



Title	Evolutionary dynamics of rDNA clusters on chromosomes of moths and butterflies (Lepidoptera)
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Citation	Genetica, 138(3), 343-354 <a href="https://doi.org/10.1007/s10709-009-9424-5">https://doi.org/10.1007/s10709-009-9424-5</a>
Issue Date	2010-03
Doc URL	<a href="http://hdl.handle.net/2115/47706">http://hdl.handle.net/2115/47706</a>
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Type	article (author version)
File Information	Gen138-3_343-354.pdf



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1 **Evolutionary dynamics of rDNA clusters on chromosomes of moths**  
2 **and butterflies (Lepidoptera)**

3  
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31 **Abstract**

32 We examined chromosomal distribution of major ribosomal DNAs (rDNAs), clustered in the nucleolar  
33 organizer regions (NORs), in 18 species of moths and butterflies using fluorescence *in situ*  
34 hybridization (FISH) with a codling moth (*Cydia pomonella*) 18S rDNA probe. Most species showed  
35 one or two rDNA clusters in their haploid karyotype but exceptions with four to eleven clusters also  
36 occurred. Our results in a compilation with previous data revealed dynamic evolution of rDNA  
37 distribution in Lepidoptera except Noctuoidea, which showed a highly uniform rDNA pattern. In  
38 karyotypes with one NOR, interstitial location of rDNA prevailed, whereas two-NOR karyotypes  
39 showed mostly terminally located rDNA clusters. A possible origin of the single interstitial NOR by  
40 fusion between two NOR-chromosomes with terminal rDNA clusters lacks support in available data. In  
41 some species, spreading of rDNA to new, mostly terminal chromosome regions was found. The  
42 multiplication of rDNA clusters without alteration of chromosome numbers rules out chromosome  
43 fissions as a major mechanism of rDNA expansion. Based on rDNA dynamics in Lepidoptera and  
44 considering the role of ordered nuclear architecture in karyotype evolution, we propose ectopic  
45 recombination, *i.e.* homologous recombination between repetitive sequences of non-homologous  
46 chromosomes, as a primary motive force in rDNA repatterning.

47

48

49 **Key words:** ribosomal DNA, nucleolar organizer region, chromosome fusion, chromosome fission,  
50 karyotype evolution, ectopic recombination

## 51 Introduction

52 Chromosomes of moths and butterflies (Lepidoptera) are small, numerous and uniform in both shape  
53 and size (Robinson 1971, Bedo 1984). They lack a distinct primary constriction (the centromere) and,  
54 as a result, sister chromatids separate by parallel disjunction during mitotic metaphase; they are thus  
55 regarded as holokinetic with kinetochores extended over a large portion of the chromosome surface  
56 (Wolf 1996). Holokinetic nature of chromosomes is supposed to facilitate chromosome fusions and  
57 fissions (Wrensch et al. 1994). However, the karyotype of Lepidoptera appears to be relatively stable  
58 as it preserves cytological similarities not only within this large and diverse order but also shares  
59 common features with the sister order Trichoptera, caddis-flies (Suomalainen 1966, Wolf et al. 1997),  
60 which diverged from a common ancestor about 200 MYA (Grimaldi and Engel 2005). Chromosome  
61 numbers range from  $n=29$  to  $n=31$  in the majority of moths and butterflies (Robinson 1971, Brown et  
62 al. 2007). The modal, i.e. the most frequent chromosome number of  $n=31$ , occurring from basal to  
63 highly derived clades, has been proposed as an ancestral karyotype of Lepidoptera (Lukhtanov 2000).  
64 Moreover, Yasukochi et al. (2006) and Pringle et al. (2007) reported unusually high degree of  
65 conserved synteny of genes between *Bombyx mori* (Bombycoidea) and *Heliconius melpomene*  
66 (Papilionoidea), which suggests evolutionary stability of lepidopteran linkage groups during last 103  
67 MY. Conserved synteny of genes including conserved gene order between chromosome 15 of *B. mori*  
68 and a chromosome of *Manduca sexta* (Sphingidae, Bombycoidea) also supports these findings  
69 (Sahara et al. 2007). Recently, this has been corroborated by Beldade et al. (2009), who observed  
70 conserved assignment of genes to linkage groups between *B. mori* and *Bicyclus anynana*  
71 (Papilionoidea).

72 Uniformity of lepidopteran chromosomes, the absence of morphological landmarks (e.g., the  
73 centromeres) together with the lack of convenient differential techniques (*cf.* Bedo 1984) disabled  
74 identification of individual chromosomes by standard cytogenetic methods and for a long time  
75 restricted their study to simple chromosome counts. Mainly because the mitotic chromosomes were  
76 unfavourable for conventional cytogenetics, much longer meiotic chromosomes in the pachytene  
77 stage were employed for the so-called pachytene mapping (Traut 1976). But even the chromomere  
78 pattern of pachytene bivalents could not be generalized for chromosome identification in Lepidoptera.  
79 Nevertheless, this approach made possible the study of sex chromosome systems (WZ/ZZ and  
80 derived variants) as the female-specific W chromosome could be distinguished by its heterochromatin

81 in a number of species (reviewed by Traut et al. 2007). More recently, modern approaches such as  
82 genomic *in situ* hybridization (GISH), comparative genomic hybridization (CGH), and fluorescence *in*  
83 *situ* hybridization (FISH) with W-chromosome painting probes allowed detailed studies on the  
84 evolution and molecular structure of the W chromosome (Traut et al. 1999, Mediouni et al. 2004,  
85 Fuková et al. 2005, 2007, Yoshido et al. 2005b, 2006, Vítková et al. 2007). However, autosomes still  
86 remain unexplored except *B. mori*, in which all chromosomes were mapped by the so-called BAC-  
87 FISH with probes prepared from bacterial artificial chromosome (BAC) clones (Sahara et al. 2003,  
88 Yoshido et al. 2005a).

89 Problems with chromosome identification, similar to those in Lepidoptera, occur in other  
90 groups of invertebrates as well. The only autosomes consistently identified in different species are  
91 chromosomes bearing tandem arrays of major ribosomal RNA (rRNA) genes (*i.e.* genes for 18S, 5.8S,  
92 and 28S rRNA). These arrays of the so-called rDNA are located in one or several nucleolar organizer  
93 regions (NORs). Series of works showed that the number and location of rDNA (or NOR) can be a  
94 useful marker for the study of karyotype evolution (*e.g.* Hirai et al. 1996, Roy et al. 2005, Bombarová  
95 et al. 2007). However, data on NORs in lepidopteran genomes are scarce, often obtained as a by-  
96 product of principal research by conventional cytogenetic methods detecting only active nucleoli. In  
97 Lepidoptera, no particular study on NOR-chromosomes has been accomplished to date. In this work,  
98 we examined the number and distribution of rDNA clusters in 18 selected species of moths and  
99 butterflies by FISH with 18S rDNA probe with the aim to understand the role of rDNA in the karyotype  
100 evolution of Lepidoptera. Our study is the first systematic survey of NOR-chromosomes not only in  
101 Lepidoptera but also in species with holokinetic chromosomes in general. The latter is of a particular  
102 interest as holokinetic systems may be fundamentally different from monocentric ones in their  
103 constraints (Wrensch et al. 1994).

104

## 105 **Material and methods**

### 106 *Insects*

107 Species examined along with their origin are given in Table 1. Specimens were either obtained from  
108 laboratory stocks or collected in natural populations. Detailed rearing conditions and diet of laboratory  
109 stocks are given in works cited in Table 1. In the field-collected species, except *Orgyia recens*,  
110 *Nymphalis xanthomelas*, and *Polyommatus bellargus*, fertilized females were captured, kept in plastic

111 containers at laboratory conditions, and let to lay eggs. Hatched larvae were reared on appropriate  
112 host plants chosen according to species bionomy, at room temperature and native day/night regime.  
113 In *O. recens* and *N. xanthomelas*, larvae were collected on a hawthorn shrub and Chinese hackberry,  
114 respectively, and then reared on the same host plant at laboratory conditions. In the case of *P.*  
115 *bellargus*, adult males collected in the field were dissected.

#### 116 *Chromosome preparations*

117 Spread preparations of mitotic and meiotic chromosomes of all species except *P. bellargus* were  
118 made from both male and female gonads of 3-5th instar larvae as described in Mediouni et al. (2004).  
119 In *P. bellargus* spread chromosome preparations were obtained from gonads of male imago following  
120 the same procedure. The preparations were passed through an ethanol series (70%, 80%, and 100%;  
121 30 s each) and stored at  $-20^{\circ}\text{C}$  until further use.

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

123 Unlabelled, about 1650 bp long 18S rDNA probe was generated by PCR from the colding moth, *Cydia*  
124 *pomonella*, genomic DNA. PCR was carried out using the 18S-Gal forward (5'-  
125 CGATACCGCGAATGGCTCAATA-3') and 18S-Gal reverse (5'-ACAAAGGGCAGGGACGTAATCAAC-  
126 3') primers as described in Fuková et al. (2005). The probes were labelled with biotin-14-dATP by nick  
127 translation using a Bionick Labeling System (Invitrogen, Life Technologies Inc., San Diego, CA, USA).

128 For rDNA-FISH, we used the procedure as previously reported in Fuková et al. (2005). Briefly,  
129 chromosome preparations were treated with 100  $\mu\text{g}/\text{mL}$  RNase A to remove an excessive amount of  
130 rRNAs. After denaturation the chromosomes were hybridized with a probe cocktail containing 15 ng of  
131 biotinylated 18S rDNA probe and 25  $\mu\text{g}$  of sonicated salmon sperm DNA per slide. Hybridization  
132 signals were detected with Cy3-conjugated streptavidin (Jackson ImmunoRes. Labs. Inc., West  
133 Grove, PA, USA), amplified with biotinylated anti-streptavidin (Vector Labs. Inc., Burlingame, CA,  
134 USA). The preparations were counterstained with 0.5  $\mu\text{g}/\text{mL}$  of DAPI and mounted in antifade based  
135 on DABCO (for composition, see Mediouni et al. 2004).

136 Preparations were inspected in a Zeiss Axioplan 2 microscope equipped with a cooled F-View  
137 CCD camera and AnalySIS software, version 3.2 (Soft Imaging System GmbH, Münster, Germany).  
138 Black-and-white images were captured separately for each fluorescent dye and then pseudocoloured  
139 (light blue for DAPI and red for Cy3) and superimposed with Adobe Photoshop, version 7.0.

140

## 141 **Results**

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

150

### 151 *Pyraloidea*

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

162

### 163 *Bombycoidea*

164 Mitotic spermatogonia of the tobacco hornworm, *Manduca sexta* (Sphingidae), showed 2n=56  
165 chromosomes of uniform shape (cf. Sahara et al. 2007). The 18S rDNA probe hybridized to two of  
166 these chromosomes (not shown). In pachytene nuclei of spermatocytes, the probe identified a single  
167 bivalent with an rDNA cluster located in an interstitial position near one end of the bivalent (Fig. 1b).  
168 This result corroborates recent finding of Sahara et al. (2007), who observed a bivalent associated  
169 with a nucleolus in a similar position after staining of pachytene oocytes with lactic acetic orcein. The

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

175

#### 176 *Papilionoidea*

177 Members of families Pieridae, Lycaenidae, and Nymphalidae were examined. In mitotic complements  
178 of the large white, *Pieris brassicae* (Pieridae),  $2n=30$  rod-shaped chromosomes were observed as  
179 previously reported by Bigger (1975). Two obviously homologous chromosomes of a similar size  
180 carried each terminal rDNA signals (Fig. 1d). In pachytene nuclei of spermatocytes, the 18S rDNA  
181 probe hybridized to a terminal region of one NOR-bivalent (Fig. 1e). Subterminal segments of some  
182 pachytene bivalents were highlighted with DAPI, indicating the presence of subtelomeric  
183 heterochromatin (see arrows in Fig. 1e). In a closely related species, the small white *Pieris rapae*  
184 (Pieridae), spermatogonial nuclei had  $2n=50$  chromosomes (*cf.* Robinson 1971). Also in this species  
185 rDNA-FISH identified two mitotic chromosomes carrying rDNA clusters. The rDNA signals appeared  
186 terminally located in these small, densely packed chromosomes (Fig. 1f). Unfortunately, we failed to  
187 get preparations of pachytene spermatocytes in this species and therefore, could not determine  
188 precisely the rDNA location at a high resolution of much longer pachytene bivalents. In spermatocytes  
189 of the pale clouded yellow, *Colias hyale* (Pieridae), four bivalents bearing rDNA clusters were regularly  
190 observed among 31 chromosome pairs of its pachytene complements. All these clusters were  
191 localized in interstitial chromosomal regions (Fig. 1g).

192 In spermatogonial metaphases of the common blue, *Polyommatus icarus* (Lycaenidae),  $2n=46$   
193 chromosomes were observed. Hybridization signals of the rDNA probe were found in two largest  
194 chromosomes, most likely corresponding to chromosome pair no. 1 in the study of Bigger (1975) (Fig.  
195 1h). A single rDNA cluster was found in pachytene nuclei of spermatocytes. It was located in an  
196 interstitial region near one end of the NOR-bivalent (Fig. 1i). In the Adonis blue, *Polyommatus*  
197 *bellargus* (Lycaenidae), only metaphase I chromosomes were found on preparations of testes from  
198 adult males. The metaphase I complement consisted of  $n=45$  bivalents. Two of the bivalents showed  
199 each two clusters of hybridization signals, each cluster corresponding to one homologous



200 chromosome. The rDNA signals of the smaller bivalent were much fainter than those of the larger  
201 bivalent, most likely indicating a small number of rDNA repeats (Fig. 1j). Positions of rDNA clusters on  
202 the bivalents could not be determined.

203 Two bivalents bearing strong terminal hybridization signals were observed in female  
204 pachytene nuclei of the yellow-legged tortoiseshell, *Nymphalis xanthomelas* (Nymphalidae), with a  
205 haploid chromosome number of  $n=31$  (see Robinson 1971). The rDNA signals were located at the  
206 ends of these NOR-bivalents and formed small spheres, probably reflecting distribution of rRNA genes  
207 in nucleoli. The two NOR-bivalents were often oriented in head-to-head manner by their rDNA (NOR)  
208 ends, obviously due to association of their nucleoli in a common nucleolus (Fig. 1k). The peacock  
209 butterfly, *Inachis io* (Nymphalidae), has also a haploid chromosome number of  $n=31$  (see Robinson  
210 1971). In the Japanese subspecies *I. io geisha*, Yoshido et al. (2006) observed at least five NOR-  
211 bivalents in pachytene oocyte complements after genomic *in situ* hybridization (GISH) with female  
212 genomic DNA probe and DAPI counterstaining. We examined rDNA distribution in *I. io* specimens  
213 originating from a Czech population. In pachytene oocytes, rDNA-FISH identified up to 11 rDNA sites  
214 localized at the ends of seven bivalents; three bivalents had rDNA signals at one end and four  
215 bivalents at both ends (Fig. 1l). Some of the hybridization signals were relatively weak, most probably  
216 due to a small number of rDNA repeats. In addition, the number of the weak rDNA signals varied in  
217 some nuclei, probably because of a lower resolution of rDNA-FISH for the rDNA sites with low  
218 numbers of repeats.

219

## 220 *Noctuoidea*

221 In pachytene oocytes of *Lymantria dispar*, rDNA-FISH identified a single rDNA cluster located  
222 interstitially in a bivalent (not shown). This finding is in a good accord with a single NOR-bivalent  
223 observed in this species by Krider and Shields (1997). The karyotype of scarce vapourer, *Orgyia*  
224 *recens*, consists of  $n=30$  chromosomes (Robinson 1971). Also in this species, we found a single rDNA  
225 cluster located interstitially in the NOR-bivalent of pachytene oocytes (Fig. 2a). Metaphase I  
226 spermatocyte nuclei of the white-marked tussock moth, *Orgyia leucostigma*, consisted of  $n=28$   
227 bivalents, which is in keeping with the earlier published data (see Robinson 1971). The 18S rDNA  
228 probe hybridized to a single bivalent of its metaphase I complement (Fig. 2b). Accordingly, one NOR-  
229 bivalent was found in pachytene nuclei of spermatocytes with an interstitial rDNA cluster (Fig. 2c).

230 Furthermore, rDNA-FISH was carried out on chromosomal preparations of *O. antiqua* males. In  
231 metaphase I spermatocyte nuclei, one out of 14 bivalents showed clear rDNA signals (not shown).  
232 The presence of a single cluster of rRNA genes was confirmed in nuclei of pachytene spermatocytes,  
233 which also showed an NOR-bivalent with an interstitial rDNA site (not shown). These results are  
234 consistent with observations of a single autosome NOR-bivalent in orcein-stained pachytene oocytes  
235 of *O. antiqua* (Traut and Clarke 1997, Traut and Marec 1997). We also examined rDNA distribution in  
236 the garden tiger, *Arctia caja*, a representative of the family Arctiidae, which is closely related to the  
237 family Lymntriidae (Mitchell et al. 2006). Male mitotic metaphase complements consisted of  $2n=62$   
238 chromosomes, two of which bore hybridization signals of the rDNA probe (Fig. 2d). Accordingly, a  
239 single chromosome pair (*i.e.* NOR-bivalent) with an interstitial rDNA cluster was observed in  
240 pachytene nuclei of both sexes. Moreover, the WZ sex-chromosome bivalent was easily discernible in  
241 oocyte pachytene nuclei according to a conspicuous block of W-heterochromatin deeply stained with  
242 DAPI (Fig. 2e). Similar to species of Lymantriidae and Arctiidae, also the cabbage moth, *Mamestra*  
243 *brassicae* (Noctuidae), with  $n=31$  (Robinson 1971) had only one NOR-bivalent in spermatocyte  
244 pachytene nuclei, with an rDNA site located in an interstitial region (Fig. 2f).

245

## 246 Discussion

### 247 *Number and location of rDNA clusters in Lepidoptera*

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

252 The basal superfamilies of Ditrysia, Gelechioidea and Tortricoidea, showed three different  
253 patterns of rDNA distribution in four species with similar chromosome numbers,  $n=28-30$  (Fig. 3). No  
254 common rDNA pattern was found in three species of Pyraloidea with  $n=30-31$ , where *E. kuehniella*  
255 and *Ectomyelois ceratoniae* have two NORs but one NOR in the latter species is interstitially located.  
256 Five rDNA clusters in another pyraloid, *O. nubilalis*, is most probably a derived state (Fig. 3).

257 One NOR in an interstitial position seems to be a typical feature of karyotypes in the  
258 superfamily Bombycoidea as it occurs in representatives of three families, *M. sexta* (Sphingidae), *B.*  
259 *mori* (Bombycidae), and *Samia cynthia* (Saturniidae) (Fig. 3). Three saturnids of the genus *Antheraea*

260 have either one NOR, probably terminal as in *A. pernyi*, or two terminal NORs as in *A. yamamai* (Fig.  
261 3). Available data including chromosome numbers do not allow us to suggest whether the ancestor of  
262 *A. pernyi* and *A. roylei* lost one NOR or *A. yamamai* acquired one NOR.

263 All Noctuoidea examined so far have only one NOR-chromosome with rDNA cluster located  
264 interstitially (Fig. 3) except *Spodoptera latifascia* and *S. descoinsi* (Monti et al. 1998). The uniform  
265 pattern of NOR distribution in species with the ancestral chromosome number of  $n=31$  and in those  
266 with reduced chromosome numbers such as in *Orgyia* sp. suggests that one interstitial NOR is an  
267 ancestral character of Noctuoidea. In *S. latifascia* and *S. descoinsi*, the interstitial rDNA cluster could  
268 move to terminal locus by inversion, which is corroborated by a single interstitial NOR observed in *S.*  
269 *litura* (H.B. Manjunatha and F. Marec, unpublished data). The important role of inversions in  
270 rearrangements of NOR-bearing chromosomes was demonstrated, for example, in thorny-headed  
271 worms of the genus *Pomphorhynchus* (Acanthocephala) (Bombarová et al. 2007) and *Myrmecia* ants  
272 (Hymenoptera) (Hirai et al. 1996).

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

283

#### 284 *Mechanisms of rDNA changes in Lepidoptera*

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

290 to assume the existence of a common mechanism responsible for changes in the number and position  
291 of rDNA clusters. However, no such consensual mechanism has been found so far.

292 In Lepidoptera, the absence of data from 'primitive' non-dytrisian families and insufficient data  
293 from basal lineages of Ditrysia do not allow for a plausible hypothesis on NOR distribution in the  
294 ancestral karyotype. Our study showed that most examined species with  $n=28$  and lower have one  
295 interstitial NOR (Fig. 3). Providing that the ancestral karyotype of  $n=31$  (Lukhtanov 2000) had two  
296 NOR-chromosomes, each bearing one NOR in a terminal region, the most parsimonious way to  
297 explain evolutionary dynamics of rDNA clusters in species with reduced chromosome numbers is by  
298 fusion of two chromosomes through their terminal NORs. Such fusion, resulting in a chromosome with  
299 an interstitial rDNA site, would resemble Robertsonian translocations between the NOR-bearing  
300 acrocentric chromosomes in man (Stahl et al. 1983, Choo et al. 1988) and would be facilitated by  
301 holokinetic nature of lepidopteran chromosomes (see discussion in Rego and Marec 2003), which  
302 overcomes challenge of forming unstable dicentric chromosomes (Stahl et al. 1983). The origin by  
303 fusion seems to be applicable in Bombycoidea, where three species with  $n=28$  have one interstitial  
304 NOR and *A. yamamai* with  $n=31$  has two terminal NORs (Fig. 3). Further, the curious NOR-autosome  
305 in the codling moth, bearing two terminal NORs, was supposed to arise by fusion of two ancestral  
306 NOR-chromosomes by their non-NOR ends (Fuková et al. 2005). In another tortricid, *Zeiraphera*  
307 *diniana*, which has the same number of chromosomes ( $n=28$ ), the only NOR-autosome with an  
308 interstitial NOR (Emelianov et al. 2004; F. Marec, unpublished data on NOR) could also originate by  
309 fusion but through NOR-ends. However, the fusion concept lacks a support in our data set as we  
310 failed to find a group of closely related species that would substantiate NOR-fusion.

311 Instead of fusions of NOR-bearing chromosomes, this study revealed an opposite trend in the  
312 karyotype evolution of Lepidoptera, *i.e.* a multiplication of rDNA clusters. Hirai et al. (1996) reported a  
313 positive correlation between a total number of chromosomes and number of NORs in ants of the  
314 genus *Myrmecia*. A similar multiplication of rDNA clusters was observed in the plant *Hypochoeris*  
315 *radicata* (Hall and Parker 1995). In both cases, rDNA was localized in pericentric heterochromatin, and  
316 the multiplication of rDNA clusters was thus a direct consequence of fission of the metacentric NOR-  
317 bearing chromosome, which formed two acrocentrics, each bearing a portion of the former rDNA  
318 cluster. Hall and Parker (1995) hypothesized that the NOR itself could cause instability of the  
319 centromeric region resulting in chromosomal fission. However, in Lepidoptera the mechanism of rDNA

320 multiplication via chromosome fissions can be applied only in the case of the blue butterfly *P.*  
321 *bellargus*. The modal chromosome number in blue butterflies is reduced to n=23-24 (Robinson 1971),  
322 the state represented by *P. icarus* which has a single interstitial NOR. Most probably, the chromosome  
323 number in *P. bellargus* increased to n=45 by a sequence of chromosome fissions (Kandul et al. 2007).  
324 During this process, the NOR-chromosome was likely to split into two fragments resulting in two NOR-  
325 chromosomes, the number observed in this study.

326         The observed increase in the number of rDNA clusters is not associated with increasing  
327 chromosome numbers in *O. nubilalis* (Pyraloidea), *C. hyale* and *I. io* (both Papilionoidea), all having  
328 the ancestral number of n=31. In these species, rDNA probably dispersed into new chromosomal  
329 regions. Such rDNA dispersion is often associated with chromosomal rearrangements such as  
330 inversions and translocations, which can result in transfer of an rDNA cluster or its part in a new  
331 chromosomal locus. Subtelomeric location of NORs was proposed to enhance rDNA mobility by  
332 reducing deleterious effects of these rearrangements. Therefore, more NORs and a higher variability  
333 in the number of NORs between closely related species are expected in species with terminally  
334 located rDNA clusters (Hanson et al. 1996). Observations in several plant genera such as *Allium*  
335 (Schubert and Wobus 1985), *Paeonia* (Zhang and Sang 1999), and *Aloe* (Adams et al. 2000), and in  
336 *Phaseolus vulgaris* (Pedrosa-Harand et al. 2006) are consistent with these predictions. Data available  
337 in Lepidoptera (Fig. 3) suggest that species with terminal NORs have a higher number of NORs as  
338 well and point thus to a similar mechanism of rDNA dispersion. However, Dubcovsky and Dvořák  
339 (1995) and Shishido et al. (2000) found that NOR loci have changed position in the genome without  
340 affecting linkage groups in two Poaceae genera, *Triticum* and *Oryza*, respectively. Also recent findings  
341 of Cabrero and Camacho (2008) contradict the above-mentioned premises of Hanson et al. (1996).  
342 According to these authors, rDNA mobility can be explained by transposition involving mobile  
343 elements rather than chromosomal rearrangements (see also Raskina et al. 2004, Datson and Murray  
344 2006).

345         Generally, chromosomal rearrangements can be ascribed to ectopic recombination, *i.e.*  
346 homologous recombination between repetitive sequences dispersed throughout genome (Mieczkowski  
347 et al. 2006), which is an efficient mechanism for double strand break (DSB) repairs. Ectopic  
348 recombination has two possible resolutions, (i) a crossover configuration resulting in chromosomal  
349 rearrangements and (ii) a non-crossover gene conversion, predicted to occur with an equal frequency

350 according to the conservative DSB repair model. However, deleterious chromosome rearrangements  
351 are under selection pressure and therefore, it is reasonable to assume the existence of mechanisms  
352 favouring non-crossover resolution (Shalev and Levy 1997), such as the synthesis-dependent strand-  
353 annealing model usually generating only gene conversion (Aylon and Kupiec 2004). Connection  
354 between rDNA genes and gene conversion is proved by their concerted evolution, *i.e.* homogenization  
355 of ribosomal gene family (reviewed in Nei and Rooney 2005). Considering these arguments, we  
356 suggest that rDNA dynamics observed in Lepidoptera can be mainly ascribed to ectopic homologous  
357 recombination which results either in rearrangements of NOR-chromosomes (by inversion, fusion or  
358 translocation) or transfer of few ribosomal transcriptional units to a new location by gene conversion  
359 followed by their amplification via unequal crossing-over (*cf.* Dubcovsky and Dvořák 1995, Cabrero  
360 and Camacho 2008). The small size of rDNA clusters in *I. io* is consistent with this hypothesis.  
361 Recognition of ectopic recombination as a primary motive force in rDNA dynamics would be of  
362 significant consequences. It would mean that changes in number and position of rDNA clusters could  
363 be promoted by the presence of ubiquitous repetitive sequences, such as satellite DNA or mobile  
364 elements in the NOR and its vicinity (Choo et al. 1988, Maggini et al. 1991, Mieczkowski et al. 2006,  
365 Song and Boissinot 2007).

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

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

387

### 388 **Acknowledgements**

389 We wish to thank Marie Korchová for technical assistance and our colleagues, Oxana Habušťová for  
390 *Ostrinia nubilalis*, Jiří Beneš, Zdeněk Fric, Josef Jaroš, and Martin Konvička for their help with  
391 collecting and determination of some species. Our thanks are also due to Zenta Kajiura (Shinshu  
392 University, Japan) for *Antheraea pernyi* through NBRP, Sandra Barns and Stuart Reynolds (Bath  
393 University, UK) for *Manduca sexta*, Josef Chytil (Přerov, Czech Republic) for *Lymantria dispar*, Tomáš  
394 Hamřík (Velká Bíteš, Czech Republic) for *Arctia caja*, and Zdeněk Hanč (České Budějovice) for  
395 valuable advice. This work was funded by grants 206/06/1860 of the Grant Agency of the Czech  
396 Republic (GACR) and IAA600960925 of the Grant Agency of the Academy of Sciences of the Czech  
397 Republic, both Prague, and from the Entomology Institute project Z50070508. P.N. acknowledges the  
398 support from the grant SGA2006/01 of the Student Grant Agency of the Faculty of Science, University  
399 of South Bohemia, Czech Republic, and the support of his Ph.D. programme by the GACR grant  
400 521/08/H042.. K.S. and A.Y. were supported from grants 18380037 and 191114 of the Japan Society  
401 for the Promotion of Science (JSPS), respectively.

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566 **Titles and legends to figures**

567

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

584

585 **Fig. 2** Localization of rDNA clusters in spread chromosome preparations from gonads of species  
586 belonging to the superfamily Noctuoidea as revealed by FISH with 18S rDNA probe (red signals,  
587 arrowheads). Chromosomes were counterstained with DAPI (blue). **a** Female pachytene complement  
588 of *Orgyia recens* (Lymantriidae). **b** Metaphase I complement of *Orgyia leucostigma* (Lymantriidae)  
589 male consisting of 2n=28 bivalents. **c** Male pachytene nucleus of *O. leucostigma*. **d** Spermatogonial  
590 mitotic metaphase of *Arctia caja* (Arctidae). **e** Female pachytene complement of *Arctia caja*; note a  
591 nucleolus (N) associated with an NOR-bivalent carrying a cluster of strong interstitial rDNA signals;  
592 also note the WZ bivalent identified by DAPI-highlighted segment of the W chromosome. **f** Male  
593 pachytene nucleus of *Mamestra brassicae* (Noctuidae). Bar=10  $\mu$ m (**a, c, e, and f**); bar=5  $\mu$ m (**b, d**).

594

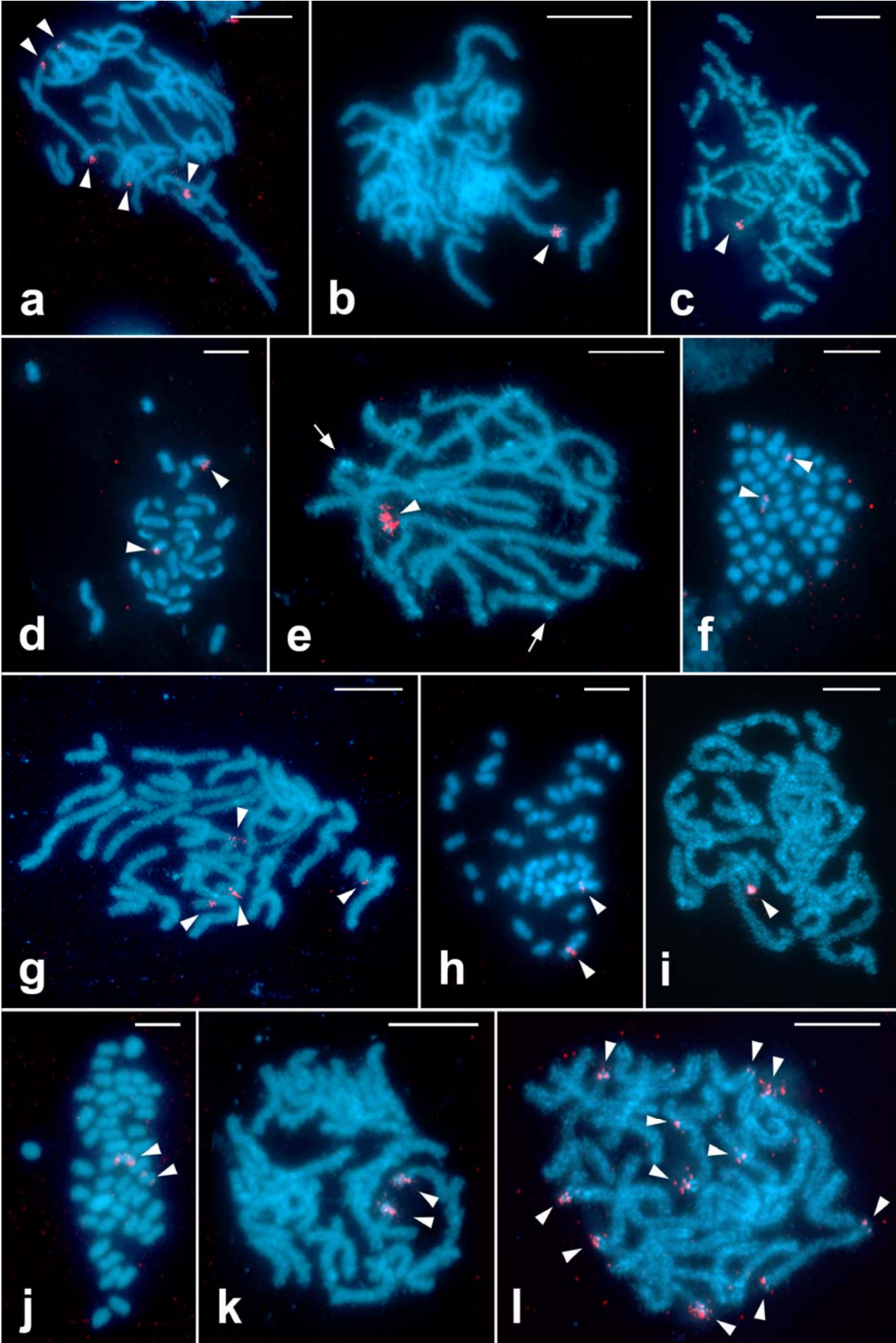
595 **Fig. 3** Summary of available data on the number and position of NORs in lepidopteran species along  
596 with their phylogenetic relationships and haploid chromosome numbers. Idiograms of NOR-bearing  
597 chromosomes show number and location (terminal or interstitial without closer specification) of rDNA  
598 clusters (red) in haploid complement. Phylogenetic relationships are based on Kristensen (1999),  
599 Wahlberg et al. (2005), Mahendran et al. (2006), Mitchell et al. (2006), and Regier et al. (2008).  
600 Colours of phylogenetic branches indicate the following superfamilies: grey, Gelechioidea; orange,  
601 Tortricoidea; violet, Pyraloidea; blue, Bombycoidea; yellow, Papilionoidea; green, Noctuoidea. \*this  
602 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);  
603 <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.  
604 (1991); <sup>10</sup>Van't Hof et al. (200\*); <sup>11</sup>Yoshido et al. (2006); <sup>12</sup>Monti et al. (1998); <sup>13</sup>Traut and Marec  
605 (1997); <sup>14</sup>Krider and Shields (1997); <sup>15</sup>Traut and Clarke (1997); <sup>16</sup>Traut and Clarke (1996); <sup>†</sup>F. Marec,  
606 unpublished; <sup>††</sup>H.B. Manjunatha and F. Marec, unpublished; <sup>‡</sup>chromosome numbers differed in  
607 subspecies (2n=25 in *S. c. sp. indet.*; 2n=26 in *S. c. walkeri*; 2n=27 in *S. c. ricini*).

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

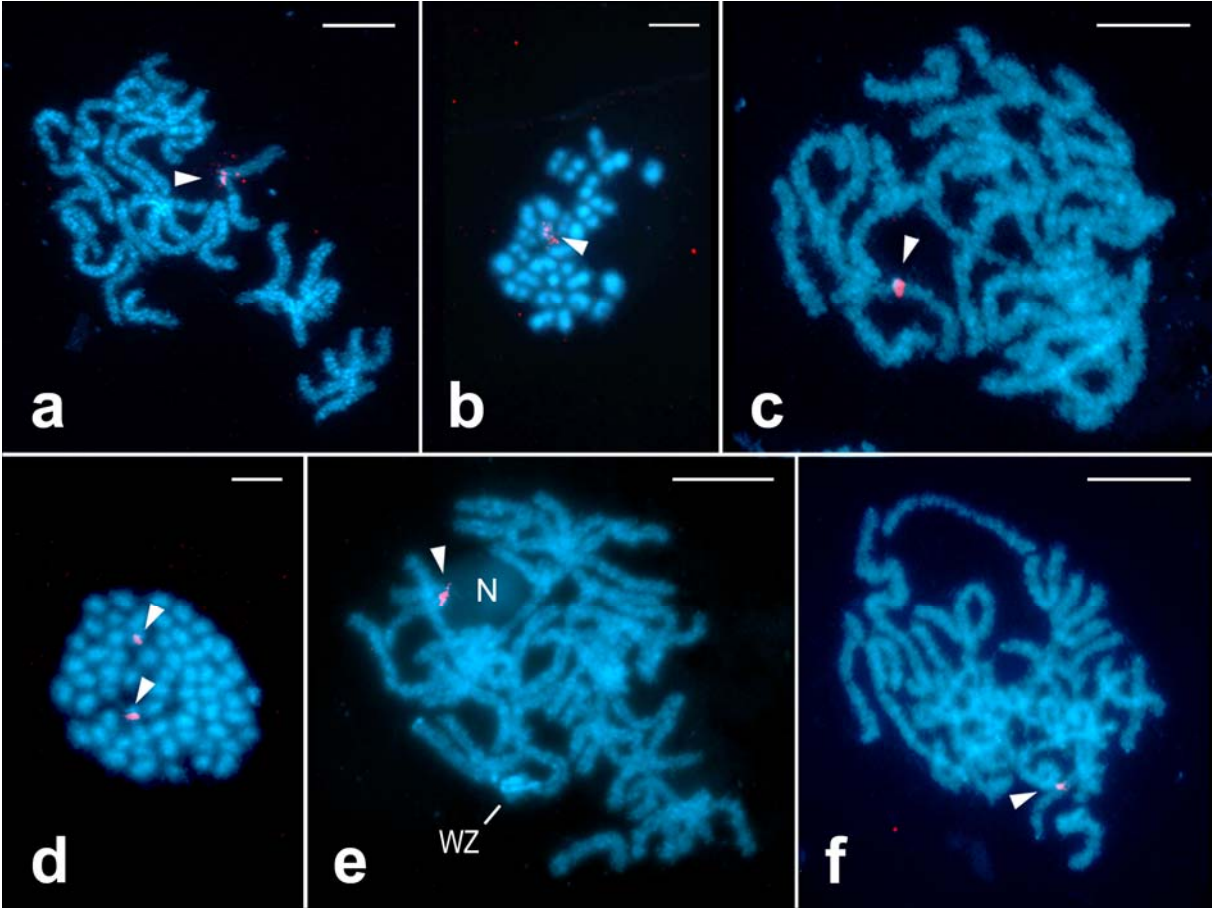
Superfamily	Family / Species	Source or locality	Rearing conditions; diet
Tortricoidea	Tortricidae		
	<i>Cydia pomonella</i>	Laboratory strain Krym-61	see Fuková et al. (2005)
Pyraloidea	Crambidae		
	<i>Ostrinia nubilalis</i>	Laboratory culture (O. Habuštová)	25°C, LD 12/12 h; wheat germ/alfalfa-based diet (Lewis and Lynch 1969)
	Pyralidae		
	<i>Ephestia kuehniella</i>	Laboratory strain WT-C	see Rego and Marec (2003)
Bombycoidea	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)



Nguyen et al.: Fig. 1



Nguyen et al.: Fig. 2



Nguyen et al.: Fig. 3

