Lotus Cytogenetics

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Abstract

Most *Lotus* species have the basic chromosome number $x = 7$. The basic number $x = 6$ is, however, characteristic for the Corniculatus group and the other species from the section *Lotus*. Polyploidy, especially tetraploidy ($2n = 4x$), is recurrent in the genus with many species showing diploid and tetraploid accessions and others known as tetraploids only, such as *L. corniculatus*, the major forage crop. Genomes are relatively small, which, together with other interesting features, led to the choice of *L. japonicus* as a model legume species. Since then, advances in molecular cytogenetics, with the mapping of repetitive and single-copy sequences, enabled the integration of chromosomes to genetic maps and genome sequence information. Comparative cytogenetic maps were established for species from the section *Lotus*, mostly from the Corniculatus groups, and have demonstrated the importance of inversions and translocations, in addition to descending dysploidy and polyploidy, to the karyotype evolution of the genus.

2.1 Introduction

The first report on *Lotus* chromosomes was from 1924 (reviewed by Grant 1965). Since then, chromosome numbers have been reported for most of its species (reviewed by Grant 1995). The economic importance of *L. corniculatus* and related species has led to more detailed analyses of *Lotus* chromosomes, especially for understanding the origin of *L. corniculatus*, a polyploid crop species (Grant 1995). More recently, with the proposal of *L. japonicus* as a legume model, the fluorescent in situ hybridization (FISH) technique was applied to *Lotus* chromosomes (Ito et al. 2000), marking the transition from the classical to the molecular cytogenetic age (Jiang and Gill 2006).

In this chapter, we review the major advances in *Lotus* cytogenetics and its contribution to understanding *Lotus* genome organization and evolution.

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2.2 Relationship Among *Lotus* Species

The genus *Lotus* comprises approximately 120–130 species and belongs to *Loteae*, a tribe of herbaceous species from temperate climates that was expanded by the inclusion of *Coronilleae* (Allan and Porter 2000). *Lotus* is the largest genus of the tribe and has the most complex taxonomic delimitation, mostly due to its high morphological and biogeographical diversity (Grant and Small 1996; Kramina and Sokoloff 2004; Kramina 2006). The circumscription of species and sections, as well as the genus itself, is controversial, but Degtjareva et al. (2006, 2008) considered the genus to be restricted to species native to Europe, Asia, Africa, and Australia, accepted the segregation of three Old World monotypic genera (*Kebirita*, *Podolotus,* and *Pseudolotus*) and included species commonly placed in *Dorycnium* and *Tetragonolobus* in *Lotus*. In this circumscription, 14 sections are recognized.

Phylogenetic analyses have contributed to elucidate the relationships among its species (Allan and Porter 2000; Arrambari 2000a, b; Allan et al. 2003; Degjareva et al. 2006, 2008). In general, those analyses have been congruent with major classical groups defined by morphological, reproductive, and cytotaxonomic approaches (Cheng and Grant 1973; Ross and Jones 1985; Arrambari et al. 2005; Barykina and Kramina 2006; Kramina 2006; Sokoloff et al. 2007).

The most investigated species of the genus belongs to the *L. corniculatus* group (Grant 1995), due to the fact that *L. corniculatus*, birdsfoot trefoil, is widely used as forage and for soil bioremediation in temperate regions (Díaz et al. 2005; Banuelos et al. 1992). Three other species were also domesticated: *L. glaber* Mill. (also known as *L. tenuis* Wald and Kit.), *L. uliginosus* Schkuhr (also considered synonymous with *L. pedunculatus* Cav.), and *L. subbiflorus* Lag. (Grant 1995; Gonnet and Diaz 2000; Scheffer-Basso et al. 2005). *Lotus glaber* and *L. uliginosus* are classically included in the Corniculatus group, together with *L. alpinus*, *L. borbassi*, *L. burttii*, *L. filicaulis*, *L. japonicus*, *L. krylovii*, *L. schoeleri,* and other species (Grant 1995). The phylogenetic analysis, based on ribosomal nuclear ITS (Internal Transcribed Spacer) and on morphologic characters, included in the same clade of *L. corniculatus* (also denominated Corniculatus group) almost all species cited above, plus *L. delortii*, *L. palustris*, *L. peczoricus*, *L. preslii,* and *L. stepposus* (Degtjareva et al. 2006, 2008). *Lotus uliginosus*, greater lotus, big trefoil or marsh birdsfoot trefoil, was, however, grouped with other species in the sister clade of the Corniculatus group, and *L. subbiflorus*, hairy birdsfoot trefoil, is now recognized as a less related species (Degtjareva et al. 2006).

2.3 Classic Cytogenetics

The species from the Corniculatus group were often investigated using classical cytogenetic methods, which were mainly aimed at contributing to the understanding of the origin of *L. corniculatus* and to its improvement (Sz-Borsos 1973; Ross and Jones 1985; Pupilli et al. 1990; Grant 1995; Grant and Small 1996; Gauthier et al. 1997). *Lotus corniculatus* is a tetraploid, with $2n = 4x = 24$ (Grant 1995). The other species of the group are diploids, also with basic chromosome number $x = 6$, which thus constitute a shared, derived character (synapomorphy) of the section *Lotus*, to which those species belong (Degtjareva et al. 2006).

Classic cytogenetics also has a long tradition in the genus *Lotus* outside the Corniculatus group, predominantly with cytotaxonomic studies comprising chromosome counts and karyotype descriptions (Cheng and Grant 1973; Freed and Grant 1976; Grant 1995). It was shown that in addition to $x = 6$ the genus also presents basic numbers $x = 5$ and 7. The basic number $x = 5$ is present in a single species of the section *Lotus*, while $x = 7$ is the most common and probably the ancestral basic chromosome number (reviewed by Grant 1995), observed in the ten sections with cytologically investigated species (Table 2.1). It probably gave rise to $x = 6$ and 5 by descending dysploidy. Supernumerary B-chromosomes have been reported in few species (Table 2.1).

Table 2.1 Basic chromosome number, ploidy level, and C-value of *Lotus* species represented in the genus phylogeny (Degtjareva et al. 2006, 2008)

Species[a]	Name status	Basic	Ploidy	1C (pg)[b]	References
***Lotus* sect. *Benedictella* (Maire) Kramina and D.D. Sokoloff (1/0)**					
***Lotus* sect. *Bonjeanea* (Rchb.) D.D. Sokoloff (3/3)**					
L. hirsutus L. [= *Dorycnium hirsutum* (L.) Ser.]	Synonym (ILDIS)	7	2*x*		IPCN (2013)
L. rectus L. [= *Dorycnium rectum* (L.) Ser.]	Synonym (ILDIS)	7	2*x*		IPCN (2013)
L. strictus Fisch. and C.A. Mey. [= *Dorycnium strictum* (Fisch. and C.A. Mey.) Lassen]	Synonym (ILDIS)	7	2*x*		Grant (1995)
***Lotus* sect Canaria (Rikli.) D.D. Sokoloff (3/0)**					
***Lotus* sect. Chamaelotus Kramina and D.D. Sokoloff (3/2)**					
L. glinoides Del. [= *L. trigonelloides* Webb and Berth.]	Accepted (ILDIS)	7	2*x*		Grant (1995)
L. schimperi Steud. ex Boiss	Accepted (ILDIS)	7	2*x*		IPCN (2013)
***Lotus* sect. Dorycnium (Mill.) D.D. Sokoloff (5/2)**					
L. dorycnium L. s.l.[= *Dorycnium herbaceum* Vill.]	Synonym (ILDIS)	7	2*x*		IPCN (2013)
L. graecus L. [= *Dorycnium graecum* (L.) Ser.]	Synonym (ILDIS)	7	2*x*		IPCN (2013)
***Lotus* sect. *Erythrolotus* Brand (0/0)**					
***Lotus* sect. *Heinekenia* Webb and Berth. (23/9)**					
Lotus arabicus group					
L. arabicus L.	Accepted (ILDIS)	6, 7	2*x*		Grant (1995)
L. lanuginosus Vent.	Accepted (ILDIS)	7	2*x*		Grant (1995)
L. laricus Rech.f., Aellen and Esfand	Accepted (ILDIS)	7	2*x*		IPCN (2013)
Lotus australis group					
L. australis Andrews	Accepted (ILDIS)	7	4*x*		Grant (1995)
L. cruentus Court	Accepted (ILDIS)	7	4*x*		Grant (1995)
Lotus discolor group					
L. discolor E. Mey	Accepted (ILDIS)	7	2*x*		Grant (1995)
Lotus gebelia group					
L. aegaeus (Griseb.) Nym	Accepted (ILDIS)	6, 7	4*x*		Grant (1995)
L. gebelia Vent.	Accepted (ILDIS)	7	2*x*		Grant (1995), IPCN (2013)
L. michauxianus Ser.	Accepted (ILDIS)	7	2*x*		IPCN (2013)

(continued)

Table 2.1 (continued)

Species[a]	Name status	Basic	Ploidy	1C (pg)[b]	References
***Lotus* sect. *Krokeria* (Moench) Ser (1/1)**					
L. edulis L.	Accepted (ILDIS)	7	2*x*	1.10	Grant (1995), IPCN (2013)
***Lotus* sect. *Lotea* (Medik.) DC. (10/8)**					
L. cytisoides L.	Accepted (ILDIS)	7	2*x*	1.40	IPCN (2013)
L. halophilus Boiss. and Spruner	Accepted (ILDIS)	7	2*x*, 4*x*		Grant (1995), IPCN (2013)
L. longiseliquosus R. Roem. [= *L. collinus* (Boiss.) Heldr.]	Accepted (ILDIS)	7	2*x*, 4*x*		Grant (1995), IPCN (2013)
L. ornithopodioides L.	Accepted (ILDIS)	7	2*x*	1.30[c]	Grant (1995), IPCN (2013)
L. peregrinus L.	Accepted (ILDIS)	7	4*x*		Grant (1995), IPCN (2013)
L. polyphyllos Clarke	Accepted (ILDIS)	6, 7	2*x*		Grant (1995)
L. tetraphyllus Murr.	Accepted (ILDIS)	7	2*x*		Grant (1995)
L. weilleri Maire	Accepted (ILDIS)	7	2*x*		Grant (1995)
***Lotus* sect. *Lotus* (31/22)**					
L. angustissimus group					
L. angustissimus L. [= *L. praetermissus* Kuprian.]	Accepted (ILDIS)	6	2*x*, 4*x*		Grant (1995), IPCN (2013)
L. castellanus Boiss. and Reut. [= *L. subbiflorus* Lag.]	Synonym (ILDIS)	6	2*x*		IPCN (2013)
L. castellanus Boiss. and Reut. [= *L. glareosus* Boiss. and Reut.]	Synonym (ILDIS)	6	2*x*		Grant (1995), IPCN (2013)
L. parviflorus Desf.	Accepted (ILDIS)	6	2*x*		Grant (1995), IPCN (2013)
L. subbiflorus Lag. [= *L. suaveolens* Pers.]	Accepted (ILDIS)	6	2*x*, 4*x*		Grant (1995), IPCN (2013)
Lotus corniculatus group					
L. alpinus (DC.) Schleicher ex Ramond	Accepted (ILDIS)	6 + B	2*x*, 4*x*, 6*x*	0.48	Grant (1995), IPCN (2013)
L. borbasii Ujhelyi	Accepted (ILDIS)	6	2*x*	0.50	Grant (1995)
L. burttii Borsos	Accepted (ILDIS)	6	2*x*	0.53	Grant (1995)
L. corniculatus L.	Accepted (ILDIS)	6	4*x*[d]	0.48, 1.05	Grant (1995), IPCN (2013)
L. delortii Timb.-Lagr. ex F.W. Schultz [= *L. pilosus* Jordan]	Accepted (ILDIS)	6	4*x*		Grant (1995)
L. filicaulis Durieu[= *L. tenuis* Waldst. and Kit. ex Willd.]	Synonym (ILDIS)	6	2*x*	0.50	Grant (1995)

(continued)

Table 2.1 (continued)

Species[a]	Name status	Basic	Ploidy	1C (pg)[b]	References
L. glaber Mill. [= *L. tenuis* Waldst. and Kit]	Accepted (ILDIS)	6[e]	2*x*, 4*x*	0.48	Grant (1995), IPCN (2013)
L. japonicus (Regel) K. Larsen 'Gifu' [= *L. corniculatus* subsp. *corniculatus* L.]	Synonym (ILDIS)	6	2*x*	0.48	Grant (1995), IPCN (2013)
L. japonicus (Regel) K. Larsen 'Miyakojima' [= *L. corniculatus* subsp. *corniculatus* L.]	Synonym (ILDIS)	6	2*x*		Grant (1995), IPCN (2013)
L. krylovii Schischk. and Serg.	Accepted (ILDIS)	6	2*x*	0.53	Grant (1995), IPCN (2013)
L. palustris Willd.	Accepted (ILDIS)	6, 7	2*x*, 4*x*	0.75	Grant (1995)
L. peczoricus Miniaev and Ulle	Accepted (ILDIS)	6	2*x*		Grant (1995)
L. preslli Tem.	Accepted (ILDIS)	6	2*x*, 4*x*		Grant (1995), IPCN (2013)
L. schoelleri Schweinf.	Accepted (ILDIS)	6	2*x*	0.50	Grant (1995)
L. conimbricensis Brot. [= *L. coimbrensis* Brot. ex Willd.]	Accepted (ILDIS)	6	2*x*	0.45	Grant (1995), IPCN (2013)
Lotus pedunculatus group					
L. pedunculatus Cav.	Accepted (ILDIS)	6	2*x*, 4*x*	0.55	Grant (1995), IPCN (2013)
L. uliginosus Schkuhr [= *L. pedunculatus* Cav.]	Synonym (ILDIS)	6	2*x*, 4*x*	0.55	Grant (1995), IPCN (2013)
***Lotus* sect. *Ononidium* Boiss. (4/0)**					
***Lotus* sect. *Pedrosia* (Lowe) Christ (29/10)**					
L. arenarius Brot.	Accepted (ILDIS)	7	2*x*, 4*x*	1.13	Grant (1995), IPCN (2013)
L. azoricus P.W. Ball [= *L. macranthus* Lowe]	Accepted (ILDIS)	7[f]	2*x*		Grant (1995), IPCN (2013)
L. campylocladus Webb and Berth	Accepted (ILDIS)	7	2*x*	0.62	Grant (1995), IPCN (2013)
L. creticus L.	Accepted (ILDIS)	7 + B	2*x*, 4*x*		Grant (1995), IPCN (2013)
L. emeroides R.P. Murray	Accepted (ILDIS)	7	2*x*, 4*x*		Grant (1995), IPCN (2013)
L. jacobaeus L.	Accepted (ILDIS)	7	2*x*		Grant (1995), IPCN (2013)
L. jolyi Battand	Accepted (ILDIS)	7	2*x*		Grant (1995), IPCN (2013)
L. lancerottensis Webb and Berth	Accepted (ILDIS)	7	2*x*		Grant (1995), IPCN (2013)
L. maroccanus Ball	Accepted (ILDIS)	7	2*x*		Grant (1995), IPCN (2013)
L. mascaensis Burchd	Accepted (ILDIS)	7	4*x*	1.25	Grant (1995), IPCN (2013)

(continued)

Table 2.1 (continued)

Species[a]	Name status	Basic	Ploidy	1C (pg)[b]	References
***Lotus* sect. *Rhyncholotus* (Manod) D.D. Sokoloff (3/2)**					
L. berthelotii Masf	Accepted (ILDIS)	7	4*x*	1.22	Grant (1995), IPCN (2013)
L. maculatus Breitf	Accepted (ILDIS)	7	4*x*		Grant (1995), IPCN (2013)
***Lotus* sect. *Tetragonolobus* (Scop.) Benth. and Hook.f. (5/2)**					
L. maritimus L. [= *Tetragonolobus maritimus* (L.) Roth.]	Accepted (ILDIS)	7 [g]	2*x*		Grant (1995), IPCN (2013)
L. tetragonolobus L. [= *T. purpureus* Moench.]	Accepted (ILDIS)	7	2*x*		Grant (1995), IPCN (2013)

[a] Species names and name status are based on The Plant List (2010). Version 1. Sections of *Lotus* are based on Degtjareva et al. (2006, 2008). Numbers after sectional names show total number of species in a section/number of species included here

[b] C-values from Bennett and Leitch (2012)

[c] C-value for *L. ornithopoides*

[d] 2*x* was reported, but is not anymore accepted

[e] Chromosome number for *L. tenuis*

[f] Chromosome number for *L. macranthus*

[g] Chromosome number for *T. maritimus*

Genome sizes are relatively small and have been estimated for 26 species (Bennett and Leitch 2012), even before the C-value was considered for estimating genome coverage in genome sequencing projects. Estimates are available for around 20 % of the species of the genus, comprising representatives from five out of the fourteen sections (see Table 2.1). Minimum and maximum genome sizes were 0.45 pg/1C for *L. conimbricensis* and 1.40 pg/1C for *L. cytisoides*, an approximate threefold difference in genome size at the diploid level within the genus.

Chromosome differential staining techniques, such as C-banding, which allows the differentiation between euchromatin and heterochromatin, have been applied to three species: *L. pedunculatus*, *L. tenuis* and *L. japonicus* (Shankland and Grant 1976; Falistocco and Piccirilli 1989; Pedrosa et al. 2002). Because heterochromatic regions remain condensed during most of the cell cycle, they appear as more condensed regions during mitotic prometaphase. Thus, imaging analysis of prometaphase chromosomes has also been used to construct idiograms for *L. japonicus* (Ito et al. 2000; Ohmido et al. 2007). Both approaches revealed that the heterochromatin is mainly located at pericentromeric regions, with terminal and intercalary blocks in few chromosomes and variation in heterochromatin distribution between genotypes of *L. japonicus* (Ito et al. 2000; Hayashi et al. 2001).

2.4 Molecular Cytogenetics in *Lotus*

Various repetitive DNA sequences have been used as probes in FISH experiments to investigate their distribution along *Lotus* chromosomes. The FISH technique consists of denaturing the chromosomes on microscopic preparations to separate the two complimentary DNA strands, followed by their renaturation in the presence of a probe, a labeled DNA fragment. The excess of available probe will compete against the chromosomal DNA strands, allowing its localization on chromosomes (Jiang and Gill 2006). For example, probes for ribosomal RNA coding sequences 5S and 45S rDNA were applied to several plants because these sequences are

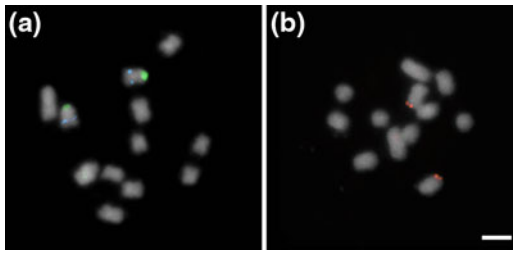

Fig. 2.1 Fluorescent in situ hybridization on mitotic metaphase chromosomes of *Lotus japonicus* 'Gifu.' **a** TAC 28L17/TM0153 (*blue*) is positioned on the opposite chromosome arm of 45S rDNA (green). **b** TAC 15K21/TM0088 (*orange*). Both TACs are located on the second largest chromosome and identify the chromosome 2. Chromosomes were counterstained with DAPI and are shown in gray. Bar in **b** = 5 μm

conserved and repeated in tandem, generating signals that are usually easily visualized on chromosomes (reviewed by Kato et al. 2005).

In *L. japonicus*, the 5S rDNA site was located interstitially in the short arm of chromosome 2, linked to a 45S rDNA site that was terminally located in the same chromosome arm (Hayashi et al. 2001; Pedrosa et al. 2002). In addition to this major 45S rDNA site on chromosome 2 (Fig. 2.1a), minor 45S rDNA sites were observed in the smallest chromosomes pairs, 5 and 6, in interstitial positions. Both probes have also been applied to other species of the Corniculatus group, showing that the linkage between 5S and 45S rDNA sites on chromosome 2 is conserved in *L. filicaulis* (Pedrosa et al. 2002), *L. burttii* (Kawaguchi et al. 2005), *L. glaber*, and *L. krilovii* (Fig. 2.2a, c). Except for *L. krilovii*, the 45S rDNA site on chromosome 6 was also present in the investigated species, but the weakest site on chromosome 5 has only been detected in *L. japonicus* 'Gifu' and 'Miyakojima'. Mapping of 5S and 45S on *L. uliginosus*, however, revealed more pronounced differences, although the rDNA sites on chromosome 2 were maintained. An additional 5S rDNA site was observed on chromosome 6, and two additional 45S rDNA sites were present on chromosomes 4 and 5, both in terminal positions (Ferreira et al. 2012).

Other repetitive DNA sequences have also been identified and localized to *Lotus* chromosomes. The *Ljcen1* repeat was identified because of its similarity to the Arabidopsis-type telomeric repeat and turned out to be centromeric, not only in *L. japonicus*, but also in other investigated species from the Corniculatus group, such as *L.*

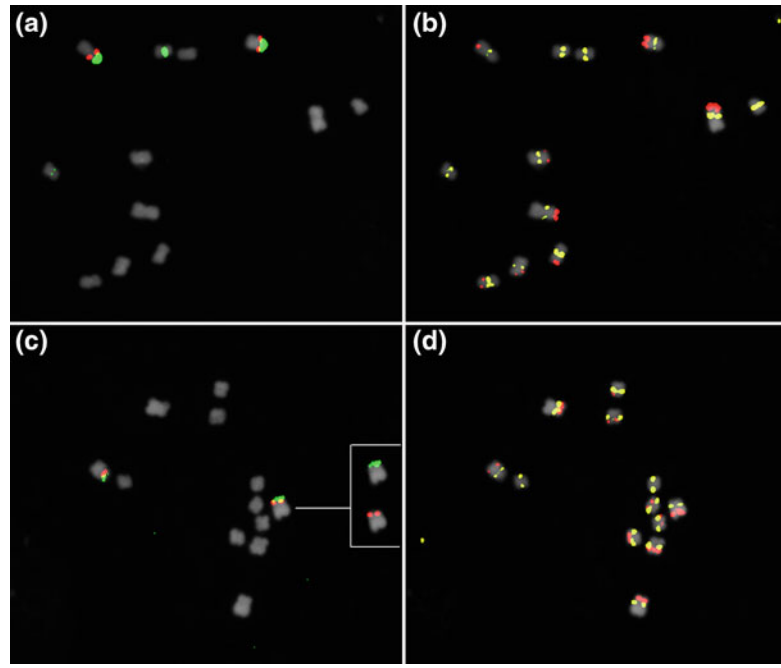

Fig. 2.2 Fluorescent in situ hybridization of repetitive sequences on mitotic metaphase chromosomes of diploids *L. glaber* (**a**, **b**) and *L. krilovii* (**c**, **d**). (**a**, **c**) 45S (*green*) and 5S (*orange*) rDNA, and (**b**, **d**) *Ljcen1* (*yellow*) and LJTR1 (*red*). Chromosomes were counterstained with DAPI and are shown in gray. Bar in (**d**) = 5 μm

filicaulis (Pedrosa et al. 2002), *L. burttii* (Kawaguchi et al. 2005), *L. glaber,* and *L. krilovii* (Fig. 2.2b, d). Later, a Ty3-*gypsy* LTR-retrotransposon, named LjRE2, was shown to have the same distribution as *Ljcen1* (Sato et al. 2008), as *Ljcen1* shows high sequence similarity to the LTR region of LjRE2 (Ohmido et al. 2010). The other characterized LTR-retrotransposon, LjRE1, a Ty1-*copia* type, showed a dispersed labeling of all chromosomes (Sato et al. 2008). Four tandem repeat sequences, LjTR1-4, were distributed in specific chromosomal regions, forming blocks associated with eu- or heterochromatin in prometaphase or pachytene chromosomes (Sato et al. 2008; Ohmido et al. 2010). LjTR1 has also been localized to *L. glaber* and *L. krilovii* mitotic metaphase chromosomes, showing similar patterns of terminal blocks of varying intensities in the short or the long chromosome arm, except for chromosome 5 (Fig. 2.2b, d).

2.5 Integrated Genetic and Cytogenetic Maps in *Lotus*

After *L. japonicus* had been chosen as a model legume, genetic maps were established as a first step toward positional cloning (Handberg and Stougaard 1992; Sato and Tabata 2006). The first maps, which included AFLPs, RAPDs, RFLPs, SSRs, and dCAPS markers, as well as mutant phenotypes, were based on mapping populations obtained from crosses between *L. japonicus* ecotypes, 'Gifu' and 'Miyakojima,' or between *L. japonicus* and a closely related species from the Corniculatus group, *L. filicaulis* (Hayashi et al. 2001; Sandal et al. 2002). The first version of these maps, however, presented distortions in the recombination frequencies, leading to maps with five or seven linkage groups, instead of the expected six.

In parallel to the genetic mapping efforts, cytogenetic maps were built using genomic DNA clones with large, single-copy inserts, such as BACs (bacterial artificial chromosomes) and TACs (transformation-competent artificial chromosomes). Cytogenetic maps are physical maps in which DNA sequences are localized on the chromosomes and positioned in relation to centromeres, telomeres, and the heterochromatin and are usually developed by FISH. The *Lotus* BACs and TACs used as probes were anchored to the genetic maps, allowing the integration of linkage groups and chromosomes (Fig. 2.1). These integrated cytogenetic maps helped to establish six linkage groups in each map, which were named according to the six chromosome pairs. Furthermore, they revealed chromosome rearrangements between the parental accessions or species, which were responsible for the observed segregation distortions (Hayashi et al. 2001; Pedrosa et al. 2002). TACs have later been used to mitotic prometaphase and meiotic pachytene chromosomes for higher resolution mapping (Sato et al. 2008; Ohmido et al. 2010). The availability of those BACs and TACs as chromosome markers and the indication of rearrangements among closely related genotypes stimulated the investigation of chromosome evolution in the genus.

2.6 Comparative Cytogenetics in *Lotus*

The establishment of cytogenetic maps for *L. japonicus* made available a set of chromosome-specific markers that could be used to build similar maps in related species. These comparative maps allow exploration of the macrosynteny and collinearity among genomes and investigation of karyotype evolution in more detail.

In *Lotus*, paracentric and pericentric inversions and translocations could be clearly demonstrated between *L. japonicus* ecotypes 'Gifu' and 'Miyakojima' and between *L. japonicus* and *L. burttii* and *L. filicaulis* (Hayashi et al. 2001; Pedrosa et al. 2002; Kawaguchi et al. 2005). Between 'Gifu' and 'Miyakojima', a reciprocal translocation has exchanged the terminal portions of chromosome 1 short arm and chromosome 2 long arm. When the same chromosome markers were mapped in *L. burttii* and *L. filicaulis*, synteny with 'Gifu' was observed, what indicates that 'Gifu' chromosomes 1 and 2 represent the ancestral (plesiomorphic) condition. On the other hand, the inversion in a small portion of the long

arm of *L. japonicus* chromosome 1, when compared to the other two species, seemed to be the derived (apomorphic) condition, as well as a pericentric inversion on *L. filicaulis* chromosome 3, which is acrocentric and has so far only been observed as acrocentric in this species.

Lotus japonicus ecotypes 'Miyakojima' and 'Gifu' present other cytogenetic differences. The TAC 28L17, mapped on 'Miyakojima' between the 5S and 45S rDNA sites on the short arm of chromosome 2, is positioned on the opposite chromosomal arm on 'Gifu' (Fig. 2.1a). Furthermore, terminal heterochromatic blocks are more frequent in 'Miyakojima' than in 'Gifu.' These ecotypes appear to have not only enough genomic differences, but also distinct morphological characters to be considered two species: *L. japonicus* (Regel) K. Larsen and *L. miyakojimae* Kramina (Barykina and Kramina 2006). In fact, it was also suggested in the first phylogeny (Degtjareva et al. 2006) and considered in the last update (Degtjareva et al. 2008).

More recently, the comparative map was expanded to *L. uliginosus*, a phylogenetically more distant species (Degtjareva et al. 2006), which does not belong to the Corniculatus group (Ferreira et al. 2012). A different translocation was observed, involving chromosomes 3 and 5. Karyotypic differences were more pronounced between *L. uliginosus* and *L. japonicus* than between any Corniculatus species, reflecting their phylogenetic distances (Fig. 2.3).

2.7 *Lotus* Polyploids

Although most *Lotus* species are diploids, polyploids, particularly tetraploids, are of relevance in the genus because polyploidy is observed in at least five sections and most of the cultivated accessions are polyploids. *Lotus corniculatus* is the classical example, but even in species known as diploid, such as *L. uliginosus*, its cultivars may be polyploid, such as 'Maku,' with $2n = 4x = 24$. Indeed, several species are reported to have diploid and tetraploid accessions, such as *Lotus subbiflorus* (see Table 2.1).

Lotus subbiflorus also belongs to the section *Lotus*, but is placed in clade A, a sister clade to clade B, where *L. corniculatus* is present (Degtjareva et al. 2006). One polyploid accession has been recently investigated using rDNA and *Ljcen1* probe and this analysis gave support for an allopolyploid origin for this species. The first evidence came from the number and distribution of 5S and 45S rDNA sites. One chromosome pair showed linked 5S and 45S rDNA sites, as observed for chromosome 2 in the Corniculatus group, but the possible homeologous pair showed a 45S rDNA cluster only. A second 5S rDNA site was in one smaller chromosome pair (Fig. 2.4a). In addition, *Ljcen1* only strongly labeled one set of chromosomes (Fig. 2.4b), suggesting that the two diploid species that hybridized to form the *L. subbiflorus* genome showed remarkable karyotype differences. Because its closely related, diploid species have not been investigated to date, it is still not possible to suggest putative ancestral species.

The origin of *L. corniculatus* has been investigated in more detail. Classical cytogenetic analysis, as well as biochemical and morphological markers, have been employed. The most recent hypothesis considered this an allotetraploid species originating from the crossing of *L. tenuis* and *L. uliginosus* (Ross and Jones 1985; Grant and Small 1996). Other possible diploids considered to be involved in the origin of *L. corniculatus* are *L. alpinus* and *L. japonicus* (Grant and Small 1996) or *L. schoelleri*, *L. stepposus*, *L. peczoricus*, *L. borbasii*, *L. krylovii,* and *L. japonicus* (Degtjareva et al. 2006).

From these, *L. glaber* (a synonym of *L. tenuis*), *L. uliginosus*, *L. japonicus,* and *L. krylovii* have been investigated cytogenetically in more detail and compared to *L. corniculatus*. *L. glaber,* and *L. japonicus* 'Gifu' have the most similar karyotypes, with 5S and 45S rDNA sites in chromosome 2 and a 45S rDNA site in chromosome 6. *L. corniculatus* chromosomes, when analyzed with the same probes, showed double the number of rDNA sites in similar positions (Fig. 2.4c). *L. krylovii* apparently lacks the 45S rDNA site in chromosome 6 and *L. uliginosus* is

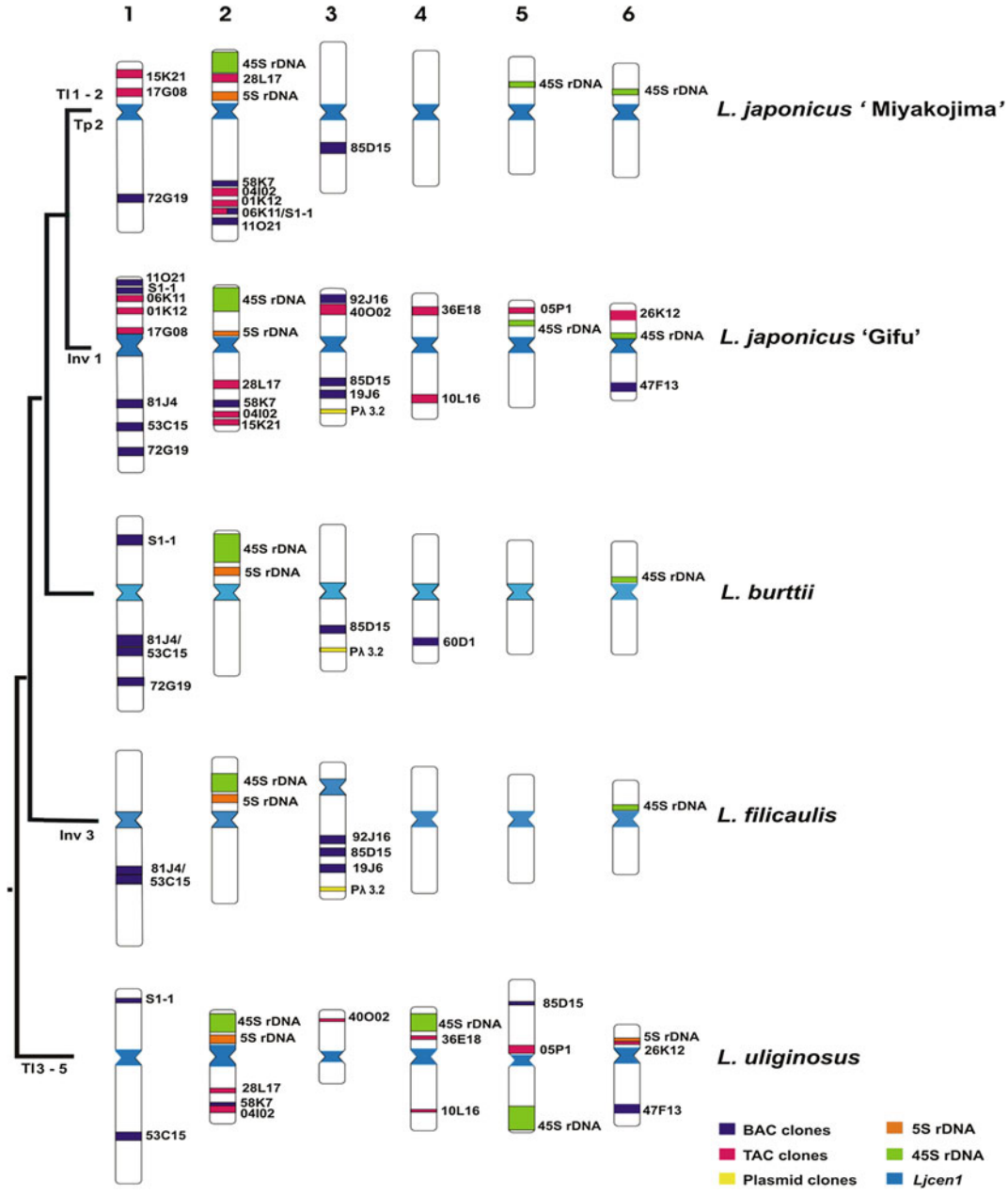

Fig. 2.3 Comparative schematic representation of the chromosome complement of *L. japonicus* 'Miyakojima' and 'Gifu', *L. burttii*, *L. filicaulis* (modified from Hayashi et al. 2001; Pedrosa et al. 2002; Sato et al. 2008), and *L. uliginosus*. Approximated positions of rDNA sites, pericentromeric repeat *Ljcen1*, and mapped TAC/BAC clones are represented. TACs are visualized in red and BACs in dark blue (thin blocks represent weaker signals in *L. uliginosus*). *Lotus uliginosus* chromosomes 3 and 5 were rotated (short arm down) to facilitate comparison. Phylogenetic relationships are based on Degtjareva et al. (2006, 2008). The proposed rearrangements (Tl = translocation, Tp = transposition, and Inv = inversion) are indicated (Ferreira et al. 2012)

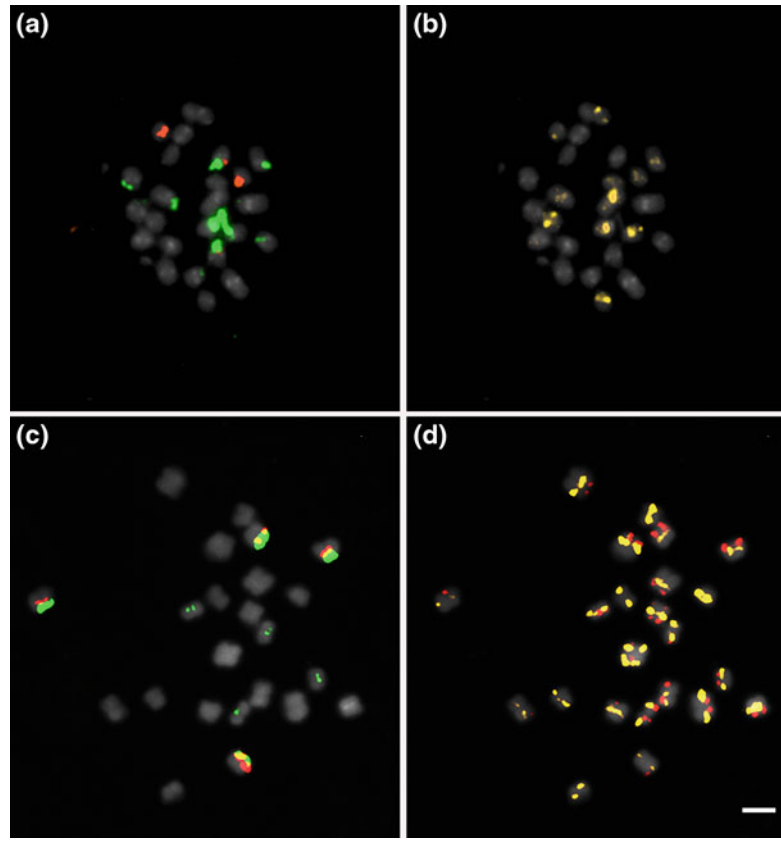

Fig. 2.4 Fluorescent in situ hybridization of repetitive sequences on mitotic metaphase chromosomes of polyploids *L. subbiflorus* (**a**, **b**) and *L.corniculatus* (**c**, **d**). (**a**, **c**) 45S (*green*) and 5S (*orange*) rDNA, (**b**, **d**) *Ljcen1* (*yellow*) and (**d**) LJTR1 (*red*). Note that *Ljcen1* signals are present in only one set of chromosomes of *L. subbiflorus*, suggesting an allotetraploid origin. Chromosomes were counterstained with DAPI and are shown in gray. Bar in (**d**) = 5 μm

clearly very different in rDNA distribution. Current cytogenetic evidence would suggest *L. glaber* and *L. japonicus* as possible ancestral species of *L. corniculatus*, or other closely related species with similar karyotypes (Fig. 2.4c–d).

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