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Genetic diversity, population structure and marker trait associations for alkaloid content and licit opium yield in India-wide collection of poppy (*Papaver somniferum* L.)

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ABSTRACT

The importance of alkaloids of opium poppy is increasing constantly worldwide to fulfill the demand of pharmaceutical industries. India is one of the countries to produce gum opium and contain many potential genotypes to attend the recent demand of alkaloids. So, for proper utilization of the available potential genotypes, the present investigation was carried out to study the genetic diversity and genetic differentiation along with marker-trait association based on quantitative traits as well as AFLP marker in a large number of germplasm lines of opium poppy which were collected from different parts of India. Various genetic parameters and correlation among different traits were also worked out to find out the particular traits enhancing yield potential. Eight AFLP primer pairs were deployed that generated 140 polymorphic bands with fragment ranging in size from 50 to 498 bp in size. The maximum gene diversity was found in the germplasm lines of UP. Most of the variability (96%) was partitioned into within populations implying that collection strategies for conservation should focus on a few populations with many individuals across the ecological amplitude of the population. Genetic differentiation was $F_{ST} = 0.0413$, that implies more or less complete panmixis. The morphine was found associated with seven AFLP loci, such as E-AAG/M-CAG_76, E-AAC/M-CAG_102, E-ACT/M-CTA_181, E-ACG/M-CTC_167, E-ACG/M-CTC_176, E-ACG/M-CTC_194, and E-ACG/M-CTC_102 which showed stability by both models (GLM, MLM) of analysis while three markers (E-ACT/M-CAA_53,E-ACT/M-CAA_243,E-AGG/M-CTG_241) for codeine, four markers (E-ACT/M-CAA_193,E-AAC/M-CAG_151, E-AAC/M-CAG_91, and E-AGC/M-CTA_75) for thebaine, six for narcotine (E-ACT/M-CAA_85, E-AGG/M-CTG_75, E-AAC/M-CAG_116, E-ACT/M-CTA_122, E-ACT/M-CTA_182, and E-ACT/M-CTA_209), two markers (E-AAG/M-CAG_303, and E-AAC/M-CAG_210) for papaverine and five markers (E-ACT/M-CAA_76, E-AAG/M-CAG_122, E-AAG/M-CAG_245, E-AGG/M-CTG_84, and E-AGG/M-CTG_219) for opium yield were found associated ($P < 0.05$). This is first report on population genetic structure and differentiation in India-wide collection of opium poppy. Based on both marker analysis, the germplasm lines BR296, BR061, BR059 and BR282 were identified utmost diverse for developing mapping populations to carry out linkage/QTL mapping of opioids in poppy. Simultaneously, these lines can also be used in multi-parent breeding program to develop new variety with targeted multi-traits. The associated AFLP markers for various alkaloids open the new avenues for alkaloid improvement breeding program with MAS, genome-wide association and QTL analysis.

1. Introduction

Opium poppy (*Papaver somniferum* L.) stands top ranking among the medicinal plants due to its distinctive ability to synthesize diverse array of pharmacologically active alkaloids. According to the latest technical report by the International Narcotics Control Board, global demand for opiates used in painkillers, is rapidly increasing that has tripled between 1993 and 2012 (Hiscock, 2015). Industry experts say demand is likely to increase further as population's age in the key consuming countries, and newly prosperous consumers in developing economics turning towards branded painkillers. Australia is the biggest legal grower of opiates from poppy husk (Hiscock, 2015) while India is one of the leading countries producing licit opium to meet out the national and international pharmaceutical demand (Priya et al., 2012; Rastogi et al., 2013). Consumers around the world approximately spend about $30 billion per year on pain medication. North America and Europe are the biggest market for codeine, thebaine, morphine and other opiates that go into branded pharmaceutical products such as Panadeine, Oxy Contin and Roxanol (Hiscock, 2015). Poppy is one of the most ancient domesticated

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medicinal crops being used since Neolithic age (Dittbrenner et al., 2008) and is believed to be originated in Asia Minor (Terry and Pellens, 1928) and moved to Egypt, Greece, Arab, China, India and Europe afterward (Schiff, 2002). It has a long history of cultivation in India and was introduced probably in 300 BCE by Alexander the Great. The concrete proof of poppy cultivation in India is traced from the Mughal's period during 1500 CE (Chopra and Chopra, 1955). Opium was also exported to China and Eastern countries during Mughal's rule. According to the Narcotic Drugs and Psychotropic Substances Act (NDPS) 1985, no one can grow poppy, produce, possess, transport, import or sell opium without the permission of Government of India (Deshpande, 2009). In India, cultivation of opium poppy is mainly confined to the particular regions of few states viz. Uttar Pradesh, Madhya Pradesh and Rajasthan (Schiff, 2002).

P. somniferum is a self-pollinating angiosperm belongs to the genus '*Papaver*' which comprises >100 species of annual, biennial and perennials nature spread all over the world. The genus commonly contains species ranging from diploid (2×) to octaploid (8×). Among nine sections of the genus, section 'Mecones' includes five species namely *P. somniferum* L., *P. setigerum* D.C., *P. glaucum* Boiss., *P. gracile* Auch. and *P. decaisnei* Hochst. with basic chromosome number X = 11 (Shukla and Singh, 2004). The species *P. somniferum*, *P. setigerum* and *P. aculeatum* have morphine but *P. somniferum* (2n = 2× = 22) is chiefly cultivated for commercial purposes due to its high opium yield potential (Rastogi et al., 2012). *P. somniferum* produces >80 alkaloids in which narcotic and analgesic morphine, antitussive codeine, vasodilator and smooth muscle relaxant papaverine, antitussive and antitumorigenic agent noscapine and starting material of semi-synthetic opiates (oxycodone, oxymorphone, etorphine and buprenorphine) thebaine are the major ones (Facchini, 2001; Facchini et al., 2008; Desgagne-Penix et al., 2009; Hagel et al., 2008).

Hence, keeping in view the global demand of opiates, the development of novel high yielding varieties is urgently needed. The development of varieties chiefly depends on judicial selection of parents/genotypes. The identification of potentially distinct lines on the basis of morphological traits is labour intensive, time taking, not always accessible for the study and influenced by environmental variations that results vague interpretation due to phenotypic plasticity. This often creates a major predicament in the selection of promising genotype for any trait targeted breeding programme. The diverse genotypes can easily be sorted out based on morphological and molecular parameters to create segregating population with maximum variability for advance selection and introgression of desirable genes from different germplasms into available germplasm. Diverse molecular markers have been used to resolve genetic diversity based on DNA analysis and each has its own requirement, sensitivity and reliability (Dubey et al., 2010). Among molecular markers, AFLP is an efficient DNA fingerprinting tool with high degree of polymorphism, sensitivity, robustness and reproducibility which confers multi locus and genome wide marker profiles (Chial, 2008). In present days, AFLP technique is being used to construct high density linkage maps, population structure determination, genetic diversity and relationship analysis (Kuck et al., 2012; Blignaut et al., 2013). It is extensively used in several medicinal plants like Jatropha (Tatikonda et al., 2009), *Aloe vera* (Tripathi et al., 2011), *Swertia* (Mishra et al., 2010), *Tribulus terrestris* (Sarwat et al., 2008), *Echinacea* species (Russi et al., 2009), *Allium sativum* (Ipek and Ipek, 2003), *Chlorophytum borivilianum* (Tripathi et al., 2012) and *Cocculus pendulus* (Shadia et al., 2014) beside other crops (Saunders et al., 2001). AFLP marker has also been used to identify and characterize the different accessions of opium poppy and has proved its utility in identification of potential genotypes (Saunders et al., 2001). Another most comprehensive study on genetic diversity was done on 300 accessions of opium poppy using AFLP marker (Dittbrenner et al., 2009). Several studies on genetic diversity based on morphological traits (Bhandari et al., 1997; Brezinova et al., 2009; Lal et al., 1996; Saini and Kaicker, 1987; Singh et al., 1998, 2003, 2004; Tiwari et al., 2001; Parmaksiz and Ozcan, 2011) and molecular markers (Saunders et al., 2001; Dubey et al., 2010; Acharya and Sharma, 2009; Parmaksiz and Ozcan, 2011; Gurkok et al., 2013; Guclu et al., 2014; Straka and Nothnagel, 2002; Darokar et al., 2014) have been done in opium poppy. However, AFLP analysis have also been widely used to study the genetic diversity and population structure of other various medicinal plant species like *Borago officinalis* (Lisi et al., 2014), *Chlorophytum borivilianum* (Tripathi et al., 2012),*Tribulus terrestris* (Sarwat et al., 2008), *Dendrobium moniliforme* (Ye et al., 2015), *Salvia miltiorrhiza* (Wang et al., 2007), *Valeriana jatamansi* (Rajkumar et al., 2011), *Nothapodytes nimmoniana* (Shivaprakash et al., 2014), *Ocimum gratissimum* (Matasyoh et al., 2011). But no such combined study based on morphological as well as molecular markers has been made on genetic diversity including a large number of germplasm lines of opium poppy collected from different parts of India. Thus, the present investigation was undertaken with the objectives (i) to study the genetic diversity among the indigenous Indian germplasm lines based on both morphological traits and AFLP markers and their comparison, and (ii) to characterize the germplasm lines for the identification of diverse lines to be used in various hybridization program for the development of novel varieties rich in specific alkaloids.

2. Materials and methods

2.1. Plant materials

Ninety five diverse germplasm lines of opium poppy (*P. somniferum*) were selected from the exclusive collection maintained at Genetics and Plant Breeding Section, CSIR-National Botanical Research Institute, Lucknow, representing four different growing regions of India [Uttar Pradesh (UP), Rajasthan (RJ), New Delhi (ND) and Madhya Pradesh (MP)] (Table 1 and see Table S1 in Supplementary material). These selected lines comprised of cultivars, landraces, developed breeding lines and varieties which are being maintained by selfing for the last many years and are pure and homozygous.

2.2. Phenotypic evaluation, alkaloids estimation and their statistical data analysis

All the germplasm lines were evaluated in a randomized block design with three replications during two consecutive years 2010–11 and 2011–12 at the experimental field of Genetics and Plant Breeding Division of CSIR-National Botanical Research Institute, Lucknow, India which is located between 26°40′ N latitude and 80°45′ E longitude with an altitude of 129 m sea level. The seeds of each germplasm line were sown in three rows per replication (each 3 m length) with row to row distance 30 cm and plant to plant 10 cm in first week of November when the day and night temperature varies from 30 °C to 20 °C respectively. Normal agricultural practices were adopted throughout the crop season (Yadav et al., 2006). Five plants were randomly selected from each replication for the documentation of mean data on days to 50% flowering, days to maturity, plant height (cm), peduncle length (cm), leaves per plant, branches per plant, capsules per plant, stem diameter (cm), capsule size (cm^2), capsule weight per plant (g), seed yield per plant (g), husk yield per plant (g) and opium yield per plant (mg).

The latex of four successive lancings of five tagged plants from each replication of each line was pooled. The air dried opium was used to quantify five major alkaloids viz. morphine, codeine, thebaine, narcotine and papaverine through HPLC (Waters Pvt. Ltd. Milford, USA) consisting of M 6000 pump, 717 autosampler, μBondapak C18 Column, 996 PDA detector and millennium 32 software. The samples were prepared by dissolving dried powdered opium latex in 10 ml of Dimethyl Sulfoxide (DMSO). The samples were filtered through Whatman filter paper followed by micro filtration through micro filtration syringe. The 5 μl of each sample was injected for analysis. The mobile phase was prepared by adding 1.1 g of 1-heptane sulphonic acid into double distilled water, methanol and glacial acetic acid (59:40:10). The samples were run at 254 nm wavelength with flow rate 2 ml/min. (Khanna and Shukla, 1986).

The pooled replicated mean data of both the experimental years of all the germplasm lines was statistically analyzed for all the quantitative