Genetic variation within and among four species of *Lotus* L. (Fabaceae)

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Abstract

Karyotype characters, mitotic metaphase chromosomes, monoploid idiograms, karyograms and pollen abortion of *Lotus creticus* L., *Lotus glaber* Mill., *Lotus halophilus* Boiss. and *Lotus peregrinus* L. were investigated. Analysis of somatic metaphases showed that the chromosome numbers of these taxa were 2n=28 for *L. creticus*, 2n=12 for *L. glaber*, 2n= 14 for both *L. halophilus* and *L. peregrinus*. Karyotype asymmetry was estimated by many different methods, the total form percent (TF %), the Rec and Syi indices, the intrachromosomal asymmetry index (A1) and interchromosomal asymmetry index (A2). In this study, there is a variation in the frequency of pollen fertility and abortive pollen grains of the *Lotus* species. The anatomical characteristics of leaf and stem of *Lotus* species were made. Various genetic markers have been used to estimate the genetic variability within and among the four *Lotus* species collected from ten different accessions. Protein profile of *Lotus* species were studied by polyacrylamide gel electrophoresis (SDS-PAGE) technique and generated seventeen bands, six of them monomorphic and eleven polymorphic bands. Five tested primers were used for RAPD technique, a total of 33 well-defined and scorable RAPD bands were obtained as a result of fingerprinting of *Lotus* species, 12 bands were common for all species and 21 were polymorphic. Perceptual mapping (PERMAP) used for the combination of taxa and attributes PERMAP-Biplot distinguished the taxa into three groups.

Keywords: *Lotus* species, karyotype, idiogram, karyotype asymmetry, genetic variation, RAPD, SDS-PAGE, anatomy.

1.Introduction

Fabaceae (Leguminoseae) is the third largest family of the flowering plants **(Mabberley, 1997),** consisting of about 463 genera and 18000 species **(Sprent, 1999).**

Lotus is a genus of an important forage legume and its members are adapted to a wide range of habitats, from coastal environment to high altitudes **(Mariana *et al.*, 2003).**. *Lotus* comprises about 100 annual and perennial species growing widely through out the world **(Polhill, 1994 a, b).** *Lotus* species are mainly distributed in the Mediterranean and NW of America **(Polhill and Raven, 1981). (Boulos, 1999**) reported that genus *Lotus* L. found in both old and new world. The old world species are distributed around the Mediterranean,

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up to Arctic and down to Nile through Ethiopia and East Africa to South Africa and Western Asia.

Cytogenetics played an essential role in studies of chromosome structure, behavior and evolution in numerous plant species. However, many advances were made in the cytogenetic mapping of crop plants **(Zhang *et al.*, 2007).** Cytogenetic studies are also related in the study of plant evolution and diversification **(Mousavi *et al.*, 2013).**

The chromosome number and the chromosome morphology are increasingly used in plant taxonomy. These data are also important to elucidate the origin, speciation and phylogenetic relationships of plants **(Cai *et al.*, 2004 & Pavlova and Tosheva, 2005).** Many karyological investigations have been performed over the last decades and provide fundamental characters for plant systematics and evolutionary analysis **(Stace, 2000).**

Karyological data can show variation in absolute and relative chromosome size, in chromosome morphology and in staining properties of the chromosomes **(Sharma and Sen 2002).** The description of the chromosome morphology has proved to be a powerful method to characterize genomes. Chromosome data provide essential information for various fields, as illustrated by the "taxonomic importance of karyology at the generic and sub generic level in Geraniaceae **(Albers and Van der Walt, 2007),** in *Pancratium* **(Soliman *et al*., 2012)** and *Launaea* **(Soliman *et al*., 2014a).** Karyological data may also help to interpret results from molecular studies **(Crawford *et al.*, 2005 and Horandle *et al.*, 2005).**

Pollen viability is an important factor for plant genetic variability, especially for those plants in which cross fertilization is prevails over self fertilization **(Divakara *et al.*, 2010).** The capability of germination of pollen depends on many factors such as nutrition conditions of species, varieties used and the environmental factors **(Khan and Perveen, 2008).**

Many evolutionary and taxonomically problems of several plants have been successfully resolved by seed proteins patterns **(Shah *et al.*, 2011 and Emre, 2011).** Seed storage protein electrophoretic is also valuable evidence in cultivar identification and wild species phylogeny studies **(EL – Rabey, 2008).**

The application of the molecular genetics methods for assessing the genetic diversity has been increased. These methods have been applied to increase our knowledge of the extent of genetic variation within and among species **(Mondini *et al.*, 2009 and Soliman *et al*., 2014b).** Molecular methods such as DNA analysis giving high indication of genetic variation

within species without interference of environmental factors **(Karp *et al.*, 1996 and Mondini *et al.*, 2009).** The detection of polymorphism in closely related species can be achieved by Random Amplified Polymorphic DNA (RAPD) technique **(Williams *et al.*, 1990).**

The anatomical structures are useful in taxonomic characterization of plants. The distribution and arrangement of cells and tissues such as sclerenchyma, vascular bundles and other anatomical features have been reported and utilized at different systematic levels for delimitation of taxa **(Agbagwa and Ndukwu, 2004; Ajuru, 2012).**

The first target of genetic conservation studies is to estimate the level of genetic variability in considered species. Genetic data may play a significant role in the formulation of appropriate management strategies for the conservation of the species **(Cardoso *et al.*, 1998).**

More studies are required on the Egyptian plant genetic resources to characterize their genetic variation. Consequently, genotypes should be properly characterized cytogenetic evaluated and documented using standard methods. Studying the evolutionary relationship between *Lotus* L. species needs work to assess their genetic relatedness.

The aim of the present investigation is to assess the genetic variation within and among four *Lotus* L. species growing in Egypt through cytological, biochemical, molecular and anatomical studies and its impact on conservation strategies of gene banks.

2. Materials and Methods

Plant materials:

In the present study, ten accessions representing four selected species of *Lotus* L. were examined. The selected four plant species were identified by Prof. Dr. Ibrahim A. Mashaly Professor of Plant Ecology and Flora, Botany Department, Faculty of Science, Mansoura University, and the herbarium specimens were deposited in the herbarium of Botany Department, Faculty of Science, Mansoura University, Egypt. Identification and nomenclature were according to **Tackholm (1974)** and up to date by **Boulos (1999)** as shown in Table (1).

Table (1): The studied accessions of selected plant species of genus *Lotus* L.

Species	Code	Locality	Habitat type
Lotus creticus L.	Lc1	El-Beihera Governorate (Idku District)	Sand dunes
Lotus creticus L.	Lc2	Kafr El-Sheikh Governorate (Motobas District)	Sand flats
Lotus glaber Mill.	Lg1	Kafr El-sheikh Governorate (Motobas District)	Canal banks
Lotus glaber Mill.	Lg2	El-Dakahlyia Governorate (Gamasa District)	Road sides
Lotus glaber Mill.	Lg3	El-Dakahlyia Governorate (Mansoura District)	Cultivated lands
Lotus halophilus Boiss.	Lh1	Kafr El-sheikh Governorate(EL-Borollus District)	Sand dunes
Lotus halophilus Boiss.	Lh2	Kafr El-sheikh Governorate(El-Shahabyia District)	Sand flats
Lotus halophilus Boiss.	Lh3	El-Dakahlyia Governorate(Gamasa District)	Sand dunes
Lotus peregrinus L.	Lp1	El-Beihera Governorate(Rashid District)	Sand flats
Lotus peregrinus L.	Lp2	El-Beihera Governorate (Idku District)	Sand dunes

Cytological analysis:

The cytological investigation followed **Chattopadhyay and Sharma (1988).** At least three metaphases were drawn for each accession. The nomenclature system used for the description of the chromosome morphology is that proposed by **Abraham and Prasad (1983).** The abbreviations nsm (-), nsm (+) and nm designate nearly submetacentric (-), nearly sub metacentric (+) and nearly metacentric respectively. Karyotype analysis was carried out using "Micro measure" Computer Program **(Reeves, 2001).**

Pollen abortion assay:

The collected young, closed flowers and flower buds from a sufficient number of randomly selected individual plants of the four species of *Lotus* were collected and fixed in a fresh mixture of absolute alcohol, chloroform and glacial acetic acid (6:3:1). The flowers were removed from the fixing solution, washed and the anthers excised. Anthers were stained and squashed in 4% alcoholic hydrochloric acid carmine **(Snow, 1963).** The large and stained pollens were counted as fertile, but the small and unstained were counted as sterile.

Protein- Polyacrylamide gel electrophoresis (SDS-PAGE):

The seeds of the four species of *Lotus* collected from ten different accessions were used to study their protein profiles. Therefore sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used according to the method of **Laemmli (1970).**

DNA isolation and RAPD analysis:

From each plant sample, 1 gm of young and fresh leaves was used for DNA extraction following the Dellaporta method **(Dellaporta *et al.*, 1983).** Five random primers listed in Table (2) were applied. PCR reaction was conducted according to **Williams *et al.*, (1990).** The gels of DNA were visualized and photographed by gel documentation system (Gel- Doc Bio-Rad 2000) under UV transilluminator. The RAPD bands were scored as present (1) or absent (0), each of which was treated as independent character regardless of its intensity. The levels of polymorphism were calculated by dividing the polymorphic bands by the total number of scored bands.

Table (2): Primers used in RAPD technique and their sequences.

Primer name	Sequence
OPA 04	5¯-AATCGGGCTG-3¯
OPA 09	5¯-GGGTAACGCC-3¯
OPA 10	5¯-GTGATCGCAG-3¯
OPA 13	5¯-CAGCACCCAC-3¯
OPA 17	5¯-GACCGCTTGT-3¯

Anatomical studies:

For anatomical investigation, samples of stem and leaves of *Lotus* L. were fixed in formalin-acetic acid-alcohol (FAA). After fixation, specimens were dehydrated in tertiary butyl alcohol series and embedded in paraffin wax according to **Johansen (1940),** and adopted by **Jensen (1962)** and **Peacock and Bradbury (1973).** Sections of 10-12 micron thick were made using a rotatory microtome and double stained with safranin and fast green, mounted in canada balsam and examined by a full automatic Olympus microscope.

Data analysis:

Data analysis was performed using the SYSTAT version 7.0 program **Wilkinson (1997).** Regarding electrophoresis techniques of SDS-PAGE and DNA- RAPD, The presence or absence of each band was treated as a binary character in a data matrix (coded 1 and 0 respectively) and the data represented by polymorphism percentage.

3. Results and Discussion

Karyotype analysis:

The karyotype analysis is an essential method of study of plants chromosome, which shows vital significance to study the origin, the evolution and the classification **(Yang *et al.*, 2006).** The karyotype analysis of plant chromosomes has various applications in plant systematics, cultivar identification and breeding purposes **(Fregonezi *et al.*, 2006 and Chengqi, 2008).** It includes the measurements of chromosomes such as the total chromosome length, chromosome area, centromeric position, presence of satellites and long/short arm ratio **(Fukui, 1988).**

Twenty-eight chromosomes were observed in somatic cells of *Lotus creticus*, twelve chromosomes were detected in *Lotus glaber* and fourteen chromosomes in *Lotus halophilus* and *Lotus peregrinus* Plate (1).

Karyotype analysis includes chromosomes number, arm ratio, centromeric index, diploid complement length (DCL), mean chromosome length (MCL), total chromosome volume (TCV), symmetry percent (S%), total form percentage (TF%), intrachromosomal asymmetry index (A1), interchromosomal asymmetry index (A2), the symmetric indices (Syi), resemblance between chromosomes (Rec), as well as karyotype formulae of all species are recorded in Table (3) for *Lotus* species. The karyotype analysis showed differences in chromosome morphology. The following chromosome types are identified: nsm (+): chromosome nearly submetacentric (+), nsm (-): chromosome nearly submetacentric(-), nm: chromosome nearly metacentric .

Karyogram of *Lotus* species is illustrated in Plate (2) as well as idiogram of haploid complement Fig. (1) based on chromosome measurement. The results of karyotype analysis of studied *Lotus* species collected from different accessions are as follow:

Lotus creticus

The chromosome numbers in Lc1 and Lc2 were diploid. It showed a chromosome number 2n= 28 Plate (1 A and B). Most previous records reported 2n=28 by **(Valds-Bermejo, 1980& Grant 1995 and IPCN 2013)** confirmed our investigation. The karyotype formula was three pairs nearly submetacentric (-), eleven pairs nearly metacentric for Lc1 and five pairs nearly submetacentric (-), nine pairs nearly metacentric for Lc2.

Lotus glaber

The chromosome numbers in Lg1, Lg2 and Lg3 observed were 2n= 12 Plate (1 C, D and E). This count agrees with the previous counts performed on this species by **(Grant, 1995 and IPCN 2013).** The karyotype formula for Lg1 was: two pairs nearly submetacentric (-), four pairs nearly metacentric. The karyotype formula for Lg2 is: one pair nearly submetacentric (-), five pairs nearly metacentric and the karyotype formula for Lg3 is: two pair nearly submetacentric (-), four pairs nearly metacentric.

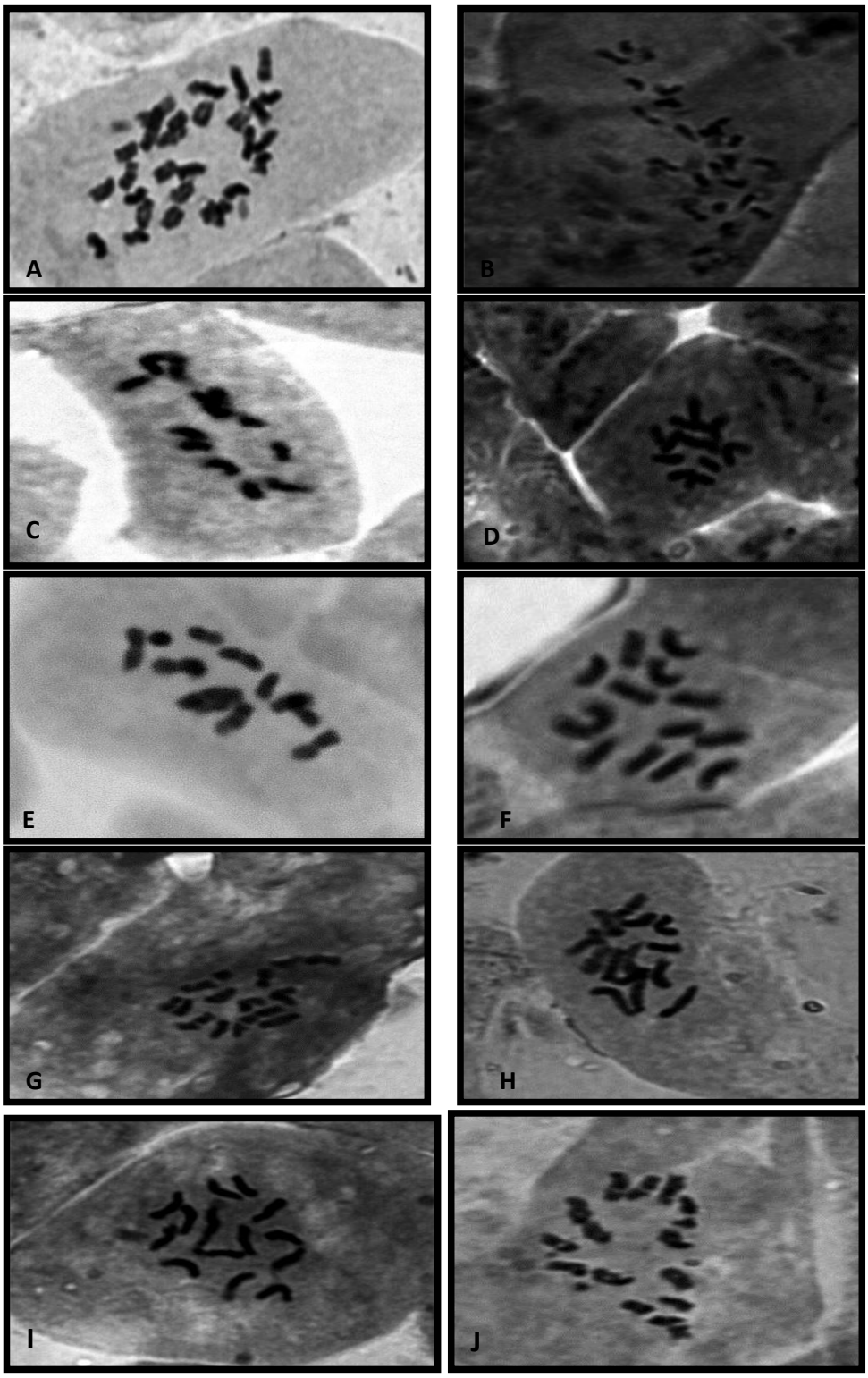
Lotus halophilus

Lh1, Lh2 and Lh3 showed a chromosome number of 2n= 14 Plate (1 F, G and H). This count agrees with previous counts by **(Bartolo *et al.*, 1979 & Grant, 1995 and IPCN 2013)**. The karyotype formula was: one pair nearly submetacentric (+), three pairs nearly submetacentric (-), three pairs nearly metacentric for Lh1. Two pairs nearly submetacentric (-), five pairs nearly metacentric for Lh2 and four pairs nearly submetacentric (-), three pairs nearly metacentric for Lh3.

Lotus peregrinus

The chromosome numbers observed in Lp1 and Lp2 were 2n= 14 Plate (1 I and J). This count agrees with previous counts by (**Gasmanova *et al.*, 2007). (Bartolo *et al.*, 1979 and Grant 1995)** reported that 2n= 28 for *L. peregrinus* which do not agree with our results, the observed variation in chromosome number as compared with other records may be due to two factors : different phytochoria and chromosomal structural alterations that might have taken place during speciation. The karyotype formula for Lp1 was: one pairs nearly submetacentric (+), four pairs nearly submetacentric (-), two pairs nearly metacentric and the formula for Lp2 was: five pairs nearly submetacentric (-) and two pairs nearly metacentric.

Karyotypic variations exist not only between different species but also within the same species. **Oyewole (1988)** stated that, karyotype variation in *Pancratium hirtum* is associated with population differentiation and ecological preference. Also karyotype differentiation involves at least chromosome breakage, presence of accessory chromosomes and differences in the total length of chromatin material per nucleus **(Soliman *et al.*, 2010)** and **(Soliman *et al.*, 2014c).**


A
B
C
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E
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J

Plate (1): Showing somatic chromosomes of four species of *Lotus* L. collected from ten different accessions in Egypt. (A and B) *Lotus creticus*, (C-E) *Lotus glaber*, (F-H) *Lotus halophilus* and (I and J) *Lotus peregrinus*, X=1000.

Table (3): Karyotype parameters of *Lotus* species collected from ten different accessions.

Codes	2n	DCL	MCL	TCV	A1	A2	S%	TF%	Syi Index	Rec Index	Karyotype formula
Lc1	28	355.46	12.70	7790.689	0.254	0.190	0.242	41.51	0.417	0.697	6nsm(-) +22nm
Lc2	28	356.86	12.745	16755.08	0.320	0.245	0.262	39.362	0.399	0.575	10nsm(-)+18nm
Lg1	12	360.27	30.023	34478.31	0.247	0.225	0.299	42.13	0.415	0.727	4nsm(-)+ 8nm
Lg2	12	141.179	11.76	4641	0.380	0.218	0.361	43.61	0.439	0.836	2nsm(-)+ 10nm
Lg3	12	190.901	15.908	8304.838	0.246	0.225	0.277	42.5	0.419	0.783	4nsm(-)+8nm
Lh1	14	148.67	10.619	2915	0.428	0.102	0.244	35.82	0.357	0.854	2nsm(+)+6nsm(-)+ 6nm
Lh2	14	172.341	12.310	3349.26	0.282	0.179	0.287	41.14	0.412	0.766	4nsm(-)+10nm
Lh3	14	258.710	18.479	7850.41	0.426	0.194	0.216	36.30	0.366	0.766	8nsm(-)+ 6nm
Lp1	14	243.951	17.425	4998.61	0.451	0.086	0.260	33.62	0.337	0.896	2nsm(+) + 8 nsm(-) + 4nm
Lp2	14	221.65	15.832	6987.33	0.408	0.149	0.289	36.39	0.369	0.776	10nsm(-)+4nm

2n : Diploid somatic chromosome number. TF%: Total form percentage.

DCL: Diploid complement length. A1: Intrachromosomal asymmetry index

MCL: Mean chromosome length. A2: Interchromosomal asymmetry index

TCV: Total chromosome volume. Syi: The symmetric index.

S%: Symmetry percent. Rec: Resemblance between chromosomes

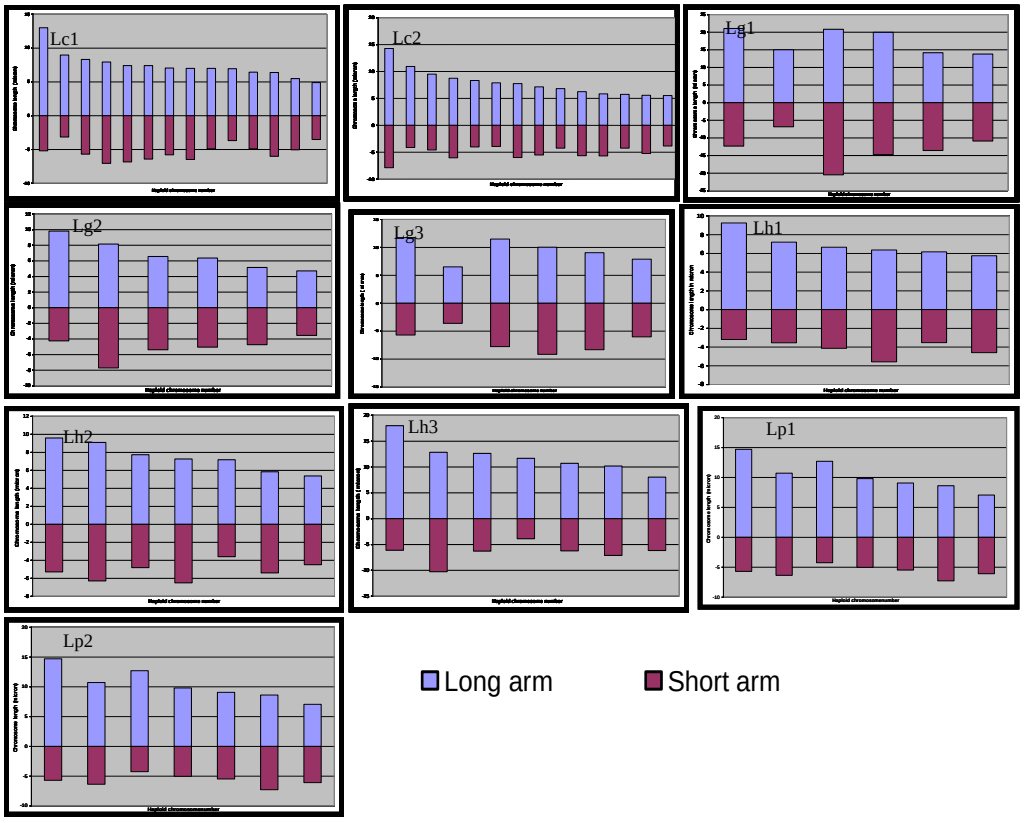

fig. (1): Idiogrammatic representation of haploid karyotype of *Lotus* species collected from different accessions. (For accession name see table 1), (Lc1, Lc2) *Lotus creticus* (Lg1, Lg2 and Lg3) *Lotus glaber* (Lh1, Lh2 and Lh3) *Lotus halophilus* and (Lp1 and Lp2) *Lotus peregrinus*.

Plate (2): Karyogram of somatic chromosomes of four species of *Lotus* L. collected from ten different accessions in Egypt. (For accession name see table 1), Bar = 26.5 µ, (Lc1, Lc2) *Lotus creticus* (Lg1, Lg2 and Lg3) *Lotus glaber* (Lh1, Lh2 and Lh3) *Lotus halophilus* and (Lp1 and Lp2) *Lotus peregrinus*.

Pollen abortion assay:

An important component in the reproductive success is the quality and the quantity of the pollen produced by the plant, pollen quality can be determined by pollen viability **(Dafni and Firmage, 2000).** The behavior of the chromosomes at meiosis affects pollen viability. If the meiosis is regular, the chromosomes pairs and segregate normally, the sterility of pollen grain is not expected to occur because of cytological reasons **(Boff and Schifino- Wittmann, 2002).** The pollen age and the exposure to the environmental stresses such as temperature and humidity are non genetic causes of decline the pollen viability **(Kelly *et al.*, 2002).**

The basic criteria of establishing abortion of pollen grains were the staining deficiency where, non stain were counted as sterile, while the stainable pollen grains were classified as fertile. As shown in Plate (3). The obtained data in Table (4) indicate the percentage of pollen fertility and abortive pollen in *Lotus* species.

It is clear that, concerning all accessions of *Lotus* species, *L. creticus* (Lc2) collected from Motobas District shown the highest percentage of pollen fertility was (92%) and the lowest percentage of abortive pollen was (8%).

Regarding *L. glaber*, (Lg2) collected from Gamasa District shown the highest percentage of fertility was (80%), and also the lowest pollen abortive percentage was (20%). Regarding *L. halophilus*, (Lh1) which collected from Borollus District shown the highest percentage of fertility was (61%), and the lowest pollen abortive percentage was (39%). Regarding *L. peregrinus*, (Lp1) which collected from Rashid District shown the highest percentage of fertility was (61%), and the lowest pollen abortive percentage was (39%).

Table (4): The percentage of pollen fertility and abortive pollen grains of *Lotus* species collected from ten different accessions.

Species		No. of Stained pollen	No. of Non stained pollen	% of pollen fertility	% of abortive pollen
Lotus creticus	Lc1	49	43	53%	47%
	Lc2	203	17	92%	8%
Lotus glaber	Lg1	29	20	59%	41%
	Lg2	110	27	80%	20%
	Lg3	326	140	70%	30%
Lotus halophilus	Lh1	44	28	61%	39%
	Lh2	55	42	57%	43%
	Lh3	33	24	58%	42%
Lotus peregrinus	Lp1	70	45	61%	39%
	Lp2	23	15	60.5%	39.5%

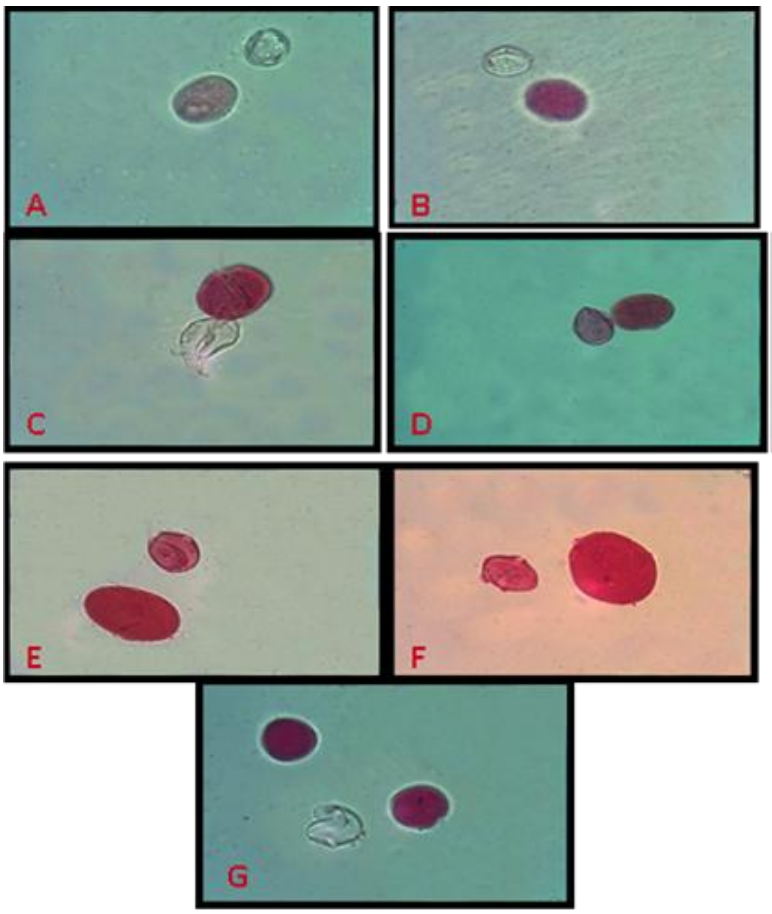

Plate (3) (A-G): Showing the types of pollen grains of four species of *Lotus* collected from ten different accessions, fertile pollen (stained or large) and sterile (unstained or small), X=40.

Seed protein analysis:

The genetic variation among the accessions of the wild species can be assessed successfully by the qualitative traits of the seed proteins obtained by electrophoresis **(Elham *et al.*, 2010 and Vishwanath *et al.*, 2011).** The genetic diversity within and between different plant species was determined by seed storage protein using Sodium dodecyl sulphate polyacrylamide gel electrophoresis **(Arsalan and Ertugrul, 2010 and Rdawan *et al.*, 2013).**

The electrophoretic analysis of total seed protein extracts using discontinuous SDS-PAGE gel for the ten collected samples of *Lotus* species, are shown in Table (5). The scanning of SDS-PAGE gel of the species is shown in Fig. (2).

Seventeen bands in *Lotus* spp. are distinguished from the scanning of seed protein gel using Doc 2000, Bio Rad Densitometer scanner. The protein profile generated six monomorphic bands, eleven polymorphic bands one of them unique band and the other ten are non-unique bands. Relationship among these taxa is based on the variation in the banding profile so that the percentage of polymorphism was 64.71% among the different species of *Lotus.* While, the percentage of polymorphism was 29.4% within *Lotus creticus*, 50% within *Lotus glaber*, 53.8 within *Lotus halophilus* and 8.33 within *Lotus peregrinus*.

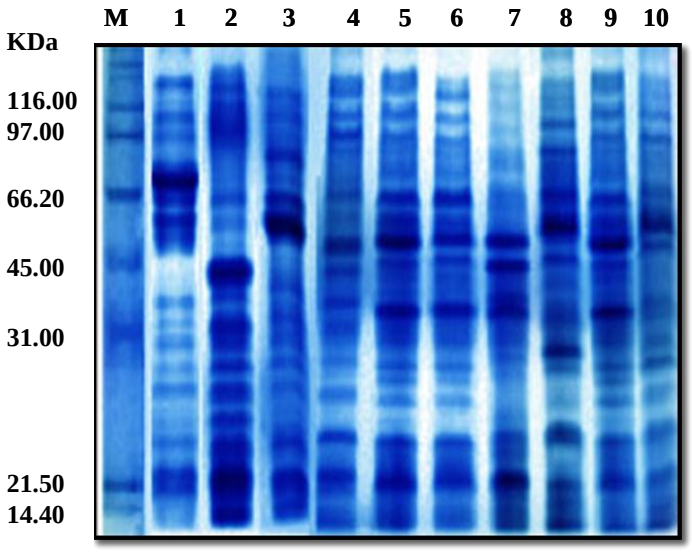

Fig. (2): SDS-PAGE of protein banding patterns of four *Lotus* L. species collected from ten different accessions.

1= (Lc1) *Lotus creticus* collected from Idku District (EL-Beihera Governorate)
2= (Lc2) Lotus *creticus* collected from Motobas District (Kafr el-Sheikh Governorate)
3= (Lg1) *Lotus glaber* collected from Motobas District (Kafr El-Sheikh Governorate)
4= (Lg2) *Lotus glaber* collected from Gamasa District (EL-Dakahlyia Governorate)
5= (Lg3) *Lotus glaber* collected from Mansoura District (EL-Dakahlyia Governorate)
6= (Lh1) *Lotus halophilus* collected from EL-Borollus District (Kafr El-Sheikh Governorate)
7= (Lh2) *Lotus halophilus* collected from EL-Shahabyia District (Kafr El-Sheikh Governorate)
8= (Lh3) *Lotus halophilus* collected from Gamasa District (EL-Dakahlyia Governorate)
9= (Lp1) *Lotus peregrinus* collected from Rashid District (EL-Beihera Governorate)
10= (Lp2) *Lotus peregrinus* collected from Idku District (EL-Beihera Governorate)

Table (5): Seed protein attributes of four species of *Lotus* collected from ten different accessions.

No.	Codes / KDa	*Lotus creticus*		*Lotus glaber*			*Lotus halophilus*			*Lotus peregrinus*	
		Lc1	Lc2	Lg1	Lg2	Lg3	Lh1	Lh2	Lh3	Lp1	Lp2
1	110.3	1	1	1	1	1	1	1	1	1	1
2	75.6	1	1	1	1	1	1	1	1	1	1
3	47.2	1	1	1	1	1	1	1	1	1	1
4	41.5	1	0	1	0	0	0	0	1	0	0
5	35.6	1	1	1	1	1	1	0	1	1	1
6	29.1	1	1	1	0	1	1	0	1	1	1
7	28.5	0	1	0	1	0	0	1	1	1	0
8	27.3	1	1	1	1	0	0	1	0	1	1
9	26.1	1	1	1	1	0	0	0	0	0	0
10	25.3	1	1	1	1	0	0	0	0	0	0
11	23.7	1	1	1	1	1	1	1	1	1	1
12	21.6	1	1	1	1	1	1	0	0	1	1
13	20.5	0	1	1	0	0	0	0	0	0	0
14	18.4	1	1	1	1	1	1	1	1	1	1
15	16.2	1	1	1	1	1	1	1	1	1	1
16	15.4	1	0	0	0	0	0	0	0	0	0
17	13.5	0	1	0	1	1	0	0	1	1	1
Total bands		14	15	14	13	10	9	8	11	12	11
% polymorphism among species		64.71									
Total bands		17		16			13			12	
No. of monomorphic		12		8			6			11	
No. of polymorphic		5		8			7			1	
% polymorphism within species		29.4		50			53.8			8.33	

DNA Fingerprinting:

Random Amplified Polymorphic DNA is rapid, simple and inexpensive method. It deduces DNA polymorphisms produced by rearrangement or deletions at or between oligonucleotide primer binding sites in the genome using short random oligonucleotide sequences mostly ten bases long **(Agarwal *et al.*, 2008).** About 100-130 species included within *Lotus* have been widely explored using molecular markers such as Random Amplified Polymorphic DNA (RAPD) **(Garcia De Los Santos and Steiner, 2003).**

In the present study, five tested primers were used to differentiate between the 10 accessions of four *Lotus* species Plate (4). A total of 33 well-defined and scorable RAPD bands were obtained as a result of fingerprinting of *Lotus* species. Accessions and amplicon size varying from 110 to 1450 bp. Of the 33 amplified bands, 12 were common to all accessions and 21 were polymorphic bands. The number of polymorphic bands varied from

three bands in OPA- 4 primer, five bands in primer OPA- 9, four bands in primer OPA- 10, five bands in OPA- 13 primer, and four bands in OPA- 17 primer.

The percentage of polymorphic bands of the studied taxa is given in Table (6). Regarding the polymorphism of all accessions of *Lotus* spp. the maximum value of polymorphism 83.3% recorded in primer OPA-9 with sequence 5-GGGTAACGCC-3 and the minimum value 42.9% recorded in primer OPA-4 with sequence 5-AATCGGGCTG-3.

Table (6): Polymorphic bands of RAPD-PCR for *Lotus* species from ten different accessions.

Primers	Monomorphic bands	Polymorphic bands		Total bands	Polymorphism %
		Unique bands	Non_unique bands		
OPA-4	4	0	3	7	42.9
OPA-9	1	1	4	6	83.3
OPA-10	3	0	4	7	57
OPA-13	2	1	4	7	71.4
OPA-17	2	2	2	6	66.66

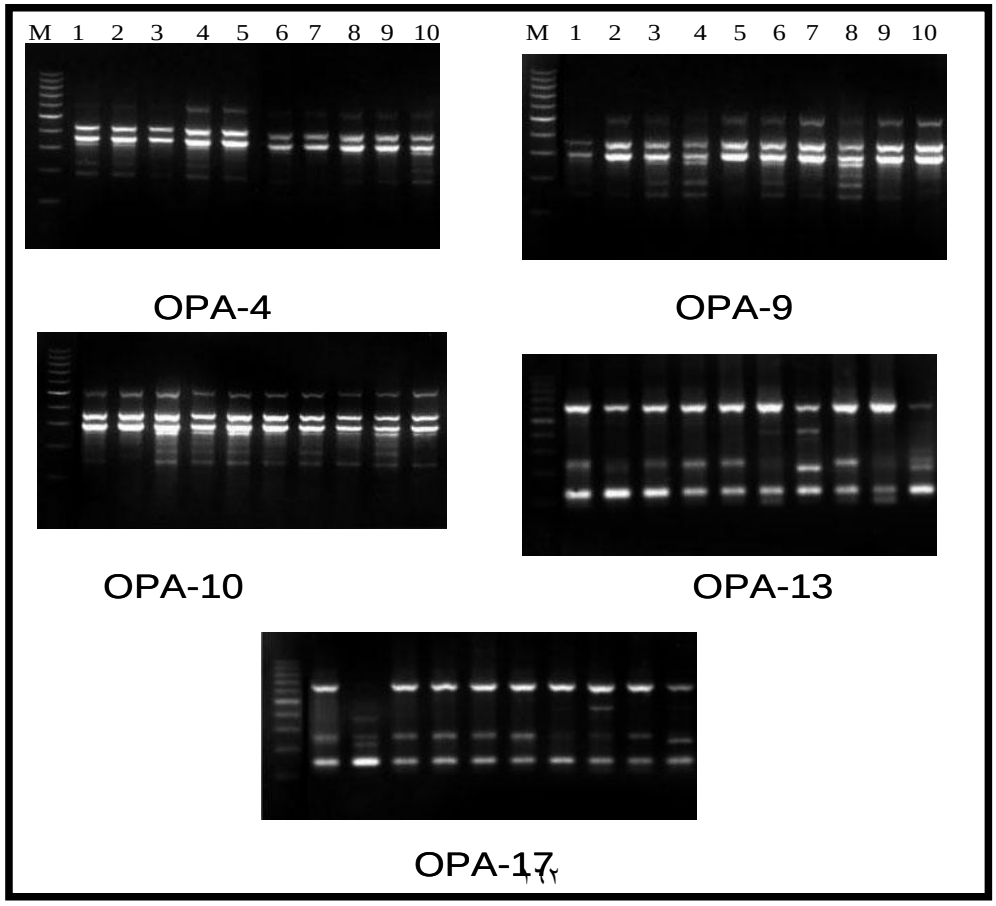

Plate (4): The amplification profiles of the ten accessions of *Lotus* species generated by five primers (OPA-4, OPA-9, OPA-10, OPA-13 and OPA-17). M – indicates molecular size marker, (1) Lc1, (2) Lc2, (3) Lg1, (4) Lg2, (5) Lg3, (6) Lh1, (7) Lh2, (8) Lh3, (9) Lp1 and (10) Lp2.

(Lc1) *Lotus creticus* collected from Idku District (EL-Beihera Governorate)
(Lc2) *Lotus creticus* collected from Motobas District (Kafr El-sheikh Governorate)
(Lg1) *Lotus glaber* collected from Motobas District (Kafr El-sheikh governorate)
(Lg2) *Lotus glaber* collected from Gamasa District (EL-Dakahlyia Governorate)
(Lg3) *Lotus glaber* collected from Mansoura District (EL-Dakahlyia Governorate)
(Lh1) *Lotus halophilus* collected from EL-Borollus District (Kafr El-Sheik Governorate)
(Lh2) *Lotus halophilus* collected from EL-Shahabyia District (Kafr El-sheik Governorate)
(Lh3) *Lotus halophilus* collected from Gamasa District (EL-Dakahlyia Governorate)
(Lp1) *Lotus peregrinus* collected from Rashid District (EL-Beihera Governorate)
(Lp2) *Lotus peregrinus* collected from Idku District (EL-Beihera Governorate)

Anatomical studies:

Anatomical studies have been used successfully to clarify the taxonomic status and the identification of different species. **(Ocak *et al.*, 2004)** made incorporation between morphological and anatomical studies to solve many taxonomic problems of monocots. There is a great need for knowledge of anatomical investigation in many plant families because of its important role in discerning natural groups and understanding phylogenetic relationships **(Sahreen *et al.*, 2010)**. Physiological and biochemical processes are also connected with anatomical structure of plant organs such as leaves and roots **(Katarzyna *et al*., 2008).**

The anatomical characteristics of *Lotus* species, stems and leaves are outlined in Tables (7 and 8) and are illustrated in Plates (5 and 6).

Stems in *Lotus* species:

From the study of the transverse sections of *Lotus* species stem, three regions of tissue were identified they are epidermis, cortex and vascular cylinder.

1- Epidermis:

The epidermis of the studied accessions consists of single layer of living cells which are closely packed. The walls are thickened and covered with thin water proof layer called cuticle. Either unicellular or multicellular hair – like outgrowths appear from the epidermis. Concerning epidermal width of *Lotus* species, the highest epidermal width recorded in *L. glaber* (Lg3) which collected from Mansoura and lowest width identified in *L. glaber* (Lg2) which collected from Gamasa.

2- Cortex:

Below the epidermis cortex was present, this region comprises the collenchymas, parenchyma and endodermis. These cells lie under the epidermis and constitute three or four layers of cells. Several tannins containing cell observed in cortex, the highest amount recorded in *L. creticus* (Lc2) collected from Motobas and *L. halophilus* (Lh2) collected from EL-Shahabyia, and not detected in *L. halophilus* (Lh1) collected from EL-Borollus.

3- Vascular cylinder:

This region comprises the vascular bundles and pith.

- Vascular bundles

The vascular bundles are situated in a ring on the inside of the pericycle of the plant. This distinct ring of vascular bundles is distinguishing characteristics of *Lotus* species, this region consists of three main tissues xylem, phloem and cambium.

- Pith (Medulla)

The pith occupies the large central part of the stem. It consists of thin-walled parenchyma cells with intercellular air spaces. Within *Lotus* species all accessions have hollow pith except the three accessions of *L. halophilus* free from the hollow pith.

Leaves in *Lotus* species:

1- Epidermis:

Both upper and lower epidermis is made up of single layers of cells that are closely packed. There was cuticle on the upper epidermis and thicker than that of lower epidermis. Concerning the epidermal width of *Lotus* species, in the upper layer it ranged from 94.40µ in *L. glaber* (Lg3) to 166.21µ in *L. halophilus* (Lh3). While, the lower layer it ranged from 85.99µ in *L. glaber* (Lg3) to 163.42µ in *L. glaber* (Lg2).

Mesophyll:

The entire tissue between the upper and lower epidermis, differentiated into palisade and spongy parenchyma.

- **Palisade parenchyma**

Below the epidermis, vertically elongated cylindrical cell in one or more layer without intercellular spaces. Regarding *Lotus* species, palisade like cells ranged from 350.17 to 653.33 µ abaxial, and 366.17 to 670 µ adaxial.

- **Spongy parenchyma**

Below palisade parenchyma towards lower epidermis, irregularly shaped, loosely arranged cells with numerous air spaces. In *Lotus* species the spongy ranged from 210.55 to 560.07 µ

2- Vascular tissue (in midrib):

Are conjoint, collateral and closed, xylem was present towards the upper epidermis, while phloem towards the lower epidermis, surrounded by a compact layer of parenchymatous cells called bundle sheath. Within *Lotus* species vascular bundles length ranged from 550.5 to 797.08 µ.

In plants, the environment plays a greater role in regulating development. The internal structure of the same plant can be slightly different when grown in different environments **(Edward, 1998)**. On the basis of stem and leaf anatomy internal structure, the *Lotus* species showed some similar adaptive characteristics while, others showed particular characters related to drought and salinity stress.

(AL-Nowaihi *et al*., 2005) reported that there is an increase in the accumulation of tannin compounds and alkaloids in xerophytic plants that grow under drought stress and this drought may cause the accumulation of tannin compounds. From results it could be concluded that, the accession of *L. creticus* (Lc2) and *L. halophilus* (Lh2) under high drought stress conditions have the highest content of tannins.

Table (7): Anatomical aspects of the stem of four *Lotus* species collected from ten different accessions, the measurements in µ.

Species	Codes	Epidermis	Cortex		Pith		Vascular bundles	
			Collenchyma	Tannins	Parenchyma	Hallow pith	Phloem	Xylem
L.creticus	Lc1	135.70	218.15	++	766.88	3642.69	277.77	610.76
	Lc2	153.87	220.20	++++	1687.99	4139.31	505.55	919.76
L. glaber	Lg1	140.64	174.77	+	1950.06	4125.61	630.8	1192.44
	Lg2	169.43	277.63	++	1346.52	3687.65	321.17	652.82
	Lg3	93.55	297.77	+	1999.96	1501.02	211.79	469.70
L. halophilus	Lh1	141.57	280.36	-	3176.44	_	271.96	369.81
	Lh2	101.83	272.36	++++	2966.16	_	221.91	450.89
	Lh3	161.09	258.00	++	2975.69	_	198.244	439.32
L. peregrinus	Lp1	158.13	293.63	++	679.91	3266.38	184.19	576.70
	Lp2	142.55	283.11	+	603.11	3107.17	165.20	550.32

Table (8): Anatomical aspect of the leaf of four *Lotus* species collected from ten different accessions, the measurements in µ.

Species	Codes	Upper epidermis	Lower epidermis	Isobilateral Mesophyll			Vascular bundles
				Abaxial Palisade	Adaxial Palisade	Spongy	height
L. creticus	Lc1	120.21	115.30	350.13	366.17	210.55	550.5
	Lc2	150.51	140.12	455.41	475.91	307.31	660.88
L. glaber	Lg1	141.21	145.31	565.31	598.09	404.21	750.11
	Lg2	157.00	163.42	653.33	670.21	532.65	797.08
	Lg3	94.40	85.99	530.90	598.05	430.65	670.70
L. halophilus	Lh1	131.87	139.90	623.65	623.32	550.51	690.00
	Lh2	111.76	114.78	598.76	566.66	490.67	607.07
	Lh3	166.12	156.74	680.90	634.56	560.07	688.88
L. peregrinus	Lp1	150.09	146.43	566.67	578.94	499.90	603.03
	Lp2	144.40	139.09	420.30	599.90	520.54	677.77

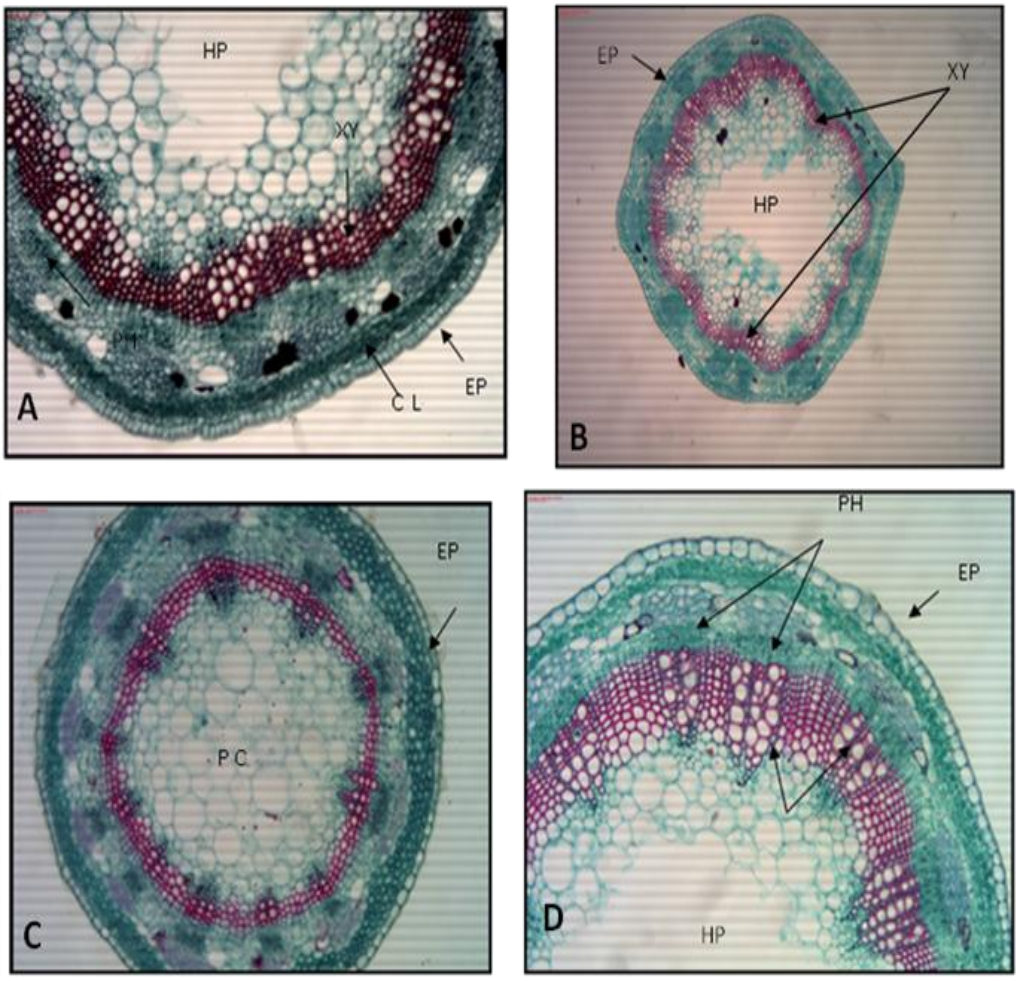

Plate (5) (A-D): Cross section in stem of *Lotus* species. A, *Lotus creticus* stem, B, *Lotus glaber* stem, C, *Lotus halophilus* stem, D, *Lotus peregrinus* stem; EP = epidermis; HP = hollow pith; CL = collenchyma layer; PH = phloem; XY = xylem; PC = parenchyma cells (A&D: X=100) (B&C: X=40).

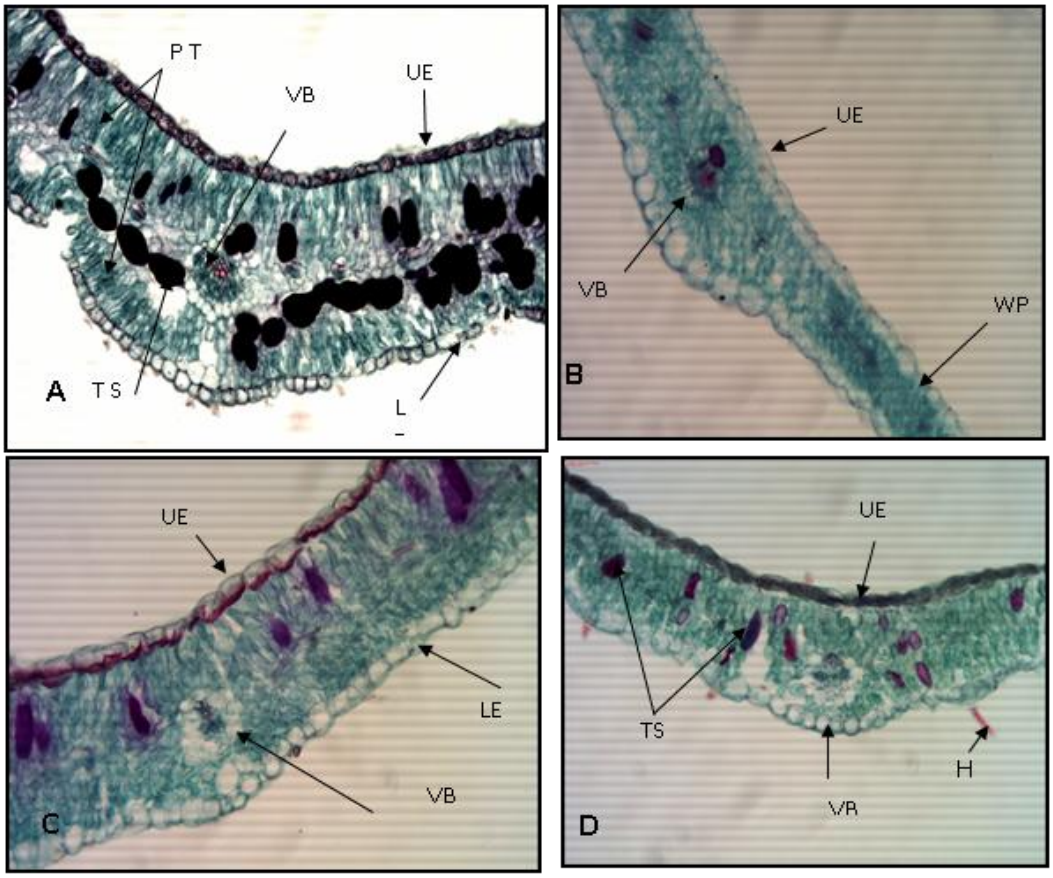

Plate (6) (A-D): Cross section in leaf of *Lotus* species. A, *Lotus creticus* leaf, B, *Lotus glaber* leaf, C, *Lotus halophilus* leaf, D, *Lotus peregrines* leaf; VB = vascular bundles, UE = upper epidermis, LE = lower epidermis, PT = palisade tissue, H = hairs, TS = tannins spots (X=40).

Data analysis:

To get linkage between the studied taxa collected, the cytological, biochemical and molecular attributes were standardized and compute coordinates for plotting Biplot mapping by using perceptual mapping (PERMAP). Perceptual mapping (PERMAP) using combination of taxa and attributes was shown in Fig. (3).

Protein polymorphism and the primer OPA-17 are important to distinguish *Lotus creticus* accessions (Lc1, Lc2)

Rec index, TF%, A1 and OPA-10 have the power to distinguish Lh1 and Lh2 of *Lotus halophilus* collected from Kafr El-sheik Governorate than the third accession (Lh3) collected from EL-Dakahlyia Governorate.

OPA-13 and S% have the importance positive value to distinguished Lg2 and Lg3 of *Lotus glaber collected* from EL-Dakahlyia Governorate, while negative value of Lg1 of *Lotus glaber collected* from collected from Kafr El-sheikh Governorate .

Protein polymorphism, OPA-17, OPA-4, Syi and A2 have the importance highly value to separate (Lp2) *Lotus peregrinus* collected from Idku District (EL-Beihera Governorate) from (Lp1) *Lotus peregrinus* collected from Rashid District (EL-Beihera Governorate).

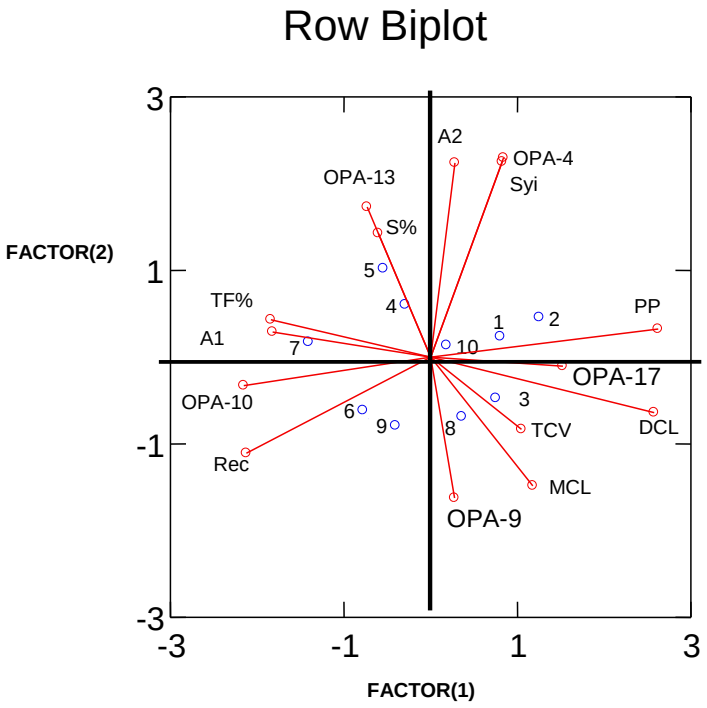

Fig. (3): Perceptual mapping (Biplot) of the studied species of *Lotus* for combination of samples and cytological parameters and polymorphisms of electrophoresis techniques of DNA- RAPD and seed protein. 1=Lc1, 2=Lc2, 3= Lg1, 4=Lg2, 5=Lg3, 6=Lh1, 7= Lh2, 8= Lh3, 9= Lp1 and 10= Lp2. Pp=protein polymorphism.

4. Conclusion

It could be concluded that the cytological parameters, biochemical, molecular markers, pollen abortion assay and anatomical features of the four species of *Lotus* which collected from ten different accessions in Egypt could be considered as suitable parameters for the evaluation of the genetic diversity within and among the four species of *Lotus*. These obtained data may play a role in the management low cost strategies of gene banks for the conservation of this species and reduced the number of accessions of plant genetic resources conserved in gene banks.

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الملخص العربى

الاختلافات الوراثية داخل وبين أربعة أنواع من اللوتس (الفصيلة القرنية)

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يهدف هذا البحث الى دراسة كل من صفات الكاريوتيب والكروموسومات والاديوجرام والكاريوجرام واجهاض حبوب اللقاح لكل من *Lotus creticus, Lotus glaber, Lotus halophilus, Lotus peregrinus* . ولقد أظهرت دراسة الكروموسومات الجسدية أن عدد الكروموسومات فى نبات *Lotus creticus* ٢٨ كروموسوم و١٢ كروموسوم فى *Lotus glaber* و ١٤ كروموسوم فى كل من *Lotus halophilus , Lotus peregrinus* . ولقد تم استخدام عدد من الطرق المختلفة لتعيين تماثل الكاريوتيب مثل TF%, Syi, Rec, A1, A2 . ولقد ظهر ايضا فى هذه الدراسة اختلافات فى نسبة حبوب اللقاح الخصيبة والعقيمة. ولقد تم عمل دراسات تشريحية لكل من الساق والورقة لأنواع اللوتس المدروسة. وقد تم استخدام عدد من المعايير الوراثية لتقدير الاختلافات الوراثية بين الاربعة أنواع المدروسة من نبات اللوتس والمجمع من مناطق مختلفة. وقد تم تعيين البروتين بطريقة التفريد الكهربى الذى اظهر ١٧ حزمة وكانت الانواع المدروسة متماثلة فى ٦ حزم ومختلفة فى ١١ حزمة. وقد تم استخدام خمسة بادئ فى طريقة التكبير العشوائى متعدد الاشكال واعطى ٣٣ حزمة منهم ١٢ حزمة موجودة فى كل الانواع المدروسة و٢١ حزمة متابينة. وتم عمل تحليل احصائيا للنتائج من خلال عمل علاقة بين الانواع المدروسة والصفات وقد ميز النباتات الى ثلاث مجموعات.