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QUARTERLY

Organization of the 18S, 5S, 4S rRNA genes and the tRNA-like repeat in the mitochondrial genomes of three lupin species

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Southern blots of mitochondrial (mt) DNAs of three *Lupinus* species cleaved with three restriction enzymes were probed with *Lupinus luteus* mtDNA fragments containing 18S, 5S rRNA genes or a tRNA-like repeat. Comparison of the number of hybridizing bands and their intensity suggested that the mt 18S and 5S rRNA genes occur mostly in one copy in the genomes of three lupin species. The exception concerned the *Lupinus angustifolius* 5S rRNA gene showing two hybridizing bands of unequal intensity. The results of hybridization of the lupin mitochondrial genomes with a probe specific for the *Lupinus luteus* tRNA-like repeat pointed to the presence of such a repeat in other parts of the genomes besides the vicinity of the 18S rRNA gene. Northern hybridization analysis showed the presence of 18S, 5S and tRNA-like repeat transcripts similar in size in all lupin species.

The characteristic feature of plant mitochondrial genomes is the presence of repeated sequences that recombine to generate structural heterogeneity in that DNA. These repeated sequences undergo recombination between inverted repeats as well as between direct repeats [1, 2]. It is known that plant mitochondrial genomes contain a variable number of families of such recombining repeats giving rise to differences in multicircular complexity. The number of copies per repeat family varies from two [3] to three [4–8]. The molecular mechanism of such an extensive heterodispersity is at present poorly understood. Palmer & Herbon [9] have shown that the identities of the recombining repeat sequences may differ even between closely related species. This implies that these sequences undergo relatively rapid turnover during evolution. Evidence that molecular heterogeneity in plant mitochondrial genomes may exist at the level of individual genes has come from studies on the arrangement of mitochondrial rRNA genes in wheat and rye [4, 5, 7]. Mitochondrial genes for 18S and 5S rRNA have been mapped as three copy sequences in *Secale cereale* [4, 5], *Petunia hybrida* [6], *Triticum aestivum* [7], and *Oryza sativa* [8], and as unique sequences in *Zea mays* [10], *Brassica oleracea* [11], and *Oenothera berteriana* [12]. The length of the

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[1]Abbreviations used: ATA, aurintricarboxylic acid; bp, base pairs; kb, kilobase pairs; mt, mitochondrial; nt, nucleotide.

18S/5S ribosomal coding fragment varies among higher plants.

The genus *Lupinus*, which comprises about 200 species, includes various species cultivated over a wide geographical range, e.g. *L. albus*, *L. angustifolius*, *L. luteus* and *L. mutabilis*. These species are characterized by a rather high chromosome number and high incompatibility of sexual crossing. All these features have stimulated our comparative studies on the organization of RNA genes and their flanking regions.

The arrangement of the 18S and 5S RNA genes in mtDNA[1] of *L. luteus* is the same as that of the corresponding genes from other sources [13, 14]. The 18S gene is located on the same strand as 5S rRNA gene and separated from it by 190 nucleotides. This 18S/5S rRNA gene region shows high sequence homology to that from other species [13, 14]. In the region of 61 nucleotides upstream of 18S rRNA *L. luteus* mtDNA contains a 90 bp long direct repeat. The repetition includes a six-nucleotide long motif (GGATTC) which can be folded into tRNA-like structures.

The purpose of the present studies was to examine the arrangement of 18S and 5S rRNA genes as well as the repeat in mtDNA of three lupin species.

MATERIALS AND METHODS

Plant material. Seeds of *L. luteus* L. cv. Topaz and *L. angustifolius* L. cv. Mirella were from the Plant Breeding Station Wiatrowo (Poland).

Seeds of *L. albus* L. cv. Kalina were obtained from the IHAR Research Station Przebędowo near Poznań (Poland).

DNA preparation. Mitochondrial DNAs of etiolated seedlings of *Lupinus* species were prepared as described by Karpińska & Augustyniak [15].

Large-scale preparation of the *L. luteus* plasmid DNA containing mtDNA fragment with the linked 18S and 5S rRNA genes was performed by the alkaline lysis method of Birnboim & Doly [16] followed by centrifugation in CsCl-ethidium bromide gradient [17]. Genomic and cloned DNAs were cleaved with various restriction endonucleases and separated by 0.8% agarose gel electrophoresis in Tris/acetate/EDTA buffer. DNA fragments were transferred to a nylon membrane (Hybond-N) and hybridized with specific ^{32}P-labelled DNA probes.

The *L. luteus* (yellow lupin) pEB8, 2 clone-subclone of pEB8 [18] used in this paper as a specific probe A had no sequence of pBR327 vector. This clone was also used for preparation of the other probes: B, C and D. Probe B contained a 1262 bp *Pst*I-*Eco*RI fragment internal to the 18S rRNA gene. Probe C consisted of a 410 bp *Ava*II-*Bam*HI fragment having the entire 5S gene and, in addition, an 116 bp intergenic spacer between the 3'-end of the 18S rRNA gene and the 5'-end of the 5S rRNA gene, as well as an 175 bp flanking sequence 3' to the 5S rRNA gene. Probe D contained a 382 bp *Bam*HI-*Pst*I mitochondrial sequence with an 90 bp tRNA-like repeat. All specific probes used in this study were recovered by extraction from low gelling temperature agarose.

Preparation of mitochondrial RNA. Mitochondria were isolated as described in "DNA Preparation" except that mitochondria were obtained without treatment with deoxyribonuclease I; mtRNA was prepared in the presence of ATA essentially as described by Stern & Newton [19].

Preparation of mitochondrial tRNA. The preparation of highly purified mitochondrial tRNA from 6-day-old etiolated lupin hypocotyls was carried out as outlined by Karpińska & Augustyniak [15].

Labelling of DNA and hybridization. The purified probes were radiolabelled by random primer extension [20]. DNA was hybridized to Southern blots at 42°C in hybridization buffer (50%, v/v, deionized formamide, 5 × Denhardt's solution, 5 × SSC, 1 mM EDTA, pH 8.0, 0.1% SDS and 0.25 mg/ml sonicated and denatured salmon sperm DNA). Southern blots were prehybridized for 2 h and hybridized for 18 h. Following hybridization, blots were washed at room temperature with 2 × SSC, 0.1% SDS (w/v) once for 5 min and three times for 20 min, then once in 0.1 × SSC, 0.1% SDS at 42°C for 60 min and finally with 2× SSC, 0.1% SDS at room temperature for 5 min. Autoradiography was usually performed at –70°C with an intensifying screen. Mitochondrial RNA (10–15 µg) was fractionated in 1.5% denaturing formaldehyde agarose gels and transferred directly to nylon membrane Hybond N. Northern blot hybridization was carried out at 42°C for 18 h in the mixture: 50% formamide, 10 × Den-

hardt's solution, 50 mM Tris/HCl, pH 7.5, 0.1% SDS and 0.1 mg/ml denatured salmon sperm DNA. Prehybridization was performed for 3 h at 42°C in a similar mixture. The prehybridization solution contained 0.5 mg/ml salmon sperm DNA and, in addition, 1 M NaCl. The membrane was washed twice in 2 × SSC at room temperature, twice in 2× SSC at 65°C for 30 min and twice in 0.1 × SSC at room temperature.

Labelling of tRNA and hybridization. ^{32}P-Labelled tRNAs were prepared using T_4 polynucleotide kinase and [γ-^{32}P]ATP, or tRNA nucleotidyl transferase and [α-^{32}P]ATP [21] were hybridized to Southern blots at 42°C in 50% formamide solution, 5 × SSC, 1 × Denhardt solution at 0.1% SDS in the manner outlined by Karpińska & Augustyniak [15].

RESULTS AND DISCUSSION

The relative arrangement of rRNA sequences in the *Lupinus* species was assessed by hybridization of mtDNA fragments covering particular sequences of genes to filter-bound DNA. Mitochondrial DNA of three lupin species cleaved with *Bam*HI, *Hin*dIII and *Eco*RI enzymes were hybridized with yellow lupin mtDNA fragment containing 18S, 5S rRNA genes and the tRNA-like repeat surrounding 18S rRNA from 5'-end of the gene. *Eco*RI digested mtDNAs were also hybridized with separate probes of 18S, 5S rRNA genes or tRNA-like repeat isolated from yellow lupin mtDNA. All hybridizations to *Eco*RI digested mtDNAs were performed with the same nylon filter which was stripped of the probe after each experiment. The distribution of tRNA genes were checked on duplicate filter.

Figure 1 shows the organization of the 5S–18S rDNA of *L. luteus* and the regions of this mtDNA spanned by each probe. The results of probing the *Bam*HI and *Hin*dIII cleaved lupin DNAs with probe A containing 18S, 5S rRNA and the tRNA-like repeat are shown in Fig. 2. Probe A hybridized to fragments of 3 kb in size in all three *Bam*HI digested lupin DNAs. This suggests a similarity in DNA cleavage in the three lupin species by *Bam*HI enzyme as well as similar arrangement of the 5S–18S rDNA region. An overexposed autoradiogram shows also a few additional weak bands. This may indicate that sequences homologous to probe A in these fragments are present in non equimolar amounts. In the case of *Hin*dIII digested mtDNAs there also appeared on autoradiogram two main bands displayed by 4.4 kb fragments in *L. luteus* and *L. angustifolius* and a 7.0 kb fragment in *L. albus*, respectively. On the

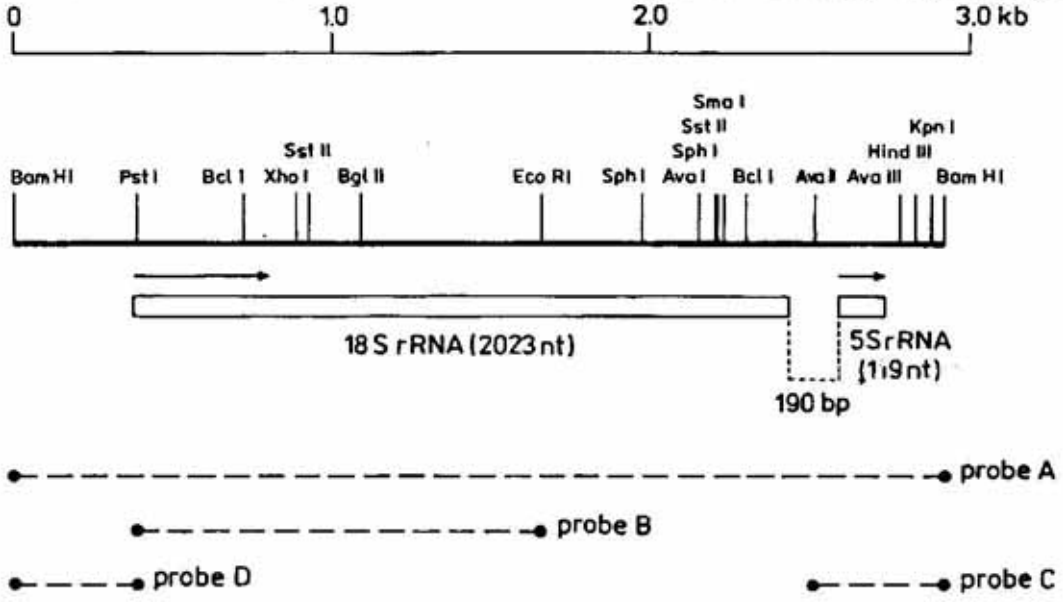

Fig. 1. Organization of the 18S–5S rDNA region in yellow lupin mitochondria.
The region spanned by each probe is indicated by dots. Probe **A** was the insert of the yellow lupin pEB8, 2 clone, which contains 18S, 5S rRNA genes and tRNA-like repeat. Probe **B** contained the yellow lupin 1262 bp *Pst*I-*Eco*RI fragment internal to the 18S rRNA gene. Probe **C** consisted of a 410 bp *Ava*II-*Bam*HI fragment of yellow lupin mtDNA having the entire 5S gene and, in addition, 116 bp of 5'-upstream flanking sequence and 175 bp of 3'-downstream flanking sequence. Probe **D** contained a 382 bp *Bam*HI-*Pst*I sequence with a 90 bp tRNA-like repeat.

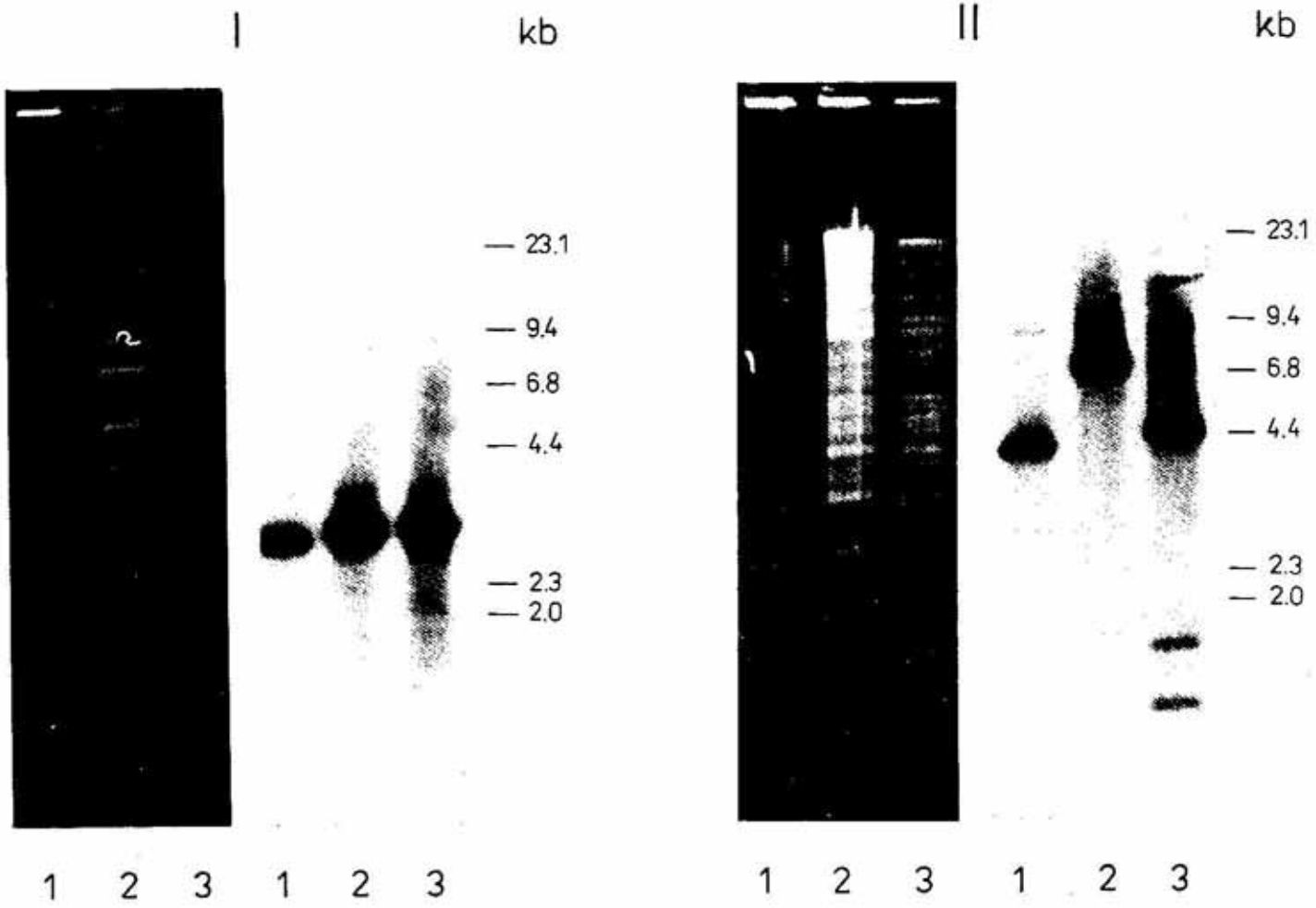

Fig. 2. Hybridization of ^{32}P-labelled probe A (18S, 5S rRNA genes and tRNA-like repeat) to lupin mtDNAs digested by BamHI and HindIII restriction enzymes. Restriction digests of total mitochondrial DNA (3 µg each) with *Bam*HI (I) and *Hind*III (II) were size fractionated in a 0.8% agarose gel, blotted, and hybridized with labelled probe **A**. For each digest, the right hand panel shows the autoradiogram of Southern blot of lupin mtDNAs. Lane 1, *L. luteus;* lane 2, *L. albus;* lane 3, *L. angustifolius*. Migration distances of molecular size markers (λ DNA cleaved with *Hind*III) are shown on the right hand side of the autoradiograms.

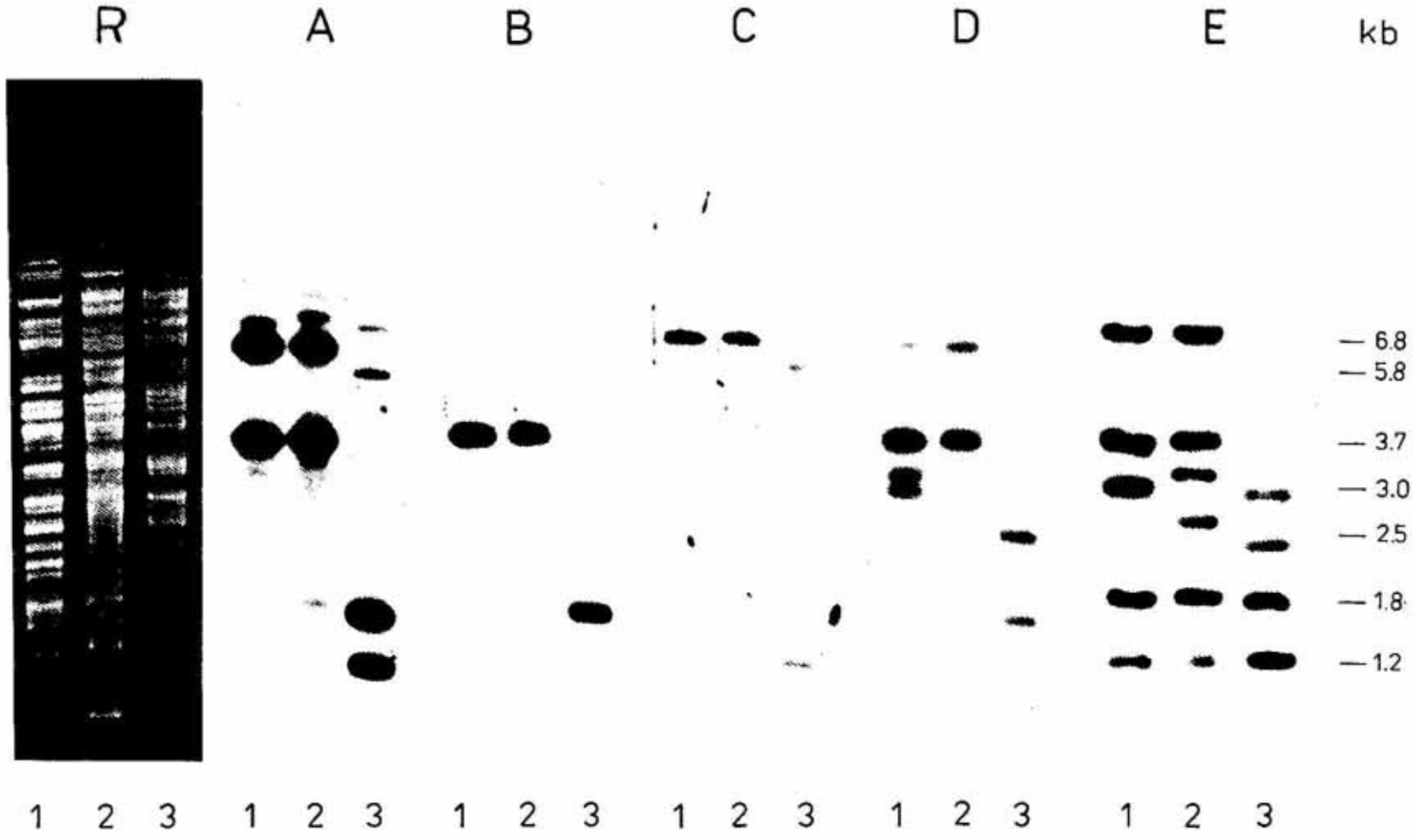

Fig. 3. Hybridization of ^{32}P-labelled probes (A, B, C, D, E) to lupin mtDNAs digested by EcoRI restriction enzyme.
Panel R the restriction paterns of mtDNA of three lupin species. Panels A, B, C, D hybridizations to the same Southern blots of lupin mtDNAs with probes A, B, C and D, respectively (cf. Fig.1). Panel E presents hybridization of the tRNA probe to lupin DNAs. The estimated size of the *Eco*RI fragments hybridizing to a particular probe is shown on the right side of panel E. Molecular size marker was λ DNA cleaved with *Hin*dIII. Lane 1, *L. luteus;* lane 2, *L. albus;* lane 3, *L. angustifolius.*

autoradiogram of this digest weaker hybridizing bands are also visible. The hybridization signals were more intense and more abundant than in the case of the *Bam*HI restriction pattern.

For purposes of direct comparison hybridization of probe A to *Eco*RI digested mtDNAs is shown in Fig. 3. As it can be seen, every lupin species gives two strong hybridization signals besides a few weaker ones. This may suggest that at least a part of the investigated sequences were present in more than one location in the mitochondrial genomes. To estimate which region of the probes (18S, 5S rRNA genes or tRNA-like repeat) can be present in multiple location in the genome, the same blot of the *Eco*RI cleaved mtDNAs was hybridized separately with probes B, C and D. As shown in panel B, *L. luteus* and *L. albus* DNAs hybridized with probe B (18S rRNA gene) displayed positive signals, predominantly to one fragment of 3.7 kb in size. A *L. angustifolius* fragment which showed a strong hybridization signal with probe B was smaller and was 1.8 kb in size. This may suggest that the 18S rRNA gene in the latter species is smaller than in yellow lupin, or that part of the gene is present in another fragment of mtDNA. When the same Southern blots of these DNAs were hybridized with probe C (5S rRNA gene) the signals in *L. luteus* and *L. albus* were the strongest at the fragments of 6.8 kb, whereas in *L. angustifolius* at the fragment of 1.2 kb. In *L. angustifolius* a fragment of 5.8 kb also showed hybridization with 5S rRNA but with lower intensity; this may suggest that the latter fragment does not contain a complete copy of 5S rRNA gene.

All the above results point to the presence of one copy of 18S/5S rRNA genes in the mitochondrial genome of all three lupin species studied.

In an attempt to extend our knowledge on the region about 5'-upstream of the 18S rRNA genes we compared the location of the tRNA-like repeat which surrounds this gene in yellow lupin. Unlike in wheat in which the 5'-end of 18S rRNA $tRNA^{fmet}$ is located in the 5'-upstream region, *L. luteus* contains a 90 nt long direct repeat, which can fold to tRNA-like structures. This direct repeat used as a hybridization probe to three filter bound lupin DNAs (probe D) showed that this sequence was present mainly close to 18S rDNA gene which seems to suggest that there is one type of arrangement in three *Lupinus* genomes of the 18S–5S unit. The comparison of hybridization signals shown in panel D confirmed the supposition that this repeat could be present not only close to 18S rRNA genes but also in other fragments of the genomes. In *L. luteus* mtDNA, strong signals displayed two fragments of about 3 kb whereas in *L. albus* and *L. angustifolius* fragments of 6.8 kb and about 2.5 kb, respectively. Fragments hybridizing less intensely to the tRNA-like repeat were also observed on the autoradiogram, which would indicate that the sequence homology in that case was lower. While the function of this conserved and reiterated sequence is unknown, its location in the 5'-flank of 18S rRNA from three *Lupinus* species may point to its regulatory function, or to a role in creation of genome diversity. Nevertheless, the presence of a tRNA-like repeat in different fragments of mtDNA is very characteristic and might be considered a marker of particular lupin cytoplasm.

To gain some idea whether tRNA-like repeats, like tRNA genes, occupy similar fragments, *Eco*RI restriction fragments were additionally probed with mttRNAs isolated from *L. luteus*. The duplicated Southern blot of *Eco*RI digested lupin mtDNAs was hybridized with tRNA probe (probe E). Radioactive tRNAs were prepared in two ways: by labelling at the 3'- or 5'-termini. No variation was observed in the hybridization pattern obtained with the two probes. Higher specific activities of the probe were obtained by labelling at the 5'-terminus with [γ^{32}-P]ATP and polynucleotide kinase. The results are shown in Fig. 3, panel E. The fragments of nylon bound mtDNAs hybridizing most strongly to the labelled yellow lupin mttRNA were similar in size. The similarities in the hybridization pattern were mostly observed between *L. luteus* and *L. albus*; similar *Eco*RI generated fragments of mtDNAs were of 6.8, 3.7, 1.8 and 1.2 kb in size. Divergence of the *L. angustifolius* pattern may point to some rearrangements that occurred in the genome of a common ancestor of the three lupin species. The same conclusion could be also drawn from the pattern of rRNA organization in this lupin species, or this pattern could reflect differential loss of duplicate loci present in particular species. The fragments having rRNA genes and tRNA-like repeat sequences showed the strongest hybridizing signals which may indicate a

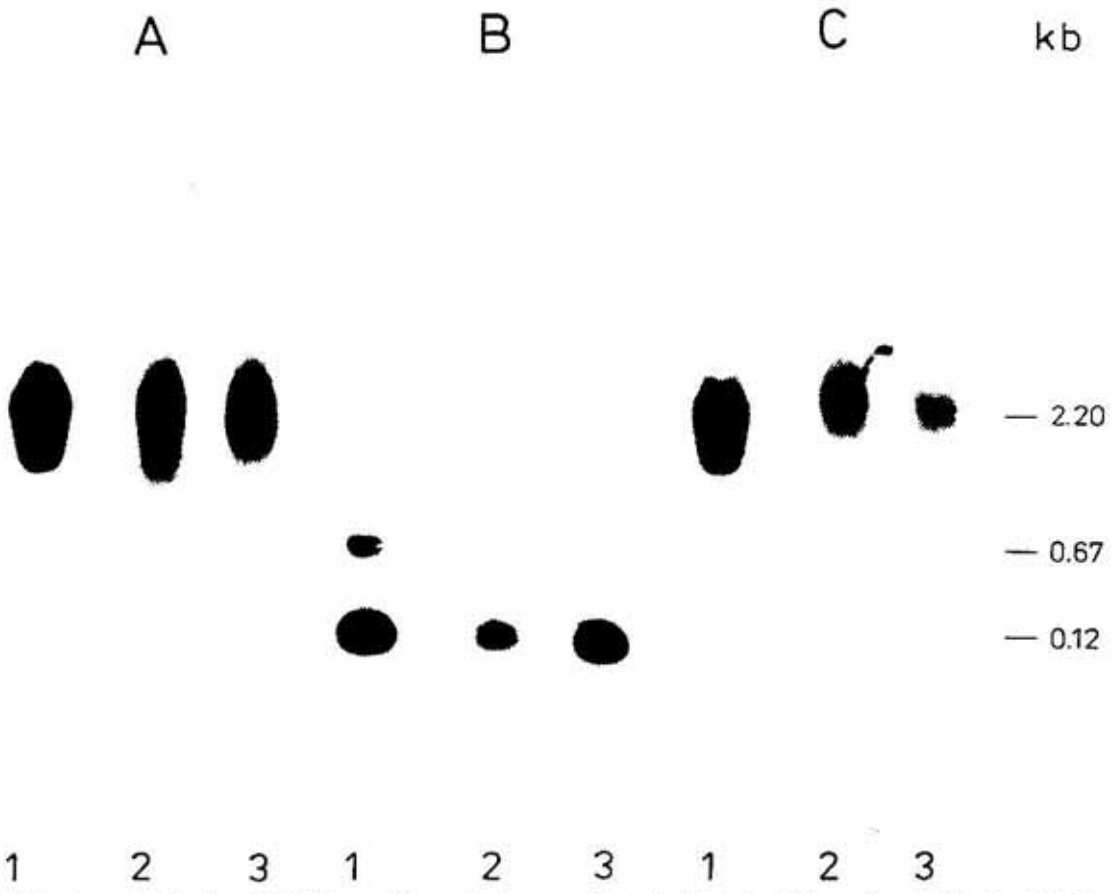

Fig. 4. Northern blot of mtRNA from three lupin species hybridized with probes **B** *(18S rRNA),* **C** *(5S rRNA) and* **D** *(tRNA-like repeat).*

The hybridization patterns of mtRNAs: panel A with probe **B**; panel B, with probe **C**; panel C, with probe **D**. Lanes 1 contained 15 μg of mtRNA from *L. luteus*. Lanes 2 and 3 contained 10 μg of mtRNAs from *L. albus* and *L. angustifolius*, respectively. The size of transcripts in kb is shown on the right hand side of panel C.

clustering of tRNA genes close to 18S, 5S rRNAs. The hybridization signal could also be exaggerated by tRNA-like sequences contained in 18S rRNA [22]. In addition to these fragments in all three lupin mtDNAs there was at least one hybridizing fragment characteristic of the particular lupin species, for example: of about 2.0 kb for *L. angustifolius*, about 2.7 kb for *L. albus* and about 3.2 kb for *L. luteus*. In general, distribution of tRNA species in three lupin mitochondrial genomes resembles the pattern reported for other plants [23].

To determine whether the 18S, 5S and tRNA-like repeat were transcriptionally active, Northern blots of RNA from mitochondria of *Lupinus* species were probed with these DNAs (Fig. 4). All the DNAs showed one predominant hybridizing band of 2.2 kb for 18S and tRNA-like repeat and of 0.12 kb for 5S rRNA. In the transcription pattern of 5S rRNA in *L. luteus* presumably a nonspecific degradation product of the main cotranscript or prematurely terminated transcription product is responsible for the presence of an additional transcript of 0.67 kb. Further analysis of the details of transcription of mitochondrial rRNA genes of three lupin species would clarify not only the transcription rate of particular genes but also the problem of cotranscription of 18S and 5S mtRNAs.

Lupin, a legume of agricultural importance, has been the subject of numerous biochemical studies. However, systematic screening of lupin cytoplasms and thorough studies on the extent of variability within and between species are lacking. The above presented results seem to indicate that the mitochondrial genome arrangement in *L. angustifolius* is the greatest among *Lupinus* species. At present we do not have any evidence which could suggest the existence of molecular heterogeneity in this species.

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