

## A comparative morphological and anatomical study of the model legume *Lotus japonicus* and related species

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**Summary:** A comparative anatomical study of main vegetative organs in three members of the genus *Lotus* section *Lotus* (namely *L. corniculatus* L., *L. japonicus* (Regel) Larsen, and a new described species *L. miyakojimae* Kramina) has been conducted. The plants investigated were collected in natural populations or grown from seeds. Quantitative data were analysed by several methods of statistics. The results obtained allow to extend anatomical and morphological descriptions of the studied species and to reveal their important diagnostic characters.

**Keywords:** Leguminosae, *Lotus*, anatomy, morphology, Japan, new species

The *Lotus corniculatus* species complex (Leguminosae-Papilionoideae-Loteae) is known as a complicated taxonomic group including both, diploid and tetraploid taxa. The typical form of *L. corniculatus* L. sensu stricto has a tetraploid chromosome number  $2n=24$  (GRANT 1965, 1995). Several other tetraploid forms described under the specific names *L. ambiguus* Bess., *L. arvensis* Pers., *L. balticus* Min., *L. callunetorum* (Juxip) Min., *L. drinensis* Min. et Ulle, *L. komarovii* Min., *L. ruprechtii* Min., *L. zbegulensis* Klok., and some others were proved to be so close to *L. corniculatus* s. str. by their morphological characters, that their recognition at the specific level has been rejected, and they have been included in *L. corniculatus* sensu lato. Several diploid races of the studied species complex with the chromosome number  $2n=12$  (i.e. *L. glaber* Mill. (syn. *L. tenuis* Walst. et Kit. ex Willd.), *L. kerylovii* Schischk. et Serg., *L. stepposus* Kramina, *L. schoelleri* Schweinf., and some others) are rather clearly separated by morphological characters from each other and from the tetraploid *L. corniculatus*, so they have been accepted as separate species (KRAMINA & TIKHOMIROV 1991; KRAMINA 1999 a,b, 2000). However, some facts do not support the concept of the existence of one large tetraploid species and several smaller diploid species. Particularly, it is known that some of the diploid races are not clearly differentiated from tetraploid ones. Such facts need special investigations.

Several species of the *L. corniculatus* complex recently attracted much attention because they are widely-used crop plants cultivated in many countries in the New and Old World (GRANT & MARTEN 1985). One diploid member of the complex, i.e. *Lotus japonicus* (Regel) Larsen, was selected as a model legume species for investigations of the mechanisms of symbiotic root nodules formation and nitrogen fixation (COOK et al. 1997) and for molecular and genetic studies (HANDBERG & STOUGAARD 1992). However, the attempts to recognize its taxonomic rank met serious problems. On one hand, *L. japonicus* is a diploid race geographically well separated from *L. corniculatus*, on the other hand, it is very close to the latter by morphological characters and was primarily described as its variety, *L. corniculatus* L. var. *japonicus* Regel. The problem of its taxonomic status is still unsolved.

Japanese researchers selected the population Gifu B-129 as a model line of *L. japonicus* for genetic studies. They also collected material from several other localities in Japan (KAWAGUCHI et al. 2001) to find an appropriate crossing partner to the model line. While collecting material they discovered an isolated population of *Lotus* (called Miyakojima MG-20) on the small island Miyako (Ryukyu Islands, South Japan). Plants of this population differ from the typical *L. japonicus* by a number of characters. Morphological differences between populations Gifu B-129 and Miyakojima MG-20 were summarized by KAWAGUCHI et al. (2001). They wrote that plants from Miyakojima differ from those in other Japanese accessions in low concentration of anthocyanin in stems and in petals, more upright habit, few trichomes, broad petals and leaflets, and large black seeds. Kawaguchi also found that plants of this population developed many flowers and pods in short-day conditions, while the typical *L. japonicus* is a long-day plant (KAWAGUCHI 2000). Genetic difference between populations Gifu B-129 and Miyakojima MG-20 consists in reciprocal translocation between the 1<sup>st</sup> and 2<sup>nd</sup> chromosome (HAYASHI et al. 2001). Basing on all mentioned peculiarities and the geographical isolation of the population Miyakojima MG-20, we suppose that it has reached a high degree of isolation and has to be described as separate species.

***Lotus miyakojimae* Kramina sp. nov.**

*Lotus japonicus* [K. Larsen in Bot. Tidsskr. LII: 13 (1955)] proxima, sed foliis latioribus, floribus longioribus (12–14 mm nec 10–12.5 mm), calycibus pedicellisque subglabris et seminibus majoribus atrofusis differt.

**Holotype:** Japan, Ryukyu islands, isle Miyakojima, Prefecture Okinawa, Miyakojima, meadow at the tip of Agarihenna cape; accession MG-20. Seeds received from M. Kawaguchi and grown in the greenhouse of the Lomonosov Moscow State University, Russia. Leg.: T. E. Kramina s.n., 17<sup>th</sup> Jun. 1999 [MW, with two isotypes].

Taking into account the insufficient knowledge about both, typical *L. japonicus* and the new taxon *L. miyakojimae*, the problem of their taxonomic relationships can not be considered as completely resolved. To enlarge the descriptions of both taxa and to specify their differences from *L. corniculatus*, a detailed biomorphological study including anatomical analysis is needed.

Data on microstructure of vegetative and generative organs of *Lotus* species are rather poor in literature. Papers dealing with anatomical structure of vegetative organs usually concern leaf epidermis (UJHELYI 1960; LARSEN & ŽERTOVÁ 1963; BORSOS 1969; ARAMBARRI & COLARES 1993; STENGLEIN et al. 2003) and stem structure (SZ.-BORSOS 1973). Authors of these publications noted a small difference of stomata guard-cell dimensions observed between diploid and tetraploid taxa. Studied species and infraspecific taxa were also different from each other in stomata density and shape of epidermis cells, as well as their anticlinal walls. On the base of results obtained from comparative anatomical studies of stems of several species of the *L. corniculatus* complex, SZ.-BORSOS (1973) concluded that the majority of the studied anatomical characters had low diagnostic and taxonomic significance.

The purpose of the present work is to conduct a comparative anatomical study of vegetative organs (i.e. shoot and root) of *L. miyakojimae* plants from the population MG-20 and the closely related species *L. japonicus* and *L. corniculatus* for obtaining more detailed anatomical and morphological descriptions and getting new diagnostic and taxonomic characters.

## Materials and methods

For anatomical studies of vegetative organs samples of plants collected in the wild in 1987, and samples of plants grown from seeds in the greenhouse of the Biological Faculty of the Lomonosov Moscow State University in 2000–2002 were used. Plant material was fixed in 70% ethanol.

### Plants grown from seeds

*Lotus japonicus* (Regel) Larsen — Japan, Honshu: Prefecture Gifu, Gifu, on the bank of Sakai river, population B-129; Prefecture Kanagawa, Totsuka, population MG-3; Prefecture Iwate, Tono, population MG-10 [specimens studied: 7];

*Lotus miyakojimae* Kramina — Japan, archipelago Ryukyu, Miyako, Prefecture Okinawa, Miyakojima, meadow at the tip of Agarihenna cape, population MG-20, the only known population in the world [specimens studied: 5].

Samples from populations B-129 and MG-20, collected in 2000 and 2002, were considered as different ones.

### Plant samples collected in the wild

*Lotus corniculatus* L. var. *birsutus* Koch — Russia, European part, Tula prov.: steppified meadow on limestone, slope by Upa river, population Nikolo-Zhupan; meadow on the compartment line in the broad-leaved forest, population Yasnaya Polyana; Ryazan prov.: meadow on the left bank of Oka river, population Izhevskoye; all samples collected in 1987 [specimens studied: 4];

*Lotus corniculatus* L. var. *corniculatus* — Russia, European part, Ulyanovsk prov., Ulyanovsk, forest margin, meadow, population Ulyanosk; sample collected in 1987 [specimens studied: 1].

Anatomical structure of vegetative organs was studied by cross sections. Temporary preparations were made by standard methods with conducting additional reactions on starch (with iodine in potassium iodide), anthocyanins (with vapour of ammonia and concentrated chlorohydric acid), and phlobaphenes (with ferric chlorid) (BARYKINA et al. 2004). From each population one to four specimens were selected for the study. Three sections from each part of the shoot (i.e. upper, middle, and lower internodes) were analyzed. On each section the characters of three bundles and three rays were measured. For the study of leaf microstructure three leaves from the middle part of a shoot were chosen from each population. Cross and surface sections were made from the middle part of the terminal leaflet. For anatomical studies light microscope, ocular micrometer, and a camera lucida were used. Seeds were investigated by means of binocular lens and a scanning electron microscope Hitachi-S405 A.

The following quantitative anatomical characters were included in the analysis:

Seventeen characters of *stem cross sections* (tables 1–3). The characters № 3 (type of additional vascular bundles), № 10 (degree of lignification of cell walls in xylem), and № 11 (degree of lignification of cell walls of ray parenchyma at xylem level) were estimated in grades. When a section was characterized by an intermediate or variable value of one of these characters, it was given an intermediate grade. Values of characters № 14–17 were measured in micrometers.

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Table 1: Means, standard errors, and significance of difference between specific and population means of characters of the upper stem internode (results of two-factor ANOVA). Significance of means difference: '\*' –  $P < 0.05$ , '\*\*' –  $P < 0.01$ , '\*\*\*' –  $P < 0.001$ , '—' – differences are not significant.

Characters	Species			Significance of means differences		Species means significantly different from each other
	<i>L. japonicus</i> species 1	<i>L. miyako-jimae</i> species 2	<i>L. corniculatus</i> var. <i>hirsutus</i> species 3	Between species	Between populations	
1. Number of main vascular bundles	10,17±0,49	8,58±0,19	11,67±0,38	*	***	all
2. Number of additional vascular bundles	0	0,33±0,22	0,33±0,22	—	**	—
3. Type of additional vascular bundles, grades (1 — complete, 2 — incomplete)	—	0,17±0,11	0,17±0,11	—	**	—
4. Number of cells in a radial xylem chain	3,65±0,19	4,78±0,21	5,24±0,31	*	***	3 from 1
5. Vascular bundle width (number of rows of xylem elements)	8,81±0,45	9,64±0,45	9,92±0,35	—	—	—
6. Ray width (number of cell rows)	9,59±0,67	12,03±0,34	8,72±0,48	**	—	3 from 2
7. Number of cells with phlobaphenes on the sides of protophloem bands	21,17±1,90	19,08±1,45	26,25±1,67	—	***	—
8. Number of cells with phlobaphenes in the cortex	0	0	0	—	—	—
9. Number of cells with phlobaphenes in the pith	7,89±1,43	5,25±0,62	11,92±0,93	—	***	—
10. Degree of lignification of cell walls in xylem (grades 0 to 3)	1,13±0,07	1,21±0,10	1,17±0,11	—	**	—
11. Degree of lignification of cell walls of ray parenchyma at xylem level (grades 0 to 3)	0,13±0,07	0,54±0,14	0,50±0,15	—	***	—
12. Number of trichomes on the transverse section of the stem	0,08±0,08	0	2,50±0,51	**	—	3 from 1 3 from 2
13. Number of cork layers	0	0	0	—	—	—
14. Thickness of the outer tangential wall of the epidermis cells (in $\mu\text{m}$ )	4,99±0,31	6,00±0,47	5,36±0,30	—	***	—
15. Thickness of the cuticle (in $\mu\text{m}$ )	1,29±0,10	1,39±0,06	1,33±0,06	—	—	—
16. Diameter of the largest tracheal elements in the main vascular bundles (in $\mu\text{m}$ )	11,46±0,56	16,15±1,29	15,41±1,22	—	***	—
17. Diameter of the largest tracheal elements in the additional vascular bundles (in $\mu\text{m}$ )	—	1,30±0,88	1,43±0,99	—	**	—

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Table 2: Means, standard errors, and significance of difference between specific and population means of characters of the middle stem internode (results of two-factor ANOVA). Significance of means difference: '\*' –  $P < 0.05$ , '\*\*' –  $P < 0.01$ , '\*\*\*' –  $P < 0.001$ , '—' – differences are not significant.

Characters	Species			Significance of means differences		Species means significantly different from each other
	<i>L. japonicus</i> species 1	<i>L. miyako-jimae</i> species 2	<i>L. corniculatus</i> var. <i>birsutus</i> species 3	Between species	Between populations	
1. Number of main vascular bundles	9,83±0,39	8,67±0,22	13,08±0,50	**	***	3 from 1 3 from 2
2. Number of additional vascular bundles	0	0,92±0,08	0,83±0,27	*	—	1 from 2 1 from 3
3. Type of additional vascular bundles, grades (1 — complete, 2 — incomplete)	—	0,92±0,08	0,50±0,15	**	*	1 from 2 1 from 3
4. Number of cells in a radial xylem chain	7,56±0,32	9,78±0,62	10,06±0,92	—	***	—
5. Vascular bundle width (number of rows of xylem elements)	10,83±0,68	13,83±0,70	13,78±0,59	—	***	—
6. Ray width (number of cell rows)	14,89±1,31	18,19±1,32	11,33±0,82	—	***	—
7. Number of cells with phlobaphenes on the sides of protophloem bands	29,00±2,94	17,67±1,34	31,17±1,14	*	***	2 from 1 2 from 3
8. Number of cells with phlobaphenes in the cortex	0	0	0	—	—	—
9. Number of cells with phlobaphenes in the pith	10,33±1,43	5,33±0,69	9,92±1,49	—	***	—
10. Degree of lignification of cell walls in the xylem (grades 0 to 3)	2,00±0,00	2,00±0,00	2,00±0,00	—	—	—
11. Degree of lignification of cell walls of ray parenchyma at xylem level (grades 0 to 3)	1,42±0,14	2,00±0,00	2,00±0,00	*	***	1 from 2 1 from 3
12. Number of trichomes on the transverse section of the stem	0,17±0,11	0	2,42±0,65	*	*	3 from 1 3 from 2
13. Number of cork layers	0	0	0	—	—	—
14. Thickness of the outer tangential wall of the epidermis cells (in $\mu\text{m}$ )	6,41±0,45	8,25±0,54	6,38±0,35	—	***	—
15. Thickness of the cuticle (in $\mu\text{m}$ )	1,33±0,06	1,41±0,08	1,20±0,05	—	—	—
16. Diameter of the largest tracheal elements in the main vascular bundles (in $\mu\text{m}$ )	22,62±1,46	27,12±1,35	26,21±1,57	—	***	—
17. Diameter of the largest tracheal elements in the additional vascular bundles (in $\mu\text{m}$ )	—	15,84±1,78	4,77±2,05	***	—	2 from 1 2 from 3

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Table 3: Means, standard errors, and significance of difference between specific and population means of characters of the lower stem internode (results of two-factor ANOVA). Significance of means difference: '\*' –  $P < 0.05$ , '\*\*' –  $P < 0.01$ , '\*\*\*' –  $P < 0.001$ , '—' – differences are not significant.

Characters	Species			Significance of means differences		Species means significantly different from each other
	<i>L. japonicus</i> species 1	<i>L. miyako-jimae</i> species 2	<i>L. corniculatus</i> var. <i>birsutus</i> species 3	Between species	Between populations	
1. Number of main vascular bundles	6,83±0,63	6,50±0,40	10,36±0,24	*	***	3 from 1 3 from 2
2. Number of additional vascular bundles	0,33±0,19	3,08±0,29	3,45±1,06	—	***	—
3. Type of additional vascular bundles, grades (1 — complete, 2 — incomplete)	0,42±0,23	1,08±0,06	1,18±0,25	—	*	—
4. Number of cells in a radial xylem chain	22,19±0,91	21,67±1,72	17,30±0,77	—	***	—
5. Vascular bundle width (number of rows of xylem elements)	19,22±1,84	24,58±2,46	18,03±1,07	—	***	—
6. Ray width (number of cell rows)	15,67±2,22	14,08±1,98	11,24±1,35	—	***	—
7. Number of cells with phlobaphenes on the sides of protophloem bands	13,50±1,14	7,92±1,64	13,55±1,36	—	***	—
8. Number of cells with phlobaphenes in the cortex	43,92±12,84	0,00±0,00	29,64±11,28	—	***	—
9. Number of cells with phlobaphenes in the pith	5,58±0,90	3,83±0,60	8,36±0,43	—	***	—
10. Degree of lignification of cell walls in the xylem (grades 0 to 3)	2,50±0,15	3,00±0,00	3,00±0,00	—	—	—
11. Degree of lignification of cell walls of ray parenchyma at xylem level (grades 0 to 3)	2,63±0,13	3,00±0,00	3,00±0,00	—	—	—
12. Number of trichomes on the transverse section of the stem	0,00±0,00	0,00±0,00	0,55±0,31	—	—	—
13. Number of cork layers	0,46±0,24	0,00±0,00	3,27±0,52	*	***	3 from 1 3 from 2
14. Thickness of the outer tangential wall of the epidermis cells (in $\mu\text{m}$ )	6,51±0,30	10,26±0,60	6,93±0,44	**	**	2 from 1 2 from 3
15. Thickness of the cuticle (in $\mu\text{m}$ )	1,43±0,08	1,44±0,08	1,10±0,05	—	—	—
16. Diameter of the largest tracheal elements in the main vascular bundles (in $\mu\text{m}$ )	19,44±0,82	26,50±1,39	28,29±1,76	—	***	—
17. Diameter of the largest tracheal elements in the additional vascular bundles (in $\mu\text{m}$ )	3,43±1,86	16,72±0,88	9,52±2,43	**	—	1 from 2 1 from 3

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Twenty-two characters of *leaf cross sections* (table 4). Values of characters № 1, 3–4, 10–11, 17–22 were measured in micrometers ( $\mu\text{m}$ ). Characters № 5–7 were estimated in grades. Characters № 14 and 15 are indices. Character № 14 shows the degree of manifestation of palisade tissue (i.e. a portion of palisade tissue in the whole mesophyll). Character № 15 demonstrates how much a midvein protrudes beyond the leaf blade. Values of character № 8 were counted on the distance of  $425\ \mu\text{m}$  from the midvein.

Fourteen characters of the *leaf epidermis* (table 5). Values of characters № 1, 3, 8, and 10 were measured in micrometers ( $\mu\text{m}$ ). Characters № 4 and 11 (protrusion of lobes of adaxial epidermal cells) are estimated in percents of cell width.

Statistical analyses were performed using the software package Statistica for Windows (Version 5.5). For all characters of stems and leaves arithmetical means and standard errors were calculated. Comparison of population and specific means was performed using two-factor MANOVA, nested design, mixed effects. ‘Species’ was a fixed factor, and ‘population’ was a random one. The factor ‘population’ was nested by the factor ‘species’. A set of characters providing significant interspecific difference was revealed. Means comparison was performed by Tukey method. *L. corniculatus* var. *corniculatus* was not included in MANOVA analysis because of the insufficiency of material (only one population was studied).

For testing the hypothesis on the discrimination of species and varieties on the basis of a set of anatomical characters the stepwise discriminant analysis was performed. The analysis was conducted separately for the characters of upper, middle, and lower internodes, and of leaf cross section.

## Results

### Morphological description

#### *Lotus japonicus*, population B-129

Perennial or short-lived perennial herbs with a main root. The main shoot of the seedling is usually well developed and later reaches generative phase of development (starts to bloom). It is often tortuous and has a tendency towards lying flat before blooming. Together with the main shoot, two lateral shoots start to develop rather early and often almost overtake the main one in size. Besides two “main” lateral shoots, some additional shoots start to rise from serial buds in cotyledonary axils. The general number of lateral shoots rising from cotyledonary axils may reach 4 and even more. The longest shoot (usually, the main) reaches 34–36 cm in blooming phase and 39 cm in fruiting phase. Leaves have five leaflets. They are green or greyish-green. The 3 upper leaflets are obovate, rarely narrow-obovate (two lateral upper ones are slightly oblique), the leaflets of the lower pair are oblique-ovate. The size of the upper impairy leaflet is  $12.4\text{--}13.8 \times 6.5\text{--}7.4\text{ mm}$  on leaves in the middle part of the shoot and  $10\text{--}12.7 \times 4.7\text{--}5.4\text{ mm}$  on those in the upper part. Leaves are glabrous or with solitary or rare trichomes  $0.1\text{--}1.0\text{ mm}$  long (indumentum develops mainly along the main vein, on the rachis and petiolules). Stems are glabrous, or covered with solitary, rare or middle-density trichomes. Peduncles are  $4.2\text{--}11.5\text{ cm}$  long. Umbels are 1–2-flowered. The corolla is yellow,  $9.8\text{--}12.5\text{ mm}$  long. The calyx is  $6.2\text{--}7.2\text{ mm}$  long, slightly zygomorphic (its teeth of unequal width); the teeth ( $3.1\text{--}3.7\text{ mm}$  long) are equal to, or slightly longer than the tube ( $2.8\text{--}3.5\text{ mm}$  long). The awl-shaped part of the calyx teeth

Table 4: Means, standard errors, and significance of difference between specific and population means of characters of the transversal leaf section (results of two-factor ANOVA). Significance of means difference: '\*' –  $P < 0.05$ , '\*\*' –  $P < 0.01$ , '\*\*\*' –  $P < 0.001$ , '—' – differences are not significant.

Characters	Means and standard errors for each character			Significance of means differences		Species means significantly different from each other
	<i>L. japonicus</i> species 1	<i>L. miyakojimae</i> species 2	<i>L. corniculatus</i> var. <i>birsutus</i> species 3	Between species	Between populations	
1. Thickness of the leaf blade near the midvein (in $\mu\text{m}$ )	246,41 $\pm$ 14,07	302,60 $\pm$ 10,67	274,27 $\pm$ 9,63	—	*	—
2. Number of vascular bundles in the midvein	1,22 $\pm$ 0,15	1,00 $\pm$ 0,00	1,00 $\pm$ 0,00	—	—	—
3. Height of adaxial epidermal cells above the midvein (in $\mu\text{m}$ )	24,18 $\pm$ 0,92	34,47 $\pm$ 2,19	30,13 $\pm$ 2,78	—	*	—
4. Height of abaxial epidermal cells below the midvein (in $\mu\text{m}$ )	39,86 $\pm$ 2,30	51,38 $\pm$ 2,65	39,67 $\pm$ 1,63	*	—	2 from 1 2 from 3
5. Interruption of palisade tissue above the midvein 1 – interrupted, 0 – uninterrupted	0	0	0	—	—	—
6. Type of mechanical tissue below the midvein 1 – collenchymatous parenchyma, 0 – no mechanical tissue developed	0,89 $\pm$ 0,11	1,00 $\pm$ 0,00	0,50 $\pm$ 0,08	*	—	3 from 1 3 from 2
7. Type of vascular bundle sheath: 1 – parenchyma, 2 – chlorenchyma, 3 – cells with phlobaphenes	1,28 $\pm$ 0,12	1,17 $\pm$ 0,08	2,16 $\pm$ 0,09	**	—	3 from 1 3 from 2
8. Number of cells with phlobaphenes in the leaf mesophyll	0,22 $\pm$ 0,22	0,00 $\pm$ 0,00	2,78 $\pm$ 0,40	***	—	3 from 1 3 from 2
9. Thickness of the leaf blade in the area of small veins (in $\mu\text{m}$ )	168,49 $\pm$ 3,98	241,02 $\pm$ 4,30	223,27 $\pm$ 5,46	***	—	all
10. Thickness of palisade tissue in the area of small veins (in $\mu\text{m}$ )	47,98 $\pm$ 3,56	92,56 $\pm$ 5,48	67,24 $\pm$ 4,04	**	*	all
11. Thickness of spongy tissue in the area of small veins (in $\mu\text{m}$ )	62,99 $\pm$ 3,08	87,27 $\pm$ 5,42	92,74 $\pm$ 4,61	*	—	1 from 2 1 from 3
12. Number of layers of palisade tissue in the area of small veins	2,00 $\pm$ 0,00	2,22 $\pm$ 0,15	2,39 $\pm$ 0,14	—	—	—
13. Number of layers of spongy tissue in the area of small veins	3,83 $\pm$ 0,14	3,94 $\pm$ 0,06	4,44 $\pm$ 0,23	—	—	—
14. Coefficient $P = \frac{\text{char10}}{(\text{char10} + \text{char11})} \cdot 100\%$	43,03 $\pm$ 2,24	51,45 $\pm$ 2,74	42,01 $\pm$ 1,89	—	*	—
15. Protrusion of the midvein $(= \frac{\text{char1} - \text{char9}}{\text{char9}} \cdot 100\%)$	46,88 $\pm$ 8,71	25,68 $\pm$ 4,48	22,98 $\pm$ 3,68	—	**	—
16. Number of trichomes on the leaf blade	0	0	0	—	—	—
17. Height of adaxial epidermal cells in the area of small veins (in $\mu\text{m}$ )	26,35 $\pm$ 0,76	28,05 $\pm$ 1,32	28,14 $\pm$ 1,89	—	*	—
18. Height of abaxial epidermal cells in the area of small veins (in $\mu\text{m}$ )	31,17 $\pm$ 1,51	33,15 $\pm$ 1,62	35,13 $\pm$ 3,05	—	**	—
19. Thickness of the outer wall of the adaxial epidermal cells in the area of small veins (in $\mu\text{m}$ )	6,08 $\pm$ 0,52	4,80 $\pm$ 0,28	5,18 $\pm$ 0,45	—	**	—
20. Thickness of the outer wall of the abaxial epidermal cells in the area of small veins (in $\mu\text{m}$ )	4,65 $\pm$ 0,38	4,98 $\pm$ 0,37	5,58 $\pm$ 0,38	—	**	—
21. Thickness of the cuticle of the outer wall of the adaxial epidermal cells in the area of small veins (in $\mu\text{m}$ )	1,33 $\pm$ 0,06	1,28 $\pm$ 0,05	1,25 $\pm$ 0,05	—	—	—
22. Thickness of the cuticle of the outer wall of the abaxial epidermal cells in the area of small veins (in $\mu\text{m}$ )	1,43 $\pm$ 0,08	1,25 $\pm$ 0,05	1,30 $\pm$ 0,05	—	—	—



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Table 5: Means, standard errors, and significance of difference between specific and population means of characters of leaf epidermis (results of two-factor ANOVA). Significance of means difference: ‘\*’ –  $P < 0.05$ , ‘\*\*’ –  $P < 0.01$ , ‘\*\*\*’ –  $P < 0.001$ , ‘—’ – differences are not significant.

Characters	Means and standard errors for each character			Significance of means differences		Species means significantly different from each other
	<i>L. japonicus</i> species 1	<i>L. miyako-jimae</i> species 2	<i>L. corniculatus</i> var. <i>hirsutus</i> species 3	Between species	Between populations	
1. Length of guard cells of the adaxial epidermis (in $\mu\text{m}$ )	23,76 $\pm$ 0,36	25,62 $\pm$ 0,37	27,34 $\pm$ 0,41	—	***	—
2. Number of adaxial epidermal cells adjoining the stoma	3,67 $\pm$ 0,11	3,50 $\pm$ 0,12	3,40 $\pm$ 0,09	—	—	—
3. Length of adaxial epidermal cells (in $\mu\text{m}$ )	68,48 $\pm$ 2,04	75,84 $\pm$ 2,01	71,72 $\pm$ 2,49	—	—	—
4. Protrusion of lobes of adaxial epidermal cells (in %)	17,07 $\pm$ 1,08	18,27 $\pm$ 0,92	18,37 $\pm$ 1,06	—	**	—
5. Number of lobes of adaxial epidermal cells	6,60 $\pm$ 0,36	7,23 $\pm$ 0,22	7,10 $\pm$ 0,21	—	*	—
6. Stoma density in the adaxial epidermis (number of stomata / $\text{mm}^2$ )	165,90 $\pm$ 5,91	170,07 $\pm$ 7,69	165,10 $\pm$ 6,71	—	**	—
7. Trichome density in the adaxial epidermis (number of trichomes / $\text{mm}^2$ )	1,67 $\pm$ 0,44	0,00 $\pm$ 0,00	8,65 $\pm$ 0,22	**	***	all
8. Length of guard cells of the abaxial epidermis (in $\mu\text{m}$ )	23,72 $\pm$ 0,62	25,37 $\pm$ 0,44	27,78 $\pm$ 0,40	—	***	—
9. Number of abaxial epidermal cells adjoining the stoma	3,60 $\pm$ 0,09	3,50 $\pm$ 0,10	3,63 $\pm$ 0,12	—	—	—
10. Length of abaxial epidermal cells (in $\mu\text{m}$ )	68,99 $\pm$ 1,81	74,83 $\pm$ 3,47	75,78 $\pm$ 2,27	—	***	—
11. Protrusion of lobes of abaxial epidermal cells (in %)	21,67 $\pm$ 1,19	19,43 $\pm$ 1,25	20,43 $\pm$ 1,03	—	**	—
12. Number of lobes of abaxial epidermal cells	6,40 $\pm$ 0,39	7,33 $\pm$ 0,26	7,43 $\pm$ 1,03	—	***	—
13. Stoma density in the abaxial epidermis (number of stomata / $\text{mm}^2$ )	163,33 $\pm$ 8,34	173,33 $\pm$ 6,34	157,50 $\pm$ 0,21	—	***	—
14. Trichome density in the abaxial epidermis (number of trichomes / $\text{mm}^2$ )	0,00 $\pm$ 0,00	0,00 $\pm$ 0,00	7,28 $\pm$ 0,48	**	***	3 from 1 3 from 2

is usually half of their length (it varies from 0.33 to 0.67). The calyx is covered by a middle- or low-density indumentum, which is more dense on its ventral side. Trichomes are slightly or greatly spreading, 0.1–1.5 mm long. On edges of the wide part of the calyx teeth, middle-density spreading trichomes 0.1–0.5 mm long develop. Legumes are straight, cylindrical, often dark-brown 15–35  $\times$  1.6–1.9 mm, or rarely light-brown 15–38  $\times$  1.8–2.7 mm.

Seeds are globose-disciform or globose-triangular-disciform, on the lateral side ovate or triangular-ovate, rarely roundish; on the hilum side ovate. Seed surface is smooth, dull. Seeds from light-brown one-coloured to brown with light-brown spots, sometimes with rare dark-purple points (dots); the radicular lobe is slightly prominent; seed size: length 1.2–1.4 mm, width 1.1–1.3 mm, thickness 0.9–1.1 mm. Hilum roundish, rather large, situated on the level of seed surface, in the middle of the ventral part of the seed, surrounded by a rim aril. Hilar rim roundish, more light than the main surface colour, yellowish-brown or light-brown. Raphe obovate, brown or greenish-brown, darker than the main surface colour. Lens prominent. Micropyle widely-

triangular. Primary exotesta sculpture papillose-foveolate on lateral side and foveolate on hilum side. Secondary exotesta sculpture smooth or slightly rugulate on lateral side and rugulate on hilum side.

*Lotus miyakojimai*, population MG-20

Short-living perennial herb with a main root or probably annual. The main shoot of the seedling is usually well developed. It is often vertical and usually lying down flat only in the fruiting phase. Together with the main shoot, one or two lateral shoots usually develop in cotyledonary axils, but they are often smaller in size than the main one. Besides two “main” lateral shoots some additional shoots sometimes arise from serial buds in cotyledonary axils, but their number is usually small (1–2) and their development weak. The longest shoot (usually, the main) reaches 17–33 cm in blooming phase and 43 cm in fruiting phase. Leaves consist of five, light-green leaflets. The 3 upper leaflets are obovate (two lateral upper ones are slightly oblique), the leaflets of the lower pair are oblique-ovate. Size of the upper impairy leaflet is  $12.2\text{--}13 \times 7.6\text{--}8.6$  mm on leaves in the middle part of the shoot and  $12.1\text{--}15.5 \times 5.4\text{--}8.3$  mm on those in the upper part. Leaves are glabrous or with solitary more or less spreading trichomes 0.1–0.8 mm long (indumentum develops mainly on the rachis and petiolules). Stems are usually glabrous. Peduncles 6.2–14.2 cm long. Umbels are 1 (rarely 2)-flowered. Corolla yellow, 12–14 mm long. Calyx 6.3–8.0 mm long, slightly zygomorphic (its teeth of unequal width), the teeth (3.2–4.7 mm long) are usually longer than the tube (2.8–3.4 mm long). The awl-shaped part of calyx teeth is usually 0.30–0.50% (sometimes 0.20%) of their length. Calyx usually glabrous, and only on the edges of the wide part of the teeth middle-density spreading trichomes 0.1–0.7 mm long develop. Legumes straight, cylindrical, light-brown  $33\text{--}42 \times 1.7\text{--}2.7$  mm.

Seeds like in the previous taxon but dark-brown, sometimes with rare lighter spots, densely covered with dark-purple spots or dots; the radicular lobe is prominent, straight. Seed size: length 1.4–1.6 mm, width 1.1–1.4 mm, thickness 0.9–1.1 mm. The secondary exotesta sculpture is like the previous phenotype or, rugulate-reticulate on the hilum side (small cells of the reticulum are formed by the cuticle above single cells of the exotesta).

### Anatomical structure of one-year generative shoots

#### Stem

All species studied are characterized by a set of common stem characters. Among them are a narrow (3–5-layered) chlorophyll-bearing primary cortex with its inner layer endodermis presented as starch sheath. The stele is fasciculate. Vascular bundles are open, the vascular cambium develops predominantly secondary xylem. Tracheal elements are in radial chains. Phloem includes conspicuous groups of thick-walled primary and secondary fibres. Bundles are separated by wide pith rays with cell walls often lignified at xylem level. Additional bundles were revealed in the majority of studied specimens. In the primary cortex, pith rays and pith idioblasts with phlobaphenes are present. Nodal anatomy is trilacunar, three-trace.

However, the stem microstructure along the shoot is not constant which arises from the peculiarities of shoot morphogenesis and different functional loads in each of its three zones (TROLL 1964).

*Upper internodes* (figs. 1 A,D; 2 A). All species studied possess 8 to 13 vascular bundles, additional bundles are usually absent but sometimes present (e.g. in *L. corniculatus* var. *hirsutus*). The degree

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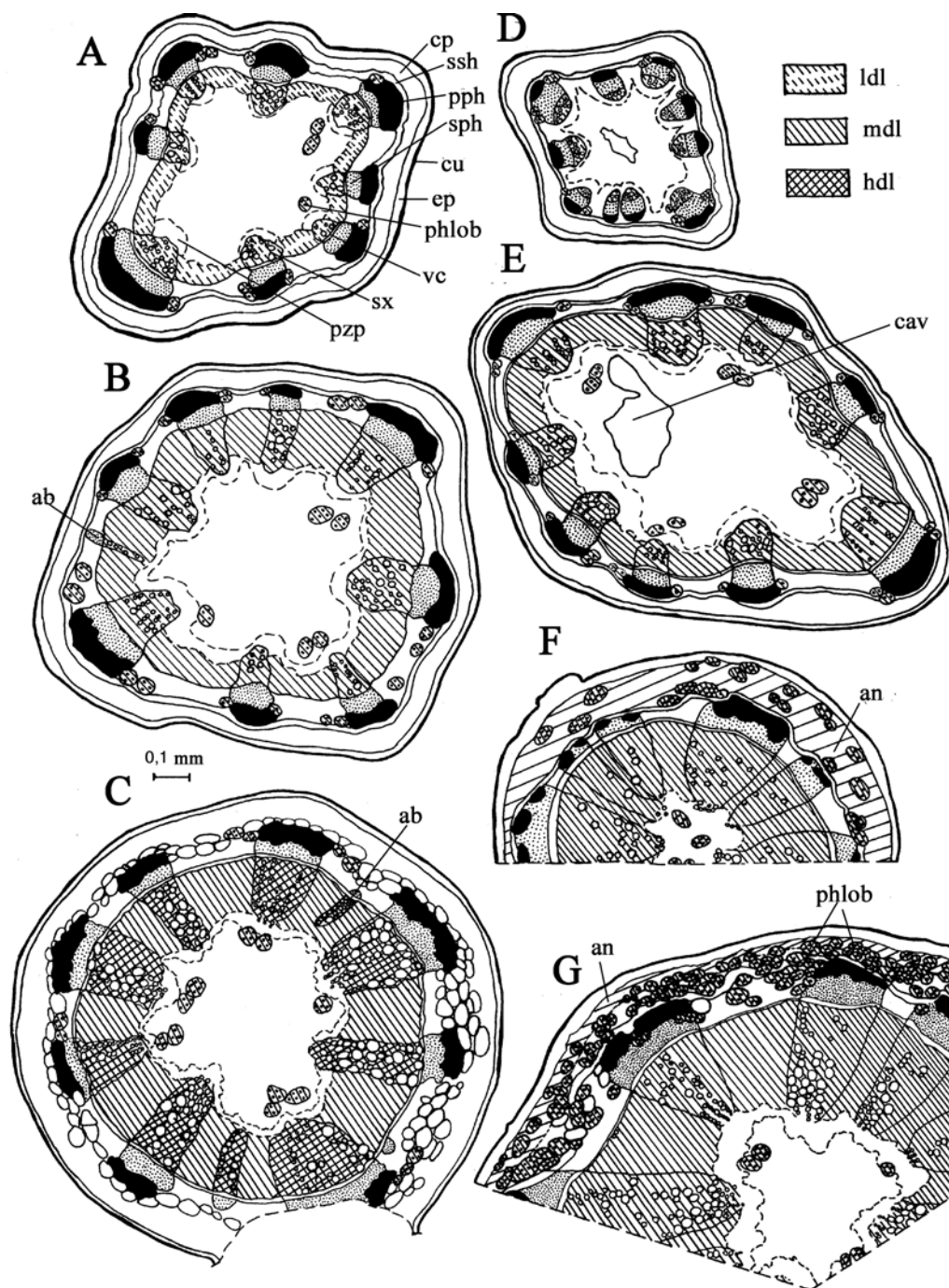


Figure 1: Transverse sections of stems; schemes: A, B, C – *Lotus miyakojimae*; D, E, F, G – *L. japonicus*; A, D – upper internode; B, E – middle internode; C, F, G – lower internode.

an – cells with anthocyanins, sx – secondary xylem, sph – secondary phloem, ab – additional bundle, ssh – starch sheath, cu – cuticle, vc – vascular cambium, pzp – peripheral zone of the pith, cp – cortex parenchyma, pph – protophloem fibers, hdl – high degree of lignification, ldl – low degree of lignification, mdl – middle degree of lignification, phlob – cells with phlobaphenes, ep – epidermis, cav – cavity.

of lignification of cell walls of xylem elements is low. On the sides of protophloem 8 to 34 idioblasts with phlobaphenes are present, and in the pith there are 1 to 17 idioblasts. Radial chains of xylem include 2 to 7 elements, the diameter of the largest varies from 8  $\mu\text{m}$  to 24  $\mu\text{m}$ . Characters of upper internodes are the variable (see tab. 1), especially the following: number and type of additional bundles, number of cells with phlobaphenes, the degree of lignification of cell walls in xylem and ray parenchyma, the thickness of outer tangential wall of epidermis cells, and the diameter of largest vessels. We revealed significant differences between species in 4 of 16 studied characters. These are the number of main bundles, the number of cells in the radial chain of xylem, ray width, and the number of trichomes on the transverse section of stem. All three species studied are significantly different in the number of main bundles: minimal values (8 to 10) were revealed in *L. miyakojimae*, maximal values (10 to 13) in *L. corniculatus* var. *hirsutus*. Ray width correlates with the number of bundles. *L. corniculatus* var. *hirsutus* is characterized by narrower rays containing 5 to 11 cells, and in *L. miyakojimae* the rays are broader, with 8 to 14 cells. Vascular cambium in the stems of *L. corniculatus* var. *hirsutus* was the most active and formed 4 to 8 xylem elements in a radial chain, while in *L. japonicus* its activity was minimal, and the number of xylem elements was 2 to 6. The highest trichome density was found in *L. corniculatus* var. *hirsutus*, the two other studied species had almost glabrous stems.

*Middle internodes* (figs. 1 B, E; 2 B), as compared to the upper ones, are characterized by the increase of the number of main bundles up to 8–15, accompanied by the enlargement of their diameter up to 8 to 18 cells, and the elongation of radial chains of xylem elements to 6–14. The diameter of the largest tracheal elements is increased up to 17–37  $\mu\text{m}$ . The degree of lignification of xylem cell walls is also increased. In all stems studied the indumentum density is comparatively low. Though the majority of characters varied on inter-population level (i.e. number of cells in radial chain of xylem, bundle and ray width, number of idioblasts with phlobaphenes in the stele, thickness of the outer wall of epidermis cells, diameter of the largest tracheal elements, see tab. 2), we revealed 7 characters of interspecific level. *L. japonicus* differs from other species in the absence of additional bundles and low degree of lignification of ray parenchyma. *L. miyakojimae* is characterized by a comparatively small number of cells with phlobaphenes and the maximal diameter of tracheal elements in additional bundles. *L. corniculatus* var. *hirsutus* has the highest number of vascular bundles (i.e. 10 to 15, as compared to 8–12 in other two species), and higher trichome density.

In *lower internodes* (figs. 1 F, G; 2 C) we revealed a decrease of the number of main bundles to 5–11 accompanied by the increase of additional bundles. Solitary additional bundles appeared in *L. japonicus* – not present in sections of other parts of the stem. The dimensions of main bundles increase, radial chains of xylem include 15 to 30 elements, the bundle width is 9 to 35 elements. The width of some rays diminishes to 4–10 cells. In stems of perennials a periderm appears. In the cortex many idioblasts with phlobaphenes are present. In the majority of characters, considerable inter-population variability was revealed (tab. 3), and only 4 features have diagnostic value and characterize individual species. Basal internodes of *L. corniculatus* var. *hirsutus* are characterized by the maximal number of main vascular bundles, and by a multilayer cork containing up to 5 layers. The phellogen initiates separate arcs deep in the cortex. In the lower internodes of *L. miyakojimae* with annual habit under culture conditions, the cork is absent. It has a thick-walled epidermis with an outer wall of 6–12  $\mu\text{m}$  thickness (4–9  $\mu\text{m}$  in the other species studied). In basal metameres, *L. japonicus* has no or solitary incomplete additional bundles with

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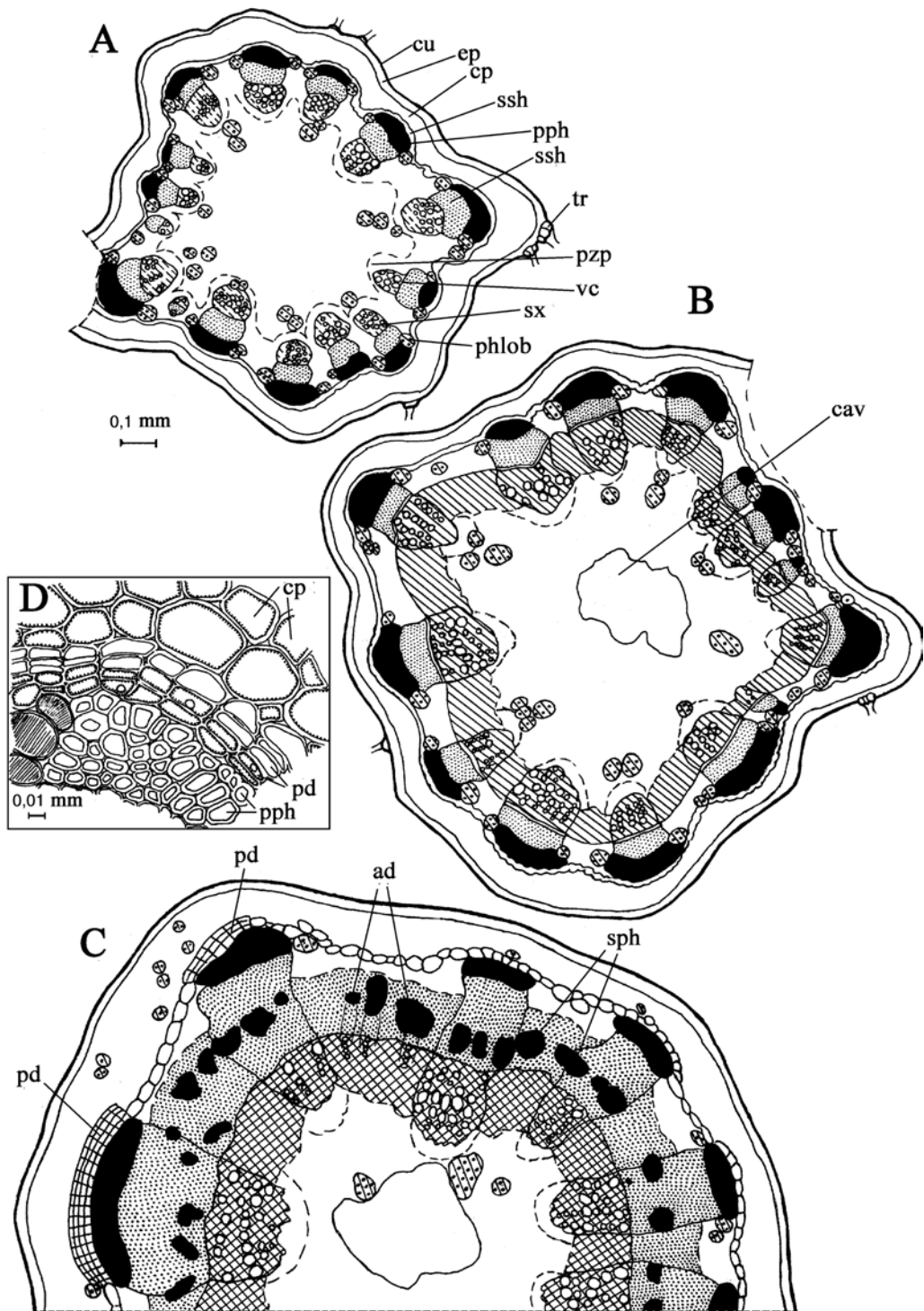


Figure 2: Transverse sections of the stem of *Lotus corniculatus* var. *hirsutus*: A – upper internode; B – middle internode; C – lower internode (schemes); D – initiation of periderm.

tr – trichome, sph – secondary phloem fibers, pd – periderm. The rest of symbols are the same as in fig. 1.

comparatively narrow conducting elements. This species is also characterized by a great amount of anthocyanins what is more abundant in plants of northern populations. Anthocyanins are located in outer layers only (fig. 1 G: plant from the population Gifu, central part of Honshu), or totally in the cortex (fig. 1 E: plant from the population Tono, northern part of Honshu). This specific feature was mentioned by other authors earlier (KAWAGUCHI et al. 2001). However, due to quick outwashing of anthocyanins, this character is very unstable and was not analyzed by quantitative methods. We conclude that anatomical characters of middle internodes of the stems were the most informative for species delimitation.

#### Multivariate analysis based on the characters of stem microstructure

To find out if the species under consideration can be delimited on the basis of the complex of anatomical features of the stem, a stepwise discriminant analysis was conducted. The analysis was carried out separately for the upper, middle, and lower internodes (fig. 3).

In the analysis of upper internodes 15 characters were included. The characters 'number of cells with phlobaphenes in the cortex' and 'number of cork layers' were excluded because their values were equal 0 in all cases. 8 characters were selected by the stepwise procedure (namely the characters № 1, 4, 5, 7, 12–16), and they were used in the final analysis. For the first root the maximal standardized coefficient (equal to -0.72) was associated with the character 'number of trichomes on the transverse section of the stem'. Those for the second root (equal to 0.75 and -0.68) were connected to the characters 'diameter of the largest tracheal elements in the main vascular bundles' and 'number of main vascular bundles' respectively. Two first canonical variables explain 86% of total among-groups variability. Clouds of the species in the subspace of the first and second roots contacted each other and overlapped partly (fig. 3 A). The studied species were not clearly separated. Specimens of *L. corniculatus* var. *corniculatus* were closer to *L. japonicus* than to *L. corniculatus* var. *hirsutus* which might be connected with the pubescence pattern. The pubescence was dense in *L. corniculatus* var. *hirsutus* and comparatively low in *L. japonicus* and *L. corniculatus* var. *corniculatus*.

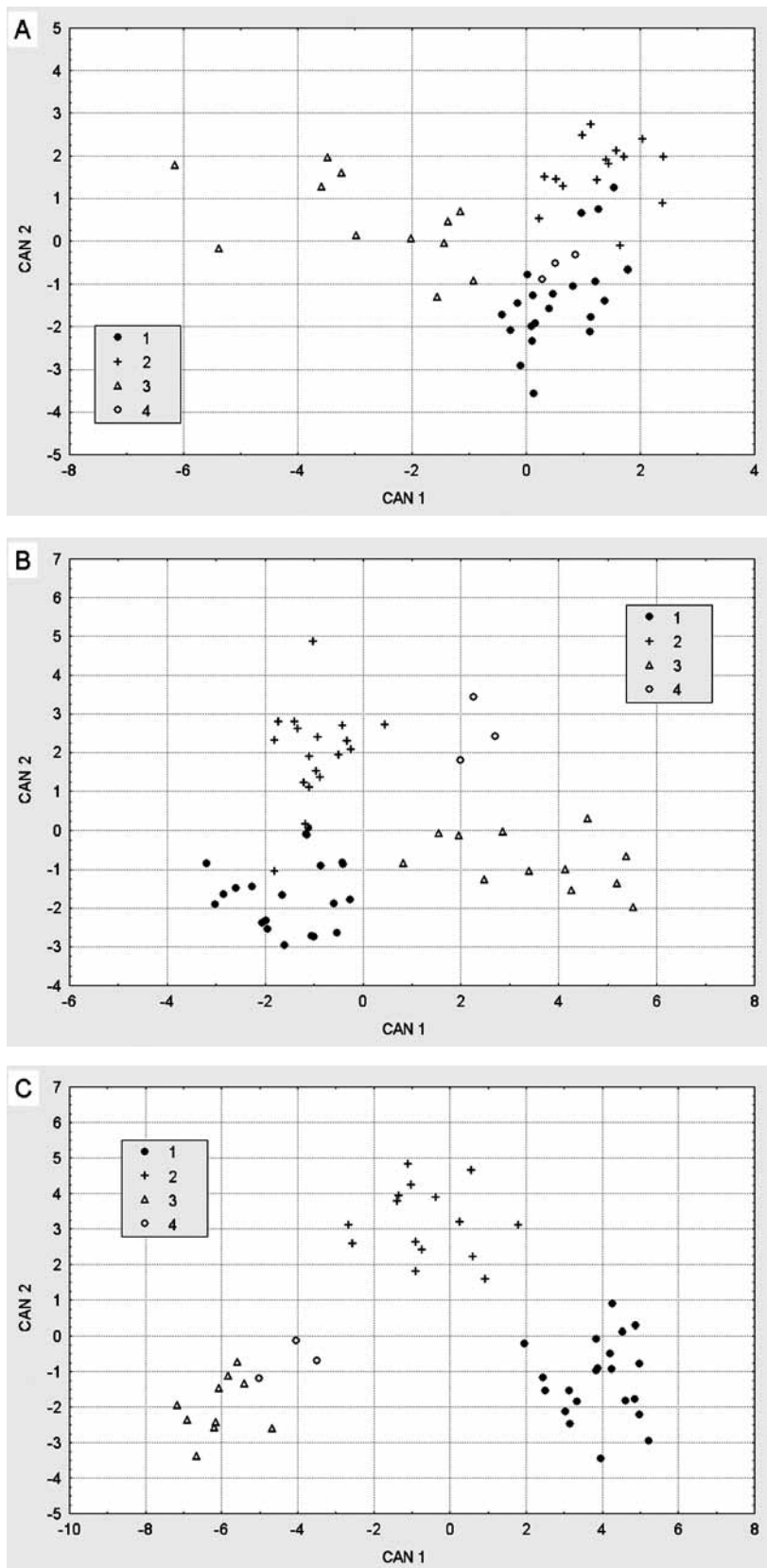
The discriminant analysis of middle internodes was conducted using 14 characters. The characters 'degree of lignification of cell walls in xylem', 'number of cells with phlobaphenes in the cortex', and 'number of cork layers' were excluded because their values were constant. 11 characters (namely the characters № 1, 2, 5–7, 9, 12, 14–17) were selected by the stepwise procedure as the most informative. This variant of analysis demonstrated more clear separation of studied species than the previous one (fig. 3 B). In the subspace of the first and second roots clouds of the species were more compact and didn't overlap. Two first canonical variables explain 90% of the total among-groups variability. The maximal influence on the root № 1 showed the characters 'ray width' (its standardized coefficient -1.10), 'number of main vascular bundles' (0.82), and 'diameter of the largest tracheal elements in the main vascular bundles' (0.89). For the second root the most valuable characters were 'number of additional bundles', 'diameter of the largest

Figure 3: The results of discriminant analysis of studied *Lotus* species made by stem characters. Two-dimension scatterplots of cases in the subspace of the first and second roots: A – by upper internode characters; B – by middle internode characters; C – by lower internode characters.

1 – *Lotus japonicus*, 2 – *L. miyakoimae*, 3 – *L. corniculatus* var. *hirsutus*, 4 – *L. corniculatus* var. *corniculatus*.

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tracheal elements in the additional vascular bundles', and 'number of cells with phlobaphenes on the sides of protophloem bands'. Their standardized coefficients were -0.81, 1.49 and -1.22, correspondingly.

The best result in species delimitation was achieved in the discriminant analysis of characters of lower stem internodes (fig. 3C). All 17 characters were initially included in the analysis, and 15 characters were selected by the stepwise procedure (the characters № 2 and № 12 were excluded). Two first canonical variables explained about 91% of total among-groups variability. Species clouds were the most compact and clearly separated. Two varieties of *L. corniculatus* formed entire clouds.

Due to the fact that too many high standardized coefficients were associated with the first and second roots, we used factor structure between roots and studied characters for the interpretation of axes. Factor loadings were not high. Maximal loading on the first root (-0.29) was found for the character 'number of main vascular bundles', and those for the second root had the characters 'number of cork layers' and 'thickness of outer tangential wall of epidermis cells' (their values were -0.46 and 0.35, respectively).

On the base of the results obtained in the quantitative study of anatomical characters of the stem, we conclude that the clearest interspecific difference can be revealed in the structure of middle and lower internodes. For delimitation of the studied species following characters are most valuable: number of main and additional vascular bundles, their histological structure, diameter of tracheal elements, degree of lignification of cell walls in ray parenchyma, number of cells with phlobaphenes in different zones of the stem, indumentum density, and, for the lower internodes, number of cork layers.

### Leaf

The *transversal section of leaves* (fig. 4). All species studied have dorsiventral amphistomatal leaf blades with differentiated palisade and spongy tissues. The number of layers in palisade tissue varies from 2 to 3, that in spongy tissue from 3 to 5. In several plants of *L. miyakojimae* and *L. corniculatus* var. *hirsutus* growing under high insolation, the blade was isolateral due to formation of an additional abaxial palisade layer. The midvein protrudes from the abaxial leaf surface by 3–73%; its palisade tissue does not interrupt. Sheath of vascular bundles may be formed by parenchyma, chlorenchyma, or phlobaphene-possessing cells.

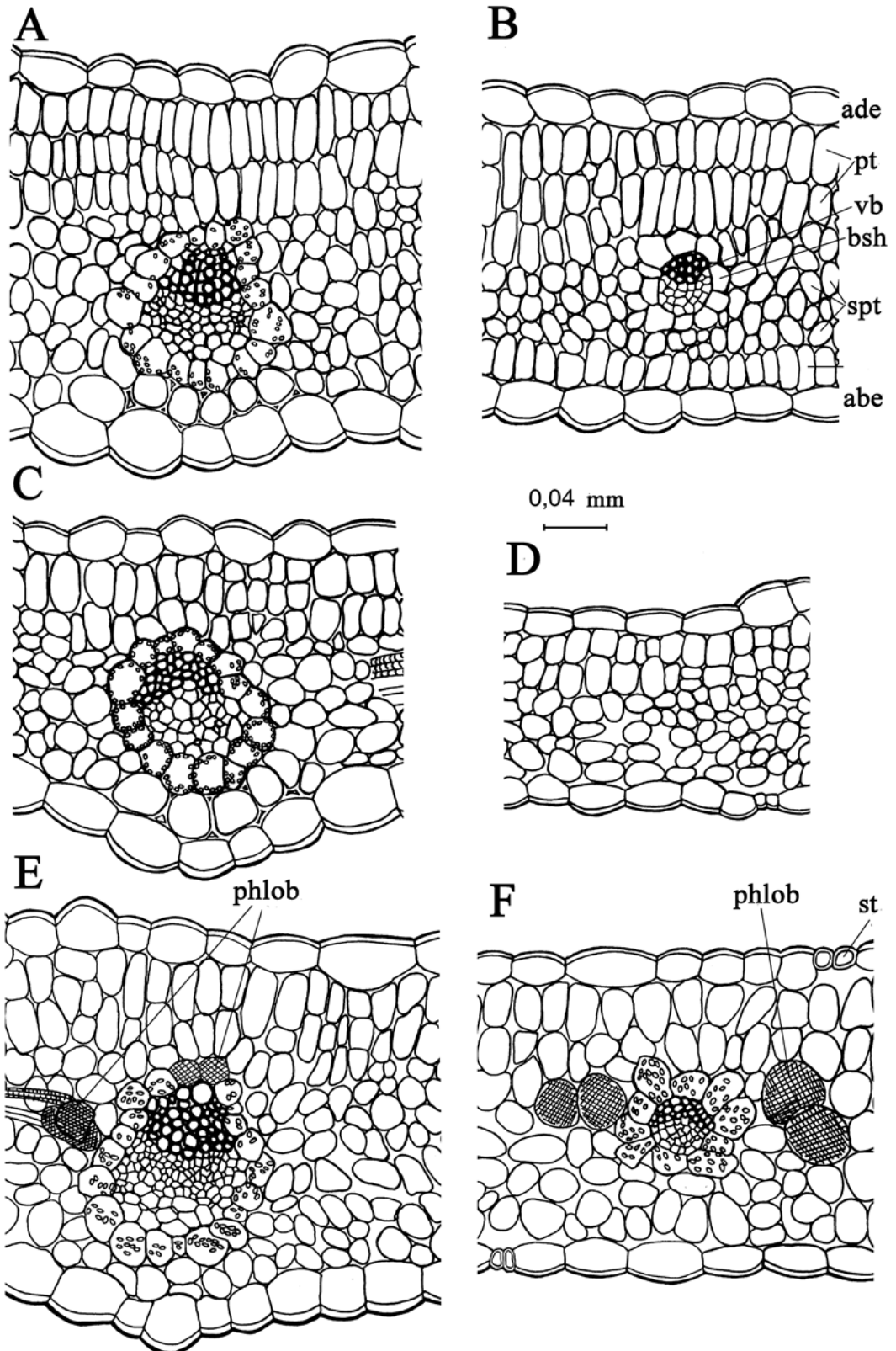
Significant differences were found in the following leaf characters (tab. 4): height of abaxial epidermal cells and the type of mechanical tissue below midvein, the type of bundle sheath, number of phlobaphene-possessing cells in the leaf tissue, thickness of leaf blade and its palisade and spongy tissues in the area of small veins. *L. corniculatus* var. *hirsutus* differs from both Japanese species, primarily in the lack or small development of mechanical tissue below the midvein, a bundle sheath formed by chlorenchyma and/or phlobaphene-possessing cells, and in the

Figure 4: Transverse sections of leaf blades: A, B – *Lotus miyakojimae*; C, D – *L. japonicus*; E, F – *L. corniculatus* var. *hirsutus*; A, C, E (left column) – in the area of midvein; B, D, F (right column) – in the area of small veins.

ade – adaxial epidermis, spt – spongy tissue, abe – abaxial epidermis, bsh – bundle sheath, pt – palisade tissue, vb – vascular bundle, st – stomata, phlob – cells with phlobaphenes. >



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presence of idioblasts with phlobaphenes in the leaf tissue. Differentiation of collenchymatous mechanical tissue below the phloem, parenchymal bundle sheath, and solitary phlobaphene-possessing cells in the leaf tissue is characteristic for both Japanese species, i.e. *L. japonicus* and *L. miyakoimae*. The three species studied are different in the thickness of leaf blade and its palisade tissue in the area of midveins. Maximal values of these characters can be found in *L. miyakoimae*, minimal ones in *L. japonicus*. Spongy tissue is more developed in leaves of *L. miyakoimae* and *L. corniculatus* var. *hirsutus*, its thickness is 61–112 µm and 73–111 µm, correspondingly, while in *L. japonicus* it is only 54–79 µm. *L. miyakoimae* is characterized by the highest and largest abaxial epidermis cells, 42–66 µm of height, as compared with the two other species, *L. japonicus* and *L. corniculatus* var. *hirsutus*, having 29–54 µm and 33–48 µm.

*Leaf epidermis* (fig. 5). Leaf epidermis has a similar structure in adaxial and abaxial surfaces. It consists of roundish or ovate 5–10-lobed surface cells with triangular or half-rounded lobes, more protruding in abaxial epidermal cells. The lobe length is 9–39% of the cell width in abaxial epidermis, and 7–31% in the adaxial one. In connection with the shape of epidermis cells, their anticlinal walls may be characterized as flexuous or zigzag, depending on the lobe shape. The walls usually possess small nodulous thickenings. The stomata type is anomocytic; number of surface cells contacting guard-cells varies from 3 to 5. Stomata density in the abaxial surface is 75–250/mm<sup>2</sup> and in the adaxial one 100–250/mm<sup>2</sup>.

In the majority of studied epidermal characters, among-population difference was significant and greater than among-species one (tab. 5) concerning the following features of adaxial and abaxial epidermises: length of guard cells, number and protrusion of lobes in surface cells, stomata density per mm<sup>2</sup>, as well as the length of surface cells. A significant difference among the species studied was only found in trichome density, both on adaxial and abaxial surfaces. All three species are significantly distinguished from each other by trichome density on the adaxial surface. The density varies from 0 in *L. miyakoimae* to 7.5–10/mm<sup>2</sup> in *L. corniculatus* var. *hirsutus*; *L. japonicus* was characterized by intermediate values of this character varying from 0 to 5. In *L. corniculatus* var. *hirsutus*, the maximal density of trichomes was also observed on the lower surface, i.e. 3.5–10.7/mm<sup>2</sup>, while in Japanese loti trichomes on the lower surface were absent. Generally, characters of leaf epidermis are extremely variable and have little taxonomic significance.

#### Multivariate analysis based on leaf characters

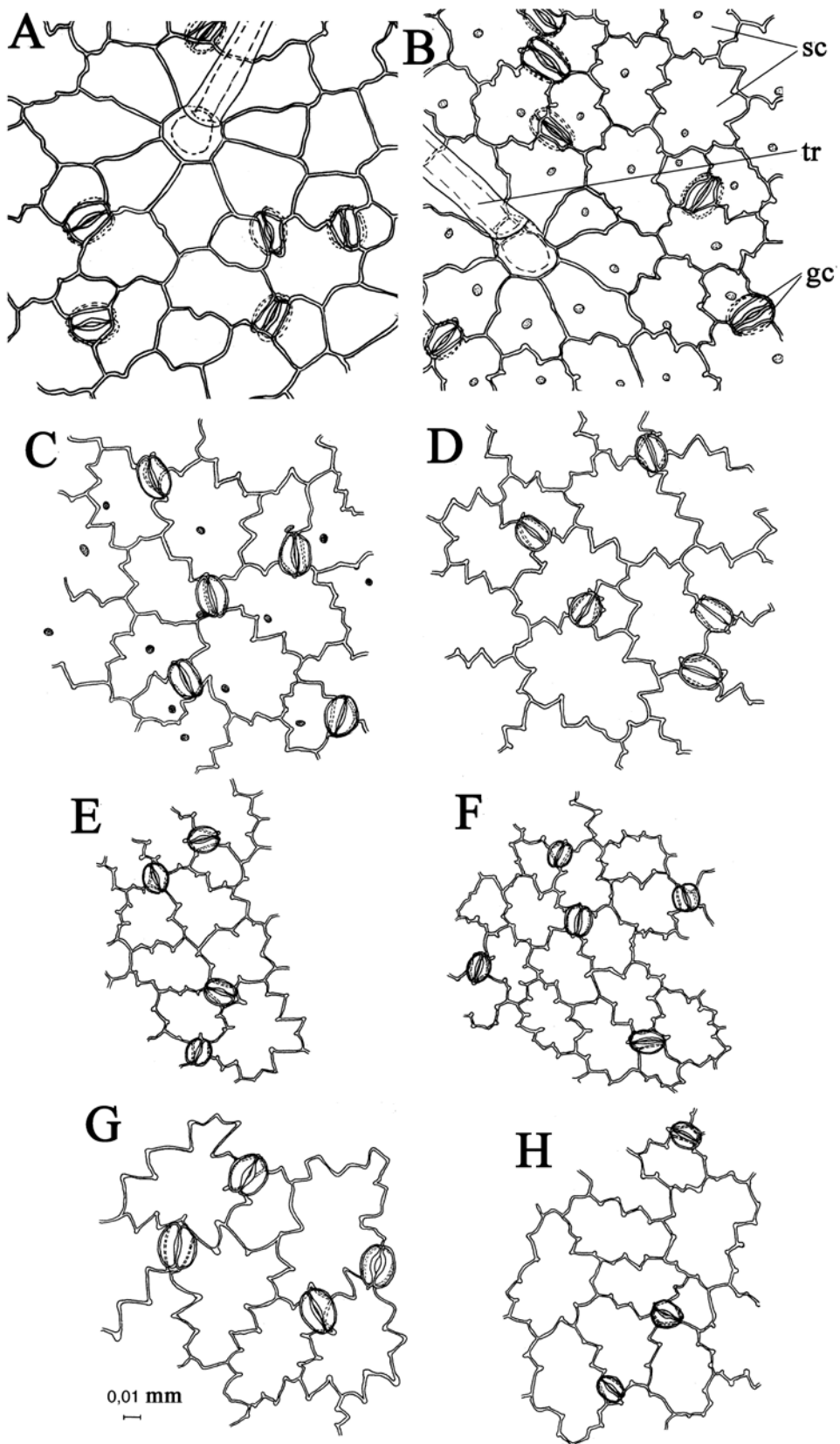
Stepwise discriminant analysis was conducted on the basis of 20 characters of the leaf cross section. The characters ‘number of trichomes on the leaf blade’ and ‘interruption of palisade tissue above midvein’ were excluded because their values were constant. 13 characters were selected by the stepwise procedure (namely the characters № 2, 4, 6–9, 11–15, 18, and 21). Two first canonical variables explain more than 95% of total among-groups variability. The pattern of specimen points in the subspace of the first and second roots show clear separation of three species (fig. 6). Both varieties of *L. corniculatus* form a common cloud. Taking into account, that

Figure 5: Epidermal characteristics: A, C, E, G (left column) – adaxial epidermis; B, D, F, H (right column) – abaxial epidermis; A, B – *L. corniculatus* var. *hirsutus*; C, D – *L. corniculatus* var. *corniculatus*; E, F – *L. japonicus*; G, H – *Lotus miyakoimae*.

tr – trichome, gc – guard cells, sc – surface cells of epidermis.

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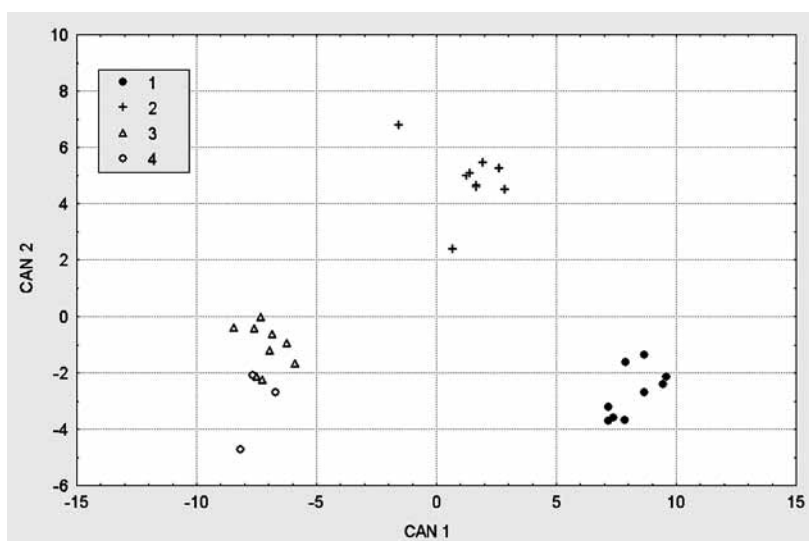


Figure 6: The results of discriminant analysis of studied *Lotus* species made by characters of leaf transverse section. Two-dimension scatterplot of cases in the subspace of the first and second roots.

1 – *Lotus japonicus*, 2 – *L. miyakoijimae*, 3 – *L. corniculatus* var. *hirsutus*, 4 – *L. corniculatus* var. *corniculatus*.

many characters have high standardized coefficients associated with two first axes, reasonable interpretation of the axes can hardly be made. Factor loadings of characters demonstrate, that 'the type of vascular bundle sheath' is mostly associated with the axis № 1 (factor loading = -0.24), and 'the thickness of leaf blade in the area of small veins' and 'the type of vascular bundle sheath' are connected to the axis № 2 (factor loadings 0.44 and -0.27, respectively).

### Root

The root structure in the species studied is determined by their life form. The root anatomical structure is more stable than the shoot one. In this connection, we did not conduct any quantitative analysis of root characters and only give a general anatomical description.

The main root in all three species is triarch and has similar structure (fig. 7). In the primary cortex of young roots idioblasts with phlobaphenes are distinguishable. Secondary thickening of the stele early develops, which corresponds with the formation of periderm and afterwards, rhytidome (in perennials). Protophloem and secondary phloem fibers are well developed. Wide 3–8-rowed primary rays and narrow 1–2-rowed secondary rays are clearly marked. Their cells are parenchymatous with non-lignified or slightly lignified cell walls. In perennial roots borders of annual rings in secondary xylem are hardly visible.

In the microstructure of the main and lateral roots several interspecific distinctions were revealed. Lateral roots of the second and third order in *L. corniculatus* are diarch, while in *L. japonicus* and *L. miyakoijimae* roots of the second order are diarch and those of the third order vary from diarch to tetrarch. For roots of the perennials *L. corniculatus* and *L. japonicus* a thick 4–5-layered cork is characteristic. We also observed a lot of reserve starch in their main roots. In roots of the annual *L. miyakoijimae* the cork was thin, 2–3-layered. For this species we can notice the absence of reserve starch in roots; only its traces are sometimes present.

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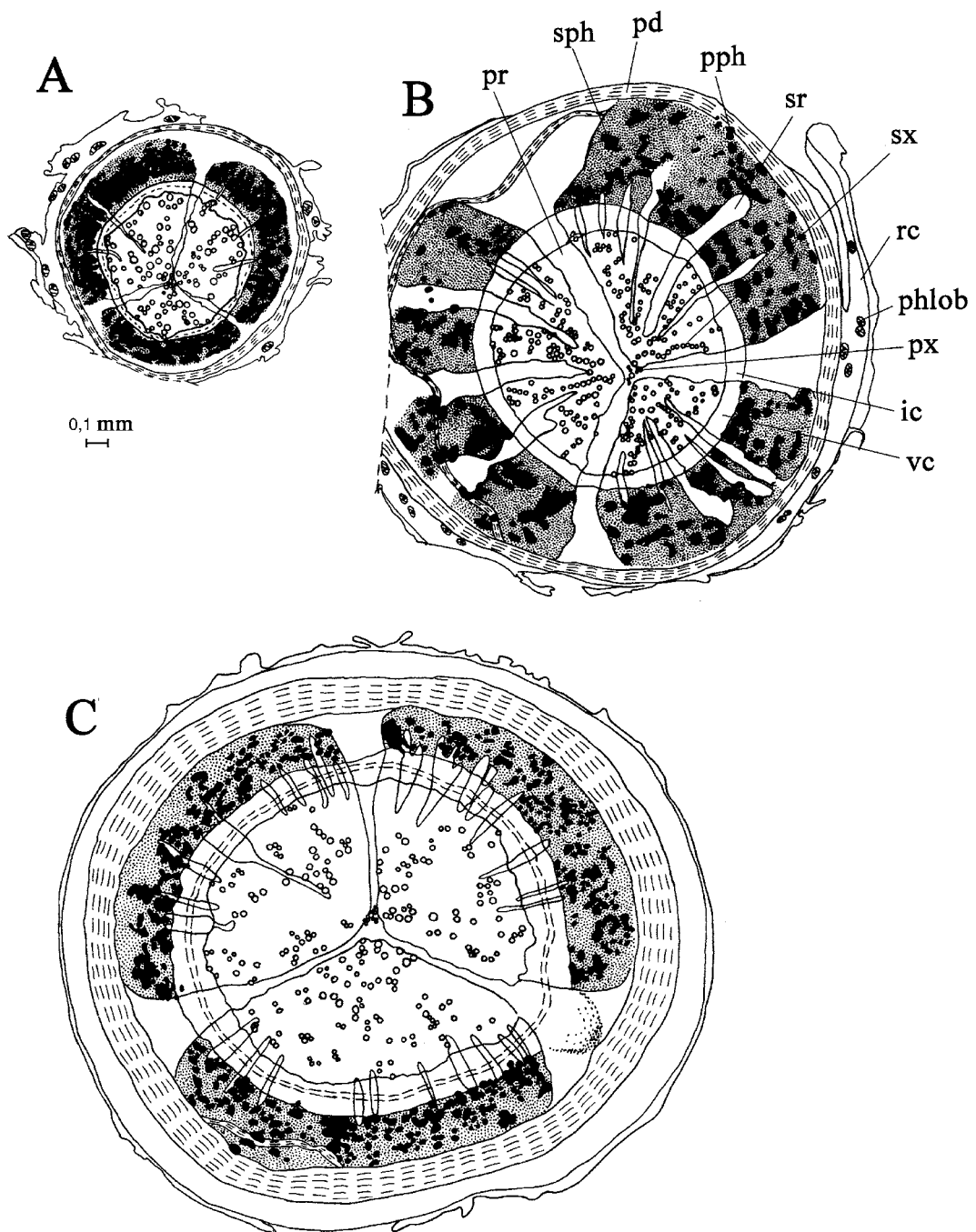


Figure 7: Transverse sections of roots, schemes: A – *L. miyakojimae*; B – *Lotus japonicus*; C – *L. corniculatus* var. *corniculatus*.

sr – secondary ray, ic – interfascicular cambium, rc – the remnants of the cortex, px – primary xylem, pr – primary ray. The rest of symbols is the same as in fig. 1 and 2.

## Discussion and conclusion

On the basis of the results obtained in morphological and anatomical studies, the most informative characters of vegetative organs in three *Lotus* species have been revealed. These new data allow to improve and precise diagnostics of the species studied and specify the ways of their adaptive specialization. The results of the investigation demonstrate that the transformation of microstructure along the annual shoot is connected both, to its morphofunctional zones (TROLL 1964), and to the taxonomic position of the plants studied. As stated above, specific properties are mostly expressed in the structure of middle and lower internodes corresponding to the 'enrichment zone' and 'innovation zone'. At the latter level, not only solitary structural characters but their interrelated combination gain importance, as it is shown in the multivariate analysis.

Comparative anatomical studies of vegetative organs demonstrate that due to the presence of thick cork and a big amount of reserve starch in roots, comparatively low stoma density in the leaf and its dorsiventral structure, as well as relatively low degree of lignification in the stele (excluding lower internodes), *L. corniculatus* and *L. japonicus* can be characterized as perennial light mesophytes. *L. japonicus* differs from the typical *L. corniculatus* in a smaller number of the main conductive bundles along the whole shoot, lack of additional bundles (or, only in lower internodes, presence of solitary incomplete ones with narrow tracheal elements), thinner cork, slight pubescence of stem and leaves, thinner leaf blades with smaller guard- and surface epidermal cells, and a bigger number of idioblasts with anthocyanins and phlobaphenes in the primary cortex of the stem. In *L. corniculatus* var. *hirsutus* cells with phlobaphenes can be also observed in the leaf tissue.

As it is known from literature data, anthocyanins and phlobaphenes, which belong to phenolic compounds may increase resistance of the plant to fungal, bacterial, or viral infections, protect it against ultra-violet radiation and low temperatures; phlobaphenes may also increase drought resistance of the plant and show antioxidant activity (KOKINA 1936; HARBORN 1968; ESAU 1969; KRETOVICH 1980; BARYKINA et al. 2004). Therefore, the presence of phlobaphenes and anthocyanins in *L. japonicus* may be considered as its important biological property, useful for adaptation to the warm-temperate and subtropical humid marine climate of Japan. It is worth mentioning that in specimens of *L. japonicus* from north Japanese areas the amount of anthocyanins in the stem was bigger than in those from southern regions. Development of idioblasts with phlobaphenes in stems and leaves of *L. corniculatus* var. *hirsutus* may be connected with the adaptation to growing conditions of dry meadows in the European part of Russia.

Unlike the two previously mentioned species, *L. miyakojimae* can be considered as a taproot annual meso-xerophyte. A thin cork layer in its roots and their loss of storage function may be connected with the short life cycle of the species. In addition, this taxon is distributed in the far south of Japan and has no anthocyanins in the stem cortex, and only a small number of cells with phlobaphenes. The stele of the stem in *L. miyakojimae* is characterized by the smallest number of main vascular bundles, unlike the other two species. In this species we observed an early development of additional vascular bundles in the stem, that may be associated with its distinct type of branching, i.e. basitonic against mesotonic in the two other taxa. Early and intense lignification of cell walls of ray parenchyma and xylem elements are characteristic for *L. miyakojimae*, as well as for several other annual species, as it was mentioned earlier (BARYKINA 1992). This peculiarity may be connected with the necessity to support a large mass of early

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developing generative structures (i.e. shoots, flowers, and fruits). In lower shoot internodes of *L. miyakojimae* the cork is not usually developed, but intense thickening of the outer cell walls of epidermal cells can be observed. Other distinctive features of this species growing on seashores of one of the southernmost Japanese islands, characterized by hot climate and intense radiation, are large glabrous leaves with slightly fleshy blades, isopalisade leaf tissue and higher stoma density.

Taking into account the ecological conditions of the locality, a number of distinctive morphological, physiological and genetical properties of the population MG-20 of *L. miyakojimae*, as well as the results obtained from microstructure of vegetative organs, the separation of this population as a new species is justified.

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