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# Improved Basic Cytogenetics Challenges Holocentricity of Butterfly Chromosomes

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## Keywords

Butterflies · Holocentric chromosomes · Monocentric chromosomes · Canonical meiosis

## Abstract

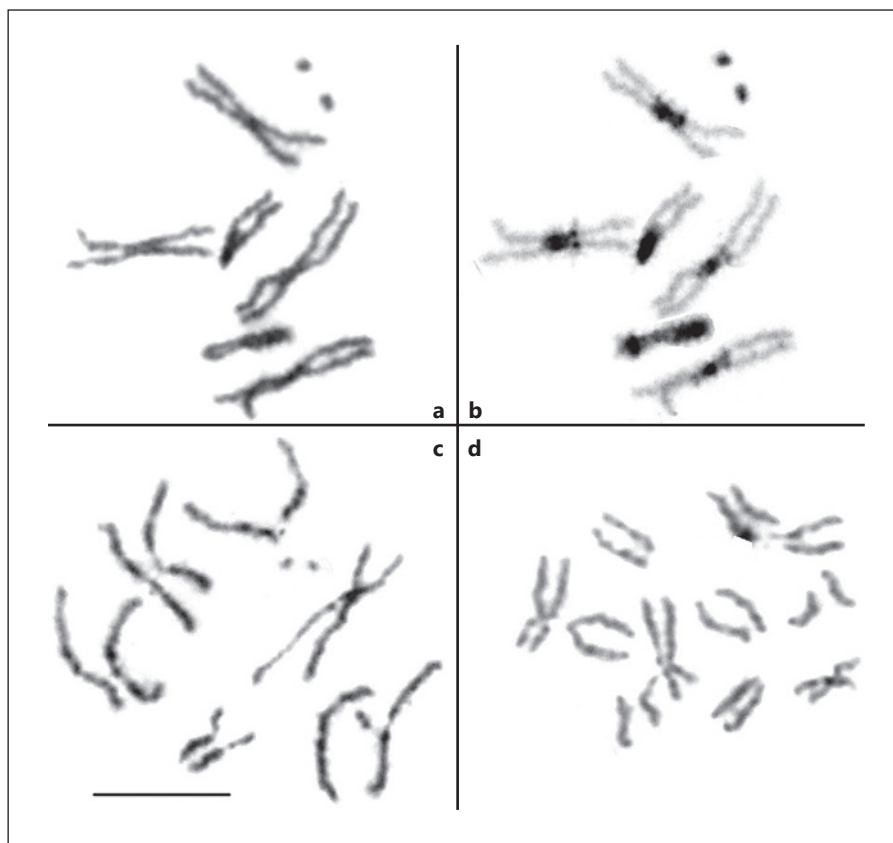
Mitotic chromosomes of butterflies, which look like dots or short filaments in most published data, are generally considered to lack localised centromeres and thus to be holokinetic. This particularity, observed in a number of other invertebrates, is associated with meiotic particularities known as “inverted meiosis,” in which the first division is equational, i.e., centromere splitting-up and segregation of sister chromatids instead of homologous chromosomes. However, the accurate analysis of butterfly chromosomes is difficult because (1) their size is very small, equivalent to 2 bands of a mammalian metaphase chromosome, and (2) they lack satellite DNA/heterochromatin in putative centromere regions and therefore marked primary constrictions. Our improved conditions for basic chromosome preparations, here applied to 6 butterfly species belonging to families Nymphalidae and Pieridae challenges the holocentricity of their chromosomes: in spite of the absence of primary constrictions, sister chromatids are recurrently held together at definite positions during mitotic metaphase, which makes possible to establish karyotypes composed of acrocentric and submetacentric

chromosomes. The total number of chromosomes per karyotype is roughly inversely proportional to that of non-acrocentric chromosomes, which suggests the occurrence of frequent robertsonian-like fusions or fissions during evolution. Furthermore, the behaviour and morphological changes of chromosomes along the various phases of meiosis do not seem to differ much from those of canonical meiosis. In particular, at metaphase II chromosomes clearly have 2 sister chromatids, which refutes that anaphase I was equational. Thus, we propose an alternative mechanism to holocentricity for explaining the large variations in chromosome numbers in butterflies: (1) in the ancestral karyotype, composed of about 62 mostly acrocentric chromosomes, the centromeres, devoid of centromeric heterochromatin/satellite DNA, were located at contact with telomeric heterochromatin; (2) the instability of telomeric heterochromatin largely contributed to drive the multiple rearrangements, principally chromosome fusions, which occurred during butterfly evolution.

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## Introduction

Lepidoptera are one of the largest insect orders, traditionally divided into 2 groups based on morphological and ecological features: butterflies and moths. While butterflies



**Fig. 1. a–c** *Drosophila melanogaster* mitotic chromosomes. **a, b** Same prometaphase after Giemsa staining (**a**) and C-banding (**b**). **c** Early anaphase. **d** Part of a Giemsa-stained early anaphase of a *Pieris brassicae* caterpillar cerebral ganglion cell, in which indisputable primary constrictions exist on both acrocentric and submetacentric chromosomes. Scale bar, 10  $\mu\text{m}$  (**a–c**) and 5  $\mu\text{m}$  (**d**).

**Table 1.** Chromosome numbers and DNA content in 4 reference species

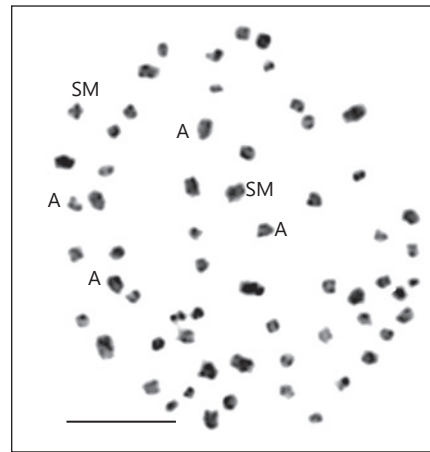
Species	Whole genome		Average chromosome (HSA8, DME2)		
	diploid chromosome number	diploid DNA content, Mb	size, Mb	percent of HSA8	percent of DME2
<i>Homo sapiens</i>	46	6,400	145 (8)	100	330
<i>Bombyx mori</i>	56	864 <sup>a</sup>	15.4	11	35
<i>Pieris rapae</i>	50	492 <sup>a</sup>	10	7	23
<i>Drosophila melanogaster</i>	8	279 <sup>b</sup>	44 (2)	30	100

A medium-sized chromosome of *P. rapae* is about 15-fold smaller than a human chromosome 8 (HSA8); 4.5-fold smaller than a *D. melanogaster* chromosome 2 (DME2), and almost 2-fold smaller than an average chromosome of *B. mori*. <sup>a</sup> Shen et al. [2016]. <sup>b</sup> Carvalho [2002].

form the superfamily Papilionoidea, which comprises about 18,000 species, moths do not form a monophyletic group of species [Misof et al., 2014]. Nevertheless, the distinction between butterflies and moths remains practical. Both butterflies and moths are classically considered to have holokinetic chromosomes [Suomalainen, 1969; Murakami and Imai, 1974; Maeki, 1980a, b, 1981]. The haploid genome of Lepidoptera, with a mean C-value of

$0.66 \pm 0.04$  pg DNA, is one of the smallest ones amongst insects, and amongst Lepidoptera, the mean size of the butterfly genome (0.4 pg) is almost half that of the moth genome (0.7 pg) [calculated from Gregory and Herbert, 2003; Gregory, 2011]. This suggests that some differences exist in the chromosome structure and composition of these 2 groups, but the question remains open because cytomolecular studies have essentially been limited to moths,

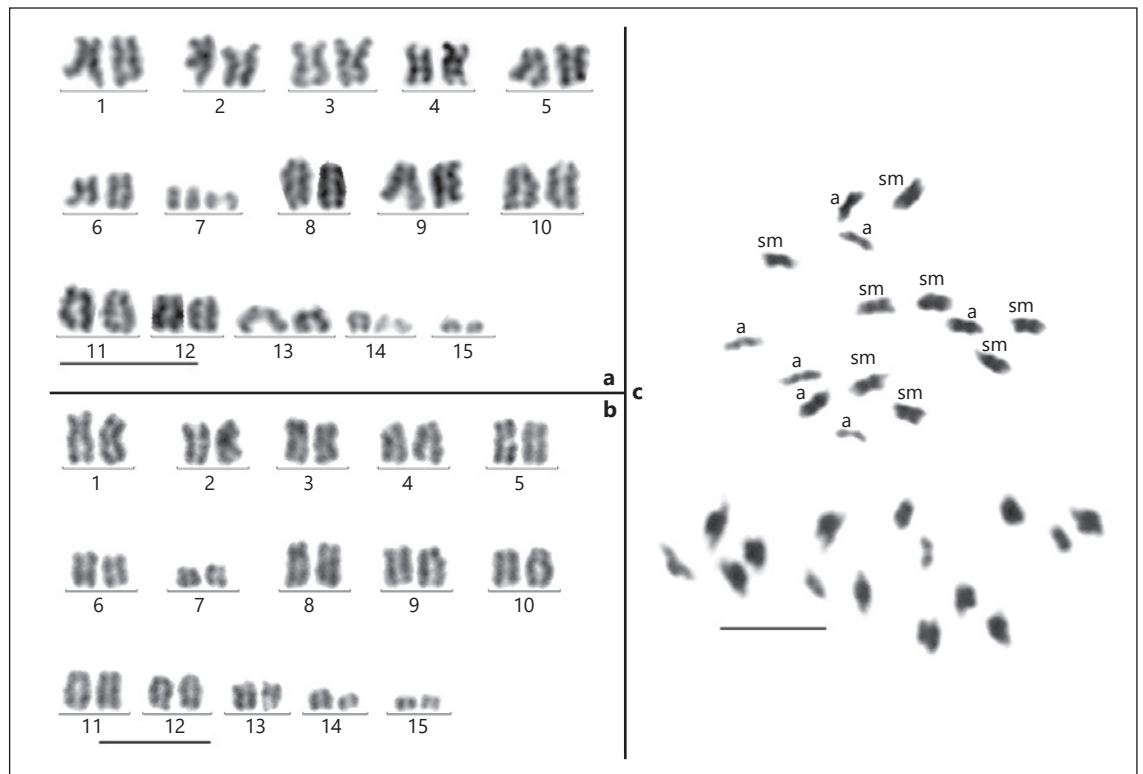
which have a larger economic importance than butterflies [Wolf et al., 1997; Mediouni et al., 2004; Fukova et al., 2005]. Compared to the mammalian genome ( $\pm 3.5$  pg), the small size of the Lepidoptera genome is partly due to the relative paucity in the various types of repeated DNA sequences, including transposable elements [d'Alençon et al., 2010]. In spite of their small genome, their modal number of chromosomes ( $2n = 62$ ) is among the highest in animals. A direct consequence is that butterfly chromosomes are very small, about 1/15 of medium-sized mammalian chromosomes, and lack structures harbouring DNA repeats such as primary constrictions (pericentromeric heterochromatin) and euchromatin banding. Most cytogenetic data on butterflies have used male gonads and the techniques often privileged squashes or direct fixation without previous hypotonic shock and colchicine treatment. In such conditions, mitotic chromosomes often look like dots at metaphase and short shapeless filaments at prometaphase. The difficulty to observe distinct sister chromatids and primary constrictions led to the notion that the chromosomes of butterflies lack localized centromeres and are thus holokinetic, a particularity reported in other insect orders such as Dermoptera, Ephemeroptera, Hemiptera, Heteroptera, Homoptera, Odonata, Psocoptera, Thysanoptera, and Trichoptera [for review, see Mola and Papeschi, 2006]. Comparisons of DNA sequences provide convincing evidence that these orders are scattered across the phylogenetic tree of insects [Drinnenberg et al., 2014; Misof et al., 2014; Kjer et al., 2016]. Thus, holocentricity has not evolved once, but rather derived from multiple convergent events from the widely spread centric chromosomes [Bauer, 1967; Melters et al., 2012]. In butterflies as in other taxa, the holocentric nature of chromosomes was confirmed by the lack of centromeric histone H3 variant CenH3 [Drinnenberg et al., 2014]. In spite of real advances, as in the nematode *Caenorhabditis elegans* [Howe et al., 2001; Zedek and Bures, 2012], the structure of holocentric chromosomes remains largely unknown, in particular in insects, and there is no strong argument to consider that identical structures replaced localised centromeres in the various taxonomic groups which independently acquired holocentricity during evolution. Consequently, holochromosomes may differ from order to order, and even among various taxa inside a given order, as butterflies and moths amongst Lepidoptera. Another difficulty is that the appearance of chromosomes may depend on several parameters such as their size, the phase in the cell cycle, the tissue studied, and the technique used. It is well known that the primary constrictions marking the centromeres,



**Fig. 2.** Mitotic metaphase of *Anaea euryppyle*. Most chromosomes look acrocentric and a few look submetacentric (some of them are labelled A and SM, respectively). The DNA content of each *D. melanogaster* metacentric autosome (Fig. 1) is equivalent to that of about 10 *A. euryppyle* chromosomes. Scale bar, 10  $\mu\text{m}$ .

hardly visible at early prometaphase, become obvious at metaphase when chromatids are condensed and cohesins are cleaved. Similar to butterflies, other insect cytogenetic studies were generally performed on male germinal cells [Smith and Virkki, 1978]. Working on both beetles and mouse male germinal cells, we were surprised to occasionally observe similar atypical morphologies of their monocentric chromosomes. A more systematic study was then performed on mouse spermatogenesis, which showed that the appearance of mitotic chromosomes deeply changes along divisions from gonocytes to late spermatogonia [Coffigny et al., 1999]. While chromosomes have hypercohesive, long and thin chromatids with hypermethylated DNA in gonocytes, they become shorter with more fuzzy and separated chromatids concomitantly with their hypermethylation loss in spermatogonia [Bernardino-Sgherri et al., 2002]. The strong relationship between DNA methylation status and chromatid compaction and cohesion remained unexplained, but the similarity of transient morphological changes of chromosomes in both mouse and beetles [personal data] suggests that they may occur in a large range of animals. These tissue and developmental stage changes may render difficult the interpretation of chromosome morphology in germ cells. Electron microscopy also showed that the size of the kinetochore plate could vary at different stages of gametogenesis [Wolf et al., 1997].

During meiosis, the behaviour of monocentric and holokinetic chromosomes was found to be different. Meiosis is described as inverted in many species with holochromo-



**Fig. 3.** *Pieris brassicae*. **a, b** Karyotypes from caterpillar brain cells. Sex chromosomes are not identified. **c** “Brother” spermatocytes II displaying acrocentric (a) and submetacentric (sm) chromosomes. Their morphology, with strongly stained and highly compacted proximal and poorly stained fuzzy distal regions, seems to be typical for butterflies. Scale bars, 10  $\mu$ m.

somes [Mola and Papeschi, 2006; Lukhtanov et al., 2018]: at metaphase I, bivalents are disposed perpendicularly to the spindle, each homologue is attached to several spindle fibres and sister chromatids, instead of homologous chromosomes, segregate at anaphase I (pre-reduction) as in the post-reduction of canonical meiosis in species with monocentric chromosomes. Consequently, the typical morphology of chromosomes at metaphase II (MII), with 1 centromere linking 2 well-separated fuzzy sister chromatids, may not be observed in species with true holochromosomes, but the difficulty to observe the morphology of the small MII butterfly chromosomes prevented assessment of the occurrence of inverted meiosis. However, some authors [Lukhtanov et al., 2020] consider that meiosis can be flexible, canonical or inverted, within the same individual. The presence of holochromosomes was questioned in some butterfly species [Rishi and Rishi, 1977, 1979; Gus et al., 1983] and monocentric mitotic chromosomes with “G-banded” chromatids were even described in Pieridae [Bigger, 1975], but the photos shown were much less convincing than the drawings, a flaw also shared by many reports on holochromosomes. With the

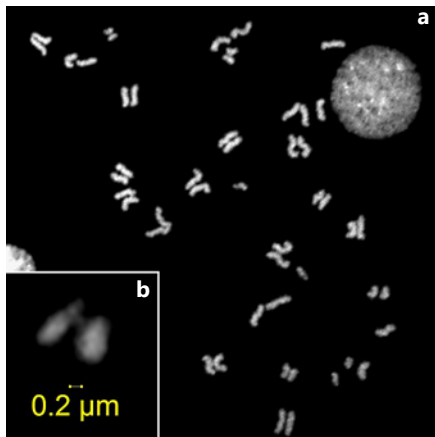
purpose of studying the karyotypes of some butterfly species, we adapted our usual technique to this particular material [McClure et al., 2017]. After some additional improvements, we describe here a very simple technique for the study of Lepidoptera chromosomes, particularly adapted to small cell samples. It allowed us to observe in mitotic cells not only atypical monocentric chromosomes, but also bi-chromatidic chromosomes at meiotic metaphase II, hardly compatible with the occurrence of an inverted meiosis. Chromosomes of some selected species of butterflies are shown and compared to those of *Drosophila melanogaster*, whose chromosomes are well known and whose DNA content is not very different. The meaning of these basic cytogenetic observations is discussed.

## Materials and Methods

### Species

*D. melanogaster* Melgen, 1930, obtained from a laboratory strain, was selected for chromosome size comparisons.





**Fig. 4.** **a** Confocal image of a DAPI-stained ganglion cell at metaphase/anaphase transition. **b** Higher magnification of a small acrocentric chromosome from another cell.

Butterfly species included in this study are the following:

*Anaea euryppyle* Hübner, 1819 (Pointed Leafwing, Nymphalidae, Charaxinae) is a Neotropical species. Specimens were obtained as pupae from a butterfly farm. This species was selected amongst those we studied because its chromosome number of 62 is presumed to be that or very close to that of butterfly ancestors.

*Pieris brassicae* Linnaeus, 1758 (Large White, Pieridae) was caught (eggs, caterpillars, and imago) at Bois-le-Roi, France (48.28.25 N, 02.41.50 E). This species was selected because the holocentricity of its chromosomes was previously questioned [Bigger, 1975; Rishi and Rishi, 1977].

*Pieris rapae* Linnaeus, 1758 (Small White, Pieridae), caught at Bois-le-Roi, was selected because its genome size is known.

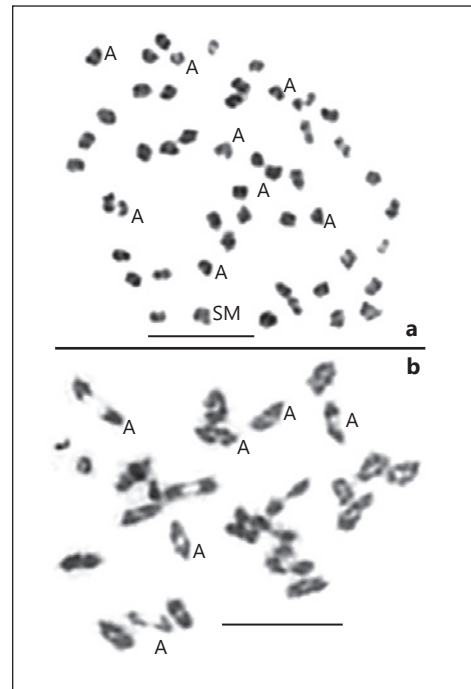
*Archaeoprepona demophon* Linnaeus, 1758 (One-spotted Demophon, Nymphalidae, Charaxinae). This neotropical species, obtained as pupae from a butterfly farm, was selected for its small number of chromosomes.

*Melinaea menophilus* n. ssp. Hewitson, 1856 (Hewitson's tiger, Nymphalidae, Danainae). Specimens of this neotropical species, obtained from eggs laid in captivity by wild-caught females, were also selected for their small chromosome number.

*Ithomia salapia aquinia* Hopffer, 1874 (Nymphalidae, Danainae). This neotropical species was selected among those studied because its chromosomes exhibit many C-bands. Specimens were obtained from eggs laid in captivity by wild-caught females.

#### Methods

Dividing cells were obtained from either cerebral ganglia of caterpillar (*Pieris* species) or testes from freshly killed male imago (all species). All dissections were performed on a glass slide in 1 or 2 drops of a solution of 0.88 g KCl in 100 mL distilled water. Important remark: fresh KCl powder is not stable and becomes more or less quickly hydrated, especially in the field. We deliberately used hydrated KCl, more stable for weighing. All centrifugations were performed at about 7 g for 7 min. After all centrifugations and settling, cells were suspended by gently tapping the tube. Carnoy I fixative was used for all fixations. Staining was performed in 2% Giemsa in tap water for 7–10 min, and occasionally with DAPI. C-banding was performed as described by Angus [1982].



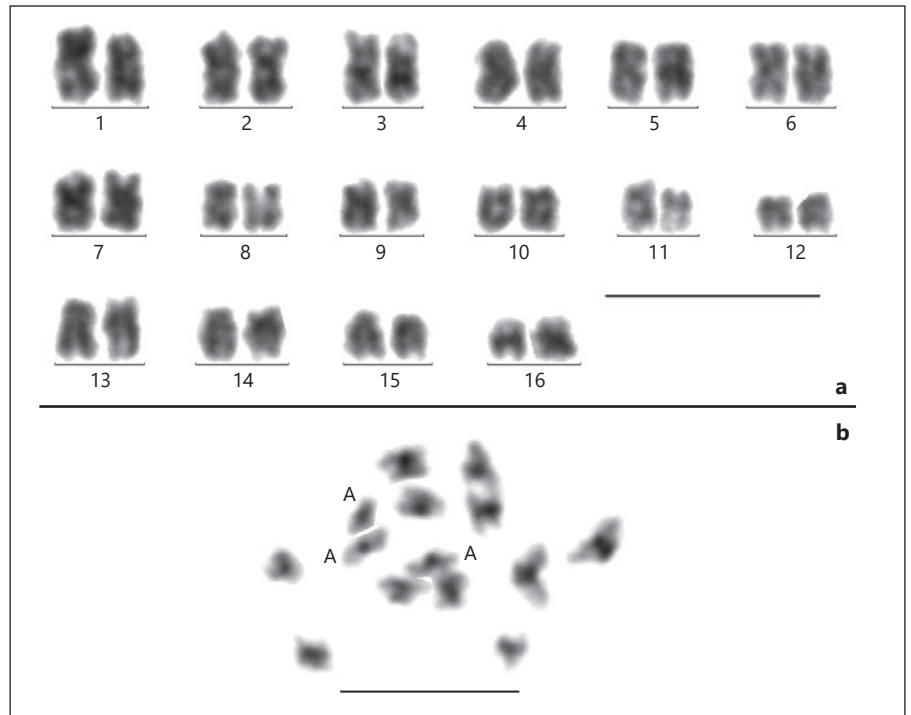
**Fig. 5.** *Pieris rapae*. **a** Metaphase of a caterpillar brain cell. Most chromosomes look acrocentric, some are indicated (A). **b** Metaphase I/anaphase I transition. Homologues in most bivalents are in end-to-end association. Some of them are more or less dissociated and look acrocentric and pulled by a single point, their centromere, and not by multiple points along the chromatids, as expected for holochromosomes. Scale bars, 10  $\mu$ m.

#### Cerebral Ganglia and Eggs

Eggs or freshly dissected ganglia cells were placed and ruptured inside an Eppendorf tube where they were maintained in 1 mL of the 0.88 g/L KCl solution added with colchicine (5  $\mu$ L of a 4 mg/L solution) for 45 min. After centrifugation, the supernatant was replaced by either an aqueous solution of 0.55 g/L KCl or foetal calf serum diluted in water (1 vol:4 vol) for 10 min. One drop of Carnoy fixative was added just before centrifugation. The pellet obtained was not always visible and about 100  $\mu$ L was left in the tube before immediate addition of 1 mL fixative. After a new centrifugation, about 75–100  $\mu$ L of supernatant were left in which cells were gently suspended with a Pasteur pipette and dropped from about 20 cm on glass slides special for FISH.

#### Testicle

Immediately after dissection, the unique testicle was placed in an Eppendorf tube containing 0.6 mL of an aqueous solution of KCl (0.85 g/L), to which colchicine was eventually added, as above. The testicle was then ruptured using a piston adapted to the internal diameter of the tube, which was gently turned for about 10 s. For salvaging adhering cells, the piston was rinsed with 2–3 drops of the 0.85 g/L KCl solution, of which 0.5 mL was added. The tube was gently tapped, left for 1 h, tapped again and 1 drop of fixative was added. Centrifugation, fixation, and spreading of the cells were as above.



**Fig. 6.** *Archaeoprepona demophon*. **a** Karyotype of a spermatogonium, which displays 10–11 submetacentric and 5–6 acrocentric pairs. **b** Spontaneous pseudo C-banding of a spermatocyte II with compacted proximal and fuzzy distal regions (2 chromosomes are missing). As in the karyotype, acrocentric chromosomes (A) are a minority. Scale bars, 10  $\mu$ m.

#### Confocal Microscopy

We employed a recent method developed by Zeiss corporation for studying specimens in thin sections, using confocal laser scanning microscopy and image processing software that allowed us to produce high-resolution 3-dimensional visualizations. We acquired confocal images with a Zeiss LSM 880 laser-scanning confocal microscope using a 63 $\times$ /1.4 oil DIC objective. The DAPI fluorescence signal was collected with an Airyscan head using a 32 GASP detector array (quantum efficiency of the detector was about 50%) in super resolution mode, which increases the SNR and the resolution by a factor 1.7. DAPI fluorescence of the samples was excited by the 405 nm diode laser (power 1% with pixel dwell 2.52  $\mu$ s). Emission was collected with a band pass filter 420–480 + 495–550 nm. Images were acquired with 16-bit depth and 0.2 airy unit for each elementary detector of the Airyscan head and recorded with pixel size of 35 nm. Typically producing 11 slice z-stacks comprising individual focal planes, each separated by a 160-nm z-step, corresponding to a z-depth of 1.75  $\mu$ m. The fluorescence signal from each z-plane was projected onto a maximum projection image by the software Zen Black version 2.3 (Zeiss corporation). Images were processed using the Fiji software [Schindelin et al., 2012].

## Results

### *Drosophila melanogaster*

Its well-known male diploid karyotype (2n = 8), obtained with a technique close to that of butterflies, is shown for comparison in Figure 1a. Its diploid DNA content is 279 Mb

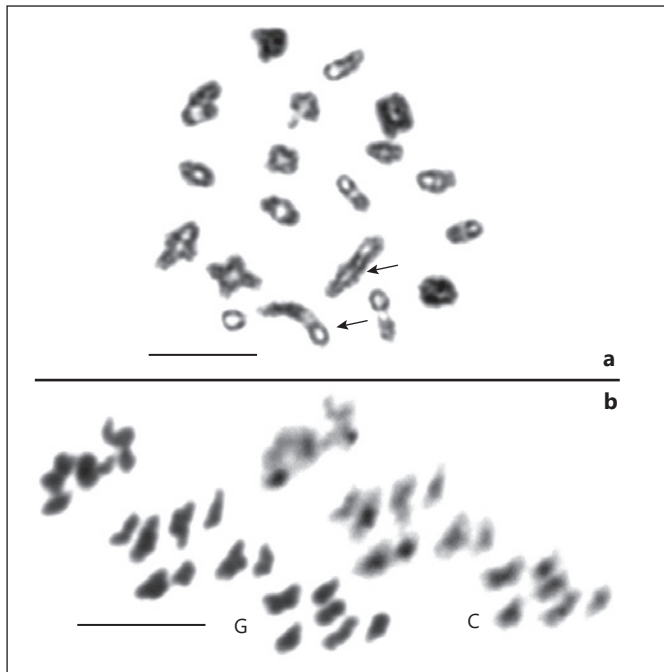
[Adams et al., 2000] (Table 1). C-banded heterochromatin, which represents about one sixth (chromosome 2) to nine tenths (chromosomes 4 and Y) of whole chromosome lengths, largely corresponds to regions of sister chromatid joining, independently of the immediate centromere proximity (Fig. 1b). At metaphase/anaphase transition (Fig. 1c), heterochromatin disjoins but sister chromatids remain loosely attached, presumably by their centromeric regions.

### *Anaea euryppyle*

The male karyotype is composed of 62 chromosomes (62,ZZ), the very small size of which makes the analysis of their morphology difficult. However, most of them look acrocentric (Fig. 2), alongside with some submetacentric chromosomes. At diakinesis, all but 2 bivalents display 1 chiasma, most frequently in interstitial position (not shown).

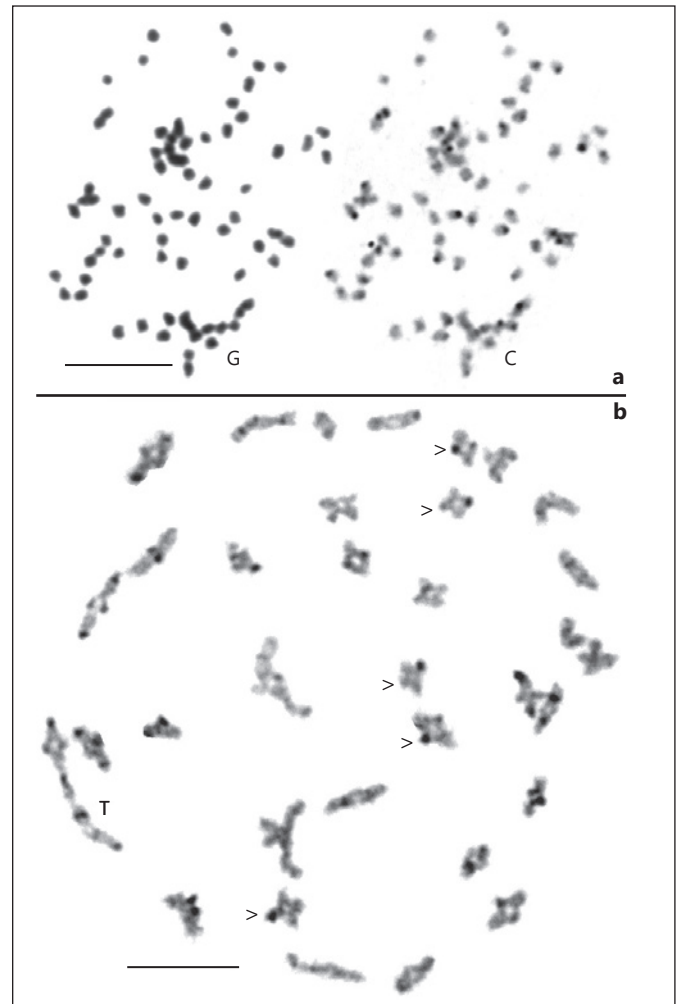
### *Pieris brassicae*

As previously described [Bigger, 1975], the male karyotype of *P. brassicae* is composed of 30 chromosomes: 30,ZZ. The discrimination of sister chromatids in mitotic chromosomes is easier in caterpillar ganglia cells and eggs, but is occasionally possible in germ cells. After Giemsa staining, chromosomes generally have no clear primary constriction, i.e., no lighter staining regions,



**Fig. 7.** *Melinaea menophilus*. **a** Metaphase I displaying 16 bivalents and 2 trivalents (arrows). **b** Sequentially Giemsa-stained (G) and C-banded (C) metaphase II in which many chromosomes look submetacentric. Scale bars, 10  $\mu$ m.

but they often display one region where sister chromatids are brought closer together, which suggests the presence of localized centromeres (Fig. 3a, b; online suppl. Fig. S1, S2; see [www.karger.com/doi/10.1159/000526034](http://www.karger.com/doi/10.1159/000526034) for all online suppl. material). Somatic and germ cells roughly display similar chromosome patterns, with 7 submetacentric and 8 acrocentric chromosomes (Fig. 3a–c). The presence of monocentric chromosomes is clearer at metaphase/anaphase transition: at this stage, the morphology of *D. melanogaster* and *P. brassicae* chromosomes is quite similar (compare with Fig. 1c, d). More or less discrete C-bands on either one or both homologues at many terminal but no interstitial regions indicate the presence of polymorphic juxtatelomeric heterochromatin. In some eggs or ganglia cells, one chromosome is almost entirely C-banded (online suppl. Fig. S3). This particularity is not seen in homogametic ZZ spermatogonia, which may indicate that the W chromosome of ZW females is largely composed of C-banded heterochromatin, which fits with the richness in repetitive DNA elements of the W described in some moths and butterflies [Sahara et al., 2012]. In meiotic cells at diakinesis/metaphase I, a majority of bivalents (10–12/15) have a ring configuration (2 chiasmata). All have at least 1 terminal



**Fig. 8.** *Ithomia salapia aquinia*. **a** Giemsa-stained (G, left) and C-banded (C, right) spermatogonial metaphase. **b** C-banded diakinesis exhibiting 33 bivalents and 1 trivalent (T). C-bands are more intense than in the spermatogonium and located in up to 4 telomeric regions per bivalent. Their frequent asymmetry (arrowheads) demonstrates strong polymorphism. They are located at intercalary positions in a few bivalents, possibly formed by 2 metacentric chromosomes. Scale bars, 10  $\mu$ m.

chiasma, and about half of the second chiasmata are interstitial. At metaphase I/anaphase I transition, chromosomes are very small and remain attached by the extremity of 1 or 2 chromatids but they have not the side-by-side position described for holochromosomes (online suppl. Fig. S4). At metaphase II, chromosomes remain very small and their compaction is unusual. Their sister chromatids are distally well separated, fuzzy, and lightly stained, but clearly linked at a position of locally highly compacted chromatin. They form submetacentric and acrocentric chromosomes in numbers comparable to



those of mitotic divisions (Fig. 3c). To further explore chromosome morphologies, DAPI-stained mitotic cells at metaphase/anaphase transition were analysed through a high-resolution confocal microscope, which evidenced discrete chromatin amounts linking the sister chromatids at the place of their putative centromere (Fig. 4).

#### *Pieris rapae*

The male karyotype is composed of 50 chromosomes: 50,ZZ. Although the small size of chromosomes makes the analysis difficult, most mitotic chromosomes look acrocentric, and thus monocentric (Fig. 5a). The acrocentric morphology of many chromosomes is confirmed at metaphase I/anaphase I transition, when the homologues begin splitting (Fig. 5b). This stage contradicts the occurrence of an inverted meiosis.

#### *Archaeoprepona demophon*

The male karyotype is composed of 32 chromosomes: 32,ZZ, including 11 pairs of submetacentrics (Fig. 6a). At diakinesis/metaphase I, most bivalents display 1 interstitial chiasma (not shown). At metaphase II, largely separated sister chromatids are pale and fuzzy, whereas they look compacted at their junction, which makes a pseudo C-banding (Fig. 6b).

#### *Melinaea menophilus* n. ssp.

An intraspecific polymorphism is present in karyotypes of the males studied: 38,ZZ and 40,ZZ. Observed mitotic cells, which have long chromosomes with tightly associated chromatids, were probably in prometaphase and chromosome morphology was hardly visible. The polymorphism is confirmed at metaphase I of meiosis by varying numbers of bivalents (16 or 17) and the presence of trivalents, which indicates the heterozygous state for two chromosome rearrangements (Fig. 7a). Metaphases II display typical submetacentric and acrocentric chromosomes (Fig. 7b). Such morphologies are hardly compatible with an inverted meiosis.

#### *Ithomia salapia aquinia*

The male mitotic karyotype is composed of 69 chromosomes. After C-banding, many chromosomes exhibit one band, most often in a terminal region (Fig. 8a). Diakinesis display 33 bivalents and 1 trivalent: the specimen studied was probably heterozygous for 1 chromosome fusion or fission, which suggests that specimens with 68 or 70 chromosomes exist in the natural population. As usual in our hands with insects, C-banding is more intense in meiotic than in mitotic cells and thus,

many more chromosomes look C-banded at diakinesis (Fig. 8b). Individual chromosomes from each bivalent display 0, 1, or 2 C-bands. Differences between homologues (arrowheads) demonstrate the existence of a polymorphism of C-banded heterochromatin repartition. Most bivalents exhibit 1 chiasma in median position and do not differ from acrocentric bivalents of canonical meiosis. A few other bivalents form a distal chiasma, and each homologue exhibits a C-band in median position. On the whole, the location of C-bands at chromosome ends characterizes telomeric rather than centromeric heterochromatin.

## Discussion

### *Mitotic Chromosomes Exhibit Centromere-Like Regions*

In a recent study of the genus *Melinaea* (Nymphalidae) in which presumed holocentric chromosomes were involved in a complex evolution by multiple fissions and fusions, we were surprised to observe that each chromosome was apparently attached to a single and not to multiple spindle fibres, as would be expected for holochromosomes at anaphase I of meiosis [McClure et al., 2017]. However, the lack of clear primary constrictions on the mitotic chromosomes prevented us to challenge their holokinetic nature. By using improved chromosome spreading techniques, the classification of mitotic chromosomes based on their acrocentric or submetacentric morphology was made possible in species with low chromosome numbers. Indeed, our images suggest that the chromosomes might not be holocentric. However, compared to classical monocentric chromosomes their morphology is particular: they do not exhibit the Giemsa-negative primary constrictions that typically mark centromeric regions. Usually, these primary constrictions harbour, in addition to the proper centromere, large amounts of highly repeated (satellite) DNA resistant to denaturation and nested into so-called constitutive heterochromatin generally stained after C-banding. The putative centromere regions of butterflies shown above are marked only by a well-localized, but loose coalescence of the chromatids. The poorness in highly repeated sequences (satellite DNA) in the genome of butterflies may explain the frequent paucity of heterochromatin, C-banding, and primary constrictions, but it does not demonstrate the lack of functional centromeres, which may be nested in other DNA sequences, more difficult to identify. At metaphase/anaphase transition of mitotic cells, the use of a high-resolution confocal microscope on DAPI-stained chromo-

somes discloses discreet chromatin, which links the 2 sister chromatids at the location of the putative centromere (Fig. 4). Yet, butterfly chromosomes lack the centromeric protein CenH3 (CENP-A of mammals), considered to be present in all centromeres of monocentric chromosomes [Drinnenberg et al., 2014]. Therefore, our observations suggest the presence of centromere-like regions, which display some centromere functions despite the loss of CenH3. Alternatively, some unknown localised chromatin components may maintain the cohesion of sister chromatids until anaphase, inducing this recurrent morphology. Only cytomolecular approaches would provide a convincing answer [Cabral et al., 2014; Heckmann et al., 2014].

The difficulty for distinguishing butterfly chromosome morphology is materialised by comparing the ( $2n = 8$ ) karyotype of *D. melanogaster* to the ( $2n = 50$ ) karyotype of *P. rapae*, which has a slightly higher DNA content (Table 1). The DNA content of an average chromosome of *P. rapae* is about 9.8 Mb, whereas chromosome 2 of *D. melanogaster* is composed of 48.8 Mb, hence a ratio of 1/5. Likewise, the average DNA content of a medium-sized butterfly chromosome represents about 8% of human chromosome 1. According to ISCN [1985] this chromosome exhibits about 27 bands at metaphase, 40 bands at prometaphase, and 50 bands at prophase. Thus, an average butterfly chromosome is scarcely larger than 2 (metaphase), 3 (prometaphase), or 4 (prophase) bands and is therefore very small compared to a mammalian chromosome. This may explain why, in the literature, butterfly chromosomes usually look like dots at metaphase and thin filaments with coalescent chromatids at prophase. As in any prophase, their chromatids are hardly discriminated. Furthermore, their putative centromeres, devoid of heterochromatin, are hardly detectable. Numerical variations of chromosomes may also be considered to challenge their holocentricity, by comparing closely related species with very different chromosome numbers, as the 2 pierid species studied here. Proportionally, more submetacentric chromosomes are expected in karyotypes with a smaller number of chromosomes than values close to the presumed ancestral number (62). Indeed, submetacentric chromosomes are observed in *P. brassicae* ( $2n = 30$ ) and *A. demophon* ( $2n = 32$ ), but hardly in *P. rapae* ( $2n = 50$ ) or *A. euryppyle* ( $2n = 62$ ) karyotypes, in which acrocentric chromosomes largely predominate. In butterfly species with such high chromosome numbers, many chromosomes are expected

to be acrocentric with their putative centromeres located near or at contact with telomeric heterochromatin [Chawla and Azzalin, 2008; Schoeftner and Blasco, 2009], whose instability [Murnane, 2012] may be responsible for multiple chromosome rearrangements, notably fusions. Another factor possibly involved in butterfly chromosome evolution is the very frequent distal position of their 18S rRNA genes on one or multiple chromosomes [Provaznikova et al., 2021]. In both beetles and primates, we showed that the terminal position of rRNA genes, which behave like fragile sites, is a major source of transmissible chromosome rearrangements [Dutrillaux et al., 2016; Gerbault-Seureau et al., 2017].

#### *Meiosis Is Not Inverted*

The large intra- and interspecific numerical variations of butterfly chromosomes usually represent indirect arguments in favour of their holocentricity, supposed to facilitate the correct segregation of asymmetrical chromosomes via an inverted meiosis [Mola and Papeschi, 2006; Lukhtanov et al., 2018]. One particularity of holocentricity is the attachment of chromosome bivalents to multiple spindle fibres at metaphase I/anaphase I of meiosis. A second particularity is that the first division is equational (separation of the two chromatids of each homologue), instead of reductional (separation of the homologues with non-cleaved centromeres). The cytological consequences should be visible at each stage of the meiotic process from the end of diplotene to metaphase II [see Fig. 9 in Lukhtanov et al., 2018], although some flexibility between canonical and inverted meiosis seems to be possible [Lukhtanov et al., 2020]. We have no argument to contest the existence of such meiosis in some species, but it does not seem to occur in the butterflies studied here for the following reasons:

- Diakinesis/metaphase I (Fig. 7a, 8b) bivalents and trivalents do not differ from those of canonical meiosis;
- metaphase I/anaphase I transition, as shown for *P. rapae* (Fig. 5b), perfectly fits with the presence of acrocentric chromosomes, as also exhibited by mitotic cells. The numerous acrocentrics have a well-marked centromere and clearly separated chromatids and homologues form bivalents in end-to-end (chiasmatic or achiasmatic?) association. This configuration is typical of a canonical meiosis;
- metaphases II display monocentric chromosomes, typical for this phase, with strongly apart and fuzzy chromatids linked by a compact centromeric region, often darker than chromatids (Fig. 3c, 6b, 7b). This

mimics a C-banding, a feature frequently observed in metaphases II of beetles (data not shown);

- and indeed, in a given species, similar proportions of acrocentric and submetacentric chromosomes can be recurrently observed in different mitotic karyotypes (compare Fig. 3a, b and online suppl. Fig. 1–3) and at both mitotic metaphase and metaphase II of meiosis (compare Fig. 3a and c).

Finally, the example of *M. menophilus*, which belongs to a genus with very high intra- and interspecific numerical polymorphisms [McClure et al., 2017], shows that such polymorphisms do not preclude gametogenesis with a canonical meiosis.

## Conclusion

The very small size of butterfly chromosomes makes the analysis of their morphology difficult. This difficulty is worsened by the lack of primary constrictions, i.e., centromeric heterochromatin, which contains highly repetitive (satellite) DNA sequences and is usually associated with particular staining, compaction, and joining of sister chromatids. In butterflies, heterochromatin is scarce, and essentially telomeric. In such conditions, putative centromeres would be marked by discrete joining of sister chromatids only, more visible in cohesin-less meiotic metaphases II than in mitotic prometaphases. We show that karyotypes with about 60 chromosomes, a number considered to be close to that of the butterfly ancestor, seem to be essentially composed of acrocentrics. Consequently, putative centromeres of butterfly chromosomes would be often located in or at close contact with telomeric heterochromatin, whose composition differs from that of centromeric heterochromatin [DeBaryshe and Pardue, 2011] and whose instability may be responsible for their numerous rearrangements. In other words, the large numerical variations of chromosomes observed in butterflies might be better explained by their telomere/centromere instability than by the facilitated transmission of rearranged chromosomes through inverted meiosis, whose occurrence is challenged here. However, these interpretations, suggested by classical cytogenetic observations, do not evidence that butterfly chromosomes are monocentric. They need to be validated by molecular approaches, but the basic technical improvements reported here could be useful for future cytological approaches.

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## Statement of Ethics

The authors have no ethical conflicts to disclose. Ethical approval is not required for this type of research.

## Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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## Author Contributions

Bernard Dutrillaux and Anne-Marie Dutrillaux conceived the study and performed the technical work. Marianne Elias obtained the funding. Bernard Dutrillaux, Anne-Marie Dutrillaux, Mélanie McClure, and Marianne Elias obtained the samples. Marc Gèze performed the confocal study. All authors discussed the project and findings. Bernard Dutrillaux wrote the manuscript with extensive contributions from Bertrand Bed'hom and Marianne Elias. All authors read and approved the manuscript.

## Data Availability Statement

All data are included in the manuscript and its supplementary data files.

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