Notes on *Astragalus* sect. *Macrophyllium* with a Cytogenetic Report on its two Tetraploid Species

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Abstract

Habit and pollen morphology were studied in four taxa belonging to *Astragalus* sect. *Macrophyllium* in Iran. Data obtained from pollen morphology support the phenetic grouping based on habit morphology. In addition, meiotic chromosome number and behavior were analyzed in two species of the section. The species were cytogenetically analyzed and found to be tetraploid and possess a $2n = 4x = 32$ chromosome number; consistent with the proposed base number of $x = 8$ for the section from the check list of Legumes of Northern Eurasia. The taxa displayed an almost regular bivalent pairing and chromosome segregation at meiosis. However, some meiotic abnormalities observed here included varied degrees of chromosome stickiness and laggards in telophase I and II, asynchronous nuclei in telophase I, multipolar cells and cytomixis.

Introduction

Astragalus L. with nearly 3000 species is one of the largest genera of flowering plants. Iran alone with more than 840 species is one of the main centers of diversity of the genus [25, 27, 29, 37]. *Astragalus* has been divided into approximately 150 sections, of which *A.* sect. *Macrophyllium* with 8 species is a small section. The species of *A.* sect. *Macrophyllium* are cushion-forming plants, mostly with paripinnate leaves and calyx covered with white hairs. They are distributed in several southwest Asian countries. However, some diversity of the species is also found in Turkey (5 species) and Iran (4 species).

KeyWords:*Astragalus* sect. *Macrophyllium*, Chromosome number, Iran, Meiosis, Morphology, pollen grain
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As one of the most heterogeneous and difficult groups of spiny *Astragali*, the section has been revised several times as a whole [7], [9] and for the area of Flora Iranica [35]. The importance of cytological information in plant systematics and evolution has attracted the attention of several researchers. Most of the cytological studies in the tribe Astragaleae have concentrated on the chromosome count [1], [2], [26], [27], [48]-[50]. The basic chromosome number (x = 8) and five ploidy levels ($2n = 2x = 16$, $2n = 4x = 32$, $2n = 6x = 48$, $2n = 8x = 64$ and $2n = 12x = 96$) are present in the genus. However, studies on the impact of cytogenetic data on the interspecific and phylogenetic relationships in the genus are still limited. Also, little is known about the nature of genetic variability and the taxonomic relationships of the different taxa in the genus. The study of pollen grains of the leguminous plants [e.g. [10], [14], [18]-[20], [23] has dealt mainly with the description of the pollen grains of certain genera or sometimes tribes.

Hence, investigations in different aspects can be useful to solve taxonomic problems of this problematic group. This work follows previous studies conducted on leguminous fodder species in Iran [36]-[43] and aims to: increase knowledge about the patterns of morphological variation, chromosome number, meiotic behavior and pollen morphology in 4 taxa of *A.* sect. *Macrophyllium* in Iran; establishes relationships between the cytogenetic data, pollen morphology and taxonomic delimitation.

Materials and methods

Morphology

Taxa belong to *Astragalus* sect. *Macrophyllium* were collected from the field in different regions of their natural geographical distributions during our several excursions in Iran. The collected materials were in vegetative or fruiting phase and deposited at BASU, Hamedan, Iran. Also several sheets of herbarium specimens have been examined for each taxon from the following herbaria: W, WU and PR. The populations studied morphologically are listed in Table 1 and used as operational

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taxonomic units (OTUs). A numerical taxonomic analysis of the different individuals from these populations were carried out based on 24 quantitative/qualitative characters related to vegetative and reproductive organs. The list of morphological characters studied here is presented in Table 2. Data were entered onto a computerized spreadsheet program, Microsoft Excel version 7. The spreadsheet was later transformed into a file format suitable for phenetic analysis. Principal coordinates analysis (PCO) was carried out using MVSP software version 3.2 [24], with a matrix of standardized data. For PCO, an average distance matrix of standardized data was obtained.

Table 1. Collection data of the investigated taxa of *A*. sect. *Macrophyllium*.

Taxa	Locality	Voucher specimen	Abbreviation
A. cephalotes Banks & Sol. (appressed hairy)	East Azerbaijan: Oshnavieh to Orumieh, after Movana, 1669 m, 26.6.2009, Ranjbar & Assadi	BASU 17677	MAC77
A. cephalotes Banks & Sol. (spreading hairy)	East Azerbaijan: Oshnavieh to Orumieh, after Movana, 1669 m, 26.6.2009, Ranjbar & Assadi	BASU 17678	MAC78
A. dipodurus Bunge	East Azerbaijan: Oshnavieh to Orumieh, 1669 m, 26.6.2009, Ranjbar & Assadi	BASU 17679	MAC79
A. octopus C. C. Towns.	Kordestan: Tazeh Abad to Sarpol-e Zahab, Dalaho mountain, 1315 m, 13.5.2008, Ranjbar & Assadi	BASU 17680	MAC80

Pollen morphology

Pollen samples were obtained from the materials collected during several excursions and prepared using the standard method described by Erdtman [15]. The pollen grains were then mounted on unstained glycerin jelly, and observations were made with a Nikon Type-2 microscope. The measurements were based on 25 readings from each specimen. Equatorial diameter (E), polar axis (P), colpus length (CL), colpus width in granule site (CG), colpus width in none granule site (CN), granule length (GL), granule diameter (GD) and shape index (P/E) were measured. Data were analyzed by MVSP software version 3.2, and the relationships between different taxa were studied. The terminology used here are according to Faegri [16].

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Cytogenetics

Chromosome number and meiotic behavior were analyzed in *A. dipodurus* and *A. octopus*. 15 flower buds from at least 5 plants at an appropriate stage of development were fixed in 96% ethanol, chloroform and propionic acid (6:3:2) for 24 h at room temperature, and then stored in 70% ethanol at 4°C until used. Anthers were squashed and stained with 2% acetocarmine. All slides were made permanent by the Venetian turpentine. Photographs of chromosomes were taken on an Olympus BX-41 photo-microscope at an initial magnification of X 1000. Chromosome counts were made from well-spread metaphases in intact cells, by direct observation and from photomicro-graphs.

Results and discussion

Morphology

PCO analysis based on morphological characters resulted in three groups. Populations MCA77 and MCA78 of *A. cephalotes* were included in group 1, *A. octopus* (MCA80) in group 2, and *A. dipodurus* (MCA79) in group 3 (Fig. 1). It seems that among the morphological characters studied here (Table 2), plant height, number of leaflet pairs, leaflet length, hair density on leaflet upper surface, hair length on leaflet lower surface, stipule length, stipule free portion length, inflorescence width, and hair length on calyx play decisive roles in differentiating taxa. Two populations of *A. cephalotes* (group 1) are separated from other groups by some morphological characters such as plant height, number of leaflet pairs, hair density on leaflet upper surface, stipule width, and stipule free portion length. However, the two populations can differentiate from each other by their leaf and leaflet indumentums. *A. octopus* differs from other taxa by its petiole length, number of leaflet pairs, leaflet length, hair density on leaflet upper surface, hair length on leaflet lower surface, hair density on leaflet lower surface, stipule length, stipule free portion length, inflorescence width, inflorescence length, and calyx length. *A. dipodurus* differs from other taxa by its plant

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height, leaflet form, number of leaflet pairs, hair density on leaflet upper surface, inflorescence width, calyx length and hair length on calyx.

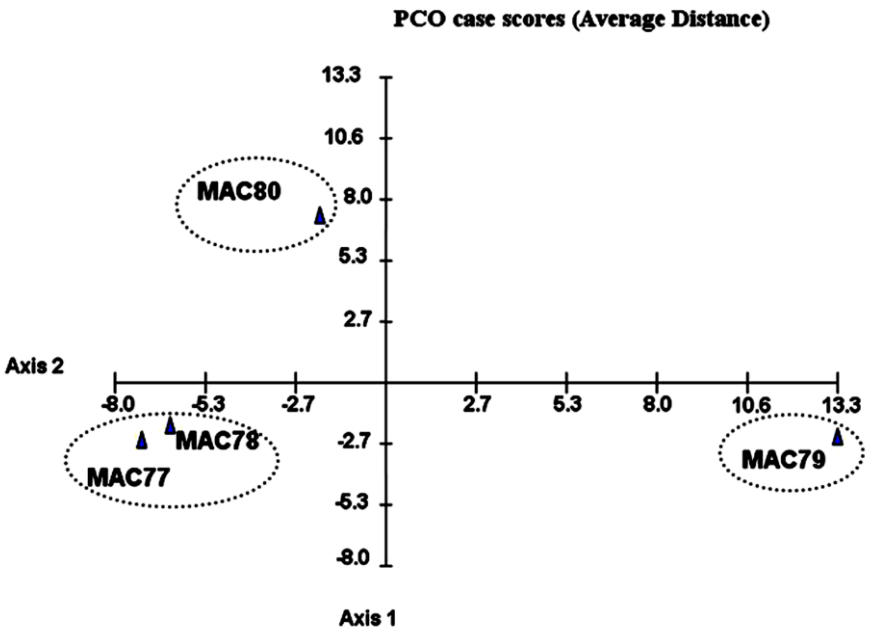

Fig. 1. PCO analysis of 4 taxa of *A*. sect. *Macrophyllium* based on morphological characters (abbreviations are as listed in Table 1).

Table 2. Morphological characters and character state matrix of 4 taxa of *A*. sect. *Macrophyllium*.

Morphological characters	MAC80	MAC77	MAC78	MAC79
Plant height (mm)	310	260	270	360
Leaf length (mm)	240	250	240	270
Petiole length (mm)	32	70	70	75
Number of leaflet pairs	9	10	10	12
Leaflet length (mm)	18.5	20	22	33
Leaflet width (mm)	11	10	10	10
Leaflet shape (Elliptic = 1, Oblong = 0)	0	0	0	1
Leaflet mucron length (mm)	1	1	2	2
Hair position on leaflet upper surface (Appressed = 1, Subappressed = 3)	3	3	1	1
Hair density on leaflet upper surface (Loose = 1, Sparse = 2)	2	0	0	1
Hair length on leaflet lower surface (mm)	0	0.8	0.5	0.7
Hair position on leaflet lower surface (Appressed = 1, Subappressed = 3)	3	3	1	1
Hair density on leaflet lower surface (Loose = 1, Glabrous = 0, sparse = 2)	0	2	2	11
Stipule length (mm)	17	23	21	21
Stipule width (mm)	16	14	14	18
Stipule free portion length (mm)	9	14	15	11
Stipule joined portion length (mm)	8	9	7	10
Inflorescence width (mm)	50	60	60	120
Inflorescence length (mm)	50	60	65	70
Calyx length (mm)	16	17	18	20
Calyx teeth length (mm)	6	9	6	11
Calyx tube length (mm)	10	10	12	10
Calyx width (mm)	6	7	6	6
Hair length on calyx (mm)	3	3	3	5

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Pollen morphology

Pollen grains in the studied taxa are large, sometimes medium, sized ranging from P = 37(40.4)42 µm, E = 31(33.8)38 µm to P = 31(33.86)37 µm, E = 25(28.93)33 µm. The smallest pollen grains belong to *A. octopus*, while the largest ones belong to *A. dipodurus* (Table 3). The pollen grains are prolate to subprolate and often protruding at the equator and tricolpate. The colpi are long, extending onto the poles with tapering ends, coarsely granulated membranes and with either smooth or ornamented margins (Fig. 2). The mean values and ranges of seven quantitative characters, which were useful in separating different populations, are given in Table 3. PCO analysis based on pollen morphology resulted in 3 groups (Fig. 3). Two populations of *A. cephalotes* (MAC77 and MAC78) were included in group 1; *A. octopus* in group 2; and *A. dipodurus* in group 3. *A. octopus* is far from the other taxa because of its small equatorial diameter, granule length, colpus width in none granule site and colpus length. *A. dipodurus* differs from the other taxa by its large polar axis, equatorial diameter, colpus width in granule and none granule sites, colpus length and granule diameter. On the other hand, with the exception of polar axis, all pollen characters show maximum minimum values in *A. dipodurus* and in *A. octopus*, respectively. Two populations of *A. cephalotes*, which show intermediate values similar in pollen size. Results from PCO analysis of different taxa based on pollen characters are in agreement with morphological grouping.

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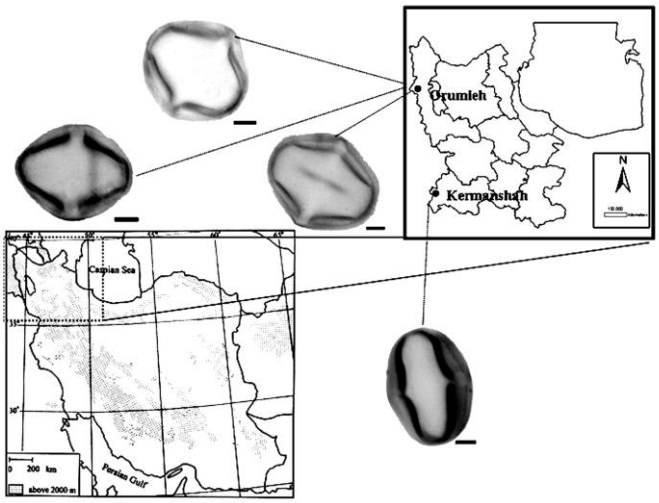

Fig. 2. Distribution and pollen photomicrographs of 4 taxa of *A*. sect. *Macrophyllium* in Iran. Scale bar = 6 µm.

Table 3. Pollen characteristics of 4 taxa of *A*. sect. *Macrophyllium*.

Taxa	P	E	GD	GL	CN	CG	CL
MAC80	35(34.53)42	23(27)31	2(2.93)5	3(3.13)5	10(9.73)13	20(22.2)27	29(30.4)35
MAC79	37(40.4)42	31(33.8)38	2(3.53)5	4(4.86)8	13(17.06)18	30(31.2)37	35(37.2)38
MAC78	33(34.8)38	25(29.86)35	3(4.13)6	3(4.8)5	11(12.6)15	23(26.13)30	30(32.8)35
MAC77	31(33.86)37	25(28.93)33	3(3.53)5	3(3.66)5	10(11.66)14	22(25.93)28	28(30.53)35

Abbreviations: E: Equatorial diameter; P: Polar axis; CL: Colpus length; CG: Colpus width in granule site; CN: Colpus width in none granule site; GL: Granule length; GD: Granule diameter; P/E: Shape index.

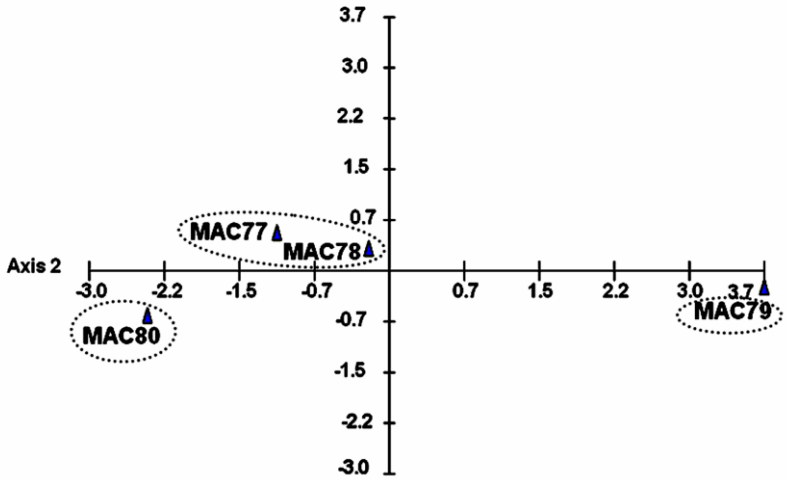

Fig. 3. PCO analysis of 4 taxa of *A*. sect. *Macrophyllium* based on pollen morphological characters (abbreviations are as listed in Table 1).

Cytogenetic study

Data with regard to meiotic chromosome number, meiotic stages, as well as abnormalities observed in each stage for *A. dipodurus* (MAC79) and *A. octopus*

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(MAC80) are presented in Table 4. A total of 2224 diakinesis/metaphases I (D/MI), 2182 anaphase I/telophase I (AI/TI), 17 methaphase II (MII), and 1808 anaphase II/telophase II (AII/TII) cells were analyzed. Both species are tetraploid and possess a $2n = 4x = 32$ chromosome number. The meiotic irregularities observed in different taxa included chromosome stickiness resulting in bridges, the occurrence of laggard chromosomes, formation of micronuclei in tetrad cells, multipolar cells and cytomixis, which are discussed bellow in detail.

Table 4. Number of pollen mother cells (PMCs) analyzed and the percentage of PMCs meiotic behavior in 4 taxa of *A*. sect. *Macrophyllium*

Meiotic characters	*A. octopus*	*A. dipodurus*
Cell number	2565	3666
D/MI	980	1244
% D/MI	38.20	33.93
% Cytomixis	1.73	0.40
% Chromosome stickiness	0	0.96
% Fragmented chromosome	0.30	2.89
AI/TI	1074	1108
% AI/TI	41.87	30.22
% Laggard chromosome	0.65	0.11
% Fragmented chromosome	0.27	0.09
% Asynchronous nucleus	1.86	1.53
% Bridge	0	0.09
% Micronucleus	0.18	0.75
% Cytomixis	1.21	0.65
MII	9	8
% MII	0	0.21
AII/TII	502	1306
% AII/TII	19.57	35.62
% Pentapolar cell	0.19	0.07
% Hexapolar cell	0	0.07
% Micronucleus	0	0.22
% Cytomixis	2.98	2.52
% Bridge	0	0.15
% Chromosome stickiness	0	0.07
n	16	16

Abbreviations: D/MI: Diakinesis/Metaphase I; AI/TI: Anaphase I/Telophase I; MII: Metaphase II; AII/TII: Anaphase II/Telophase II; *n* = Chromosome number.

Laggards and fragmented chromosomes

Fragmented chromosomes, being unable to orient at the metaphase plate, were observed during metaphase I or metaphase II (Fig. 4H). The highest frequency of fragmented chromosomes of metaphase I cells was observed in *A. dipodurus*. Laggard chromosomes were observed during anaphase I in *A. dipodurus* and *A. octopus* (Table 4,

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Figs. 4K & 4L). According to Nicklas and Ward [32], non-oriented bivalents may be related to impaired attachment of kinetochores to the spindle fibers. Pagliarini [33] reported that laggards may result from late chiasma terminalization [51]. These laggards might have degenerated or may have resulted in the formation of polyads, particularly at the resting phase [6].

Cytomixis

Cytomixis was first observed by Gates [21], in which cytoplasmic channels were formed between cells and through which chromatin material can migrate from one cell to another. The phenomenon was reported to occur in pollen mother cells at a higher frequency, although it was observed rarely between mitotic cells [8, 46] or even between meiotic and mitotic cells [12]. To date cytomixis has been studied in a variety of angiosperm taxa [3], [5], [11], [17], [30], [33], [44], [45], [47], [52]-[54]. Cytomixis in PMCs during meiosis was proposed by Falistocco et al. [17] as an origin for polyploid plants in a diploid population of *Dactylis*. In this respect, it is worth mentioning that the mechanisms leading to changes in gametic chromosome number are widely recognized as tools playing an important evolutionary role in the plant kingdom. Among these mechanisms is the alteration in the genetic mechanism controlling homologous chromosome pairing in meiosis. Similarly, cytomixis, which is principally a type of meiotic abnormality resulting in changes in gametic chromosome number through migration of chromosomes between adjacent PMCs, could be considered as a process of evolutionary significance in plant populations. This deduction agrees with conclusions reached by Zheng et al. [54], Falistocco et al. [17] and Morikawa and Leggett [30]. In agreement with de Souza and Pagliarini [52], we found that cytomixis occurred in normal diploid species and not just in genetically unbalanced individuals such as haploids, polyploids, hybrids or apomicts as assumed by other authors [3], [5], [13]. It is clear that there is need for further research on cytomixis to solve this controversy. No single stick-like chromosome was observed to migrate in a broad channel. The cytomixis was not limited to certain stages. It occurred even at prophase I.

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This is consistent with the investigations on some angiosperm taxa [51]. Moreover, cytologically, physiologically and biochemically imbalanced plants like haploids, triploids, aneuploids and apomicts show cytomixis more often than normal cytogenetically balanced and established plants [53]. It was assumed that this phenomenon is of an evolutionary importance, as it leads to aneuploidy and polyploidy of the gametes. The present study emphasized the investigation of unusual microsporogenesis, especially the cytomixis in *A.* sect. *Macrophyllium*. Cytomixis was the most conspicuous chromosomal abnormality occurring in *A.* sect. *Macrophyllium.* A very high percentage of cytomixis was obtained in *A. dipodurus* in comparison to many other taxa of the genus *Astragalus*, which were analyzed (36, 38, 42), including *A. octopus* in the present study. It occurred between two neighboring cells of the same meiotic phase. At prophase I (Fig. 5E), these cytoplasmic connections were visible. The cytoplasmic channels appeared to be narrow and stretched in most cases. In most cases, the chromatin material connections between two microspore mother cells were maintained by a fine chromatin fiber. Even when the kidney-shaped microspores were formed, such connections still remained. Finally, they disappeared gradually when the fertile microspores were formed, metaphase I (Fig. 5F), telophase I (Figs. 5D & 5H) and telophase II (Figs. 5C, 5I, 5J & 5K).

Multipolar cells

The spindle apparatus is normally bipolar and acts as a single unit, playing a crucial role in chromosome alignment during metaphase. Any distortion or breakage in the spindle may result in random sub-grouping of the chromosome [31]. Pentapolar cells were observed in *A. dipodurus* and *A. octopus* (Fig. 4O). Such cells may lead to the formation of abnormal tetrads and infertile pollen grains.

Micronucleus

Chromosomes that produced micronuclei during meiosis were eliminated from microspores as microcytes. The micronucleus reached the microspore wall and formed a

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kind of bud, separated from the microspore. The eliminated microcytes gave rise to small and sterile pollen grains [4]. Micronuclei were seen in both species during anaphase I/telophase I and only in *A. dipodurus* during anaphase II/telophase II (Table 4), with a higher percentage in *A. dipodurus* (Fig. 4M).

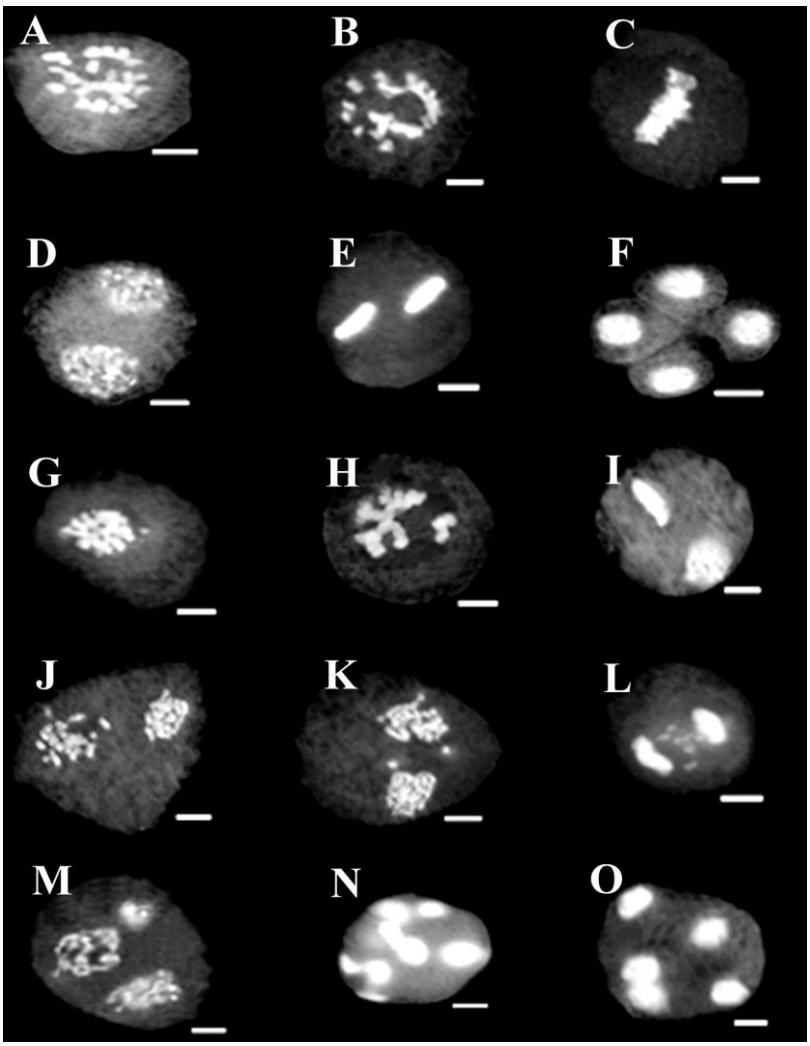

Fig. 4. Representative meiotic cells in two species of *A.* sect. *Macrophyllum*. A: Diakinesis in *A. octopus*; B: Chromosome stickiness in *A. dipodurus*; C: Metaphase I in *A. dipodurus*; D: Telophase I in *A. octopus*; E: Metaphase II in *A. octopus*; F: Telophase II in *A. dipodurus*; G, Metaphase I with fragmented chromosomes in *A. octopus*, H: Metaphase I with fragmented chromosomes in *A. dipodurus*; I: Asynchronous nuclei in *A. dipodurus*, J: Asynchronous nuclei in *A. octopus*; K: Laggards in *A. octopus*, L: Laggards in *A. dipodurus*; M: Micronucleus in *A. dipodurus*; N: Pentapolar cell in *A. octopus*; O: Pentapolar cell in *A. dipodurus*. Scale bar = 6 µm.

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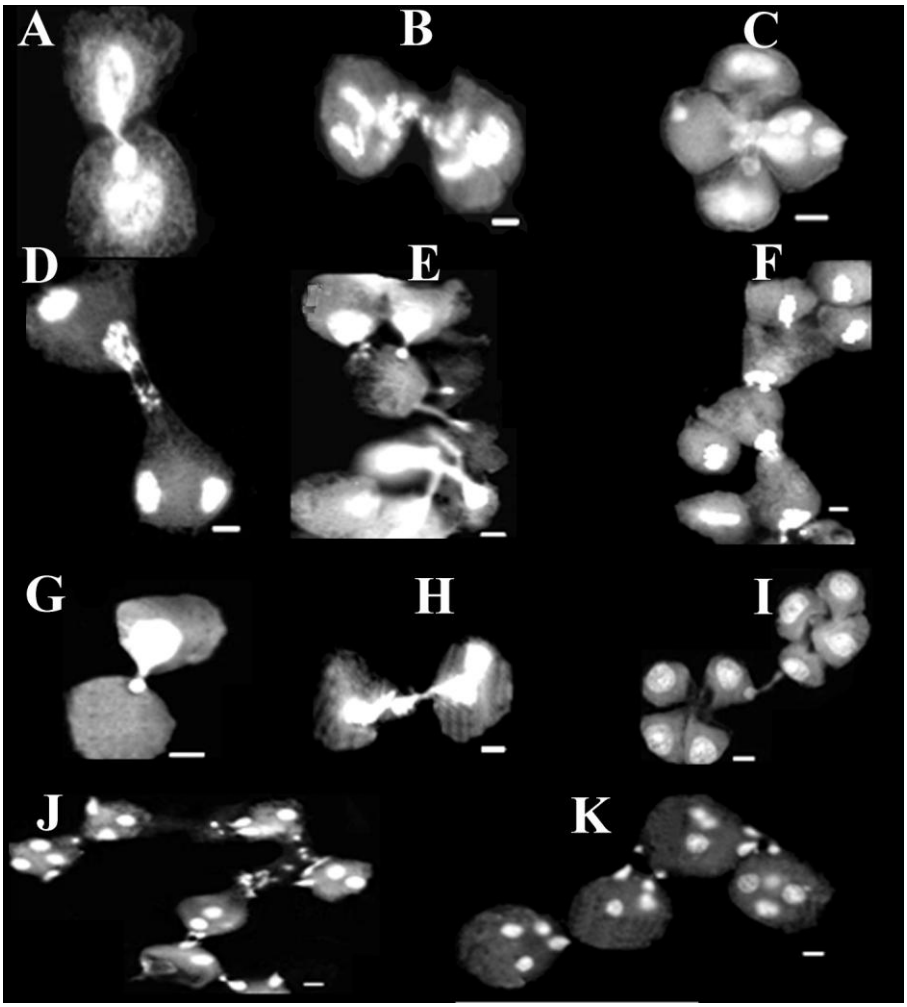

Fig. 5. Cytomixis in two species of *A*. sect. *Macrophyllum*. A, E, G, H, K: Cytomixis in *A. dipodurus*; B, C, D, F, I, J: Cytomixis in *A. octopus*. Scale bar = 6 µm.

Chromosome bridges

Chromosome bridges resulting from stickiness were only observed in *A. dipodurus* at anaphase II (Table 4). The number of chromosomes involved in their formation varied among different meiocytes. Genetics as well as environmental factors have been considered as reasons for chromosome stickiness in different plant species [31].

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