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P. Chène, M. Bourge & R. Verlaque

Study of the *Digitalis* genus 5: the species *Digitalis lutea*

Abstract

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The chromosome number of species belonging to the genus *Digitalis* L. is mainly 2*n* = 56, except for *D. lutea* L. In the latter, distinct counts and ploidy levels have been reported: 2*n* = 48-56 and 2*n* = 96-112. Depending on the authors, this taxon is treated either as a single species with two subspecies: *D. lutea* subsp. *lutea* and subsp. *australis* (Ten.) Arcang., or as two distinct species in Italy: *D. lutea* and *D. micrantha* Schweigg. However, in S. France and Corsica, classical discriminant characters of these taxa seem less reliable than in Italy. In addition, a phylogenetic study sets plants of subsp. australis from Tuscany and Corsica in two distinct clades. To clarify this situation, flow cytometry, karyological, morphological and pollen studies were carried out on a large sample from different geographical sources. Results confirmed the validity of the two infra-specific taxa, which differ in their ploidy levels (*D. lutea* subsp. *lutea* 2*n* = 112; *D. lutea* subsp. *australis* 2*n* = 56), 2C DNA content and macro- and micro-morphological traits.

Key words: *Plantaginaceae*, flow cytometry, chromosome number, morphology, pollen.

Introduction

In the enlarged *Plantaginaceae* Juss. (Olmstead & al. 2001), the small tribe *Digitalideae* (two genera) occupies a marginal position on the basis of its genetics, phytochemistry and morphology (Albach & al. 2005). In addition, we might mention very rare karyological characters for the family, such as the basic chromosome number *x* = 7 and strong polyploidy in the genus *Digitalis*. Among the 20-25 species of this genus, *D. lutea* L. is the only one that presents 2*n* = 56, 112. According to the morphological systematics, it belongs to the section *Tubiflorae* Benth., characterized by cylindrical small corollas: 8-25 mm (Werner 1960; Heywood 1972; Luckner & Wichtl 2000). However, recent genetic studies rejected this polyphyletic section, including *D. atlantica* Pomel (Algeria), *D. lutea* (W Europe) and *D. viridiflora* Lindl. (Balkans) in the section *Macranthae* Heyw. (clade I), while the systematic position of other species remains unsolved in clade II (Bräuchler & al. 2004; Herl & al. 2008).

In Italy, two related taxa were historically recognized as separate species *D. lutea* L. and *D. micrantha* Roth ex Schweigg. (Béguinot 1902; Fiori & al. 1905; Pignatti 1982), or as

two different subspecies, *D. lutea* subsp. *lutea* and *D. lutea* subsp. *australis* (Ten.) Arcang. (Werner 1960; Heywood 1972; Zangheri 1976). According to the literature, they possess distinct morphological features and geographical ranges: *D. lutea* subsp. *lutea* with long corollas (15-25 mm), in northern latitudes (N-E. Spain, continental France, N. Italy to Germany), and *D. lutea* subsp. *australis* with smaller corollas (9-15 mm), in the Apennines and Corsica (Werner 1960; Heywood 1972; Pignatti 1982; Luckner & Wichtl 2000). This distinctive character, useful to identify Italian plants, seems less relevant in S. France and Corsica, where flowers tend to be smaller: <16mm (Litardière 1928; Gamisans & Jeanmonod 2007; Tison & Foucault 2014; Tison & al. 2014). This may explain why old French floras only indicate *D. lutea* (without mention of subspecies). In addition, a genetic study has shown that both *D. lutea* subsp. *australis* from Corsica and *D. lutea* subsp. *lutea* from continental France belong to clade I, while *D. lutea* subsp. *australis* from Tuscany belongs to clade II (Bräuchler & al. 2004).

The frequent unknown origin (perhaps botanic gardens) of some plants used for karyological studies makes data on chromosome accounts of *D. lutea* quite confusing. Under the name *D. micrantha* Schrad., the subsp. *australis* was counted as $2n = 48$ (Haase-Bessell 1921) and $2n = 56$ from Italy (Campania: Larsen 1955; Ombria: Pedrotti & Cortini Pedrotti 1971). All other reports as "*D. lutea"* are $2n = 56$ in Spain (Angulo Carpio 1957; Olgun 1979) and Italy (Tuscany: Löve & Löve 1982), but also: $2n = 96$ (Haase-Bessell 1921; Delay 1947) and $2n = 112$ (Michaelis 1931; Buxton & Dark 1934) of unknown origin.

The aim of this study is to assess the validity of infra-specific taxa of *D. lutea* in Italy and France, using flow cytometry, karyological, morphological, pollen and epidermal data.

Materials and methods

Sampling, culture and observations

The seeds used in this study were collected at stations geographically isolated from each other, in France and Italy (Table 1). Seeds were sown in standard garden compost and allowed to germinate during 2-3 weeks, at 15-18°C, under constant light period (9 h/day, 2×30 W neon lights; Solar Natur T8, 9000K), and with regular watering. Each seedling was transferred into an individual pot for 2-3 months, then planted in a garden (Alsace, France), under the same culture conditions in order to avoid any morphological variations linked to environmental parameters.

Morphological studies of plants of different origins, *D. lutea* subsp. *lutea* from France (Ardennes) and Italy (Piedmont) and *D. lutea* subsp. *australis* from Italy (Apennine) and Corsica, were carried out in the middle part of inflorescences (5 individuals/site, and, for each parameter, 6 to 10 measurements and observations/plant). For the epidermis, dried cauline leaves (two per locality) were hydrated for 2 h in hot water; fragments of the lower epidermis were sampled with tweezers, stained with iodine green, and then mounted in glycerol between slide and cover-slide. On each slide, more than 30 stomata were measured; glandular hairs and wall cells were drawn.

Alexander's stain was used to distinguish between abnormal (blue-green coloured) and normal (magenta-red) pollen grains. Aperture numbers and polar axis sizes of mature grains were established (30 measures/sample) to assess the relationships between ploidy

level and cell size. Epidermis and pollen samples were observed by light microscopy (15×50, Leitz Dialux 20).

Flow cytometry

The total nuclear DNA amount was assessed by flow cytometry according to Marie & Brown (1993). *Lycopersicon esculentum* cv “Montfavet” (2C = 1.99 pg) or *Petunia hybrida* PxPc6 (2C = 2.85 pg) were used as an internal standard. Leaves of the internal standard and *Digitalis* were chopped using a razor blade in a plastic Petri dish with 1 ml of Gif nuclei-isolation buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 60 mM MOPS, 1% (w/v) polyvinylpyrrolidone 10,000, pH 7.2), containing 0.1% (w/v) Triton X–100, supplemented with 5 mM sodium metabisulphite and RNAse (2.5 U/ml). The suspension was filtered through 50 µm nylon mesh. The nuclei were stained with 50 µg/ml propidium iodide (a specific DNA fluorochrome intercalating dye), and kept for 5 min at 4°C. DNA content of 5,000–10,000 stained nuclei was determined for each sample using a cytometer (CyFlow SL3, Partec-Sysmex. Excitation 532 nm, 30 mW; emission through a 630/30 nm band-pass filter). The total 2C DNA value was calculated using the linear relationship between the fluorescent signals from the stained nuclei of *Digitalis* taxa and the internal standard. The mean value was calculated from measurements of samples comprising 6 to 14 individuals, according to populations. Statistical analysis was carried out by Mann-Whitney Rank Sum Test (SigmaPlot v. 11.0).

Chromosome numbers

As the 2C-DNA content is not always correlated with chromosome numbers (Suda & al. 2006), chromosome counts were also performed. For the two subspecies of *D. lutea* (Table 1), seedlings were obtained from seeds germinating in Petri dishes. After a cold pretreatment at 4°C for 24 h, root tips were fixed with an ethanol-acetic acid solution (4:1, v/v), kept at room temperature for two weeks, and then stored at -18°C until used. Seedlings were stained in 45% aceto-carmine-ferriacetate, boiled for 3 min, and then squashed between slide and cover-slide. Five seedlings per locality were observed by light microscopy (15×100, Leitz Dialux 20) and the best mitotic metaphases were drawn using a camera lucida.

Results and Discussion

Flow cytometry analysis reveals that in each subspecies, all plants present similar 2C DNA content, regardless of their geographical origin: coefficient of variation (standard deviation/mean) < 0.5 for both taxa (Table 1). In addition, subsp. *australis* and subsp. *lutea* significantly differ in their DNA content ($p<0.001$), with an average of 3.12 ± 0.13 pg (n = 42) and 5.23 ± 0.16 pg (n = 58), respectively. These data present a high taxonomic value, because closely related taxa of clade I (Bräuchler & al. 2004), with $2n = 56$, possess a constant but very distinct genome size from each other: *D. mariana* Boiss. 1.12 pg, *D. purpurea* L. 1.87 pg and *D. thapsi* L. 2.08 pg (Castro & al. 2012). To our knowledge, no 2C DNA value has been reported for clade II taxa.

Table 1. Geographical origin, 2C DNA content and chromosome numbers of *D. lutea* L. studied.
[1] Average followed by standard deviation; (n): number of plants studied. [2] Seeds from a commercial source.

subsp.	**Origin**	**2C DNA content (pg)**[1]	**Chromosome counts**
australis	France: Corsica, San Nicolao	3.08 ± 0.13 (n = 14)	2*n* = 56
	Italy: Campania, Roccamonfina	3.07 ± 0.09 (n = 12)	2*n* = 56
	Italy: Abruzzo (Sant'Eufemia)	3.23 ± 0.05 (n = 6)	
	Italy: Apennine[2]	3.16 ± 0.14 (n = 10)	
lutea	France: Ardennes, Vendresse	5.32 ± 0.18 (n = 8)	
	France: Vosges, Wattwiller	5.35 ± 0.21 (n = 10)	2*n* = 112
	France: Alps, Gières	5.11 ± 0.09 (n = 12)	
	France: Massif-Central, Cussac sur Loire	5.14 ± 0.06 (n = 8)	
	France: Pyrénées, Melles	5.19 ± 0.12 (n = 7)	
	France: Provence, Massif de Ste Baume and N-D. du Figuier		2*n* = 112 2*n* = 112
	Italy: Liguria, Val Nervia	5.29 ± 0.07 (n = 7)	2*n* = 112
	Italy: Piedmont, Monte Leco	5.25 ± 0.25 (n = 6)	

The karyological study of *D. lutea* was rather difficult, owing to the numerous, small and often agglutinated chromosomes. This may explain some differences in published numbers (48 *vs* 56, and 96 *vs* 112). Nevertheless, in good metaphases we always found: 2*n* = 56 (L = 1.2 to 2.2 µm) in *D. lutea* subsp. *australis* and twice that number in *D. lutea* subsp. *lutea* 2*n* = 112 (L = 0.9 to 1.8 µm). Thus, chromosome counts show two ploidy levels in *D. lutea*, each of them corresponding to one subspecies, as suggested by cytometric data. It should be noted that 2C DNA values of subsp. *lutea* are only 1.7 fold higher than those of subsp. *australis*. Similar results have been found in other genera (*e.g.* Fridlender & al. 2002), and depending on the techniques used. As previously reported for many taxa (Favarger 1967), these two cytotypes show a clear geographical structuring: subsp. *australis* in the southern area (Corsica and Apennines), and the higher ploidy level, subsp. *lutea,* widespread in the north. These distinct chromosome numbers may explain the sterility of natural hybrids between the latter (2*n* = 112) and some European species with 2*n* = 56 (Tison & Foucault 2014), but also with subsp. *australis* in Tuscany (Fiori 1925).

Our results confirm and complete previous chromosome counts, with the exception of 2*n* = 56 for *D. lutea* from Spain (Angulo Carpio 1957; Olgun 1979). The plants cultivated by Olgun, in the botanical garden of Istanbul university, came from 'Spain-France, Pyrenees'. However, our individuals which also originated from the Pyrenees have the same 2C DNA content as all our samples of subsp. *lutea* with 2*n* = 112 (Table 1), and the description of Spanish plants (Benedi & Hinz 2009) corresponds exactly to this subsp. Two hypotheses can be proposed, either rare populations of subsp. *australis* persist in N Spain (S-W limit of the species), or the accounts of Angulo Carpio (1957) and Olgun (1979) refer to the closely related NW Spain endemic: *D. parviflora* Jacq. (2*n* = 56).

Pollen grains of *D. lutea* subsp. *lutea* differ from those of subsp. *australis* by having higher polar axis, aperture number (3-4 *vs* 3) and anomaly rate (Table 2). An increase in these parameters is usually linked with higher ploidy level (Fukushima & Shoichi 1964;

Table 2. Distinctive characters between the two subspecies of *Digitalis lutea*.

	subsp. *lutea*	subsp. *australis*
Raceme	Densely flowered Unilateral tendency	Very densely flowered Multilateral tendency
Sepals	Non-overlapping, without scarious margin	Overlapping at their lower part, with narrow scarious margin
Corolla : length diameter	 20.8 ± 1.3 mm 5.7 ± 0.5 mm	 15.5 ± 1 mm 4.2 ± 0.4 mm
Corolla shape in posterior view from base to top	Diameter increases near 1/3 of the length, and remains the same until lobes	Diameter increases near 1/2 of the length, and decreases below the lobes
Corolla ornamentation	No ornamentation observed	Red macula present at the junction between the lobes
Fruit	Ovoid generally	Sub-globular generally
Pollen grains : polar axis length aperture number abnormal pollen	 24 ± 2 µm 3-4 30-55%	 19.8 ± 1.1 µm 3 <10%
Length of stomata	37.5 ± 2.5 µm	26.9 ± 2 µm

Joshi & Raghuvanshi 1966). The slight difference between polar axis lengths suggests that both taxa are high polyploids, probably 16x for subsp. *lutea* and 8x for subsp. *australis*. In the genus *Digitalis,* leaf epidermal structures possess great taxonomic value (Serrano & al. 2014). Regardless of their origin, all plants studied share similar characters: scarce glandular hairs (short with a unicellular stalk and a uni- or bicellular head) and sinuous cell walls (*vs* nearly straight in clade II). However, as expected, they significantly differ by their stomata lengths, *D. lutea* subsp. *lutea* (37.5 ± 2.5 µm) and subsp. *australis* (26.9 ± 2 µm), which are correlated with ploidy levels (Bidault 1971).

Morphological study of numerous plants, of various origins and cultivated under the same conditions, highlights distinctive characters between subsp. *lutea* and subsp. *australis* (Table 2). Most of them have already been described in monographs (Béguinot 1902; Werner 1960 Luckner 2000) and floras (*e.g.* Fiori 1925; Heywood 1972; Pignatti 1982), confirming their discriminant value. We also found new quantitative and qualitative characters – shape of the corolla (in posterior view, Fig. 1), epidermis and pollen - that could be used to identify these two taxa. On the other hand, the strong morphological likeness between Corsican subsp. *australis* and French subsp. *lutea*, and the high rate of abnormal pollen on the mainland, may suggest an autopolyploid origin of subsp. *lutea* from Corsican plants (while isolated Apennine populations could have derivated).

In conclusion, our biosystematics study of *D. lutea* shows that subsp. *lutea* and subsp. *australis* are two distinct taxa that mainly differ by their ploidy levels. Our data also indicate that Corsican and Apennine populations possess certain similar taxonomic traits (chromosome number, 2C DNA content, pollen, epidermis and morphology), suggesting that they belong to the same taxon: subsp. *australis.* This result is not in agreement with the first genetic tree setting Corsican and Apennine plants of *D. lutea* subsp. *australis* in two different clades (Bräuchler & al. 2004). In a second study, using a new molecular maker with the

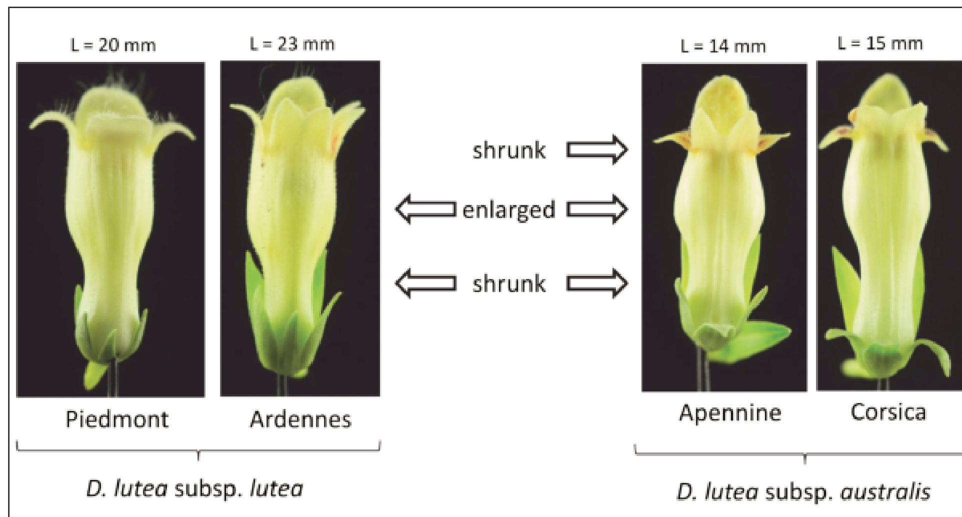

Fig. 1. Photographs of corolla, in posterior view, of *D. lutea* subsp. *lutea* and subsp. *australis,* of different geographical origins. Differences in the shape of the corolla are highlighted by arrows. Lengths (in mm) of the corolla are indicated.

same GenBank accessions, some taxa (such as *D. lutea* subsp. *australis* and *D. atlantica* Pomel) were not included (Herl & al. 2008), making any comparison or confirmation impossible. It should be noticed that the phylogeny of polyploids is often very difficult to assess (Mansion & al. 2005). Thus, the systematics of *D. lutea* needs further genetic investigation, using clearly identified plants, collected outside hybridization zones (such as Tuscany, where subsp. *australis* may produce hybrids with subsp. *lutea* and with *D. ferruginea* L. of clade II), and with more than one individual (herbarium exsiccata) per taxon.

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Addresses of the authors:
Patrick Chène[1], Mickael Bourge[2] & Régine Verlaque[3]
[1]24, rue des carrières F-68100 Mulhouse. E-Mail: patrick_chene@yahoo.com
[2]Institute of Integrative Biology of the Cell (I2BC), CEA, CNRS, Univ. Paris-Sud, Université Paris-Saclay, 91198, Gif-sur-Yvette, France.
[3]Université d'Aix-Marseille, Institut Méditerranéen de Biodiversité et Ecologie, IMBE CNRS UMR 7263, 13331 Marseille cedex 03, France.