Bangladesh J. Bot. **45**(1): 133-141, 2016 (March)

GENETIC DIVERSITY AND CONSERVATION STRATEGIES OF SOME *LILIUM CANDIDUM* L. POPULATION IN TURKEY

FAZIL ÖZEN, GÜLŞAH E AKA[1] AND ÖZLEM AKSOY[*]

Kocaeli University, Faculty of Arts and Sciences, Department of Biology, 41380 Kocaeli, Turkey

Key words: *Lilium candidum,* Genetic diversity, Conservation, Endangered

Abstract

Lilium candidum L. is an economically important plant. Due to overcollection from nature and destruction of its habitats, it is included in the "vulnerable" category of danger. Genetic diversity of five different populations of *L. candidum*, growing in Marmara region of Turkey naturally was investigated. These populations were compared with Kuşadası population that was selected for comparasion group in Aegean region of Turkey. It is aimed to developed conservation strategies in order to protect the species. RAPD-PCR analysis was performed with genomic DNA extracted from the leaves of each replicate of samples. Twenty-one primers were screened, but only six primers gave clear, reproducible banding patterns and selected were further analysis. Jaccard's genetic distances were calculated and dendogram was generated using the UPGMA algorithm. The dendogram obtained were classified into two main groups and three subgroups. Genetic distance and the polymorphic band ratio was determined as 0.0464 - 0.3619 and 74.47%, respectively between the populations. The populations that had the lowest polymorphism ratio were, Nusret (19.15%) and Şabla (19.15%). The populations that had the highest polymorphism ratio was found as Keçidere (25.53%). According to our findings the population of Keçidere and then all of the other populations in Marmara region of Turkey should be conserved *in situ*. In addition conservation of this species in botanical gardens, gene banks and agrosystems will support *in situ* management.

Introduction

Lilium candidum L. is a herbaceous, perennial and monoic species which belongs to Liliaceae family. This is also known as "Madonna Lily" and it is distributed in Balkans, Lebanon, Syria, Palestine, Greece Islands and Turkey on the world (Davis 1984). It was used in medicine and perfumery industry and aslo cultivated as ornamental plant commonly. This plant is endangered due to overcollection and destruction of its habitat. So, *L. candidum* is included in vulnerable category (VU) of danger according to Red Data Book of Turkish Plants (Ekim *et al*. 2000). Additionally *L. candidum* is listed under CITES (The Convention on International Trade in Endangered Species) where it is stated that it is illegal to harm or transport the plant without permission (Roche 1999). Eearlier studies have reported some results of research on its biochemical (Uhrin *et al*. 1989, Satou 1999; Erdoğan *et al*. 2000; Eisenreichova *et al*. 2000), medicinal (Vachalkova *et al*. 2002), antimicrobial (Mucaji *et al*. 2000) cytological (Reznikova 1973), karyotypic (West and Lechmere 1915 Sauerland 1956), physiological (Khawar *et al*. 2005) and ecological characters of the species; neither genetic diversity nor conservation were clear. The purpose of the present study is to assess genetic diversity within and among the populations of the species using RAPD markers and developing conservation strategies. Another important aim is to provide genetic data and a theoretical basis for protection of the species.

[*]Author for corresponding author: <ozlem.aksoy@kocaeli.edu.tr>. [1]Umde Engineering Contracting Industry and Trade Collective Company, 34540 Çatalca-İstanbul/Turkey.

Materials and Methods

Plants were sampled randomly from five selected populations located in Southern Marmara Region of Turkey. The outgroup was selected from Kuşadası (Aydin) population located in Aegean Region of Turkey (Fig. 1). Fresh, young, intact and damage-free leaves were collected. Leaves were dried in plastic bags with silica gel until extracted in the laboratory at Balikesir University, Turkey.

Fig. 1. Geographic location of six *L. candidum* populations sampled from Turkey.
1. Karakuz, 2. Keçidere, 3. Nusret, 4. Şabla, 5. Edremit, 6. Kuşadası.

A total of 30 individuals from six populations (Karakuz, Keçidere, Nusret, Şabla, Edremit, Kuşadas) were included in the study (Table 1). The distance between plants collected was at least 5 - 10 m to increase the possibility of detecting the variation potential within each population. Samples were stored at –80ºC until DNA extraction.

Genomic DNA (gDNA) was extracted from plants collected in the field. Total gDNA from basal leaves was isolated using the EZ-10 Spin Column gDNA Minipreps Kit (For Plant Samples) (Bio Basic, Canada). Extracted DNA was quantified spectrophotometrically (UNICAM Heλios α). The quality of DNA samples were determined by observing the ratio of absorbance at A_{260}/A_{280}.

The DNA quality was also checked by electrophoresis in a 1% agarose gel. Extracted DNA was stored at –25ºC until RAPD-PCR amplification.

Table 1. Names and locations of the six populations studied.

Population	Latitude (N)	Longtitude (E)
Karakuz (Kepsut)	39° 45'	28° 13'
Keçidere (Kepsut)	39° 42'	28° 12'
Nusret (Kepsut)	39° 37'	28° 10'
Şabla (Kepsut)	39° 46'	28° 13'
Kaşıkçı (Edremit)	39° 38'	26° 58'
Kuşadası (Aydın)	37° 41'	27° 13'

RAPD reactions were performed using random primers (Operon Technologies, Alameda, CA, USA). Twenty-one primers were screened, only six primers gave clear, reproducible banding patterns and selected were further analysis (Operon OPA-13, OPA-10, OPA-12, OPB-12, OPC-11 and OPC-02) (Table 2).

Table 2. Primers used in RAPD analyses.

Primer	Sequences 5' to 3'
OPA-13	CAGCACCCAC
OPA-10	GTGATCGCAG
OPA-12	TCGGCGATAG
OPB-12	CCTTGACGCA
OPC-11	AAAGCTGCGG
OPC-02	GTGAGGCGTC

RAPD-PCR reactions were performed in a final volume of 25 µl using a Progene thermal cycler (Techne Ltd. UK), containing 10 ng of genomic DNA, 1 µM primer, 1 µM dNTPs, 2.5 mM $MgCl_2$, 2.5 µl 10x magnesium-free PCR buffer (10x PCR buffer: 500 mM KCl, 100mM Tris-HCl at pH 8.3), 1 U Taq DNA polymerase (TaKaRa Bio Inc., Japan) and 13.35 µl ddH_2O. Amplification was carried out with one cycle of initial strand separation at 94ºC for 1 min followed by 42 cycle of 1 min at 94ºC, 1 min at 36ºC and 2 min at 72ºC. The last cycle was followed by an additional extension at 72ºC for 5 min. The PCR products were separated on a 1.5 % (w/v) agarose gel at 80 volts. DNA fragments were visualised by UV transilluminator (UVP GelDoc-It Imaging System, CA) after staining the gel with ethidium bromide and photographed. A 1 kb DNA ladder (GeneRuler, MBI Fermentas, CA) was used to estimate the approximate molecular weight of the amplified products. Reactions were replicated at least twice to control reproducibility of patterns. Only reproducible RAPD primers were included in the analysis.

The RAPD bands on the agarose gel were examined and recorded as present (1) or absent (0) and assembled into a data matrix. The POPGENE Version 1.31 software was used to investigate the genetic diversity within and between populations and draw a dendrogram based on Nei’s 1972 genetic distances using the Unweighted Pair Group Method and Arithmetic Average (UPGMA) (Yeh *et al.* 1997).

Results and Discussion

A total of 47 bands were scored for the six RAPD primers of which 74.47% (35 bands) were polymorphic (Table 3). Primer OPB-12 produced many polymorphic RAPD bands in all populations (Table 3).

Table 3. RAPD products generated by 6 primers in six *Lilium candidum* populations.

Primer	Total number of reproducable bands	Total number of polymorphic bands	Number of polymorphic bands					
			Karakuz	Keçidere	Nusret	Şabla	Kaşıkçı	Kuşadası
OPA-13	7	5	0	1	1	2	2	2
OPA-10	11	7	1	1	0	1	4	1
OPA-12	7	5	1	0	0	0	0	0
OPB-12	9	8	3	7	6	2	1	4
OPC-11	7	4	2	3	2	3	1	1
OPC-02	6	6	3	0	0	1	2	3
Total	47	35	10	12	9	9	10	11
Polymorphism (%)		21.28	25.53	19.15	19.15	21.28	23.40	

On the other hand, primer OPA-12 gave only one polymorphic band that was monomorphic in five populations. The percentage of polymorphic bands ranged from 19,15 in Şabla and Nusret to 25.53 in Keçidere. The average diversity within populations (Hs) was 0.1 and the total diversity (Ht) was 0.25 (Table 4). The mean level of genetic differentiation (Gst) between populations over all loci was 0.63. The markers contributed differently to the observed degree of population differentiation, varying from a low differentiation of 18% for OPB-12/F and OPC-11/F to a high 100% for OPA-13/A, OPA-10/D, OPA-12/C, OPA-12/D, OPA-12/F and OPA-12/G (Table 4). Shannons's index of phenotyping diversity was used to find diversity within and among populations. Table 5 shows that the diversity within populations related to different primers and different populations. Primer OPB-12 detected the highest genetic diversity within these populations while primer OPA-12 detected the lowest.

The population of Keçidere showed greater average variation (0.1635) than the other populations while the population of Şabla showed less variation (0.1081) than the others. Genetic distances calculated from the Nei's (1972) genetic diversity index average was 0.205 and varied from 0.047 between Kasikci and Şabla and was 0.362 between Kasikci and Kuşadası (Table 6).

A dendrogram constructed on the basis of Nei's genetic distances and the UPGMA method showed two main clusters: the outgroup Kuşadası population (population of Aegean Region of Turkey) and the five remaining populations (populations of Marmara Region of Turkey). Marmara populations were classified into three subgroups; Kasikci and Şabla are in the first subgroup, Karakuz is in the second subgroup and Nusret and Keçidere are in the third subgroup (Fig. 2).

The four known natural populations of *L. candidum* were used for the RAPD-PCR analysis. 21 random primers were tested and reproducible bands were obtained with 6 primers (Table 3). Dendrogram obtained from RAPD-PCR results allowed two main groups to be distinguished. The upper group was composed of Kasikci, Şabla, Nusret, Keçidere and Karakuz populations. The lower group was Kuşadasi population (Fig. 2).

Table 4. Genetic diversity between *L. candidum* populations for 35 RAPD marker.

Marker	Ht*	Hs*	Gst*
OPA-13/A	0.4444	0.0000	1.0000
OPA-13/C	0.3833	0.1498	0.6091
OPA-13/E	0.5000	0.2126	0.5747
OPA-13/F	0.1739	0.0813	0.5324
OPA-13/G	0.3618	0.0813	0.7752
OPA-10/B	0.1310	0.0813	0.3789
OPA-10/C	0.5000	0.2126	0.5747
OPA-10/D	0.2778	0.0000	1.0000
OPA-10/F	0.4630	0.0499	0.8921
OPA-10/G	0.1739	0.0813	0.5324
OPA-10/J	0.1739	0.0813	0.5324
OPA-10/K	0,4815	0.0813	0.8311
OPA-12/C	0.2778	0.0000	1.0000
OPA-12/D	0.2778	0.0000	1.0000
OPA-12/E	0.3167	0.0499	0.8423
OPA-12/F	0.2778	0.0000	1.0000
OPA-12/G	0.2778	0.0000	1.0000
OPC-11/B	0.3108	0.1627	0.4766
OPC-11/D	0.4107	0.2440	0.4058
OPC-11/F	0.3225	0.2626	0.1857
OPC-11/G	0.4412	0.2126	0.5180
OPC-02/A	0.1739	0.0813	0.5324
OPC-02/B	0.1739	0.0813	0.5324
OPC-02/C	0.1816	0.1313	0.2772
OPC-02/D	0.4412	0.2126	0.5181
OPC-02/E	0.4901	0.0813	0.8340
OPC-02/F	0.4901	0.0813	0.8340
OPB-12/B	0.3618	0.0813	0.7752
OPB-12/C	0.2421	0.1627	0.3280
OPB-12/D	0.2841	0.2126	0.2517
OPB-12/E	0.4412	0.2126	0.5181
OPB-12/F	0.3225	0.2626	0.1857
OPB-12/G	0.4975	0.1812	0.6358
OPB-12/H	0.3668	0,2940	0.1985
OPB-12/I	0.2841	0.2126	0.2517
Means	0.2495	0.0922	0.6305

* Ht: Genetic diversity over all groups. Hs: Genetic diversity within populations. Gst: Proportion of genetic diversity between populations.

The ovearall values for mean observed number of alleles (na) and mean effective number of alleles (ne) were 2.000 and 1.661, respectively. Assuming the Hardy-Weinberg equilibrium, the average gene diversity was 0.394 (Ht) at the species level. The mean value of the Shannon's information index (I) was 0.582 at the species level. Nei's genetic distance between populations varied from 0.046 to 0.342. The lowest genetic distance value (0.046) was found between

populations Şabla and Kasikci while the highest (0.342) between populations Kusadasi and Nusret (Table 6). An UPGMA dendrogram was constructed based on Nei's genetic distance (Fig. 2). The length between the populations is shown in Table 6.

Table 5. Genetic diversity within populations for *L. candidum* from six locations with Shannon's index.

Primers	Karakuz	Keçidere	Nusret	Şabla	Kaşıkçı	Kuşadası
OPA-13	0.00	0.09	0.06	0.13	0.16	0.19
OPA-10	0.04	0.06	0.00	0.06	0.24	0.04
OPA-12	0.06	0.00	0.00	0.00	0.00	0.00
OPC-11	0.16	0.29	0.19	0.26	0.09	0.06
OPC-02	0.34	0.00	0.00	0.11	0.19	0.30
OPB-12	0.22	0.52	0.38	0.12	0.05	0.23
Average	0.14	0.16	0.10	0.11	0.12	0.14

Table 6. Genetic distances between six *Lilium candidum* populations.

Population	Kasikci	Şabla	Karakuz	Kuşadası	Nusret	Keçidere
Kasikci	0					
Şabla	**0.046**	0				
Karakuz	0.210	0.165	0			
Kuşadası	0.361	0.331	0.255	0		
Nusret	0.207	0.194	0.292	**0.342**	0	
Keçidere	0.287	0.229	0.230	0.330	0.074	0

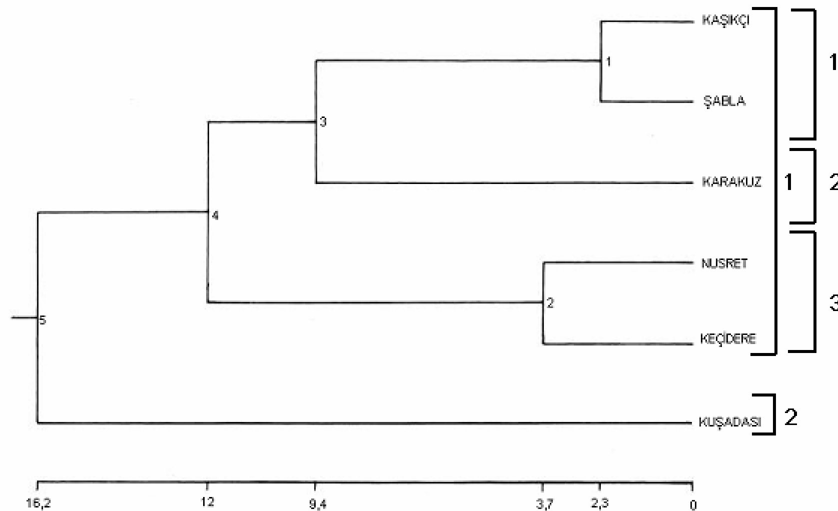

Fig. 2. Dendrogram based on Nei's (1972) genetic distance for six *L. candidum* populations constructed by the UPGMA method.

Genetic markers are important tools in the determination of genetic diversity, which is the first step for breeding projects and the protection of genetic resources (Bardakci 2001). Rare endangered plants may have high diversity, which attributed to the following factors: insufficient length of time for reduction of genetic diversity following a natural reduction of population size and isolation (Zawko *et al.* 2001, Maguire and Sedgley 1997). Generally, geographically restricted species exhibit lower levels of genetic variation than widely distributed species. The breeding systems, life forms and seed dispersal mechanisms of plant species have been regarded as the main factors affecting levels of genetic diversity, genetic divergence, and genetic structure within and among plant populations (Loveless and Hamrick 1984). Outcrossing perennial species commonly have higher levels of genetic diversity than selfing and clonal plants. The population history and habitat type are also important factors regulating the degree of genetic diversity and differentiation. High to moderate levels of genetic differentiation among populations is a common pattern in endemic or narrowly distributed plant species (Nybom 2004, Cruse-Sanders and Hamrick 2004). Due to the technical simplicity and speed of the RAPD-PCR method, it has been successfully used for the generation of genetic similarities and phylogenetic analysis (Surgun *et al.* 2012). In their recent review on estimation of genetic diversity obtained by RAPD-PCR markers, Nybom and Bartish (2000) compiled mean GST values of 0.59, 0.19 and 0.23 for selfing, mixed mating and outcrossing plant species, respectively. As is concluded, populations with high genetic diversity should be priorly protected in conservation biology. Small populations are more prone to be extinct for environmental fluctuation and lose a large of genetic variation because of genetic drift (Aksoy *et al.* 2013).

One of the most important natural resources of the century is genetic resources. These resources have an important place in the renewable natural resources as they are indispensable for the balance of ecosystems. Determination, conservation, evaluation, availability and efficiency of genetic variability should be done to protect natural resources. The ideal way to protect the population is actually protecting the ecosphere as a whole. However, it is impossible to achieve this in today's conditions so at least species and populations of species must be protected. Before developing any strategy for the conservation of a species, it must be determined if the population size of that species depending on time remains constant or not.

The conservation of plant genetic resources can be achieved by two strategies; *in situ* and *ex situ* conservation. *In situ* conservation is the best conservation method that allows the species to complete their evolution. However, implementation of this method is particularly difficult due to anthropogenic influences (Zawko *et al.* 2001). *Ex situ* conservation can be applied in the form of various methods such as seed storage, *in vitro* propogation and storage, DNA storage, pollen storage, botanical gardens, arboretums and agroecosystems (Nybom 2004).

Because of its economical importance *L. candidum* is used intensively for hundreds of years. Natural populations of the species are decreased and limited in narrow areas because its cultivation is not realized enough and the bulbs are uprooted from nature. So it is clear that, development of conservation strategies fot the species is urgently needed. The degree of danger of extinction of *L. candidum* is higher when we compared it with other endangered plant species. The percentage of polymorphic bands (74.77) of RAPD in *L. candidum* was higher than in other endangered plants, e.g. *Lactoris fernandeziana* (Lactoridaceae) 24.5 (Brauner *et al.* 1992), *Cathaya argyrophylla* 32 (Wang *et al.,* 1996), *Paeonia suffruticosa* 22.5 and *P. rockii* 27.6 **%** (Pei *et al.* 1995), *Dacydium pierrei* 33.3 (Su *et al.* 1999), *Changium smyrnioides* 69 (Fu *et al.* 2003). This result shows that the genetic diversity in *L. candidum* is high and indicates that this species has the ability to adapt easily to environmental variation. The main reason of this type of population size reduction and "island-like" distribution in habitats is both because of human activities on habitats, as well as damage to the population by removing them extremely from nature. Among the

populations Keçidere showed higher genetic diversity (Table 5) when compared wih the other we suggested that this population should be primarily conserved *in situ*. The *ex situ* conservation of *L. candidum* in gene banks, agroecosystems and botanical gardens may be recommended as additional techniques beside *in situ* conservation. The samples from the population of Keçidere will be the best choice for cultivation in agroecosystems and botanical gardens. *L. candidum* grows better in neutral and alkaline soils, full sun and average water conditions and also the cultivation area should be considered to be compatible with the ecological needs of the plant (Özen *et al.* 2012).

Acknowledgements

The authors are thankfull to Balikesir University Research Fund (Grant No. 2004/26), Balikesir University Central Researches Laboratory and The Scientific and Technical Research Council of Turkey (TÜBİTAK) for supports (Grant No. 105T021).

References

Aksoy Ö, Erbulucu T, Özen F and Deveci A 2013. Genetic variation in critically endangered plant *Amsonia orientalis* Decne. J. Bio. Environ. Sci. **3**(1): 44-53.

Bardakçı F 2001. Random amplified polymorphic DNA (RAPD) markers. Turk. J. Biol. **25**:185-196.

Brauner S, Crawford DJ and Stuessy TF 1992. Ribosomal DNA and RAPD variation in the rare plant family Lactoridaceae. Amer. J. Bot. **79** (12): 1436-1439.

Cruse-Sanders JM and Hamrick JL 2004. Genetic diversity in harvested and protected populations of wild American ginseng, *Panax quinquefolius* L. (Araliaceae). Amer. J. Bot. **91**(4): 540-548.

Davis PH 1984. Flora of Turkey and East Aegean Islands, Edinburg University Press, Vol. 8, pp. 280, Edinburg.

Eisenreichova E, Haladova M, Mucaji P, Budesinsky M and Ubik K 2000. A new steroidal saponin from the bulbs of *Lilium candidum* L. Pharmazie **55**(7): 549-50.

Ekim T, Koyuncu M, Vural M, Duman H, Aytaç Z and Adıgüzel N 2000. Türkiye Bitkileri Kırmızı Kitabı, Türkiye Tabiatını Koruma Derneği ve Van Yüzüncü Yıl Üniv. Yayını, Barışcan Ofset, Ankara.

Erdoğan İ, Şener B and Rahman A 2000. Alkaloids from the bulbs of *Lilium candidum* L. of Turkish Origin. GUEDE- J. Fac. Pharm. Gazi **17**(1): 25-8.

Fu CX, Qiu YX and Kong H 2003. RAPD analysis for genetic diversity in *Changium smyrnioides* (Apiaceae), an endangered plant. Bot. Bull. Acad. Sin. **44**: 13-18.

Khawar KM, Cocu S, Parmaksız İ, Sarıhan EO and Özcan S 2005. Mass proliferation of Madonna Lily (*Lilium candidum* L.) under *in vitro* conditions. Pak. J. Bot. **37**(2): 243-248.

Loveless MD and Hamrick JL 1984. Ecological determinants of genetic structure in plant populations. Ann. Rev. Ecol. Sys. **15**: 65-95.

Maguire TL and Sedgley M 1997. Genetic diversity in *Banksia* and *Dryandra* (Proteaceae) with emphasis on *Banksia cuneata*, a rare and endangered species. Heredity **79**: 394-401.

Mucaji P, Haladová M, Eisenreichová E, Budsínský E and Ubik K 2000. Ceska a Slovenska Farmacie: Casopis Ceske Farmaceuticke Spolecnosti a Slovenske Farmaceuticke Spolecnosti. Sterols in *Lilium candidum* L. Neoplasma. **47**: 313-318.

Nei M 1972. Genetic Distance Between Populations. Amer. Nat. **106**: 283-292.

Nybom H and Bartish IV 2000. Effects of life history traits and sampling strategies on genetic diversity estimates obtained with RAPD markers in plants. Perspectives in Plant Ecology, Evol. Sys. **3**(2): 93-114.

Nybom H 2004. Comparison of different nuclear DNA markers for estimating intraspecific genetic diversity in plants. Mol. Ecol. **13**(5): 1143-1155.

Özen F, Temeltaş H and Aksoy Ö 2012. The anatomy and morphology of the medicinal plant, *Lilium candidum* L. (Liliaceae), distributed in Marmara region of Turkey. Pak. J. Bot. **44**(4): 1185-1192.

Pei YL, Zou YP, Yin Z, Wang XO, Zhang ZX and Hong DY 1995. Preliminary report of RAPD analysis in *Paeonia suffruticosa* subsp. *spontanea* and *P. rockii.* Acta Phytotax. Sin. **33**: 350-356.

Reznikova SA 1973. Interrelations of anther tissues of *Lilium candidum* L. during microsporogenesis. Sov. J. Develop. Biol. **4**(1):64-72.

Roche NA 1999. A follow up report concerning the impacts of recent and planned development on Mounda Nesting Beach (Kaminia and Potamakia), in Kefallonia, Greece, Council of Europe. Report no. T-PVS (99) **72**: 1-12.

Satou T, Kuroda M, Sashida Y and Hatakeyama Y 1999. Steroidal saponins from the bulbs of *Lilium candidum.* Phytochem. **51**(4): 567- 573.

Sauerland H 1956. Quantitative studies on the effects of roentgen rays after irradiation of various meiosis stages in *Lilium candidum* L. Chromosoma **7**(8):627-54.

Su YJ, Wang T and Huang C 1999. RAPD analysis of different population of *Dacydium pierrei.* Acta Sci. Nat. Univ. Sun. **38**: 99-101.

Surgun Y, Çöl B and Bürün B 2012. Genetic diversity and identification of some Turkish cotton genotypes (*Gossypium hirsutum* L.) by RAPD-PCR analysis. Turk. J. Biol. **36**:143-150.

Uhrin D, Buckova A, Eisenreichova E, Haladova M and Tomko J 1989. Constituents of *Lilium candidum* L. Chem. Pap. **43**: 793-796.

Vachalkova A, Eisenreichova E, Haladova M, Mucaji P, Jozova B and Novotny L 2002. Potential carcinogenic and inhibitory activity of compounds isolated from *Lilium candidum* L. Ces. Slov. Farm. **51**(6):297-300.

Wang XQ, Zou YP, Zhang DM and Hong DY 1996. RAPD analysis for genetic polymorphism in *Cathaya argyrophylla.* Sci. China **26**: 437-441.

West C and Lechmere AE 1915. On chromatin extrusion in pollen mother-cells of *Lilium candidum.* Linn. Soc. Ann. Bot. **29** (2): 285-291.141141

Yeh FC, Young RC, Timothy B, Boyle TBJ, Ye ZH and Mao JX 1997. POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Center, University of Alberta, Canada.

Zawko G, Krauss SL, Dixon KW and Sivasithamparam K 2001. Conservation genetics of the rare and endangered *Leucopogon obtectus* (Ericaceae). Mol. Ecol. **10**(10): 2389-2396.

(Manuscript received on 9 August, 2015; revised on 2 September, 2015)