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Genetic diversity and molecular evolution of Naga King Chili inferred from internal transcribed spacer sequence of nuclear ribosomal DNA

Mechuselie Kehie [a,*], Suman Kumaria [b], Khumuckcham Sangeeta Devi [b], Pramod Tandon [b]

[a] Post Graduate Department of Environmental Science, Patkai Christian College (Autonomous), Chumukedima, Seithekema, Dimapur 797103, India
[b] Plant Biotechnology Laboratory, Centre for Advanced Studies in Botany, North-Eastern Hill University, Shillong 793022, India

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ABSTRACT

Sequences of the Internal Transcribed Spacer (ITS1-5.8S-ITS2) of nuclear ribosomal DNAs were explored to study the genetic diversity and molecular evolution of Naga King Chili. Our study indicated the occurrence of nucleotide polymorphism and haplotypic diversity in the ITS regions. The present study demonstrated that the variability of ITS1 with respect to nucleotide diversity and sequence polymorphism exceeded that of ITS2. Sequence analysis of 5.8S gene revealed a much conserved region in all the accessions of Naga King Chili. However, strong phylogenetic information of this species is the distinct 13 bp deletion in the 5.8S gene which discriminated Naga King Chili from the rest of the *Capsicum* sp. Neutrality test results implied a neutral variation, and population seems to be evolving at drift–mutation equilibrium and free from directed selection pressure. Furthermore, mismatch analysis showed multimodal curve indicating a demographic equilibrium. Phylogenetic relationships revealed by Median Joining Network (MJN) analysis denoted a clear discrimination of Naga King Chili from its closest sister species (*Capsicum chinense* and *Capsicum frutescens*). The absence of star-like network of haplotypes suggested an ancient population expansion of this chili.

1. Introduction

Chili is one of the most important crops of the world and has the distinction of being the first plant to be cultivated in the new world. It has been a part of the human diet since at least 7500 BC. Though tropical South America is believed to be the original home of chili (Greenleaf, 1986), chilies are now grown worldwide. The cultivated species of the genus are *Capsicum annuum*, *Capsicum chinense*, *Capsicum frutescens*, *Capsicum baccatum*, *Capsicum pubescens*. Chili fruits are rich sources of metabolites such as carotenoids (provitamin A), vitamins (C and E), flavonoids and capsaicinoids that are beneficial for human health (Maga, 1975; Ramchiary et al., 2014). Naga King Chili is considered as India's hottest chili and was formerly acknowledged as the world's hottest chili measuring 1,001,304 Scoville Heat Units (SHU) (Guinness Book of World Records, 2006; Kehie et al., 2012a). However, this chili was superseded by the Infinity chili in 2011, followed by the Naga Viper, the Trinidad Moruga Scorpion in 2012, and the Carolina Reaper in 2013 (Hottest Chili, 2015). Naga King Chili which is known in local Angami dialect as 'Kedi Chüsi' which literally means the 'King of Chilies', is also known by other local names such as Naga Mirchi, Bhut Jolokia, and Umorok (Kehie et al., 2013). Naga King Chili is native to the Northeastern states of India. Since time immemorial, people of Northeastern region, particularly the Naga people have close sodality with this chili. Nagas are kenned to have utilized this chili as remedial agents for treating myriads of ailments. The Government of Nagaland has patented this chili and has registered as the proprietor with the Government of India under Geographical Indication Registry (Kehie et al., 2014). Although earlier studies have treated this chili as *C. frutescens*, the taxonomic relationship of 'Naga King Chili' based on RAPD markers have placed 'Naga King Chili' in a taxonomic position between *C. chinense* and *C. frutescens* with 'Naga King Chili' clustering more closely to the *C. chinense* group (Bosland and Baral, 2007). Internal Transcribed Spacers (ITS) sequence analysis reported by Purkayastha et al. (2012) showed distinct genetic differences between Bhut Jolokia and the species of *C. frutescens* and *C. chinense*. The occurrence of high cross pollination and adaptation to micro-climatic conditions has led to the formation of variants and landraces within the species (Kehie et al., 2012b). However, study on its genetic diversity and molecular evolution remains unexplored as far as our knowledge is concerned. Molecular approach using ITS of nuclear ribosomal DNA (nrDNA) has been widely used for resolving phylogenetic relationships among closely related species of angiosperms. Eukaryotic nrDNA has two internal transcribed spacers ITS1 and ITS2. The two spacers and the 5.8S subunit are collectively known as the ITS region and it has become an important nuclear locus for molecular systematic investigations of flowering plants. They have frequent insertions/deletions which could be phylogenetically informative (Baldwin et al., 1995). The popularity of the ITS region can

* Corresponding author.
E-mail address: kehie10@gmail.com (M. Kehie).

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Table 1
Sources of *Capsicum* ITS sequences and their geographical origin.

Species/cultivar	GenBank accession no.	Geographic origin	Elevation (m)	References
		Source/location		
Naga King Chili	KP006659	Piphema, Nagaland, India	750	This study
Naga King Chili	KP006658	Kohima, Nagaland, India	1444	This study
Naga King Chili	KP006660	Ruzaphema, Nagaland, India	500	This study
Naga King Chili	KP006657	Jalukie, Nagaland, India	347	This study
Naga King Chili	KP006661	Shillong, Meghalaya, India	1525	This study
Naga King Chili	KP006656	Imphal, Manipur, India	786	This study
GenBank sequences				
Bhut Jolokia	HQ705983	Dibrugarh, Assam, India	108	Purkayastha et al. 2012
Bhut Jolokia	HQ705984	Jorhat, Assam, India	91	Purkayastha et al. 2012
Bhut Jolokia	HQ705985	Sonitpur, Assam, India	21	Purkayastha et al. 2012
Bhut Jolokia	HQ705986	Karbianglong, Assam, India	1600	Purkayastha et al. 2012
Bhut Jolokia	HQ705987	Ukhrul, Manipur, India	1662	Purkayastha et al. 2012
Bhut Jolokia	HQ705988	Kohima, Nagaland, India	1500	Purkayastha et al. 2012
Capsicum frutescens	HQ705989	Tezpur, Assam, India	48	Purkayastha et al. 2012
Capsicum chinense	HQ705990	Sonitpur, Assam, India	21	Purkayastha et al. 2012
Capsicum eximium	AY665841	Mexico, North America	–	Whitson and Manos, 2005
Capsicum annum	GU944973	Badajoz, Spain	–	Hernández et al. 2010
Capsicum baccatum	AF244708	Utah, USA	–	Bohs and Olmstead, 2001
Capsicum pubescens	AY875749	Madison, USA	–	Spooner et al. 2005
Capsicum lycianthoides	DQ314158	Madison, USA	–	Smith and Baum, 2006

= Not specified.

be attributed to the relatively high rate of nucleotide substitution in the transcribed spacers, permitting the systematic comparison of relatively recently diverged taxa (Ghada et al., 2013). The ITS region is influenced by concerted evolution which homogenizes the tandem copies within individuals, thus making ribosomal DNA amenable for phylogenetic inference (Hillis and Davis, 1988; Chiang and Schaal, 2000). However, variations within individuals have been reported primarily as a result of slow concerted evolution (Harris and Crandall, 2000; Coté and Peculis, 2001). In this study, we attempted to explore and investigate the genetic diversity and molecular evolutionary history of Naga King Chili using nrDNA.

2. Methods

2.1. Taxon sampling and genomic DNA isolation

Samples of Naga King Chili were collected from different geographical regions of Northeastern India viz., Nagaland, Manipur and Meghalaya (Table 1). Total genomic DNA was extracted from frozen fruits of Naga King Chili following the method described by Doyle and Doyle (1987) with some minor modifications. The isolated DNA concentration was estimated using a UV spectrophotometer (Perkin Elmer Lambda 35) and its integrity was checked by agarose (1%) gel electrophoresis.

2.2. PCR targeting ITS region and sequencing

A polymerase chain reaction (PCR) was used to amplify the ITS regions. The ITS1, 5.8S, and ITS2 regions of Naga King Chili were amplified using PCR primers ITS 4 and ITS 5 (White et al., 1990). The DNA amplification was performed in an Applied Biosystems Gene Amp® PCR System 2700, Rotkreuz, Switzerland. PCR reaction volume was 25 μL, containing 5 μL of template DNA (50ng/ μL), 2 μL 10× PCR buffer (Tris with 15 mM $MgCl_2$), 1.5 mM $MgCl_2$, 5 μL primer at 10 pmol, 5 μL dNTPs at 2 mM, 0.2 μL *Taq* DNA polymerase at 3 U/μL, and 1.3 μL ddH_2O. The thermocycling protocol consisted of a single denaturation step at 95 °C for 5 min, followed by 40 cycles of 1 min denaturation at 94 °C, 1 min annealing at 57 °C, 2 min extension at 72 °C, with a final incubation step at 72 °C for 10 min. To confirm successful amplification and size of the amplified fragment, 10 μL of the PCR product was run on 1% agarose gel in 1× TAE buffer, stained with ethidium bromide, and visualized under UV light. GeneRule 100 bp Plus DNA Ladder, ready-to-use, 100–3000 bp (Fermentas, In., Glenn Burnie, MD, USA). The PCR amplified product approximately 750 bp corresponding to 18S partial, ITS1, 5.8S, ITS2 and 26S partial sequence were sequenced at the Macrogen Inc. (Seoul, Korea). Amplicons were sequenced directly in both sense and antisense directions. The amplification primers were used as the sequencing primers.

2.3. Sequence alignments and phylogenetic analyses

All sequence information has been deposited in the GenBank database (accession no. KP006656, KP006657, KP006658, KP006659, KP006660, KP006661). Sequences of the whole ITS region of Naga King Chili were used to determine ITS1, 5.8S and ITS2 boundaries by homologous blast with published ITS sequences in the GenBank database. The ITS sequence alignments were performed using ClustalX version 2.1 (Thompson et al., 1997). Alignments were subsequently adjusted manually using BioEdit version 7.2.5 (Hall, 1999). The length and GC content

Table 2
Length and G + C content of ribosomal DNA sequences of Naga King Chili.

Species/cultivar	GenBank accession number	ITS1		5.8S		ITS2		ITS Entire region	
		%GC	Length	%GC	Length	%GC	Length	%GC	Length
Naga King Chili	KP006659	65.5	240	53.52	142	68.48	237	63.87	620
Naga King Chili	KP006658	62.5	240	53.52	142	68.64	236	62.78	618
Naga King Chili	KP006660	65.14	241	53.52	142	68.64	236	63.81	619
Naga King Chili	KP006657	65	240	52.81	142	68.64	236	63.59	618
Naga King Chili	KP006661	64.16	240	53.52	142	68.64	236	63.43	618
Naga King Chili	KP006656	65	240	53.52	142	69.06	236	63.19	618

Table 3
Maximum composite likelihood estimate of the pattern of nucleotide substitution.

Original nucleotide				
	A	T	C	G
(a)				
A	–	*0.09*	*0.21*	**42.89**
T	**0.13**	–	**17.96**	*0.18*
C	*0.13*	**7.34**	–	*0.18*
G	**30.61**	*0.09*	*0.21*	–
(b)				
A	–	*0.04*	*0.05*	**51.54**
T	*0.05*	–	**0.05**	*0.05*
C	*0.05*	**0.04**	–	*0.05*
G	**48**	*0.04*	*0.05*	–
(c)				
A	–	**0.04**	*0.08*	**62.5**
T	*0.03*	–	**7.89**	**0.06**
C	*0.03*	**3.92**	–	*0.06*
G	**25.28**	*0.04*	*0.08*	–
(d)				
A	–	**1.74**	*3.36*	**35.86**
T	*1.86*	–	**14.21**	*2.93*
C	*1.86*	**7.35**	–	**2.93**
G	**22.81**	*1.74*	*3.36*	–

(a) ITS1 spacer, (b) 5.8S gene and (c) ITS2 spacer, (d) ITS entire region of ribosomal DNA. Each entry shows the probability of substitution (r) from one base (row) to another base (column). For simplicity, the sum of r values is made equal to 100. Rates of different transitional substitutions are shown in bold and those of transversionsal substitutions are shown in italics.

for each sequence were estimated using the Endmemo software (http://www. endmemo.com/bio/gc.php) (Table 2). Pairwise sequence divergence in ITS1, 5.8S and ITS2 regions was calculated according to the Maximum Composite Likelihood (MCL) (Tamura et al., 2007) (Table 3).

The basic genetic parameters of variation within species were calculated. For Naga King Chili data, we estimated nucleotide polymorphism (θw) (Watterson, 1975) and diversity (π) (Nei, 1978). Genetic diversity was quantified by indices of haplotypes diversity (Hd) (Nei and Tajima, 1983), pairwise estimates of nucleotide divergence (*Pi*) (Jukes and Cantor, 1969), and the average of nucleotide differences (k). The transition/transversion ratio ti/tv was estimated using the following formula $R = [A * G * k1 + T * C * k2] / [(A + G) * (T + C)]$ with A, G, C, T as the corresponding frequencies of four nucleotides (Tamura et al., 2007) (Table 4).

Selection neutrality was tested by both Tajima's D (Tajima, 1989) and Fu and Li's D* and F* methods (Fu and Li, 1993). Demographic parameters were assessed using the distribution of pairwise sequence differences (mismatch distribution) of Rogers and Harpending (1992) and site frequency spectra (distribution of the allelic frequency at a site) of Tajima (1989) using the program DnaSP software version 4.0 (Rozas et al., 2003). Median joining (MJ) network analysis was conducted using software NETWORK 4.6.1.0 (Bandelt et al., 1999; available at http://www.fluxus-engineering.com).

3. Results

3.1. ITS sequence analysis

ITS sequence alignment of Naga King Chili and other *Capsicum* sp. sequences from the GenBank revealed sequence variability in *Capsicum* sp. It was observed that the variable of ITS1 exceeded that of ITS2. Sequence alignment results of Naga King Chili showed two sites deletion viz., 10 bp and 5 bp (indicated by arrow) in the ITS1 sequence region (Fig. 1); these deletions were also observed in sister species viz., *C. chinense* and *C. frustescens*, which were clustered more closely to Naga King Chili. Sequence analysis of 5.8S gene which is 142 bp length showed a much conserved region in all the accessions of Naga King Chili. However, sequence comparison of 5.8S with other *Capsicum* sp. revealed a distinct phylogenetic signal which discriminated Naga King Chili from the rest of the *Capsicum* sp. This phylogenetic information is attributable to its distinct 13 bp deletion (indicated by arrow) in the 5.8S gene.

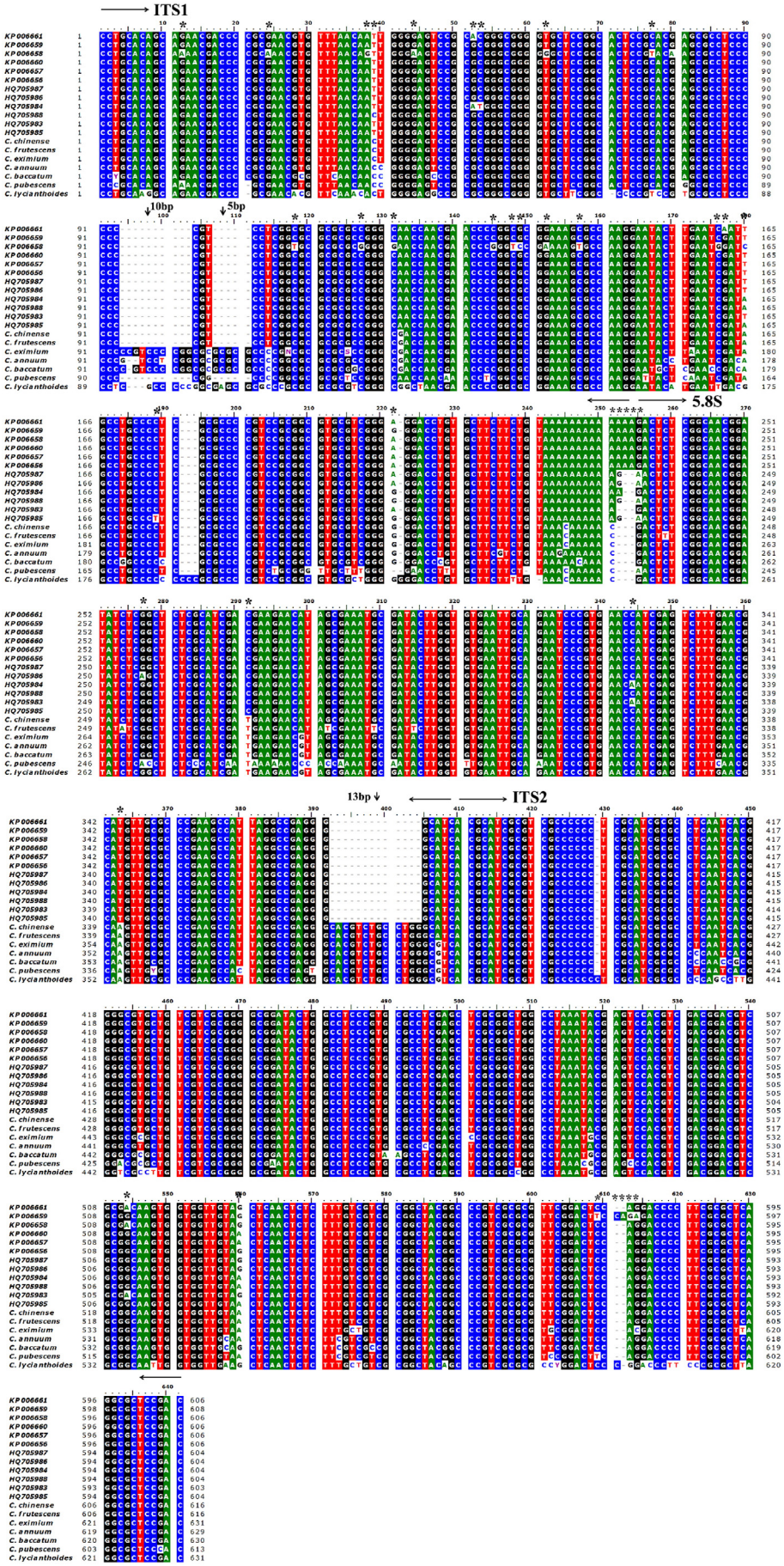
3.2. Sequence diversity, length variation and GC content of ITS sequences

The ITS sequence (ITS1-5.8S-ITS2) obtained from Naga King Chili showed variations in both length and composition (Table 2). The ITS1 spacer was 240 bp in all accessions except accession KP006660, which was 241 bp, with an average of 240.16 bp. The length of ITS2 spacers in all the accession was found to be 237 bp except the accession KP006659, which was 236 bp with an average of 236.16 bp. The GC content of ITS1 ranged from 52.5 to 55.5%, while in ITS2 the GC content ranged from 68.48 to 69.06%. The 5.8S rDNA sequence region had a conserved length of 142 bp, and its GC content varied from 52.81 to 53.52% and was significantly lower than ITS1 and ITS2 in length and GC content. The length of the entire ITS region ranged from 618 to 620 bp with GC content ranging between 62.78 to 63.87%.

Table 4
Nucleotide diversity, sequence polymorphism and neutrality test based on ribosomal DNA of Naga King Chili.

	ITS1	5.8S	ITS2	ITS Entire region
N	6	6	6	6
S	20	1	5	26
h	4	2	4	6
h_d	0.867	0.333	0.867	1
θw	0.03650 (SD = 0.01855)	0.00308 (SD = 0.00308)	0.00928 (SD = 0.00568)	0.01843 (SD = 0.00920)
π	0.02861(*SD = 0.01488*)	0.00235 (*SD = 0.00152*)	0.00876 (*SD = 0.00260*)	0.01499 (*0.00573*)
k	6.867	0	2.067	9.267
P_i(JC)	0.02995	0.00236	0.00883	0.01525
k (i)	0.333	0.333	0.333	0.667
Pi(i)	0.00138	0	0.00140	0.00107
Pi(s)	0.02861	0.00235	0.00876	0.01499
Tajima's D	−0.93302(*P < 0.10*)	0.93302 (*P > 0.10*)	1.03194 (*P > 0.10*)	−1.13197(*P > 0.10*)
Fu and Li's D*	−1.36967(*P < 0.10*)	−0.95015 (*P > 0.10*)	−0.21471 (*P > 0.10*)	−1.17008(*P > 0.10*)
Fu and Li's F*	−1.48775(*P < 0.10*)	−0.96473 (*P > 0.10*)	−0.25135 (*P > 0.10*)	−1.27884(*P > 0.10*)
Fu's Fs	1.798	−0.003	−0.439	−1.075

Fig. 1. Aligned nrDNA ITS sequence (ITS1-5.8S-ITS2) of *Capsicum* sp. The polymorphic sites in all accessions of Naga King Chili in comparison with other species are shown with (*). Sites deletion of 10 bp, 5 bp and 13 bp are shown with arrow.


ITS1
10bp
5bp
5.8S
13bp
ITS2
KP006661
KP006659
KP006658
KP006660
KP006657
KP006656
HQ705987
HQ705986
HQ705984
HQ705988
HQ705983
HQ705985
C. chinense
C. frutescens
C. eximium
C. annuum
C. baccatum
C. pubescens
C. lycianthoides

3.3. Nucleotide composition variation, mutational events and test for neutrality

The ITS sequence (ITS1-5.8S-ITS2) analysis showed that the nucleotide frequencies are 18.85% for adenine (A), 17.56% for thymine (T), 33.95% for cytosine (C), and 29.64% for guanine (G). The transition/transversion rate ratios are k1 = 12.233 (purines) and k2 = 4.231 (pyrimidines). The overall transition/transversion bias is R = 3.746. There was a total of 618 positions in the final dataset.

The analysis in Table 3 indicated that in Naga King Chili, transitions were more frequent than transversions at the intergenic spacer in ITS. The results showed that G/A and A/G transition were more frequent than C/T and T/C. The composition variation of nucleotide sequence alignment of ITS within a matrix of 621 characters showed the existence of 592 conserved sites, 26 variable sites containing 3 informative sites and 23 singleton sites. The sequence variation in the intergenic spacer ITS of the ribosomal DNA was detected with 6 haplotypes observed for 6 accessions of Naga King Chili. The haplotype diversity (Hd) was found to be 1 whereas nucleotide diversity (π) was 0.01499. Furthermore, the average difference between pairs of nucleotide (k) was calculated (9.267) which showed polymorphism (Table 4).

Neutrality test of Tajima (Tajima, 1989) and Fu and Li (Fu and Li, 1993) statistics were employed to assess whether the sequencing data shows evidence of deviation from neutrality. We obtained negative values which were not significant, which is an indication of neutral variation, and population seems to be evolving at drift–mutation equilibrium with no evidence of selection.

Further examination to assess deviation from neutrality, Fu statistics (Fu, 1997) was employed to detect neutrality. Sequence analysis showed (Table 4; Fig. 2) positive and negative values which were statistically not significant for this parameter. Fu's Fs = 1.798 for ITS 1, Fu's Fs = −0.003 for 5.8S gene, Fu's Fs = −0.439 for ITS2 and Fu's Fs = −1.075 for ITS entire region. Fu and Li and Fu statistics indicated no evidence for changes in population size or any particular pattern of selection in the regions examined in Naga King Chili. Furthermore, our suggestions were confirmed by the values of raggedness index "r" higher than 0.05 showing values of 0.1067 for the intergenic spacer of ribosomal DNA (ITS) (Fig. 3).

3.4. Mismatch distribution

Analysis of mismatch distribution in the ITS sequences of rDNA confirmed a neutral variation. A frequency graph between alleles showing multimodal aspect of the curve of mismatch indicated demographic equilibrium and not a recent demographic expansion (Fig. 3).

3.5. Haplotype network analysis

The haplotype network was reconstructed based on the whole ITS sequence data of Naga King Chili combining with ITS sequences of *Capsicum* sp. downloaded from GenBank. Median joining networks were drawn for the haplotypes identified in 19 ITS sequences of *Capsicum* sp. The network illustrated the relationship between 18 haplotypes (Fig. 4). Evolutionary relationships among haplotypes were inferred using MJN approach, which showed a clear separation between lineages of Naga King Chili (H1, H2, H3, H4, H6, H7, H8, H9, H11, H12, H13) and the rest of the other haplotypes of *Capsicum* sp. (H5, H10, H14, H15, H16, H17, H18). Haplotype network showed that majority of the Naga King Chili accessions seem to emerge from H3, which is also represented by two accessions (KP006657, KP006660). It may also be inferred from the network analysis that haplotype represented by H13 (KP006656) is the founder haplotype for H6 (KP006659), H7 (HQ705983), H9

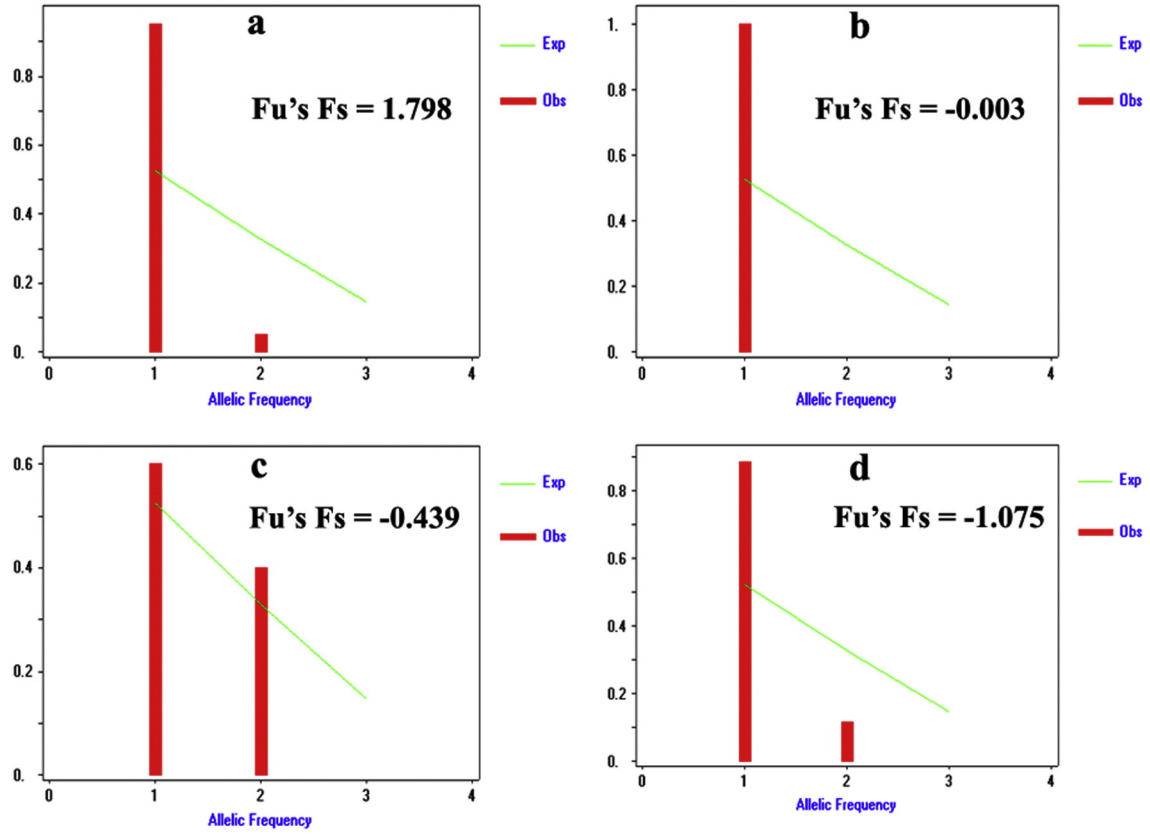


Fig. 2. Spectrum of frequencies of sites at the ribosomal DNA sequences in Naga King Chili. (a) Intergenic spacer of ribosomal DNA (ITS1), (b) 5.8S gene of ribosomal DNA, (c) intergenic spacer of ribosomal DNA (ITS2) and (d) intergenic spacer of ribosomal DNA (ITS). The solid lines in the spectrum indicate the distributions under neutrality and balance (mutation–drift). The index value of Fu is given.

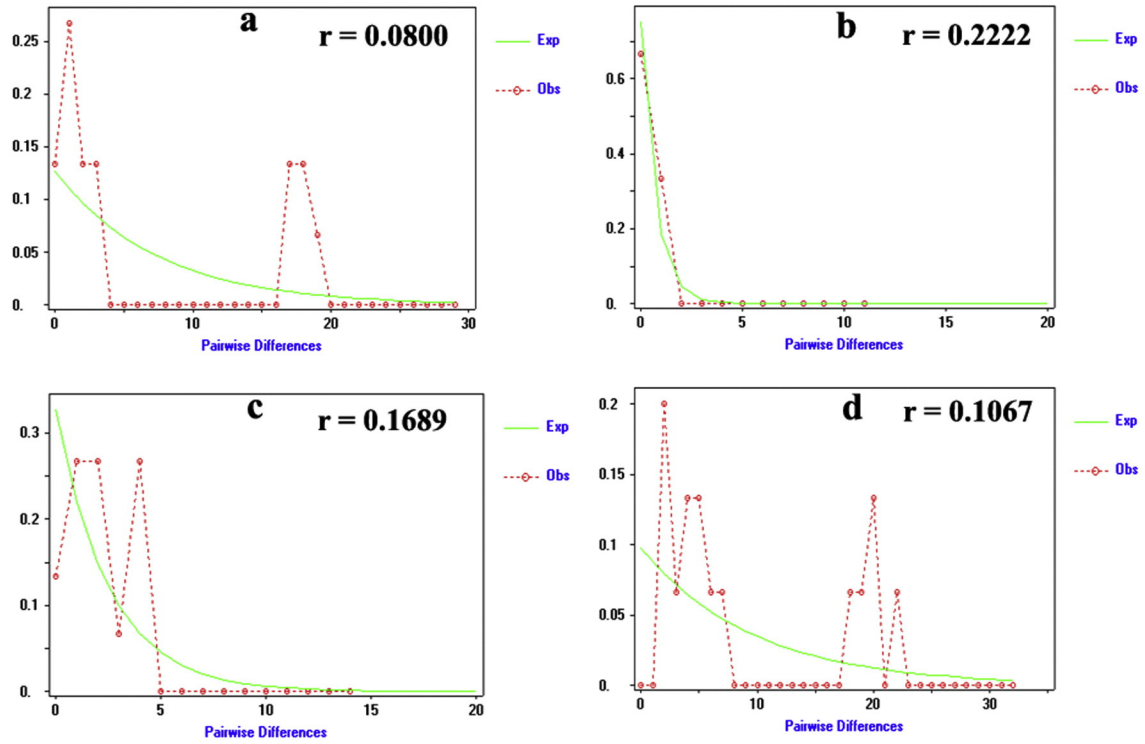


Fig. 3. Graphs depicting the results of the mismatch distribution analysis for the sequences of rDNA based on the differences between pairs of sequences. (a) Intergenic spacer of rDNA (ITS1), (b) 5.8S gene of rDNA, (c) intergenic spacer of rDNA (ITS2) and (d) intergenic spacer of rDNA (ITS). The parameter for raggdness (r) is given. The observed frequencies were represented by red dotted line. The solid green line corresponds to the frequency expected (Exp) under the hypothesis of population expansion model. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(KP006658), and H4 (KP006661) as basic branches were seen to be connected to this haplotype. Network analysis indicated that haplotype H5 (*C. chinense*) and closely related haplotype 10 (*C. frutescens*) are likely to be the ancestral haplotype of Naga King Chili accessions as they were more closely clustered around these haplotypes.

4. Discussions

The ITS sequence region is one of the most popular loci used in molecular phylogenetic studies (Alvarez and Wendel, 2003). It is known to evolve relatively quickly and is used for determining inter-specific (Jorgenson and Cluster, 1988) and intra-specific relationships (Baura et al., 1992). Our results showed that the variability of ITS1 with respect to nucleotide diversity and sequence polymorphism exceeded that of ITS2. Two sites deletion (10 bp and 5 bp) in the ITS1 sequence region of Naga King Chili were also observed in sister species viz., *C. chinense*, *C. frustescens* and *C. pubescens*, which were clustered more closely to Naga King Chili. Within the ITS region, the ITS1 sublocus evolved slightly more rapidly with a more variable length than the ITS2 sublocus (Hillis and Dixon, 1991; Hershkovitz and Lewis, 1996). Much attention has been focused on ITS1 as the more variable sublocus of the two and thereby, presumably, the better species marker (Chen et al., 2001; Narutaki et al., 2002; Hinrikson et al., 2005). Sequence analysis of 5.8S gene revealed a much conserved region in all the accessions of Naga King Chili. As known, the 5.8S rRNA region is highly evolutionary conserved in interspecific and intraspecific levels (Hřibová et al., 2011). However, it is important to note that the 13 bp deletion in the 5.8S gene which discriminated Naga King Chili from the rest of the *Capsicum* sp. provided strong phylogenetic information of this species. Our findings are in agreement with the earlier report of Purkayastha et al., (2012), thus corroborated with more sequence sources of ITS region and denoted a remarkable genetic difference of Naga King Chili with other *Capsicum* sp.

In our study to identify sequences which do not fit the neutral theory model at equilibrium between mutation and genetic drift, we used Tajima (Tajima, 1989) and Fu and Li tests (Fu and Li, 1993). Our results showed negative values which were not significant, an indication of neutral variation and population evolving at drift–mutation equilibrium with no evidence of selection. The study was further supported by the assessment of Fu statistics (Fu, 1997). In addition, mismatch analysis of the frequency graph between alleles showing multimodal aspect of the curve indicated a demographic equilibrium and not a recent demographic expansion. Phylogenetic methods may not lead to the desired resolution at the intraspecific level due to lower genetic diversity and non-hierarchical nature of intraspecific data sets (Posada and Crandall, 2001) and complementary network approaches might be valuable alternatives to study phylogenetic structures and haplogroups at the population level. Simulations (Woolley et al., 2008) have shown that MJN (Bandelt et al., 1999) outperforms minimum spanning networks (MSN) because the former is able to infer ancestral haplotypes (Woolley et al., 2008). In the present study, haplotype network analysis showed connections between haplotypes and summaries of all the shortest trees possible. Haplotypes of Naga King Chili accessions were clustered separately suggesting that they are a descendant population derived from a common ancestor. Majority of the Naga King Chili accessions seem to emerge from H3. Haplotype H5, H10 clustering closest to haplotypes of Naga King Chili are likely to be the ancestral haplotype. The absence of star-like cluster of nodes in the network indicated a non-demographic expansion and further supported a demographic equilibrium.

5. Conclusion

The results of this study contributed to the knowledge of the existing genetic status of *Capsicum* sp. Based on the sequence analysis of the nrDNA ITS region, the phylogenetic relationship of Naga King Chili showed a clear grouping from *C. chinense* and *C. frutescens*. This work

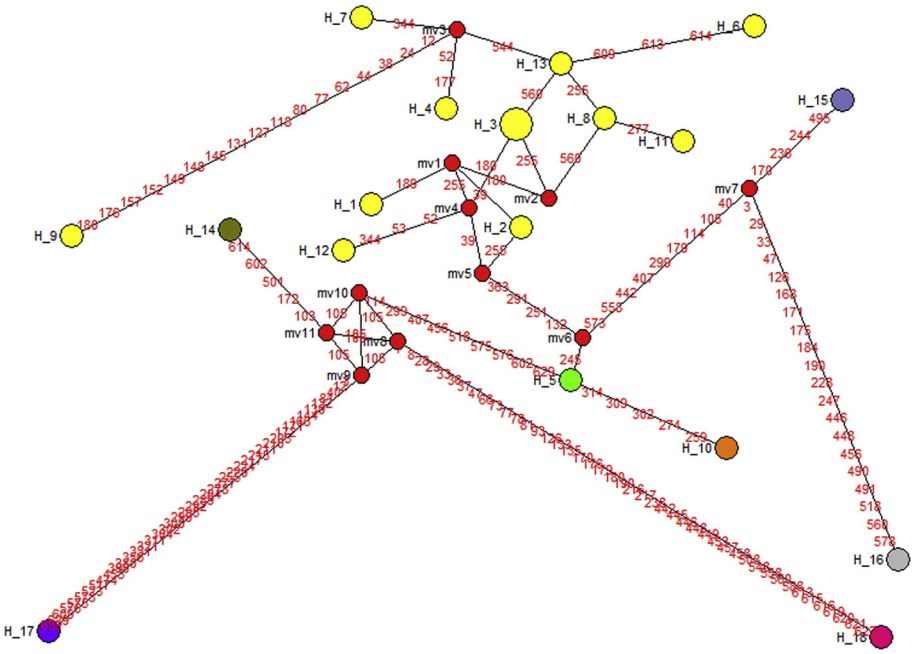


Fig. 4. Median-joining network of the haplotypes inferred from ITS sequences. Nodes are proportional to haplotypes frequencies and branches length is proportional to the number of mutations. The red dots represent theoretical median vectors introduced by the network software. H1: HQ705985, H2: HQ705988, H3: KP006657; KP006660, H4: KP006661, H6: KP006659, H7: HQ705983, H8: HQ705987, H9: KP006658, H11: HQ705986, H12: HQ705984, H13: KP006656, H5: *C. chinense*, H10: *C. frutescens*, H14: *C. eximium*, H15: *C. annum*, H16: *C. baccatum*, H17: *C. pubescens*, H18: *C. lycianthoides*. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

not only provided more sequence sources of ITS region of *Capsicum* species but the basis for clearer discrimination of Naga King Chili from other related chili species. It appeared that Naga King Chili population is evolving at drift–mutation equilibrium and free from directed selection pressure. Furthermore, our findings revealed an ancient evolutionary history of this species.

Conflicts of interest

The authors declare no conflict of interest.

Authors' contributions

MK conceived and designed research. MK and KSD conducted experiments. MK, SK, PT contributed reagents or analytical tools. MK analyzed data. MK. SK, PT wrote the manuscript. All authors read and approved the manuscript.

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