Journal of Cytology & Histology
ISSN: 2157-7099

Zayed and Zeinab, J Cytol Histol 2016, 7:2
DOI: 10.4172/2157-7099.1000401

Research Article Open Access

Karyotype Analysis and Protein Profile for Three *Trifolium* Species

Zayed EM[1]* and Zeinab ME[2]

[1]Cell Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt
[2]Forage Crops Research Department, Field Crops Research Institute, Agricultural Research Center, Giza, Egypt

Abstract

The aim of the present investigation was cytological and protein comparison among three species within the genus *Trifolium*. The results revealed, Idiogram of the haploid complements of *T. alexandrinum*, *T. refeigratum* and *T. repens*. Besides *T. refeigratum* and *T. repens* have 16 pairs of chromosomes with a pair of satellites located at the end of their short arm in chromosome 16. *T. alexandrinum* has 8 pairs of chromosomes whose karyotyping formula is 2 nsm (+), 10 nsm (-) + 4 nm. The *T. alexandrinum* was clustering alone as well as having polymorphic bands that were different from *T. refeigratum* and *T. repens*.

Keywords: Clover and cytological; *T. alexandrinum; T. refeigratum; T. repens*

Introduction

Trifolium is one of the most important genera of the Leguminosae family in Egypt and most countries of the world [1-3]. Cytological characters, including chromosome number and karyotype analysis have been considered important tool for taxonomic and evolutionary relationships [4]. The number, size and shape of chromosomes were used to characterize the karyotype and define taxonomic differences.

Zarco [3] used the intra-chromosomal and inter-chromosomal asymmetry indices (A1 and A2, respectively) to define differences among cultivars while mentioned that the cultivar with high A1 and A2 values are considered more advanced than others [5]. In general, high A1 and A2 values are scored in cultivars with higher degrees of variation in chromosome length [6]. These variations might be due to chromosome deletions or due to different levels of condensation and differential contraction of chromosomes as suggested by El-Nahas [7]. Soliman et al. [8] in Egypt identified the karyotype formula for the Egyptian clover (*Trifolium alexandrinum L.*). They also, determined the somatic chromosome counts for two cultivars as 2n = 16. Karyotype analysis showed differences in chromosome morphology. They studied chromosomes nsm (+) that were observed in cv Helaly. Furthermore, they stated that the karyotype formula for Helaly multiple cut as 2 nsm (+) + 2 nsm (−) + 12 nm but it was 6 nsm (−) + 10 nm for Fahl single cut cultivar.

In addition, Chen and Pryce [9] compared karyotypes of 15 species of *Trifolium* belonging to the section *Amoria,* on the basis of chromosome size, centromere position, number of satellite chromosomes, and size of satellites. They found that some species having similar or indistinguishable karyotypes, while others differed from one to another by one or more cytological characters. Beside they pointed out the similarity of karyotypes of *T. nigrescens*, *T. occidentale*, *T. petrisavii*, and *T. repens L.* Sudipta et al. [10] found amphidiploid (allotetraploid) *Trifolium repens L.*, diploid-like meiotic behaviour of chromosomes, with no multivalent formation, and a normal karyotype with a single pair of chromosome having a secondary constriction. They explained that these characteristics might be occurred due to by favorable genetic and cytological stability in nature, and high pollen fertility.

George et al. [9] found the dendrogram, resulted from the hierarchical cluster analysis of SDS-PAGE profiles of seed proteins conform, with some restrictions, to the present splitting of the genus *Trifolium* into the sections but not into the subsections and series. In addition, the importance of electrophoretic evidence in plant systematics has been discussed in details by many workers [11-24].

Therefore, the aim of this study was to investigate karyotype and protein profile of three species of *Trifolium*.

Materials and Methods

Cytology and karyotyping: Viable seeds of the three species of *T. alexandrinum*, *T. refeigratum* and *T. repens* were kindly obtained from the Forage Crops Research Department and Egyptian Museum as part of the Egyptian flora in 2014/2015.

Seeds were germinated and actively growing root tips were pretreated for 2-4 h in 0.002 M 8- hydroxyquinoline, fixed in 3:1 (absolute ethanol : acetic acid), hydrolyzed for 5 min in 1 N HCl at 60°C and stained in aceto-orcein according to Lacour and Chattopadhyay and Sharma [25,26]. Well spread five metaphase plates were selected and photographed. Karyograms were drawn and lengths of long arm (L) and short arm (S) were measured for karyotype analysis. Karyotype analysis was carried out using Micro Measure Computer Program [27]. Mean chromosome length (MCL) in μ, the total chromosome volume (TCV) and total chromosome length (TCL) were determined. To estimate karyotype asymmetry, two numerical parameters, namely intra-chromosomal asymmetry index (A1) and inter-chromosomal asymmetry index (A2) were used according to Zarco [3]. Symmetry percent (S%), resemblance between chromosomes (Rec. index), the symmetric indices (SYI index) and total form percent (TF%) which is the average degree of symmetry over the whole karyotype were calculated according to Huziwara [28].

Protein analysis

Extraction of seedling total proteins SDS-PAGE was performed following the method of Laemmli which was modified by Studier [29,30].

***Corresponding author:** Zayed EM, Cell Research Department, Field Crops Research Institute, Agricultural Research Center, 12619 Giza, Egypt, Tel: 20235716301; E-mail: ehabzayed31@hotmail.com

Received October 19, 2015; **Accepted** February 05, 2016; **Published** February 08, 2016.

Citation: Zeinab, Zayed (2016) Karyotype Analysis and Protein Profile for Three Trifolium Species. J Cytol Histol 7: 401. doi:10.4172/2157-7099.1000401

Results and Discussion

Cytological analysis

The study confirmed existence of remarkable degree of chromosome variability among the three species. Near sub-metacentric (nsm) and near metacentric (nm) chromosomes were common in both karyotypes (Figures 1-3) (Tables 1-4). Idiogram of the haploid complements is shown in Figures 1-3 for *T. alexandrinum*, *T. refeigratum* and *T. repens,* respectively. The evolution of karyotype is estimated by indices of symmetry. These values theoretically ranged from 0 to 100 for Rec and Syi indices and from 0 to 50 for both TF% and symmetry (S%). A karyotype with high indices is considered as evolving slowly [31].

Chromosome pair number	Chromosome length (µm)			Arm Ratio	Centromere Index	S/L	Karyotype
	Length each	Long arm	Short arm	(L/S)	(S/(L+S))		
1	12.37	7.34	5.03	1.46	0.41	0.69	nsm(-)
2	11.19	6.99	4.2	1.66	0.38	0.6	nsm(-)
3	10.65	6.49	4.16	1.56	0.39	0.64	nsm(-)
4	9.87	6.04	3.83	1.58	0.39	0.63	nsm(-)
5	9.52	5.95	3.57	1.67	0.38	0.6	nm
6	8.99	5.85	3.14	1.86	0.35	0.54	nm
7	8.84	5.66	3.18	1.78	0.36	0.56	nsm(-)
8	8.27	5.46	2.81	1.94	0.34	0.51	nsm(+)
9	7.9	5.29	2.61	2.03	0.33	0.49	nsm(-)
10	7.69	5.03	2.66	1.89	0.35	0.53	nm
11	7.37	4.83	2.54	1.9	0.34	0.53	nsm(-)
12	6.88	4.66	2.22	2.1	0.32	0.48	nsm(-)
13	6.02	4.31	1.71	2.52	0.28	0.4	nsm(-)
14	5.74	4.11	1.63	2.53	0.28	0.4	nsm(-)
15	5.26	3.86	1.4	2.76	0.27	0.36	nsm(-)
16	4.46	3.45	1.01	3.42	0.23	0.29	nm

Nsm = nearly sub-metacentric; nm = nearly metacentric

Table 1: The average measurements and arm ratios of somatic chromosomes of *T.repens.*

Chromosome pair number	Chromosome length (µm)			Arm Ratio	Centromere. Index	S/L	Karyotype
				(L/S)	(S/(L+S))		
	Length each	Long arm	Short arm		nsm(-)		
1	10.27	7.16	3.11	2.3	nsm(-)	0.43	nsm(-)
2	8.8	6.1	2.7	2.26	nsm(-)	0.44	nsm(-)
3	8.54	5.61	2.93	1.91	nsm(-)	0.52	nsm(-)
4	8.14	5.4	2.74	1.97	nsm(-)	0.51	nsm(-)
5	7.80	5.12	2.68	1.91	nsm(-)	0.52	nsm(-)
6	7.59	4.93	2.66	1.86	nsm(-)	0.54	nsm(-)
7	7.37	4.79	2.58	1.86	nsm(-)	0.54	nsm(-)
8	7.28	4.69	2.59	1.81	nsm(-)	0.55	nsm(-)
9	7.05	4.59	2.46	1.87	nsm(-)	0.54	nsm(-)
10	6.7	4.52	2.18	2.07	nsm(-)	0.48	nsm(-)
11	6.64	4.51	2.13	2.11	nsm(-)	0.47	nsm(-)
12	6.36	4.4	1.96	2.24	nsm(-)	0.45	nsm(-)
13	6.26	4.31	1.95	2.22	nsm(-)	0.45	nsm(-)
14	5.81	3.99	1.82	2.19	nsm(-)	0.46	nsm(-)
15	5.14	3.79	1.35	2.82	nsm(-)	0.36	nsm(-)
16	2.99	1.59	1.4	1.14	nsm(-)	0.88	nsm(-)

*nsm = nearly sub-metacentric; nm = nearly metacentric

Table 2: The average measurements and arm ratios of somatic chromosomes of *T. refeigratum.*

Chromosome pair number	Chromosome length (µm)			Arm ratio	Centromere. Index		Karyotype
	Length each	Long arm	Short arm	(L/S)	(S/(L+S))	S/L	
1	8.96	6.7	2.26	2.97	0.25	0.34	nm(-)
2	8.12	5.77	2.34	2.46	0.29	0.41	nm(-)
3	7.86	5.51	2.35	2.34	0.3	0.43	nm(-)
4	7.4	5.08	2.32	2.19	0.31	0.46	nm
5	6.54	4.37	2.18	2	0.33	0.5	nm
6	6.43	4.04	2.39	1.69	0.37	0.59	nm
7	5.62	3.76	1.86	2.02	0.33	0.49	nm
8	4.3	2.91	1.39	2.1	0.32	0.48	nm

nsm = nearly sub-metacentric; nm = nearly metacentric

Table 3: The average measurements and arm ratios of somatic chromosomes of *T. alexandrinum.*

Most species of *Trifolium* are diploid (2n = 16) and only 16% of the 248 species are polyploidy [9]. About 70% of the known polyploids occur in the subgenus *Amoria*, which is considered to be the most primitive and unspecialized subgenera.

Both *T. refeigratum* and *T. repens* have16 pairs of chromosomes with a pair of satellites located at the end of their short arm in chromosome 16, confirming the earlier results reported by Chen and Pryce [8] (Figure 4). Somatic chromosome karyotype was constructed from 11 mitotic cells by arranging the chromosome pairs on the basis of decreasing size and centromere position, with classification on the basis of the arm ratio using the criteria of Levan et al. [32].

Karyotype analysis data from Table 1 and Figure 1 revealed that the 11 pairs of chromosomes were nearly sub-metacentric [(nsm) (-)] (chromosomes 1, 2, 3, 4, 7, 8, 9, 11, 12, 13, 14, and 15) and 4 pairs of chromosome were nearly meta centric (nm) (chromosomes 5, 6, 10 and 16). Chen and Pryce [8] in a less detailed analysis, reported that 4 pairs of the *T. repens* chromosomes were meta centric, 11 pairs sub-meta centric, and 1 pair telocentric. Furthermore, *T. alexandrinum* has 8 pairs of chromosomes with karyotyping formula is 2 nsm (+), 10 nsm (-) + 4 nm.

Egizia et al. found that the changes in chromosome number have played an important role in the evolution of the genus *Trifolium* [33]. Along with a few species of polyploidy origin, there are several cases of diploid as evidenced by the presence of four basic chromosome numbers (x = 8, 7, 6, 5). *T. subterraneum* and *T. israeliticum* are related species with chromosome complements of 2n = 16 and 2n = 12, respectively. Although they represent an interesting case of speciation based on chromosome number reduction, no attempts to demonstrate their cytogenetic affinity have been carried out so far. The present study performed a comparative cytogenetic study with the purpose of clarifying the evolutionary relationship between these species and to verify whether genomic rearrangements, other than modification of the chromosome number, are associated with the speciation process. Although karyo-morphological analysis supports the hypothesis that chromosome rearrangements had a role in the reduction of the chromosome number, the physical mapping of the rDNA sequences revealed a significant re-modeling of the 45S and 5S rDNA sites that greatly contributed to the differentiation of the 2n = 16 and 2n = 12 karotypes. The nucleotide analysis of 5S rDNA repeats confirmed that the two species are related but distinct. The observed genomic changes lead to the hypothesis that the 2n = 12 species is the result of an evolutionary pathway that passed through intermediate forms. It cannot be excluded that the most direct ancestor of *T. israeliticum* was a species with 2n = 14.

No.	Species	TCL(µ)	MCL(µ)	S%	TF%	A1	A2	Syi index ± SE	Rec index ± SE	Karyotype formula
1	*T. alexandrinum*	110.5	6.9	0.15	44.81	0.53	0.21	0.31	0.75	2 nsm(+),10 nsm
								± 1.46	± 0.16	(-)+4 nm
2	*T. refeigratum*	227.9	7.12	0.15	48.55	0.52	0.21	0.32	0.65	4 nsm(+), 24 nsm
								± 1.47	± 0.14	(-)+4 nm
3	*T. repens*	262	8.19	0.128	53.53	0.48	0.27	0.33	0.64	8 nsm(+), 12 nsm
								± 2.20	± 0.19	(-)+12 nm

*Mean chromosome length (MCL) in µ, the total chromosome volume (TCV) and the total chromosome length (TCL) were determined. To estimate karyotype asymmetry, two numerical parameters, namely intra-chromosomal asymmetry index (A1) and (inter-chromosomal asymmetry index (A2), Symmetry percent (S%), resemblance between chromosomes (Rec. index), the symmetric indices (SYi index) and the total form percent (TF%).

Table 4: Karyotype parameters of somatic chromosomes of three species of *Trifolium.*

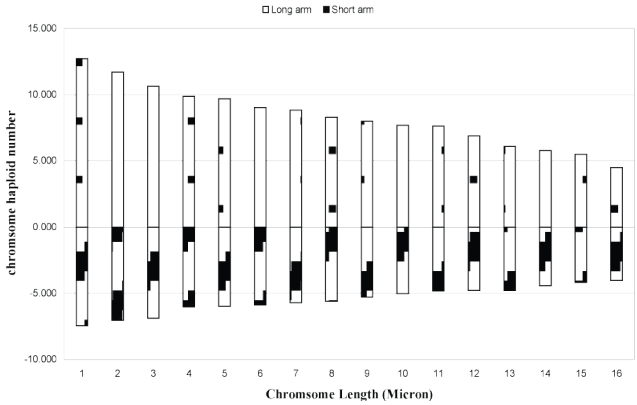

Figure 1: Idiogrammatic representation of the karyotype of *Trifolium repens*.

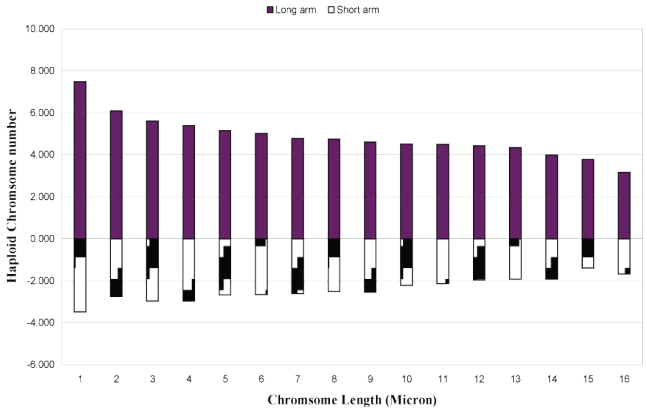

Figure 2: Idiogrammatic representation of the karyotype of *Trifolium refeigratum*.

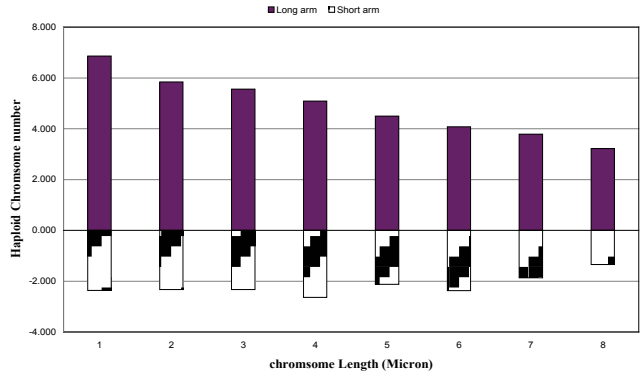

Figure 3: Idiogrammatic representation of the Karyotype of *Trifolium alexandrin*.

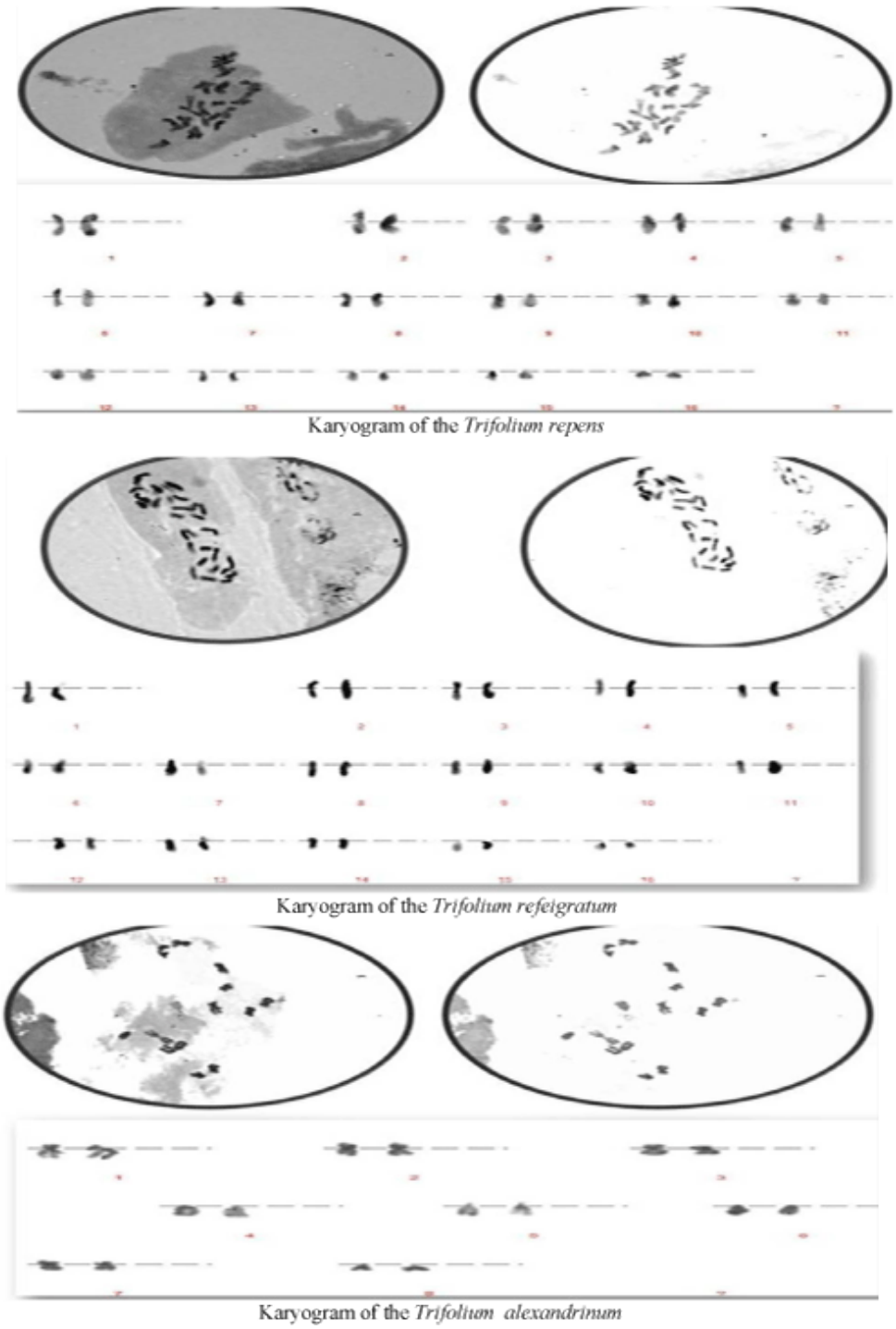

Figure 4: Karyogram of the three *Trifolium* species, chromosomal abnormalities non- congression at metaphase and late separation at anaphase (X=1000).

From Table 4 note the contrast among the three species and Egyptian clover in different values along the chromosome in total length, as well as describing the average length of the chromosome. Egyptian clover has equal *T. refeigratum* ratio in the S% while differed from them in white clover (*T. repens*). Also, TF% was 44.81 in Egyptian clover (*T. alexandrinum*) while values in *T. refeigratum* and *T. repens* were 48.55 and 53.53, respectively. These results reflect the size of the chromosome and the length of the chromosome in each species. Furthermore, values in A1 and A2 that give the duplication in *T. alexandrinum* was diploid and tetraploid in *T. refeigratum* and *T. repens*.

Protein Profile

The data in (Table 5 and Figures 5 and 6) revealed that the *T. alexandrinum* was clustering alone and has polymorphic bands that differ from *T. refeigratum* and *T. repens*. Kumar et al. and Lange and Schifino [34,35], who studied *Trifolium* species and, related

MW (KDa)	*T. repens*	*T. refeigratum*	*T. alexandrinum*
120	+	+	-
100	+	+	-
95	+	+	+
90	+	+	-
80	+	+	+
75	+	+	-
70	+	+	+
68	-	-	+
65	+	+	-
60	+	+	+
40	+	+	+
35	+	+	+
30	+	+	+
22	+	+	+
19	+	-	-
17	+	+	+
10	+	+	+

Table 5: Polymorphism among three species of *Trifolium* based on seed storage protein.

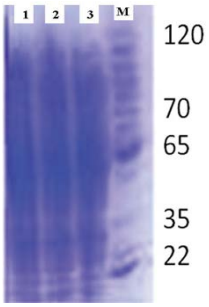

Figure 5: SDS-PAGE seedling protein profile M= marker, (1) =*Trifolium repens*, (2) = *Trifolium refeigratum,* (3) = *Trifolium alexandrinum.*

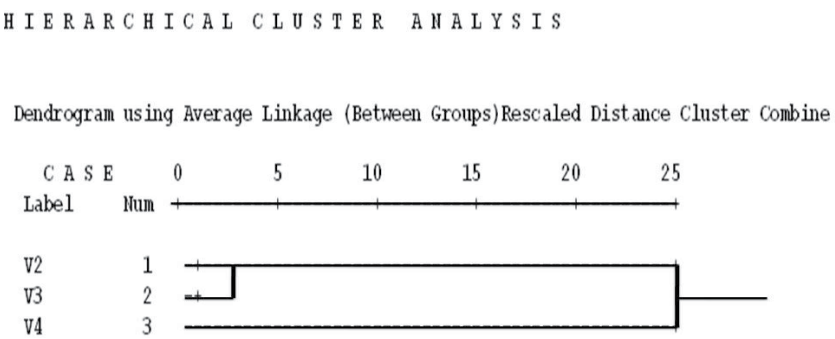

Figure 6: Dendrogram among three species of *Trifolium* based on seed storage protein, 1= *T. repens, 2* = *T. refeigratum* and 3 = *T. alexandrinum.*

the narrow genetic base of *Trifolium* species due to incompatibility barriers. Isozyme variation in wild and cultivated species of the genus *Trifolium* was noticeable among eight *Trifolium* species. Malaviya and Rao [35] evaluated some lines of *T. alexandrinum* for pollination behavior, morphology and yield. Biochemical markers, especially the electrophoretic profiles of isozymes and proteins, have been widely used for identification of cultivars. They find that electrophoretic methods have been standardized for a large number of crops and were found useful for the purpose of Indian cultivar identification and characterization. It is shown that the two clover lines as well as their plasma radiation treatments had different protein profiles, which reflects their genetic diversity.

Conclusion

Karyotype analysis and protein profile can explore taxonomy via polyploidy and genetic distance among three species of *Trifolium, T. alexandrinum* have 8 pairs of chromosomes whose karyotyping formula is 2 nsm (+), 10 nsm (-) + 4 nm. The *T. alexandrinum* was clustering alone as well as having polymorphic bands that were different from the two other species *T. refeigratum* and *T. repens.*

References

1. Radwan MS, Shabana R, Rammah AM, El-Nehrawi NA (1983) Proceedings of the 1st conferences on agriculture botany sciences 27-28 April, 86-105.
2. Tundis R, Marrelli M, Conforti F, Tenuta MC, Bonesi M, et al. (2015) Trifolium pratense and T. repens (Leguminosae): Edible Flower Extracts as Functional Ingredients. Foods 4: 338-348.
3. Zarco CR (1986) A new method for estimating karyotype asymmetry. Taxon 35: 526-530.
4. Moore DM (1968) The karyotype in taxonomy. In: Modern methods of plant taxonomy, Heywood V. H. (ed.) Academic Press, London and New York pp: 61-75.
5. Kamel EA (1999) Karyological studies on some taxa of the Asteraceae in Egypt. Comp News 33: 1-18.
6. El-Nahas AI (2000) Chromosomal criteria in relation to seed protein of some Egyptian cultivars of Vicia faba. Egypt J Biotechnol 7: 276 - 292.
7. Soliman I, Magda, Zayed EM, Ramadan GA (2010) Cytological comparison of two cultivars of Egyptian clover (Trifolium alexandrinum L). Range Mgmt & Agroforestry 31: 7-10.
8. Chen CC, Gibson PB (1971) Karyotypes of fifteen Trifolium species in section Amoria. Crop Sci 11: 441- 445.
9. Majumdar S, Banerjee S, De KK (2004) Viviapary in white clover (Trifolium repens L.) Current Science 86: 29-30.
10. Sabudak T, Guler N (2009) Trifolium L - A review on its phytochemical and pharmacological profile. Phytother. Res 23: 439- 446.
11. George NM, Ghareeb A, Fawziand NM, Saad S (2013) Electrophoretic pattern of seed proteins in Trifolium L. and its taxonomic implications. Bangladesh J Plant Taxon 20: 19-26.
12. Boulter D, Derbyshire E (1971) Taxonomic aspects of the structure of legume proteins. In: Harborne JB, Boulter D, Turner BL (eds.) Chemotaxonomy of the leguminosae, London.
13. Gottlieb LJ (1977) Electrophoretic evidence and plant systematics. Ann Miss Bot Gard 64: 161-180.
14. Ghareeb A, Khalifa SF, Nael F (1999) Molecular systematic of some Cassia species. Cytologia 64: 11-16.
15. Ladizinsky G, Hymowitz T (1979) Seed protein electrophoresis in taxonomic and evolutionary studies. Theor Appl Genet 54: 145-151.
16. Potokina E, Duncan A, Vaughan A, Eggi EE, Tomooka N (2000) Population diversity of the Vicia sativa agg. (Fabaceae) in the flora of the former ISSR deduced from RAPD and seed protein analysis. Genetic Resource and Crop Evolution 47: 171-183.
17. Ghafoor A, Arshad M (2008) Seed protein profiling of Pisum sativum L., germplasm using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) for investigation of biodiversity. Pak J Bot 40: 2315-2321.
18. Celebi A, Acik L, Aytic Z (2009) Biosystematics studies among Ebenus L. species based on morphological, RAPD-PCR and seed protein analysis in Turkey. Pak J Bot 41: 2477-2486.
19. Chattopadhyay D, Sharma AK (1988) A new technique for orcein banding with acid treatment. Stain Technol 63:283-287.
20. Nikolic Z, Vasiljevic S, Karagic D, Vujakovic M, Jovicic D, et al. (2010) Genetic diversity of red clover cultivars (Trifolium pratense L.) based on protein polymorphism. Genetika 42: 249-258.
21. Badr A, El-Shazly HH, Abou El-Enain MM (2000) Seed protein diversity and its implications on the relationships in the genus Lathyrus L. (Fabaceae). Proceedings of the 1st International Conference Biological Sciences, 7-8 May Tanta University Egypt pp: 333-346.
22. Badr A (1995) Electrophoretic studies of seed proteins in relation to chromosomal criteria and the relationships of some taxa of Trifolium. Taxon 44: 183-191.
23. Zecevic B, Stevanovic D, Mladenovic-Drinic S, Konstantinov K, Jakovjevic T (2000) Proteins as genetic markers in the study of phylogenetic relations in pepper. Acta Hortic 579: 113-116.
24. Kamel EA (2005) Biochemical and molecular variations in the genus Raphanus L. based on SDS-PAGE seed proteins and isozymes patterns. Bull Fac Sci Assut Univ 34: 95-113.
25. LaCour L (1941) Acetic orcein. Stain Technol 16: 169-174.
26. Reeves A (2001) MicoMeasure: A new computer program for the collection and analysis of cytogenetic data. Genome 4: 439-443.
27. Huziwara Y (1962) Karyotypic analysis in some genera of compositae, VIII. Further studies on the chromosomes of Aster. Amer J Bot 49: 116-119.
28. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the heat of bacteriophage T4. Nature 227: 680-685.
29. Studier FW (1973) Electrophoretic patterns of esterase and acid phosphates in the grain of registered varieties of barley (Hordeum vulgare L.). Genetika Slechteri 31: 113-122.
30. Greilhuber J, Septa F (1976) C-banded karyotypes in the Scilla hohenackeri group. S. persica and S. Puschkinia (Liliaceae). Plant Syst Evol 126: 149-188.
31. Levan A, Fredgra K, Sandberg AA (1964) Nomen-clature for centromeric position on chromosomes. Hereditas 52: 201-220.
32. Falistocco E, Marconi G, Falcinelli M (2013) Comparative cytogenetic study on Trifolium subterraneum (2n = 16) and Trifolium israeliticum (2n = 12). Genome 56: 307-313.
33. Kumar B, Malaviya DR, Roy AK, Kaushal P (2008) Isozyme variability in Trifolium alexandrinum accessions. Indian J Genet and Plant Breed 68: 195-200.
34. Lange O, Schifino-Wittmann MT (2000) Isozyme variation in wild and cultivated species of the genus Trifolium L. (Leguminosae). Annals of Botany 86: 339-345.
35. Malaviya DR, Rao AK (1997) Evaluation of exotic lines of Trifolium alexandrinum L. for pollination behaviour, morphology and yield. Crop Improvement 24: 160-166.