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

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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 However, this chili was superseded by the Infinity chili in 2011, followed by the Naga Viper, the Trinidad Moruga Scorpion in 2012, and the Carolina Reaper in 2013 (Hottest Chili, 2015).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

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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