Screening of cytoplasmic DNA diversity between and within *Lupinus mutabilis* Sweet and *Lupinus albus* sensu lato by restriction fragment length polymorphism (RFLP)

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Abstract. Seven populations and five mutant lines of the Andean lupin and four species from the section *Albus* were screened for their mitochondrial and chloroplast polymorphisms. For this purpose the RFLP method with *Eco*RI as a restriction enzyme was used. *Lupinus luteus*, *Lupinus albus* and *Phaseolus vulgaris* organellar clones as well as amplified fragments were used as probes. We found that mitochondrial probes were more suitable than chloroplast probes for identification of inter- and intra-specific variations within the examined material. Most mitochondrial probes differentiate the two species investigated. A high level of mitochondrial polymorphism was observed among the populations of *L. mutabilis* in contrast to monomorphism among the species in the section *Albus*. A limited polymorphism was detected between the mutant lines of *L. mutabilis*. We conclude from this study that the mitochondrial RFLP analysis is a valuable tool for identification of variability among Andean lupin populations.

Key words: cytoplasmic DNA, *Lupinus mutabilis*, *Lupinus albus*, RFLP.

Introduction

The Andean lupin (*Lupinus mutabilis* Sweet) and the white lupin (*Lupinus albus* L.) are promising crops for Central Europe due to their valuable seed com-

position characterized by a high protein and oil content (HUYGHE 1997, HARDY et al. 1998). The major problem with introducing the cultivated Andean lupin as an alternative high protein crop to European conditions is the indeterminate growth of this lupin and its unstable yield (ROEMER 1993, SAWICKA-SIENKIEWICZ, KADŁUBIEC 1998, 1999). To overcome these problems selection of earlier maturing plants of the Andean lupin are needed because they are more suitable for the climate of Europe. With the goal of selecting such genotypes of *L. mutabilis*, a breeding programme was started at the University of Giessen in 1983. The selection of early maturing plants from a high number of collected ecotypes did not lead to distinction of proper lines, since the feature was not genetically fixed (ROEMER, JAHN-DEESBACH 1992). In order to increase the variability of the chosen material, a mutation program using ethyl methane sulphonate (EMS) was started in 1989. This approach was successful in finding a plant, named KW, with fully determinate growth (ROEMER 1993).

Nowadays, attempts are being made to adapt *L. mutabilis* Sweet to Polish conditions. Our recent programme has involved the preparation of new mutants, interspecific hybridization with lupins from the section *Albus*, and identification of early maturing, higher yielding genotypes. The preliminary evaluation (SAWICKA-SIENKIEWICZ et al. 1998) of the interspecific hybrids made by molecular markers prompted us to analyse DNA polymorphism in the collected material in order to develop organellar molecular markers that could be helpful in desirable plant selection. As the first step organellar genomes – chloroplast DNA (ctDNA) and mitochondrial DNA (mtDNA) – were screened for diversity. The copy number of the organellar genomes in crude DNA preparation in plant material is much greater than the number of copies of nuclear genome. Therefore, the required amount of green plant tissue for analyses of organellar DNA is rather small. Mitochondrial DNA is mainly investigated for phylogeny and population genetics in animals. In plants, however, chloroplast DNA is chosen more often for molecular phylogenetic studies. The reason is the distinctive evolutionary dynamics of both genomes in plant. In contrast to ctDNA, mitochondrial DNA is characterized by a high rate of rearrangement and a low rate of point mutation. Consequently, even closely related plant species differ in mtDNA organization. However, this feature could be helpful for population genetic studies, which has been recently shown by DESPLANQUE et al. (2000). In the present study, we applied comparative restriction fragment length polymorphism (RFLP) to determine the level of ctDNA and mtDNA diversity between and within *L. mutabilis* and the species of the section *Albus*.

Material and methods

Plant material. The plant material, listed in Table 1, consisted of seven populations of *L. mutabilis*, five mutant lines of *L. mutabilis* and four species belonging to the section *Albus*.

DNA extraction. Young leaves harvested from several field-grown plants (Table 1) were collected and immediately frozen in liquid nitrogen, and then stored at −80°C. Total DNA was extracted from 2 g of leaves following the procedure described by VALLEJOS et al. (1992).

RFLP analysis. Five micrograms of total DNA was digested with *Eco*RI, under conditions specified by the enzyme supplier (MBI Fermentas) and loaded onto 0.8% agarose gel. The DNA fragments were transferred to a nylon membrane (MSI). DNA gel blotting and hybridization with the common bean mtDNA fragments were performed as reported by JAŃSKA and MACKENZIE (1993). In the case of other probes, hybridization was carried out at 42°C for 18 h in the mixture of 50% formamide, 5 × Denhardt solution, 5 × SSC, 0.1% SDS and 1 mM EDTA (pH 8.0). The prehybridization was performed for 2 h, followed by membrane washing in 2 × SSC, 0.1% SDS at room temperature for 5 min., three times in 2 × SSC, 0.1% SDS at room temperature for 20 min., then in 0.1 × SSC and 0.1% SDS at 42°C for 1 h, and 2 × SSC, 0.1% SDS at room temperature for 10 min. Exposure time of the membranes to an X-ray film (Foton, Poland) was adjusted as required. When necessary, membranes were stripped by boiling twice for 15 min. in 0.1 × SSC, 0.1% SDS, and then rinsed twice for 1 min. in this solution.

Probes for ctDNA polymorphism. All probes were generated by PCR amplification using total DNA from the yellow lupin as a template and primers homologous to the following sequences: (1) the sequence separating the *psa*A gene and the *trn*S gene in chloroplast genome (DEMESURE et al. 1995); (2) the fragment of the *psb*A gene from tobacco (GAWROŃSKI et al. 1992); (3) the sequence of the yellow lupin *ndh*C/K/J operon (OCZKOWSKI et al. 1997); (4) the 5' flanking sequences of the yellow lupin *ndh*C/K/J operon (OCZKOWSKI et al. 1997); (5) the 3' flanking sequences of the yellow lupin *ndh*C/K/J operon (OCZKOWSKI et al. 1997); (6) the intron of the common bean tRNA alanine gene (WOŁOSZYŃSKA 2000).

Probes for mtDNA polymorphism. A total of eighteen probes representing mitochondrial clones and PCR amplified fragments were used. The following probes were derived by PCR amplification using total DNA from the yellow lupin (with the exception of the mt K1 plasmid which originated from the white lupin was used) as a template and specific primers to: (1) the fragment of the mitochondrial K1 plasmid from white lupin (GORĄCZNIAK, AUGUSTYNIAK 1989); (2) the fragment of the common bean *atp*A gene (CHASE, ORTEGA 1992); (3) the fragment of apocytochrome *b* (*cob* gene) (DAWSON et al. 1984); (4) the intron sequence of the *nad*4 gene (DEMESURE et al. 1995).

Sequences of all PCR primers are available upon request.

The following mitochondrial clones of the yellow lupin were used: (1) the *Eco*RI, a fragment of the pEB1 insert (AUGUSTYNIAK et al. 1983); (2) the *Eco*RI/ *Ava*II fragment of the pEB8 insert containing *rrn*18S and *rrn*5S intergenic region and the part of *rrn*5S gene (AUGUSTYNIAK et al. 1983, MARTIN et al. 1992); (3) the *Pst*I fragment of the pEB8 insert containing tRNA–like repeat-

ing sequecs (MARTIN et al. 1992); (4) the eleven cosmid clones from previously developed common bean mitochondrial libraries (JAŃSKA, MACKENZIE 1993): 12G5, 16A4, 9D10, 11H1, 7E1, 12F10, 5B5, 15A8, 12C4, 17G5, 12D5.

The labelling of the probes with $^{32}P[\alpha dCTP]$ was performed by the FEINBERG, VOGELSTEIN (1983) method.

Results

Total cellular DNAs isolated from seven populations of *L. mutabilis*, five mutant lines of *L. mutabilis* and four species from the section *Albus* (Table 1) were digested with *Eco*RI, and the Southern blots were hybridized with 18 mitochondrial and 6 chloroplast DNA probes. *Pst*I and *Hind*III enzymes were used, but as we did not observe differences in the first trials, we decided to utilise one enzyme and more probes in all hybridization experiments.

Table 1. Characterization of the investigated plant material

| Species	Origin
Section *Albus*	
Lupinus albus L.	cv. Wat, Plant Breeding Station, Wiatrowo, Poland
Lupinus albus L.	cv. Bardo, Plant Breeding Station, Wiatrowo, Poland
Lupinus graecus Boiss. et Spruner	line no. 562 (095606, Plant Breeding Station, Wiatrowo, Poland)
Lupinus termis Forsk.	line no. 518 (095631, Plant Breeding Station, Wiatrowo, Poland)
Lupinus vavilovii Atab. et Maiss.	line no. 519 (095671, Plant Breeding Station, Wiatrowo, Poland)
L. mutabilis Sweet	
Populations: XM-5	Germany (from Dr. Roemer)
LM-13	INRA – France (from Dr. Huyghe)
LM-34	INRA – France (from Dr. Huyghe)
182	United Kingdom (from Prof. Caligari)
CM-157	INRA – France (from Dr. Huyghe)
Potosi	cv. Peru (Portugal, from Prof. Martins)
Mutal	United Kingdom (from Prof. Caligari)
Mutant lines: Mut-136	Mutant obtained after γ-irradiation of population 21756 ^{137}Cs, Poland
Mut-220	Mutant obtained after γ-irradiation of population 21756 ^{137}Cs, Poland
Mut-628	Mutant obtained after γ-irradiation of population 21758 ^{137}Cs, Poland
Mut-943	Mutant obtained after γ-irradiation of population 20993 ^{137}Cs, Poland
KW	Dr. Roemer's mutant (ROEMER 1993)

kb M 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 M 2 1
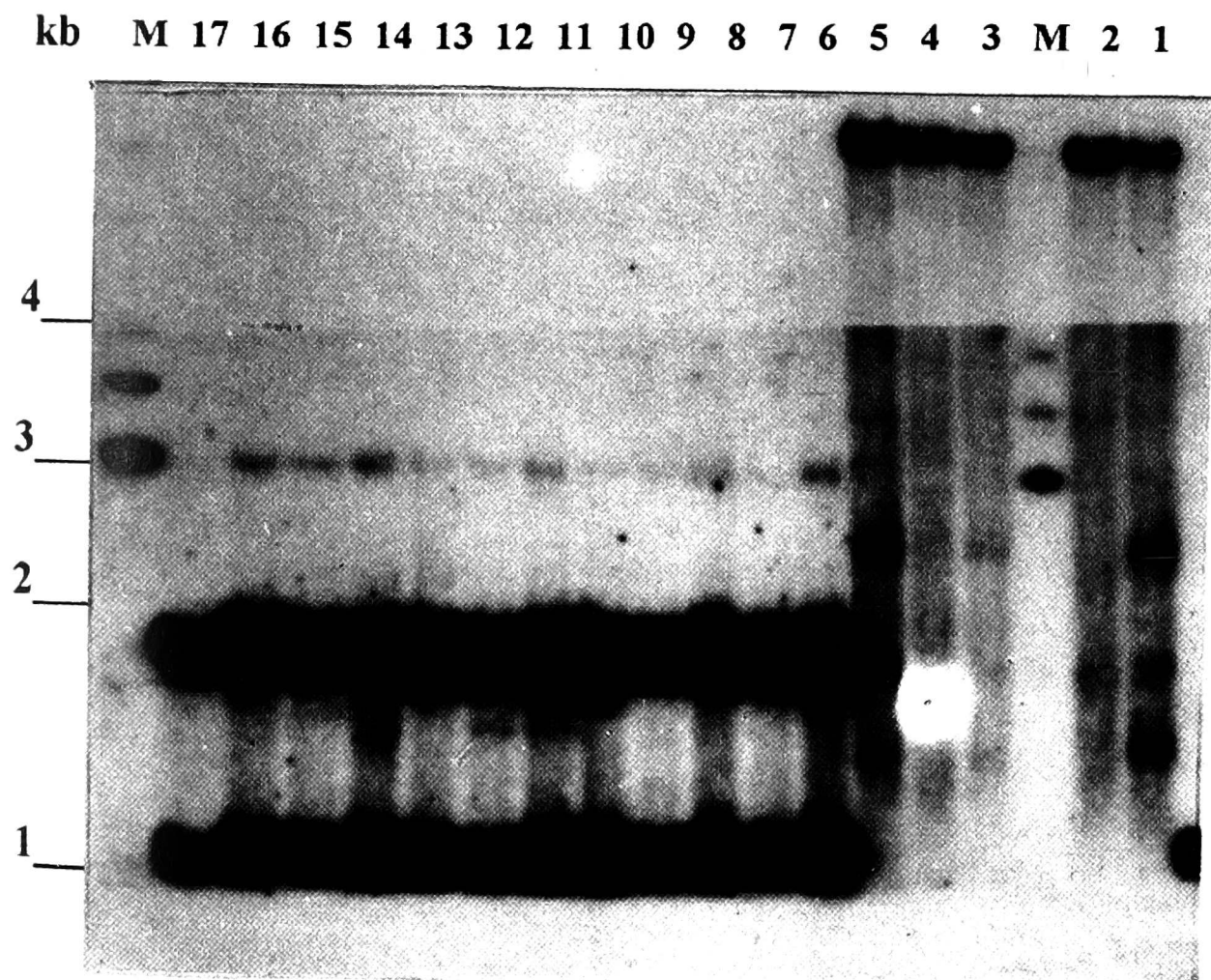
Figure 1. Southern blot hybridization of total DNA from *L. mutabilis* and lupins of the section *Albus*. The blot was hybridized with the *L. luteus* ct DNA 3' flanking sequence of the *ndh*C/K/J operon.

M = molecular mass marker; Lupins of the section *Albus*: 1 – *L. vavilovii*; 2 – *L. albus,* cv. Bardo; 3 – *L. graecus*; 4 – *L. albus,* cv.Wat; 5 – *L. termis*; *L. mutabilis* Sweet: 6 – CM-157; 7 – CM-182; 8 – Mutal; 9 – KW; 10 – Mut-220; 11 – Mut-943; 12 – Potosi; 13 – Mut-628; 14 – Mut-136; 15 – LM-34; 16 – LM-13; 17 – XM-5.

Extent of polymorphisms between two investigated species. No RFLP polymorphism was detected between *L. mutabilis* and lupins of the section *Albus* using five out of the six chloroplast probes. The difference was observed only when the probe covering the 3' flank of the *ndh*C/K/J operon was applied (Figure 1). In contrast, the two species can be distinguished with all mitochondrial probes except the *cob* gene (Figure 2) and *nad*4 intron probes. The main hybridization patterns were sufficiently distinct to presume a correct species classification (Figures 3, 4, 5).

Lack of polymorphisms among lupins of section *Albus*. The detection of polymorphism failed while RFLP analyses were used for the identification of the heterogenity of cytoplasmic DNA among lupins of the section *Albus*.

Observed polymorphisms within populations and mutant lines of *L. mutabilis*. No polymorphisms were detected with the chloroplast probes for *L. mutabilis* populations and mutant lines. Again, the mitochondrial probes were more suitable to differentiate the analysed material. Polymorphisms were found among
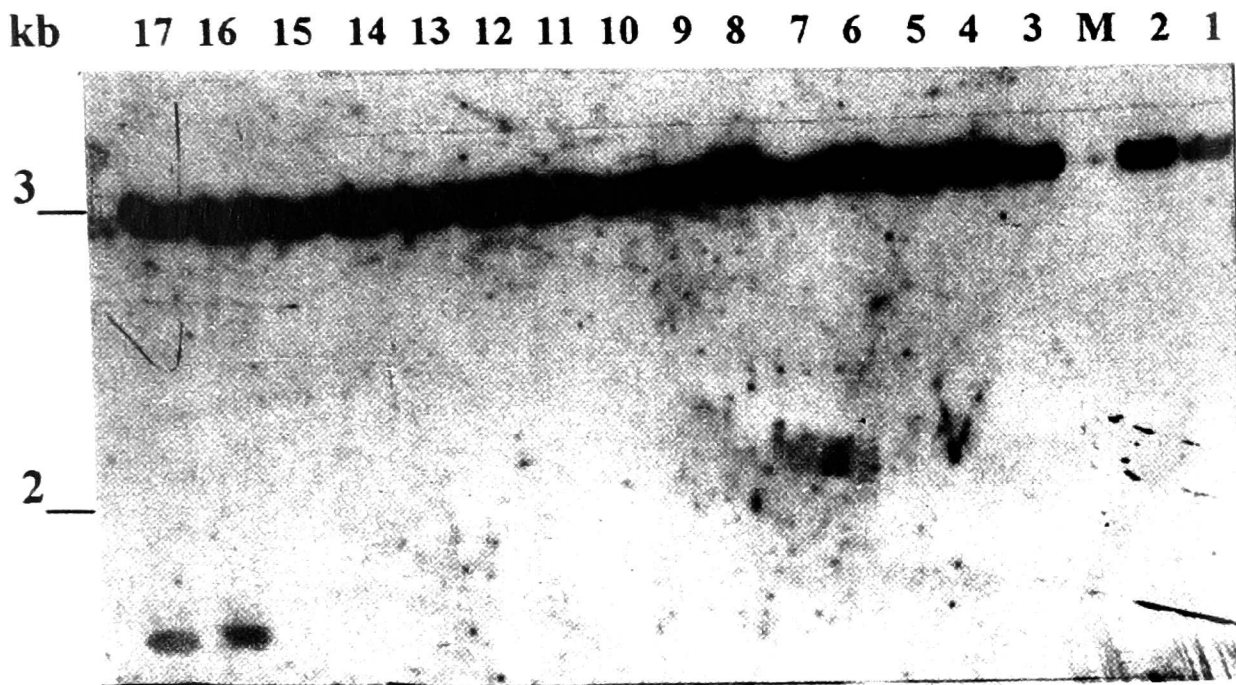
Figure 2. Southern blot hybridization of total DNA from *L. mutabilis* and lupins of the section *Albus*. The blot was hybridized with the *L. luteus* mt DNA *cob* probe. The order of lupins is the same as in Figure 1. The molecular mass marker is not detected by this probe.
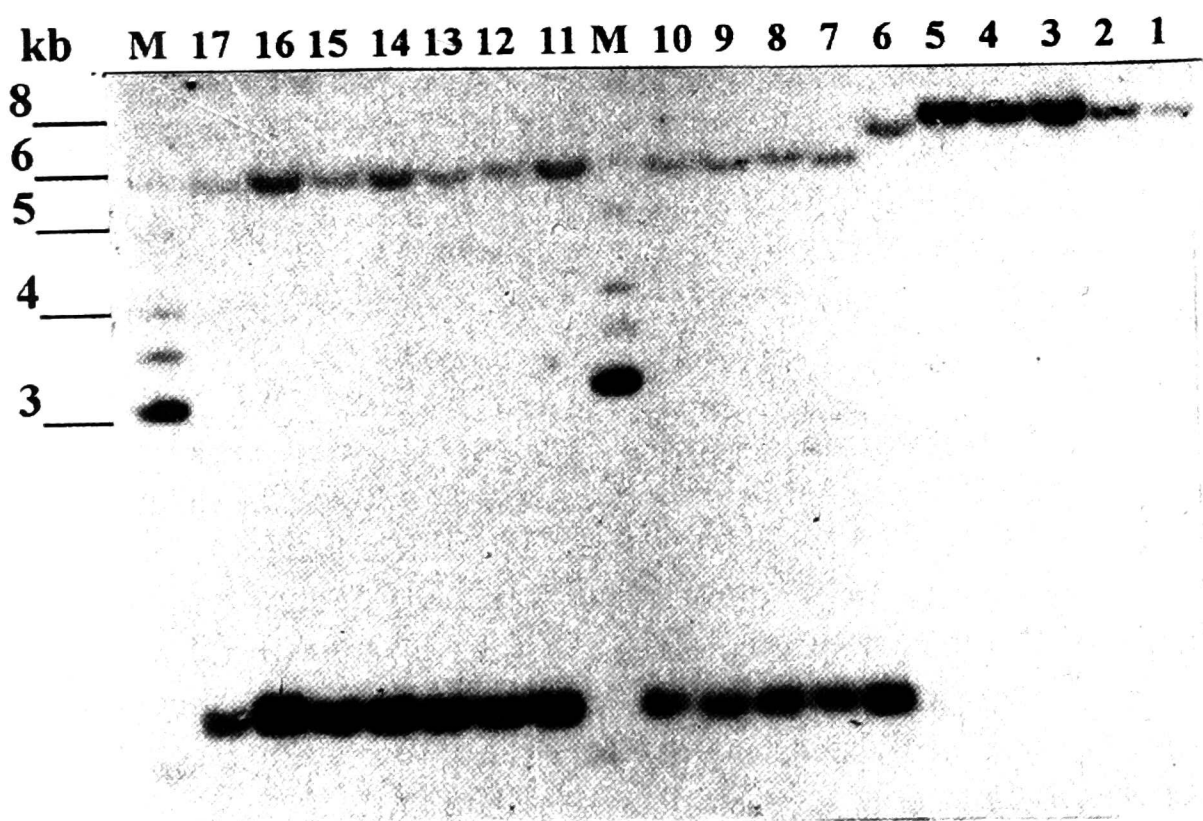

Figure 3. Southern blot hybridization of total DNA from *L. mutabilis* and lupins of the section *Albus*. The blot was hybridized with the *L. luteus* mt DNA probe containing the intergenic region 18S/5S rRNA and the part of 5S rRNA. The order of lupins is the same as in Figure 1, but the molecular mass marker is located between Mut-220 (no. 10) and Mut-943 (no. 11) instead of between *L. albus* (Bardo, no. 2) and *L. graecus* (no. 3).
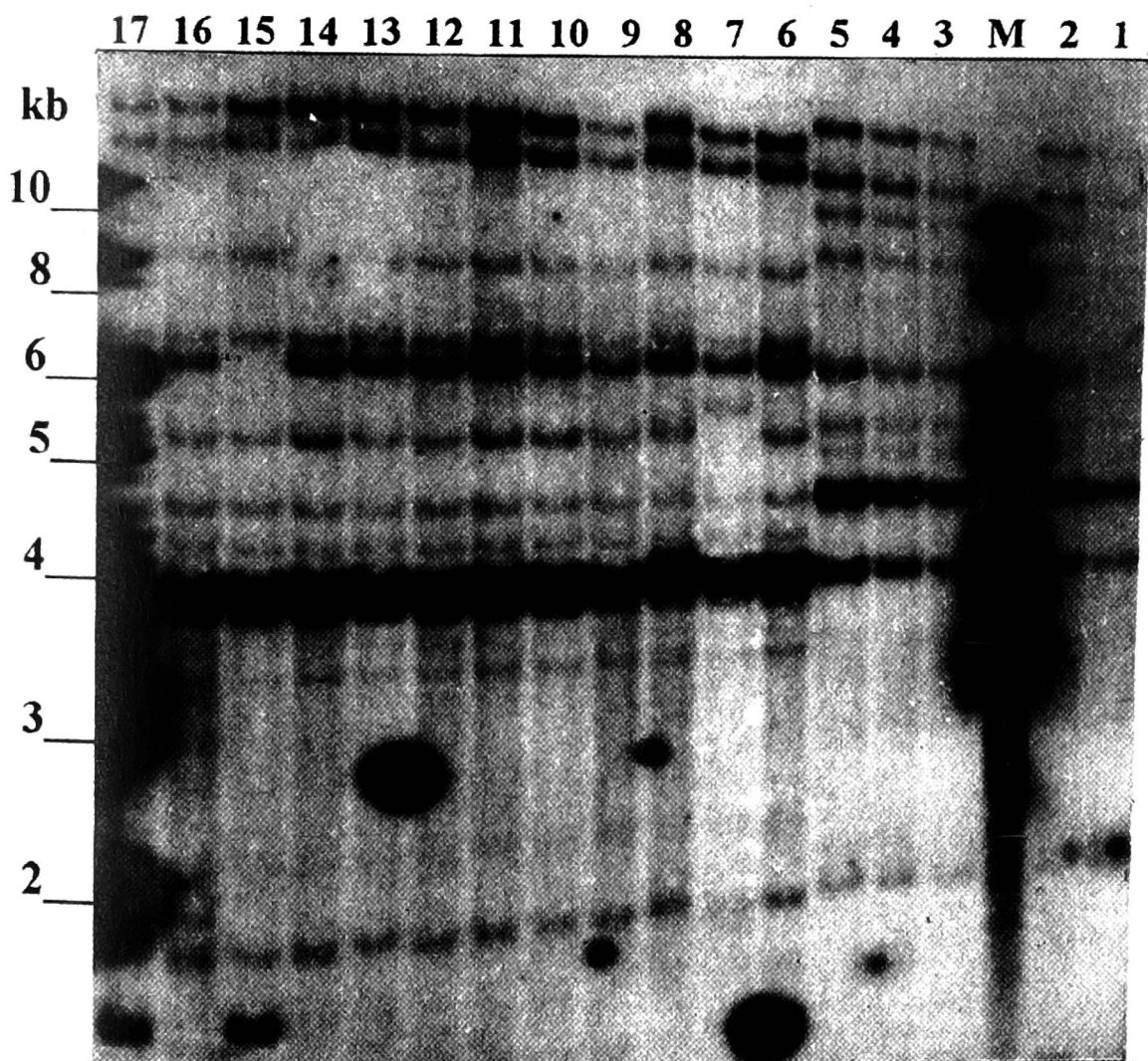
Figure 4. Southern blot hybridization of total DNA from *L. mutabilis* and lupins of the section *Albus*. The blot was hybridized with the *P. vulgaris* mt DNA 15A8 probe. The order of investigated lupins is the same as in Figure 1 but the molecular mass marker after XM-5 was cut.

the DNAs of *L. mutabilis* that were hybridized with *atp*A, the sequences of the pEB8 insert and with the four cosmid clones from the bean mitochondrial libraries. The results of these hybridizations are summarised in Table 2. For instance, the sequence containing the intergenic region 18S/5S rRNA and the part of 5S rRNA produced a distinct pattern of DNA from CM157 (Figure 3), while the clone 15A8 distinguished three populations: XM-5, LM-34 and 182 (Figure 4, Table 2). The same additional fragment, as compared to the other *L. mutabilis* populations and mutant lines, was found in mutant KW, when probed with two overlapping clones: 12G5 and 9D10 (Figure 5). Considering all polymorphisms simultaneously, there are at least nine mitotypes within *L. mutabilis* populations and mutant lines. This result is only based on the predominant hybridization signals, indicating that the observed polymorphisms reflect the differences in the mitochondrial genomes of the investigated plants. We observed a high degree of heterogeneity among the mitochondrial genomes of Andean lupin populations, but marked similarity among the mutants lines (Table 2). However, in some hybridization experiments the predominant bands were accompanied by weaker bands, which also differentiate the examined material. An example is presented
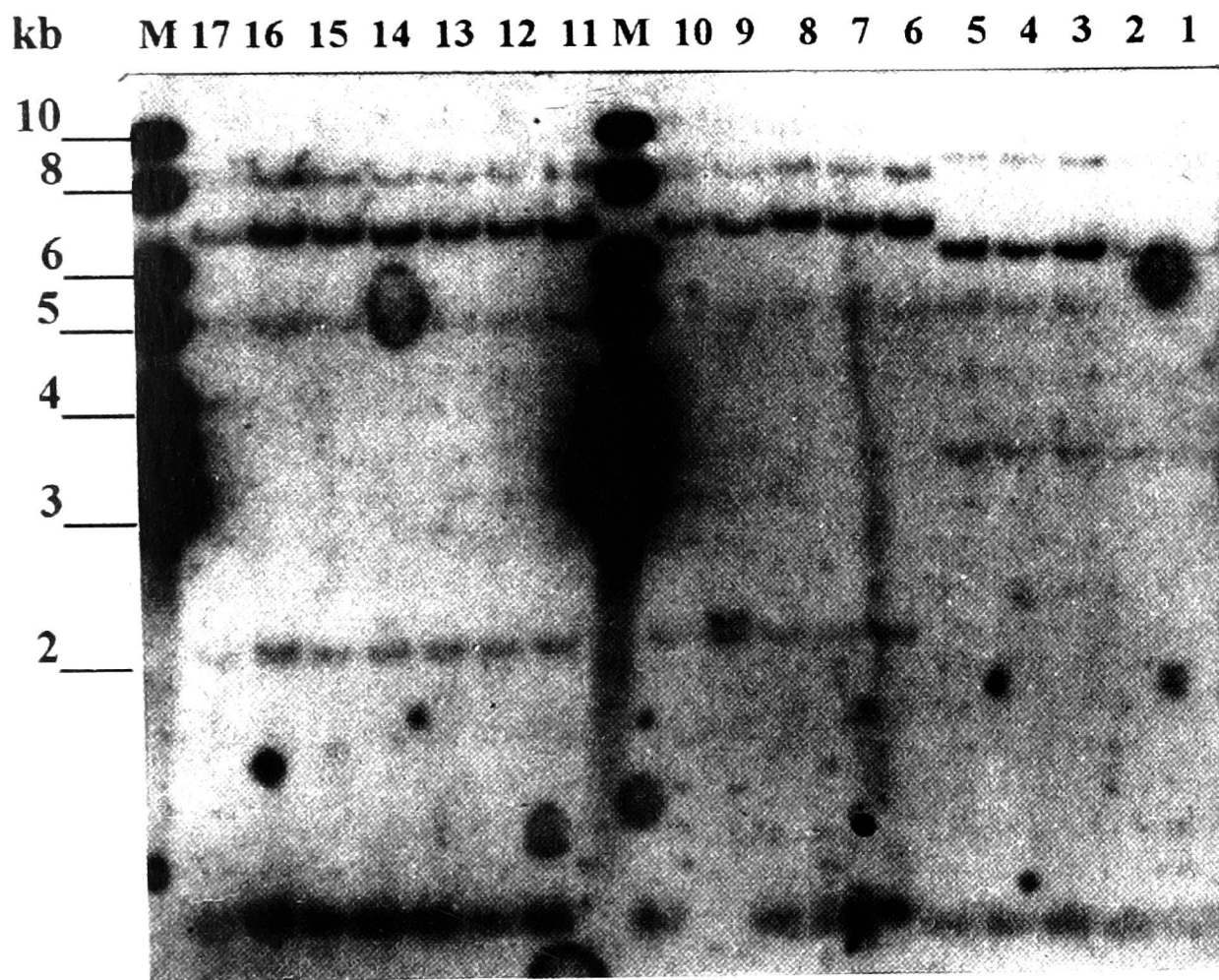
Figure 5. Southern blot hybridization of total DNA from *L. mutabilis* and lupins of the section *Albus*. The blot was hybridized with the *P. vulgaris* mt DNA 12G5 probe. The order was the same as in Figure 3

in Figure 2. In addition to a strong signal characteristic for all trials, the *cob* probe gave a very weak band of the same size for XM-5 and LM-13. These weaker bands could represent the substoichiometric mitochondrial DNA arrangements of the *cob* sequence coexisting in the mitochondrial genomes of XM-5 and LM-13 (Figure 2). Alternatively, these bands could represent a homology of the probe to other mitochondrial arrangements or to nuclear or chloroplast DNA.

Discussion

In an attempt to detect DNA diversity useful for lupin selection, we used ctDNA and mtDNA probes capable of detecting cytoplasmic differences in total cellular DNA. We found that mitochondrial probes are more suitable for identification of inter- and intra-specific variations in the examined lupin material. On the other hand, the polymorphism between the chloroplast genomes of *L. mutabilis* and lupins of the section *Albus* detected by the probe covering the 3' flanking sequences of *ndh*C/K/J operon could be used to develop a simple method for a rapid species identification. On the autoradiogram, which is shown in Figure 1, the two bands are visible for *L. mutabilis* versus the one for lupins of the section *Albus*. Since

Table 2. Restriction fragment length polymorphism in mtDNAs of *L. mutabilis*. Sizes (kb) of the additional (+) and missing (–) fragments, as compared to the common hybridization pattern (C)

Source	Probe							Mito-type
	12G5	15A8	9D10	7E1	atpA	Intergenic 18S/5S region*	tRNA like repeats	
XM-5	C	+1.6	C	–6.3; –2.2	C	C	C	1
LM-13	C	C	C	C	C	C	+2	2
LM-34	C	–6.1;+1.6	C	–6.3, –2.2	C	C	C	3
182	C	+5.5, –5.2	+5.8	+5.6; –5.3	C	C	C	4
CM-157	C	C	C	C	C	+7; –6.1	C	5
Potosi	C	C	C	C	+3	C	C	6
Mutal	C	C	C	C	C	C	C	7
Mut-136	C	C	C	C	C	C	+2	8
Mut-220	C	C	C	C	C	C	C	7
Mut-628	C	C	C	C	C	C	C	7
Mut-943	C	C	C	C	C	C	C	7
KW	+2.2;–1	C	+2.2	C	C	C	C	9

* This probe contains also the part of the 5S rRNA sequence.

the probe was generated by PCR amplification and applied to detect polymorphism by RFLP, the PCR-RFLP approach could be adapted.

No polymorphism was detected among lupins of the section *Albus* using either chloroplast or mitochondrial probes. In the light of the view that no two species of flowering plants posses the same mitochondrial gene order (PALMER 1992), this result is surprising. It is important to note that a large part of the lupin's mtDNA was checked because the heterologous mitochondrial probes cover more than half of the bean mitochondrial genome. Thus we agree with GLADSTONES (1974, 1998) that the investigated species of the section *Albus* should be considered as one species rather than four different species.

A high mitochondrial polymorphism was observed among the populations of *L. mutabilis* in comparison with the mutant lines, but in both groups chloroplast monomorphism was detected. All the investigated populations of *L. mutabilis* could be distinguished by mtDNA RFLP. The high level of observed mtDNA polymorphism is in agreement with the other studies concerning its diversity within plant populations (KOIKE et al. 1998, DESPLANQUE et al. 2000).The limited polymorphism exhibited by the mutant lines indicates a mutation programme should be directed to broaden cytoplasmic participation.

One of our Polish lupin research programmes is now oriented towards selection of lines with determinate growth. Therefore, the mtDNA polymorphism detected for the KW mutant is a valuable finding. The 12G5 probe distinguishes only

KW from other studied plants. The same polymorphic fragment appeared also with the probe 9D10. These two clones share a common fragment of the common bean mitochondrial genome. This fragment could be a potential molecular DNA marker helpful in identification of a morphological trait characteristic for mutant KW, namely determinate growth, particularity that a programme of introducing this type of morphological trait from mutant KW to other Andean lupin materials has been already undertaken.

It was shown previously that *L. mutabilis* populations are characterised by wide variation in phenological and morphological features (HARDY et al. 1998). The RFLP analysis used in this study revealed that the phenotypic variation is accompanied with mtDNA diversity. This knowledge may help to improve the breeding selection of this species.

REFERENCES

AUGUSTYNIAK H., BORSUK P., HIRSCHLER I., STĘPIEŃ P.P., BARTNIK E. (1983). Mitochondrial DNA from lupine: restriction analysis and cloning of fragments coding for tRNA. Gene 22: 69-74.

CHASE C.D., ORTEGA V.M. (1992). Organization of *atp*A coding and 3' flanking sequences associated with cytoplasmic male sterility in *Phaseolus vulgaris* L. Curr. Genet. 22: 147-153.

DAWSON A.J., JONES V.P., LEAVER C.J. (1984). The apocytochrome b gene in maize mitochondria does not contain introns and is preceded by a potential ribosome binding site. EMBO J. 3: 2107-2113.

DEMESURE B., SODZI N., PETIT R.J. (1995). A set of universal primers for amplification of polymorphic non-coding regions of mitochondrial and chloroplast DNA in plants. Mol. Ecol. 4: 121-139.

DESPLANQUE B., VIARD F., BERNARD J., FORCIOLO D., SAUMITOU-LAPRADE P., CUGUEN J., VAN DIJK H. (2000). The linkage disequilibrium between chloroplast DNA and mitochondrial DNA haplotypes in *Beta vulgaris* ssp. *maritima* (L.): the usefulness of both genomes for population genetic studies. Mol. Ecol. 9:141-154.

FEINBERG A.P., VOGELSTEIN B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specificity. Annal. Biochem. 132: 6-13.

GAWROŃSKI S.W., SUGITA M., SUGIURA M. (1992). Mutation of *psb*A gene in herbicide resistant populations of *Erigeron canadensis*. In: Research Photosynthesis (N. Murata ed.). Kluwer Acad. Pub. Dordrecht III: 405-407.

GLADSTONES J.S. (1974). Lupin of the Mediterranean region and Africa. Technic. Bull. 26: 1-48.

GLADSTONES J.S. (1998). Distribution, origin, taxonomy, history and importance. In: Lupins as Crop Plants: Biology, Production and Utilization (J.S. Gladstones, C.A. Atkins, J. Hamblin, eds.). CAB International: 1-39.

GORĄCZNIAK R.M., AUGUSTYNIAK H. (1989). Characterization and sequence of lupin mitochondrial plasmid-like DNA. Curr. Genet. 16: 469-471.

HARDY A., HUYGHE C., RAHIM M.A., ROEMER P., NEVES-MARTINS J.M., SAWICKA-SIENKIEWICZ E.J., CALIGARI P.D.S. (1998). Effects of genotype and environment on architecture and flowering time of indeterminate Andean lupin (*Lupinus mutabilis* Sweet). Aust. J. Agric. Res. 49: 1241-1251.

HUYGHE C. (1997). White lupin (*Lupinus albus* L.). Field Crops Res. 53: 147-160.

JAŃSKA H., MACKENZIE S.A. (1993). Unusual mitochondrial genome organization in cytoplasmic male sterile common bean and the nature of cytoplasmic reversion to fertility. Genetics 135: 869-879.

KOIKE T., KATO S., SHIMAMOTO K., KITAMURA K., KAWANO S., UEDA K., MIKAMI T. (1998). Mitochondrial DNA variation follows a geographic pattern in Japanese beech species. Bot. Acta 111: 87-92.

MARTIN R.P., SCHWOB E., AUGUSTYNIAK H., MAKAŁOWSKI P., STIGLER P., DIRHEIMER G. (1992). Nucleotide sequence of the mitochondrial 18S rRNA gene from lupine (*Lupinus luteus*). Plant Mol. Biol. 19: 509-511.

OCZKOWSKI M., PASZKIEWICZ J., PIĄTYSZEK M., AUGUSTYNIAK H. (1997). Cloning and transcription analysis of the *ndhC-ndhK-ndhJ* genes of lupin plastid DNA. Plant Sci. 127: 171-177.

PALMER J.D. (1992). Mitochondrial DNA in plant systematics: application and limitations. In: Molecular Systematics of Plants (Soltis P.S., Soltis D.E., Doyle J.J., eds.). Chapman and Hall, New York: 36-49.

ROEMER P. (1993). A determinated mutant of *Lupinus mutabilis* as a possible source of early maturity. Abstracts 7th Int. Lupin Conference "Advances in Lupin Research", 18-23 April 1993, Evora, Portugal: 90-92.

ROEMER P., JAHN-DEESBACH W. (1992). Eight years of experiences in breeding *Lupinus mutabilis* Sweet under Middle European conditions. In: Agrimed Research Programme – *Lupinus mutabilis*: its adaptation and production under European pedoclimatic conditions. Commission of European Community edn. EUR 14102 EN, Luxembourg: 79-85.

SAWICKA-SIENKIEWICZ E., KADŁUBIEC W. (1988). Ocena zmienności cech użytkowych materiałów kolekcyjnych *Lupinus mutabilis* Sweet. [Estimation of yield characters variability in *Lupinus mutabilis* Sweet collections.]. Zesz. Probl. Post. Nauk Roln. 463: 451-460.

SAWICKA-SIENKIEWICZ E.J., RUREK M., AUGUSTYNIAK H. (1998). Searching for molecular markers in interspecific hybrids of *Lupinus mutabilis* Sweet. Abstract 3rd European Conference on Grain Legumes, 14-19 November 1998, Valladolid, Spain: 460.

SAWICKA-SIENKIEWICZ E., KADŁUBIEC W. (1999). Evaluation of *Lupinus mutabilis* Sweet in respect to yield characters. Lupin: an Ancient Crop for the New Millenium. Abstracts 9th Int. Lupin Conference, 20-24 June 1999, Klink, Germany: 293-294.

VALLEJOS C.E., SAKIYAMA N.S., CHASE N.D. (1992). A molecular marker-based linkage map of *Phaseolus vulgaris* L. Genetics 131: 733-740.

WOŁOSZYŃSKA M. (1999). Heteroplazma u fasoli zwykłej – geneza i zmienność. [Heteroplasm in *Phaseolus vulgaris*: genesis and variability]. Ph. D. thesis, Wrocław University.