

# Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera)

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**Abstract** We examined chromosomal distribution of major ribosomal DNAs (rDNAs), clustered in the nucleolar organizer regions (NORs), in 18 species of moths and butterflies using fluorescence in situ hybridization with a codling moth (*Cydia pomonella*) 18S rDNA probe. Most species showed one or two rDNA clusters in their haploid karyotype but exceptions with 4–11 clusters also occurred. Our results in a compilation with previous data revealed dynamic evolution of rDNA distribution in Lepidoptera except Noctuoidea, which showed a highly uniform rDNA pattern. In karyotypes with one NOR, interstitial location of rDNA prevailed, whereas two-NOR karyotypes showed mostly terminally located rDNA clusters. A possible origin of the single interstitial NOR by fusion between two NOR-chromosomes with terminal rDNA clusters lacks support in available data. In some species, spreading of rDNA to new, mostly terminal chromosome regions was found. The multiplication of rDNA clusters without alteration of chromosome numbers rules out chromosome fissions as a major

mechanism of rDNA expansion. Based on rDNA dynamics in Lepidoptera and considering the role of ordered nuclear architecture in karyotype evolution, we propose ectopic recombination, i.e., homologous recombination between repetitive sequences of non-homologous chromosomes, as a primary motive force in rDNA repatterning.

**Keywords** Ribosomal DNA · Nucleolar organizer region · Chromosome fusion · Chromosome fission · Karyotype evolution · Ectopic recombination

## Introduction

Chromosomes of moths and butterflies (Lepidoptera) are small, numerous and uniform in both shape and size (Robinson 1971; Bedo 1984). They lack a distinct primary constriction (the centromere) and, as a result, sister chromatids separate by parallel disjunction during mitotic metaphase; they are thus regarded as holokinetic with kinetochores extended over a large portion of the chromosome surface (Wolf 1996). Holokinetic nature of chromosomes is supposed to facilitate chromosome fusions and fissions (Wrens et al. 1994). However, the karyotype of Lepidoptera appears to be relatively stable as it preserves cytological similarities not only within this large and diverse order but also shares common features with the sister order Trichoptera, caddis-flies (Suomalainen 1966; Wolf et al. 1997), which diverged from a common ancestor about 200 MYA (Grimaldi and Engel 2005). Chromosome numbers range from  $n = 29$  to 31 in the majority of moths and butterflies (Robinson 1971; Brown et al. 2007). The modal, i.e. the most frequent chromosome number of  $n = 31$ , occurring from basal to highly derived clades, has been proposed as an

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ancestral karyotype of Lepidoptera (Lukhtanov 2000). Moreover, Yasukochi et al. (2006) and Pringle et al. (2007) reported unusually high degree of conserved synteny of genes between *Bombyx mori* (Bombycoidea) and *Heliconius melpomene* (Papilionoidea), which suggests evolutionary stability of lepidopteran linkage groups during last 103 MY. Conserved synteny of genes including conserved gene order between chromosome 15 of *B. mori* and a chromosome of *Manduca sexta* (Sphingidae, Bombycoidea) also supports these findings (Sahara et al. 2007). Recently, this has been corroborated by Beldade et al. (2009), who observed conserved assignment of genes to linkage groups between *B. mori* and *Bicyclus anynana* (Papilionoidea).

Uniformity of lepidopteran chromosomes, the absence of morphological landmarks (e.g., the centromeres) together with the lack of convenient differential techniques (cf. Bedo 1984) disabled identification of individual chromosomes by standard cytogenetic methods and for a long time restricted their study to simple chromosome counts. Mainly because the mitotic chromosomes were unfavourable for conventional cytogenetics, much longer meiotic chromosomes in the pachytene stage were employed for the so-called pachytene mapping (Traut 1976). But even the chromomere pattern of pachytene bivalents could not be generalized for chromosome identification in Lepidoptera. Nevertheless, this approach made possible the study of sex chromosome systems (WZ/ZZ and derived variants) as the female-specific W chromosome could be distinguished by its heterochromatin in a number of species (reviewed by Traut et al. 2007). More recently, modern approaches such as genomic in situ hybridization (GISH), comparative genomic hybridization (CGH), and fluorescence in situ hybridization (FISH) with W-chromosome painting probes allowed detailed studies on the evolution and molecular structure of the W chromosome (Traut et al. 1999; Mediouni et al. 2004; Fuková et al. 2005, 2007; Yoshido et al. 2005b, 2006; Vítková et al. 2007). However, autosomes still remain unexplored except *B. mori*, in which all chromosomes were mapped by the so-called BAC-FISH with probes prepared from bacterial artificial chromosome (BAC) clones (Sahara et al. 2003; Yoshido et al. 2005a).

Problems with chromosome identification, similar to those in Lepidoptera, occur in other groups of invertebrates as well. The only autosomes consistently identified in different species are chromosomes bearing tandem arrays of major ribosomal RNA (rRNA) genes (i.e., genes for 18S, 5.8S, and 28S rRNA). These arrays of the so-called rDNA are located in one or several nucleolar organizer regions (NORs). Series of works showed that the number and location of rDNA (or NOR) can be a useful marker for the study of karyotype evolution (e.g., Hirai et al. 1996; Roy et al. 2005; Bombarová et al. 2007). However, data on NORs in lepidopteran genomes are scarce, often obtained as

a by-product of principal research by conventional cytogenetic methods detecting only active nucleoli. In Lepidoptera, no particular study on NOR-chromosomes has been accomplished to date. In this work, we examined the number and distribution of rDNA clusters in 18 selected species of moths and butterflies by FISH with 18S rDNA probe with the aim to understand the role of rDNA in the karyotype evolution of Lepidoptera. Our study is the first systematic survey of NOR-chromosomes not only in Lepidoptera but also in species with holokinetic chromosomes in general. The latter is of a particular interest as holokinetic systems may be fundamentally different from monocentric ones in their constraints (Wrensch et al. 1994).

## Materials and methods

### Insects

Species examined along with their origin are given in Table 1. Specimens were either obtained from laboratory stocks or collected in natural populations. Detailed rearing conditions and diet of laboratory stocks are given in works cited in Table 1. In the field-collected species, except *Orgyia recens*, *Nymphalis xanthomelas*, and *Polyommatus bellargus*, fertilized females were captured, kept in plastic containers at laboratory conditions, and let to lay eggs. Hatched larvae were reared on appropriate host plants chosen according to species bionomy, at room temperature and native day/night regime. In *O. recens* and *N. xanthomelas*, larvae were collected on a hawthorn shrub and Chinese hackberry, respectively, and then reared on the same host plant at laboratory conditions. In the case of *P. bellargus*, adult males collected in the field were dissected.

### Chromosome preparations

Spread preparations of mitotic and meiotic chromosomes of all species except *P. bellargus* were made from both male and female gonads of 3–5th instar larvae as described in Mediouni et al. (2004). In *P. bellargus* spread chromosome preparations were obtained from gonads of male imago following the same procedure. The preparations were passed through an ethanol series (70, 80, and 100%; 30 s each) and stored at  $-20^{\circ}\text{C}$  until further use.

### Fluorescence in situ hybridization with 18S rDNA probe (rDNA-FISH)

Unlabelled, about 1,650 bp long 18S rDNA probe was generated by PCR from the colding moth, *Cydia pomonella*, genomic DNA. PCR was carried out using the

**Table 1** List of lepidopteran species used in the present study

Superfamily	Family/species	Source or locality	Rearing conditions; diet
Tortricoidea	Tortricidae		
	<i>Cydia pomonella</i>	Laboratory strain Krym-61	See Fuková et al. (2005)
Pyraloidea	Crambidae		
	<i>Ostrinia nubilalis</i>	Laboratory culture (O. Habušťová)	25°C, LD 12/12 h; wheat germ/alfalfa-based diet (Lewis and Lynch 1969)
Bombycoidea	Pyralidae		
	<i>Ephestia kuehniella</i>	Laboratory strain WT-C	See Rego and Marec (2003)
	Sphingidae		
	<i>Manduca sexta</i>	Laboratory strain (S. Barns and S. Reynolds, Bath Univ., UK)	See Sahara et al. (2007)
	Saturniidae		
	<i>Antheraea pernyi</i>	Laboratory strain (Z. Kajiura, Shinshu University, Japan)	Room conditions; chestnut leaves
	Papilionoidea		
	Pieridae		
	<i>Colias hyale</i>	Collected in České Budějovice, Czech Republic	Room conditions; alfalfa
	<i>Pieris rapae</i>	Collected in Ohrazení near České Budějovice, Czech Republic	Room conditions; rapeseed leaves
	<i>Pieris brassicae</i>	Collected in Ohrazení near České Budějovice, Czech Republic	Room conditions; rapeseed leaves
	Lycaenidae		
	<i>Polyommatus icarus</i>	Collected in České Budějovice, Czech Republic	Room conditions; bird's-foot trefoil
	<i>Polyommatus bellargus</i>	Collected in environs of Brno, Czech Republic	–
	Nymphalidae		
	<i>Nymphalis xanthomelas</i>	Collected in Hiroshima, Japan	Room conditions; Chinese hackberry leaves
	<i>Inachis io</i>	Collected in České Budějovice, Czech Republic	Room conditions; stinging nettle
Noctuoidea	Noctuidae		
	<i>Mamestra brassicae</i>	Collected in České Budějovice	Room conditions; dandelion leaves
	Arctiidae		
	<i>Arctia caja</i>	Collected near Velká Bíteš, Czech Republic	Room conditions; dandelion leaves
	Lymantriidae		
	<i>Lymantria dispar</i>	Collected near Mikulov in Milovice forest, Czech Republic	Room conditions; oak leaves
	<i>Orgyia recens</i>	Collected in Biharugra, Körös-Maros Nemzeti Park, Hungary	Room conditions; hawthorn leaves
	<i>Orgyia leucostigma</i>	Laboratory culture purchased from Insect Production Unit at Great Lakes Forest Research Centre, Sault Ste. Marie, Ontario, Canada	22°C, LD 12/12 h; birch leaves
	<i>Orgyia antiqua</i>	Laboratory strain	See Rego and Marec (2003)

18S-Gal forward (5'-CGATACCGCGAATGGCTCAAT A-3') and 18S-Gal reverse (5'-ACAAAGGGCAGGGACG TAATCAAC-3') primers as described in Fuková et al. (2005). The probes were labelled with biotin-14-dATP by nick translation using a Bionick Labeling System (Invitrogen, Life Technologies Inc., San Diego, CA, USA).

For rDNA-FISH, we used the procedure as previously reported in Fuková et al. (2005). Briefly, chromosome preparations were treated with 100 µg/mL RNase A to remove an excessive amount of rRNAs. After denaturation the chromosomes were hybridized with a probe cocktail

containing 15 ng of biotinylated 18S rDNA probe and 25 µg of sonicated salmon sperm DNA per slide. Hybridization signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West Grove, PA, USA), amplified with biotinylated anti-streptavidin (Vector Labs. Inc., Burlingame, CA, USA). The preparations were counterstained with 0.5 µg/mL of DAPI and mounted in antifade based on DABCO (for composition, see Mediouni et al. 2004).

Preparations were inspected in a Zeiss Axioplan 2 microscope equipped with a cooled F-View CCD camera

and AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany). Black-and-white images were captured separately for each fluorescent dye and then pseudocoloured (light blue for DAPI and red for Cy3) and superimposed with Adobe Photoshop, version 7.0.

## Results

Biotin-labelled probe, prepared from the 18S rRNA gene of the codling moth, *Cydia pomonella* (Tortricidae: Tortricidae), was first hybridized by FISH to mitotic and meiotic chromosomes of the source species. In accordance with results of Fuková et al. (2005), rDNA-FISH revealed two clusters of rDNA, localized in both subtelomeric regions of a single chromosome pair. Subsequently, the *C. pomonella* 18S rDNA probe was hybridized to chromosomal preparations of other species from four large lepidopteran superfamilies, Pyraloidea, Bombycoidea, Papilionoidea, and Noctuoidea. Due to evolutionary stability of the 18S rRNA gene, reliable hybridization signals were obtained in all species examined.

### Pyraloidea

In order to verify published data on NOR-chromosomes obtained by other cytogenetic methods, several previously examined lepidopteran species were included in this study. One of these species was the Mediterranean flour moth, *Ephestia kuehniella* (Pyralidae). In male pachytene complements of the flour moth consisting of 30 chromosome pairs, rDNA-FISH revealed two rDNA clusters localized at the ends of two bivalents (not shown). This confirmed electron microscopy observation of Marec and Traut (1993), who designated these NOR-bearing chromosomes for their distinctive length as NOR<sup>S</sup> (“small”) and NOR<sup>L</sup> (“large”). Pachytene nuclei of another representative of the superfamily Pyraloidea, the European corn borer (*Ostrinia nubilalis*) (Crambidae), showed 31 bivalents, which is consistent with the number stated by Keil et al. (1990). Five of them showed rDNA signals at their terminal regions (Fig. 1a).

### Bombycoidea

Mitotic spermatogonia of the tobacco hornworm, *Manduca sexta* (Sphingidae), showed  $2n = 56$  chromosomes of uniform shape (cf. Sahara et al. 2007). The 18S rDNA probe hybridized to two of these chromosomes (not shown). In pachytene nuclei of spermatocytes, the probe identified a single bivalent with an rDNA cluster located in an interstitial position near one end of the bivalent (Fig. 1b). This result corroborates recent finding of Sahara et al. (2007),

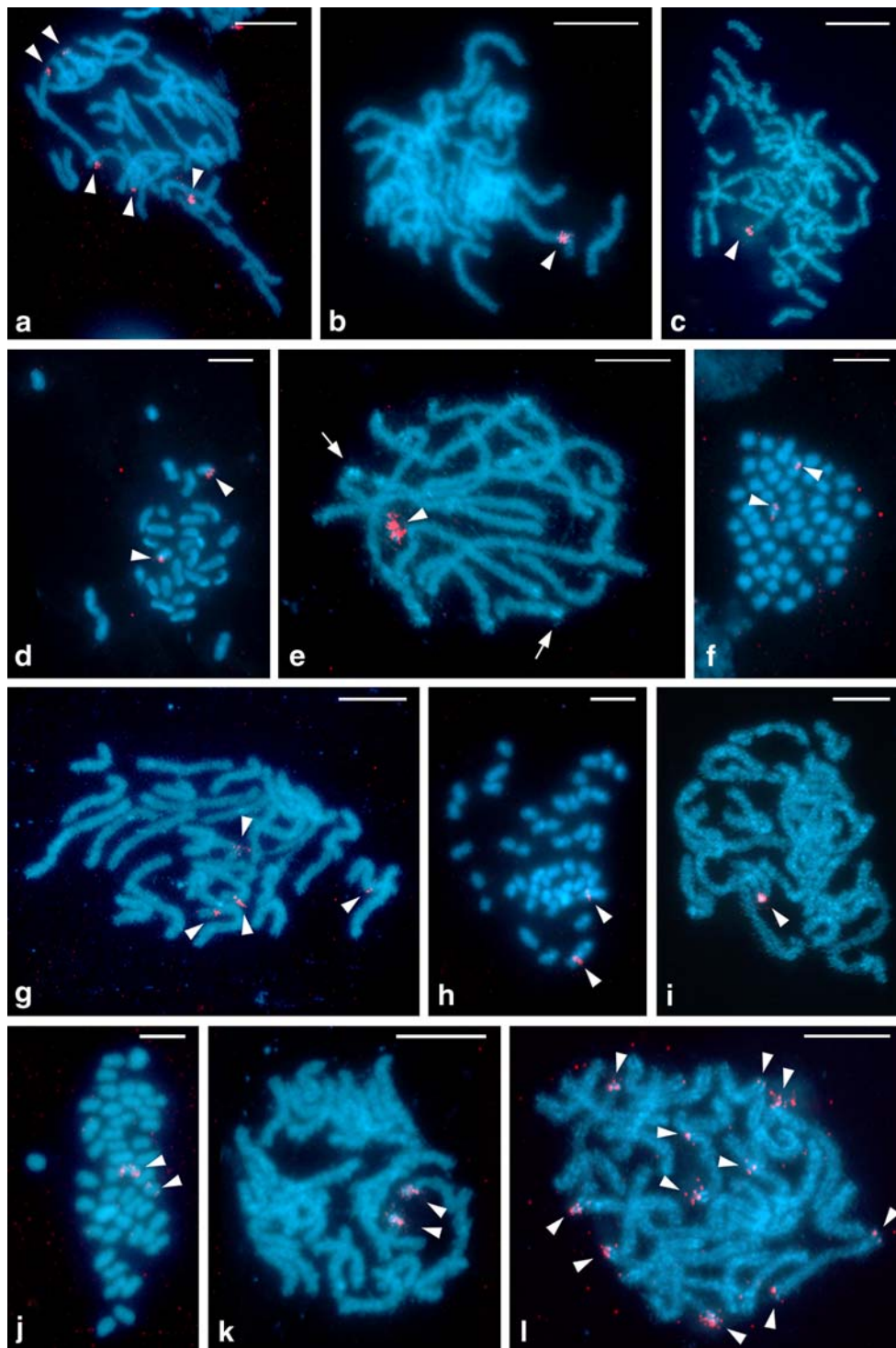
**Fig. 1** Localization of rDNA clusters in spread chromosome preparations from gonads of species belonging to superfamilies Pyraloidea (a), Bombycoidea (b, c), and Papilionoidea (d–l) as revealed by FISH with 18S rDNA probe (red signals, arrowheads). Chromosomes were counterstained with DAPI (blue). **a** Male pachytene complement of *Ostrinia nubilalis* (Crambidae). **b** Male pachytene complement of *Manduca sexta* (Sphingidae). **c** Female pachytene nucleus of *Antheraea pernyi* (Saturniidae). **d** Mitotic metaphase of male *Pieris brassicae* (Pieridae) consisting of  $2n = 30$  chromosomes. **e** Male pachytene complement of *P. brassicae*; note small DAPI-positive subterminal blocks of heterochromatin in some bivalents (arrows). **f** *Pieris rapae* (Pieridae) spermatogonial metaphase with  $2n = 50$  chromosomes. **g** Male pachytene complement of *Colias hyale* (Pieridae). **h** Spermatogonial metaphase of *Polyommatus icarus* (Lycaenidae) consisting of  $2n = 46$  chromosomes. **i** Male pachytene complement of *P. icarus*. **j** Meiotic metaphase I of *Polyommatus bellargus* male (Lycaenidae) with  $n = 45$  bivalents. **k** Female pachytene complement of *Nymphalis xanthomelas* (Nymphalidae); note the proximity of two rDNA clusters, each located at the end of a different NOR-bivalent; the two NORs form a single common nucleolus. **l** Female pachytene complement of *Inachis io* (Nymphalidae) originating from a Czech population showing eleven rDNA clusters. Bar = 10  $\mu\text{m}$  (a–c, e, g, i, k, and l); bar = 5  $\mu\text{m}$  (d, f, h, and j)

who observed a bivalent associated with a nucleolus in a similar position after staining of pachytene oocytes with lactic acetic orcein. The Chinese oak silkworm, *Antheraea pernyi* (Saturniidae), is a very interesting species for its high chromosome number of  $2n = 98$  (Robinson 1971). Pachytene complements of spread oocytes indeed contained numerous, relatively short bivalents, which were difficult to count. However, there was only one bivalent bearing a single rDNA cluster (NOR) in a terminal region (Fig. 1c). The NOR-bivalent belonged to the longest bivalents in *A. pernyi* pachytene complements.

### Papilionoidea

Members of families Pieridae, Lycaenidae, and Nymphalidae were examined. In mitotic complements of the large white, *Pieris brassicae* (Pieridae),  $2n = 30$  rod-shaped chromosomes were observed as previously reported by Bigger (1975). Two obviously homologous chromosomes of a similar size carried each terminal rDNA signals (Fig. 1d). In pachytene nuclei of spermatocytes, the 18S rDNA probe hybridized to a terminal region of one NOR-bivalent (Fig. 1e). Subterminal segments of some pachytene bivalents were highlighted with DAPI, indicating the presence of subtelomeric heterochromatin (see arrows in Fig. 1e). In a closely related species, the small white *Pieris rapae* (Pieridae), spermatogonial nuclei had  $2n = 50$  chromosomes (cf. Robinson 1971). Also in this species rDNA-FISH identified two mitotic chromosomes carrying rDNA clusters. The rDNA signals appeared terminally located in these small, densely packed chromosomes (Fig. 1f). Unfortunately, we failed to get preparations of





pachytene spermatocytes in this species and therefore, could not determine precisely the rDNA location at a high resolution of much longer pachytene bivalents. In spermatocytes of the pale clouded yellow, *Colias hyale* (Pieridae), four bivalents bearing rDNA clusters were regularly observed among 31 chromosome pairs of its pachytene

complements. All these clusters were localized in interstitial chromosomal regions (Fig. 1g).

In spermatogonial metaphases of the common blue, *Polyommatus icarus* (Lycaenidae),  $2n = 46$  chromosomes were observed. Hybridization signals of the rDNA probe were found in two largest chromosomes, most likely

corresponding to chromosome pair no. 1 in the study of Bigger (1975; Fig. 1h). A single rDNA cluster was found in pachytene nuclei of spermatocytes. It was located in an interstitial region near one end of the NOR-bivalent (Fig. 1i). In the Adonis blue, *Polyommatus bellargus* (Lycaenidae), only metaphase I chromosomes were found on preparations of testes from adult males. The metaphase I complement consisted of  $n = 45$  bivalents. Two of the bivalents showed each two clusters of hybridization signals, each cluster corresponding to one homologous chromosome. The rDNA signals of the smaller bivalent were much fainter than those of the larger bivalent, most likely indicating a small number of rDNA repeats (Fig. 1j). Positions of rDNA clusters on the bivalents could not be determined.

Two bivalents bearing strong terminal hybridization signals were observed in female pachytene nuclei of the yellow-legged tortoiseshell, *Nymphalis xanthomelas* (Nymphalidae), with a haploid chromosome number of  $n = 31$  (see Robinson 1971). The rDNA signals were located at the ends of these NOR-bivalents and formed small spheres, probably reflecting distribution of rRNA genes in nucleoli. The two NOR-bivalents were often oriented in head-to-head manner by their rDNA (NOR) ends, obviously due to association of their nucleoli in a common nucleolus (Fig. 1k). The peacock butterfly, *Inachis io* (Nymphalidae), has also a haploid chromosome number of  $n = 31$  (see Robinson 1971). In the Japanese subspecies *I. io geisha*, Yoshido et al. (2006) observed at least five NOR-bivalents in pachytene oocyte complements after genomic in situ hybridization (GISH) with female genomic DNA probe and DAPI counterstaining. We examined rDNA distribution in *I. io* specimens originating from a Czech population. In pachytene oocytes, rDNA-FISH identified up to 11 rDNA sites localized at the ends of seven bivalents; three bivalents had rDNA signals at one end and four bivalents at both ends (Fig. 1l). Some of the hybridization signals were relatively weak, most probably due to a small number of rDNA repeats. In addition, the number of the weak rDNA signals varied in some nuclei, probably because of a lower resolution of rDNA-FISH for the rDNA sites with low numbers of repeats.

#### Noctuoidea

In pachytene oocytes of *Lymantria dispar*, rDNA-FISH identified a single rDNA cluster located interstitially in a bivalent (not shown). This finding is in a good accord with a single NOR-bivalent observed in this species by Krider and Shields (1997). The karyotype of scarce vapourer, *Orgyia recens*, consists of  $n = 30$  chromosomes (Robinson 1971). Also in this species, we found a single rDNA cluster located interstitially in the NOR-bivalent of pachytene oocytes (Fig. 2a). Metaphase I spermatocyte nuclei of the

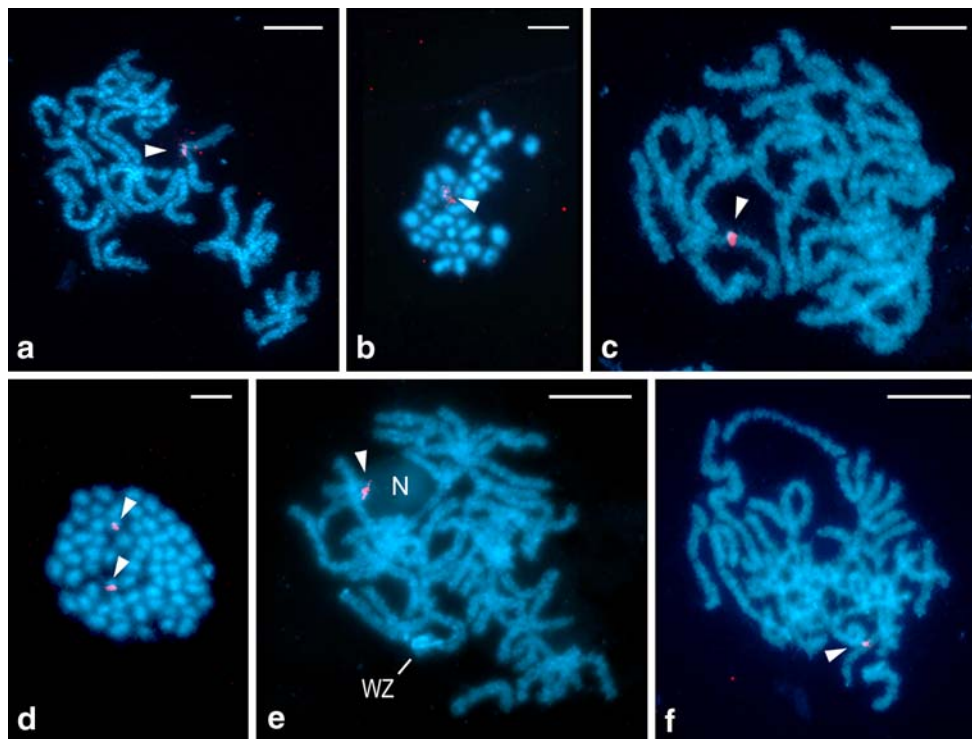
white-marked tussock moth, *Orgyia leucostigma*, consisted of  $n = 28$  bivalents, which is in keeping with the earlier published data (see Robinson 1971). The 18S rDNA probe hybridized to a single bivalent of its metaphase I complement (Fig. 2b). Accordingly, one NOR-bivalent was found in pachytene nuclei of spermatocytes with an interstitial rDNA cluster (Fig. 2c). Furthermore, rDNA-FISH was carried out on chromosomal preparations of *O. antiqua* males. In metaphase I spermatocyte nuclei, one out of 14 bivalents showed clear rDNA signals (not shown). The presence of a single cluster of rRNA genes was confirmed in nuclei of pachytene spermatocytes, which also showed an NOR-bivalent with an interstitial rDNA site (not shown). These results are consistent with observations of a single autosome NOR-bivalent in orcein-stained pachytene oocytes of *O. antiqua* (Traut and Clarke 1997; Traut and Marec 1997). We also examined rDNA distribution in the garden tiger, *Arctia caja*, a representative of the family Arctiidae, which is closely related to the family Lymntriidae (Mitchell et al. 2006). Male mitotic metaphase complements consisted of  $2n = 62$  chromosomes, two of which bore hybridization signals of the rDNA probe (Fig. 2d). Accordingly, a single chromosome pair (i.e., NOR-bivalent) with an interstitial rDNA cluster was observed in pachytene nuclei of both sexes. Moreover, the WZ sex-chromosome bivalent was easily discernible in oocyte pachytene nuclei according to a conspicuous block of W-heterochromatin deeply stained with DAPI (Fig. 2e). Similar to species of Lymantriidae and Arctiidae, also the cabbage moth, *Mamestra brassicae* (Noctuidae), with  $n = 31$  (Robinson 1971) had only one NOR-bivalent in spermatocyte pachytene nuclei, with an rDNA site located in an interstitial region (Fig. 2f).

## Discussion

### Number and location of rDNA clusters in Lepidoptera

In the present study, we examined chromosomal distribution of rDNA (and thus also potential NORs) in 18 species of the lepidopteran clade Ditrysia using FISH with a codling moth (*C. pomonella*) 18S rDNA probe. Our results in a compilation with previous data on rDNA/NORs in 32 lepidopteran species from five large superfamilies are summarized in Fig. 3 along with haploid chromosome numbers.

The basal superfamilies of Ditrysia, Gelechioidea and Tortricoidea, showed three different patterns of rDNA distribution in four species with similar chromosome numbers,  $n = 28$ –30 (Fig. 3). No common rDNA pattern was found in three species of Pyraloidea with  $n = 30$ –31, where *E. kuehniella* and *Ectomyelois ceratoniae* have two NORs but one NOR in the latter species is interstitially located.



**Fig. 2** Localization of rDNA clusters in spread chromosome preparations from gonads of species belonging to the superfamily Noctuoidea as revealed by FISH with 18S rDNA probe (red signals, arrowheads). Chromosomes were counterstained with DAPI (blue). **a** Female pachytene complement of *Orgyia recens* (Lymantriidae). **b** Metaphase I complement of *Orgyia leucostigma* (Lymantriidae) male consisting of  $2n = 28$  bivalents. **c** Male pachytene nucleus of *O.*

*leucostigma*. **d** Spermatogonial mitotic metaphase of *Arctia caja* (Arctidae). **e** Female pachytene complement of *Arctia caja*; note a nucleolus (N) associated with an NOR-bivalent carrying a cluster of strong interstitial rDNA signals; also note the WZ bivalent identified by DAPI-highlighted segment of the W chromosome. **f** Male pachytene nucleus of *Mamestra brassicae* (Noctuidae). Bar = 10  $\mu$ m (**a**, **c**, **e**, and **f**); bar = 5  $\mu$ m (**b**, **d**)

Five rDNA clusters in another pyraloid, *O. nubilalis*, is most probably a derived state (Fig. 3).

One NOR in an interstitial position seems to be a typical feature of karyotypes in the superfamily Bombycoidea as it occurs in representatives of three families, *M. sexta* (Sphingidae), *B. mori* (Bombycidae), and *Samia cynthia* (Saturniidae; Fig. 3). Three saturnids of the genus *Antheraea* have either one NOR, probably terminal as in *A. pernyi*, or two terminal NORs as in *A. yamamai* (Fig. 3). Available data including chromosome numbers do not allow us to suggest whether the ancestor of *A. pernyi* and *A. roylei* lost one NOR or *A. yamamai* acquired one NOR.

All Noctuoidea examined so far have only one NOR-chromosome with rDNA cluster located interstitially (Fig. 3) except *Spodoptera latifascia* and *S. descoinsi* (Monti et al. 1998). The uniform pattern of NOR distribution in species with the ancestral chromosome number of  $n = 31$  and in those with reduced chromosome numbers such as in *Orgyia* sp. suggests that one interstitial NOR is an ancestral character of Noctuoidea. In *S. latifascia* and *S. descoinsi*, the interstitial rDNA cluster could move to terminal locus by inversion, which is corroborated by a single interstitial NOR observed in *S. litura* (H. B. Manjunatha and

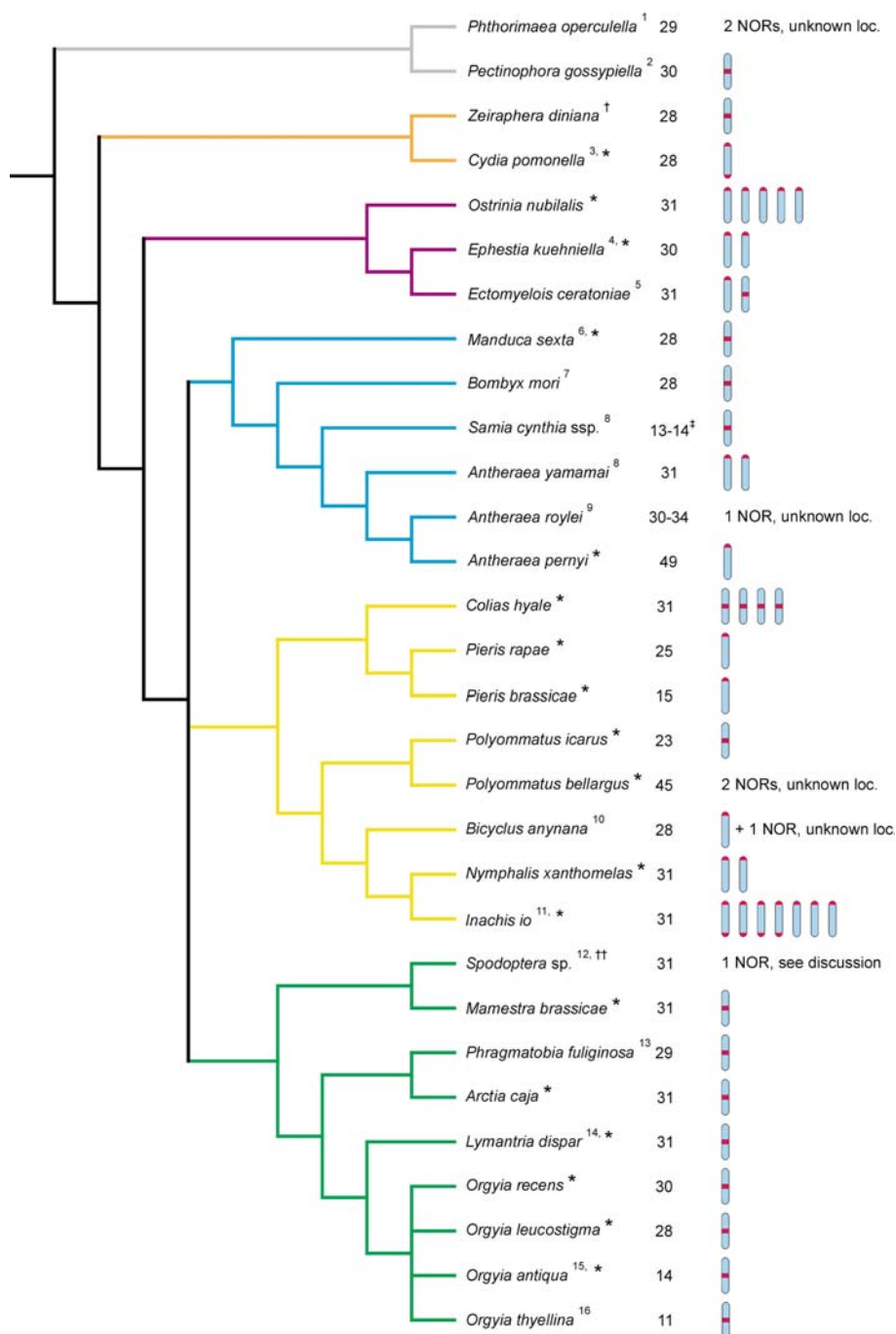
F. Marec, unpublished data). The important role of inversions in rearrangements of NOR-bearing chromosomes was demonstrated, for example, in thorny-headed worms of the genus *Pomphorhynchus* (Acanthocephala) (Bombarová et al. 2007) and *Myrmecia* ants (Hymenoptera) (Hirai et al. 1996).

The Papilionoidea, in contrast to Noctuoidea, are diverse in the distribution of rDNA site both within and between families (Fig. 3). In Lycaenidae, the occurrence of one interstitial NOR in *P. icarus* versus two NORs in *P. bellargus* is probably related to the doubled chromosome number in the latter species. In Nymphalidae, two terminal rDNA sites as in *N. xanthomelas* multiplied to 11 terminal clusters in *I. io*. Recently, two NORs were also reported in the butterfly *B. anynana*, one autosomal NOR in a terminal position and the other associated with the WZ bivalent but its location was not determined (Van't Hof et al. 2008). In Pieridae, the karyotype of *C. hyale* with  $n = 31$  but four interstitial rDNA loci demonstrates unique and definitely derived pattern of rDNA distribution, which contrasts with the single terminal NOR in the closely related species of the genus *Pieris* with reduced chromosome numbers, *P. brassicae* ( $n = 15$ ) and *P. rapae* ( $n = 25$ ).



**Fig. 3** Summary of available data on the number and position of NORs in lepidopteran species along with their phylogenetic relationships and haploid chromosome numbers.

Idiograms of NOR-bearing chromosomes show number and location (terminal or interstitial without closer specification) of rDNA clusters (*red*) in haploid complement. Phylogenetic relationships are based on Kristensen (1999), Wahlberg et al. (2005), Mahendran et al. (2006), Mitchell et al. (2006), and Regier et al. (2008). Colours of phylogenetic branches indicate the following superfamilies: *grey*, Gelechioidea; *orange*, Tortricioidea; *violet*, Pyraloidea; *blue*, Bombycoidea; *yellow*, Papilionoidea; *green*, Noctuoidea. \*this study, <sup>1</sup>Bedo (1984); <sup>2</sup>Bartlett and Del Fosse (1991); <sup>3</sup>Fuková et al. (2005); <sup>4</sup>Marec and Traut (1993); <sup>5</sup>Mediouni et al. (2004); <sup>6</sup>Sahara et al. (2007); <sup>7</sup>Traut (1976); <sup>8</sup>Yoshido et al. (2005b); <sup>9</sup>Kundu et al. (1991); <sup>10</sup>Van't Hof et al. (2008); <sup>11</sup>Yoshido et al. (2006); <sup>12</sup>Monti et al. (1998); <sup>13</sup>Traut and Marec (1997); <sup>14</sup>Krider and Shields (1997); <sup>15</sup>Traut and Clarke (1997); <sup>16</sup>Traut and Clarke (1996); †F. Marec, unpublished; ††H.B. Manjunatha and F. Marec, unpublished; ‡chromosome numbers differed in subspecies ( $2n = 25$  in *S. c. sp. indet.*;  $2n = 26$  in *S. c. walkeri*;  $2n = 27$  in *S. c. ricini*)



### Mechanisms of rDNA changes in Lepidoptera

Ability of rDNA clusters to change their number and position was first described in the genus *Allium* by Schubert (1984). Later Schubert and Wobus (1985) proved that NORs jump between some preferential chromosome sites. Since then, evidence for “the mobile NOR hypothesis” has been growing (Zhang and Sang 1999; Shishido et al. 2000; Roy et al. 2005; Datson and Murray 2006; Cabrero and Camacho 2008). Mobility of rDNA seems to

be rather common and thus, it is reasonable to assume the existence of a common mechanism responsible for changes in the number and position of rDNA clusters. However, no such consensual mechanism has been found so far.

In Lepidoptera, the absence of data from ‘primitive’ non-dytrisian families and insufficient data from basal lineages of Ditrysia do not allow for a plausible hypothesis on NOR distribution in the ancestral karyotype. Our study showed that most examined species with  $n = 28$  and lower have one interstitial NOR (Fig. 3). Providing that the



ancestral karyotype of  $n = 31$  (Lukhtanov 2000) had two NOR-chromosomes, each bearing one NOR in a terminal region, the most parsimonious way to explain evolutionary dynamics of rDNA clusters in species with reduced chromosome numbers is by fusion of two chromosomes through their terminal NORs. Such fusion, resulting in a chromosome with an interstitial rDNA site, would resemble Robertsonian translocations between the NOR-bearing acrocentric chromosomes in man (Stahl et al. 1983; Choo et al. 1988) and would be facilitated by holokinetic nature of lepidopteran chromosomes (see discussion in Rego and Marec 2003), which overcomes challenge of forming unstable dicentric chromosomes (Stahl et al. 1983). The origin by fusion seems to be applicable in Bombycoidea, where three species with  $n = 28$  have one interstitial NOR and *A. yamamai* with  $n = 31$  has two terminal NORs (Fig. 3). Further, the curious NOR-autosome in the codling moth, bearing two terminal NORs, was supposed to arise by fusion of two ancestral NOR-chromosomes by their non-NOR ends (Fuková et al. 2005). In another tortricid, *Zeiraphera diniana*, which has the same number of chromosomes ( $n = 28$ ), the only NOR-autosome with an interstitial NOR (Emelianov et al. 2004; F. Marec, unpublished data on NOR) could also originate by fusion but through NOR-ends. However, the fusion concept lacks a support in our data set as we failed to find a group of closely related species that would substantiate NOR-fusion.

Instead of fusions of NOR-bearing chromosomes, this study revealed an opposite trend in the karyotype evolution of Lepidoptera, i.e. a multiplication of rDNA clusters. Hirai et al. (1996) reported a positive correlation between a total number of chromosomes and number of NORs in ants of the genus *Myrmecia*. A similar multiplication of rDNA clusters was observed in the plant *Hypochoeris radicata* (Hall and Parker 1995). In both cases, rDNA was localized in pericentric heterochromatin, and the multiplication of rDNA clusters was thus a direct consequence of fission of the metacentric NOR-bearing chromosome, which formed two acrocentrics, each bearing a portion of the former rDNA cluster. Hall and Parker (1995) hypothesized that the NOR itself could cause instability of the centromeric region resulting in chromosomal fission. However, in Lepidoptera the mechanism of rDNA multiplication via chromosome fissions can be applied only in the case of the blue butterfly *P. bellargus*. The modal chromosome number in blue butterflies is reduced to  $n = 23$ –24 (Robinson 1971), the state represented by *P. icarus* which has a single interstitial NOR. Most probably, the chromosome number in *P. bellargus* increased to  $n = 45$  by a sequence of chromosome fissions (Kandul et al. 2007). During this process, the NOR-chromosome was likely to split into two fragments resulting in two NOR-chromosomes, the number observed in this study.

The observed increase in the number of rDNA clusters is not associated with increasing chromosome numbers in *O. nubilalis* (Pyraloidea), *C. hyale* and *I. io* (both Papilionoidea), all having the ancestral number of  $n = 31$ . In these species, rDNA probably dispersed into new chromosomal regions. Such rDNA dispersion is often associated with chromosomal rearrangements such as inversions and translocations, which can result in transfer of an rDNA cluster or its part in a new chromosomal locus. Subtelomeric location of NORs was proposed to enhance rDNA mobility by reducing deleterious effects of these rearrangements. Therefore, more NORs and a higher variability in the number of NORs between closely related species are expected in species with terminally located rDNA clusters (Hanson et al. 1996). Observations in several plant genera such as *Allium* (Schubert and Wobus 1985), *Paeonia* (Zhang and Sang 1999), and *Aloe* (Adams et al. 2000), and in *Phaseolus vulgaris* (Pedrosa-Harand et al. 2006) are consistent with these predictions. Data available in Lepidoptera (Fig. 3) suggest that species with terminal NORs have a higher number of NORs as well and point thus to a similar mechanism of rDNA dispersion. However, Dubcovsky and Dvořák (1995) and Shishido et al. (2000) found that NOR loci have changed position in the genome without affecting linkage groups in two Poaceae genera, *Triticum* and *Oryza*, respectively. Also recent findings of Cabrero and Camacho (2008) contradict the above-mentioned premises of Hanson et al. (1996). According to these authors, rDNA mobility can be explained by transposition involving mobile elements rather than chromosomal rearrangements (see also Raskina et al. 2004; Datson and Murray 2006).

Generally, chromosomal rearrangements can be ascribed to ectopic recombination, i.e., homologous recombination between repetitive sequences dispersed throughout genome (Mieczkowski et al. 2006), which is an efficient mechanism for double strand break (DSB) repairs. Ectopic recombination has two possible resolutions, (1) a crossover configuration resulting in chromosomal rearrangements and (2) a non-crossover gene conversion, predicted to occur with an equal frequency according to the conservative DSB repair model. However, deleterious chromosome rearrangements are under selection pressure and therefore, it is reasonable to assume the existence of mechanisms favouring non-crossover resolution (Shalev and Levy 1997), such as the synthesis-dependent strand-annealing model usually generating only gene conversion (Aylon and Kupiec 2004). Connection between rDNA genes and gene conversion is proved by their concerted evolution, i.e. homogenization of ribosomal gene family (reviewed in Nei and Rooney 2005). Considering these arguments, we suggest that rDNA dynamics observed in Lepidoptera can be mainly ascribed to ectopic homologous recombination

which results either in rearrangements of NOR-chromosomes (by inversion, fusion or translocation) or transfer of few ribosomal transcriptional units to a new location by gene conversion followed by their amplification via unequal crossing-over (*cf.* Dubcovsky and Dvořák 1995; Cabrero and Camacho 2008). The small size of rDNA clusters in *I. io* is consistent with this hypothesis. Recognition of ectopic recombination as a primary motive force in rDNA dynamics would be of significant consequences. It would mean that changes in number and position of rDNA clusters could be promoted by the presence of ubiquitous repetitive sequences, such as satellite DNA or mobile elements in the NOR and its vicinity (Choo et al. 1988; Maggini et al. 1991; Mieczkowski et al. 2006; Song and Boissinot 2007).

Furthermore, preferable movement of rDNA clusters in subtelomeric regions could be explained not only by deleterious effect of chromosomal rearrangements but also by the ordered spatial organization of nucleus (for reviews, see Marshall 2002; Cremer et al. 2006). If two loci are to interact physically, they must be located at the same place in the nucleus. Thus, non-random association of nonhomologous NOR-chromosomes within the same nucleolus, which was also observed in Lepidoptera (Fig. 1k), is likely to facilitate the interaction of the chromosomes through their rDNA repeats (Stahl et al. 1983; Marshall 2002). Similarly, it was shown that an efficiency of ectopic recombination is influenced by proximity of dispersed homologous sequences to telomeres (Goldman and Lichten 1996). Attachment of telomeres to the nuclear envelope is thought to create a separate compartment restricting their movement to two dimensions. Ectopic recombination between subtelomeric regions is thus facilitated in comparison with interstitial loci (Schlecht et al. 2004). Hence, a low efficiency of ectopic recombination in interstitial regions is likely to be responsible for the conserved rDNA pattern observed in Noctuoidea.

In conclusion, our study revealed dynamic evolution of rDNA distribution in most lepidopteran clades examined, particularly in Pyraloidea and Papilionoidea, which contrasts with the static rDNA pattern in Noctuoidea. Considering the above mechanisms of rDNA mobility the present data on the number and position of NORs in Lepidoptera suggest that repatterning of rDNA can be mainly ascribed to ectopic recombination. Evolutionary dynamics of rDNA clusters and chromosomes bearing them thus presumably reflects the ordered nuclear architecture and distribution of repetitive sequences. This assumption could be used as a framework to disclose the role of NOR-bearing chromosomes in the karyotype evolution.

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