

Intra-specific variability in cytotoxic and meiotic aspects of underutilized *Vitex negundo* L. from North-West Himalaya: putative causes and consequences

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Abstract

Vitex negundo Linn., a widely distributed medicinal species of North-western Himalaya, has been studied for detailed cytological aspects. Present studies on eight populations reveals this species having 34 chromosomes, which largely show regular behaviour. Besides, studied populations of this species are unusual in exhibiting cytomixis largely between paired meiocytes at prophase and rarely between 3-4 meiocytes at anaphase. Comparison of frequency of cytotoxic cells prepared using fixed and fresh anthers reveals scarce influence of anther fixation on percentage of cells showing cytoplasmic connections and chromatin transfer. Analysis of cytotoxic index of plants inhabiting varying altitudes indicate overall less cytotoxic index in populations from lower altitudes. Further analysis of dividing meiocytes for chromosomal number and behaviour reveals that out of eight populations, five (Govind-sar, Palli-morh, Kharoot-morh, Billawar and Rajouri) populations contains all and three contain maximum diploid cells, where as minority of cells of latter three populations exhibit near haploidy and polyploidy. Similarly translocations, manifested by occurrence of multivalent have been noted in 16-33% euploid PMCs of 4 populations (Palm, Bilr, Khrm, and Kol). Comparison of frequency of cells showing chromatin migration and meiotic anomalies reveal existence of inverse relations in these parameters. Existence of cytotoxic and meiotically aberrant cells also account for the production of few deviant sized pollen grains along with normal ones. All these anomalies along with their possible cause and consequences are presented in communication.

Introduction

Vitex negundo Linn., (nirgundi), is a commonly grown medicinal species of South-east Asian countries (Bramley *et al.*, 2009). During different surveys made in Jammu and Kashmir (J&K) and Himachal Pradesh (H.P.), this species was found inhabiting wastelands, sides of agricultural fields and roads along with river banks. As leaves of this species are rich in anti-inflammatory, anti-cancerous, anti-rheumatic, hepato-protective and anti-oxidant components (Vimal *et al.*, 2011), locals consume their extracts for treating cough, cold, body aches, wounds and snake bite (Ladda and Magdum, 2012; Basri *et al.*, 2014), and feed to their cattle to cure bloating and dysentery (Kaur and Sidhu, 2014).

Despite wide distribution and importance, limited cytological data are on record and that pertain to chromosome number. While most Indian accessions and those from China and Pakistan reportedly contain 34 chromosomes (Sharma and Mukhopadhyay, 1963; Gill *et al.*, 1983; Zhang and Shangguan, 1990; Ma *et al.*, 1990; Khatoon and Ali, 1993; Kaur and Sidhu, 2014), fragmentary reports describe its having 24 (Malik and Ahmed, 1963)/ 26 chromosomes (Sobti and Singh, 1961 and Chatha and Bir, 1988) and 32 chromosomes (Mehra and Bawa, 1969; Gill *et al.*, 1983; Sandhu and Mann, 1988; Bala and Gupta, 2012; Singh *et al.*, 2018). Reporting of variable chromosome counts for Indian nirgundi accessions and lack of cytological knowledge of populations inhabiting North-Western Himalaya prompted us to establish its chromosome number by carrying out detailed studies. Presently, nirgundi plants of eight populations growing in different altitudes of J&K and Himachal Pradesh have been studied for three years (2019-2021). As preliminary meiotic studies revealed existence of cytomixis in *V. negundo*, these have been critically scrutinized for different cytotoxic aspects along with possible causes and consequences. So, the specific objectives of our study were:

- i. Establishing diploid count of *V. negundo* by investigating populations inhabiting varying altitudes.
- ii. Determining population-wise frequency of cells depicting cytomixis and chromatin migration during different meiotic stages using fixed and unfixed anthers.
- iii. Finding out chromosomal anomalies both numerical and/structural prevalent in the plants.
- iv. Determining impact of chromatin migration and structural/numerical chromosomal variability on pollen viability.

Materials and Methods

Plant materials:

Field visits were made in different parts of Jammu and Kashmir and Himachal Pradesh (Fig 1) during 2019–2021, and plants of *V. negundo* growing at different altitudes were geo-tagged (Table 1). Because this species also propagates by vegetative means, plants selected per population were at least 1-2 meters apart so as to avoid resampling. Dried specimens of inflorescences bearing leafy branch of each accession has been deposited in Jammu university herbarium. Voucher number of different accessions, their identity, geo-coordinates and habitat are given in table 1.

Cytological studies:

Four treelets per population were analysed for meiotic details using fresh and fixed anthers. For fixation purpose, young inflorescences were put in freshly prepared 3 alcohol:1 acetic acid solution for 24 hours, followed by their shifting to 70% alcohol. Anthers excised from buds, were squashed in 1% propionocarmine and good meiotic spreads were analysed for chromosome number and behaviour at diakinesis, meta and anaphase I. Since PMCs of *Vitex* show cytomixis, for determining the effect of fixation on cytomixis, meiotic spreads prepared using fixed and unfixed anthers were scrutinized for various cytotoxic aspects and frequency of cells showing structural and numerical chromosomal variations. Pollen stainability was estimated using 1% acetocarmine and by counting stained and healthy pollen against unstained pollen. For estimating pollen viability, each anther was crushed in glass slide in a drop of freshly prepared 1% TTC solution (2,3,5 triphenyltetrazolium chloride), followed by its incubation in a dark humid chamber for 24 hours. Viable pollens were red-colored whereas non-viable were transparent.

Using ocular meter, size of viable pollens was determined. While pollens having size ranging from 22×19 to $26 \times 25 \mu\text{m}$ were taken as normal, those of 27×26 to $28 \times 31 \mu\text{m}$ were considered large and a few of size 19×17 to $22 \times 18 \mu\text{m}$ were considered small. Photomicrographs of meiotic spreads and pollens were taken using microscope (Nikon make) fitted with Nikon FM3 A camera. The bar on all the photographs represents $10 \mu\text{m}$.

Statistical analysis

PaST – Paleontological Statistic ver. 1.64 (Hammer et al. 2007) software was used to analyze the correlation between meiotic anomalies associated with cytomixis and pollen viability.

Results and discussion

Chromosome number determination from 8 *V. negundo* populations reveal all/maximum meiocytes having 34 chromosomes, with minority of these (3-4%) in a few populations (Botanical garden, Kolka and Domana) having either 30/36 chromosomes or haploid ($2n=20$), tetra- ($2n=64$) and aneuploid counts (Domana). Persistent finding of $2n=34$ chromosomes in eight *V. negundo* cytotypes existing between 315-915 masl of North-west Himalaya establishes 34 as its chromosome count.

Cytotoxic indices of fixed and unfixed anthers:

V. negundo plants also reveal existence of cytoplasmic connections between adjacent meiocytes. As cytomixis in many plant taxa is reportedly influenced by pesticide treatment (Boback and Herich, 1978), fixation (Takats, 1959; Haroun, 1995) and altitudinal variations (Malik et al., 2010), we attempted to determine possible factors affecting it. Pesticide treatment cannot be taken as a cause of cytomixis as current studies are performed using wild populations. For determining the possible role of anther fixation underlying cytomixis, fixed and unfixed anthers of studied

populations were scrutinized for percentage frequency (%) of cells showing cytoplasmic connections and chromatin transfer. The observations made on these aspects and number of cells concurrently involved at different meiotic stages are tabulated (Table 2).

Table 2 reveals that in the studied populations, greater percentage of cells display cytomictic connections than those depicting chromatin migration both for fixed and unfixed anthers. This disparity must have resulted because of greater tendency of broader channels to permit chromatin transfer than narrow ones as has been viewed by Mallah and Attia (2003) in *Diplotaxis harra*.

In so far as frequency of cells showing chromatin migration in fixed/unfixed anthers is concerned (Table 2), it is higher in untreated anthers in 4 populations (Palli-morh, Rajouri, Botanical-garden and Domana), lesser in similar anthers in 3 populations (Govind-sar, Billawar, Kolka) and nearly same in a single population (Kharoot-morh). As such this comparison reveals no/scarcely influence of fixation on extent of cytomixis of a species. Similar findings have previously been reported in *Leonorussibricus* and *Leucas aspera* by Kundu and Sharma (1988).

To determine whether/not the frequency of cytomictic cells is influenced by specific meiotic stage, frequency of such cells have been compared population-wise at different meiotic stages. This analysis reveals that cytoplasmic connections, largely between cells at early prophase I and rarely between those at anaphase I. Like *V. negundo*, *Mentha* species (*M. arvensis*, *M. spicata* and *M. piperita*) depict cytomixis between cells at pro- and metaphase I, though in few species such as in *Leonorussibricus*, *Leucas aspera* and *Hedeoma multiflorum*, connections occur in cells from prophase to tetrad stage (Kundu and Sharma, 1988; Tyagi, 2003; Kaur and Singhal, 2014; Lin *et al.*, 2017; Liebana *et al.*, 2017). Cytomictic channels between pectin-cellulosic walls of connected cells at early prophase might have originated by the activity of hydrolytic enzymes produced by golgi bodies and endoplasmic reticulum or enlargement of single plasmodesma/fusion of proximate ones viewed as de novo formation in cell wall. In so far as simultaneous involvement of cells in cytomixis concerned, cytomictic connections have been noted largely between paired cells and occasionally between group of three to four cells (Fig. 2).

Frequency of cells depicting chromatin migration in population inhabiting varying altitudes.

Existence of chromatin material in the intercellular channels, compared to cytoplasmic connections provides clear evidence of cytomixis. For determining relationship between altitudinal sites inhabited by a population and frequency of cells depicting chromatin migration, the two parameters of nirgundi plants have been compared (Fig. 4).

This comparison depicts that populations growing at lower altitudes viz. 300-600 masl (Domana, Palli-morh, Kharoot-morh, Botanical-garden and Govind-sar) contain more cells showing chromatin migration, whereas those found at higher (>600 masl) altitudes (Billawar, Kolka and Rajouri) possess less such cells. Unlike *V. negundo*, *Martensia echodia* cytotypes from higher altitudes contain more cytomictic cells than these growing at lower altitudes (Malik *et al.*, 2010).

Deviation in Chromosome number and behaviour

As mentioned earlier, except for meiocytes of Govind-sar and Rajouri which contain 34 chromosomes showing regular behaviour, remaining populations contain free meiocytes with variable count and/or multivalent (Table 3). While 3-9% cells of Billawar, Botanical-garden and Domana populations were aneuploid/haploid/polyploidy, 14-18% cells of remaining populations contain trivalent and hexavalent (Fig. 3).

Though aneuploid cells with few additional/missing chromosomes are on record in many cytomictic taxa like *Mertensia echoides* (Malik *et al.*, 2014), near haploidy ($2n=20$) and polyploidy ($2n=64$) noted currently in Domana

plants is rarely reported. Quardi and hexavalent as noted presently in few cells of four nirgundi populations, must have resulted by exchange of segments between at least two and three non-homologous chromosomes. Tyagi (2003) and Malik *et al.* (2014) and Devi and Sharma (2021) also found in multivalent in cytotoxic *Mentha spicata*, *Mertensia echoides* and *Mentha longifolia*.

Relation between frequency of deviant meiocytic cells and pollen viability

Preservence of cell viability after involvement in cytomixis is a decisive factor for determining the outcome of this phenomenon. Barring few taxa like *Dactylis glomerata*, *Avena canariensis* and *Salvia perzewalskii* (Falistocco *et al.*, 1995; Morikawa and Leggett, 1996; Lin *et al.*, 2017) whose pollen viability remain unaffected by cytomixis, in other like *M. echoides* (Malik *et al.*, 2014) and *Papaver somniferum* (Patra *et al.*, 1987), it is greatly reduced *Mentha arvensis* var. *javonica* depicted no pollen formation (Kundu and Sharma, 1988). To determine impact of meiotic aberrations on pollen viability in *Vitex negundo*, data gathered on cytotoxic PMCs and those exhibiting aneuploidy and multivalent formation have been collated and compared with pollen viability (Table 4).

A perusal of table 4 reveals the least pollen viability in Domana population of *V. negundo*. Across the remaining populations, those from Palli-morh possessing maximum percentage of cytotoxic cells (75.92) produce least viable pollens (22.9%). For this and remaining studied populations slight ($r=0.44$) correlation exist between cytotoxic plus aneuploid cells and pollen viability.

Frequency of cytotoxic meiocytes versus variable sized pollen grains

Meiocytes involved in cytomixis after gaining/loosing chromatin may continue development and mature into fertile pollens with varying genetic content and size. Pollen size determination in nirgundi plants reveals these ensembling in normal, large and small categories.

While maximum deviant pollens (large/small) are found in Palli-morh having highest number of cells showing chromatin transfer and meiotic aberration, the least ones are in Kolka plants possessing minimum cytotoxic index. Probably small and large pollen in *Vitex* plants seem to have developed from PMCs that might have lost/gained genetic material through cytomixis. Same view has been put forth by Singhal *et al.* (2011) and Saggoo *et al.* (2011) to explain existence of deviant sized pollens in *Thalictrum foetidum*, *Nepeta discolor* and *N. gowaniana*. There is every probability that such deviant pollens may differentiate into chromosomally/genetically imbalanced male gametes which on fertilization can produce aneuploid offsprings.

As such, present cytological studies reveal that (i) established chromosome number of *V. negundo* is $2n=34$ (ii) cytotoxic connection occur largely between 2 meiocytes at prophase I stage and rarely between 3-4 meiocytes at Metaphase/Anaphase I stage. (iii) cytotoxic indices of nirgundi populations are scarcely influenced by fixative treatment, though to some extent by altitudinal variations (iv) Numerical and structural chromosomal anomalies also exist in few population. (v) populations having greater percentage of cells showing chromatin migration and meiotic aberrations exhibit comparatively low viability. Besides these findings persistent occurrence of cytomixis and associated anomalies for two consecutive years indicates that cytomixis is not a fortuitous event but a natural phenomenon under direct genetic control. Nevertheless, the role of physiological factors in its manifestation cannot be excluded (Mantu and Sharma, 1983).

Declarations

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Tables

Table 1. Geo-coordinate of different populations, their habitat and sites inhabited by eight *V. negundo* populations

State/U.T.	District	Locality	Identity	Habitat	Accession Number JU/BOT/160	Elevation		Altitude (masl)
						Latitude N	Longitude E	
J&K	Kathua	Palli-morh	Palm	Field and road-side	16007	32°25.510'	75°26.432	344
	Kathua	Govind-sar	Govs	Waste-land	16008	32°24.122'	72°32.73	424
	Kathua	Kharoot-morh	Khrm	Road-side	16009	32°32.510'	72°26.395	343
	Kathua	Billawar	Bilr	Road-side	16010	32°25.472'	75°25.274	630
	Jammu	Jammu University	Botg	Botanical-garden	16011	32°25.433'	75°25.433	402
	Jammu	Domana	Dom	Fields and Waste-land	16012	32°25.433'	75°25.433	315
	Rajouri	BGSBU	Rajr	Road-side	16013	33°23.624'	74°20.432	915
H.P.	Bilaspur	Kolka	Kol	Waste-land	16014	32°24.223'	74°28.399	673

Table 2. PMCs (%age) of fixed and unfixed anthers showing cytoplasmic connections and chromatin transfer across populations

Populations		PMCs displaying cytotoxic connections /channels (Number/total)	PMCs (%) exhibiting chromatin transfer (Number/total)	Meiotic stage	PMCs concurrently involved in cytomixis
Govs (424 masl)	Untreated	54.63 (212/388)	7.21 (28/388)	EPI (100)	2 (48.11%)
	Anther				3 (31.13%)
					5 (20.75%)
	Treated Anther	62.25 (254/408)	12.5 (51/408)	EPI (93.38) Diakinesis(6.69)	2 (100%)
Palm (343 masl)	Untreated	75.92 (412/542)	9.96 (54/542)	EPI (100)	2 (100%)
	Anther				
	Treated	69.25 (408/590)	5.4 (32/590)	EPI (90.68) Diakinesis(9.31)	2 (100%)
	Anther				
Khmr (344 masl)	Untreated	61.29 (152/248)	7.25 (18/248)	EPI (100)	2 (97.36%)
	Anther				4 (2.63%)
	Treated	62.87 (188/299)	7.35 (22/299)	EPI (90.42) Diakinesis(8.51) Anaphase(1.06)	2 (100%)
	Anther				
Bilr (630 masl)	Untreated anther	36.54 (148/405)	6.17 (25/405)	EPI (100)	2 (97.29%)
					4 (2.70%)
	Treated	48.32 (188/389)	12.85 (50/389)	EPI (100)	2 (100%)
	Anther				
Rajr (915 masl)	Untreated	34.95 (172/492)	34.95(172/492)	EPI (100)	2 (40.69%)
	Anther				3 (59.30%)
	Treated	12.45 (64/514)	6.42 (33/514)	EPI (100)	2 (100%)
	Anther				
Botg (402 masl)	Untreated	69.41 (270/389)	8.74 (34/389)	EPI (100)	2 (96.29)
	Anther				5 (3.70)
	Treated	13.62 (68/499)	5.81 (29/499)	EPI (100)	2 (100%)
	Anther				
Kol (678 masl)	Untreated anther	30.21 (58/192)	6.25 (12/192)	EPI (96.55) Diakinesis (3.44)	2 (89.65%)
					3 (10.34%)
	Treated	37.25(76/204)	13.72 (28/204)	EPI (100)	2 (100%)
	Anther				

Dom (315 masl)	Untreated	49.58 (178/359)	6.41 (23/359)	EPI (94.94)	2 (93.25)
	Anther			Metaphase I (1.68)	3(6.74)
				Anaphase I (3.37)	
	Treated Anther	26.73 (108/404)	4.20 (17/404)	EPI (100)	2 (100%)

Table 3. Percentage of cytotoxic and aneuploid cells showing different chromosomal associations at diakinesis/metaphase I.

Populations	PMCs (%) migration	showing chromatin	Chromosome Number	Associations	% age (Number/Total)
Govind-sar	54.63 (7.21)		34	17II	100 (68/68)
Palli-morh	75.92(9.96)		34	17II	67.03 (61/91)
				1IV+14II+2I	32.96 (30/91)
Kharoot- morh	61.29(7.25)		34	17II	84.48 (49/58)
				16II+1III+1I	10.34 (6/58)
				1IV+15II	5.17 (3/58)
Billawar	36.54(6.17)		34	17II 1VI+1IV+12II	82.53 (52/63)
			36	1VI+14II+2I	14.28(9/63)
Rajouri	34.95(100%)		34	17II	100 (34/34)
Botanical- garden	69.41(8.71)		34	17II	96.87 (62/64)
			40	20II	3.12 (2/64)
Kolka	30.21(6.25)		34	17II	81.25 (26/32)
				1VI+14II	18.75 (6/32)
Domana	49.58(6.41)		34	17II	94.64 (50/56)
			29	14II+1I	3.57 (2/56)
			20	10II appearing morbid	3.57 (2/56)
			64	32II	3. 57 (2/56)

Table 4. Population-wise comparison of cytotoxic cells, pollen stainability and viability in *Vitex negundo*

Populations	PMCs (%) showing chromatin transfer and meiotic abnormalities	Pollen stainability	Pollen viability (%)	Normal vs. deviant (small/large pollens)	
				Normal	Small/Large
Govs	54.63	72.5	21.95	92.59	3.70/3.70
Palm	75.92	75.8	23.95	79.16	16.66/4.16
Khrm	61.29	73.6	25.25	91.66	4.17/4.17
Bilr	36.54	80.06	24.1	88.23	5.88/5.88
Rajr	34.95	71.7	23.5	90	6.66/3.33
Botg	69.41	77	24.2	88.88	7.40/3.70
Dom	49.58	59.9	18.1	91.30	4.34/4.34
Kol	30.21	89.6	45.4	93.75	6.25/0

Figures

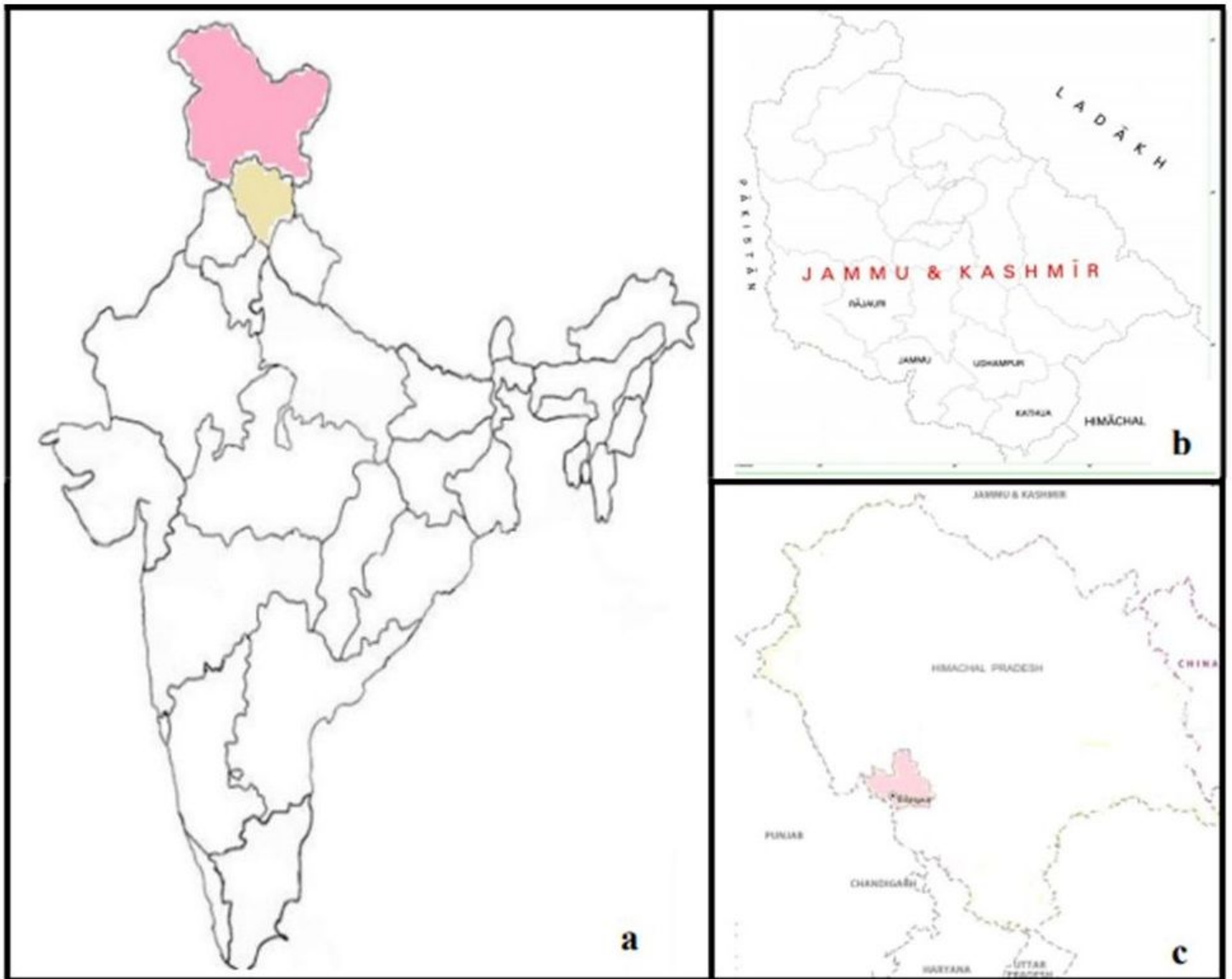


Figure 1

Legend not included with this version.

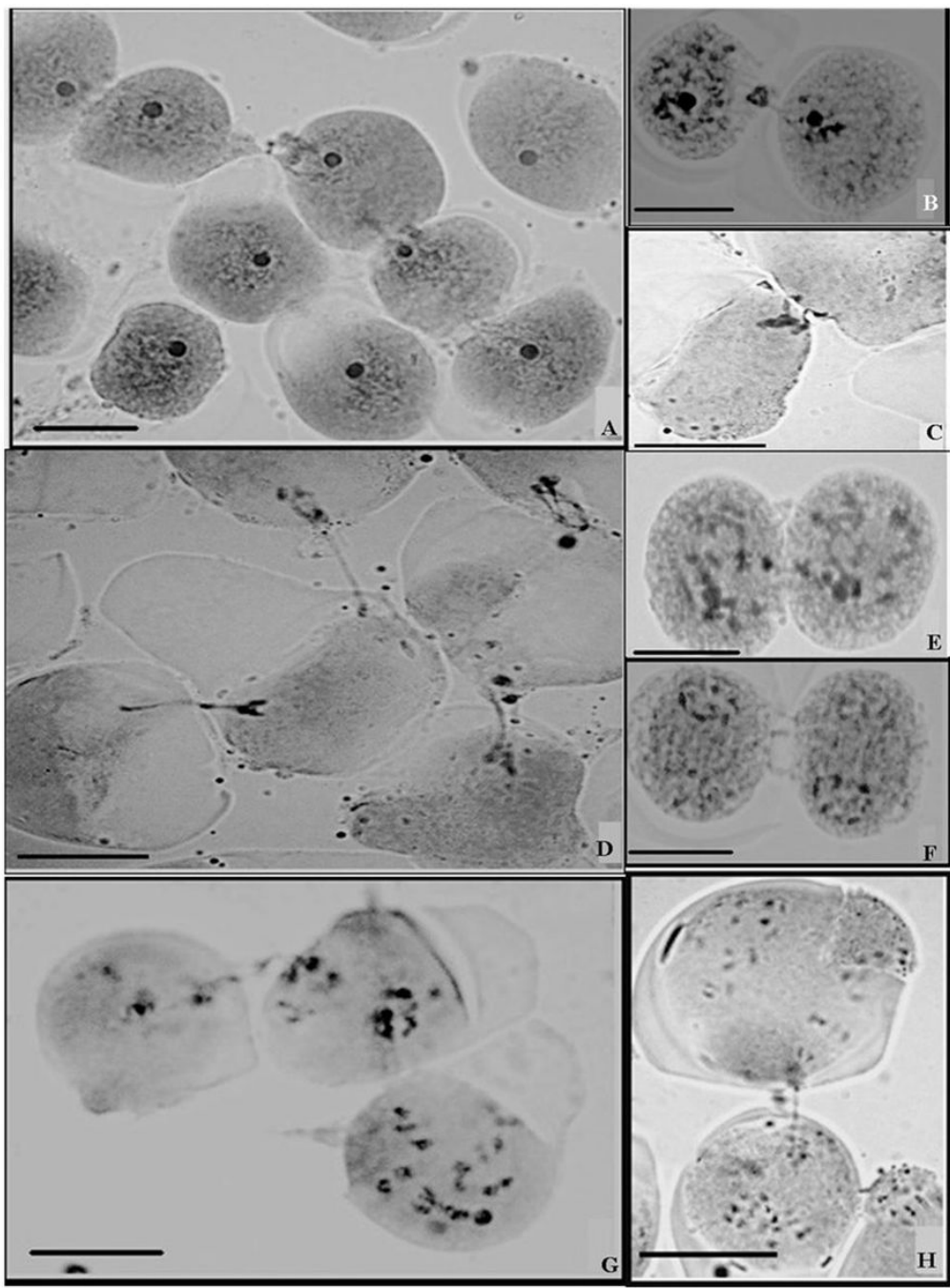


Figure 2

Legend not included with this version.

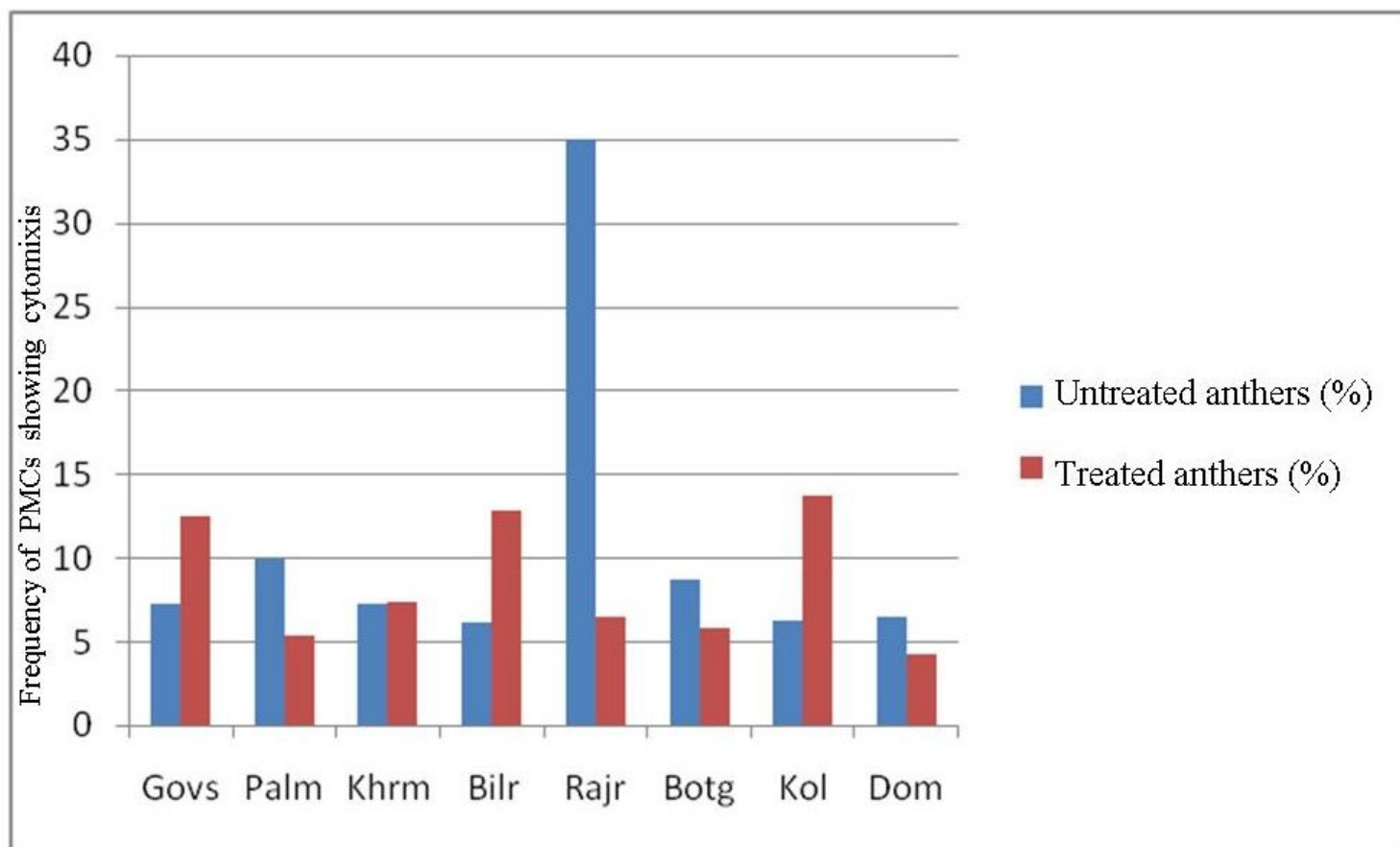


Figure 3

Legend not included with this version.

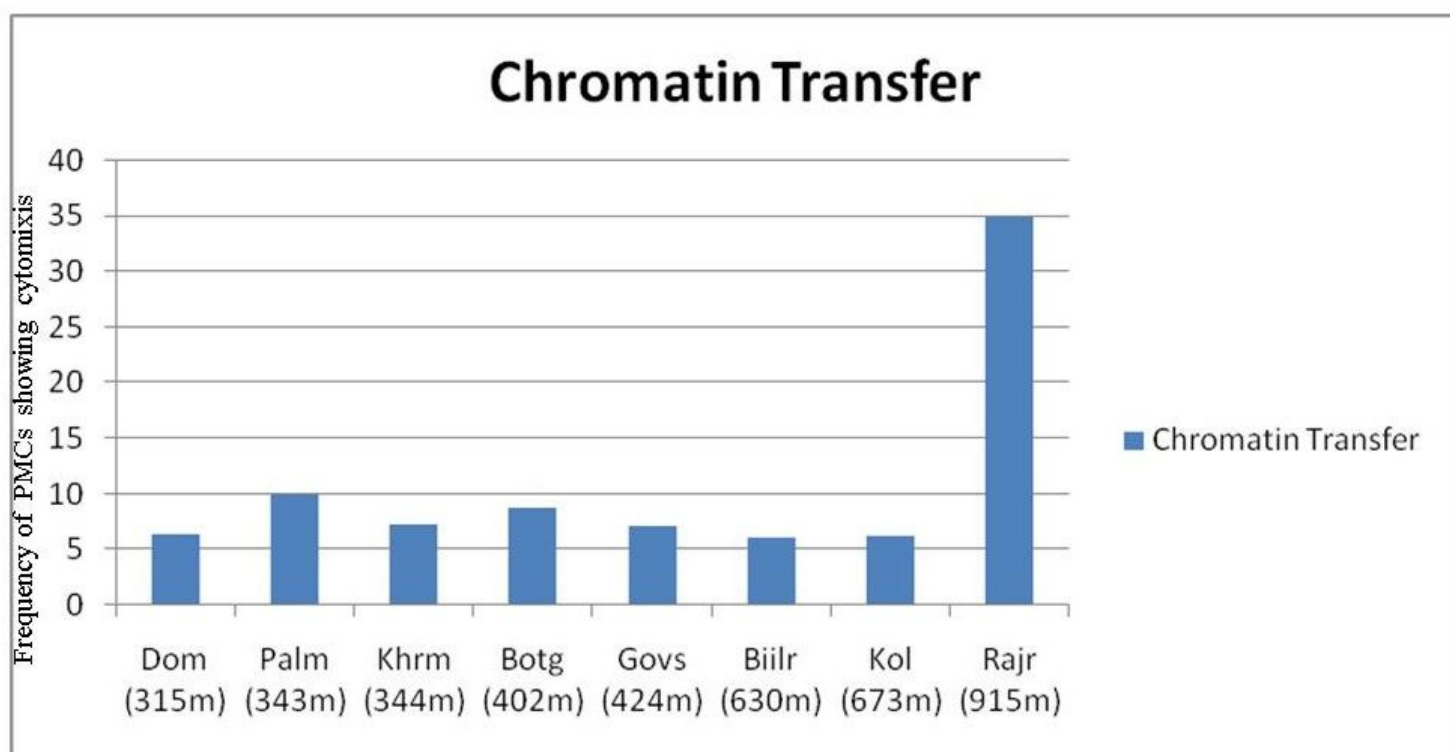


Figure 4

Legend not included with this version.

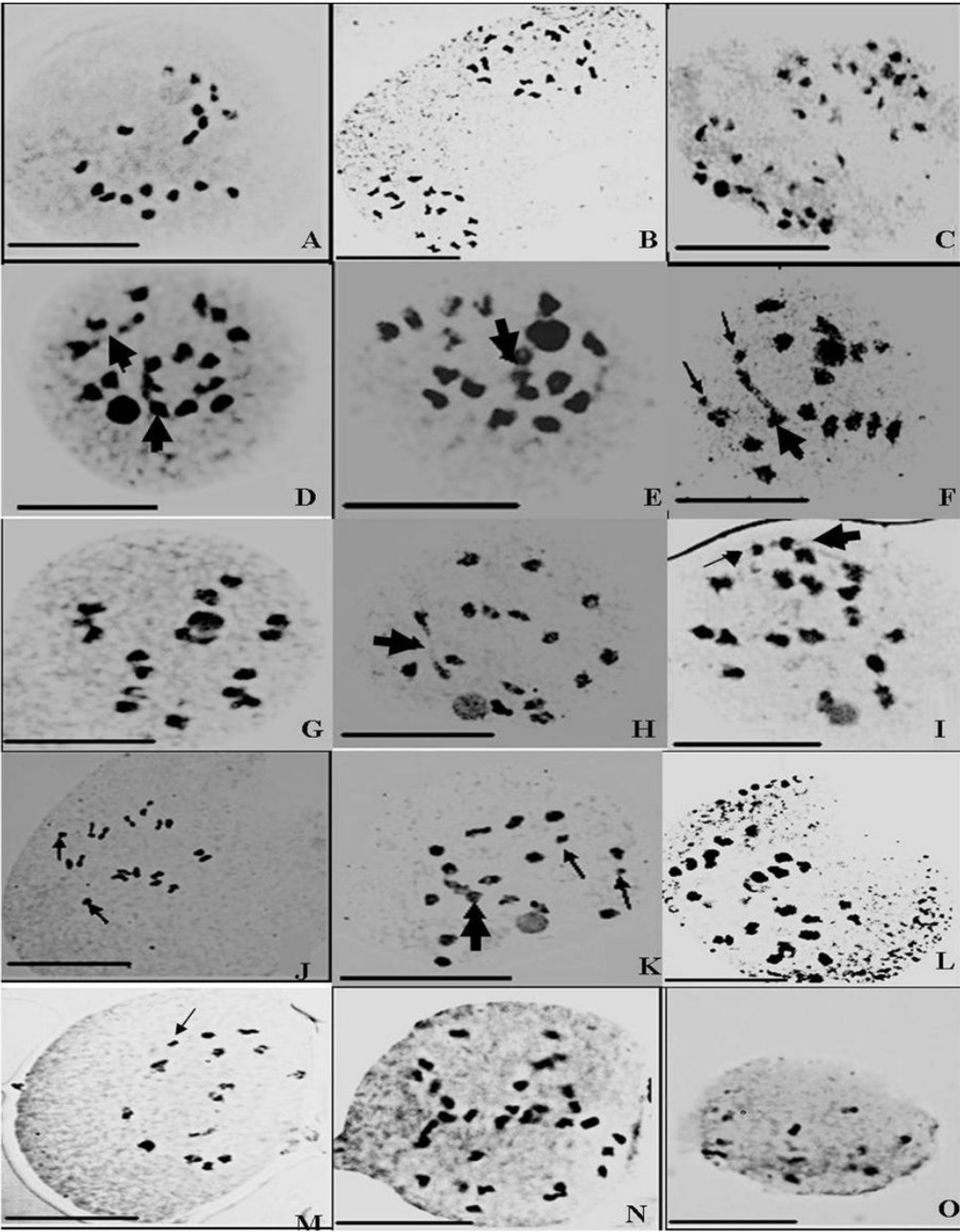


Figure 5

Legend not included with this version.

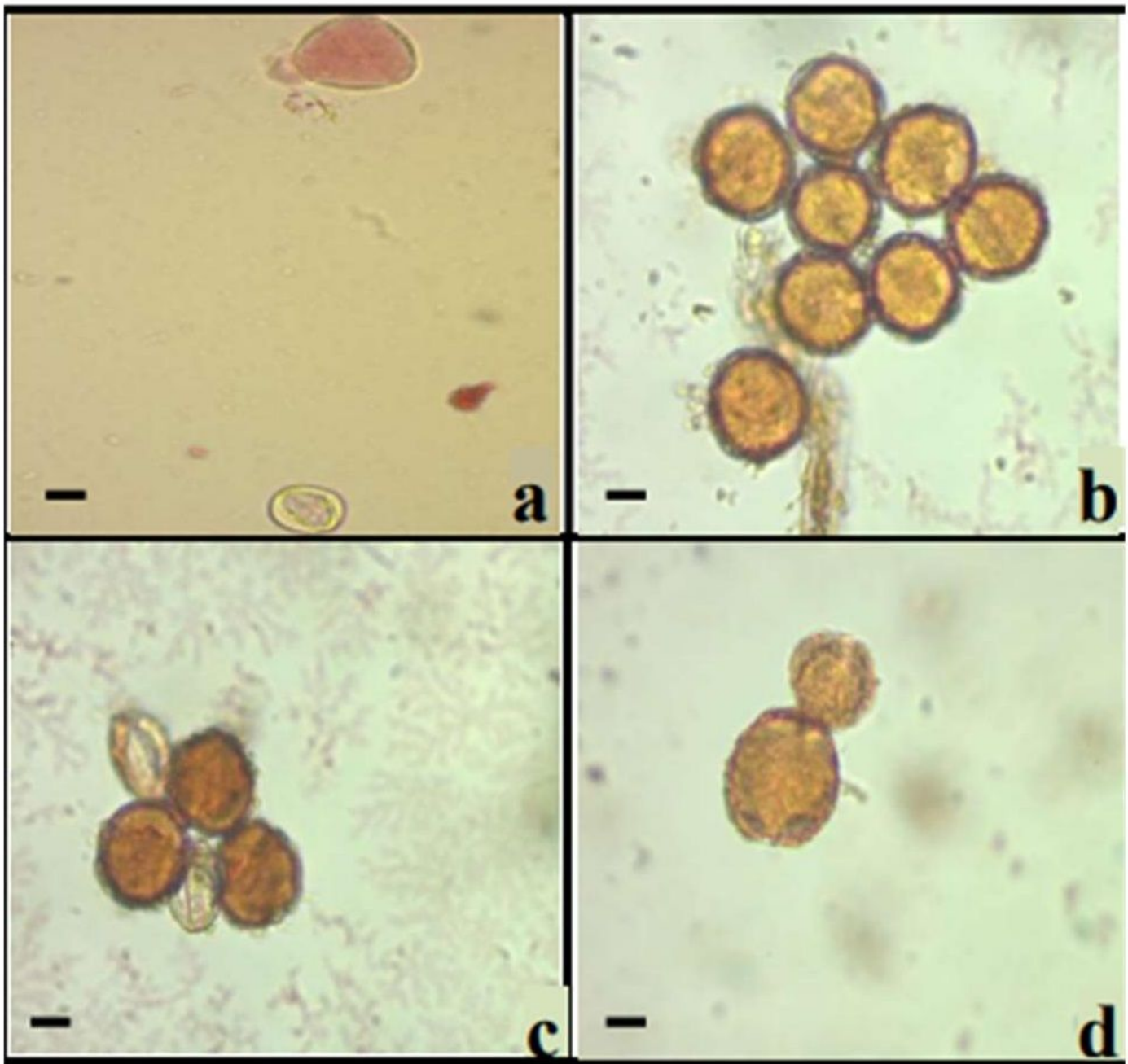


Figure 6

Legend not included with this version.