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Secondary constrictions and NORs of *Lathyrus* investigated by silver staining and *in-situ* hybridization

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A variety of karyotypic differences were seen among six *Lathyrus* species. In part these differences are the result of different numbers of metacentric and acrocentric chromosomes in the complement but there were also clear differences in the number(s) and position(s) of secondary constrictions. Silver staining and in-situ hybridization were used to identify the nucleolar organizer regions (NORs). Silver staining also occurred at the centromere in most species and in addition at a number of non-centromeric sites in *L. tingitanus*. Some of these additional sites coincide with allocyclic regions revealed by the use of air-dried preparations. Unusual 'extended' centromeres were observed in these air-dried preparations of two of the species.

Keywords: *in-situ*, hybridization, karyotypes, *Lathyrus*, silver staining.

Introduction

The importance of karyotype studies to plant systematics has been stressed by many authors (e.g. Stebbins, 1950; Jackson, 1971), and a knowledge of karyotypes is also an important adjunct to any programme of interspecific or wide hybridization. The Leguminosae is a family of major economic importance (Heywood, 1978) and it is a priority family for cytogenetic and other research at the Royal Botanic Gardens, Kew. We are also involved in the development of interspecific hybrids for the introduction of new genes into the sweet pea, *Lathyrus odoratus* (Murray & Hammett, 1989).

As plants often have highly symmetrical karyotypes where many of the chromosomes are of very similar morphology, it is often necessary to use cytological markers to aid indentification and comparison. The nucleolar organizer region (NOR) and the constriction that it forms along the chromosome arm, known as a secondary constriction (Darlington, 1926), make it one of the more useful markers for karyotype analysis. It is clear, however, that not all secondary constrictions are the sites of NORs or that all NORs form obvious constrictions in the chromosome arm. The development of techniques such as silver staining and in-situ hybridization with rRNA gene probes has helped to clarify the situation. For example, in a group of Australian frogs, King *et al.* (1990) showed that in some of these species, which can have five secondary constrictions, not all are the sites of rRNA genes. These methods were also used to show that NOR regions appear to be highly mobile (Schubert & Wobus, 1985) and recently to demonstrate additional sites of rRNA genes even in a species as well known as *Triticum aestivum* (Mukai *et al.*, 1991).

Several hundred papers have described chromosome number and karyotype in a significant proportion of the species of the genus *Lathyrus*. Despite statements such as those of Narayan & Durrant (1983) and Lavania & Sharma (1980) that the genus is karyotypically uniform, these studies have often produced a variety of conflicting observations. In some cases this may be due to taxonomic confusion, use of different techniques or tissue at different stages of development. However, in well known species such as *L. odoratus*, (the sweet pea) and *L. sativus*, a huge range of different karyotypes have been described. Often these are reported to differ in the number and position of secondary constrictions (compare Bhattacharjee, 1954; Sharma & Datta, 1959; Roy & Singh, 1967; Fouzdar & Tandon, 1975; Verma & Ohri, 1979; Nazeer *et al.*, 1982). In *L. odoratus*, Sharma & Datta (1959) describe cultivars with as many as eight second-

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ary constrictions whereas Fouzdar & Tandon (1975) reported some with none at all. *Lathyrus* therefore appears to be a good candidate for applying techniques such as silver staining and in-situ hybridization with rRNA gene probes and we have done this for a sample of six species from *Lathyrus* section *Lathyrus*.

Materials and methods

The species used in this study and their origin are listed in Table 1; all are diploids with $2n = 2x = 14$. In most cases only a single accession was grown as we have found very limited evidence of intraspecific karyotype variation despite extensive studies in several *Lathyrus* species (Murray *et al.*, 1992 and unpublished). The two accessions of *L. blepharicarpus* are an exception as they were found to differ in the structure of their NOR regions. Duplicate herbarium specimens are deposited in the herbaria of the University of Auckland, New Zealand (AKU) and Royal Botanic Gardens, Kew (K).

Mitotic chromosomes were observed either in conventional squash preparations or more frequently following the air-drying technique of Geber & Schweizer (1988). Air-dried preparations for silver staining were incubated in 50 per cent aqueous silver nitrate at 60°C for 1.5–2 h, rinsed in 1 per cent acetic acid for 15 min, dried and mounted in immersion oil. The rRNA gene probe used for in-situ hybridization contains a single wheat 18S.26S rRNA gene repeat unit (8.9 kb) which originated from plasmid pTa71 (Gerlach & Bedbrook, 1979). Probe DNA was labelled by nick translation using the BioNick labelling system (BRL Life Technologies Inc.) as recommended by the manufacturer. Slides were treated with RNase (100 μg ml^{-1} in 2 × SSC) then dehydrated in an ethanol series and air-dried. They were then denatured in 70 per cent formamide + 30 per cent 2 × SSC for 2 min at 72°C followed by dehydration in an ethanol series at 0°C. The hybridization mixture consisted of 50 per cent formamide, 20 per cent dextran sulphate and 20 per cent biotinylated probe DNA (200 ng DNA per slide) in Tris EDTA, pH 8, which was denatured for 20 min at 80°C followed by the addition of 10 per cent 20 × SSC. The probe was cooled for 2 min on ice prior to adding it to the slides. The slides were then incubated for 18–20 h at 37°C in a humid chamber. After hybridization the slides were washed in 2 × SSC for 10 min at 37°C, then 50 per cent formamide + 50 per cent 2 × SSC for 10 min at 37°C and again in 2 × SSC for 10 min at room temperature. Sites of in-situ hybridization were detected by the alkaline phosphatase method using the BRL DNA detection system and following the manufacturers instructions. Slides were counterstained with 1 per cent acetocarmine and photographed with Pan F film.

Results

The karyotypes

There are similarities between the karyotypes of the six species but they do differ in the number of metacentric, sub-metacentric and acrocentric chromosomes, and in the number(s) and position(s) of the secondary constrictions. *L. odoratus*, *L. hirsutus* and *L. cassius* were very similar with a large and a small sub-metacentric pair and five pairs of acrocentrics. In *L. odoratus* and *L. hirsutus* the largest of the acrocentrics had a secondary constriction very close to the telomere of the short arm but this was not visible in *L. cassius* (Fig. 1a and b). *L. blepharicarpus* also had a secondary constriction on a large acrocentric chromosome but this delimited a larger and more distinct satellite. The two accessions of this species differed in the structure of their secondary

Table 1 Species of *Lathyrus* used in this study

Species	Origin
L. blepharicarpus Boiss.	Syria ex. ICARDA, IFLA 380
	Syria ex. ICARDA, IFLA 793
L. cassius Boiss.	Syria ex. ICARDA, IFLA 603
L. hirsutus L.	Egypt ex. Institut für Genetik und Kulturpflanzenzuchtung, Gatersleben, Germany, LAT 144/75
L. odoratus L.	Commercial cv. Apricot Queen and unnamed cv. ex. R. B. G. Kew 000-69-10215
L. sativus L.	Cyprus ex. Agricultural Research Institute, Nicosia, Cyprus, ARI 00262
L. tingitanus L.	Warkworth, Northland, New Zealand

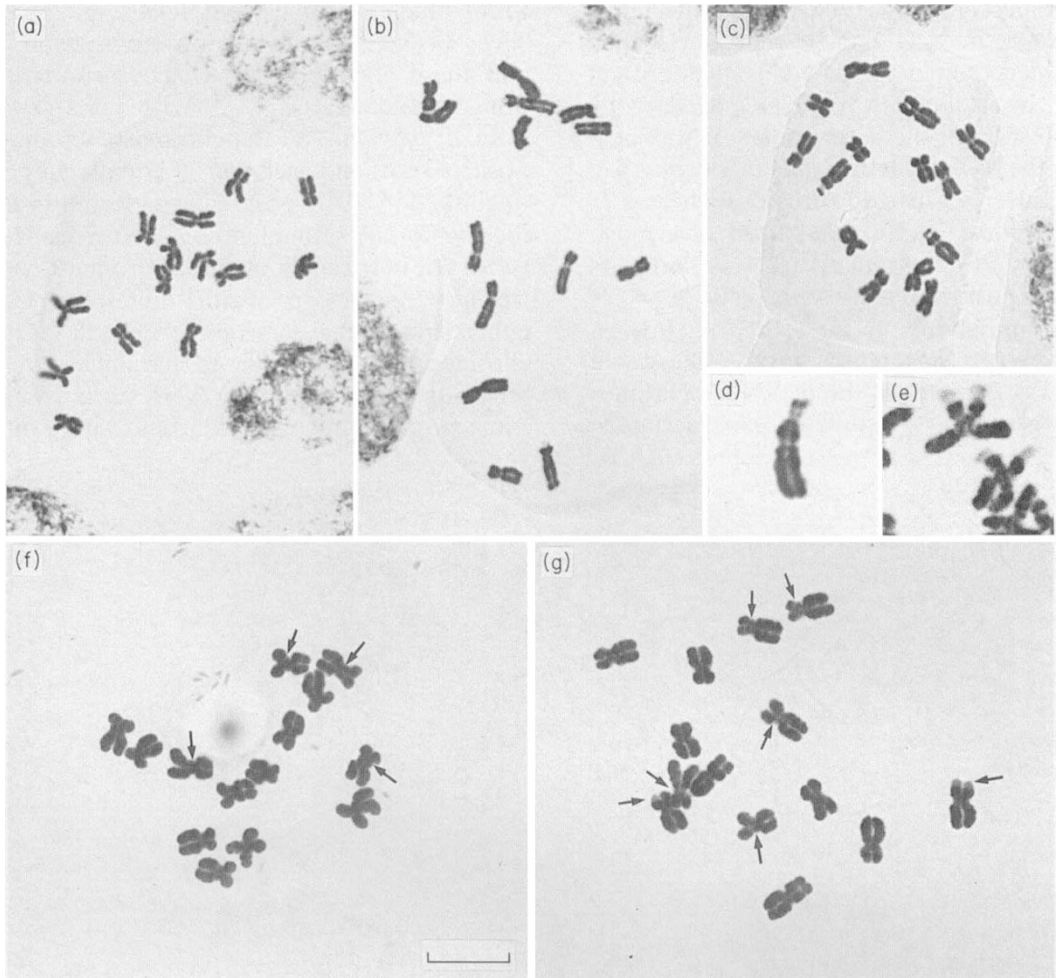

Fig. 1 Karyotypes of *Lathyrus* species, a–d prepared from orcein squashes, e–g from air-dried preparations. (a) *L. hirsutus*, (b) *L. cassius*, (c) *L. blepharicarpus*, (d) NOR chromosome of *L. blepharicarpus* showing the tandem duplication, (e) enlargement of the pair of allocyclic chromosomes in *L. tingitanus* (f) *L. tingitanus*, arrows showing allocyclic segment and 'extended' centromeres, (g) *L. sativus* with some of the 'extended' centromeres arrowed. Scale = 10 μm (a, b, c, f and g) and = 5 μm (d and e).

construction with one showing what appeared to be a tandem duplication of all, or part, of the structure (Fig. 1c and d). In general the chromosomes of *L. blepharicarpus* were more symmetric than those of *L. odoratus* as it had one small metacentric pair and three pairs of sub-metacentrics. In *L. tingitanus* the secondary constriction was on a metacentric chromosome and the rest of the complement was sub-metacentric or acrocentric. In one of the sub-metacentric pairs the terminal third of the short arm was allocyclic and less densely staining than the rest of the chromosome (Fig. 1e and f) and in several others pairs the centromere region appeared 'extended' (Fig. 1f). Similar 'extended' centromeres were seen even more clearly in three pairs of chromosomes in *L. sativus* (Fig. 1g). This species also had secondary constrictions in a metacentric pair and also in the long arm of an acrocentric pair (Fig. 1g). As in *L. tingitanus*, this acrocentric pair also had a lightly straining region at the terminal third of its short arm. Because of the very clear expression of the 'extended' centromeres it is difficult to decide whether or not some of the chromosomes in *L. sativus* are metacentric.

Silver staining of NORs

Several interesting observations emerged from the use of silver staining to identify NORs. In many instances, in addition to staining at the secondary constrictions, there was staining at or near the centromere. This is particularly clear in the two cells of *L. odoratus* shown in Fig. 2a and b. Figure 2a shows four well-stained NORs at the ends of the short arms of two acrocentric pairs and faint staining of the centromeres of several

other chromosomes and Fig. 2b shows only lightly staining NORs but densely stained centromers. The stained centromeres often appeared as two distinct dots. However, in all species except *L. tingitanus*, by knowing the position of the centromeres, it was possible to identify the NORs clearly. *L. blepharicarpus* had a single pair of silver positive constrictions whereas *L. cassius* and *L. hirsutus* had a single pair of silver positive terminal spots. In *L. odoratus* there were two pairs of terminal, silver positive regions at the ends of two of the largest acrocentric pairs. In the F_1 hybrid between the two accessions of *L. blepharicarpus* the intensity of silver staining was different in the two NOR chromosomes, the larger spot presumably corresponding to the tandemly duplicated NOR (Fig. 2c). Only three silver positive constrictions were seen in *L. sativus* and these corresponded to the secondary constrictions (Fig. 2d).

In *L. tingitanus* all the chromosomes showed silver positive spots and each pair of homologues can be distinguished by its silver staining pattern (Fig. 2e). Unlike the *L. odoratus* example in Fig. 2b these silver positive spots are not usually at the centromere and in some chromosomes a pair of dots quite clearly existed on either side of the centromere (Fig. 2e). One pair of chromosomes had a large terminal silver positive region but in others there were silver spots in interstitial positions along the chromosome arms. One of

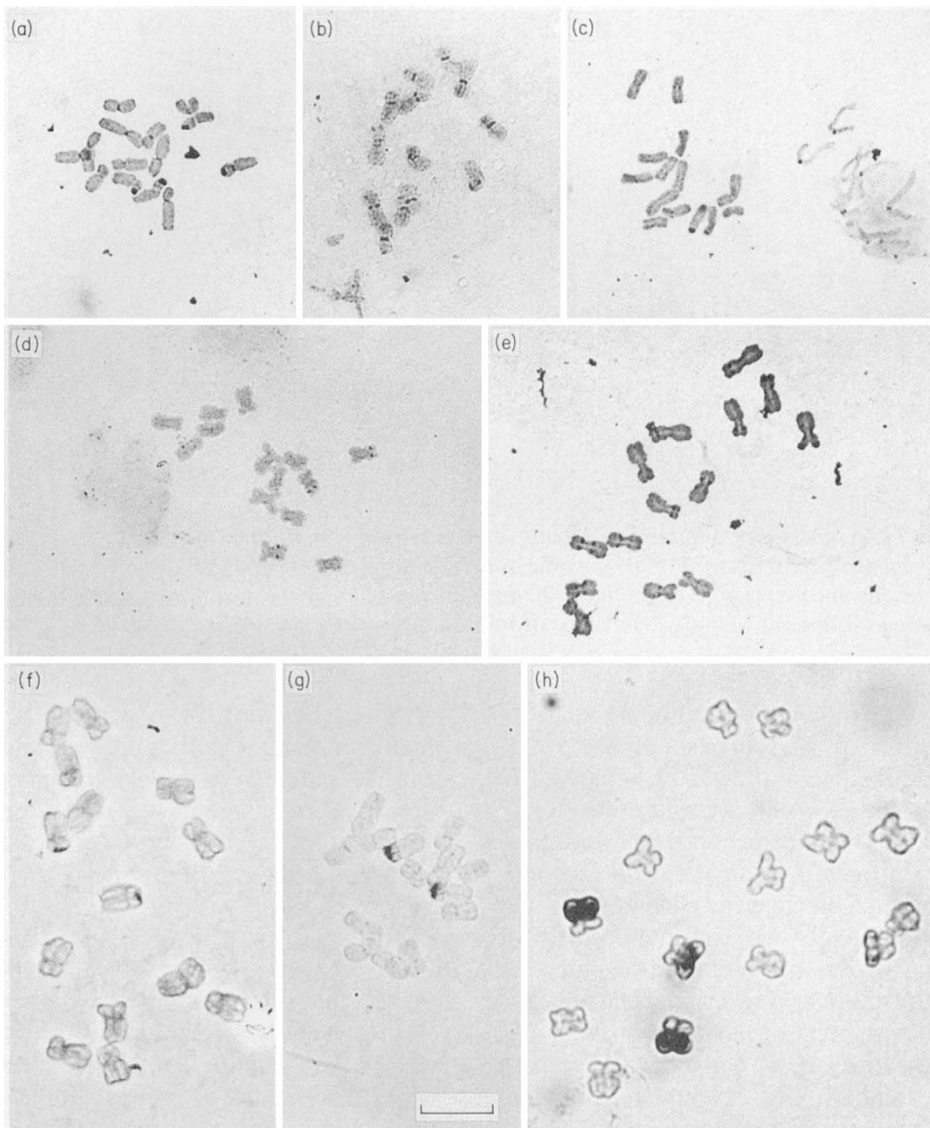

Fig. 2 Silver-stained chromosomes of (a and b) *L. odoratus*, (c) *L. blepharicarpus*, (d) *L. sativus*, and (e) *L. tingitanus* and in-situ hybridization of the probe pTa71 on the chromosomes of (f) *L. cassius*, (g) *L. blepharicarpus*, and (h) *L. sativus*. Scale = 10 μm.

these interstitial spots corresponds to the secondary constriction and the large terminal spot corresponds to the less densely staining chromosome region described above.

In-situ hybridization

The six species can be divided into two groups depending on whether they have two or four sites of in-situ hybridization. *L. blepharicarpus*, *L. cassius* and *L. hirsutus* all had two in-situ hybridization sites and these correspond to the silver positive sites and the secondary constrictions when they are present (Fig. 2f and g). *L. tingitanus* also had a pair of hybridization sites which corresponded to the secondary constriction and one of its many silver positive sites. There was no evidence of hybridization at the ends of any of the chromosomes and, therefore, no in-situ site that corresponded to the prominent silver positive region on the largest acrocentric pair. In *L. odoratus* the four terminal hybridization sites correspond to the silver positive sites whereas *L. sativus*, which had only three silver positive sites, showed four sites of in-situ hybridization (Fig. 2h). In both *L. sativus* and *L. odoratus* two of the sites of hybridization were clearly larger than the other two.

Discussion

The karyotypes of these six *Lathyrus* species differ in several respects. They have different numbers of metacentrics and acrocentrics, different numbers of constrictions on different chromosomes and in some of the species there are differences in the staining intensity of parts of the chromosomes and in the nature of their centromeres. Even if these last two features are ignored, this very limited sampling of the karyotypes of *Lathyrus* shows that they are not uniform. Additional evidence to support this statement comes from our recent study of two other *Lathyrus* species (Murray & Hammett, 1989) which have yet another clearly different karyotype. Consequently, at this level of detail we disagree with the statements of Lavania & Sharma (1980) and Narayan & Durrant (1983), that the genus is karyotypically uniform.

The use of both silver staining and in-situ hybridization has greatly improved the identification of the NOR regions in many plants and shows that these occur in varying numbers and positions in different species. However, in our material the results obtained with the two techniques are not always in agreement due to the silver staining of centromeric and allocyclic regions of the air-dried chromosomes. For *L. odoratus*, our results with in-situ hybridization confirm the report of Nazeer *et al.* (1982), that this species has two pairs of NOR chromosomes but in *L. sativus* we could only confirm the existence of two pairs of NOR chromosomes, as found by Verma & Ohri (1979), rather than the three reported by Bhattacharjee (1954). Interestingly, in our material of *L. sativus*, only three of the four sites of in-situ hybridization show silver staining. When this is taken in conjunction with the observations on silver staining in some of the other species, it is clear that silver staining is not necessarily as reliable as in-situ hybridization for locating NORs. Clearly some caution is needed when interpreting the results of silver staining. Where it is the centromeres that are the additional silver staining sites, as in the illustrated example of *L. odoratus*, ambiguity may be easily resolved, but in *L. tingitanus* the situation is less clear. The extensive silver staining in this species occurs at a variety of sites, only some of which are near the centromeres whereas others are associated with chromosome regions that have different orcein staining properties.

In this study, unlike previous ones in *Lathyrus*, we have used the air-drying technique of Geber & Schweizer (1988) rather than the more conventional squash one. This has revealed a number of interesting features of the karyotype, such as the allocycly of parts of some chromosome arms and 'extended' centromeres in *L. sativus* and *L. tingitanus*, and the silver staining of chromosome regions other than the NORs. As Geber & Schweizer (1988) point out, chromosomes prepared by the air-drying method are free from overlying cell wall material and cytoplasm and they are also larger than those prepared by the squash method.

There are other reported examples of silver-stained centromeres or kinetochores in the literature. Sudman & Greenbaum (1989) showed in several mammalian species that kinetochores are silver positive at the meiotic divisions but not at mitosis. They suggested that this indicates that there are fundamental structural differences between mitotic and meiotic kinetochores, possibly related to different protein associations. They also give several examples where pretreatment of mitotic chromosomes with basic solutions such as NaOH and phosphate buffer, results in the silver staining of kinetochores and suggest that the staining results from the removal or alteration of some of the proteins associated with the mitotic kinetochore. Apparently the air-drying method used here makes previously inaccessible regions of the chromosome available for silver staining. Presumably the non-NOR regions that stain with silver are associated with similar proteins or structures to those found at the NOR region. Perhaps the silver staining comes about as a consequence of differential chromosome condensation, since in *L. tingitanus* one example of it is associated with the allocyclic ends of one pair of

homologues. There is evidence to suggest that chromosome decondensation is not the only precondition required for silver staining (Jimenez *et al.*, 1988) and in the case of NORs, transcriptional activity in the previous interphase is another possible prerequisite. However, Haaf *et al*, (1984) cast doubt on the idea that transcriptional activity is important or that decondensation *per se* also results in silver staining. They treated chromosomes at interphase with AT-binding ligands such as distamycin A and DAPI, which then show large undercondensed regions that are Ag+ at the following metaphase but the heterochromatic regions undercondensed with the cytidine analogue 5-azacytidine were Ag−. They concluded that the silver staining proteins are acidic (non-histone) proteins that are associated with and may regulate the decondensation of some types of heterochromatin.

The nature of the 'extended' centromeres in *L. sativus* and *L. tingitanus* remains unclear. These were not mentioned in other studies on these species, although Verma & Ohri (1979), Lavania & Sharma (1980) and Narayan & Durrant (1983) all showed that there is an extensive band of heterochromatin associated with the centromere of some or all chromosomes in these species. Again it would appear that the air-drying technique has revealed a new aspect of the chromsome structure of *L. tingitanus* and *L. sativus* and further studies are underway to investigate the nature and distribution of heterochromatin in these species. It is also possible that these species have complex centromeres with tandemly duplicated kinetochores and these could then appear as the pairs of silver staining dots on either side of the centromeric constriction, as illustrated in *L. tingitanus*.

Clearly it is important not to ascribe functions to constrictions unless these are known and in the description of karyotypes it would be better to call constrictions, other than the centromere, secondary constrictions unless it is clearly demonstrated that they are the NORs.

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