Compendium of Plant Genomes
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Editors

The *Lotus japonicus* Genome

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The *Lotus japonicus* Genome

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This book series is dedicated to
my wife Phullara, and our children Sourav, Carena, and Devleena

Chittaranjan Kole

Preface to the Series

Genome sequencing has emerged as the leading discipline in the plant sciences coinciding with the start of the new century. For much of the twentieth century, plant geneticists were only successful in delineating putative chromosomal location, function and changes in genes indirectly through the use of a number of 'markers' physically linked to them. These included visible or morphological, cytological, protein, and molecular or DNA markers. Among them, the first DNA marker, the RFLPs, introduced a revolutionary change in plant genetics and breeding in the mid-1980s, mainly because of their infinite number and thus potential to cover maximum chromosomal regions, phenotypic neutrality, absence of epistasis, and codominant nature. An array of other hybridization-based markers PCR-based markers, and markers based on both facilitated construction of genetic linkage maps, mapping of genes controlling simply inherited traits and even gene clusters (QTLs) controlling polygenic traits in a large number of model and crop plants. During this period a number of new mapping populations beyond F_2 were utilized and a number of computer programs were developed for map construction, mapping of genes, and for mapping of polygenic clusters or QTLs. Molecular markers were also used in studies of evolution and phylogenetic relationship, genetic diversity, DNA-fingerprinting and map-based cloning. Markers tightly linked to the genes were used in crop improvement employing the so-called marker-assisted selection. These strategies of molecular genetic mapping and molecular breeding made a spectacular impact during the last one and a half decades of the twentieth century. But still they remained 'indirect' approaches for elucidation and utilization of plant genomes since much of the chromosomes remained unknown and the complete chemical depiction of them was yet to be unraveled.

Physical mapping of genomes was the obvious consequence that facilitated development of the 'genomic resources' including BAC and YAC libraries to develop physical maps in some plant genomes. Subsequently, integrated genetic-physical maps were also developed in many plants. This led to the concept of structural genomics. Later on emphasis was laid on EST and transcriptome analysis to decipher the function of the active gene sequences leading to another concept defined as functional genomics. The advent of techniques of bacteriophage gene and DNA sequencing in the 1970s was extended to facilitate sequencing of these genomic resources in the last decade of the twentieth century.

As expected, sequencing of chromosomal regions would have led to too much data to store, characterize and utilize with the-then available computer software could handle. But development of information technology made the life of biologists easier by leading to a swift and sweet marriage of biology and informatics and a new subject was born—bioinformatics.

Thus, evolution of the concepts, strategies and tools of sequencing and bioinformatics reinforced the subject of genomics—structural and functional. Today, genome sequencing has traveled much beyond biology and involves biophysics, biochemistry and bioinformatics!

Thanks to the efforts of both public and private agencies, genome sequencing strategies are evolving very fast, leading to cheaper, quicker and automated techniques right from clone-by-clone and whole-genome shotgun approaches to a succession of second generation sequencing methods. Development of software of different generations facilitated this genome sequencing. At the same time newer concepts and strategies were emerging to handle sequencing of the complex genomes, particularly the polyploids.

It became a reality to chemically—and so directly—define plant genomes, popularly called whole-genome sequencing or simply genome sequencing.

The history of plant genome sequencing will always cite the sequencing of the genome of the model plant *Arabidopsis thaliana* in 2000 that was followed by sequencing the genome of the crop and model plant rice in 2002. Since then, the number of sequenced genomes of higher plants has been increasing exponentially, mainly due to the development of cheaper and quicker genomic techniques and, most importantly, development of collaborative platforms such as national and international consortia involving partners from public and/or private agencies.

As I write this preface for the first volume of the new series "Compendium of Plant Genomes", a net search tells me that complete or nearly-complete whole-genome sequencing of 45 crop plants, eight crop and model plants, eight model plants, 15 crop progenitors and relatives, and three basal plants are accomplished, the majority of which are in the public domain. This means that we nowadays know many of our model and crop plants chemically, i.e. directly, and we may depict them and utilize them precisely better than ever. Genome sequencing has covered all groups of crop plants. Hence, information on the precise depiction of plant genomes and the scope of their utilization is growing rapidly every day. However, the information is scattered in research articles and review papers in journals and dedicated web pages of the consortia and databases. There is no compilation of plant genomes and the opportunity of using the information in sequence-assisted breeding or further genomic studies. This is the underlying rationale for starting this book series, with each volume dedicated to a particular plant.

Plant genome science has emerged as an important subject in academia, and the present compendium of plant genomes will be highly useful both to students and teaching faculties. Most importantly, research scientists involved in genomics research will have access to systematic deliberations on the plant genomes of their interest. Elucidation of plant genomes is not only of interest for the geneticists and breeders, but also for practitioners of an array of plant science disciplines, such as taxonomy, evolution, cytology,

physiology, pathology, entomology, nematology, crop production, biochemistry, and obviously bioinformatics. It must be mentioned that information regarding each plant genome is ever-growing. The contents of the volumes of this compendium are therefore focusing on the basic aspects of the genomes and their utility. They include information on the academic and/or economic importance of the plants, description of their genomes from a molecular genetic and cytogenetic point of view, and the genomic resources developed. Detailed deliberations focus on the background history of the national and international genome initiatives, public and private partners involved, strategies and genomic resources and tools utilized, enumeration on the sequences and their assembly, repetitive sequences, gene annotation and genome duplication. In addition, synteny with other sequences, comparison of gene families and, most importantly, potential of the genome sequence information for gene pool characterization through genotyping by sequencing (GBS) and genetic improvement of crop plants have been described. As expected, there is a lot of variation of these topics in the volumes based on the information available on the crop, model or reference plants.

I must confess that as the series editor it has been a daunting task for me to work on such a huge and broad knowledge base that spans so many diverse plant species. However, pioneering scientists with life-time experience and expertise on the particular crops did excellent jobs editing the respective volumes. I myself have been a small science worker on plant genomes since the mid-1980s and that provided me the opportunity to personally know several stalwarts of plant genomics from all over the globe. Most, if not all, of the volume editors are my long-time friends and colleagues. It has been highly comfortable and enriching for me to work with them on this book series. To be honest, while working on this series I have been and will remain a student first, a science worker second and a series editor last. And I must express my gratitude to the volume editors and the chapter authors for providing me the opportunity to work with them on this compendium.

I also wish to mention here my thanks and gratitude to the Springer staff, Dr. Christina Eckey and Dr. Jutta Lindenborn in particular, for all their constant and cordial support right from the inception of the idea.

I always had to set aside additional hours to edit books besides my professional and personal commitments—hours I could and should have given to my wife, Phullara, and our kids, Sourav, Carena, and Devleena. I must mention that they not only allowed me the freedom to take away those hours from them but also offered their support in the editing job itself. I am really not sure whether my dedication of this compendium to them will suffice to do justice to their sacrifices for the interest of science and the science community.

Chittaranjan Kole

Preface to the Volume

Progress in plant genomics and genetics has been rapid and sustained in recent years. Focused research efforts on model plants have spearheaded this development and laid the foundation for subsequent investigations in the major crop species that are often less amenable. In the legume family (Fabaceae) *Lotus japonicus* (birdsfoot trefoil) was adopted as a model species more than 20 years ago and a considerable body of knowledge has since been built using genomic and genetic analyses in this species. Without being exhaustive, this volume presents some of the achievements made and provides a timely overview of topics relevant for future developments using legume genomics to improve our understanding of legume biology.

With more than 18,000 species represented, Fabaceae comprises the third largest family among the flowering plants and only grasses are more important in agriculture. Legumes are very diverse, ranging from tropical trees to temperate herbs. In addition to food and feed, they provide products from secondary metabolites and protein to oil and timber. The symbiosis with nitrogen fixing bacteria, rhizobia, enables legumes to obtain reduced dinitrogen for their own growth and is a major source of nitrogen in ecosystems and crop rotations. Like many other plants, legumes can also form symbiotic association with mycorrhizal fungi, which are important for phosphate uptake, and recent studies have identified a common symbiosis pathway for mycorrhizal and rhizobial symbiosis. Encompassing these biological and agricultural features, central topics in endosymbiosis, development, hormone regulation, carbon/nitrogen, and secondary metabolism, together with progress in high throughput genomic and genetic approaches, will be covered in this volume on the *Lotus japonicus* model system.

The world population is rapidly growing and an increase in food production is needed to match this increased food demand. Given the importance of legumes in sustainable agriculture, mining the model legume genomes and translation of knowledge from model legumes to crop legumes is important for our future. This volume provides an overview of some of the pertinent topics. We thank all the authors for their excellent contributions to this volume and hope that the expert's overview they have provided will serve as inspiration and encouragement for the future.

Satoshi Tabata
Jens Stougaard

Contents

Part I
The Importance of Lotus as a Model and a Crop

Background and History of the *Lotus japonicus* Model Legume System

1

Jens Stougaard

Abstract

The combination of favourable biological features, stable transformation procedures, application of genetics and genome-based global approaches has established *Lotus japonicus* as a model legume and provided a platform for addressing important biological questions often, but not exclusively, focusing on endosymbiosis. Several important discoveries have been made, and the *Lotus* community has contributed novel results, promoting our understanding of plant biology as well as our understanding of properties and characteristics typical for plants belonging to the legume family. Progress has been fast since *L. japonicus* was first promoted as a model plant yet there are many challenges for the coming years. This introductory chapter will set the stage for some of these challenges, while possibilities and challenges emerging from specific research projects will be addressed in the chapters that follow.

1.1 The *Lotus japonicus* Model Legume System

Mendel worked with garden peas for his groundbreaking work that established genetics as a science (Reid and Ross 2011). For many years, pea plants were also the workhorse in classical plant physiology. The ethylene-induced triple response of pea seedlings was, for example, one of the key observations leading to the identification of ethylene as a plant hormone. Continuing the genetic approaches, large collections of pea mutants and morphological variants were isolated, and substantial effort was invested in their phenotypic characterisation. Included in this collection was a sizeable subset of symbiotic plant mutants, with phenotypes ranging from non-nodulation to hypernodulation (Borisov et al. 2007; Tsyganov et al. 2002). From a historical perspective, the need for a model legume may therefore not have been obvious when the quest for a model legume started. However, prospects for combining genetics with stable transformation and emerging methodologies for genome-based studies inspired a search for a legume better suited to these global approaches. One of the outcomes was the proposal of *Lotus japonicus* as a model legume in 1992 (Handberg

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S. Tabata and J. Stougaard (eds.), *The Lotus japonicus Genome*, Compendium of Plant Genomes,
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and Stougaard 1992). Without aiming to be exhaustive, this volume highlights some of the achievements reached within the 20 years that followed and sketches the possibilities lying ahead.

Early botanical work on morphological features of the *Loteae* tribe in the 1950s led to the proposal of *L. japonicus* as a separate species (Larsen 1955). Further biological studies and karyotyping of chromosomes found *L. japonicus* to be self-fertile and diploid with a chromosome number of $n = 6$ (Cheng and Grant 1973). Subsequently, fluorescent measurements of 1C values for DNA content in nuclei of individual root cells indicated a genome size among the lowest in the legume family (Bennett and Smith 1976). These features distinguished *L. japonicus* from the morphologically very similar tetraploid outbreeder *Lotus corniculatus* ($n = 12$) that had previously been used for investigating regulation and promoter function of nodulin genes in transgenic roots and transgenic plants (Stougaard Jensen et al. 1986; Stougaard et al. 1990). Fortunately, some of the tissue culture and transformation techniques developed in *Lotus corniculatus* could be refined and transferred to *L. japonicus* (Stougaard et al. 1987; Hansen et al. 1989). A list of these model plant features was published previously (Handberg and Stougaard 1992).

Model features are to some extent technology and time dependent; however, it appears that *L. japonicus* has passed the test of time. An updated version of this list of "raison d'être" is shown in Table 1.1. Almost all of the features in the list have in one way or another been exploited in experimental procedures addressing important biological questions often, but not exclusively, focusing on endosymbiosis. Several different transformation procedures for regeneration of transgenic and composite plants have been established and used experimentally (Handberg and Stougaard 1992; Hansen et al. 1989). The number of selectable markers that can be used has been expanded, and both positive and negative selection schemes have been developed on this basis (Lohar et al. 2001; Lombari et al. 2003; Stougaard 1993). RNAi technologies have been used successfully (Kumagai et al. 2006; Soyano et al. 2013), and stable lines, such as pNin-GUS that inducibly express promoter reporter fusion for use as symbiotic response markers, have been made available (Radutoiu et al. 2003). Exploiting the favourable culture characteristics of *L. japonicus*, grafting procedures for root–shoot grafts and Y grafts have been used for investigating systemic plant responses mainly in the context of autoregulation of nodulation (Magori et al. 2009; Takahara et al. 2013). The small size of *L. japonicus* plantlets allowed for the development of in vitro mycorrhization in petri dishes using a filter sandwich set-up (Novero et al. 2002). Taking a whole plant approach, the vegetative growth pattern has been described and the role of strigolactone investigated. In contrast to Arabidopsis, *L. japonicus* develops multiple axillary shoots, and the ontogeny of these cotyledonary shoot meristems has been characterised and the influence of strigolactone on shoot architecture described (Alvarez et al. 2006; Lui et al. 2013). The reproductive life phase has also been studied, and analysis of the genetic background for the development of asymmetric flowers is ongoing (Xu et al. 2013). Another line of investigation has taken advantage of easy access to seeds in the simple straight seedpods of *L. japonicus* to follow seed development and the seed proteome from early-stage green seeds to mature dry seeds (Dam et al. 2009; Credali et al. 2013).

Forward genetic approaches based on mutant populations and gene discovery starting from interesting phenotypes have been a core activity for the *L. japonicus* community (Kouchi et al. 2010; Kistner et al. 2005; Sandal et al. 2006). Several breakthroughs have been achieved, and combined with the parallel efforts in *Medicago truncatula,* this has, in a relatively short time span, revealed the molecular backbone of both rhizobial and mycorrhizal endosymbioses. Key components of the legume signal perception/transduction genetic network mediating the rhizobial and endomycorrrhizal interactions have been defined and the functional aspects of symbiosis opened for analysis (Madsen et al. 2010; Desbrosses and Stougaard 2011; Oldroyd 2013).

Table 1.1 Features and characteristics of *Lotus japonicus*

Growth characteristics	Small primary plant Auxiliary shoots, bushy plant architecture Perennial 7-week period from seed to flowering Generation time from seed to seed, 3–4 months Small seeds: ~1.2 g per 1,000 seeds Fast regrowth from stem base/tap root Fast plant multiplication from nodal sections Root/shoot grafting and Y grafts possible
Propagation	Continuous flowering Large flowers allow for controlled crossings Self-fertile Simple spikeless and straight seedpod—like soybean and pea Approximately 20 seeds per pod Ample seed production, up to 6,000 seeds per plant Relative humidity above 65 % prevents seed shattering Hand pollination possible
Genome characteristics	Diploid, $2n = 12$ Genome size of ~478 Mb Cytogenetics developed Genespace fully sequenced Gene models based on mRNA and small RNAseq Genome re-sequenced in different ecotypes and diploid Lotus species High-resolution genetic maps available Recombinant inbred populations available Large collection of ecotypes available Diploid Lotus species for interspecific crosses available
Tissue culture	Regeneration from callus Stable transformation with *Agrobacterium tumefaciens* Positive selection: Hygromycin, kanamycin, geneticin and Basta Negative selection: 5-fluorocytosine Composite plants with *Agrobacterium rhizogenes*
Nodulation	Primary symbiont: *Mesorhizobium loti* Alternative often less-efficient symbionts: *Azorhizobium coulinodans*, *Sinorhizobium fredii*, IRGB74, NGR234 Several symbiont genomes sequenced Determinate nodules Sequential nodule development Primarily invasion mode via infection threads Crack entry and intercellular invasion observed in absence of infection threads
Mycorrhiza	Mycorrhized by *Rhizophagus irregularis* and *Gigantia margarita* and more
Pathogens	Leaf rust, *Uromyces loti* Clover rot, *Sclerotinia trifoliorum* Root-knot nematodes, *Meloidogyne incognita*
Parasites	Weed parasites, compatible and incompatible *Striga* and *Orobanche* spp
Insect interactions	Burnet moth, *Zygaena* filipendulae

Interestingly, the pea mutant collection has frequently been drawn into this work upon identification of causative genes using the model legume discovery tools (Madsen et al. 2003: Zhukov et al. 2008; Borisov et al. 2003). Taking a broader view of plant interactions, *L. japonicus* has been used for studies of nematode invasion (Poch et al. 2007; Weerasinghe et al. 2005), emerging investigations of root colonisation by parasitic weeds like Striga spp (Hiraoka et al. 2009) and specialised insect interactions (Zagrobelsky et al. 2007).

Aiming at continuing this success, development of additional genetic resources has remained a focal point. To enable reverse genetics, a TILLING population was established from EMS mutagenised *L. japonicus* seeds and made available to the plant community (Perry et al. 2009). Later, an endogenous retrotransposon called *Lotus retrotransposon 1* (LORE1) enabled the organisation of an insertion mutant population for reverse genetics. LORE1 has several unique characteristics making it particularly suitable for this (Urbanski et al. 2012; Fukai et al. 2012). The element was initially found to be activated by tissue culture; however, it is only transposed in the pollen line. In regenerated plants, these features give rise to seeds with independent patterns of insertions (Fukai et al. 2010). This has paved the way for identification of insertions in genes of interest by a simple sequence search, and together with the annotated genome sequence available, this resource was a quantum leap in legume research and a resource matching the best among model plant systems. The already established studies of primary and secondary metabolism that can be difficult to approach using forward genetics are likely to benefit from this resource (Vriet et al. 2010; Clemente et al. 2012; Perez-Delgado et al. 2013).

1.2 Challenges Ahead

Much has been accomplished, yet there are many challenges for the coming years. The *L. japonicus* genespace has been sequenced and re-sequenced in different ecotypes and *Lotus* species to uncover the biodiversity, and a well-annotated genome has been established as a basis for comparative genome analysis within the genus and the legume family. So far, around 30 *L. japonicus* ecotypes and related species such as *Lotus burttii* have been re-sequenced providing single nucleotide polymorphisms and thereby setting the stage for genome-wide association studies accessing natural variation and biodiversity (Kai et al. 2010; Andersen and Sato, pers.com). Epigenetic regulation is another level of control that can now be addressed on a comparative basis. Further improvements in the annotation are likely to come from participatory genome annotation, and this will be useful for functional analysis in the more complex genomes of crop legumes.

Reverse genetic resources are available and the gene coverage is high. However, inactivation of small genes that by nature have a limited target size could still be improved. Likewise, genetic linkage is also an obstacle for functional analysis of individual members of gene families. Redundancy may shield the effects of inactivation, and because of the linkages, double mutants may be difficult or impossible to obtain by crossing. Gene-specific inactivation procedures based on transcription activator-like effector nucleases (TALEN), Zinc finger nucleases (ZFN) or clustered regulatory interspaced short palindrome repeat-based technologies (CRISPR) could nicely supplement TILLING and LORE1 mutants for studies of small genes and gene families. Studies of miRNAs and other small RNAs that do not lend themselves easily to molecular genetic studies may particularly benefit from these technologies (De Luis et al. 2012). Biochemistry and physiology are the brothers of genetics, and it is now time to bring biochemical and physiological analysis back to centre stage. Molecular genetics is a powerful tool for the identification of central components in processes of interest. However, other approaches are needed for detailed understanding of cellular processes and pathways. It is thus important to advance approaches integrating genetic, biochemical and physiological analyses. Finally, the *L. japonicus* model system with all the resources available and the knowledge generated from analysis of fungal and bacterial endosymbiosis should be in a prime position to contribute to a better understanding of plant–endophyte interactions as well as interactions with microbial populations in the rhizosphere.

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Lotus Cytogenetics

2

Joana Ferreira and Andrea Pedrosa-Harand

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

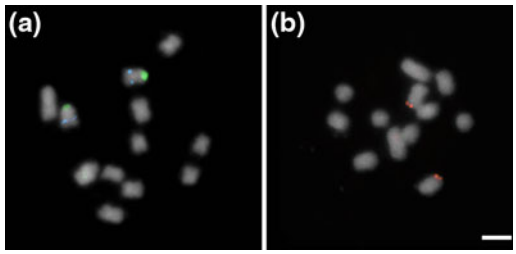

Fig. 2.1 Fluorescent in situ hybridization on mitotic metaphase chromosomes of *Lotus japonicus* 'Gifu.' **a** TAC 28L17/TM0153 (*blue*) is positioned on the opposite chromosome arm of 45S rDNA (green). **b** TAC 15K21/TM0088 (*orange*). Both TACs are located on the second largest chromosome and identify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

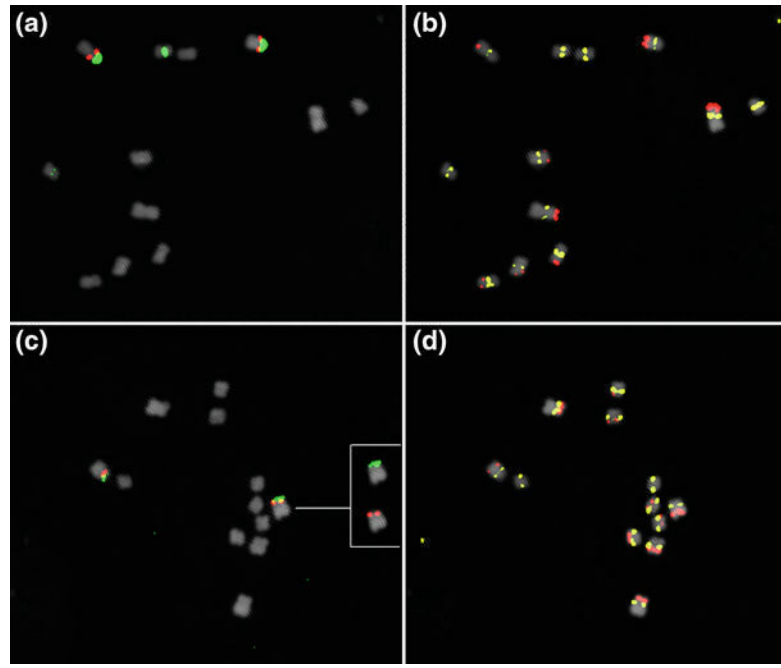

Fig. 2.2 Fluorescent in situ hybridization of repetitive sequences on mitotic metaphase chromosomes of diploids *L. glaber* (**a**, **b**) and *L. krilovii* (**c**, **d**). (**a**, **c**) 45S (*green*) and 5S (*orange*) rDNA, and (**b**, **d**) *Ljcen1* (*yellow*) and LJTR1 (*red*). Chromosomes were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