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Comparison of the genome size, endoreduplication, and ISSR marker polymorphism in eight *Lotus* (Fabaceae) species

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Abstract: Several species within the genus *Lotus* are important forage crops, and many are endangered or rare. Despite the high genetic diversity of the genus, identification of *Lotus* species is problematic because of the limited number of reliable morphological markers. In search of a quick, inexpensive, and steady method for species identification, genome size and cell cycle/endoreduplication intensity of 14 accessions belonging to eight *Lotus* species were estimated by flow cytometry. ISSR-PCR was also applied to find sensitive molecular markers for genetic diversity estimation. Genome size estimation revealed that *Lotus* species possess very small genomes and this characteristic enables the identification of five out of eight species. However, a flow cytometric study of cell ploidy/endopolyploidy in seeds and seedlings enabled us also to distinguish the remaining species. Thus, it is proposed here that combined flow cytometric analyses (the estimation of genome size and cell cycle/endoreduplication pattern) can be applied for screening of *Lotus* species. Nonetheless, ISSR markers provided a more precise identification of studied accessions, including detection of genetic diversity within a species. Most of the tested primers revealed polymorphism between species, and three primers, $(GACA)_4$, $(CA)_7G$, and $(CTC)_4RC$, also revealed polymorphism between accessions within a species. The ISSR markers revealed high polymorphism between eight *Lotus* species and low intraspecific variation between accessions within the same species (*L. maritimus* and *L. uliginosus*).

Key words: DNA content, endopolyploidy, flow cytometry, Leguminosae, molecular markers

1. Introduction

The genus *Lotus* (Fabaceae; previously Leguminosae) comprises nearly 200 annual and perennial species that are distributed worldwide, except in very cold regions and in some tropical areas of Southeast Asia and Central America (Allan et al., 2004; Escaray et al., 2012). Within this genus, more than 15% of the species are endangered, vulnerable, or rare (IUCN, 2015). Several species are important forage crops (e.g., *L. corniculatus*, *L. uliginosus*, *L. tenuis*, and *L. subbiflorus*) since their nutritional value is similar or even superior to that of white clover and alfalfa (Jones and Earle, 1966; Escaray et al., 2012).

Lotus taxonomy is one of the most problematic within the tribe Loteae due to the limited number of appropriate discernable morphological traits, insufficient genetic description, and high morphological and biogeographical diversity (Grant and Small, 1996; Allan et al., 2004; Escaray et al., 2012). Therefore, in addition to morphological analyses, karyological and molecular studies should be applied for species identification and establishing taxonomic assignment and phylogenetic relationships.

The majority of *Lotus* species have the basic chromosome number $x = 6$ or 7 and are mostly diploids. However, in some species both diploid and tetraploid accessions occur, while others include only tetraploid forms (e.g., *L. corniculatus*; Ferreira and Pedrosa-Harand, 2014). Genome sizes of the genus *Lotus* have been reported for 39 species only (20%), with a range from 0.56 to 2.80 pg/2C (Gasmanová et al., 2007; Bennett and Leitch, 2012; Ferreira and Pedrosa-Harand, 2014; Tanaka et al., 2016). Polymorphism and phylogenetic relationships among species and cultivars have been studied using isoenzymes, RAPD, AFLP, nrITS, ISSR, and SSR markers (Raelson and Grant, 1988; Campos et al., 1994; Allan and Porter, 2000; Alem et al., 2011; Kawaguchi et al., 2001; Kramina et al., 2012; Kramina, 2013; Tanaka et al., 2016). Several diploid species (*L. alpinus*, *L. japonicus*, and *L. tenuis*) have been proposed as ancestors of *L. corniculatus*; however, it is still unclear if this species is autotetraploid or allotetraploid. Studies on phylogenetic relationships in the Loteae have revealed that the genus *Lotus* is not monophyletic and consists of two geographically distinct lineages, originating

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from the Old and New Worlds (Allan and Porter, 2000). Furthermore, high allelic variability was confirmed within and among *L. corniculatus* cultivars and *L. japonicus* accessions (Jiang and Gresshoff, 1997; Kawaguchi et al., 2001; Alem et al., 2011). ISSR and nrITS markers used to study genetic variability between *L. corniculatus*, *L. stepposus*, and *L. ucrainicus* revealed that *L. ucrainicus* is a hybrid between *L. corniculatus* and *L. stepposus* (Kramina et al., 2012; Kramina, 2013).

Many species belonging to the Fabaceae family, e.g., *Cicer arietinum*, *Glycine max*, *Lens culinaris*, *Lupinus angustifolius*, *Phaseolus vulgaris*, *Pisum sativum*, *Vicia sativa*, *V. faba*, and *Medicago sativa*, are polysomatic; in addition to cells with 2C and 4C DNA they contain endopolyploid ones, i.e. they possess cells with DNA content higher than 4C (Barow and Meister, 2003; Kocová and Mártonfi, 2011; Rewers and Sliwinska, 2012; Kocová et al., 2014; Straková et al., 2014). This phenomenon is a consequence of endoreduplication, a process during which nuclei undergo repeated rounds of DNA replication without mitosis. Knowledge of endopolyploidy in the genus *Lotus* is very scarce; it has been detected in roots of *L. corniculatus* and *L. uliginosus*, and in root nodules of *L. japonicus*, but not in leaves, petioles, stems, and petals of these species (Blair et al., 1988; González-Sama et al., 2006; Bainard et al., 2012; Suzaki et al., 2014). Endoreduplication is a genetically determined process and is species-specific (Sliwinska and Lukaszewska, 2005; Lukaszewska and Sliwinska, 2007; Sliwinska et al., 2012; Rewers and Sliwinska, 2012, 2014). Consequently, the level of endopolyploidy is similar in cultivars/accessions of the same species, for example in sugar beet and Arabidopsis (Sliwinska and Lukaszewska 2005; Lukaszewska and Sliwinska, 2007; Sliwinska et al., 2012). Since the endopolyploidy pattern can be different in species of the same genus (Rewers and Sliwinska, 2012, 2014), it can be helpful in species identification.

In the present study, genome size (2C DNA content), seed and seedling cell ploidy/endopolyploidy patterns, and intersimple sequence repeat (ISSR) molecular markers were established for 14 accessions belonging to eight *Lotus* species to determine inter- and intraspecific variation within this genus. The species were selected due to their importance in agriculture and potential usefulness as breeding material. Also, they are closely related phylogenetically, and therefore most problematic in identification and taxonomy. The usefulness of the proposed characteristics for *Lotus* species identification is discussed and the efficiency of flow cytometry (FCM) and ISSR-PCR for species distinguishing is compared. For *L. tetragonolobus* this is the first report on genome size. Endopolyploidy in different regions of the seed and young seedlings of *Lotus* species was also estimated for the first time.

2. Materials and methods

2.1. Plant material

The seeds of 14 accessions of eight *Lotus* species were used as plant material (Table 1). All accessions obtained from botanical gardens were additionally morphologically verified independently by two experienced taxonomists. Since the mature, dry seeds were dormant, scarification was performed to permit germination upon imbibition. First, seeds were scarified with sand paper, then incubated for 5 min in 75% ethanol, washed with distilled water, and sterilized for 20 min in 2% sodium hypochlorite. After washing three times with sterile distilled water, seeds were incubated in water in Eppendorf tubes for 12 h. Sterilized seeds were placed on wetted filter paper (65% relative substrate moisture content) and germinated at 22 °C in darkness (Melchiorre et al., 2009). To obtain leaves for genome size estimation, young seedlings were transferred to pots with garden soil and grown in a growth chamber under a 16-h photoperiod at 22 °C.

2.2. Genome size estimation

For FCM, samples of young leaves of *Lotus* accessions and of internal standard *Vicia villosa* 'Minikowska' (2C = 3.32 pg; Dzialuk et al., 2007) were prepared as previously described (Sliwinska and Thiem, 2007) using Galbraith's buffer (Galbraith et al., 1983) supplemented with propidium iodide (PI; 50 µg/mL) and ribonuclease A (50 µg/mL). Nuclear DNA content was estimated directly using a CyFlow SL Green flow cytometer (Partec GmbH, Münster, Germany) equipped with a high-grade solid-state laser with green light emission at 532 nm, long-pass filter RG 590 E, DM 560 A, as well as with side (SSC) and forward (FSC) scatters. For each sample, the nuclear DNA content in 5000–8000 nuclei was measured using linear amplification. Analyses were performed on five individuals per accession. Histograms were collected as FCS files and evaluated manually using a FloMax program (Partec GmbH). The coefficient of variation (CV) of the G_0/G_1 peak of *Lotus* species ranged between 2.92% and 5.90%. Nuclear DNA content was calculated using the linear relationship between the ratio of the 2C peak positions of *Lotus*/*V. villosa* on a histogram of fluorescence intensities.

2.3. Endopolyploidy estimation

Endopolyploidy was analyzed in each species; however, when more than one accession represented the species, only one of them was used after being randomly chosen (*L. maritimus* C2.442 and *L. uliginosus* LE-627). FCM analysis was conducted at three developmental stages: (I) mature dry seeds, (II) young seedlings after radicle protrusion, and (III) seedlings with unfolded cotyledons. Seeds and young seedlings after radicle protrusion were dissected into the embryo axis and cotyledons, and seedlings with unfolded cotyledons into the root, hypocotyl, and cotyledons. Samples of each seed/seedling

Table 1. List and origin of studied *Lotus* accessions.

Species	Code	Origin
L. burttii Borsos	B-303	Kabul River, Peshawar, Pakistan
L. corniculatus L.	'San Gabriel'	National Institute of Agricultural Research (INIA), La Estanzuela, Colonia, Uruguay
L. filicaulis Durieu	B-37	National Institute of Agricultural Research (INIA), La Estanzuela, Colonia, Uruguay
L. japonicus (Regel) K. Larsen	MG-20	Agari-henna point, Miyakojima Island, Okinawa, Japan
L. maritimus L.	3723	Botanical Garden, Goethe University of Frankfurt, Germany (native)
L. maritimus L.	C2.442	Botanical Garden of Faculty of Science, Masaryk University, Brno, Czech Republic (native)
L. tenuis Waldst. & Kit. ex Willd.	'La Esmeralda'	Institute of Biotechnology Research (INTECH), San Martin, Buenos Aires, Argentina
L. tetragonolobus L.	-	Botanical Garden, Christian Albrechts University, Kiel, Germany (native)
L. uliginosus Schkuhr	7	Grimbosq (forest property of the city Caen), Calvados, France
L. uliginosus Schkuhr	8	Briouze, l'Orne Caen, France
L. uliginosus Schkuhr	203	La Plaine, pond of Thinaudières, France
L. uliginosus Schkuhr	204	La Rabatelière, Notre-Dame de la Salette, France
L. uliginosus Schkuhr	205	Saint-Etienne de Montluc, les Perrières, France
L. uliginosus Schkuhr	LE-627	National Institute of Agricultural Research (INIA), La Estanzuela, Colonia, Uruguay

part were prepared as previously described (Rewers et al., 2009) using 4',6-diamidino-2-phenylindole (DAPI; 2 µg/mL) for DNA staining. Analyses were performed on five biological replicates using a Partec CCA flow cytometer (Partec GmbH), equipped with an HBO lamp, KG1 heat protection filter, BG12 and UG1 short-pass filters, GG435 long-pass filter, and a dichroic mirror TK420, using a logarithmic amplification, with no gating. For each sample (organ at a particular stage of development in an individual), fluorescence of 5000–7000 nuclei was analyzed. Histograms were collected as DYN files and evaluated manually using the DPAC v. 2.2 program (Partec GmbH). The proportion of nuclei with different DNA contents, the number of endocycles, and the mean C-value (Lemontey et al., 2000) were calculated.

The results of both FCM experiments were estimated using a one-way analysis of variance and Duncan's test ($P < 0.05$). In this work, only nuclei with DNA content higher than that in the G_2 phase of the mitotic cycle (>4C) were considered endopolyploid (Rewers and Sliwinska, 2012, 2014).

2.4. ISSR-PCR

Genomic DNA was extracted from 0.12 g of fresh leaf material from five randomly selected plants per accession using a Plant DNA GPB Mini Kit (GenoPlast Biochemicals, Poland) according to the manufacturer's instructions. DNA quality and quantity were established by spectrophotometric measurements and agarose gel electrophoresis. Only samples of high quality were used for ISSR-PCR.

ISSR-PCR amplifications were performed in reaction volumes of 25 µL, containing 30 ng of genomic DNA template, 0.1 U/µL Taq DNA polymerase, 4 mM $MgCl_2$, 0.5 mM of each dNTPs, 10 µM primer, and sterile deionized water. Reactions were performed using a T100 Thermal Cycler (Bio-Rad, Poland) under the following conditions: initial denaturation at 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, annealing at 47.1–67.2 °C (depending on the primer) for 1 min, and 72 °C for 2 min. The last cycle was followed by a final extension step of 7 min at 72 °C. Thirty-nine ISSR primers (Genomed, Poland; previously reported by Rewers and Jedrzejczyk,

2016) were tested, out of which 28 generated stable band patterns and were selected for further studies (Supporting Information, Table S1). All reactions with the selected primers were repeated twice. Amplification products were separated using 1.5% (w/v) agarose gel electrophoresis. A DNA ladder of 3000 bp was used to determine the size of the fragments. The bands were visualized using GelDoc XR+ (Bio-Rad).

The ISSR bands were counted using a binary scoring system that recorded the presence or absence of bands as 1 and 0, respectively. The number of monomorphic and polymorphic amplification products generated by each primer was determined. The polymorphism information content (PIC) was calculated according to Ghislain et al. (1999). Estimates of genetic distances were calculated according to Nei and Li (1979) and a dendrogram was constructed using the unweighted pair group method with arithmetic average (UPGMA) by the Treecon v. 3.1 program (Van de Peer and De Wachter, 1994). Statistical support of the branches was tested with bootstrap analysis using 2000 replicates. The distance matrix was used for principal coordinate analysis (PCoA).

3. Results

3.1. Genome size

The 2C DNA contents of the investigated species ranged from 1.04 pg in *L. uliginosus* to 2.36 pg in *L. corniculatus*, which is a tetraploid species ($2n = 4x = 24$; Table 2; Figure 1). According to the categorization proposed by Soltis et al. (2003), all species possessed very small genomes (<2.8 pg/2C). However, significant differences between some of the species were detected. Although *L. filicaulis*, *L. tenuis*, and *L. tetragonolobus* possessed genomes of the same size (2C = 1.16 pg), all other species could be distinguished based on their 2C-value. In *L. burttii* 2C DNA content was 1.12 pg, in *L. japonicus* 1.24 pg, in both *L. maritimus* accessions 1.40 pg, and in all *L. uliginosus* accessions about 1 pg. There was no intraspecific variation in genome size among different accessions of *L. maritimus* and it was very low between accessions of *L. uliginosus* (3% difference, some of them were statistically significant).

3.2. Endopolyploidy

Out of the eight studied species, polysomaty was not detected only in *L. corniculatus*; in all the others, nuclei with DNA content higher than 4C (8C and 16C; an effect of one or two endocycles, respectively) occurred (Table 3; Figure 2). The intensity of endoreduplication in the seven species expressing polysomaty depended on the species, embryo/seedling region, and developmental stage.

No endopolyploid nuclei were detected in the dry seeds of any species (Figure 2). However, species varied in the proportion of 2C and 4C nuclei. In one of them, *L. uliginosus*, only 2C nuclei were present in both the cotyledons and embryo axis of the seed, but in the others

Table 2. Genome size and chromosome number of 14 *Lotus* accessions.

Species	Nuclear DNA content			Number of chromosomes**
	2C (pg ± SD)		1Cx (pg)	
L. burttii	1.116 ± 0.009	e*	0.558	12
L. corniculatus	2.360 ± 0.029	a	0.590	24
L. filicaulis	1.164 ± 0.009	d	0.582	12
L. japonicus	1.240 ± 0.019	c	0.620	12
L. maritimus (3723)	1.400 ± 0.014	b	0.700	14
L. maritimus (C2.442)	1.396 ± 0.017	b	0.698	14
L. tenuis	1.162 ± 0.016	d	0.581	12
L. tetragonolobus	1.156 ± 0.021	d	0.578	14
L. uliginosus (7)	1.064 ± 0.009	fg	0.532	12
L. uliginosus (8)	1.052 ± 0.022	fgh	0.526	12
L. uliginosus (203)	1.044 ± 0.011	gh	0.522	12
L. uliginosus (204)	1.046 ± 0.015	fgh	0.523	12
L. uliginosus (205)	1.036 ± 0.017	h	0.518	12
L. uliginosus (LE-627)	1.068 ± 0.011	f	0.534	12

* Values followed by the same letter are not significantly different at $P < 0.05$ (Duncan's test).
** According to Ferreira and Pedrosa-Harand (2014).

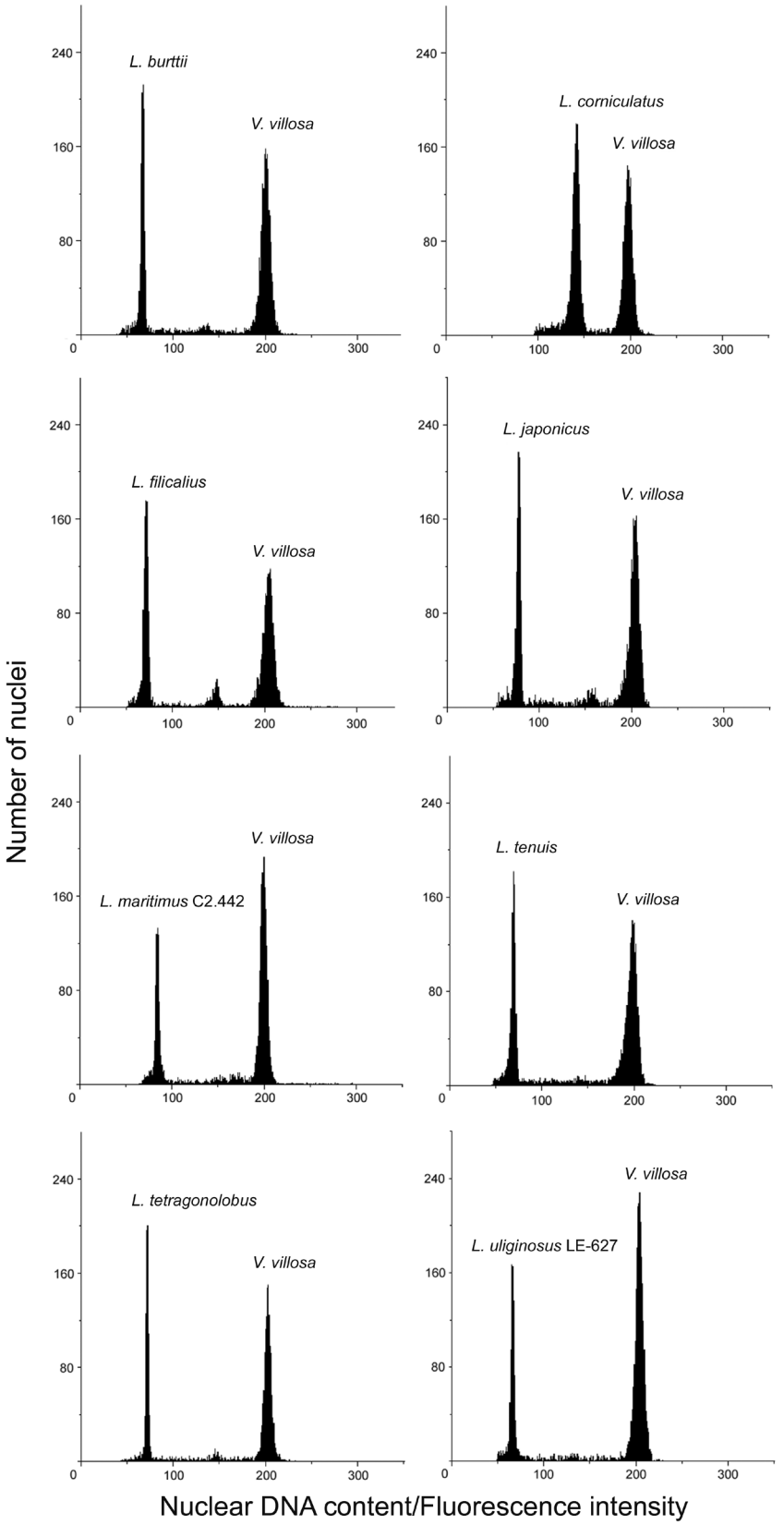

Figure 1. Selected histograms of DNA contents in nuclei isolated from leaves of *Lotus* species and *Vicia villosa* (internal standard).

Table 3. The mean C-value (±SD) and number of endocycles (in parentheses) in different regions of: I, dry seed; II, seedling after radicle protrusion; III, seedling with unfolded cotyledons, in eight *Lotus* species.

Species	Cotyledons			Embryo/seedling axis		Root	Hypocotyl
	I	II	III	I	II	III	III
L. burttii	2.12 ± 0.03 b* (0)	2.16 ± 0.05 bc (0)	2.38 ± 0.04 b (0)	2.25 ± 0.03 b (0)	3.15 ±0.29 a (1)	3.55 ± 0.12 b (1)	3.57 ± 0.09 c (1)
L. corniculatus	2.06 ± 0.01 c (0)	2.12 ± 0.04 bc (0)	2.12 ± 0.02 d (0)	2.11 ± 0.02 c (0)	2.26 ± 0.12 c (0)	2.88 ± 0.14 c (0)	2.70 ± 0.19 f (0)
L. filicaulis	2.06 ± 0.02 c (0)	2.17 ± 0.11 bc (0)	2.30 ± 0.10 bc (0)	2.11 ± 0.03 c (0)	2.72 ± 0.04 b (0)	3.58 ± 0.04 b (1)	3.58 ± 0.11 c (1)
L. japonicus	2.12 ± 0.04 b (0)	2.11 ± 0.04 c (0)	2.18 ± 0.09 cd (0)	2.18 ± 0.02 c (0)	2.73 ± 0.18 b (1)	3.60 ± 0.05 b (1)	3.40 ± 0.11 cd (1)
L. maritimus (C2.442)	2.10 ± 0.02 b (0)	2.17 ± 0.04 bc (0)	2.29 ± 0.13 bc (0)	2.30 ± 0.09 b (0)	3.12 ± 0.20 a (1)	3.60 ± 0.08 b (1)	3.92 ± 0.15 b (1)
L. tenuis	2.06 ± 0.04 c (0)	2.12 ± 0.08 bc (0)	2.41 ± 0.08 b (0)	2.12 ± 0.04 c (0)	2.18 ± 0.15 c (0)	3.55 ± 0.04 b (1)	3.34 ± 0.05 de (0)
L. tetragonolobus	2.32 ± 0.04 a (0)	2.58 ± 0.10 a (1)	3.95 ± 0.24 a (1)	2.97 ± 0.07 a (0)	3.35 ± 0.14 a (1)	3.95 ± 0.08 a (1)	5.16 ± 0.24 a (2)
L. uliginosus (LE-627)	2.00 ± 0.00 d (0)	2.23 ± 0.12 b (0)	2.16 ± 0.04 cd (0)	2.00 ± 0.00 d (0)	3.23 ± 0.16 a (1)	3.50 ± 0.03 b (1)	3.20 ± 0.08 e (0)

* Values for particular species and stages (in columns) followed by the same letter are not significantly different at $P < 0.05$ (Duncan's test).

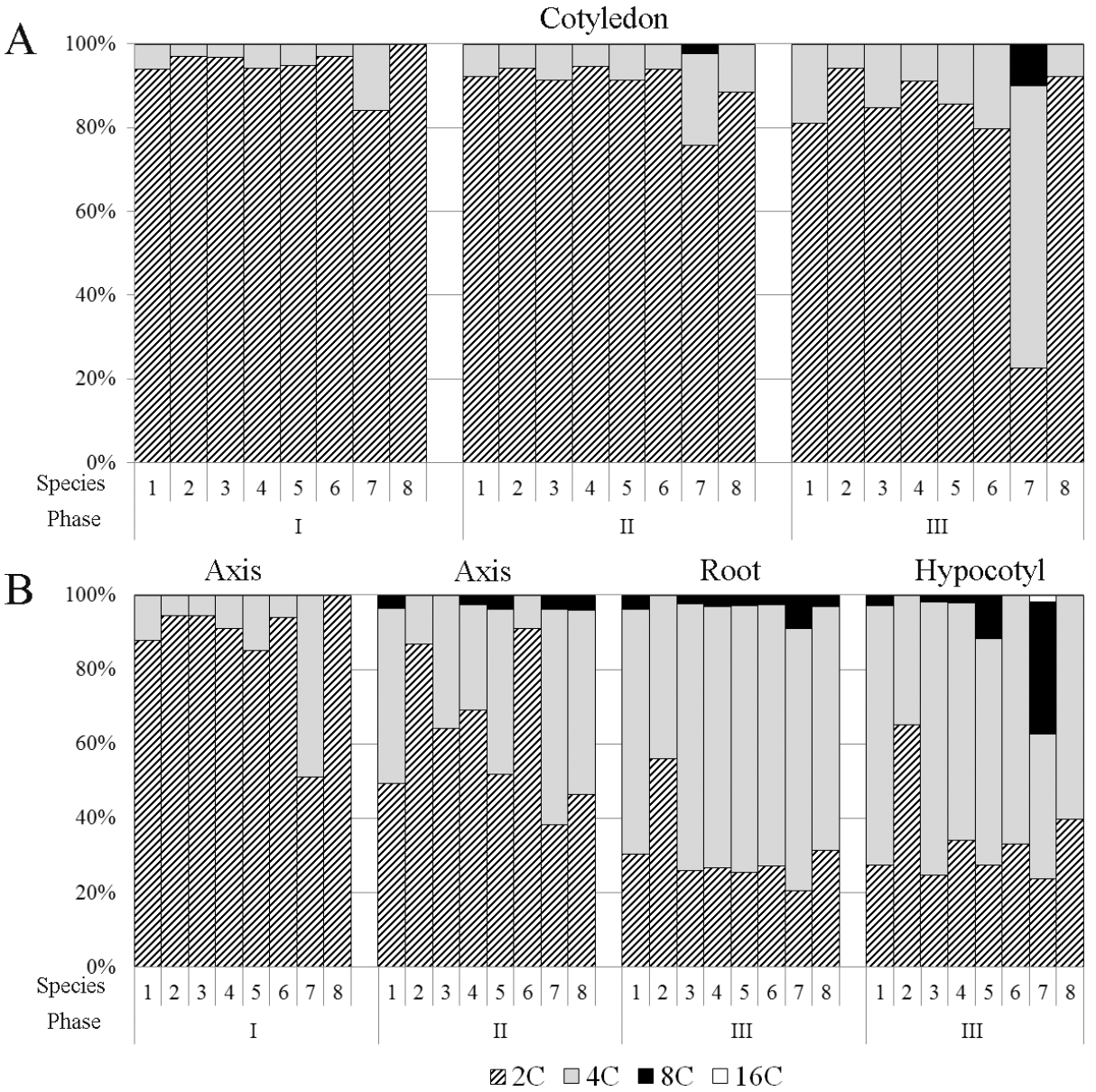

Figure 2. Proportion of nuclei with different DNA contents in the cotyledons (A) and embryo axis (B) of I, dry seed; II, seedling after radicle protrusion; III, seedling with unfolded cotyledons, of eight *Lotus* species: 1, *L. burttii*; 2, *L. corniculatus*; 3, *L. filicaulis*; 4, *L. japonicus*; 5, *L. maritimus*; 6, *L. tenuis*; 7, *L. tetragonolobus*; 8, *L. uliginosus*.

the proportion of 4C nuclei varied from 3% to 16% in the cotyledons and from 6% to 49% in the axis. The highest proportion of 4C nuclei occurred in *L. tetragonolobus*. This species also expressed the highest endopolyploidy in the seedling; it was the only one possessing 8C nuclei in the seedling cotyledon (2% and 10% at stages II and III, respectively) and 16C nuclei (2%) in the hypocotyl of the seedling with unfolded cotyledons (stage III).

The DNA synthesis pattern differed more in the seedling axis than in the cotyledons (Figure 2). In the young seedling after radicle protrusion (stage II), 8C nuclei were not detected only in three species, *L. corniculatus*, *L. filicaulis*, and *L. tenuis*. However, the proportion of 2C nuclei decreased to about 60% as compared to 95% in the dry seed axis of *L. filicaulis*, while it remained high in the two other species (87% in *L. corniculatus*, 91% in *L. tenuis*). This proportion decreased further in the roots of older seedlings (at stage III), but in that of *L. filicaulis* and *L. tenuis* some 8C nuclei (about 2%) appeared, while only in *L. corniculatus* did the proportion of 4C nuclei increase

to 44%. In the hypocotyl of seedlings of *L. tetragonolobus* with unfolded cotyledons, endoreduplication was the most intensive (36% of endopolyploid nuclei, two endocycles).

The mean C-value varied from 2 to over 5; it was highest in the hypocotyl of *L. tetragonolobus* at the stage of unfolded cotyledons (Table 3). In this species, the value was significantly higher than in the other species, and also than in the other organs and stages. This parameter was as high as in *L. tetragonolobus* only in the seedling axis at stage II of *L. maritimus*, *L. uliginosus*, and *L. burttii*. In the seedlings of *L. corniculatus* at stage III, in both the root and hypocotyl, and of *L. maritimus* in the hypocotyl, the mean C-value distinguished these species from the others.

3.3. ISSR marker polymorphism

The species were screened using 28 ISSR primers, which produced reproducible polymorphic banding patterns that allowed for their identification. The primers resulted in amplification of 438 loci; 437 were polymorphic with an average polymorphic percentage of 99.8% (Figure 3; Table S1). Only one primer, $(CAG)_5$ (ISSR-29), exhibited lower

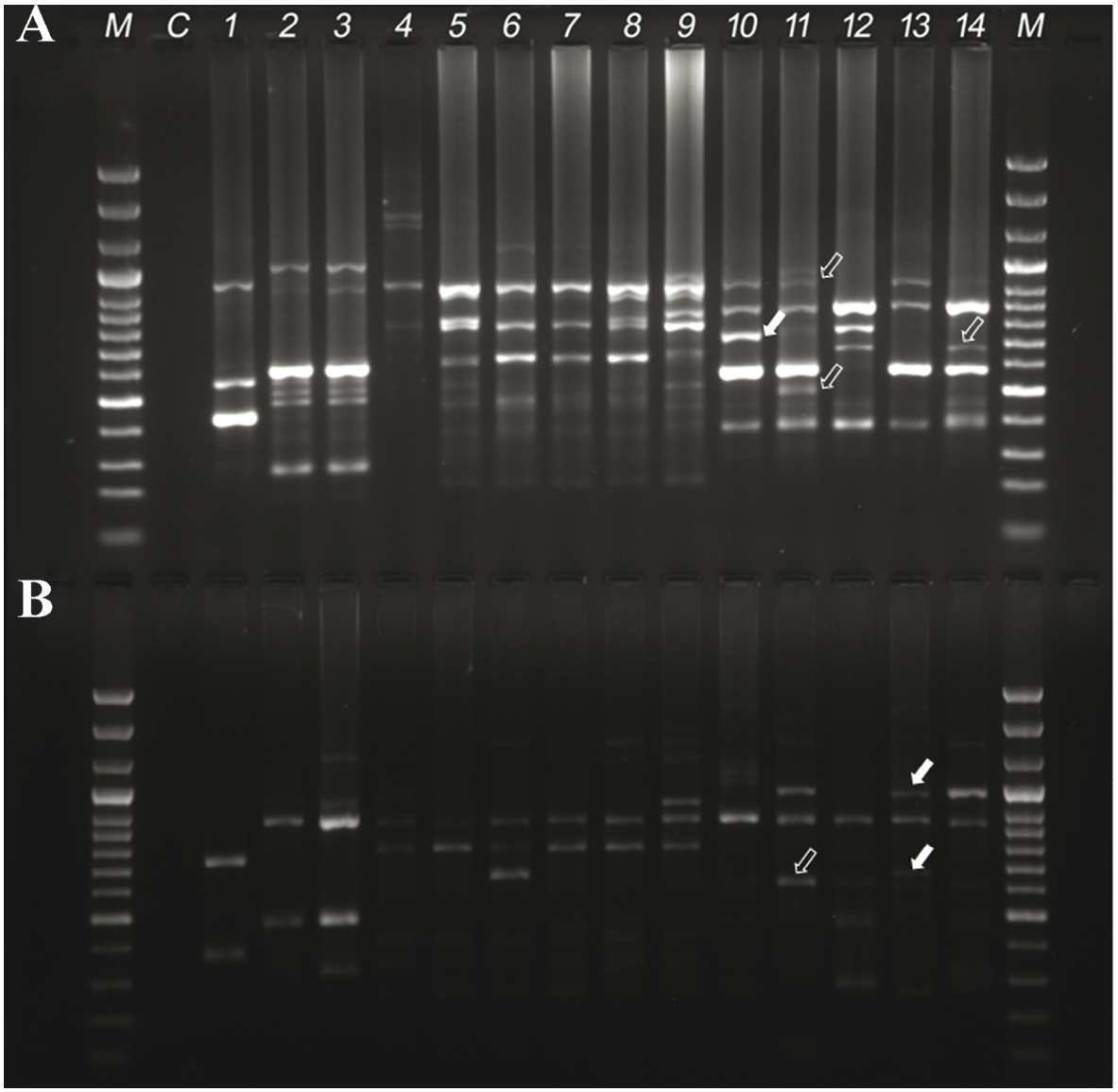

Figure 3. Banding profiles generated by ISSR-PCR using (A) primer ISSR-12 and (B) primer ISSR-24. M, 3000-bp ladder; C, negative control; 1, *L. tetragonolobus*; 2, *L. maritimus* (3723); 3, *L. maritimus* (C2.442); 4, *L. uliginosus* (7); 5, *L. uliginosus* (8); 6, *L. uliginosus* (203); 7, *L. uliginosus* (204); 8, *L. uliginosus* (205); 9, *L. uliginosus* (LE-627); 10, *L. japonicus*; 11, *L. corniculatus*; 12, *L. filicaulis*; 13, *L. burttii*; 14, *L. tenuis*. Arrows indicate bands that differentiate *L. japonicus* from *L. burttii* (filled arrow), and *L. corniculatus* from *L. tenuis* (unfilled arrow).

than 100% polymorphism (94%). The number of bands generated per primer varied from 7 for $(GA)_6CC$ (ISSR-36) to 23 for $(GTG)_6T$ (ISSR-7). The approximate size of the amplified products ranged from 190 to 2600 bp. The PIC value, which describes the informativeness of the primer, ranged from 0.26 for $(GA)_9T$ (ISSR-21) to 0.48 for $(GAC)_6$ (ISSR-3), $(GAG)_3GG$ (ISSR-37), and $(GTC)_3GC$ (ISSR-38) primers, with an average of 0.38 (Table S1). Three primers, $(GACA)_4$ (ISSR-1), $(CA)_7G$ (ISSR-24), and $(CTC)_4RC$ (ISSR-30), revealed polymorphism not only between species but also between accessions collected at different locations within a species.

The relationship between the tested accessions was reflected by genetic distance estimation (Supporting Information, Table S2; a low value represents a low degree of genetic distance and consequently a close relationship between accessions). The lowest distance, 0.16, was between two *L. maritimus* accessions, whereas the highest, 0.82, was between *L. maritimus* (C2.442) and *L. uliginosus* (LE-627). The UPGMA clustering algorithm grouped accessions into three clusters, but one species (*L. tetragonolobus*) was not clustered into any of the created groups (Figure 4). All six accessions belonging to *L. uliginosus* were included in group I and two *L. maritimus* accessions in group III, while *L. japonicus*, *L. burttii*, *L. filicaulis*, *L. corniculatus*, and *L. tenuis* were in group II. Within this group, the cluster analysis revealed a close relationship between *L. japonicus* and *L. burttii*, as well as between *L. corniculatus* and *L. tenuis*. PCoA analysis revealed a similar grouping of accessions (Supporting Information, Figure S1). The first two coordinates explained 71% (46% for axis PCoA1 and 25% for axis PCoA2) of the total variance based on ISSR data.

4. Discussion

The high number of *Lotus* species and the occurrence of interspecific hybridization result in the wide genetic diversity of this genus. However, the recognition of species based on morphological traits can be erroneous and cause confusion during taxonomic classification, propagation, and germplasm collection (Drobná, 2010). Correct species identification is necessary not only for taxonomical purposes but also to allow selection of closely related species for the introgression of agricultural traits into fodder *Lotus* species. Therefore, an inexpensive, fast, and accurate method to diversify accessions and characterize their genetic diversity is needed. In the present research a relatively cheap and fast method, FCM, is proposed for screening different *Lotus* species as an alternative to the molecular method of ISSR-PCR.

4.1. Identification of *Lotus* species by flow cytometry

FCM estimation of genome size has been successfully used previously for the identification of various species, such as

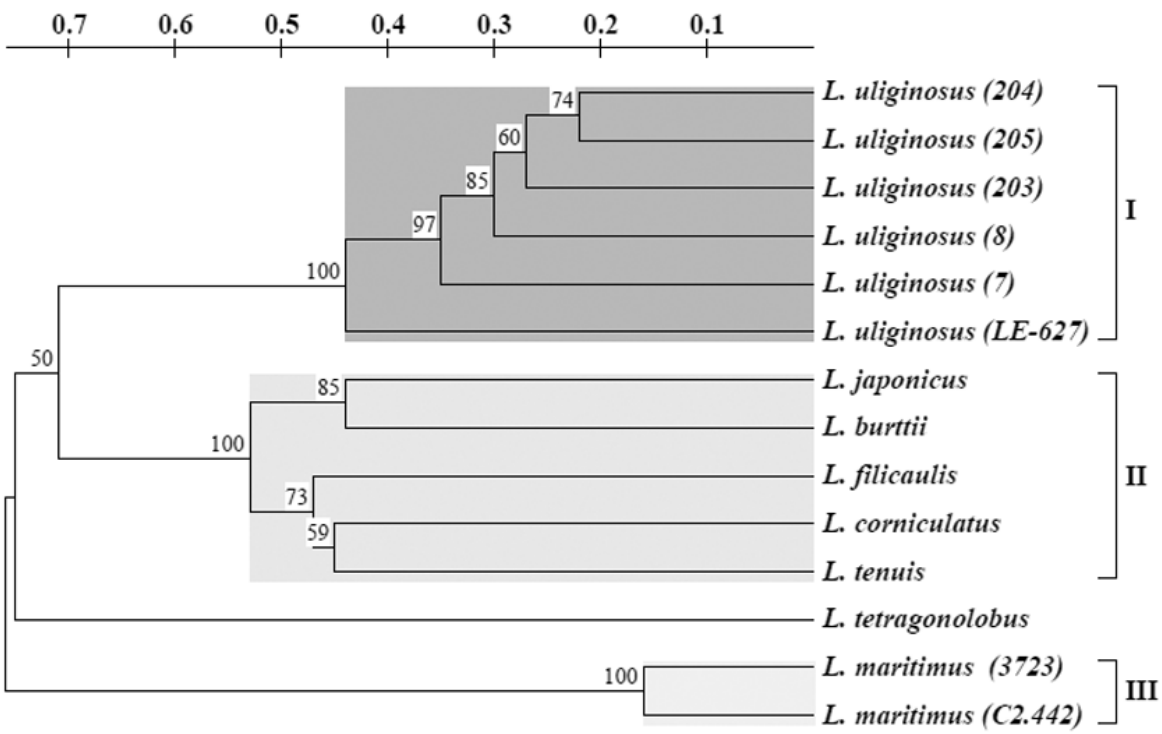

Figure 4. The UPGMA dendrogram computed using genetic distance matrix based on ISSR-PCR data. Only bootstrap values of >50% are indicated; scale indicates genetic distance.

those belonging to genera *Curcuma*, *Nasturtium*, *Petunia*, *Lactuca*, and *Papaver* (Koopman, 2000; Mishiba et al., 2000; Leong-Škorničková et al., 2007; Aragane et al., 2014; Morozowska et al., 2015). Also, as shown here, based on the 2C-values it was possible to identify the only tetraploid species, *L. corniculatus*, as well as four diploid ones, *L. burttii*, *L. japonicus*, *L. maritimus*, and *L. uliginosus*. However, since *Lotus* species whose nuclear DNA content is known (Bennett and Leitch, 2012; Tanaka et al., 2016; present research) all possess very small genomes, the differences in their sizes can be too small for the identification of some species with similar DNA amounts. Indeed, in the present experiments the differences between 2C DNA contents of *L. filicaulis*, *L. tetragonolobus*, and *L. tenuis* were not statistically significant (1.16 pg for all three, despite the fact that *L. tetragonolobus* possesses $2x = 14$ chromosomes and the two other species possess $2x = 12$), and hence this characteristic did not allow for discrimination between them. Therefore, additional FCM characteristics related to cell cycle/endoreduplication intensity in the seeds and young seedlings were applied to check if the combined analyses of genome size and DNA synthesis pattern would enable an unambiguous identification of *Lotus* species. Since the number of endocycles is genetically determined and species-specific (Galbraith et al., 1991; Sliwinska and Lukaszewska, 2005; Lukaszewska and Sliwinska, 2007; Sliwinska et al., 2012; Rewers and Sliwinska, 2012, 2014), endoreduplication intensity seems to be a suitable parameter for species identification. According to our literature review, such an approach has not been reported before. Within the Fabaceae, endopolyploidy has also been detected in seeds and young seedlings of *P. vulgaris*, *P. sativum*, *V. faba* var. *minor*, and *V. sativa*, whereas no endopolyploid nuclei occurred in *Olneya tesota* or *Parkinsonia aculeata* (Sliwinska et al., 2009; Rewers and Sliwinska, 2012).

As shown here, even without evaluation of their FCM histograms, just by the appearance or lack of a unique peak corresponding to nuclei possessing a certain DNA content, it was possible to identify three out of eight species: *L. corniculatus* (lack of 8C nuclei in the seedling root at stage III), *L. tetragonolobus* (presence of 8C nuclei in the cotyledons at stages II and III, and of 16C nuclei in the hypocotyl at stage III), and *L. uliginosus* (lack of 4C nuclei in the cotyledons in the dry seed). The three species possessing very similar genome sizes, *L. filicaulis*, *L. tetragonolobus*, and *L. tenuis*, had different endoreduplication patterns that can be used to differentiate between them. In *L. tetragonolobus*, not only was the presence of additional peaks corresponding to endopolyploid nuclei evident, but also the mean C-value was higher than in all other species at all stages and parts, except for the seedling axis at stage II. As for the other two species with the genome of 1.16 pg/2C, they expressed different cell cycle/endoreduplication patterns in the seedling at stage III; in both the root and the hypocotyl of *L. filicaulis* some nuclei had undergone one endocycle (possessing 8C DNA content), while in the hypocotyl of *L. tenuis* only nuclei with 2C and 4C DNA were present. The mean C-value also allowed for identification of *L. corniculatus* at stage III using the root and/or hypocotyl, and of *L. maritimus* using only the hypocotyl. As summarized in Table 4, when genome size and cell cycle/endoreduplication intensity estimation were combined, all the species could be distinguished by FCM. Here, in order to obtain clarity of the results, those two analyses establishing the absolute nuclear DNA content and different nuclei ploidies were performed separately. However, they can be performed in one run of an individual sample (with an internal standard) by an experienced FCM user who would not confuse the peaks corresponding to the nuclei of different ploidies of a sample species with those of an internal standard.

Table 4. The possibility of identifying *Lotus* species using genome size, endopolyploidy, and ISSR markers (+, identification possible; -, identification not possible).

Species	Genome size	Cell ploidy/ endopolyploidy	ISSR markers
L. burttii	+	-	+
L. corniculatus	+	+	+
L. filicaulis	-	+	+
L. japonicus	+	-	+
L. maritimus (C2.442)	+	+	+
L. tenuis	-	+	+
L. tetragonolobus	-	+	+
L. uliginosus (LE-627)	+	+	+

The present results also revealed that endopolyploidy does not occur in the tetraploid species *L. corniculatus*. This agrees with previous observations on sugar beet seedlings of different ploidies, where fewer endocycles occurred in triploid and tetraploid plants than in diploid ones (Sliwinska and Lukaszewska, 2005). It was hypothesized that in some species a certain maximum nuclear DNA content is programmed, rather than the number of endocycles, and this seems to be the case for the *Lotus* species (in 4C nuclei of *L. corniculatus* the DNA content of about 4.7 pg is similar to that of 8C nuclei of diploid species). Thus, the lack of endoreduplication in other studied *Lotus* species can be an indication of its polyploidy.

Our analyses of the genome size of different accessions of *L. maritimus* and *L. uliginosus* confirm the suggestion from studies of 60 genotypes of *L. corniculatus* collected from different ecogeographical sites (Soltis et al., 2003; Gasmanová et al., 2007) that there is no or low intraspecific variation in the genome size of *Lotus* species. Although significant differences were detected in nuclear DNA contents between *L. uliginosus* accessions LE-627, and 205 and 203, it was only 3%. This knowledge can be important for species identification by FCM.

4.2. Identification and classification of *Lotus* species by ISSR-PCR

ISSR-PCR revealed high polymorphism of the investigated species, confirming the high discriminating power of ISSR markers. Based on the banding profiles, it was possible to identify all species (Table 4). Specific bands enabled the distinction even between very closely related species like *L. japonicus* and *L. burttii*, or *L. corniculatus* and *L. tenuis*. It confirmed the usefulness of ISSR markers as reported in previous studies, e.g., on *Dendrobium* (Wang et al., 2009), *Ocimum* (Chen et al., 2013; Rewers and Jedrzejczyk, 2016), and *Miscanthus* (Cichorz et al., 2014). Most of the primers tested here for *Lotus* revealed polymorphism between species, and three of them (ISSR-1, ISSR-24, and ISSR-30) also between accessions of the same species of different origin; therefore, they are recommended for identification of accessions of this genus.

The results of ISSR-PCR also allowed for the establishment of relationships between accessions, reflected by genetic distance estimation and results of PCoA analysis. A close relationship was found between accessions of *L. maritimus* from Germany and the Czech Republic. Similarly, *L. uliginosus* accessions collected at different locations were clustered into one group, although genetic diversity between populations was evident. At the same time, the highest genetic distance was observed between *L. maritimus* and *L. uliginosus*. *L. japonicus*, *L. burttii*, *L. filicaulis*, *L. corniculatus*, and *L. tenuis*, all possessing $x = 6$ chromosomes, although not of the same ploidy, were included in the same group. However, the closest relationship was found between *L. japonicus* and *L. burttii*, and between *L. corniculatus* and *L. tenuis*. This, together with the observation of meiotic chromosome behavior in hybrids between these last two species (Wernsman et al., 1964), would suggest autotetraploidy of *L. corniculatus*, with *L. tenuis* as its ancestor, rather than allotetraploidy. Also, *L. alpinus* has been previously proposed to be an ancestor of autotetraploid *L. corniculatus* (Somaroo and Grant, 1971; Campos et al., 1994); however, synthetic autotetraploids of these species did not resemble *L. corniculatus* in morphology and fertility (Somaroo and Grant, 1971). Therefore, more detailed studies are needed to confirm the genetic background of *L. corniculatus*. *L. tetragonolobus* was separated from the remaining accessions, which confirms that there were chromosome rearrangement(s) during speciation, resulting in the presence of 14 chromosomes and not 12 in all other species except *L. maritimus* (Table 2). The results support the classification of the genus *Lotus* provided by Ferreira and Pedrosa-Harand (2014), where *L. burttii*, *L. filicaulis*, *L. japonicus*, *L. corniculatus*, and *L. tenuis* are included in the section *Lotus* and *L. uliginosus* into a separate *L. uliginosus* group. The remaining species, *L. maritimus* and *L. tetragonolobus*, are included into the *Lotus* section *Tetragonolobus*. Similarly to the present results, RAPD and nrITS analyses also grouped *L. corniculatus*, *L. japonicus*, and *L. tenuis* into one cluster, which was distinct from *L. uliginosus* (Campos et al., 1994; Escaray et al., 2012).

Although molecular markers are usually reliable, ISSR-PCR can sometimes produce diverse results depending on the number and informativeness of the primers used for DNA amplification. In contrast to the present study, which revealed the highest genetic distance between *L. maritimus* and *L. uliginosus*, the results reported by Tanaka et al. (2016) clustered these two species into one group. Such grouping is also not in agreement with the conclusions of Ferreira and Pedrosa-Harand (2014). The FCM results presented here verify that *L. maritimus* and *L. uliginosus* should be placed into separate groups (their 1Cx DNA contents were different: 0.7 and 0.5 pg, respectively). However, interpreting taxonomy data, it has to be considered that differences between individuals/populations obtained by molecular methods, sampled from two different species or the same species, may not always represent an inter- or intraspecific polymorphism, respectively.

In conclusion, the precise identification of *Lotus* species is possible using FCM or/and ISSR markers (Table 4). The differences in genome size alone allowed identification of five out of eight species; however, additionally establishing cell cycle/endoreduplication intensity made it possible

to find characteristics that distinguished between the remaining species. This combined (estimation of genome size and cell cycle/endoreduplication pattern) system of FCM analysis has not been used before for species identification and our results reveal that it is recommended for screening of species of the genus *Lotus*. Even if it may not allow for identification of all species, it will significantly reduce the number of accessions to be verified by molecular methods. For very precise species identification, however, ISSR-PCR is a more sensitive method and it creates markers that can identify even accessions of different origin within a species. One has to be aware, however, that depending on the primers selected, the results of different ISSR-PCR experiments may not be entirely comparable, and supporting them by FCM is desirable. The identification system reported in the present study can be applied to breeding, conservation, germplasm collection, and taxonomy of *Lotus* species.

Acknowledgements
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Table S1. Features of ISSR primers used in molecular characterization of 14 *Lotus* accessions.

Primer code	Primer sequence (5'-3')	Annealing temperature (°C)	No. of total bands	No. of polymorphic bands	Percentage of polymorphism	PIC
ISSR-1	$(GACA)_4$	49.0	12	12	100	0.37
ISSR-3	$(GAC)_6$	63.4	12	12	100	0.48
ISSR-4	$(GTG)_6A$	58.4	21	21	100	0.36
ISSR-5	$(GACA)_4T$	49.0	22	22	100	0.33
ISSR-6	$(GACA)_4A$	49.0	11	11	100	0.36
ISSR-7	$(GTG)_6T$	58.4	23	23	100	0.36
ISSR-11	$(GA)_8T$	54.3	14	14	100	0.45
ISSR-12	$(CA)_8A$	52.5	16	16	100	0.39
ISSR-13	$(TC)_8A$	54.0	15	15	100	0.36
ISSR-14	$(AG)_8YT$	54.0	14	14	100	0.38
ISSR-15	$(AG)_8YA$	54.0	17	17	100	0.42
ISSR-20	$(TGAG)_4$	52.5	20	20	100	0.37
ISSR-21	$(GA)_9T$	60.2	17	17	100	0.26
ISSR-22	$(AGG)_6$	67.2	20	20	100	0.36
ISSR-23	$(AG)_{10}T$	63.4	13	13	100	0.30
ISSR-24	$(CA)_7G$	61.1	18	18	100	0.32
ISSR-25	$(AC)_8T$	56.6	13	13	100	0.40
ISSR-27	$(AC)_9T$	56.6	10	10	100	0.35
ISSR-28	$(CA)_6AC$	48.5	17	17	100	0.42
ISSR-29	$(CAG)_5$	61.0	17	16	94	0.41
ISSR-30	$(CTC)_4RC$	52.2	21	21	100	0.35
ISSR-31	$(CAA)_5$	47.1	13	13	100	0.42
ISSR-33	$(CT)_8TG$	50.0	15	15	100	0.32
ISSR-34	$(GA)_6GG$	57.3	13	13	100	0.37
ISSR-36	$(GA)_6CC$	54.3	7	7	100	0.37
ISSR-37	$(GAG)_3GG$	50.0	17	17	100	0.48
ISSR-38	$(GTC)_3GC$	50.0	19	19	100	0.48
ISSR-39	$(CT)_8GC$	50.0	11	11	100	0.39
Average			16	16	99.8	0.38

Table S2. Genetic distances between 14 *Lotus* accessions established based on ISSR markers. The lowest and highest values are given in bold.

No.	Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1.	*L. burtii*	0.00													
2.	*L. corniculatus*	0.55	0.00												
3.	*L. filicaulis*	0.48	0.49	0.00											
4.	*L. japonicus*	0.44	0.52	0.52	0.00										
5.	*L. maritimus* (3723)	0.81	0.74	0.74	0.77	0.00									
6.	*L. maritimus* (C2.442)	0.79	0.78	0.74	0.77	**0.16**	0.00								
7.	*L. tenuis*	0.55	0.45	0.46	0.56	0.74	0.74	0.00							
8.	*L. tetragonolobus*	0.70	0.73	0.65	0.76	0.75	0.77	0.72	0.00						
9.	*L. uliginosus* (7)	0.71	0.66	0.72	0.73	0.74	0.75	0.71	0.80	0.00					
10.	*L. uliginosus* (8)	0.73	0.69	0.71	0.71	0.78	0.80	0.74	0.81	0.33	0.00				
11.	*L. uliginosus* (203)	0.75	0.72	0.75	0.73	0.74	0.75	0.77	0.81	0.37	0.32	0.00			
12.	*L. uliginosus* (204)	0.74	0.72	0.71	0.68	0.74	0.74	0.74	0.81	0.35	0.26	0.27	0.00		
13.	*L. uliginosus* (205)	0.70	0.69	0.73	0.68	0.77	0.76	0.75	0.79	0.36	0.32	0.27	0.22	0.00	
14.	*L. uliginosus* (LE-627)	0.65	0.62	0.66	0.72	0.78	**0.82**	0.71	0.72	0.50	0.44	0.43	0.44	0.37	0.00

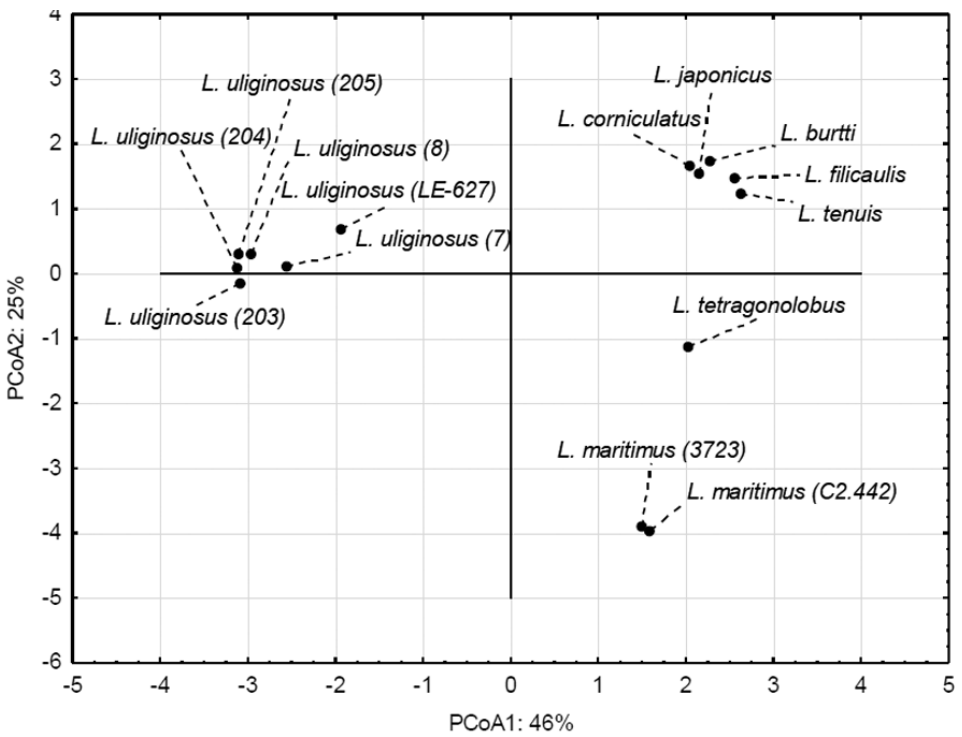

Figure S1. PCoA plot of 14 *Lotus* accessions, based on ISSR genetic distance data.