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Morphological and molecular characterization of two distinct chilli cultivars from North Eastern India with special reference to pungency related genes

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ABSTRACT

King chilli and Dalle Khursani are the two hottest chilli (*Capsicum sp.*) cultivars widely grown in NE region of India but a well-organized study pertaining to King chilli and Dalle Khursani is unavailable. We have characterized twenty-two accessions of King chilli and Dalle Khursani, collected from different states of NE region with respect to 27 morphological parameters and using 30 SSR markers. The allelic status of three key pungency related genes viz, putative aminotransferase (*pAmt or Pun1*), acyl-CoA synthetase (*Acs1*) and β-ketoacyl-ACP synthase (*Kas1*) was also determined. Maximum morphological variation was observed for fruit traits and traits like plant growth habit, plant height, etc., which can be used to distinguish between the two cultivars. SSR markers HpmsE062 and HpmsE063 were able to distinguish between the three different cultivars (including a nonpungent bell pepper), while HpmsE116 and HpmsE139 distinguish King chilli from Dalle Khursani. Ward clustering method grouped all the eleven Dalle Khursani accessions together with non-pungent *C. annuum* accession in one cluster. Sequencing across *Acs1* revealed total of eight SNPs and three InDels. Seven SNPs including three within exonic regions were conserved between bell pepper, Dalle Khursani and the reference pungent *C. annuum* accession. A 2 bp InDel at position 50–51 bp and a 3 bp InDel at position 6313–6315 bp were found to be uniquely conserved haplotypes in both Dalle Khursani and King Chilli. A total of 16 SNPs were found within the sequenced region of *Pun1* gene, of which eight SNPs lie within the coding region. Three novel exonic SNPs were found in Dalle Khursani. All eight SNPs found in the intronic region for *Pun1* were conserved across Dalle Khursani and the two pungent reference sequences of *C. annuum*. Our SSR and SNP data suggests that Dalle Khursani is genetically closer to pungent *C. annuum*. This work provides insights into the genotype of two hottest chillies and paves way for their utilization in chilli improvement programme.

1. Introduction

Chilli (*Capsicum sp.*) is herbaceous, flowering plant belonging to the Nightshade family (Solanaceae). It originated in South and Central America where it has been cultivated for thousands of years. The crop is extensively cultivated throughout tropical Asia and equatorial America for its edible, pungent fruits. It is a self-pollinated crop; however, considerable cross-pollination (up to 10%) may occur due to bees, ants and thrips (Roy, 2016). India is the largest producer of dry chilli fruits, accounting for more than 43% of the world's total dry chilli production (Rai et al., 2013).

King chilli (Bhut Jolokia) and Dalle Khursani are the two chilli cultivars popularly grown in North Eastern (NE) region of India due to their highly pungent fruits. King chilli which is categorized as one of the hottest chilli cultivars, along with Trinidad Moruga Scorpion and Carolina Reaper, is indigenous to NE region of India (Kehie et al., 2016) while Dalle Khursani is believed to be indigenous to Sikkim state and its surrounding places where it has been extensively cultivated over a period of time (Jha et al., 2017). Triangular fruit bearing King chilli is now known to be an interspecies hybrid (Bosland and Baral, 2007) but the taxonomic classification of Dalle khursani is still not clear. The cherry-type or round fruits of Dale Khursani suggest that it might be *Capsicum annuum* but other morphological parameters have shown significant differences with known/type species of *C. annuum* complex viz. *C. annuum*, C. *frutescens* and *C. chinense* (Jha et al., 2017; Yumnam et al., 2012).

At present, 38 species of *Capsicum* are reported (USDA-ARS, 2011). Five domesticated species (*C. annuum*, *C. frutescens*, *C. chinense*, *C. pubescens*, and *C. baccatum*) are considered to be derived from at least five independent events (Andrews, 1984). The domesticated taxa *C. annuum*, *C. frutescens* and *C. chinense* are considered members of a species complex that were each independently derived from wild progenitors that may or may not be independent species (Eshbaugh, 1993; Pickersgill, 1971). Chilli exhibits a considerable amount of diversity

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within and among species and their landraces, especially for traits like plant growth habit, fruit color, shape, size and pungency. However, several phenotypic traits are known to be influenced by the environment (Baral and Bosland, 2002). Thus, it necessitates the use of molecular markers for ascertaining diversity between and within species.

Several molecular markers (RFLP, RAPD, AFLP, SSR, CAPS and SNP) (Lee et al., 2004; Yi et al., 2006; Nagy et al., 2007; Lee et al., 2009; Lu et al., 2011; Yumnam et al., 2012; Hill et al., 2013; Liu et al., 2013; Hulse-Kemp et al., 2016) have been developed in chilli over the past few decades. Among these markers, SSRs, also known as microsatellite markers are the most attractive ones (Ashrafi et al., 2012). They have been widely used in marker assisted selection in breeding programmes, mapping of important genes, construction of linkage maps, study of genetic diversity and evolution in many organisms because of their locus-specific, codominant, PCR based, multi-allelic nature, high level polymorphism between different individuals and accessions, significant abundance in genome and high rates of transferability across the species (Ahn et al., 2014).

Pungency is the most important quality attribute of the chilli fruit and is due to the presence of alkaloid capsaicin and its analogs, collectively known as capsaicinoids (Stewart et al., 2005; Chakradhar et al., 2013; Liu et al., 2013). Bennett and Kirby (1968) outlined capsaicinoid biosynthetic pathway for the first time which involves two metabolic pathways along with the enzymes corresponding to the structural genes. In recent years, several putative genes coding for enzymes in capsaicinoid biosynthetic pathway have been isolated (Mazourek et al., 2009; Aza-Gonzalez et al., 2011), however, it is necessary to demonstrate their specific roles in the capsaicinoid biosynthetic pathway.

Till date, *Pun1* encoding putative aminotransferase *(pAmt)* is the only gene that has been reported to be directly responsible for presence or absence of capsaicinoids in chilli fruit (Stewart et al., 2005, 2007). Moreover, Aluru et al. (2003) isolated three candidate genes *(Kas, Acl* and *Fat* genes) which are the components of FAS (fatty acid synthase) complex based on their differential expression from pungent habanero and the transcript level of these three candidate genes in the placental tissue was proved to be positively correlated with the degree of pungency.

Although most of the genes involved in biosynthesis of capsaicinoids have been identified recently, very little is known about the actual role played by these genes or the enzymes they code for catalyzing the reactions in capsaicinoid biosynthetic pathway. Therefore, molecular characterization of two of the hottest chilli cultivars with respect to allelic status of the genes participating in the capsaicinoid biosynthesis is a key step towards understanding the role these genes play in capsaicinoid biosynthesis. Since King chilli and Dalle Khursani are the two hottest chilli cultivars popularly grown in the NE region of the country, their characterization with the help of SSR markers with respect to diversity within and between different accessions, and determination of allelic status of pungency related genes would help in protection of GI (Geographical Index) of these economically important species; and also aid in further improvement and utilization of the crop. The current study was undertaken to ascertain the diversity within and between King chilli (Bhut Jolokia) and Dalle Khursani using morphological and molecular markers (SSRs), and also wanted to characterize King chilli and Dalle Khursani with respect to allelic status for pAmt (putative aminotransferase), Acs (Acyl-CoA synthetase) and Kas (β-ketoacyl-ACP synthase) genes.

2. Materials and methods

2.1. Plant material

Twenty two different chilli accessions were used in the present study (Table 1). The collected germplasm included ten King chilli accessions and eleven Dalle Khursani accessions. One non-pungent chilli (bell pepper) sold by Sultan seeds farm, Srinagar (Jammu & Kashmir) was used in this study as non-pungent check. King chilli accessions were collected from different parts of North Eastern states including Meghalaya, Manipur, Nagaland and Arunachal Pradesh. The collected plant samples include four accessions each from Meghalaya and Manipur, one accession each from Nagaland and Arunachal Pradesh. Dalle Khursani accessions were exclusively collected from Sikkim state except one accession which has been maintained at CPGS campus from an earlier research (Supplemental file S1). The accessions used in this study were assigned alphanumerical code such as 'K' for King chilli, 'D' for Dalle Khursani and 'C' for bell pepper as indicated in Table 1. Seeds were soaked in water overnight prior to sowing and sown in small pots filled with soil and FYM @ 1:1. Seeds began to germinate after around 10 days of sowing and were retained in pots until they attained 2–3 leaf stage. Transplanting was done in the evening hours in order to minimize the effect of high temperature during the day.

2.2. Morphological characterization

Morphological characterization was done for 27 characters according to the guidelines given by Protection of Plant Varieties and Farmers' Rights Authority for chilli (hot pepper), bell (sweet pepper) and paprika (*Capsicum annuum* L.) (Gazette of India: extraordinary, 2015). Morphological descriptors were modified for traits including plant height, days to 50% flowering and fruit colour at mature ripe stage as the trait values were beyond the descriptors provided. A minimum of five observations were recorded for each trait on fully grown plants. Data for all the quantitative characters like plant height, length of leaf blade, fruit length, etc. were recorded on a minimum of five plants, for the calculation of trait. The data for plant height was taken from randomly selected plants by measuring the length between the above ground level of the plant to the tip of the plant. All the observations on leaf and flower characters were recorded starting from initiation of the first flower till the beginning of harvesting. Assessment of colours such as leaf colour, flower petal colour, etc. were done according to Royal Horticulture Society chart (Voss, 2002). Data on length and width of the leaf blade was taken from the leaf obtained in the first branch for each plant. Measurement on the leaf length was taken by measuring the length between the leaf base and the leaf tip. Width of leaf blade was taken at the widest point of the leaf. Observations on leaf pubescence were taken by visual assessment of a group of plants for each accession. Based on flower/fruit orientation, plants were divided into drooping, semi-drooping and erect. Observation on flower petal colour was done from fully open flower while anther colour was observed at the time of anthesis. All the observations on fruit traits were taken at the time of harvesting or after harversting except for fruit colour at mature unripe stage, fruit orientation and fruit bearing habit. Observations for traits like fruit curvature, fruit glossiness and fruit colour at ripe maturity were taken by visual assessment. Pericarp thickness of the fruit was measured when fruit attained physiological maturity. Fruit length was taken by measuring the length of the fruit from the neck of the fruit to the tip of the fruit while fruit diameter was taken on the widest part of the fruit. Fruit shape, fruit neck at the base, shape of apex, etc. were recorded by visual assessment of the fruits as per the guidelines.

2.3. DNA extraction and SSR genotyping

DNA was extracted from young leaves and SSR markers were run on all the twenty-two accessions following previously reported protocol (Yumnam et al., 2012). Three representative genotypes namely K-3 (King chilli), D-11 (Dalle Khursani) and C-22 (bell pepper) were used for the initial screening of the primers. A total of 60 set of primers spanning across 14 different linkage groups were checked. A minimum genetic distance of 10 cM between any two markers was maintained i.e. if the selected markers lie within a genetic distance of 10 cM, only one