Available online on www.ijcpr.com (ISSN: 0976 822X)

International Journal of Current Pharmaceutical Review and Research

Volume 1, Issue 3, November 2010 - January 2011

Research Article

Phylogenetic relationship between *Argemone mexicana* and *A. ochroleuca*

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Combined data based on morphology, cytology, cross ability and interspecific fertility are secured in *Argemone mexicana and A,ochroleuca ssp ochroleuca* and *A.ochroleuca ssp stenopetla* with an intent to understand their relationship. Distinct differences between the two species merit their separate species status. *Argemone mexican* is diploid with 14 bivalents and normal meiotic behavior whereas *A.ochroleuca ssp. ochroleuca* and *A. ochroleuca ssp stenopetla* ssp had 28 bivalents.

Hybrids between *mexicana x ochroleuca* ssp. *ochroleuca* were triploid with 2n=42 chromosomes. At meiosis variable behavior was noticed and it varied from 42 univalent to 6-8 bivalents and rarely 2-3 trivalent. Pollen fertility ranged from 90 % (*mexicana*), 93% (*ochroleuca ssp. ochroleuca*) and 30% (hybrid). In the triploids 4-8 seeds were collected per capsule. The Neighbour-Joining tree of population of *A. mexicana* (AM), *A. ochrolecuca* ssp *ochroleuxca* (AO), *A. ochrolecuca ssp stenopetla* (AS) and *mexicana x ochroleuca ssp ochroleuca* (AHy) are observed .The AO and AS exhibit maximum similarity, suggesting that they have the common origin. We also assume that ssp *stenopetla* has originated from ssp *ochroleuca* despite the occurrence of some features which vary in the two ssp.

The genus *Argemone* comprises 32 species[1,2,3] and have been reduced to 24[4] . *Argemone* is widely distributed in tropical regions and in India three species and one forma are reported [5]. In Jaipur only two species, *Argemone mexicana* Lin and *A. ochroleuca* ssp *ochroleuca* and *A.ochroleuca* ssp *stenopetala* are found and both are weeds, and are being used as minor medicinal plants. Some studies on the cytogenetic relationship of the two species have been published [6, 7] . The interspecific relationships between the two species have been controversial. Some authors suggest that *A.ochroleuca* may have arisen as an autoteraploid of *A. mexicana* [1].

Argemone mexicana and *A. ochroleuca* ssp *ochroleuca* have been crossed under controlled conditions [8.] Additionally, crossing of the two species in nature has also been reported [9, 8], [and 10.] Schwarzbach and Kadereit [4] examined intrageneric relationships on the basis of nuclear ribosomal DNA (nrDNA) sequence data. And have included *A.ochroleuca* ssp *ochroleuca* and *A.ochroleuca* ssp *stenopetala* as ssp of *A. mexicana* ssp *ochroleuca* .The different plant parts, both fresh and dried are of immense medicinal importance. Leaves are used as expectorant; biliousness; infections; pruritis; antidote for poisons; gastric distention; haematemesis; leprosy; dyuria; lithiasis; oedema. Seed oil is used to cure colic; chest pain; asthma; jaundice, malaria and several other ailments [11].

DNA bar-coding is the use of a short DNA sequence or sequences from a standardized locus (or loci) as a species identification tool. Three plastid genes currently used in bar coding. trnH- psbA spacer- is a non-coding intergenic spacer region found in plastid DNA [12]. This region is one of the most variable non-coding regions of the plastid genome in angiosperms in terms of having the highest percentage of variable site [12]and can offer high levels of species discrimination.

With a view to delineate genetic relationship between the two species a molecular study was undertaken using chloroplast bar code. The chief intent of this analysis was to integrate this information with general morphological analyses and cytogenetic of the two species and their hybrid to determine the phylogenetic relationships within '*mexicana-ochroleuca'* complex.

Material and Methods

A. mexicana, A. ochroleuca ssp ochroleuca and A. ochroleuca ssp stenopetala used presently were sampled locally and identified using different flora [13,1].Triploids were raised by reciprocal crossing *mexicana x ochroleuca ssp ochroleuca*. Seeds were only obtained in *mexicana x ochroleuca* crosses.

The diploid has 2 n = 28: *A. mexicana L.*, and one tetraploid, 2n= 56 *A. ochroleuca* ssp. *ochroleuca* Sweet species were used in the present studies. Flower buds were emasculated at a young stage and subsequently cross pollinated as desired. As many as 20 flowers per species were emasculated. To study meiosis, anthers were fixed in Carnoy(1 : 3:6) and stained in 1% aceto-carmine.

DNA Extraction

DNA extraction was performed with Qiagen DNA miniprep Kit, after the tissue disruption with liquid nitrogen DNA extraction followed manufacturer's protocol. Two microlitres of the isolated total Genomic DNA were analysed using 0.8 per cent agarose gel with ethidium bromide.

Amplification of psbA- trnH spacer region by PCR:

Amplification of psbA- trnH spacer by PCR was performed with universal primers, psbA3'f (5'GTTATGCATGAACGTAATGCTC3') and trnHR (5-CGCGCATGGTGGATTCACAATCC3') specific to conserved regions.

The PCR amplified psbA- trnH spacer region DNA were eluted from the agarose gel and sent to ILS Bio Sciences, New Delhi and sequenced with the specific primers used for amplification. Sequences of partial psbA- trnH spacer region DNA were subjected to a Genbank BLASTN 2.2.22 analysis to retrieve closely related sequences [14]. and was used to construct phylogenetic tree by using Cluster W [15].

The Phylogenetic tree was constructed for the aligned sequences using TREEVIEW [16] along with bootstrap valves predicted using NJPLOT software .

Results

The morphology in the interspecific triploid hybrids and the two parents is described briefly. Figure I show young flowers in the two species and their hybrid. The figure also displays mature capsules and the leaves in the two species as well as hybrid. The length and shape of the sepal horns is of great diagnostic value. In *mexicana* the flower buds are sub spherical, sepal horns are terete, 5-10 mm, long including the apical spine, smooth.. In *ochroleuca* the flower buds are oblong, 12-16 mm long, with 2-4 spines on each sepal, terminal spine slender. In the hybrid the buds were larger than the two species, 18-22 mm long, having 3-7 spines per sepal.

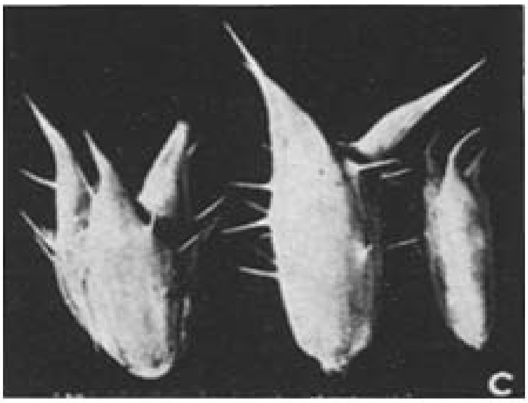


Fig.1. Unopened flower buds in *mexicana* (left), hybrid (middle) and *ochroleuca* (right)

Petals: In *mexicana* petals are bright yellow, the outer obovate with 30-50 stamens, filaments pale lemon yellow, anthers yellow; *ochroleuca* has pale lemon yellow, lemon yellow or rarely white , obcuneate-obovate, stamens 20-75 with pale yellow filaments, anthers dark yellow; in *ochroleuca* ssp *stenopetla* petals narrowly elliptical, pale lemon yellow or white , flower diameter smaller than *ocroleuca* ssp *ochroleuca.*

In the triploid the petals are light lemon color

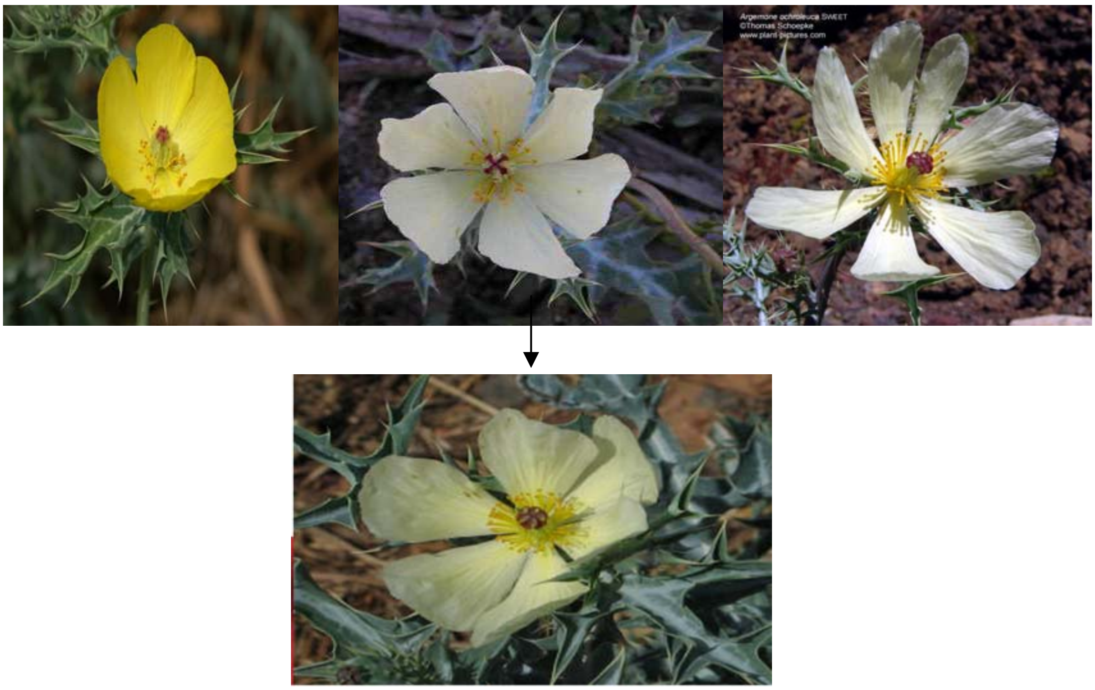


Fig. 2. Fully opened flowers of *A. mexicana* (a), *ochroleuca ssp ochroleuca* (b),*ochroleuca ssp stenopetla* (c) and triploid (*mexicana x ochroleuca ssp ochroleuca*).

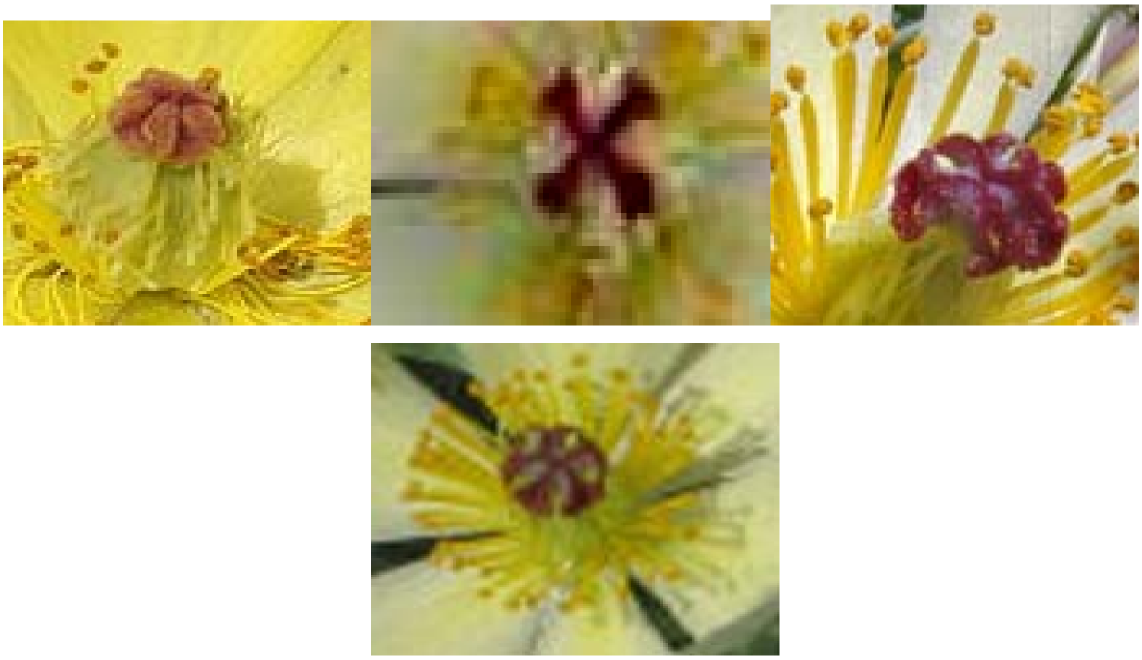


Fig. 3 (a-d). Stigmatic lobes in *mexicana* (a), *ochroleucha ssp. ochroleuca* (b), *ochroleuca ssp stenopetla* (c) and triploid (*mex X ochroleuca* ssp o*chroleuca*).

Stigma:

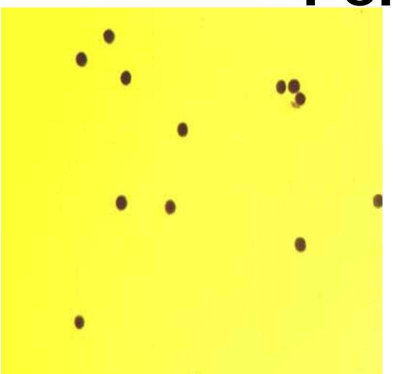
According to [1] stigmatic character is extremely useful in delineating the species. In *mexicana* the stigmatic lobes are appressed rto the style and the n on-receptive areas between them are generally completely hidden by the receptive surfaces (Fig 3 a). In *ochrolecuca* ssp *ochroleuca* the stigmatic lobes are divergent/ or crossed shaped (Fig. 3 c).The lobes are not appressesd to the style. The non-receptive areas between them are clearly visible and pinkish blue in color. In *ochroleuca* ssp *stenopetala* the stigmatic lobes are divergent with non-receptive areas visible.

Capsules: *mexicana* has capsules oblong to elliptical, 12-20 mm wide 25-40 mm long, surface spine scent even sized spines in comparison, *ochroleuca* ssp *ocroleuca* capsules are lanceolate or rarely oblong-elliptic, 20-25 mm long, spines stout, even-sized. In *ochroleuca* ssp *stenopetla* having spines large like *ochroleuca* ssp *ochroleuca* with small spines and prickles intermingled. In the triploid capsule shape and sizes varied (Figs. 5 a-e) and the shape of the capsule depended on the female parent used.

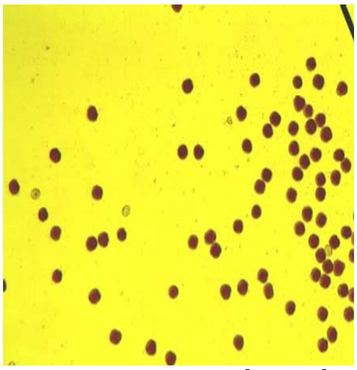
Fig. 4. **Mature capsules in-**

Stenopetla **ochroleuca** **triploid**

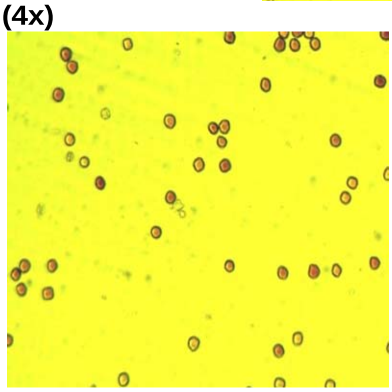
Pollen grains



***A.ochroleuca* (4x)**



***A.mexicana*(2x)**



Triploid(3x)

Fig. 5. Pollen grains in *mexicana* (left top), *ochroleuca* (top right and triploid (middle). Notice sterile grains in the triploid hybrid.

Pollen grains: Pollen grains in the two species are 3 (-4)-zonocolpate with reticulate exine surface, and are distinguishable only on the basis of pollen size, the *ochroleuca ssp ochroleuca* species had larger pollen grains. In the triploid plant, a large number of grains deviated from the normal 3-zonocolpate condition showing a number of apertural types, such as 1-aperturate-operculate, 2-colpate, 3-syncolpate, 4-zonocolpate and spiraperturate. Besides, plan aperturate 3-colpate-trilobed grains also occur. The triploid is also characterized by `small grains (size 13 ± 19 mm), a feature absent in *mexicana and ochroleuca.*

Discussion:

Both the species had normal meiosis and nearly perfect pollen fertility. The triploid hybrid had 2n=-42 chromosomes which showed variable pairing i.e. 6-8 bivalents and rarely 2-3 trivalent. Malhotra [9] observed varying number of univalent and bivalents and rarely trivalents as well .Chaturvedi et al.[10] reported as many as 14 bivalents and 14 monovalents in the natural triploid. Crosses between diploid *(A. mexicana)* and one tetraploid *(A. ochroleuca) Argemnone* species were made. The Fl's were cytogenetically analysed. The raised triploid hybrids were sterile and did not set any seed. In the species there was predominantly bivalent pairing (14ii; 28It) and high pollen and seed fertility. The Fl's displayed different configurations, e.g. I, II and III, and pollen fertility was low; the capsules were shrunken and did not contain many seeds.

In the hybrid pairing was both auto- and allosyndetic and the number of univalents, bivalents and trivalents varied . In general a negative correlation was observed between univalents and chiasmata per cell. However, chiasma frequency and paired associations displayed a positive correlation. It is deduced that sufficient similarities existed between one of the *ochroleuca* and the diploid species genome; the remainder of the *ochroleuca* genome had homologous chromosomes. Apparently *A. ochroleuca* carried enough cryptic intergenomal honlologies which ordinarily remained unexposed. Earlier Malik and Grover [8a] in the *albiflora x ochroleuca* combination as many as 13 triyalents reported. It seems that
In the hemizygous state however, as in the Fl's, there was intergenomal pairing. In all attempt to resolve the conflict between homology and bivalent pairing in the species, a diploid zing genetic mechanism is envisaged. Alternatively an acute propensity to preferential

pairing caused bivalent formation. Such a system or systems caused meiotic isolation of various genomes and instituted normal fertility.

Furthermore, the segmental allotetraploid nature of *A. ochroleuca* is proposed.

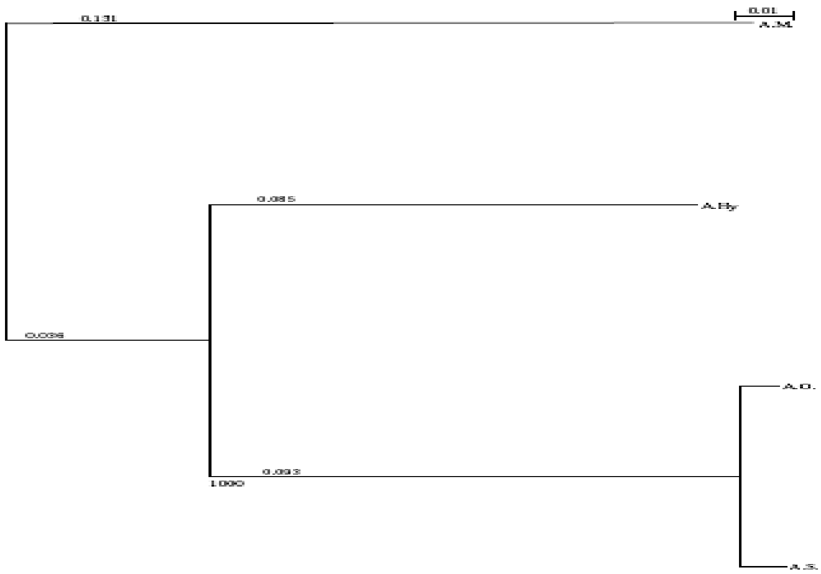


Fig.7. Phylogenetic analysis of *psbA*- *trnH* spacer region

The Neighbour-Joining tree of population of *A. mexicana* (AM), *A. ochrolecuca* ssp *ochroleuxca* (AO), *A.e ochrolecuca ssp stenopetla* (AS) and *mexicana x ochroleuca ssp ochroleuca* (AHy) are shown in Fig. 7. The AO and AS exhibit maximum similarity, suggesting that they have the common origin. We also assume that ssp *stenopetla* has originated from ssp *ochroleuca* despite the occurrence of some features which vary in the two ssp.

The AH (Hybrid between AM and AO) showed the relationship with both AM and AO confirming the results of Malik and Grover [8a]. The AM sequence when subjected to BLAST, it showed the maximum similarity with already submitted sequence of AM (accession no. GQ435427) confirming the amplification of correct DNA fragment by PCR. The high boot strap valve in the NJ tree shows the accuracy of the analysis. Furthermore, AO and AS are tetraploid spp. and may have the common ancestral origin from the diploid AM spp., suggests that AO and AS diverged through mutations. It may be added that we failed to sample any intermediates in the field pointing towards incompatibility barriers between them. The inference is further confirmed by *psb*A- *trn*H spacer region sequence analysis of hybrid of

AM and AO, a triploid, which showed the similarity with AO and AS cluster (4x) and AM (2x). The AH had most similarity with the AO and AS cluster, which confirms that the AH is having more characteristics of 4x AO compared with 2x AM. This postulate is further confirmed by morphological analysis such as flower shape, petals color, stigma shape and capsule, etc

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