Journal of Medicinal Plants Research Vol. 4(18), pp. 1888-1894, 18 September, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR10.050
ISSN 1996-0875 ©2010 Academic Journals

Full Length Research Paper

Genetic diversity in two *Lilium* (Liliaceae) species from different regions of Greece based on a random amplified polymorphic DNA (RAPD) analysis

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Accepted 25 August, 2010

Distribution of genetic variation between populations of *Lilium albanicum* and *Lilium chalcedonicum* was investigated using RAPD (random amplified polymorphic DNA) profiles. Plants for the analysis were collected from four different populations, containing 33 individuals. Eleven primers yielded 88 polymorphic bands. UPGMA clustering and principle coordinate analysis indicated a clear separation between the populations of the two species and all the analyzed individuals clustered in three groups, one comprising *L. chalcedonicum* and the other two groups encompassing three populations of *L. albanicum*. The analysis of molecular variance (AMOVA) indicated that 33% of the total variance is between species. The highest amount of genetic differentiation, 51% of the total variance, is found within the individual populations.

Key words: Liliaceae, *Lilium albanicum*, *Lilium chalcedonicum*, random amplified polymorphic DNA (RAPD), genetic variation.

INTRODUCTION

The Balkan Peninsula with eight taxa is one of the centers of species diversity for the genus *Lilium* L. One of those species is *Lilium albanicum* Griseb. which has yellow unspotted turk's-cap flowers and no hairs on the abaxial leaf surfaces but the leaf margins have short, silver hairs. It is the smallest of the *Lilium carniolicum* group of lilies with stems 30 to 40 cm and one or four (but sometimes up to 10) flowers per stem. *Lilium albanicum* is distributed on subalpine slopes or steep meadows in northern Greece, Macedonia, Montenegro, Bulgaria and in Albania. Other closely related species are *L. carniolicum* Bernh., *Lilium jankae* A. Kern and *Lilium bosniacum* G. Beck. *Lilium carniolicum* with orange-red or light red flowers and hairs on the abaxial leaf surfaces has the widest distribution amongst these four species. It occurs in the South East Alps and the Balkan Peninsula (Rešetnik et al., 2007; Synge, 1980). The yellow flowering species, *L. jankae* has larger flowers with leaves equally pubescent on the veins beneath (Synge, 1980). Orange or red flowering *L. bosniacum* has hairs unevenly pubescent only on nerves underneath the leaf surfaces (Meyer 1937; Synge, 1980). Matthews (1984) recognised *Lilium pyrenaicum* Gouan, *L. carniolicum* and *Lilium ponticum* K. Koch from North East Turkey and the adjacent Caucasus at the subspecies level. He divided *L. pyrenaicum* into three subspecies; subsp. *pyrenaicum*, subsp. c*arniolicum* and subsp. *ponticum*. Subspecies *carniolicum* was divided into four varieties; var. *carniolicum*, var. *albanicum*, var. *bosniacum* and var. *jankae* (Matthews, 1984). Davis and Henderson (1984) regarded *L. ponticum* K. Koch as a subspecies of *L. carniolicum*. Ascherson and Graebner (1905) united *L. jankae*, *L. carniolicum*, *L. albanicum* and *Lilium chalcedonicum* and treated *L. albanicum* as a variety of *L. chalcedonicum*. On the other hand, Popova (1981) treated *L. albanicum* as a separate species.

L. chalcedonicum L. has brilliant orange-red turk's-cap flowers with red anthers and up to 10 flowers per stem (Woodcock and Stearn, 1950). It takes its name from Chalcedon, now Kadiköy, a district of Istanbul. This species is found in Greece, Macedonia and southern Albania on limestone, grasslands and in deciduous and coniferous forests (McRae 1998; Micevski and Mayer 1980; and pers. observations). *Lilium heldreichii* Freyn is regarded as a synonym of *L. chalcedonicum*. The spotted form of *L. chalcedonicum* was described as var. *maculatum*. Linneaus in his *Species Plantarum* (1762) did not distinguish *L. carniolicum* from *L. chalcedonicum*

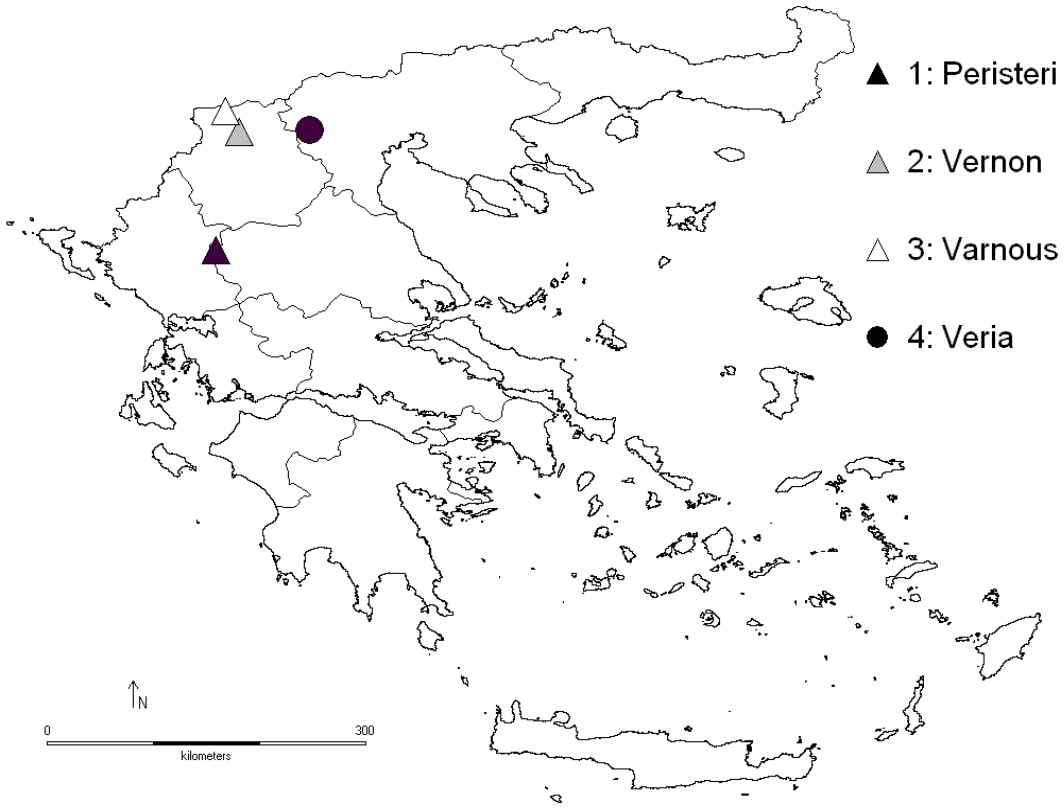

Figure 1. Geographic origin of the studied *L. albanicum* and *L. chalcedonicum* populations. Detailed information about each population is provided in Table 1.

and included *L. carniolicum* within *L. chalcedonicum*.

Baker (1871) and Wilson (1925) included *L. albanicum* and *L. chalcedonicum* in section *Martagon* Rchb. Comber (1949) classified *L. albanicum* and *L. chalcedonicum* under the section *Liriotypus* Asch. and Graebn. Baranova (1988) placed two species in section *Eurolirium* M. Baranova. Recent molecular studies showed that two species are in section *Liriotypus*, together with other European lilies except for *Lilium martagon* and *Lilium bulbiferum* (Rešetnik et al., 2007; İkinci et al., 2006; Nishikawa, 2001). Rešetnik et al. (2007) demonstrated that *L. albanicum* and *L. chalcedonicum* have very similar nuclear ribosomal DNA internal transcribed spacer (ITS) sequences.

The RAPD technique provides a high number of marker loci and high levels of polymorphisms and allows detection of genotypes in a population. Different methods were tested to reveal the relationships and species boundaries amongst these *Lilium* species utilizing DNA fingerprinting techniques. Chloroplast microsatellites (Weising and Gardner, 1999) tested for these species did not provide polymorphic bands and AFLP analysis did not generate reproducible amplification products. RAPD markers were employed to study genetic relationships amongst six north eastern Turkish *Lilium* species (İkinci and Oberprieler, 2010), for the systematic of Korean *Lilium* species (Bin et al., 1993), for identification of Asiatic lily hybrids (Lee et al., 1996; Yamagishi, 1995), and for measuring genetic diversity in *L. martagon* populations (Persson et al., 1998) and in two different varieties of *L. longiflorum* (Wen and Hsiao, 1999; 2001).

The objectives of this study are to investigate the pattern of genetic variation of *L. albanicum* and *L. chalcedonicum* populations at the molecular level and to resolve the relationships between these closely related species via RAPD profiles from four populations.

MATERIALS AND METHODS

The study material was collected from wild populations in Greece (Figure 1) in 2004. Silica gel preserved leaf tissue and herbarium voucher specimens were collected from each population (Table 1). Voucher specimens representing all populations were deposited in the herbarium of Abant Izzet Baysal University (AIBU). A total of 33 individuals from four different populations (six to ten individuals per population) of *L. albanicum* and *L. chalcedonicum* were included in the present study.

DNA extraction and RAPD PCR amplification

Total genomic DNA was extracted from around 4 mg of silica-gel dried leaf tissue using a modified CTAB protocol (Wagner et al., 1987) and Qiagen Dneasy Plant Mini Kit following

Table 1. Sampled populations of *Lilium* species. All of the populations are from Greece and the voucher specimens are deposited at AIBU.

Species	Population number	Locality name, voucher number	Longitude, latitude	Altitude (m)	Sample size
Lilium albanicum	1	Trikalon, Kalambakas, Mt. Peristeri. *Th. Constantinidis 11182*	39°37´N, 21°10´E	1650	6
	2	Between Florinis and Kastorias, Mt Vernon (Vitsi), *İkinci 2269*	40°39´N, 21°22´E	1900-2000	10
	3	Florinis, Eparchia Florinis, Mt Varnous, Summit Kalo Nero (Bela Voda), *İkinci 2272*	40°50´N, 21°15´E	1950-2000	9
L. chalcedonicum	4	Veroia (Veria), Metamorphis Village, *İkinci 2255*	40°40´N, 21°58´E	1160	8
Total					33

manufacturer's protocol. Concentrations of the extracted DNA were adjusted as 10 ng/µl prior to PCR reactions. Random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) was performed with the method as detailed in İkinci and Oberprieler (2010).

Screening of primers

Initially 108 decamer primers were used for screening (A, AB, B, C, J, and I Kits from Roth Company) on individuals from different populations to identify primers that exhibited scorable polymorphisms and gave reproducible results. After the initial screening, 11 primers were selected for further analysis (Table 2). For each selected primer, all the PCR reactions and agarose gels were run simultaneously to prevent errors resulting from ambient temperature and other circumstances that could lead to low reproducibility. Some of the reactions were repeated to confirm the reproducibility. Faint bands and individuals with strongly deviating band profiles were excluded from further analyses. Determination of band identity was aided by the computer program BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium). Bands with the same size were considered homologous and bands with different sizes were assumed to represent different loci. Data were organized as binary matrices (0 for absence and 1 for presence of a given band).

Statistical analysis

Unweighted pair group method with arithmetic mean (UPGMA) cluster analysis of Euclidean distances was calculated and a dendogram was obtained. Additionally, principle coordinate analysis (PCO) was performed also based on Euclidean distances amongst OTUs using the software program MVSP, version 3.13n (Kovach, 2005). Population structure was investigated by analysis of molecular variance (program AMOVA; Excoffier et al., 1992) in ARLEQUIN version 3.1 (Excoffier et al., 2005) in order to quantify the distribution of RAPD variance components between species among populations, and within populations. Variance components were calculated from a matrix of squared Euclidian distances among all pairs of RAPD phenotypes (Excoffier et al., 1992; Huff et al., 1993). The significance of the variance components and Φ-statistics was tested using a permutation approach (Excoffier et al., 1992; 1023 permutations). Pairwise genetic distances (Φ_{ST}) among four populations were also obtained from AMOVA.

RESULTS

After excluding bands that were monomorphic for thewhole dataset, a total of 88 bands were generated by 11 primers for 33 individuals from four different populations of the two *Lilium* species (Table 2). The number of bands per primer ranged from five to 13. All 33 individuals had unique band patterns.

In principle coordinate analysis (PCO) based on Euclidean distances the first principal co-ordinates accounted for a total of 46.361% of the total variance (24.42, 16.37 and 5.57%, respectively) (Figure 2). PCO divided the four populations into three distinct groups and the analysis indicates a clear separation between the individuals of *L. albanicum* and *L. chalcedonicum*. All representatives of *L. chalcedonicum* (population 4) formed a single distinct group, and members of *L. albanicum* were clustered in two groups. The first group comprised all the individuals from Varnous Mountain (population 3) and three individuals from Vernon Mountain (population 2). The second group is made up of all members of Peristeri Mountain (population 1) and the remaining Vernon Mountain populations.

The dendogram obtained from the UPGMA analysis based on Euclidean distances revealed three groups similar to the pattern as with PCO. The results indicate that members of the *L. chalcedonicum* form a separate cluster. Individuals from three different populations of *L. albanicum* were clustered in two groups. The first group contains all the representatives of Varnous Mountain population and three individuals from Vernon Mountain populations. The second group contains all individuals of Peristeri Mountain population and seven individual from Vernon Mountain population. There is no clear-cut difference between the populations of Peristeri Mountain and Vernon Mountain

Variation in RAPD patterns was analyzed by analysis of molecular variance (AMOVA). This analysis allows us to calculate variance components and their significance levels for variation between species, among populations, within species and within populations. AMOVA

Table 2. Primer sequences and the number of polymorphic fragments generated by 11 arbitrary primers.

Primer	Nucleotide sequence (5'-3')	Number of fragments
I-15	TCA TCC GAG G	13
AB-02	GGA AAC CCC T	8
AB-11	GTG CGC AAT G	11
AB-14	AAG TGC GAC C	6
AB-16	CCC GGA TGG T	9
C-04	CCG CAT CTA C	5
C-10	TGT CTG GGT G	6
C-11	AAA GCT GCG G	7
C-14	TGC GTG CTT G	7
C-16	CAC ACT CCA G	10
C-20	ACT TCG CCA C	6
Total		88

Table 3. Summary of analysis of molecular variance (AMOVA).

Source of variation	d.f.	Sum of squares	Variance	% Total	*Φ*-Statistics	(*P*-value)
Between species	1	115.827	6.490	33	Φ_{CT} = 0.33	0.241
Among populations/ within species	2	74.298	3.320	17	Φ_{SC} = 0.25	< 0.001
Within populations	29	291.572	10.054	51	Φ_{ST} = 0.49	< 0.001
Total	32	481.697	19.864	100		

Summary of analysis of molecular variance (AMOVA) for the partitioning of RAPD variation among species and among populations within groups based on 88 bands states. Statistics include degrees of freedom (d.f.), AMOVA sum of squares, variance components, percentage of variation (% Total), differentiation values (Φ_{CT}, Φ_{SC}, Φ_{ST}) and their levels of significance (P-value) which are based on 1000 permutations.

computations were based on the pairwise squared Euclidean distance matrix and the results of Φ-statistics are given in Table 3. The analysis of molecular variance showed a 33% genetic differentiation between species. The genetic differentiation among the populations within the different species is 17%. The highest amount of genetic differentiation is found within the individual populations (51% of the total variance). All three variance components are highly significant.

DISCUSSION

Results of random amplified polymorphic DNA (RAPD) analysis provided high-resolution for the detection of genetic variation among and within natural populations of *L. albanicum* and *L. chalcedonicum*. Additionally, the results obtained contributed to understanding the relationships between these closely related species. UPGMA cluster and principle coordinate analyses (PCO) indicated a clear separation between the populations of the two species. A recent molecular phylogenetic study utilizing plastid and nuclear DNA markers about section *Liriotypus* of the genus *Lilium* also included *L. albanicum* and *L. chalcedonicum* (İkinci, 2011). In this study, *L. albanicum* and *L. chalcedonicum* were clustered together with other European and Caucasian lilies. However, employed molecular marker did not provide further resolution concerning the species relationships.

The AMOVA showed a considerable level of genetic differentiation between species (33%). Genetic variation among populations within species was low (17%). High percentage of within population variation was reported from different out-crossing species based on RAPD markers (Huff et al., 1998; Kolliker et al., 1998; Wen and Hsiao, 1999; Wen and Hsiao, 2001). On the other hand, species with self breeding systems have higher levels of among population diversity (Sun and Wong, 2001). However, *Lilium* species are cross-pollinated, and they show strong self-incompatibility (McRae, 1998; Pelkonen et al., 2007). More than half of the total variance (51%) is found among individuals within populations. Similar genetic differentiation values were also obtained from a recent population level study on north eastern Turkish *Lilium* species (Ikinci and Oberprieler, 2010).

In addition, the study of Wen and Hsiao (2001) on *L. longiflorum* var. *formasanum* using RAPD markers indicated that 92.09% of the total variation was found among individuals within populations. A study on *L. martagon* L. showed a high level of genetic variability at intrapopulational level even for the populations introduced into cultivation (Persson et al., 1998). The authors' explanation is that this species has an outcrossing breeding system and a long generation time due to its perennial habit. In terms

Table 4. Pairwise genetic distances (Φ_{ST}) and geographical distances (in paranthesis; km) among four populations of *L. albanicum* and *L. chalcedonicum.*

Population	Population			
	1	2	3	4
1	0.0000			
2	0.0970 (115)	0.0000		
3	0.3371 (135)	0.2349 (24)	0.0000	
4	0.5408 (135)	0.4947 (50)	0.5096 (65)	0.0000

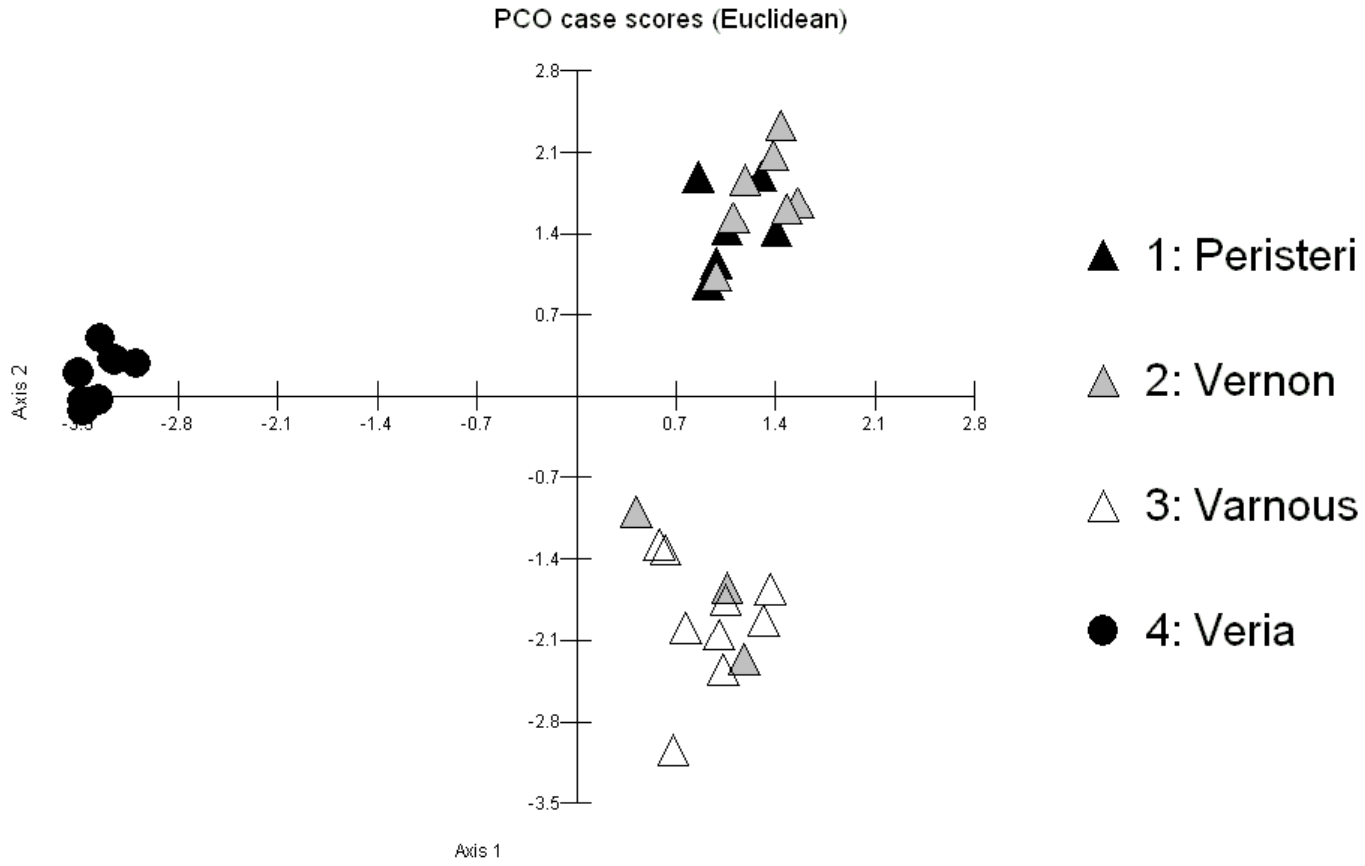

Figure 2. Ordination of OTUs in a principle coordinate analysis (PCO) based on Euclidean distances inferred from RAPD fragments. Space defined by the first two coordinates. Detailed information about each population is provided in Table 1.

of pairwise genetic distances, the population of *L. chalcedonicum* has a higher genetic distance with the three other populations of *L. albanicum* also correlated with geographic distance (Table 4). Among all three populations of *L. albanicum*, the highest genetic distance (0.3371) is between populations 3 and 1 which are also the most geographically separated (135 km) populations. Although populations 3 and 2 are only 24 km apart from each other, they have a higher genetic distance (0.2349) than populations 2 and 1 which are 115 km apart and have the lowest genetic distance (0.0970). These results indicate that genetic connectivity among populations of *L. albanicum* does not decrease with increasing spatial distance.

Conclusion

Our results based on analysis of RAPD profiles produced basic genetic information about natural populations of *L. albanicum* and *L. chalcedonicum*. Future studies, covering the complete range of geographical distribution of the two *Lilium* species, will allow better understanding of the genetic variation of those species.

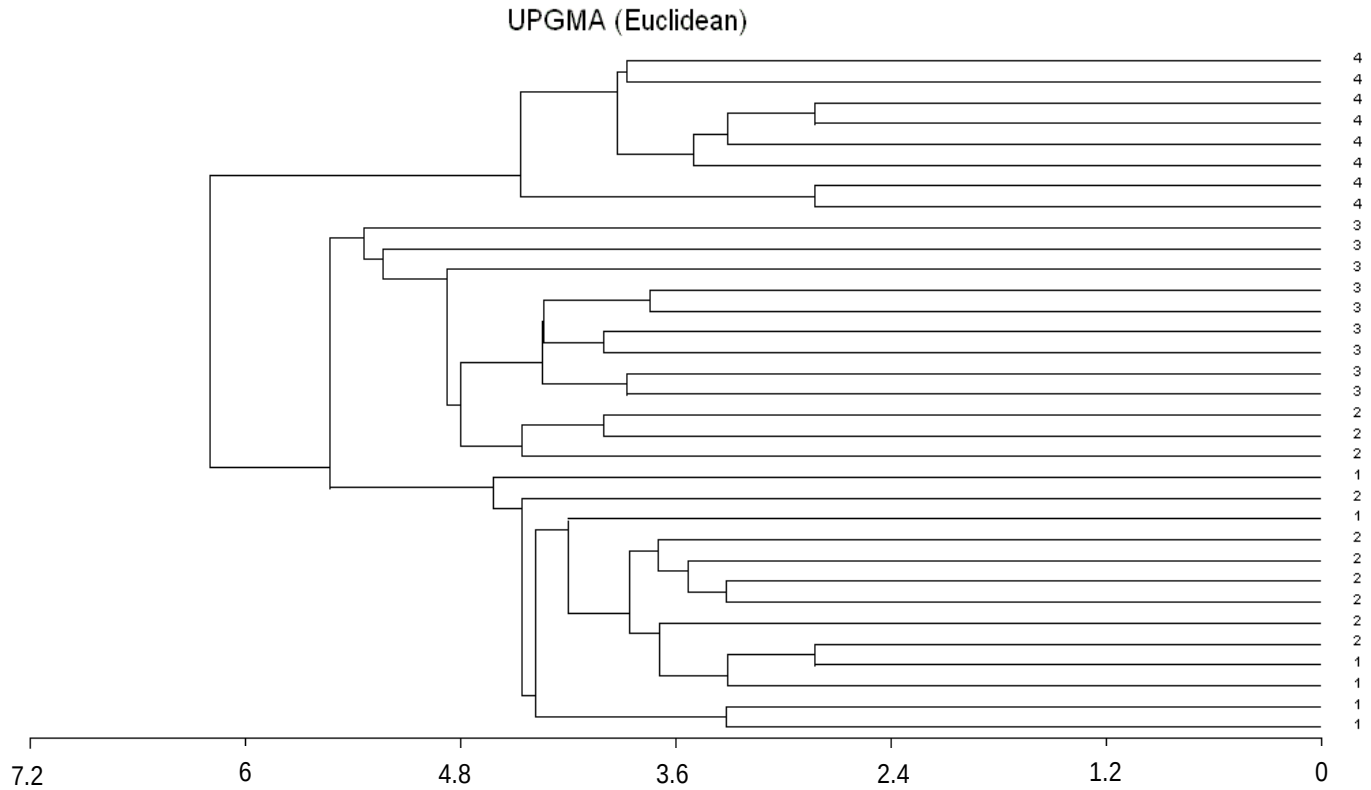

Figure 3. Dendogram of UPGMA cluster analysis based on the Euclidean measure of genetic distance. Numbers indicate populations as in Table 1

ACKNOWLEDGEMENTS

The author thanks the Ali Nihat Gökyigit Foundation for their partial financial support for the laboratory expenses, Christoph Oberprieler and Christoph Reisch (both from the Botanical Institute, Regensburg University) for their help with the RAPD technique, Alexandra Vasiliou and Theophanis Constantinidis (Athens) for providing assistance during the field work in Greece and Margaret Johnson for checking the manuscript.

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