India.

In the present study, we have chosen 16S rDNA for evaluating the haplotype diversity and phylogenetic relationships among the isolates of *A. fulica* sequenced in this study in relation with other global populations.

Materials and methods

In the present study, a total of eight individuals of *A. fulica* were collected from September 2014 to December 2015 from different geographical regions of India covering four different states, viz., Andhra Pradesh, Karnataka, Odisha, and Bihar (Table 1; Fig. 1) for studying their phylogenetic relationships.

Isolation of genomic DNA

Interference of mucopolysaccharides and proteins from the gastropod tissues makes genomic DNA unsuitable for PCR amplification (Wade and Mordan 2000; Ayyagari et al. 2017) and (personal observation). To overcome this, we have standardized different isolation procedures for the isolation of good quality genomic DNA from the foot muscle tissue of *A. fulica* suitable for the amplification of 16S rDNA. They include the protocol of Ayyagari et al. (2017) and slightly modified procedure of Sokolov (2000) for the isolation of genomic DNA from the foot muscle tissue of *A. fulica*. For few isolates, small piece of foot muscle tissue was cut noninvasively. For these, genomic DNA isolation was carried out by employing NucleoSpin® Tissue kit (Macherey–Nagel) following the manufacturer's instructions.

Amplification and sequencing of 16S rDNA

Genomic DNA was amplified for 16S rDNA using the primers of Palumbi et al. (2002). PCR conditions were as that of Ayyagari et al. (2017). Alternatively, PCR was performed in a 20 µl reaction volume which contained 1× Phire PCR buffer (contains 1.5 mM $MgCl_2$), 0.2 mM dNTPs, 1 µl DNA, 0.2 µl Phire Hot start II DNA polymerase enzyme, 5 µM of forward and reverse primers, 0.1 mg/ml BSA, 3% DMSO, and 0.5 M betaine. The cycling conditions consisted of denaturation at 98 °C for 30 s followed by 40 cycles of denaturation at 98 °C for 5 s, annealing at 48 °C for 10 s, extension at 72 °C for 15 s, and a final extension at 72 °C for 1 min (Fig. 2a–d). PCR amplification was carried out in thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

PCR products were cleaned using ExoSAP-IT™ (GE Healthcare) following the manufacturer's instructions. Purified amplicons were sequenced in both the directions with the same primers used for PCR at Regional facility for

Table 1 List of NCBI accession numbers, places of collection, and haplotypes of the isolates of *Achatina fulica* sequenced in the present study

Sr. no.	Organism (isolates)	Locality	Collector	NCBI GenBank™ accession number	No. of isolates sequenced	Haplotype
1	*Achatina fulica* (Af-Ban_1 and Af-Ban_2)	Bangalore, Karnataka	Vijaya Sai A	KP317640	2	C
			Vijaya Sai A	KX060743		C
2	*Achatina fulica* (Af-Od_1 and Af-Od_2)	Bharatpur, Odisha	Jena	KP317641	2	S
			Jena	KP119753		S
3	*Achatina fulica* (SKN-Af-Bhr_1, SKN-Af-Bhr_2 and SKN- Af-Bhr_3)	Bihar	Kishore Kunal	KX514436	3	C
			Kishore Kunal	KX514437		C
			Kishore Kunal	KX514438		C
4	*Achatina fulica* (Af-SSSPN-PTP_1)	Puttaparthi, Andhra Pradesh	Vijaya Sai A	KX514435	1	C

Fig. 1 India map depicting the states from where *Achatina fulica* was sampled for the present study. *A.P* Andhra Pradesh state

DNA fingerprinting, India. The chromatograms were visualized and edited for obtaining consensus sequences in DNA Baser v4.20.0 (www.DnaBaser.com). Resulting consensus sequences were submitted to NCBI GenBank™ and were assigned accession numbers KP317640–KP317641, KX514435–KX514438, KX060743, and KP119753 (Table 1).

Sequence analysis

We first report the longest 16S rDNA sequence of *A. fulica* so far submitted to the NCBI GenBank™/DDBJ/EMBL. The primers used in our study were different from the primers used by Fontanilla et al. (2014). The primers of Fontanilla et al. (2014) bind to two conserved regions that lies within the regions of 16S rDNA amplified by the primers used in our present study. To include only the common regions amplified by both the primers in our analyses, we have removed few nucleotide sequences obtained in our study lying outside the primer sequences of Fontanilla et al. (2014). Therefore, while performing multiple sequence alignment, only the common regions were included.

Apart from the nucleotide sequences obtained from the present study, we have retrieved 16S rDNA sequences of *A. fulica* from NCBI GenBank™ for studying the phylogenetic relationships of the sequenced specimens with those from the retrieved (Table 2).

Multiple sequence alignment was performed in MEGA v6.06 (Tamura et al. 2013) using the inbuilt muscle (Edgar 2004) programme by default parameters. The sequence alignment data were exported to fasta format using MEGA (Tamura et al. 2013), which was then converted to Nexus format suitable for usage in MrBayes v3.03 (Huelsenbeck and Ronquist 2001) by MESQUITE v3.2.5 (Maddison and Maddison 2015). The suitable substitution model for the data set implemented in MrBayes was set to HKY, which

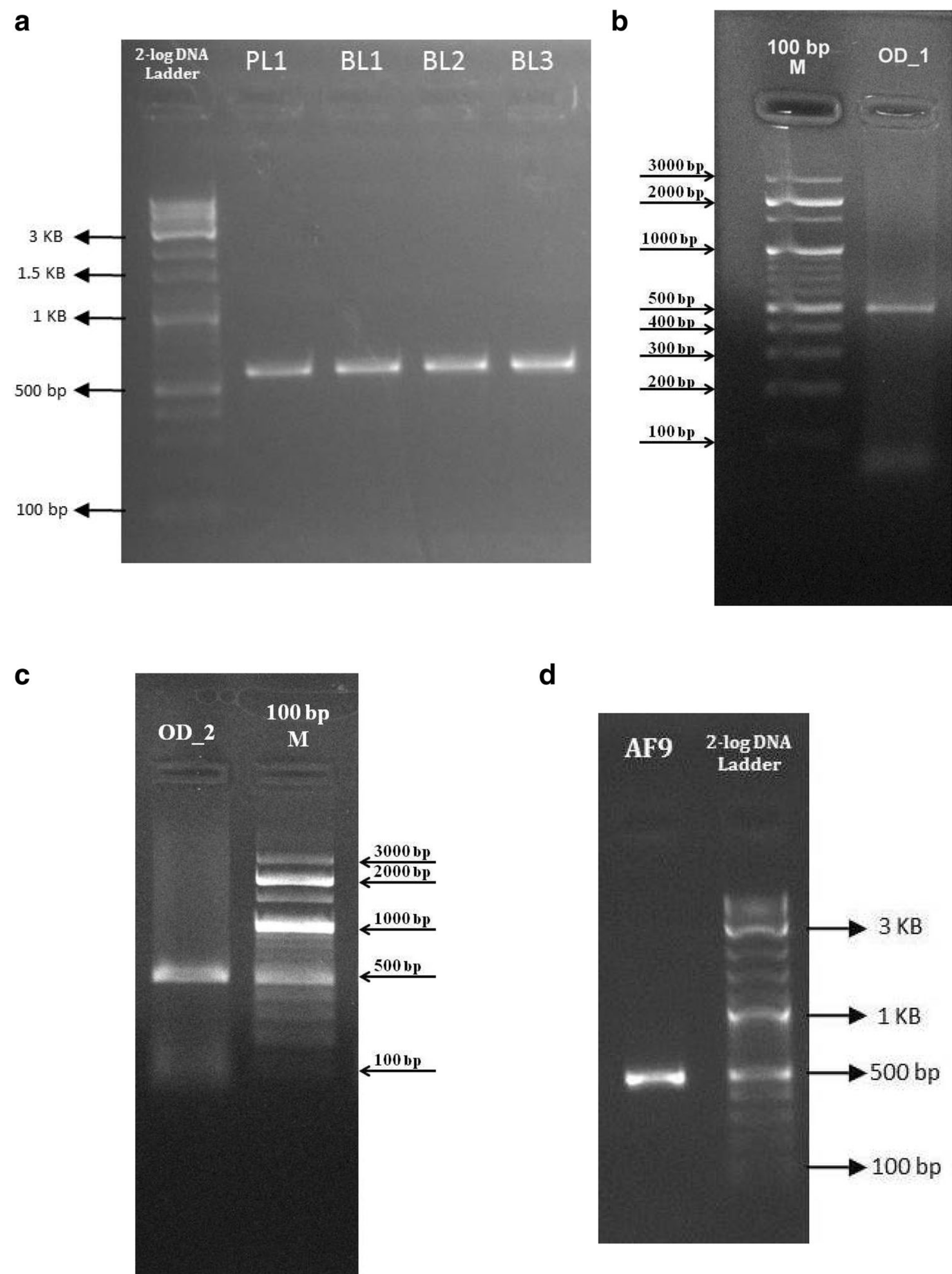

Fig. 2 **a** PCR amplification of 16S rDNA from the isolates of *Achatina fulica. 2-Log DNA ladder* is molecular weight marker. *PL1*, *BL1*, *BL2,* and *BL3* are the different isolates of *Achatina fulica.* **b** PCR amplification of 16S rDNA from the isolate of *Achatina fulica. 100 bp M* is molecular weight marker. *OD_1* is an isolate of *Achatina fulica.* **c** PCR amplification of 16S rDNA from the isolate of *Achatina fulica. 100 bp M* is molecular weight marker. *OD_2* is an isolate of *Achatina fulica.* **d** PCR amplification of 16S rDNA from the isolate of *Achatina fulica. 2-Log DNA ladder* is molecular weight marker. *AF9* is an isolate of *Achatina fulica*

was determined by importing the Nexus file created by Mesquite to jMODELTEST v2.1.3 (Darriba et al. 2012) by choosing the Bayesian inference criterion (BIC).

Phylogenetic analyses

Bayesian inference (BI) of phylogenetic relationships of the haplotypes was carried out in MrBayes for 10,000,000 generations with two independent runs. Each run was carried out with four chains (3 heated and 1 cold) with default heating parameters. The chains were sampled at the end of every 1000th generation. Of the 10,000 trees generated, initial 2500 trees were discarded as burnin. The average standard deviation of the split frequencies was <0.05 and the potential scale reduction factor (PSRF) was 1.000. The phylogram was visualized using the 'sumt' command. The consensus tree generated was visualized in FigTree v1.4.2 (Rambaut 2012) by importing the '.con' file. The convergence of markov chains towards the target distribution was assessed by importing the 'P' files into

Table 2 List of nucleotide sequences retrieved from NCBI GenBank™ (Fontanilla et al. 2014)

S. no.	Locality	Haplotype(s)	NCBI accession no.	Reference
1	East Africa: Kampala, Uganda	O	JQ436767	Fontanilla et al. (2014)
2	East Africa: Dar Es Salaam, Tanzania	I, J, K, L, M, N	JQ436761–JQ436766 KC682495	Fontanilla et al. (2014)
3	Indian Ocean Islands, Mayotte	A, B	JQ436753–JQ436754	Fontanilla et al. (2014)
4	*Indian Ocean Islands* Mayotte, Mauritius, Seychelles and Madagascar, Nepal, Sri Lanka	C	JQ436755	Fontanilla et al. (2014)
	India			
	Pune, Talegaon			
	South East Asia			
	Burma, Thailand, Philippines, Singapore, Malaysia, Vietnam			
	Pacific Islands			
	Hahasima, Ogasawara, Polynesia, Hawaii			
	Caribbean			
	Martinique			
	North America			
	Florida, USA			
	South America			
	Ecuador, Bolivia, Brazil			
12	*Indian Ocean Islands* Mayotte and Mauritius, *South America*	D	JQ436756	Fontanilla et al. (2014)
	Guayas, Ecuador, La Mana, Cotopaxi, Ecuador and Puerto Suarez, Bolivia			
13	Mayotte	G	JQ436759	Fontanilla et al. (2014)
14	Mayotte and Mauritius	H	JQ436760	Fontanilla et al. (2014)
15	*Indian Sub-continent*	P	JQ436751	Fontanilla et al. (2014)
	Nagpur and Nashik			
16	*South East Asia*	E	JQ436757	Fontanilla et al. (2014)
	Philippines			
17	*Pacific Islands*	F	JQ436758	Fontanilla et al. (2014)
	New Caledonia			
	Caribbean			
	Barbados			
18	*South America*	Q	JQ436752	Fontanilla et al. (2014)
	Ecuador			

TRACER v1.6 (Rambaut et al. 2014) for estimating the effective sample size. The sample size was found to be >500.

Apart from Bayesian inference (BI), maximum-likelihood (ML) analysis was carried out based on HKY model in MEGA with 1000 bootstrap replicates.

Both rooted and unrooted trees were constructed by ML and BI methods. Isolates sequenced in the present study were searched against the NCBI GenBank database using Blast algorithm (Basic Local Alignment Search Tool; http://www.blast.ncbi.nlm.nih.gov) for choosing an appropriate outgroup. On the basis of percentage similarity, *Achatina reticulata* was chosen to root the phylogenetic trees.

Haplotype analyses

After multiple sequence alignment, haplotypes were identified by carefully observing the nucleotide differences between the sequences. Each haplotype was designated by an alphabet code. The nomenclature of haplotypes used was the same as that was used by Fontanilla et al. (2014), except for the newly observed haplotype 'S' (Table 3).

Haplotype network of the isolates of *A. fulica* was drawn using TCS v1.21 (Clement et al. 2000; Fig. 3) by importing the alignment file in Nexus format into this software. Apart from this, median-joining network of haplotypes was constructed using SplitsTree v4.14.3 (Huson and Bryant 2006; Fig. 4).

Results

Till date, there is a little systematic evaluation of the genetic diversity of this snail collected from different parts of India. This study aims in evaluating the haplotype diversity of *A. fulica* collected from different parts of India and also to study the phylogenetic relationships of the isolates sequenced in this study in relationship with the isolates of *A. fulica* from different parts of the world.

Various parameters relating to the nucleotide sequences were estimated in MEGA (Tamura et al. 2013). Pairwise distance between the sequences was analyzed using the maximum composite likelihood (MCL) model in MEGA v6 (Table 4). The nucleotide frequencies for A, T, C, and G were 28.50, 35.79, 15.44, and 20.27%, respectively. The transition/transversion rate ratios were $k_1 = 8.019$ (purines) and $k_2 = 6.022$ (pyrimidines), and the overall transition/transversion bias was $R = 3.186$. The rates of substitution of one nucleotide to another were estimated using the MCL method. The rates of transitional substitutions computed for $A \rightarrow G = 18.07$, $T \rightarrow C = 10.33$, $C \rightarrow T = 23.96$ and $G \rightarrow A = 25.4$ and transversional substitutions computed for $A \rightarrow T = 3.98$, $A \rightarrow C = 1.72$, $T \rightarrow A = 3.17$, $T \rightarrow G = 2.25$, $C \rightarrow A = 3.17$, $C \rightarrow G = 2.25$, $G \rightarrow T = 3.98$, and $G \rightarrow C = 1.72$ (Table 4).

The pairwise distance between the haplotypes varied from a maximum of 0.025 to a minimum of 0–0.003. With respect to the isolates sequenced in the present study, haplotype S was highly distant (0.025) from haplotype L and least distant (0.003) from haplotype C (Table 5).

There were a total of 293 nucleotide positions after multiple sequence alignment. Of these, 274 were conserved and 19 were variable. Of the 19 variable sites, six sites were parsimony informative.

Table 3 Nucleotide sequence variations in the 19 haplotypes (A–S) of *Achatina fulica*

Haplotype	Nucleotide position																				
	19	42	71	102	103	106	145	151	153	155	156	158	197	205	210	217	258	277	278	282	283
A	G	A	T	C	C	C	C	A	A	T	A	A	T	T	C	A	A	T	T	T	T
B	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
C	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
D	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.
E	A	C	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
F	A	.	.	.	.	.	.	.	.	C	.	G	.	.	.	.	.	-	.	.	.
G	A	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	-	.	.	.
H	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	.
I	A	.	.	T	.	.	.	G	.	.	T	.	C	.	.	.	.	.	.	.	.
J	A	.	.	T	.	.	.	G	.	.	T	.	.	.	.	.	.	-	.	.	A
K	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	-	-	.	.
L	A	.	.	G	.	.	.	G	.	.	T	.	C	C	.	.	.	-	-	.	.
M	A	.	.	T	.	.	.	G	.	.	T	.	c	.	.	.	.	-	.	.	.
N	A	.	.	T	.	.	.	G	.	.	T	.	.	.	.	G	.	-	.	.	.
0	A	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	-	.	A	.
P	A	.	.	.	T	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
Q	A	.	C	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
R	A	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	-	-	.	.
Odisha_S	A	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.	.	.	.	.
Odisha_S	A	.	.	.	.	.	.	.	G	.	.	G	.	.	.	.	.	.	.	.	.
Bangalore_C	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
Bangalore_C	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
Bihar_C	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
Bihar_C	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
Bihar_C	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.
Puttaparthi_C	A	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	-	.	.	.

In Odisha_S, Bangalore_S, Bihar_C, and Puttaparthi_C, the prefix indicates the geographical location of the isolate sequenced in the present study and suffix indicates the haplotype of the isolate

Fig. 3 TCS network of the haplotypes of *Achatina fulica*. Network tree depicted was obtained by selecting the 'spring tree' option in the TCS software. Indicated on the *lines* connecting different haplotypes is the position of the nucleotide sequence variation. The size of the oval or square box do not exactly correspond to the haplotype frequency. The frequency of each haplotype was rounded off to one, except for the isolates sequenced in the present study

Fig. 4 Median-joining network of haplotypes of *Achatina fulica* drawn using SplitsTree

Table 4 Maximum composite likelihood estimate of the pattern of nucleotide substitution

	A	T	C	G
A	–	3.98	1.72	**18.07**
T	3.17	–	**10.33**	2.25
C	3.17	**23.96**	–	2.25
G	**25.4**	3.98	1.72	–

The rates of transitional substitutions were highlighted in bold

Phylogenetic analyses

Bayesian Inference of phylogeny was carried out in MrBayes (Fig. 5). Bayesian tree consisted of three distinct clades. Clade 1 consisted of all the individuals of *A. fulica* that were sequenced in the present study. Apart from the isolates sequenced in our present study, it also harboured all the previously sequenced individuals of *A. fulica* by Fontanilla et al. (2014) that were originated

Table 5 Estimates of evolutionary divergence between sequences (the number of base substitutions per site from between sequences is shown)

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	s*	s*	C*	c*	c*	c*	c*	c*
A																										
B	0.007																									
C	0.007	0.007																								
D	0.003	0.003	0.003																							
E	0.010	0.007	0.003	0.007																						
F	0.010	0.010	0.003	0.007	0.007																					
G	0.007	0.007	0.007	0.003	0.010	0.010																				
H	0.007	0.007	0.000	0.003	0.003	0.003	0.007																			
I	0.017	0.017	0.017	0.014	0.021	0.021	0.017	0.017																		
J	0.017	0.017	0.017	0.014	0.021	0.021	0.017	0.017	0.007																	
K	0.007	0.007	0.007	0.003	0.010	0.010	0.007	0.007	0.017	0.017																
L	0.021	0.021	0.021	0.017	0.025	0.025	0.021	0.021	0.007	0.014	0.021															
M	0.017	0.017	0.017	0.014	0.021	0.021	0.017	0.017	0.000	0.007	0.017	0.007														
N	0.017	0.017	0.017	0.014	0.021	0.021	0.017	0.017	0.007	0.007	0.017	0.014	0.007													
0	0.010	0.010	0.010	0.007	0.014	0.014	0.010	0.010	0.021	0.021	0.010	0.025	0.021	0.021												
P	0.010	0.010	0.003	0.007	0.007	0.007	0.010	0.003	0.021	0.021	0.010	0.025	0.021	0.021	0.014											
Q	0.010	0.010	0.003	0.007	0.007	0.007	0.010	0.003	0.021	0.021	0.010	0.025	0.021	0.021	0.014	0.007										
R	0.007	0.007	0.007	0.003	0.010	0.010	0.007	0.007	0.017	0.018	0.007	0.021	0.017	0.017	0.010	0.010	0.010									
Odisha_S	0.010	0.010	0.003	0.007	0.007	0.007	0.010	0.003	0.021	0.021	0.010	0.025	0.021	0.021	0.014	0.007	0.007	0.010								
Odisha_S	0.010	0.010	0.003	0.007	0.007	0.007	0.010	0.003	0.021	0.021	0.010	0.025	0.021	0.021	0.014	0.007	0.007	0.010	0.000							
Bangalore_C	0.007	0.007	0.000	0.003	0.003	0.003	0.007	0.000	0.017	0.017	0.007	0.021	0.017	0.017	0.010	0.003	0.003	0.007	0.003	0.003						
Bangalore_C	0.007	0.007	0.000	0.003	0.003	0.003	0.007	0.000	0.017	0.017	0.007	0.021	0.017	0.017	0.010	0.003	0.003	0.007	0.003	0.003	0.000					
Bihar_C	0.007	0.007	0.000	0.003	0.003	0.003	0.007	0.000	0.017	0.017	0.007	0.021	0.017	0.017	0.010	0.003	0.003	0.007	0.003	0.003	0.000	0.000				
Bihar_C	0.007	0.007	0.000	0.003	0.003	0.003	0.007	0.000	0.017	0.017	0.007	0.021	0.017	0.017	0.010	0.003	0.003	0.007	0.003	0.003	0.000	0.000	0.000			
Bihar_C	0.007	0.007	0.000	0.003	0.003	0.003	0.007	0.000	0.017	0.017	0.007	0.021	0.017	0.017	0.010	0.003	0.003	0.007	0.003	0.003	0.000	0.000	0.000	0.000		
Puttaparthi_C	0.007	0.007	0.000	0.003	0.003	0.003	0.007	0.000	0.017	0.017	0.007	0.021	0.017	0.017	0.010	0.003	0.003	0.007	0.003	0.003	0.000	0.000	0.000	0.000	0.000	

* Haplotypes obtained in the present study

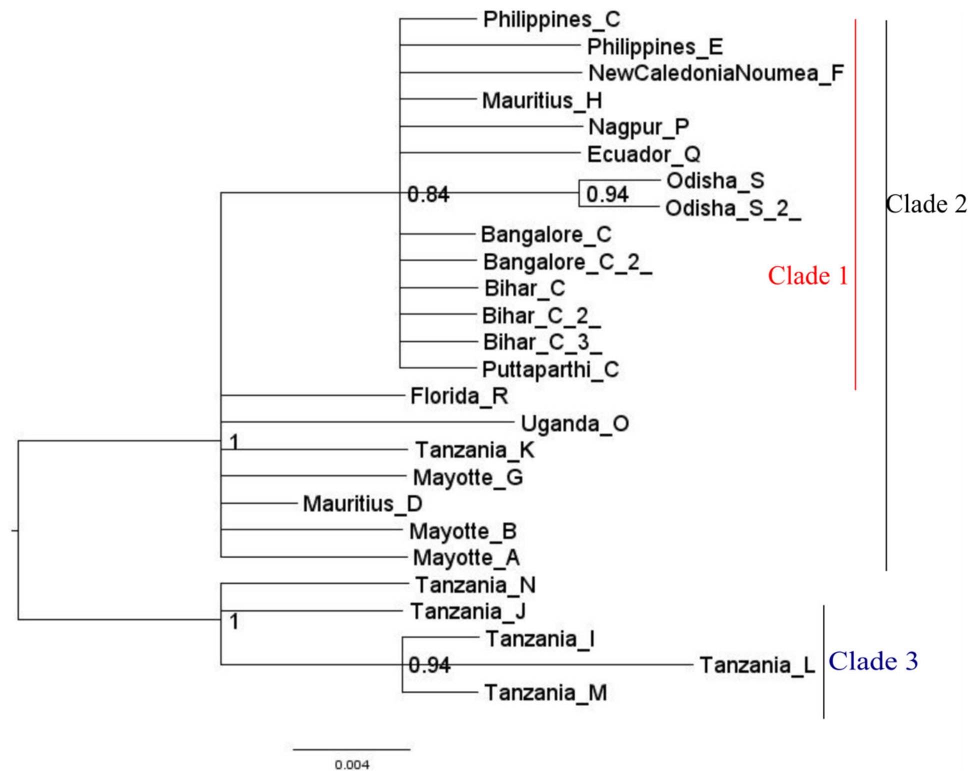

Fig. 5 *Midpoint*—rooted Bayesian tree depicting the phylogenetic relationships of the isolates of *Achatina fulica* based on 16S rDNA. Indicated on the nodes are the Bayesian probability values. *Scale bar* represents 0.004 substitutions per site

from the Indian sub-continent that harboured haplotypes 'C' and 'P'. Nested within Clade I were the haplotypes C, H, F, E, Q, and P that were evolved outside East Africa.

Of the eight isolates of *A. fulica* sequenced in the present study, six haplotypes were of a previously reported haplotype 'C' and the remaining two isolates from Odisha were of a new haplotype 'S'. These two isolates formed a distinct clade within Clade 1.

Outside Clade 1, within Clade 2 were haplotypes A, B, D, G, K, O, and R that showed polytomy. It is interesting to note that haplotypes K, O, and R were from Tanzania. Instead of clustering with the remaining Tanzanians in Clade 3, they were emerged with nonAfrican haplotypes in Clade 2. This is similar to the observation made by Fontanilla et al. (2014), when they performed median-joining network analysis for studying the evolution of different haplotypes; they noticed that the Tanzanian haplotypes K, O, and R were emerged outside the remaining haplotypes from Africa. The same was observed in the network analysis carried out in the present study. All the clades in the Bayesian tree were strongly supported (posterior probability >0.9).

Clade 3 emerged out from clade 2 and consisted of five different Tanzanian haplotypes. Within this clade, Tanzanian haplotypes L, I, and M formed a separate clade.

In addition to the Bayesian inference, phylogenetic relationships between the haplotypes were also studied using ML method (Fig. 6). Both of them have yielded similar topologies but differed in the node support values (Figs. 5, 6).

Apart from this, ML and Bayesian analyses were also carried out using *Achatina reticulata* as outgroup. In ML analysis (Fig. 7), it was observed that inner nodes are not clearly resolved when compared to the one constructed without outgroup (Fig. 6). However, it recovered two of the major clades consisting of Tanzanian haplotypes J, N, L, I, and M as a distinct clade and haplotypes C, H, Q, S, F, P, and E as another clade. In contrast, Bayesian tree constructed with an outgroup (Fig. 8) inferred the same relationships with the one constructed without outgroup (Fig. 5).

Network analyses

Haplotype network drawn using TCS v1.21 (Fig. 3) showed that haplotype C gave rise to haplotypes E, F, P, and Q each with a single-nucleotide difference from haplotype C. Apart from these, haplotypes H and D were linked to Haplotype C each with a single mutation step. Haplotype S from the Indian sub-continent emerged from Mauritius haplotype 'H' by a single mutation step. Haplotypes H and D were linked by a hypothetical haplotype which was distant from these two haplotypes by a single-nucleotide difference. This hypothetical haplotype gave rise to two haplotypes A and B which were distant from it by a single mutation step. Emerged from haplotype D were the Tanzanian haplotypes I, J, L, M, and N which

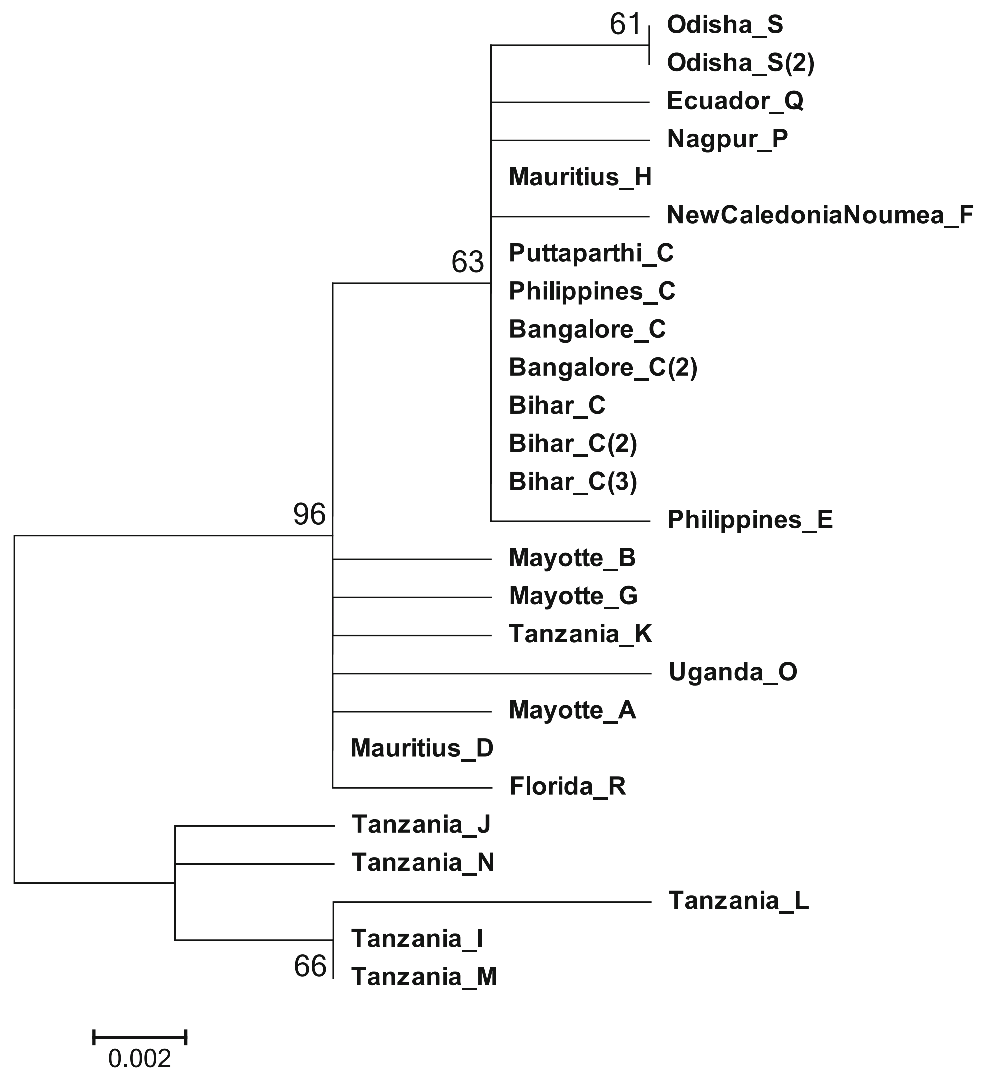

Fig. 6 Phylogenetic relationships of the haplotypes of *Achatina fulica* constructed by maximum likelihood (midpoint rooted) in MEGA. Indicated on the nodes are the bootstrap values. *Scale bar* represents 0.002 nucleotide substitutions per site

were linked to haplotype D by three mutation steps. Apart from these haplotypes, Tanzanian haplotypes R and K were linked to haplotype D each with two mutation steps. Furthermore, Tanzanian haplotype O and a Mayotte haplotype G linked to haplotype D with two and one mutation steps, respectively.

Apart from this, median-joining network analysis of haplotypes was carried out using SplitsTree (Fig. 4). The topologies of haplotype networks drawn using SplitsTree and TCS were similar except that haplotypes K and R emerged directly from haplotype D in the former instead of emerging from a hypothetical haplotype as seen in the latter.

Discussion

The comprehensive observations regarding the haplotype diversity of *A. fulica* in India were that two haplotypes C and P were observed from the studies of Fontanilla et al. (2014). In the present study, a new haplotype S was observed along with the haplotype C, which is the predominant one in the Indian Ocean islands (Fontanilla et al. 2014). Thus, in total, three haplotypes of *A. fulica* were observed in India to the extent it was sampled.

It is to be noted that there is limited genetic variation outside the East Africa as evident from the studies carried out by Fontanilla et al. (2014). However, it is also to be noted that the extent of sampling plays an important role in determining the haplotype diversity and in uncovering new haplotypes. High extent of sampling sites with increased sampling may possibly give a clear picture on the prevalence of new haplotypes.

Achatina fulica is native to East Africa and was spread to the entire world by introductions. The time scale or the time in which introductions started is very less compared to the evolution of *A. fulica* itself (Raut and Baker 2002). Hence, the prevalence of diverse numbers of haplotypes in the native regions of *A. fulica* is of no wonder. However, such a high

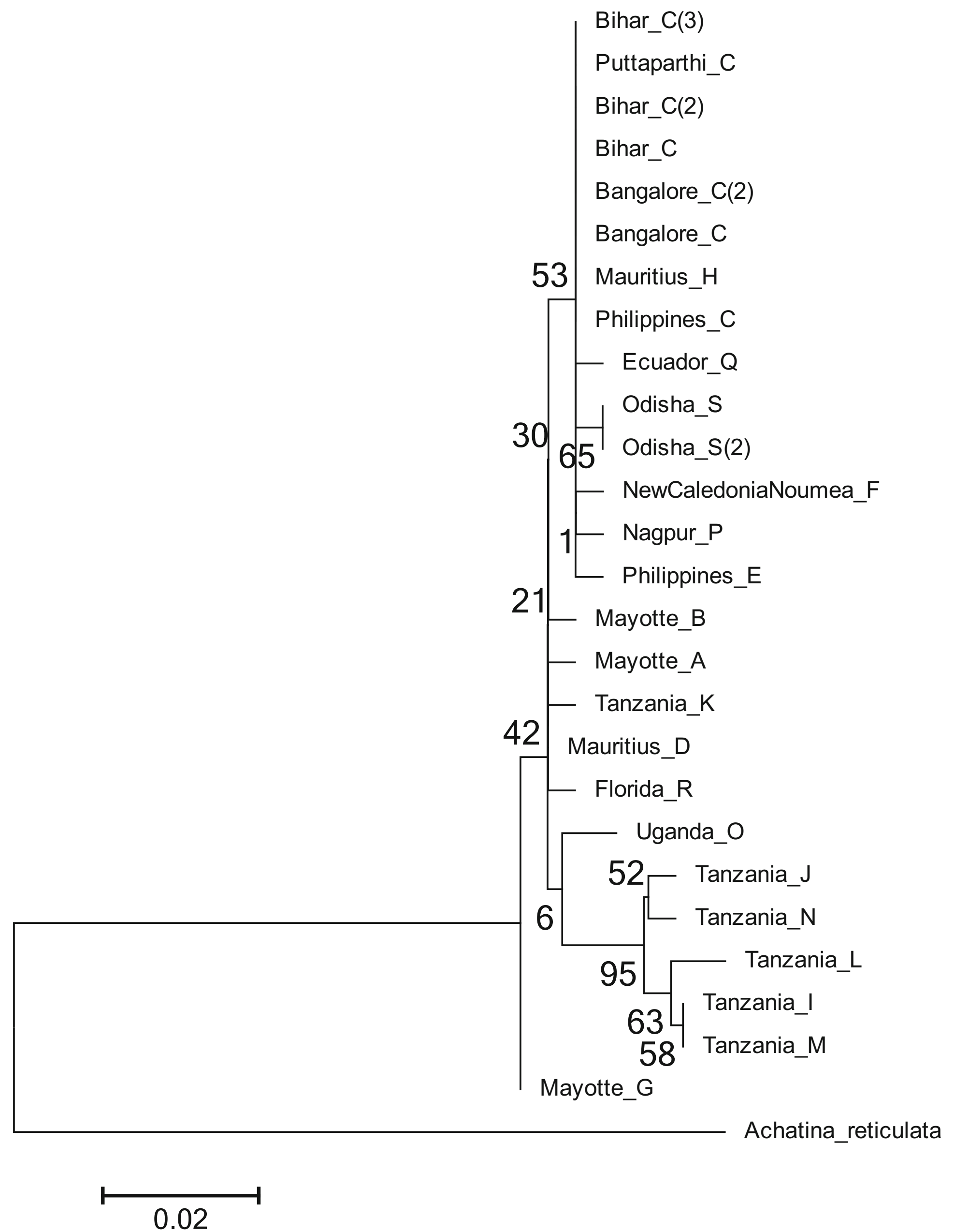

Fig. 7 Phylogenetic relationships of the haplotypes of *Achatina fulica* constructed by maximum likelihood in MEGA. The phylogenetic tree is rooted by *Achatina reticulata*. Indicated on the nodes are the bootstrap values. *Scale bar* represents 2 substitutions per 100 nucleotides

prevalence of different haplotypes in the introduced regions of Mayotte and Madagascar (Fontanilla et al. 2014) raises several important questions as to how these haplotypes emerged in a short period of time in these countries when compared to the other regions of introduction. A possible explanation to this is that there could have been multiple introductions from Africa to Madagascar and Mauritius in the past which might have given rise to the existing haplotype diversity along with the emergence of new haplotypes during the course of evolution.

Lack of fossil evidence in Madagascar suggests East Africa to be the place of origin of this snail (Raut and Baker 2002). As per Raut and Baker (2002), *A. fulica* was introduced to Madagascar prior to 1800 from Kenya. However, Fontanilla et al. (2014) did not sample this snail from Kenya. Sampling from Kenya may give a clear picture about the prevalence of different haplotypes in that region. Further increased sampling from Madagascar also gives more insights regarding the prevalence of haplotypes in relationship to Kenya. Especially, it may be known whether haplotype C is prevalent in Kenya or not.

In the Indian populations of *A. fulica*, haplotype C is the predominant one. However, it cannot be established definitely that the two individuals of *A. fulica* introduced into the country a century ago were of C haplotype. On one

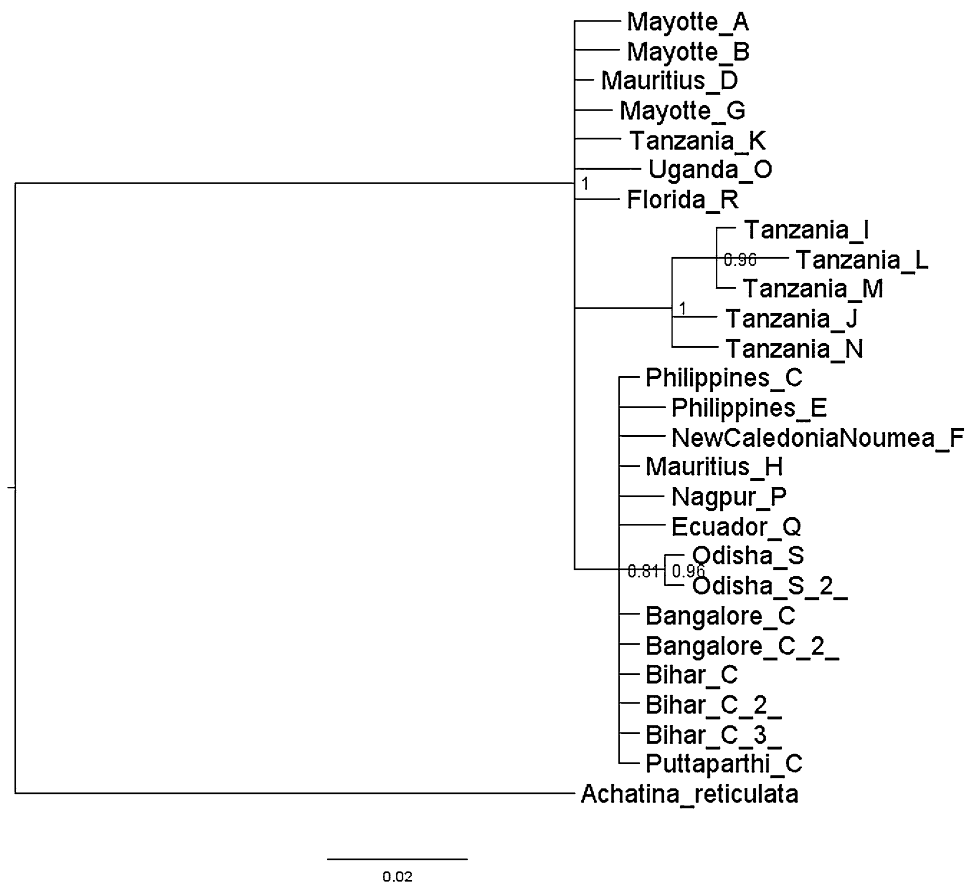

Fig. 8 Bayesian inference of phylogenetic relationships of the haplotypes of *Achatina fulica*. The phylogenetic tree is rooted by *Achatina reticulata*. Indicated on the nodes are the posterior probability values. *Scale bar* represents 2 substitutions per 100 nucleotides

hand, it may seem likely because haplotype C is the predominant one amongst the haplotypes sampled from Mauritius by Fontanilla et al. (2014) from where these were introduced into the country a century ago.On the other hand, it may be unlikely because there might had an ancestral haplotype which might have given rise to the present day 'C' haplotype in India which might have gone extinct or was not sampled. Another possibility which cannot be ruled out is that the two individuals of *A. fulica* introduced in India a century ago might have consisted of different haplotypes.

Haplotype network analyses showed that haplotype 'S' is linked to a Mauritius haplotype 'H' by a single mutation. Mauritius is considered to be the place from where *A. fulica* was introduced into the country (Raut and Baker 2002). In contrast, haplotype 'P' from India emerged from haplotype 'C'. Further understanding is required with regard to the association of haplotype S observed from the Indian state of Odisha with Mauritius haplotype 'H'. Is this because of unseen multiple introductions to India from Indian Ocean islands? As opined by Fontanilla et al. (2014), the possibility of unseen introductions into the country also cannot be ruled out. It is yet to be known whether *A. fulica* from Kolkata harbours haplotype(s) C, P, S or a new haplotype. This shall be known only after this snail could be sampled from Kolkata in future.

As this snail was introduced into the country deliberately by human introductions, strict quarantine checks prevent its further dispersal from one country to another country. Apart from this, public awareness needs to be created about the potential damage that it causes to the crops plants as well as to human beings. This limits the dispersal of this snail from one region to another region within a country. With the changing climatic patterns, there are chances that new areas may be prone for further invasions (Rekha et al. 2015). In this scenario, increased surveillance with respect to controlling its further spread in the country needs to be carried out. In conclusion, this study gives insights regarding the genetic diversity of this snail population in India despite several logistical constraints in sampling snails from different regions. In future, further sampling from different geographical regions gives more insights into the genetic diversity of this snail population in India.

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Compliance of ethical standards

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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