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Giemsa staining and fluorescent chromosome banding in some *Vitis* L. species

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Abstract — Giemsa staining technique and fluorescent chromosome banding with CMA_3 and DAPI were applied to the mitotic chromosomes of *Vitis champinii*, *V. cinerea*, *V. girdiana*, *V. labrusca*, *V. rotundifolia*, *V. rupestris* and *V. vinifera* for the purpose of chromosome measurement and constitutive heterochromatin characterization at the cytochemical level, respectively. Both fluorescent CMA and conventional Giemsa staining constituted a valuable tool for chromosome characterization. Karyomorphometric data obtained after Giemsa staining allowed for an average ideogram and karyotype formulae based on chromosome length for the species. *V. champinii* and *V. girdiana* distinguished from the other five species by means of the total haploid chromosome length, by the longest chromosome length and by the average chromosome length. The seven grape species have moderate chromosome asymmetry values and were classified on 2A Stebbins' category. Positive CMA bands were seen in all species. *V. girdiana* distinguished from the other species solely by the presence of two CMA^+ bands, while *V. champinii*, *V. cinerea*, *V. labrusca*, *V. rotundifolia* and *V. rupestris* had four bands. *V. vinifera* showed chromosome heteromorphism for CMA bands. No clearly visible $DAPI^+$ band was seen in the species. According to the present observations, it seems that the evolutionary process of speciation involving North American and European *Euvitis* species studied, resulted in some discrete changes in chromosome measurements and also in heterochromatin base composition of at least one species. These data enlarge the chromosomal information of the genus *Vitis* and make possible further comparative studies into the Vitaceae family.

Key words: Fluorescent banding, karyotype, mitotic chromosomes, Vitaceae, *Vitis*.

INTRODUCTION

Vitis L. (Vitaceae) is an economical important genus of wide geographical distribution over lands of the North Hemisphere (North American, European and Asiatic groups). The southeast region of North America is especially rich in wild *Vitis* species (Olmo 1979) while Central America and the north of South America present few native *Vitis* species. The Old World *V. vinifera* is undoubtedly the most important species and its ancient culture has given rise to thousands of different varieties adapted to different regions and soil, not only in temperate lands but also in subtropical and tropical ones where the grape culture has been growing very well. Though not holding the same importance as *V. vinifera*, some of the wild grape species such as *V. rupestris* or *V. rotundifolia*, for instance, have been used as rootstock to select *V. vinifera* varieties. Others such as *V. labrusca* are employed in breeding programs resulting in many cultivars such as 'Concord', 'Niagara Rosada', cultivated as table grapes in Brazil or as 'Isabel', employed in the juice industry (Souza 1996). Wild species such as *V. cinerea* and *V. rupestris* are also considered a potential source of gene-resistant to diseases and drought which may be further cloned and transferred to some vinifera cultivars (Pommer 1993; Reisch and Pratt 1996; Mahanil *et al.* 2007; Angelotti *et al.* 2008).

Molecular studies for grape characterization are carried out either subjected to analysis by

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RFLP, RAPD for genomic DNA (BOURQUIN *et al.* 1991; GOTO-YAMAMOTO *et al.* 1998), or by chloroplast microsatellites (ARROYO-GARCIA *et al.* 2002) or by amino acid content (ASENSIO *et al.* 2002) and are mainly aimed at the more important *V. vinifera* varieties on the purpose of establishing the origin of those varieties or the degree of relationship among them. Recently, grapevine genome was sequenced (JAILON *et al.* 2007) opening new perspectives for improvement in grape breeding programs.

Vitis classification is still a controversial subject, especially concerning American species, where number of valid species varies according to the author (SOUSA 1996; ALVARENGA *et al.* 1998). The genus encompasses approximately 60 species which is divided in two sections, *Euvitis* and *Muscadinia* according to chromosome number and external morphological characteristics. *Euvitis* section comprises American and also Euro-Asiatic species with 2n = 38 chromosomes, including the well-known *V. vinifera*, while *Muscadinia* section comprises only three species with 2n = 40, *V. munsoniana*, *V. popenoei* and *V. rotundifolia*, natives of Mexico and southwestern USA (MOORE 1991; JACKSON 1994; SOUSA 1996; THIS *et al.* 2006).

Even though Vitaceae is not considered a large family, less than 7% of its 945 species have the chromosome number determined and less than 1% of these species have some information about chromosome morphology, according to data compiled from GOLDBLATT and JOHNSON (2006). The first chromosome count in the genus *Vitis* was done by Ghimpu, in 1927, who established 2n = 38 for *V. vinifera* (cited by OLMO 1937). Later, BRANAS (1932) determined 2n = 40 for *V. rotundifolia*. Since then, cytological work on grapes have been predominantly centered on the detection of chromosome number, with the exception of *V. vinifera* and *V. rotundifolia* which have the karyotype determined (RAJ and SEETHAIAH 1969, 1973; PATIL and JADHAV 1985; PATIL and PATIL 1992). Moreover, little is known about the interspecific genomic affinities of *Vitis* by cytological comparative studies (ALLENWELDT and POSSINGHAN 1988; VILJOEN and SPIES 1995). Some attempts towards *Vitis* chromosomal characterization were carried out by some authors who tried various procedures to get a satisfactory chromosome spreading and staining for chromosome characterization (RAJ and SEETHAIAH 1969, 1973; MARTENS and REISCH 1988; PATIL and PATIL 1992, for instance). Despite efforts, all of these authors were unanimous in their conclusion that the species had very small and numerous chromosomes. The in situ hybridization performed by HAAS *et al.* (1994) in mitotic chromosomes of *V. vinifera* using 45S rDNA sequence evidenced only the positive hybridization signals without discriminating which chromosomes were involved.

Some attempts of C-banding in grape mitotic chromosomes were also carried out by researchers such as ME *et al.* (1984) without reaching any satisfactory results. However, it is known that the heterochromatic regions, which are hard to detect after C-banding, may be differentiated by the use of some fluorochrome banding techniques which allow for the characterization of species populations, varieties and also cultivars (NIGER and ALAM 2007; KHANDAKER *et al.* 2007).

Knowing these difficulties and also that the karyotype analysis is a useful tool for characterizing germplasm, chromosomal studies were carried out on seven *Vitis* species by employing CMA_3 and DAPI fluorochromes as well as the conventional Giemsa staining technique in an attempt towards species characterization, aiming at further knowledge on a possible relationship among them at chromosomal level, therefore amplifying the chromosomal data on the Vitaceae family.

TABLE 1 — Section, Series and species classification (Species) of *Vitis* species studied

Section	Series	Species
Euvitis	*Candicansae*	*V. champinii* Planchon.
	Cinerae	*V. cinerea* (Engelm.in Gray) Engelm ex Millardet
	Labruscae	*V. labrusca* L.
	Arizonae	*V. girdiana* Munson
	Ripariae	*V. rupestris* Scheels
	Viniferae	*V. vinifera* L. var. Italia
Muscadinia		*V. rotundifolia* Michaux var. Regale

MATERIALS AND METHODS

The materials employed are listed on Table 1 and belong to the *Vitis* collection of the Vegetable Genetic Resources data Center at the Agronomical Institute of Campinas - IAC (CPD Recursos Genéticos Vegetais-Instituto Agronômico de Campinas). The Galet classification and nomenclature was considered (1967, cited in ALVARENGA *et al.* 1998) for *Vitis* species.

Roots from rooted hardwood cuttings were collected, pre-treated with a saturated solution of *para*-dichlorobenzene (*p*-DB) at 16° C for 3 hours, fixed at 3:1 (ethanol and acetic acid, respectively) solution and stored at -20° C until the cytological analyses. Fixed roots were briefly washed in citrate buffer, transferred to an enzymatic mixture of 20% pectinase and 2% cellulase at 37° C for 1 hour, for cell wall softening and then squashed in 45% acetic acid solution. The cover slips were removed after freezing in liquid nitrogen and the slides were dried and aged for 1 week or more.

Some slides were stained with a fresh 2% Giemsa solution (Giemsa stock solution diluted in Sörensen buffer) for 2 to 5 minutes at room temperature, dried and mounted with Permount (Fisher). Ten metaphase cells for each species were chosen for chromosome measurements. Chromosomes were classified according to LEVAN *et al.* (1964) concept. The mean values were calculated and the standard deviation for the total haploid chromosome length (THCL), the longest (L) and the shortest chromosome (S) length, the ratio of the longest to the shortest chromosomes (L/S), the average chromosome length in the metaphase (χm) and the Huziwara karyotype asymmetry index TF% (HUZIWARA 1956) for each *Vitis* species. The F- and Tukey-tests were applied onto the karyomorphometric data. The species were also analyzed by employing the Stebbins' two-way system of classification for karyotype asymmetry (STEBBINS 1958). Karyotype formulae and an average ideogram common to the seven species were obtained by using chromosome measurements. Some slides stained with Giemsa were photomicrographed under an Olympus Vanox photomicroscope with Kodak Ultra 400 film.

The fluorescent banding technique was also employed with fluorochromes chromomicin A_3 (CMA_3) and DAPI according to protocols described in SCHWEIZER (1976) with minor adaptations as described in PINTO-MAGLIO *et al.* (2000) aiming cytomolecular characterization of constitutive heterochromatin. The fluorescent images were captured under Olympus BX-50 epifluorescent photomicroscope connected to an image analysis system.

RESULTS

The chromosome number 2n = 38 was confirmed for *Euvitis* species studied and 2n = 40 for *V. rotundifolia* var. Regale. It has been the first time that *V. champinii*, *V. cinerea*, *V. girdiana*. *V. labrusca* and *V. rupestris* has been characterized by chromosome measurements as well as *V. rotundifolia* var. Regale and *V. vinifera* var. Italia. The chromosomes are small, almost similar to each other and did not surpass 2.12μm (Fig. 1, Table 2). It was observed that the total haploid chromosome length varied from 20.45 ± 1.17μm in *V. labrusca* to 28.34 ± 2.45μm in *V. girdiana*. The mean value for the longest chromosome of the genome varied from 1.53 ± 0.13μm in *V. rotundifolia* to 2.09 ± 0.03μm in *V. girdiana* and the mean value for the shortest chromosome varied from 0.79 ± 0.07μm in *V. labrusca* to 1.00 ± 0.05μm in *V. girdiana*. The ratio of the longest to shortest chromosome length (L/S) varied from 1.93 ± 0.26 in *V. champinii* to 2.08 ± 0.09 in *V. girdiana*. The average chromosome length in the metaphase per genome (χ μm) varied from 1.08 ± 0.06μm in *V. labrusca* to 1.49 ± 0.13μm in *V. girdiana*. The TF% asymmetry mean values varied from 37.18 ± 1.09 in *V. girdiana* to 39.96 ± 1.48 in *V. cinerea*. All *Vitis* species studied were classified as 2A Stebbins' category for karyotype symmetry (Table 2). A predominance of submetacentric chromosomes (11 to 18 pairs) was observed, as well as some metacentric in *Vitis* species genome. Due to the small chromosome size, a modified karyotype formulae was chosen, in which chromosomes were divided into four groups (A to D) based on their mean length variations (Table 2). The average ideogram obtained for seven *Vitis* species studied showed a gradation in chromosome size (Fig. 2). One pair of satellite chromosomes were observed in some cells, however, due to its inconstancy it was not possible to determine to which chromosome category it belonged to, therefore not included in the average ideogram. Within comparing the data after Tukey test it was possible to notice that *V. champinii* and *V. girdiana* species could be distinguished from other species by the total haploid chromosome length, the longest chromosome length and the

average chromosome length. *V. champinii* and *V. cinerea* are the unique species with chromosomes distributed into all four categories (A to D) (Table 2).

Two pairs of chromosomes were observed with one CMA-positive terminal band in *V. champinii*, *V. labrusca*, *V. rotundifolia* and *V. rupestris* and one pair with one CMA-positive terminal band in *V. girdiana* which distinguished it from the others (Fig. 3C). The *V. vinifera* species var. Italia depicted one pair, plus one, chromosome with one CMA-positive terminal band (Table 2, Fig. 3F). However, it was not possible to determine which pairs of chromosomes held these bands. Chromosomes stained with DAPI did not display any contrastable band.

DISCUSSION

Karyotype analyses are still of great importance and have allowed for the recognition of chromosomal variations within species (SHAN *et al.* 2003). In the genus Vitis, chromosome data available in literature for ten *V. vinifera* varieties (RAJ and SEETHAIAH 1969, 1973; PATIL and JADHAV 1985; PATIL and PATIL 1992) have shown chromosomal variations among them concerning chromosome measurements and centromere position. Therefore, each of these vinifera varieties described in the literature displayed a particular karyotype formula with prevalence of metacentric chromosomes. This prevalence however was not observed in the 'Italia' variety studied, which showed a predominance of submetacentric chromosomes. Variations such as these reported in grapes, are not rare among other plants and they may appear in combination with differences in chromosome number, size and/or morphology, eventually leading to different karyotype formulae, ideogram and asymmetry index values, as exemplified in two populations of *Brachyscome basaltica* and in five varieties of *B. dichromosomatica* (WATANABE *et al.* 1999), in two varieties of *Boronia heterophylla* (SHAN *et al.* 2003), in five varieties of *Tripleurospermum oreades* (INCEER and BEYAZOGLU 2004), or in five populations of *Trigonobalanus doichangensis* (CHEN *et al.* 2007), for instance. They also occur in cultivated plants such as in 24 dessert varieties of *Cucumis melo* (RAMACHANDRAN *et al.* 1985), or in *Colocasia esculenta*, the popular taro, (SREEKUMARI and MATHEW 1991a; 1991b).

Since *V. vinifera* is an Old Eurasian domesticated species encompassing thousands of va-

TABLE 2 — Karyotype parameters for seven species of *Vitis*: diploid chromosome number (2n), total haploid chromosome length (THCL µm), longest chromosome mean value (L µm), shortest chromosome mean value (S µm), longest/shortest chromosome rate (L/S), average chromosome length (χ length µm), Huziwara asymmetry index (TF%), length variation of chromosome complement (length var), Karyotype formula (KF), Stebbins' classification (Steb.) and number of CMA_3 positive bands (CMA_3+).

Species	2n	THCL (µm)	L (µm)	S (µm)	L/S	χ length (µm)	TF%	length var.	KF	Steb.	CMA_3+
V. champinii	38	26.75 ± 1.40 ^{a}	1.96 ± 0.08 ^{a}	0.98 ± 0.00 ^{a}	1.93 ± 0.26 ^{a}	1.41 ± 0.07 ^{a}	37.45 ± 0.24 ^{a}	4A + 7B + 7C + 1D	7m +12sm	2A	4
V. cinerea	38	23.40 ± 1.10 ^{c}	1.70 ± 0.13 ^{b}	0.88 ± 0.08 ^{bc}	1.94 ± 0.26 ^{a}	1.23 ± 0.05 ^{c}	39.96 ± 1.48 ^{b}	1A + 6B + 9C + 3D	7m +12sm	2A	4
V. girdiana	38	28.34 ± 2.45 ^{a}	2.09 ± 0.03 ^{c}	1.00 ± 0.05 ^{ab}	2.08 ± 0.09 ^{a}	1.49 ± 0.13 ^{a}	37.18 ± 1.09 ^{a}	5A + 9B + 5C	7m +12sm	2A	2
V. labrusca	38	20.45 ± 1.17 ^{b}	1.57 ± 0.11 ^{b}	0.79 ± 0.07 ^{c}	1.99 ± 0.18 ^{a}	1.08 ± 0.06 ^{b}	38.87 ± 1.92 ^{ab}	3B + 9C + 7D	8m +11sm	2A	4
V. rotundifolia	40	21.74 ± 1.08 ^{bc}	1.53 ± 0.13 ^{b}	0.80 ± 0.07 ^{c}	1.95 ± 0.08 ^{a}	1.09 ± 0.05 ^{b}	37.61 ± 0.56 ^{a}	3B + 10C + 7D	9m +11sm	2A	4
V. rupestris	38	21.83 ± 1.67 ^{bc}	1.57 ± 0.11 ^{b}	0.81 ± 0.07 ^{c}	1.94 ± 0.12 ^{a}	1.15 ± 0.09 ^{bc}	37.32 ± 1.67 ^{ab}	5B + 9C + 5D	1m +18sm	2A	4
V. vinifera	38	22.57 ± 1.83 ^{bc}	1.67 ± 0.16 ^{b}	0.85 ± 0.06 ^{c}	1.96 ± 0.18 ^{a}	1.19 ± 0.09 ^{bc}	38.96 ± 1.40 ^{ab}	5B + 10C + 4D	8m +11sm	2A	3

Mean values followed by the same letter = not significant at 1% level after F-test.
Mean values followed by different letters = significant at 1% level after F-test
Chromosome categories based on mean length variation: Type A= 2.09 –1.70µm; B = 1.69–1.30 µm; C = 1.29–1.00 µm; D = 0.99 – 0.79 µm.

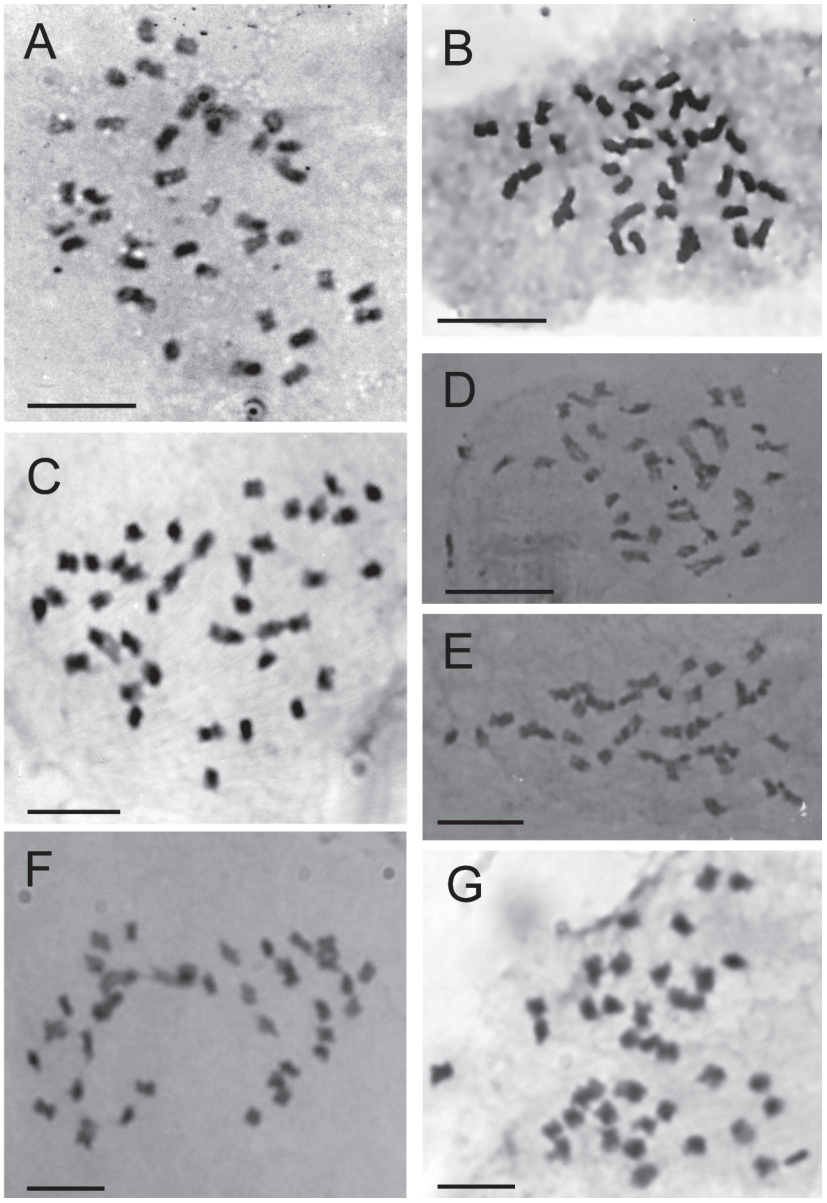

Fig. 1 — Photomicrographies of mitotic chromosomes of (A) *V. labrusca* var. Isabel; (B) *V. rupestris* var. du Lot; (C) *V. girdiana*; (D) *V. champinii*; (E) *V. vinifera* var. Italia; (F) *V. rotundifolia* var. Regale; (G) *V. cinerea.* Bar = 5 µm. A to E and G: 2n = 38; F: 2n = 40.

rieties, most of them intercrossed and adapted to different climatic and soil conditions, these karyomorphometric differences reported may be interpreted as a reflection of agronomical selective pressures that these varieties have undergone. It is likely that structural changes such as small translocations, deletions or duplications might have taken place leading to better adaptive clusters of interacting genes and also to visible changes in chromosome size and in centromere position of some chromosomes. These small variations however were not strong enough to disrupt the variety of intercrossing which has given rise to fertile hybrids among them as inferred by some *Vitis* revisions (ALLENWELDT and POSSINGHAN 1988; JACKSON 1994; SOUSA 1996, for instance). However, it has not been disregarded, that the influence of anti-mitotic pre-treatments in chromosome contraction may lead to differences in chromosome lengths. Interestingly, that GOTO-YAMAMOTO *et al.* (1998) have also observed differences among fourteen Eurasian *V.*

vinifera varieties, although at a molecular level by means of RFLP and RAPD analyses.

The chromosomal differences observed between *V. girdiana* and the other American grape species studied could likely be associated to geographical distribution. According to REISCH and PRATT (1996) geographical representation of North American wild grapes, *V. girdiana* occurs almost isolated in Southwest California and Arizona (USA) and in Northwest Baja California (Mexico) and apart from the native *V. champinii*, *V. cinerea*, *V. labrusca*, *V. rotundifolia* and *V. rupestris* which have different levels of overlapping in the southeast and east USA.

V. champinii is considered a controvertible species and according to some authors, *V. champinii* or simply *Champini* is not a true species, but a hybrid between *V. candicans*, the mustang grape, and *V. rupestris*, the sand grape, that Planchon described as a species (MOORE 1991; SOUSA 1996). *V. champinii* holds the ability to grow on calcareous soil and shows resistance to drought (MOORE 1991; SOUSA 1996; ALVARENGA *et al.* 1998). According to REISCH and PRATT (1996), *V. candicans*, *V. champinii* and *V. rupestris* have small overlapping areas in geographical distribution and *V. candicans* and *V. champinii* belong to the same *Candicansae* series. According to karyomorphological data, such as total haploid chromosome length, the mean values of the longest and the shortest chromosome of the genome, and karyotype formula, *V. champinii* differed significantly from the supposedly parental *V. rupestris* and also from *V. cinerea*, *V. labrusca* and *V. rotundifolia*, suggesting it is not a hybrid plant. The supposedly parental *V. rupestris* possesses more submetacentric chromosomes (18sm) than *V. champinii* (12sm) and more than other species analyzed. However, this is still an open question which calls for more accurate as well as more refined karyological studies on these species and also on other supposedly parental, *V. candicans*.

The species *V. champinii* and *V. cinerea* occur sympatrically in North America (REISCH and PRATT 1996) seeing that the former distribution area is smaller and enclosed by *V. cinerea* populations. The differences observed in some chromosome measures between these species may be related to differences on external morphology and habitat preferences or to differences in the flowering and fruit ripening time.

Although *V. rotundifolia* has a different chromosome number (2n = 40) which is characteristic of *Muscadinia* Section, this species did not show any significant difference concerning chromosome measurement, when compared to *V. labrusca*, *V. rupestris* and *V. vinifera*. However, based on morphological observations and on the intersterile hybrids *V. rotundifolia* and *V. vinifera* are considered very distantly related species (PATEL and OLMO 1955; OLMO 1979). They also show strong differences when compared by using some isozyme profiles (IDH, GOT, EST, PGI) and with RAPDs marks, as observed by SAWASAKI *et al.* (1996). Despite these differences, *V. rotundifolia* was classified alongside *V. champinii*, *V. cinerea*, *V. girdiana*, V. *labrusca*, *V. rupestris*

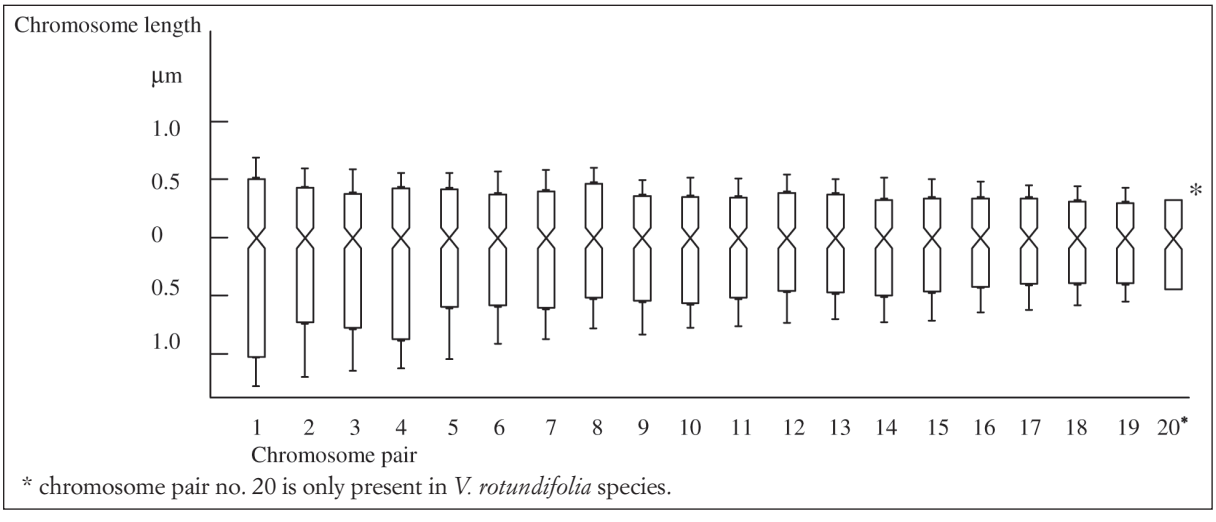

Fig. 2 — The average ideogram obtained for seven *Vitis* species studied and chromosome maximum variation represented by bars over and under each chromosome arm.

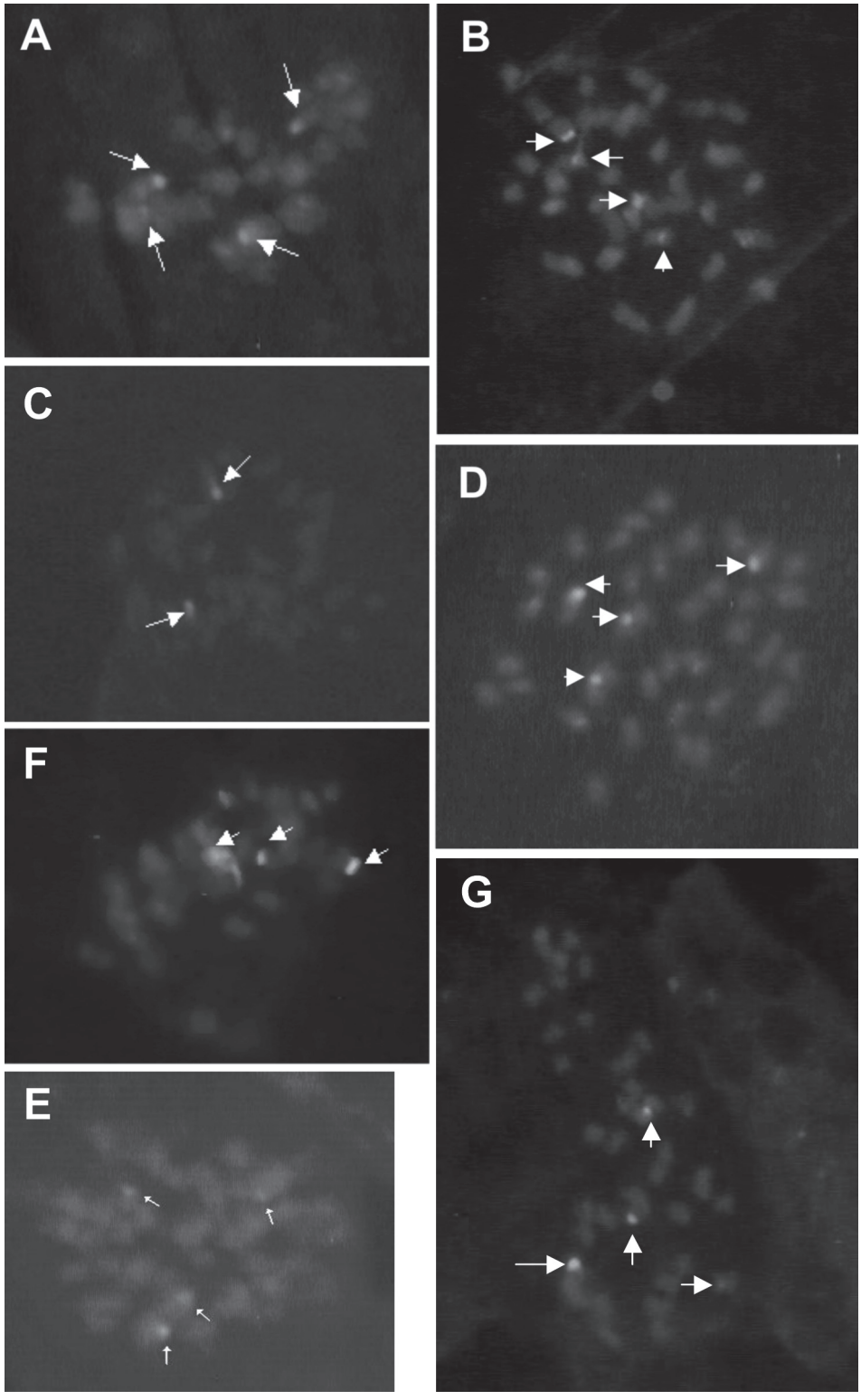

Fig. 3 — Images of mitotic chromosomes of (A) *V. rotundifolia*; (B) *V. champinii*; (C) *V. girdiana*; (D) *V. rupestris*; (E) *V. cinerea*; (F) *V. vinifera*; (G) *V. labrusca*, after CMA_3 staining procedure. Arrows = CMA + bands.

and also with *V. vinifera* on 2A Stebbins' category for karyotype symmetry due to the prevalence of small submetacentric chromosomes. These findings are emphasized by TF% mean values for the seven *Vitis* species studied which showed a moderated karyotype asymmetry.

The absence of B chromosome in these *Vitis* species has already been reported by other researchers not only in grapes but also in other Vitaceae genera (GOLDBLATT 1981; 1984; 1985; 1988, for instance).

The brilliant CMA-positive bands denoted heterochromatic GC-rich regions at terminal localization in *Vitis* species. Knowing that nucleolar organizer regions (NOR) are very often GC-rich and stain positively to chromomicin, it is possible that at least one of these chromosome pairs with CMA-positive bands observed in *Vitis* species could be related to NOR, although only one pair of satellite chromosomes was observed in some cells after Giemsa staining technique. The presence of one heteromorphic pair of chromosomes presenting a terminal CMA-positive band in only one of the homologues in *V. vinifera* var. Italia may be interpreted as (1) the presence of a weak or a small signal which was not seen; (2) highly condensation of heterochromatic region at metaphase that prevented CMA fluorochrome to bind; (3) probable occurrence of small structural changes as observed in *Vigna radiata* cultivars by MAHBUB *et al.* (2007); or also (4) may be a reflection of structural changes related to extensive crossings or to cultivation pressures. Other cultivated plants such as *Lens culinaris* (KHANDAKER *et al.* 2007) or *Gossypium hirsutum* and *G. arboreum* (NIGER and ALAM 2007), for instance, also showed chromosome heteromorphism for chromomicin and DAPI bands.

V. girdiana, also known as desert grape, is the unique species, up to now, that has only one pair of CMA-positive band. However, it is not known if the presence of only one pair of CMA-positive band is (1) a characteristic of this species; or (2) a characteristic of the *Arizonae* series which it belongs to; or (3) related to the geographical distribution of *V. girdiana* and its tolerance for growing in drier sandy or clay soils. Further studies are highly necessary on other species of *Arizonae* series.

Finally, regarding all karyomorphometric data and fluorescent banding results recorded for the seven *Vitis* species analyzed, it is likely that during the speciation process, the differences among them, except for the chromosome number, may have taken place at gene level, constitutive heterochromatin cytochemical composition and also at chromosomal level expressed as variations associated to chromosome length. However, these differences do not seem to be strong enough in order to avoid intercrossing, since inter-fertile *Euvitis* hybrids are easily to obtain, although the existence of geographical, phenological or ecological barriers were reported among most of them (REISCH and PRATT 1996). Nevertheless, other species ought to be studied and also more refined chromosomal studies should be carried out aiming at some other banding techniques on the purpose of a betterment of chromosome characterization, that may allow for the understanding of relationships among species, besides the enlargement of Vitaceae characterization at chromosomal level.

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