

*Full Length Research Paper*

# Determination of the relationship among Old World Lupin (*Lupinus* sp.) species using RAPD and ISSR markers

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Randomly Amplified Polymorphic DNA (RAPD) and Inter Simple Sequence Repeat (ISSR) markers were used to determine the genetic relationships among 20 Old World lupin genotypes from 3 lupin species (*Lupinus albus*, *L. angustifolius*, *L. luteus*) using 15 primers in each case. Polymorphism was observed in reactions with 9 out of 15 tested RAPD primers, and 11 out of 15 tested ISSR primers. Relationship was determined on the basis of polymorphic products analysis which was shown in the form of dendrograms by UPGMA method. Both RAPD and ISSR markers worked well to identify distinct grouping among three species and among genotypes within a species. The parallel use of both data set seems to allow precise estimation of cultivars relationship and diminishing mistakes connected with method's technical limitations. Such marker systems may potentially be used in the development of new lupins cultivars.

**Key words:** White lupin, genetic diversity, RAPD, ISSR.

## INTRODUCTION

Originally the name lupin is derived from the latin "*Lupus*" (wolf) thinking that they deplete soil nutrients, but the opposite is true (Christou, 1992) as they are among legumes. The Ancient Greeks referred to call lupin as Thermes, while it is called Turmus in most Arab countries and India (Belteky and Kovacs, 1984). The plant is named Termiye or Acibakla in Turkiye.

The genus *Lupinus* comprises more than 300 species, but only four of them have gained agricultural importance. These are *L. albus* (white lupin), *L. angustifolius* (blue or narrow-leafed lupin) and *L. luteus* (yellow lupin) of the "Old World" lupin species, and one "New World" species namely *L. mutabilis* (Pearl lupin or Tarwi). The first three species originate from the Mediterranean area, while *L. mutabilis* belongs to South America. The genus is comprised of geographically separated centres of diversity (Hondelmann, 1984). Old World lupin species are all large-seeded and annuals. They are also divided into 2 sub-groups being rough-seeded, which are usually of

Mediterranean origin and smooth-seeded of both Mediterranean or African origin (Plitmann and Heyn, 1984).

Hutchison (1964) treated *Lupinus* as a separate tribe, but Plitmann (1981) placed *Lupinus* in an intermediate position between the *Genisteae* and the *Thermopsidaeae* although *Lupinus* is the only grain legume genus that is included the the tribe *Genisteae*. Christofolini (1989) agrees with the presence of *Lupinus*, as a natural genus, in this tribe after immunological comparison of seed reserve proteins. However, Badr et al. (1994) criticised the inclusion of lupins in the *Genisteae* after studies on restriction site polymorphism in chloroplast DNA (cpDNA) of 35 legumes in the tribe *Genisteae* and suggests that American and Mediterranean *Lupinus* species should belong to a monophyletic group which arose from a single centre of diversification. Such molecular studies, in comparison with other species of the genera, should help understanding the evolution of the genus.

Recently, Naganowska et al. (2006) studied the evolution of the genus *Lupinus* (*Fabaceae*) by analysing 2C-DNA values of 38 species and accessions from the Old and New World using flow cytometry. They found

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**Table 1.** The plant materials used in RAPD and ISSR analyses.

No	Genotypes	Form	Place of origin	No	Genotypes	Form	Place of origin
1	<i>L. albus</i> line EGY-37	Bitter	Egypt*	11	<i>L. albus</i> cv. Giza-I	Bitter	Egypt*
2	<i>L. albus</i> line EGY-53	Bitter	Egypt*	12	<i>L. albus</i> cv. Giza-II	Bitter	Egypt*
3	<i>L. albus</i> line EGY-99	Bitter	Egypt*	13	<i>L. albus</i> cv. Local population	Bitter	Turkiye
4	<i>L. albus</i> line EGY-103	Bitter	Egypt*	14	<i>L. angustifolius</i> cv. Kubesa	Bitter	Germany***
5	<i>L. albus</i> line EGY-105	Bitter	Egypt*	15	<i>L. angustifolius</i> cv. Belera	Bitter	Australia**
6	<i>L. albus</i> line EGY-121	Bitter	Egypt*	16	<i>L. angustifolius</i> cv. Gunguru	Bitter	Australia**
7	<i>L. albus</i> line USA-6310 B	Bitter	USA**	17	<i>L. angustifolius</i> cv. Tanjil	Bitter	Australia**
8	<i>L. albus</i> line USA-6313 B	Bitter	USA**	18	<i>L. angustifolius</i> cv. Marri	Bitter	Australia**
9	<i>L. albus</i> cv. Lublanc	Sweet	France*	19	<i>L. angustifolius</i> cv. Yorrel	Bitter	Australia**
10	<i>L. albus</i> cv. Lutop	Sweet	France*.#	20	<i>L. luteus</i> cv. Topaz	Sweet	Germany***

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#Small grain size.

That *L. albus*, *L. angustifolius* and *L. luteus* had mean 2C-DNA values of 1.16, 1.89 and 2.44 pg, respectively, and they are phylogenetically separate although 3 species were placed together in the smooth seeded lupin group of Old World species.

During recent years, sequence specific polymerase chain reaction (PCR) based markers have been developed and implemented for marker-assisted selection in lupin breeding programmes (Ainouche et al., 2004; Jürgen and Weder, 2002; Yang et al., 2001, 2002, 2004; You et al., 2005, Yuan et al., 2005). For gene tagging, RAPD methodologies are now routinely handled by several laboratories involved in lupin breeding (Qiu et al., 1995).

Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR) and Amplified Fragment Length Polymorphism (AFLP) markers could be used for the assessment of genetic diversity among *Lupinus* spp. AFLP, ISSR and RAPD markers were used to identify the genetic differences among lupin species (*L. albus*, *L. angustifolius*, *L. cosentinii*, *L. hispanicus*, *L. luteus*, *L. mutabilis*, *L. pilosus* and *L. polyphyllus*) (Talhinhas et al., 2003). Accordingly, Old and New World Lupin species were distinguished from each other with similarity existing among *L. angustifolius*, *L. hispanicus*, *L. luteus*. The results of these markers matched well with the previous ITS (rDNA) and *rbcL* (chloroplast gene) sequence analyses. Thus, highly reliable genetic analysis within the *Lupinus* genus (inter-specific level) could be performed with less demanding and more economical techniques like RAPD and possibly more preferably ISSR.

Yuan et al. (2005) tested 77 cultivar-specific markers in 23 blue lupin (*L. angustifolius* L.) cultivars and a dichotomous fingerprinting key was developed for the molecular identification of lupin cultivars. Cluster analysis of pairwise distance matrix computed from RAMP profiles

grouped the 23 cultivars into 4-5 clusters, which generally agreed with their pedigree relationships.

In this study, genetical relationships of some Old World lupins were investigated using RAPD and ISSR primers aiming to contribute to lupin breeding programmes involving marker-assisted breeding of the species. *L. albus* alkaloid containing (bitter) local population used in this study is the only cultivated lupin in Turkiye in a limited area (400 ha) of Central Anatolia having lower soil pH compared to many other regions with high lime soils and neutral to alkali soil pH limiting lupin cultivation. Although Egypt and Turkiye have frequently been the Old World lupin germplasm collection sites for many World-wide lupin breeders, such studies are rarely done in the countries of origin. The other aim of this study was to form a basis for molecular lupin breeding in Turkiye at least at the methodic level.

## MATERIALS AND METHODS

### Plant material

A total of 20 lupin genotypes from *Lupinus albus* (13) ( $2n=4x=50$ ), *L. angustifolius* (6) ( $2n=4x=40$ ) and *L. luteus* (1) ( $2n=4x=52$ ) kindly provided by Australian, French and German researchers (Table 1), and one Turkish local *L. albus* landrace were used (Mulayim et al., 2002). To study higher number of genotypes was not at our option particularly from *L. angustifolius* and *L. luteus*. In addition, we mainly concentrated studying on a broader range of *L. albus* genotypes as this species is the only cultivated Turkish species. Available genotypes from the other two species were used for the purpose of comparisons. Seventeen out of 20 genotypes are bitter (alkaloid-producing), while 3 are sweet forms (alkaloid free) (Table 1). Plant materials were grown in pot in a controlled glass-house.

### DNA isolation, RAPD and ISSR assays

Leaves of 10 plants (3 wk old) were mixed to create a bulk from

**Table 2.** Characteristics of oligonucleotides used in degenerate and inverse PCR reactions for RAPD assays.

RAPD	Sequence 5'-3'	T <sub>m</sub> (°C)	bp	G/C (%)	Number of bands	Polymorphic bands	Polymorphism (%)
B3	GATGACCGCC	34	10	70	5	5	100
B4	CTCACCGTCC	34	10	70	8	8	100
B11	CCTCTGACTG	32	10	60	6	6	100
B12.2	TCCGATGCTG	32	10	60	6	6	100
B13	TTCAGGGTGG	32	10	60	2	2	100
B14	TCCTGGTCCC	34	10	70	7	7	100
B15	ACCGTTCCAG	32	10	60	6	6	100
B17	GTCGTTCTG	32	10	60	5	5	100
B18	GAGTCAGCAG	32	10	60	4	4	100
Total					49	49	100

T<sub>m</sub>, Temperature melting; bp, base pair.

B3, B4 (Volis et al., 2001); B11-B18 (Fernández et al., 2002).

which 200 mg were used for DNA isolation. Genomic DNA was extracted using a standard protocol (2x CTAB method) with minor modifications. DNA concentrations were determined by an Eppendorf BioPhotometer. DNA samples were run on 1% (w/v) agarose (Promega) gels in the presence of *EcoRI* *HindIII*-digested  $\lambda$  DNA (Fermentas Life Sciences).

Out of 15, 9 RAPD and 11 ISSR the most suitable primers (in terms of repeatability, scorability and the ability to distinguish between accessions) were selected for identification (Tables 2 and 3).

a) RAPD: Each reaction mix contained 2.5 mM MgCl<sub>2</sub> (Bioron), 1x PCR Buffer (Bioron), 2.5  $\mu$ M of each of dNTPs (Bioron); 0.5  $\mu$ M primer, 2  $\mu$ L of 25 ng DNA template and 0.3 units of Taq DNA Polymerase (Bioron) in a final reaction volume of 25  $\mu$ L. After a pre-denaturation step of 5 min at 94°C, amplification reactions were cycled 35 times at 94°C for 1 min, at 32-34°C annealing temperature for 1 min and 72°C for 2 min in mastercycler gradient thermocycler (Eppendorf). A final extension was allowed for 10 min at 72°C.

b) ISSR: Each reaction mix of ISSR primers was prepared as RAPD. The PCR reaction was carried out in a touchdown fashion with a first denaturation at 94°C for 5 min, followed by 15 cycles of; (1) denaturation at 94°C for 1 min, (2) annealing at Temperature melting (T<sub>m</sub>) for 1 min, and (3) extension at 72°C for 2 min, with the annealing temperature being reduced by 0.5°C per cycle. This procedure was followed by 35 cycles of; (1) denaturation at 94°C for 1 min, (2) annealing at T<sub>m</sub> for 1 min, and (3) extension at 72°C for 2 min, and a final extension at 72°C for 10 min. PCR applications were repeated at least twice. Eleven primers producing consistent and polymorphic fragments were selected for PCR amplification.

Upon completion of the reaction, amplified products were electrophoresed onto a 2% (w/v) agarose/1x Tris-Borate EDTA gel and followed by visualization in a Vilber Lour-mat (France) device.

#### Data analyses

RAPD and ISSR assays were repeated at least twice for each primer and only the reproducible fragments were scored, with special emphasis on the repeatability of the bands that present polymorphism. Each DNA fragment generated were treated as a separate character and scored accordingly (1 for the presence and 0 for the absence). A rectangular binary data matrix of 15 x 64 was prepared and its statistical analysis was performed using the NTSYS-pc (Ver.

1.7; Rohlf 1992). In cluster analysis the unweighted pairgroup method with the arithmetic mean (UPGMA) procedure was followed.

## RESULTS AND DISCUSSION

Bands of RAPD (49) and ISSR (76) were scored (Tables 2 and 3). The individual and combined RAPD and ISSR UPGMA dendrograms are given in Figures 1 to 3.

#### RAPD assay

Although RAPD assay resulted in low number of polymorphic fragments, a total of 49 scorable bands were obtained from 9 selected RAPD primers, which were able to reveal polymorphism among the genotypes with genetic distances calculated ranging from 0.42 to 0.98 as per the Jaccard coefficient. Among 9 primers, B4 yielded in the highest number of bands (8) whereas B13 had the poorest (2) band yield (Table 2).

The results of cluster analyses indicate that three species distinctly disintegrates with variations existing among the genotypes within each species. Among *L. albus* genotypes; local bitter *L. albus* showed closer genetic distance to Lublanc than other genotypes collected from Egypt, USA and Germany (Figure 1, Table 1). On the other hand two American genotypes were genetically closer to Egyptian genotypes.

#### ISSR assay

A total of 76 amplification loci were obtained from 11 selected ISSR primers, of which 98.6% revealed polymorphism among the genotypes (Table 3). Genetic distances calculated with the Jaccard coefficient ranged

**Table 3.** Characteristics of oligonucleotides used in PCR reactions for ISSR assays.

ISSR	Sequence 5'-3'	Tm (°C)	bp	G/C (%)	Number of bands	Polymorphic bands	Polymorphism (%)
F1	GAG(CAA) <sub>5</sub>	49.1	18	38.9	8	8	100
F2	CTC(GT) <sub>8</sub>	56.7	19	52.6	5	5	100
F3	(AG) <sub>8</sub> CG	56.0	18	55.6	21	21	100
F4	(AG) <sub>8</sub> TG	53.7	18	50.0	9	9	100
F5	(AG) <sub>8</sub>	49.2	16	50.0	6	6	100
F6	C(CAC) <sub>4</sub> CA	53.3	15	66.7	5	4	80
F7	(AC) <sub>8</sub>	49.2	16	50.0	5	5	100
F8	(GCC) <sub>5</sub>	67.0	15	100.0	8	8	100
F9	(GAA) <sub>5</sub>	39.6	15	33.3	6	6	100
M5	(GA) <sub>9</sub> C	56.7	19	52.6	1	1	100
M7	(AG) <sub>9</sub> C	56.7	19	52.6	2	2	100
Total					76	75	98.6

Tm, Temperature melting; bp, base pair.

F1-F9, (Galvan et al., 2003); M5, M7 (Domenyuk et al., 2002)

from 0.37 to 0.96. The primer yielding the highest number of polymorphic bands (21) was F3, whereas M5 produced the fewest number of polymorphic bands (1) (Table 3). The dendrogram produced as a result of genetic relationship analysis conducted through scoring of fragments is given in Figure 2.

The dendrogram generated with the NTSYS-pc packaged software when the RAPD and ISSR data were used together are given in Figure 3. Combined evaluation of RAPD and ISSR results for the three genera namely *L. albus*, *L. angustifolius* and *L. luteus* have further confirmed individual results of both techniques by clearly showing 3 separate groups (Figure 3) but with more conforming results to those obtained from ISSR assays.

Talhinhas et al. (2003) attempted to determine genetic differences among old (*L. albus*, *L. angustifolius*, *L. cosentinii*, *L. hispanicus*, *L. luteus*) and new world (*L. mutabilis*, *L. pilosus* and *L. polyphyllus*) lupin genotypes using AFLP, ISSR and RAPD techniques, and stated that the genotypes of the old and the new world differed from one another through molecular methods as confirmed again by the current study. Both RAPD and ISSR as dominant markers and their combination revealed that *L. albus*, *L. angustifolius*, *L. luteus* were placed in 3 distinct groups with minor genetical distances between the members of each group. White lupins (*L. albus*) genotypes from USA seemed that they were originally introduced to USA from Egypt.

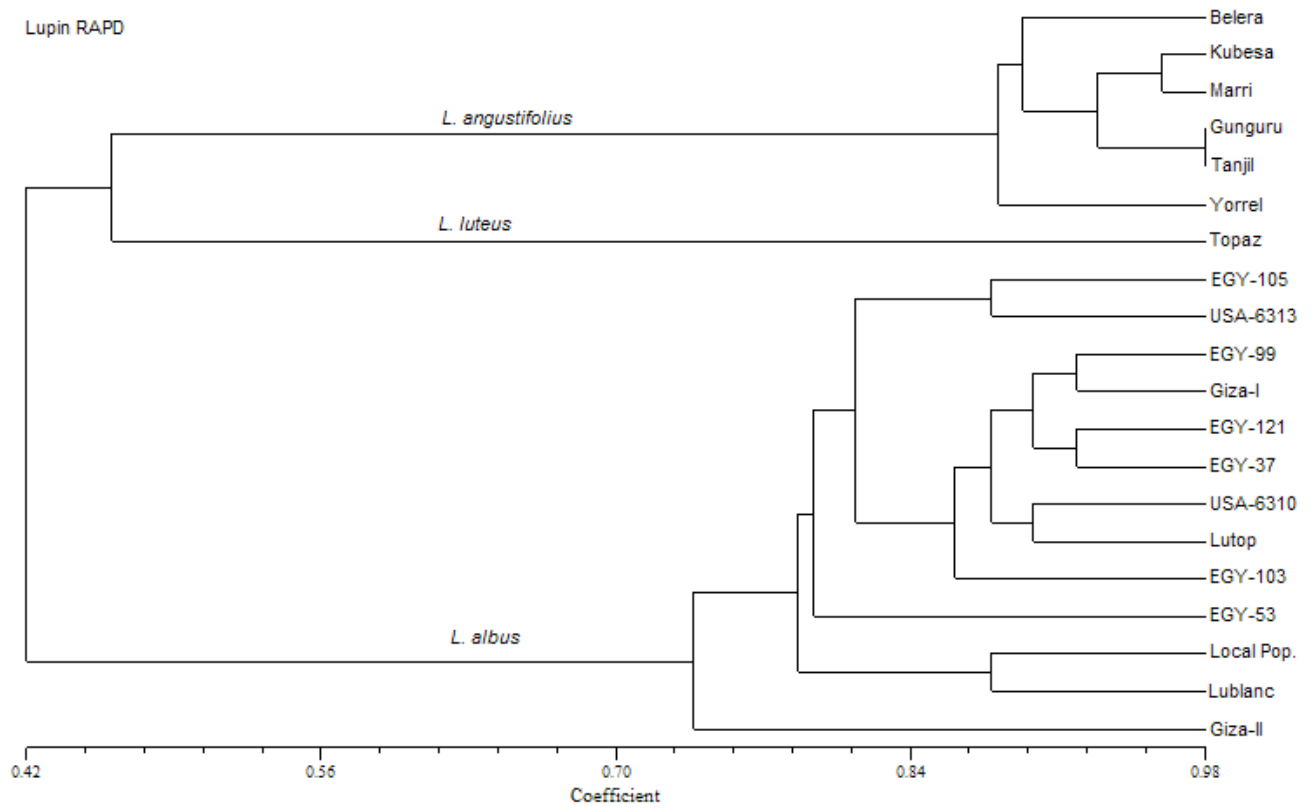
Similarly, Lublanc can be a selection form of sweet type from bitter local *L. albus* genetic pool (Turkiye or bordering countries) considering that this species belongs to Mediterranean region as the center of origin. However, according to RAPD assays, very close genetic distance of Lutop and USA-6310 with small and large grain sizes, respectively may either show that RAPD is not a sensitive method or primers used are not sufficient to reveal

genetic relationships with respect to grain size.

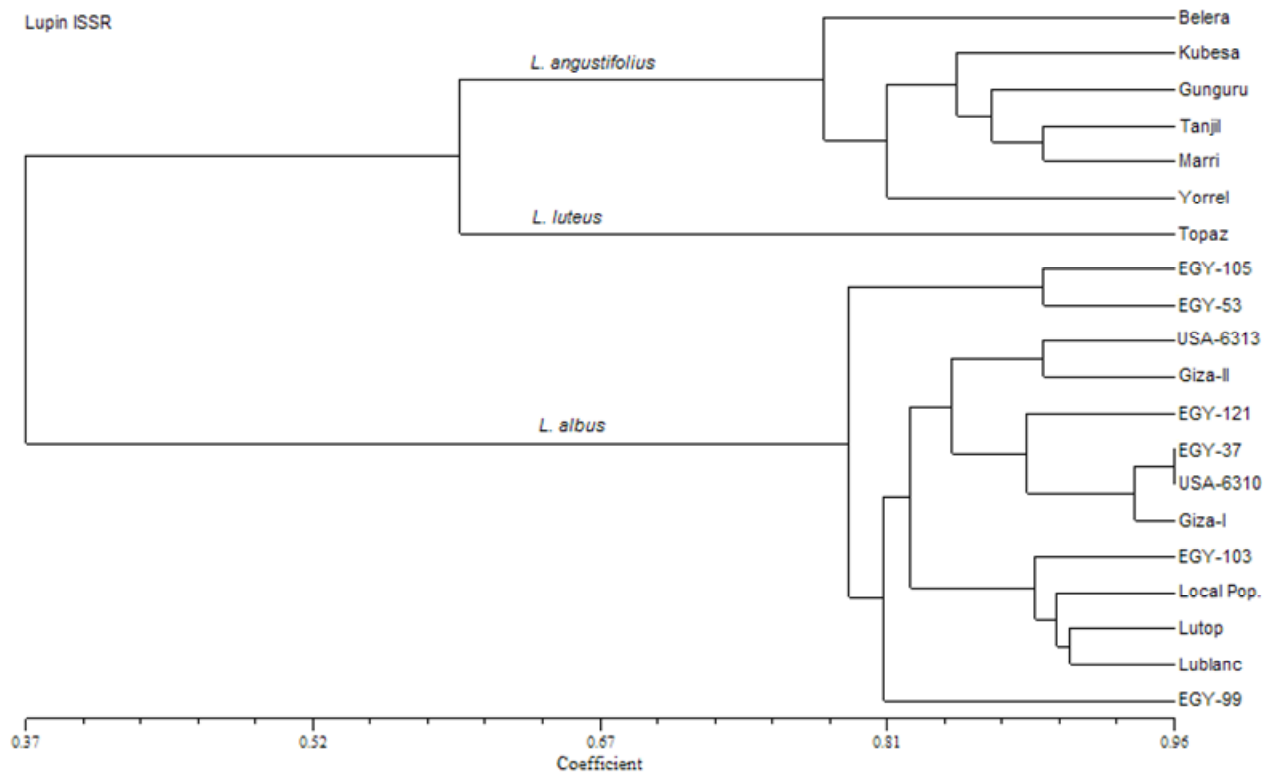
According to ISSR assays, three distinct groups of lupin genera clearly differed from one another in parallel to RAPD results but with minor variations. The ISSR data also demonstrated the close genetic relationships between the genotypes from Egypt and USA confirming RAPD results. However, the genotype USA-6310 was found to be similar in nature to the Egyptian genotype sample of EGY-37 in contrast to Lutop which was shown to be too close to this genotype by RAPD. The other Egyptian genotypes in closest relationship with these was Giza-I. Giza-II, on the other hand, was in close relationship with the genotype of USA-6313. Accordingly it can be speculated that USA-6313 genotype was selected from Egyptian origin materials (e.g. from Giza-II). ISSR data revealed that Turkish local white lupin genotype appeared to be equal in but closer genetical distance to both Lutop and Lublanc in contrast to RAPD results revealing only closer coefficient of genetical variation of Lublanc with Turkish genotype. This group consisting of three (Local population, Lublanc and Lutop) genotypes was in close relationship to EGY-103.

Conforming results were obtained by the combined analysis of RAPD and ISSR data as revealed by the joint dendrogram. However, combined dendrogram was more conforming with the results of ISSR rather than RAPD results. This may be due to more bands produced by ISSR markers than RAPD markers. In fact ISSR markers are known to be more sensitive than RAPD which is again confirmed here.

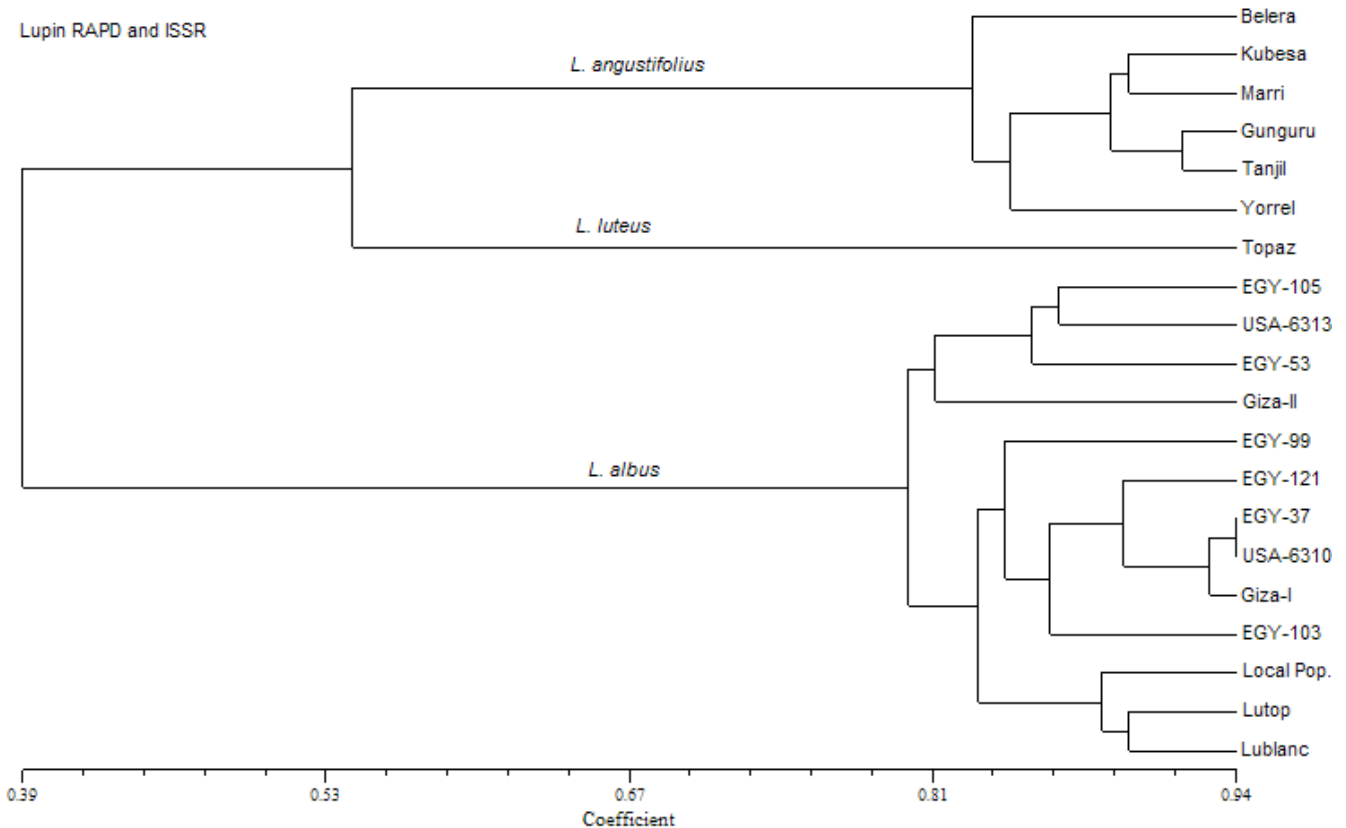
During recent years, sequence specific PCR based markers have been developed and implemented for marker-assisted selection in lupin breeding programmes (Aïnouche et al., 2004; Jürgen and Weder, 2002; Yang et al., 2001, 2002, 2004; You et al., 2005; Yuan et al., 2005). Yorgancılar et al. (2007) studied high pH and free



**Figure 1.** RAPD dendrogram showing the genetic relationships among 20 lupin genotypes from three species.



**Figure 2.** ISSR dendrogram showing the genetic relationships among 20 lupin genotypes from three species.



**Figure 3.** Combined RAPD and ISSR dendrogram showing the genetic relationships among 20 lupin genotypes from three species.

lime tolerances of some genotypes used in this study. Accordingly, high pH and lime tolerant genotypes were placed in the same group as revealed by both RAPD and ISSR analyses. Since lupins are sensitive to free lime in soil, to widen lupin cultivated areas tolerant cultivars are needed. According to the results of this study, it seems that molecular markers can contribute to early selection of tolerant genotypes in lupin breeding programs. If it is taken into consideration that the native genotype contains alkaloid (bitter) whereas Lutop and Lublanc are sweet (do not produce alkaloid), it turns out that molecular techniques could be safely used in efforts aimed at early selection of low alkaloid producing genotypes too.

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