Cytogenetic analysis of European *Cassida* (Coleoptera, Chrysomelidae)

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Petitpierre, E., Carreras, I. and Gómez-Zurita, J. 1998. Cytogenetic analysis of European *Cassida* (Coleoptera, Chrysomelidae). — *Hereditas 128*: 1–8. Lund, Sweden. ISSN 0018-0661. Received May 16, 1997. Accepted October 9, 1997

Sixteen species of European cassidines, all but one of the genus *Cassida*, have been cytogenetically investigated, twelve of them for the first time. The diploid number of 18 chromosomes and $8 + Xy_p$ meioformula is the prevalent karyotype of these species. The DNA content of Feulgen-stained spermatids shows a moderate variation, with most species having 0.8–1.1 pg. The possible trends of the chromosomal evolution in *Cassida* are discussed in the light of the present subgeneric taxonomy, host-plant selection, and other characteristics. The value of cytogenetics for the recognition and distinguishing between sibling species of *Cassida* is also dealt with.

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Most European cassidines belong to the genus *Cassida*, a taxon of almost worldwide distribution with more than 250 species (SPAETH 1914), of which some forty are found in Europe (WINKLER 1930; BORDY and DOGUET 1987). Our previous studies on the chromosome number, karyotypes, and sex-chromosome systems of cassidines (PETITPIERRE 1977, 1985), were concerned with ten species, and later we listed the numbers and meioformulas for a total of 55 species of Cassidinae, nineteen of them included in the genus *Cassida* (PETITPIERRE et al. 1988). The subfamily as a whole is relatively heterogeneous in the chromosome numbers, ranging from $2n = 16$ to $2n = 51$, although $2n = 18$ is strikingly prevalent (PETITPIERRE et al. 1988; YADAV et al. 1995). The main characteristics of the karyological evolution of cassidines have been recently discussed by several authors (PETITPIERRE 1988; VIRKKI et al. 1992; YADAV et al. 1995). In the genus *Cassida*, thirteen among the nineteen species studied had $2n = 18$, $8 + Xy_p$ ("parachute" association), the modal and presumably ancestral chromosome number and meioformula for the genus; however, for the entire subfamily much more analyses should be performed before assuming this karyotype as the plesiomorphic state for Cassidinae. Herein, we report chromosomal findings on fifteen species of *Cassida*, all but three checked for the first time, and on *Oxylepus deflexicollis*, a species pertaining also to the same tribe, Cassidini, as the former ones (SEENO and WILCOX 1982). Furthermore, the DNA content of Feulgen-stained spermatids was measured in twelve of these species of cassidines. The aim of the present paper is to have a more clear insight into the taxonomic and evolutionary relationships among the species of *Cassida*.

MATERIAL AND METHODS

The species studied and their geographical sources are given in Table 1. All these species were captured and analyzed in the years 1993–97. The chromosomal analyses were conducted using the following technique: (a) fixation of dissected testes in a freshly made solution of ethanol-acetic acid (3:1) for 15–20 min; (b) tearing of testes on slides covered with some drops of 45% acetic acid in distilled water; (c) squashing by strong pressure with the two thumbs on a cover slip under a filter paper; (d) slides dipped into liquid nitrogen for some 20–30 s and the frozen cover slip immediately removed by a sharp blade; and (e) slides finally stained in 4% Giemsa diluted in Sorensen buffer for 10–15 min, and excessive staining briefly rinsed in tap water.

The procedure of Feulgen staining reported elsewhere (JUAN and PETITPIERRE 1989), was followed to measure the nuclear DNA content of spermatids, the flour beetle *Tribolium castaneum* ($1C = 0.208$ pg) being used as an internal standard in each experiment. The light absorbance and the spermatid areas were provided by an image analyzer VIDAS-21 coupled to a ZEISS AXIOSKOP microscope.

RESULTS

Most surveyed species provided good metaphases I to analyze their meioformulas. The most widespread meioformula, $8 + Xy_p$, was found in *Cassida bergeali* (Fig. 1), *C. hexastigma* (Fig. 2), *C. azurea* (Fig. 3), *C. sanguinolenta* (Fig. 4), *C. sanguinosa* (Fig. 7), *C. leucanthemi* (Fig. 8), *C. rufovirens* (Fig. 9), *Oxylepus deflexicollis* (Fig. 12), and *C. panzeri* (not shown). The $9 + Xy_p$ meioformula appeared in *C. hemisphaerica*

Table 1. *Chromosomal and genome size data in sixteen species of cassidines from France (Fr.) and Spain (Sp.)*

Species	Geographic source	No. ind.	2n	Meioformula	No. ind.	1C (pg)
C. algirica Luc.	Pto. del Cabrito, Cádiz (Sp.)	1	18	–	–	–
C. azurea F.	Bellefontaine, Jura (Fr.)	1	–	$8+Xy_p$	–	–
C. bergeali Bordy	Dampvalley, Hte. Saône (Fr.)	2	18	$8+Xy_p$	1	1.11
C. deflorata Suffr.	Cauvert, Gard (Fr.)	1	18	$8+Xy_p$	2	0.66
C. hemisphaerica Herbst	Sierente, Ht. Rhin (Fr.)	2	20	$9+Xy_p$	1	1.07
C. hexastigma Suffr.	Montpeyroux, Hérault (Fr.)	3	18	$8+Xy_p$	–	–
C. leucanthemi Bordy	Bellefontaine, Jura (Fr.)	4	18	$8+Xy_p$	1	0.93
C. pannonica Suffr.	Breitenbrunn (Austria)	2	20	$9+Xy_p$	1	1.31
C. panzeri Weise	St. Clément, Meurthe & Moselle (Fr.)	2	18	$8+Xy_p$	–	–
C. rubiginosa Müll.	Val St. Eloi, Hte. Saône (Fr.)	1	–	$8+Xy_p$	6	1.07
C. rubiginosa Müll.	Lauroux, Hérault (Fr.)	1	–	$8+Xy_p$	2	1.05
C. rubiginosa Müll.	La Couvertoirade, Aveyron (Fr.)	1	–	$8+Xy_p$	4	0.94
C. rufovirens Suffr.	Val St. Eloi, Hte. Saône (Fr.)	1	–	$8+Xy_p$	2	0.92
C. sanguinolenta Müll.	Mersuay, Hte. Saône (Fr.)	2	18	$8+Xy_p$	2	0.95
C. sanguinosa Suffr.	Altenach, Ht. Rhin (Fr.)	2	18	$8+Xy_p$	1	0.74
C. subreticulata Suffr.	Les Rousses, Jura (Fr.)	2	30	$14+Xy_p$	1	0.71
C. viridis L.	Val d'Aràn, Lérida (Sp.)	1	24	$11+Xy_p$	3	0.86
C. viridis L.	Valle de Irati, Navarra (Sp.)	–	–	–	3	0.91
C. viridis L.	El Charcón, Granada (Sp.)	1	–	$14+Xy_p$	–	–
O. deflexicollis Boh.	Isla de Tabarca, Alicante (Sp.)	1	–	$8+Xy_p$	–	–
O. deflexicollis Boh.	Pina de Ebro, Zaragoza (Sp.)	–	–	–	1	0.67

(Fig. 6) and *C. pannonica* (Fig. 11), whereas *C. viridis* from the Pyrenees had $11+Xy_p$ (Fig. 10), but from Granada in south Spain, had $14+Xy_p$ (Fig. 18), the same meioformula displayed by *C. subreticulata* (Fig. 5). The unichiasmate bivalents were clearly predominant or the only ones found in these species, and a maximum of two bichiasmate bivalents was observed, for example in *C. hemisphaerica* and *C. sanguinosa* (Fig. 6–7).

The spermatogonial metaphases were obtained in five species, *C. panzeri* (Fig. 13), *C. hexastigma* (Fig. 14), *C. deflorata* (Fig. 16), *C. sanguinolenta* (not shown), and *C. algirica* (Fig. 17), which excepting *C. algirica* allowed to set up their karyotypes (Fig. 19a–d). All these karyotypes are composed of metacentric or submetacentric chromosomes of gradually decreasing sizes, the X and y-chromosomes are smaller than any of the autosome pairs, the X being a bit larger than the tiny y-chromosome, and both apparently being metacentrics.

The DNA content of Feulgen-stained spermatids was studied in 31 individuals of fifteen populations and twelve species of cassidines (Table 1). Almost all data for each individual were obtained from more than fifteen spermatid measurements. The range of variation of genome sizes in the sampled cassidines was not very striking, from 0.66 pg in *C. deflorata* to near two-fold 1.33 pg in *C. pannonica*. An intraspecific analysis of genome size was performed in three populations of *C. rubiginosa*, whose average values were very close, 1.07, 1.05 and 0.94 pg, as well as those shown by two Spanish Pyrenean populations of *C. viridis*, 0.86 and 0.91 pg. Moreover, a high and significant correlation ($r = 0.78$, $p < 0.001$), was found between the average genome sizes and spermatid areas in the fifteen sampled populations of cassidines (Fig. 20).

DISCUSSION AND CONCLUSIONS

The chromosome numbers and the sex-chromosome system of the sixteen examined species of cassidines show a remarkable uniformity, twelve sharing $2n = 18$ and $8+Xy_p$ male meioformula. This number and formula are also the most common ones in the 75 chromosomally surveyed species of cassidines, and among them in the 31 known species of *Cassida* (Fig. 21). The taxonomy within the genus *Cassida*, based on morphological traits, is far from clear (BORDY and DOGUET 1987). From cytogenetic standpoints, five out of the nineteen described subgenera of *Cassida* (SEENO and WILCOX 1982) are checked, and among them only the subgenus *Hypocassida* seems well-substantiated because *C.* (*Hypocassida*) *subferruginea* has a high diploid number plus a derived and unusual sex-chromosome system of $18+$neoXY meioformula (PETITPIERRE 1985; PETITPIERRE et al. 1988; VIRKKI et al. 1992).

The subgenus *Cassida* s.str., including most European species of *Cassida*, is known for 22 species and among them the current *C. algirica*, *C. bergeali*, *C. deflorata*, *C. hexastigma*, *C. leucanthemi*, *C. pannonica*, *C. panzeri*, *C. rubiginosa*, *C. rufovirens*, *C. sanguinolenta*, and *C. sanguinosa*, all but two sharing

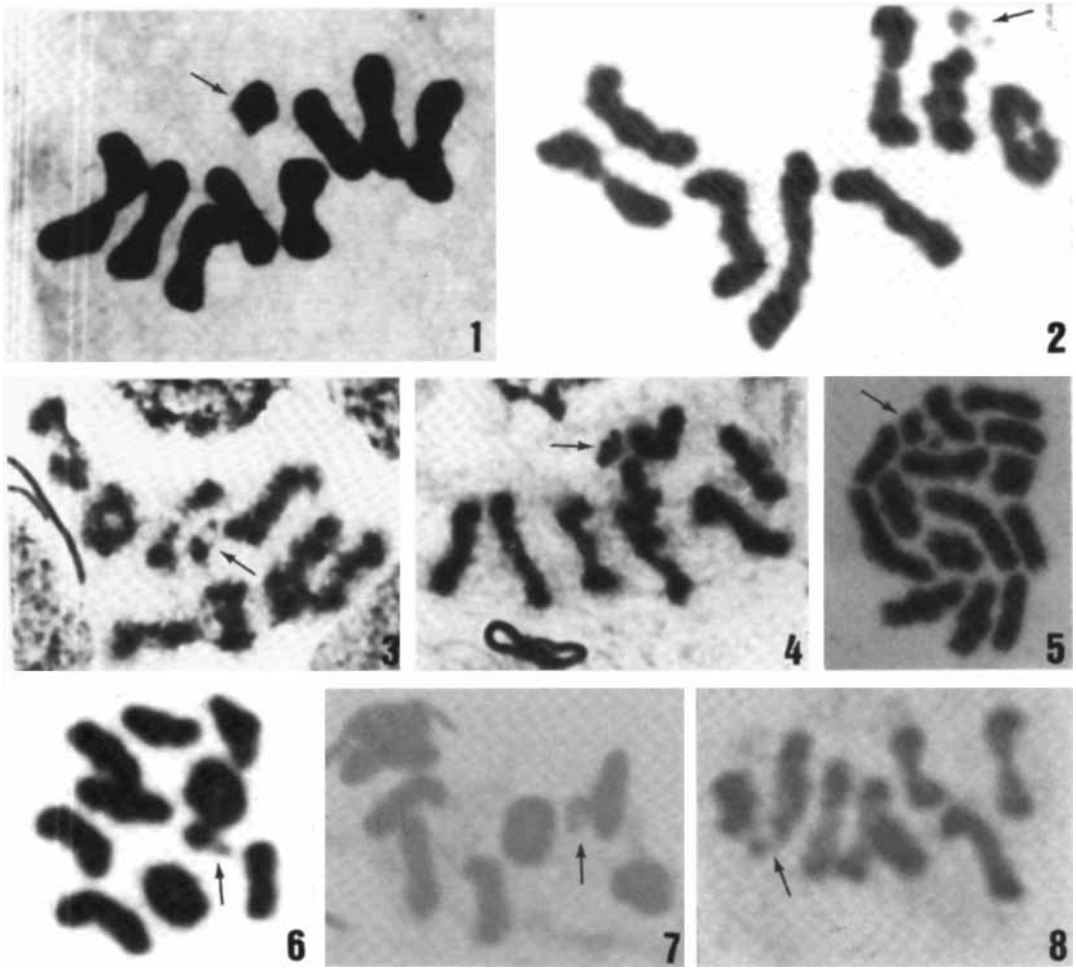

Fig. 1–8. Metaphases I of *Cassida bergeali* **(1)**, *C. hexastigma* **(2)**, *C. azurea* **(3)**, *C. sanguinolenta* **(4)**, all with $8 + Xy_p$, *C. subreticulata* **(5)** with $14 + Xy_p$, *C. hemisphaerica* **(6)** with $9 + Xy_p$, *C. sanguinosa* **(7)** and *C. leucanthemi* **(8)** with $8 + Xy_p$. The Xy_p is indicated by an arrow. All figures at $\times 2000$.

$8 + Xy_p$ meioformula. Exceptions to this conservatism are the present *C. pannonica* and the already known *C. vibex* (PETITPIERRE 1977), which have $9 + Xy_p$, although $9 + Xyy_p$ has also been recorded in the second (YADAV et al. 1995). Among the European species of *Cassida* s. str., in France there are at least two clusters of sibling species, one constituted by *C. bergeali*, *C. pannonica*, and *C. vibex* (BORDY 1995a), and another by *C. leucanthemi* and *C. sanguinosa* (BORDY 1995b). While in the first cluster *C. bergeali* can be distinguished from the other two species by having 18 instead of 20 chromosomes, in the second, *C. leucanthemi* and *C. sanguinosa* display the same number and sex-chromosome system, $2n = 18(Xy_p)$.

Three out of the five species belonging to the subgenus *Mionycha* (SPAETH 1914), have been cytogenetically analyzed. The current *C. subreticulata* has the same meioformula $14 + Xy_p$ as the previously studied *C. margaritacea* (PETITPIERRE 1977), but the third species, *C. azurea*, shows the modal one of $8 + Xy_p$ and separates clearly from the former two. A similar case appears for the two checked species of the subgenus *Cassidulella*, *C. pusilla* and *C. vittata*, the first having $8 + Xy_p$ whereas the second has $9 + Xy_p$. In addition, the subgenus *Odontionycha* is also chromosomally heterogeneous: *C. viridis* is composed of two sibling species, one in Central Europe and northern Spain with $11 + Xy_p$ (PETITPIERRE 1988), and the other (ab. *nigriceps* Fairm.) in Andalucía (Southern Spain) with $14 + Xy_p$, as demonstrated earlier (PETITPIERRE 1977, 1988), and in the present study too. Finally, the unique species of the subgenus *Mionychella*, *C. hemisphaerica*, has a meioformula of $9 + Xy_p$, also found in some species of *Cassida* s. str., as reported above.

The trophic selection of French *Cassida*, on five different plant families (BORDY and DOGUET 1987),

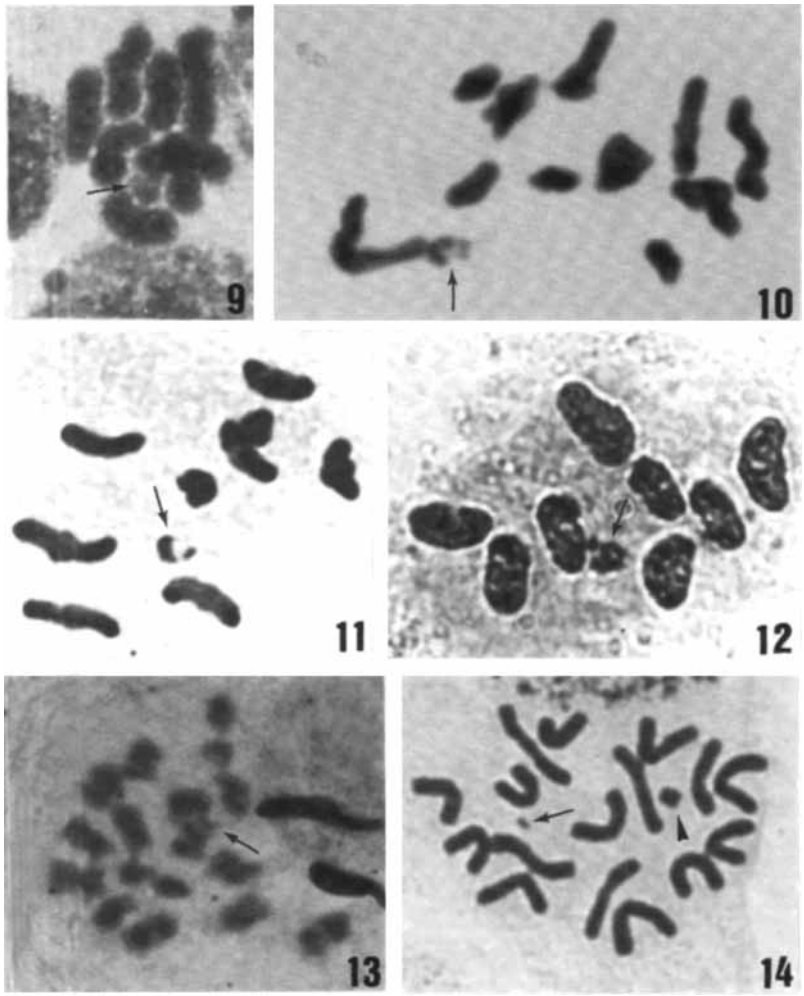

Fig. 9–14. Metaphases I of *Cassida rufovirens* (9) with $8 + Xy_p$, *C. viridis* Northern sibling **(10)** with $11 + Xy_p$, *C. pannonica* **(11)** with $9 + Xy_p$, and *Oxylepus deflexicollis* **(12)** with $8 + Xy_p$. The Xy_p is indicated by an arrow. Spermatogonial metaphases of *C. panzeri* **(13)** and *C. hexastigma* **(14)** showing 2n = 18 chromosomes. The X is indicated by an arrowhead and the y-chromosome by an arrow. All figures at ×2000.

does not provide, either, a suitable frame to interpret their chromosomal evolution, with a few exceptions only. Most European *Cassida* live on Asteraceae (=Compositae) and among them the majority have 2n = 18 but two, *C. vibex* and *C. pannonica*, share 2n = 20 chromosomes. The species living on Caryophyllaceae, such as *C. margaritacea* and *C. subreticulata* display 2n = 30, but *C. azurea* has 2n = 18, and *C. hemisphaerica* 2n = 20. *C. vittata* living on either Caryophyllaceae or Chenopodiaceae shows 2n = 20, whereas the two sibling species of *C. viridis* live on *Mentha* spp. (Lamiaceae) one having 2n = 24 and another, 2n = 30. The only remarkable exception is again that of *C. subferruginea* with a strikingly apomorphic meioformula of 18 + neoXY; this species and the closely related *C. meridionalis* are the sole representatives of the European *Cassida* feeding on Convolvulaceae. Therefore, although the presumed ancestral feeding selection of European *Cassida* with 2n = 18 was probably the Asteraceae, shifts to other plant families might have taken place associated with increases of diploid number in several cases, but not in others, as reported above, and the reverse option of shifting chromosome number without any change of plant family is also proved. The adaptive radiation of *Cassida* concerned with the botanic choice is, consequently, rather eclectic if we try to understand this pattern from our cytogenetic findings.

The spermatogonial metaphases obtained in four species of *Cassida* allow us to compare their karyotypes but no clear-cut differences appeared due to the prevalent metacentric shape of their chromo-

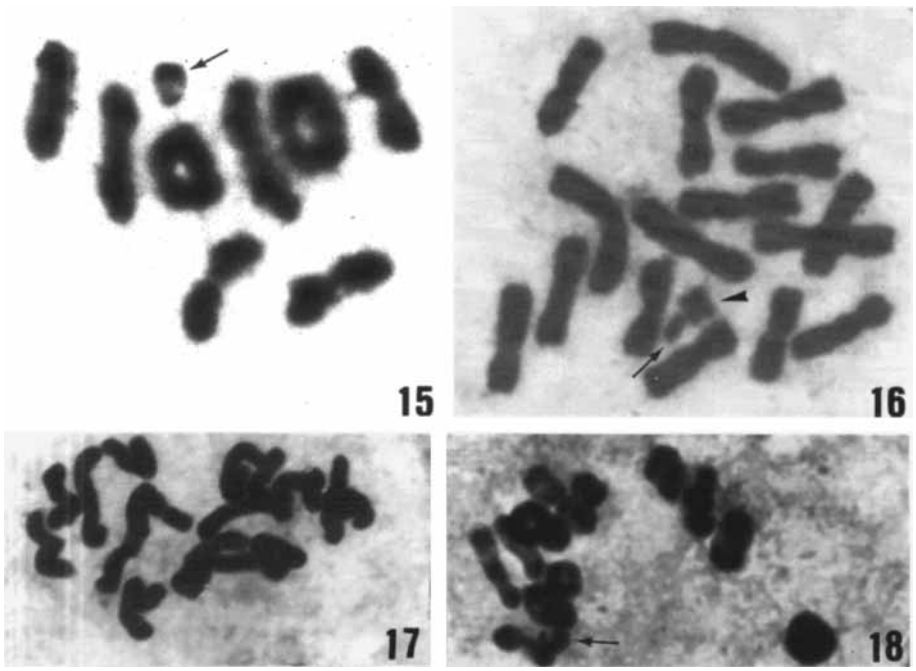

Fig. 15–18. Metaphase I **(15)** with $8 + Xy_p$ and spermatogonial metaphase **(16)** with $2n = 18$ of *Cassida deflorata*. The Xy_p is indicated by an arrow in Fig. 15, the X by an arrowhead, and the y-chromosome by an arrow in Fig. 16. Spermatogonial metaphase **(17)** of *C. algirica* with $2n = 18$. Metaphase I **(18)** of *C. viridis* Southern sibling, with $14 + Xy_p$. The Xy_p is indicated by an arrow. All figures at ×2000.

somes of gradually decreasing sizes. These four species present sex chromosomes of very small size, shorter than the smallest autosome pair, contrary to *C. vibex* (PETITPIERRE 1977), and *C. circumdata* (YADAV et al. 1995), where the X-chromosome is more than three-fold larger than the y-chromosome. Thus, two types of sex-chromosomes are found in the species of *Cassida*, one with a rather large X and another with a small X-chromosome. With the present state of knowledge it is, however, uncertain to assume what is the plesiomorphous condition in cassidines of these two types of simple sex-chromosomes. Indeed, the neo-XY of *C. subferruginea* and the multiple sex-chromosome systems, such as those found in South American stolaine cassidines (VAIO and POSTIGLIONI 1974; PANZERA et al. 1983; VIRKKI et al. 1992), are derivative types from the common "parachute" association, Xy_p, of most beetle sex-chromosomes and in particular for those of the suborder Polyphaga (SMITH and VIRKKI 1978; VIRKKI 1984).

The DNA content of spermatids does not provide a valuable tool of taxonomic and evolutionary interest for the sampled cassidines. Like the chromosomes, the genome sizes do not agree with the subgenus taxonomy of *Cassida*, because most species have quite similar amounts of haploid nuclear DNA (0.8–1.1 pg). These values resemble those found in many leaf beetles of the subfamily Chrysomelinae and, on the contrary, they are well-differentiated with respect to the smaller genomes (0.2–0.6 pg) observed in species of other subfamilies, such as Clytrinae, Cryptocephalinae, and Criocerinae (PETITPIERRE et al. 1993).

The intraspecific variation of genome size in Coleoptera has been documented in *Tribolium* flour beetles (ÁLVAREZ-FUSTER et al. 1991) and in another tenebrionid *Phylan semicostatus* (PALMER and PETITPIERRE 1996). Our studies on different geographic populations of *Cassida rubiginosa* and *C. viridis*, also give small differences within each species, which do not exceed the 15 % of maximum specific value, and are of the same magnitude as those obtained between some species of *Cassida*.

As a corollary, it seems clear that to assess the chromosomal evolution of the whole Cassidinae an enlarged analysis should be awaited, mainly directed

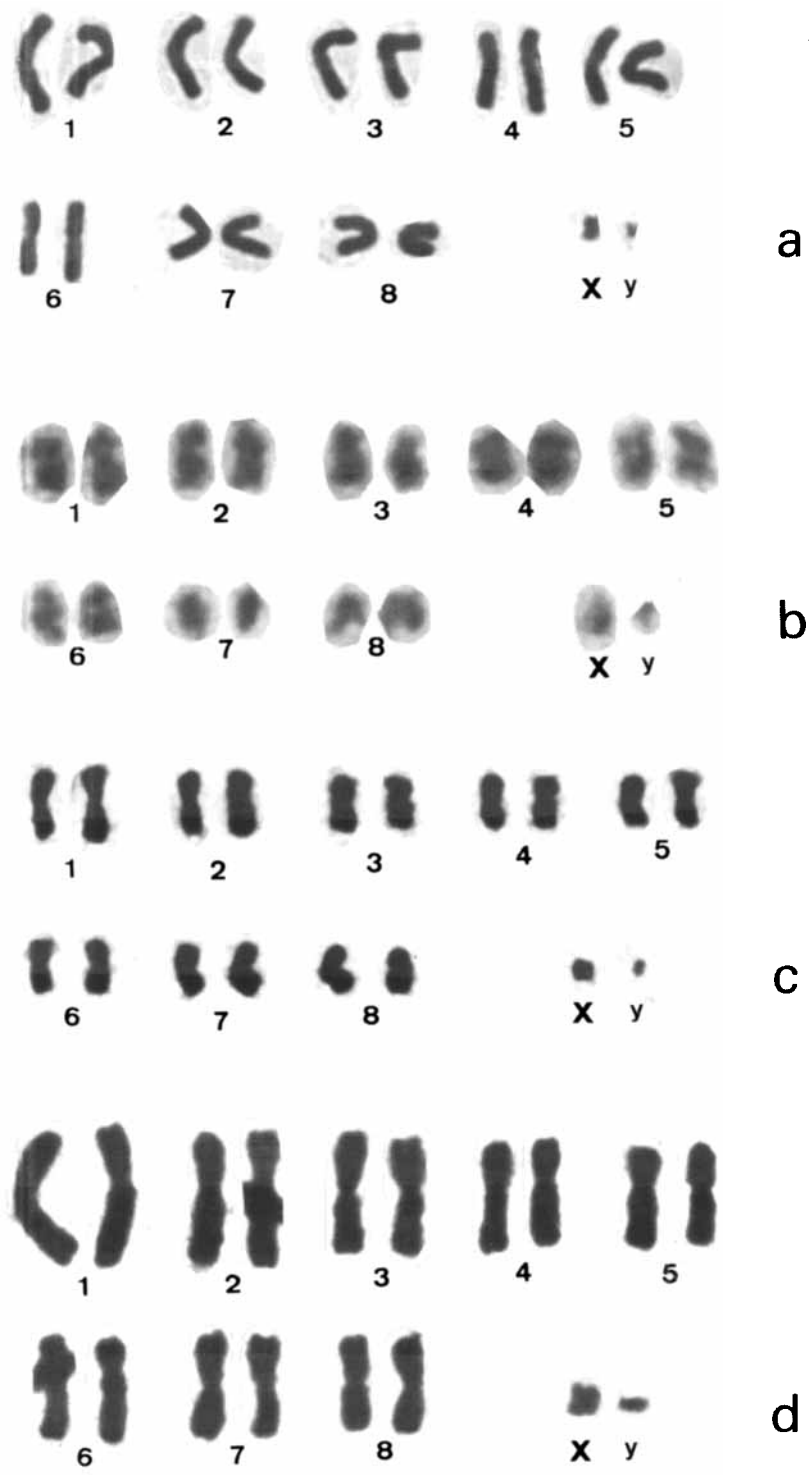

Fig. 19a–d. Karyotypes of: *Cassida hexastigma* **(a)**, *C. panzeri* **(b)**, *C. sanguinolenta* **(c)**, and *C. deflorata* **(d)**. All at ×2000.

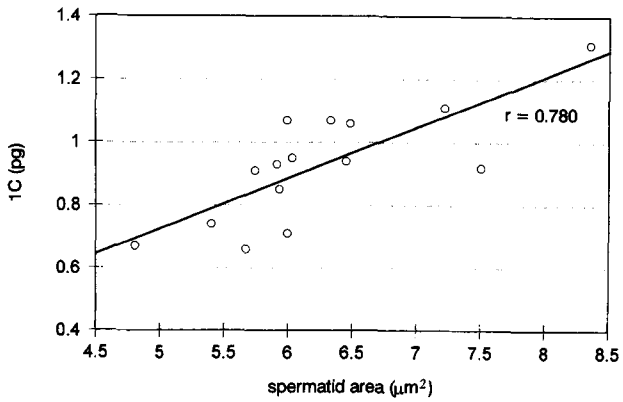

Fig. 20. Plotting of average spermatid areas (μm^2) against their genome sizes (pg) in fifteen geographic samples of twelve species of cassidines (see Table 1).

to the Neotropical and African species, since solely nine out of the nineteen present tribes (SEENO and WILCOX 1982), have been surveyed for at least one species.

ACKNOWLEDGEMENTS

This work has been supported by DGICYT project no. PB93-0419, Ministry of Education and Science. Our most sincere thanks are given to Mr. Bernard Bordy (Le Val Saint Eloi, Hte. Saône, France), who provided living individuals of most species of cassidines used in the present study.

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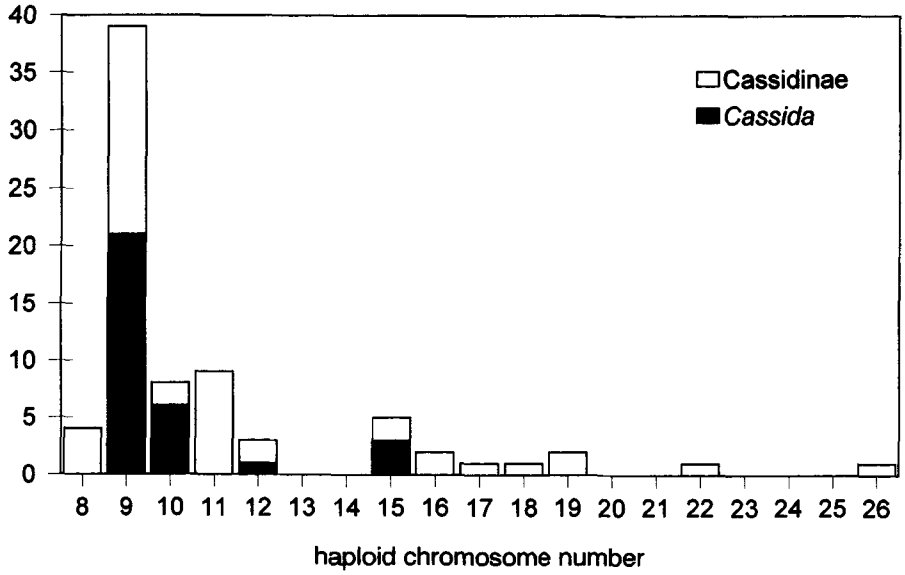

Fig. 21. Histogram of the haploid chromosome numbers of the whole checked species of Cassidinae and *Cassida*.

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