Comparative Karyotype Analysis of Grasshoppers in the Genus *Oxya* (Orthoptera, Catantopidae) by Differential Staining Techniques

Aya Yoshimura1,*, Yoshitaka Obara1, Yoshikazu Ando2 and Hiroshi Kayano3

1 Department of Biofunctional Science, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki 036-8561, Japan
2 Department of Bioproduction, Faculty of Agriculture and Life Science, Hirosaki University, 3 Bunkyo-cho, Hirosaki 036-8561, Japan
3 Kujyu-Highland Institute of Biology, Akagawa 4026, Kujyu-machi, Oita 878-0201, Japan

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Summary The chromosomes of 4 species of grasshopper in the genus *Oxya* were analyzed by conventional and five differential-staining methods. All 4 species had a chromosome complement of 2n=22+XX(female)/XO(male), with very similar karyotypes. Both *O. hyla intricata* and *O. japonica japonica* had acrocentric chromosomes with minute short arms, while the chromosomes of *O. chinensis formosana* and *O. yezoensis* were almost all telocentric. In all 4 species, a secondary constriction (SC) was detected at a proximal site in chromosome 8, which had the same staining properties (positive for C- and CMA-banding; negative for G- and QM-banding) in all 4 species. Our C-banding analysis revealed that the short arms of *O. hyla intricata* and *O. japonica japonica* consisted of C-heterochromatin. The 4 species yielded species-specific patterns of C-bands, and there appeared to be a close relationship between *O. chinensis formosana* and *O. yezoensis*, as suggested by the polymorphism of the distal C-bands of chromosome 2. In all 4 species of *Oxya*, Ag-NORs were detected on the centromeric regions of all the chromosomes, but not detected on the SC of chromosome 8. The karyosystematic relationships among the 4 species are discussed on the basis of the results of differential staining.

Key words Grasshopper, *Oxya*, Karyotype, A secondary constriction, C-heterochromatin.

Seven species of the genus *Oxya*, known as rice-field grasshoppers, are found in Japan (Fukuhara 1982a, b). *O. hyla intricata* and *O. chinensis formosana* are endemic to the Nansei Archipelago, while *O. japonica japonica* is found from Honshu to Kyushu and *O. yezoensis* is found from Hokkaido to Kyushu. Except for *O. yezoensis* these species are also found in many parts of the world, such as China and Southeast Asia (Fukuhara 1982a, b). The 4 species can be separated by the characters of the male genitalia and the female subgenital plate (Fukuhara 1982a, b). The male genitalia of *O. hyla intricata* is short, stubby and weakly sclerotized, whereas that of the other species is long and strongly sclerotized, especially in *O. yezoensis*, it is thick and robust (Fukuhara 1982a, b). They have also been investigated in detail from an ecological perspective, such as the ability of parthenogenesis (Zhu and Ando 1998) and interspecies mating behavior (Zhu and Ando unpublished). Furthermore, recent studies of their molecular genetics have focussed on the phylogenetic relationships among them (Zhu *et al.* 2001, Aikawa *et al.* 2003). According to these results, *O. hyla intricata* is the oldest of the 4 species, and *O. japonica japonica*, *O. chinensis formosana*, and *O. yezoensis* diverged from it in that order. In addition, mating of *O. chinensis formosana* and *O.
yezoensis gives rise to fertile offspring of intermediate morphology (Zhu and Ando unpublished). Therefore, these 2 species seem to have diverged more recently than *O. hyla intricata* and *O. japonica japonica*.

To our knowledge, the only reported cytogenetic study of the *Oxya* grasshoppers of Japan describes the conventional Giemsa-stained karyotypes of *O. japonica japonica* and *O. yezoensis* (Inoue 1985). According to his report the chromosome number of the 2 species is 2n=22+XO(male) and their karyotypes were very similar, though some pairs of chromosomes remained to be identified. The present study was carried out to determine the detailed karyotypes of these species using 5 differential-staining techniques (C-, G-, Ag-NOR-, QM- and CMA-banding) in order to clarify the karyosystematic relationships among them.

Materials and methods

Adults of the 4 species were caught in several areas of Japan (Fig. 1) and bred in the laboratory. Chromosome preparations were made by a modified method of Crozier’s method (Crozier 1968). The egg cases obtained from these laboratory-bred specimens were incubated at 25°C for 10 to 15 days after laying, and embryos were dissected out with fine forceps and immersed in a hypotonic solution of 0.075 M KCl at room temperature for 20 min. Embryos fixed in Carnoy’s fixative (methanol : acetic acid = 3 : 1 [v/v]) for 30 min. were placed on a slide and crushed with fine forceps in a drop of 50% acetic acid to free cells. Two drops of Carnoy’s fixative poured onto the freed cells allowed chromosomes to spread on slides.

C-banding was performed by the BSG technique of Sumner (1972) with slight modifications. The slides were kept at room temperature for approximately one month before C-banding. G-banding was performed with trypsin, as described by Burgos *et al.* (1986) and Camacho *et al.* (1991). Quinacrine mustard (QM)-banding and chromomycin A3 (CMA)-banding were performed as described by Caspersson *et al.* (1971) and Amemiya and Gold (1987), respectively. Nucleolus organizer regions (NORs) were selectively stained by the one-step method of Howell and Black (1980).

Results and discussion

Conventional Giemsa-staining

The numbers of embryos and cells analyzed are shown in Table 1. The chromosome number of *Oxya* species has been reported to be 2n=22+XX(female)/XO(male) by previous reports (Inoue 1985, John *et al.* 1985). Also in this study, the 4 species of *Oxya* (*O. hyla intricata*, *O. japonica japonica*, *O. chinensis formosana* and *O. yezoensis*) were regarded to carry 23(male)/24(female) chromosomes. The karyotypes of the 4 species consisted of 2 pairs of long chromosomes nos. 1 and 2, 7 pairs of medium chromosomes nos. 3–9, and 2 pairs of short chromosomes nos. 10 and 11, plus a single X chromosome in males or 2 X chromosomes in females (Fig. 2a–d). The X chromosome was the longest of the medium-sized group in all species. The karyotypes of *O. japonica japonica* and *O. yezoensis* were almost identical to those reported by Inoue (1985).

All the chromosomes of *O. hyla intricata,*...
Table 1. Numbers of embryos (and cells) examined in the present study

<table>
<thead>
<tr>
<th>Species</th>
<th>Conv.</th>
<th>C-band</th>
<th>G-band</th>
<th>QM</th>
<th>CMA</th>
<th>Ag-NOR</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Oxya hyla intricata</em></td>
<td>9 (11)</td>
<td>8 (27)</td>
<td>2 (23)</td>
<td>4 (9)</td>
<td>5 (8)</td>
<td>5 (8)</td>
</tr>
<tr>
<td><em>O. japonica japonica</em></td>
<td>14 (24)</td>
<td>4 (10)</td>
<td>5 (22)</td>
<td>3 (23)</td>
<td>3 (20)</td>
<td>5 (12)</td>
</tr>
<tr>
<td><em>O. chinensis formosana</em></td>
<td>9 (11)</td>
<td>10 (10)</td>
<td>2 (3)</td>
<td>13 (16)</td>
<td>9 (11)</td>
<td>3 (5)</td>
</tr>
<tr>
<td><em>O. yezoensis</em></td>
<td>5 (6)</td>
<td>20 (34)</td>
<td>6 (21)</td>
<td>5 (7)</td>
<td>5 (7)</td>
<td>6 (21)</td>
</tr>
</tbody>
</table>

Conv.: Conventional Giemsa-staining, QM: quinacrine mustard-staining, CMA: chromomycin A₃-staining. The numbers in parentheses are the numbers of cells examined.

Fig. 2. Conventional Giemsa-stained and C-banded karyotypes (from female embryos) of *O. hyla intricata* (a, c), *O. japonica japonica* (b, f), *O. chinensis formosana* (c, g) and *O. yezoensis* (d, h). a–d, Conventional Giemsa-staining; e–h, C-banding. XO in a rectangle is the monosomic X chromosome from a male specimen. Arrows indicate a secondary constriction (SC). Asterisks indicate crossing of chromosomes.
with the exception of chromosome 11, had minute short arms (Fig. 2a). All the chromosomes of *O. japonica japonica* also had minute short arms, which appeared somewhat shorter than those of *O. hyla intricata* (Fig. 2b). By contrast, none of the chromosomes had short arms in *O. chinensis formosana* (Fig. 2c), and only chromosomes 6 and 7 had minute short arms in *O. yezoensis* (Fig. 2d). In all 4 species examined, a small secondary constriction (SC) was observed at a proximal site in chromosome 8 as indicated by arrows in Fig. 2a–d. Inoue (1985) did not, apparently, notice this SC in the karyotypes of *O. japonica japonica* and *O. yezoensis*. Chromosomes 10 and 11 of *O. chinensis formosana* were indistinguishable from each other, while those in the other three species could be distinguished from each other. As a whole, the conventional Giemsa-stained karyotypes of the 4 species of *Oxya* were similar even though chromosomes 1–7 could not be identified individually.

**C-banding**

The C-banded karyotypes of the 4 species are shown in Fig. 2e–h. Centromeric C-bands were common to all chromosomes in these species, but there were some interchromosomal and interspecific size variations. The centromeric C-bands of *O. hyla intricata* and *O. japonica japonica* were larger, for the most part, than those of *O. chinensis formosana* and *O. yezoensis*, because the minute short arms of the former 2 species were also heavily stained. These minute short arms might have been formed by the amplification of repetitive DNA sequences in the centromeric C-heterochromatin. If this hypothesis is correct, the ancestral karyotype of these species might have consisted of exclusively telocentric chromosomes. In fact, the majority of acridid and catantopid grasshoppers examined to date have a chromosome complement of 2n=22+XX(female)/XO(male) and all the chromosomes are telocentric (Hewitt 1979, Inoue 1985).

All pairs of chromosomes could be identified on the basis of size and the patterns of centromeric, proximal and distal C-bands in these *Oxya* species. Thus, C-banding is useful for the identification of individual chromosomes and also for considerations of phylogenetic relationships. However, we have to take C-bands polymorphism and the presence of different chromosome morphotypes in other isolated populations into account. In this study, C-bands were polymorphic in chromosome 2 of *O. chinensis formosana* and *O. yezoensis* (Fig. 3). Specimens of *O. chinensis formosana* from Okinawa yielded 3 types of homomorphic pairs of double or triple distal C-bands and a heteromorphic pair with double and triple C-bands. In the case of *O. yezoensis*, the embryos from Aomori and Osaka were the double/double homomorphic type, while those from Oita were double/triple heteromorphic type in addition to double/double homomorphic type. This polymorphism might be caused by deletion or addition of one of the distal C-band.

The interstitial C-band of chromosome 8 was common to all 4 species (Fig. 2e–h), and it corresponded to the SC that was detected after conventional Giemsa-staining. While C-banding patterns of chromosome 2 were species-specific in particular: a single proximal C-band in *O. hyla intricata*, 2 distal C-bands in *O. japonica japonica*, and 2 proximal and 2 or 3 distal C-bands in *O. chinensis formosana* and *O. yezoensis* (Fig. 2e–h). Very similar C-banding patterns of chromosome 2 between *O. chinensis formosana* and *O. yezoensis* strongly suggest the phylogenetic kinship, and it agrees with the previous reports (Zhu et al. 2001, Aikawa et al. 2003). These 2 species are isolat-
ed geographically, but not reproductively (Zhu and Ando unpublished), therefore, the differences in morphological, ecological and karyological seem be result of geographical isolation.

John et al. (1985) reported that Australian specimens of *O. japonica* had large C-blocks in the distal regions of chromosomes 4–10 that were missing from ours. *O. japonica* is divided into 2 subspecies, *O. japonica japonica* and *O. japonica vitticollis* (Fukuhara 1982a). Judged from the range of habitats, the Australian specimens examined by John et al. (1985) probably belong to the latter subspecies. Thus, the chromosomes of the present 4 species might have undergone considerable modification with respect to the size and location of C-heterochromatin, in spite of the conservative features of karyotypes revealed by the conventional staining. Species-specific patterns of C-banding have also been reported in the acridid grasshoppers, whose karyotypes are similar one another (King and John 1980, John et al. 1985, Cabrero and Camacho 1986, Yoshimura et al. 2003).

**G-banding and fluorescence staining**

The G-banded haploid karyotypes of the 4 species are shown in Fig. 4, together with the fluorochrome (QM- and CMA)-banded ones. No clear bands were generated in the euchromatic regions, irrespective of the types of differential staining. However, some C-band regions, such as the distal and interstitial C-heterochromatin of chromosome 2, the SC of chromosome 8 and some centromeric regions, were differentially stained by G-banding and/or fluorochrome banding. The G-

![Fig. 4. Differentially stained haploid karyotypes of *O. hyla intricata* (a, b, c), *O. japonica japonica* (d, e, f), *O. chinensis formosana* (g, h, i) and *O. yezoensis* (j, k, l). a, d, g and j, G-banding; b, e, h and k, QM-staining; and c, f, i and l, CMA-staining. Arrowheads and bars indicate negative and positive staining, respectively.](image-url)
bands on chromosome 2 of *O. chinensis formosana* were not clearly defined, probably as a result of inappropriate treatment of cells with trypsin, but they appeared to be basically the same as those of *O. yezoensis*.

In all species, the SC of chromosome 8 and the centromeric regions of some medium- to small-sized chromosomes yielded no fluorescent bands after QM-staining (Fig. 4). These QM-negative regions were G-negative or very weakly stained. Relatively bright QM-fluorescence was evident in the centromeric and interstitial regions of chromosomes 2 and 9 of *O. japonica japonica* (Fig. 4e). Some of the C-heterochromatic regions, such as the SC of chromosome 8 and the centromeric regions of some medium- to small-sized chromosomes emitted bright fluorescence after CMA-staining, but these regions fluoresced weakly after QM-staining (Fig. 4). Since CMA is known to bind specifically to GC base pairs (Schweitzer 1976, Schmid 1980), these heterochromatic regions are likely to be highly GC-rich.

In general, it is quite difficult to obtain well-differentiated G-bands in the euchromatic regions of grasshopper chromosomes. The same is also true of QM- and CMA-bands. In fact, neither G-banded nor QM-banded karyotypes have been reported for this group, with the exception of 2 reports in which faint bands are recognizable (John *et al.* 1985, Camacho *et al.* 1991). It is possible that the euchromatic regions of grasshopper chromosomes have not sufficiently defined AT- or GC-rich subchromosomal segments so that the entire chromosomes appear more or less homogeneous.

As shown in Figs. 2 and 5, the staining properties of the SC of chromosome 8 were basically the same in all 4 species of *Oxya*: positive for C-band and CMA-staining; negative for G-band, QM- and CMA-staining. These results suggest that the base composition of the C-heterochromatin in the SC might be strongly conserved among species. The C-heterochromatin in the proximal G-bands and fluorochrome bands, on the other hand, were not conserved among species, as summarized in Table 2. All the species, with exception of *O. hyla intricata* had G- and QM-negative and CMA-positive proximal bands in common in 2 to 3 pairs of medium-sized chromosomes, probably 5–7. Thus, *O. hyla intricata* might be phylogenetically less closely related to remain-

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**Table 2.** Differential staining of the proximal C-band areas in four species of *Oxya*

<table>
<thead>
<tr>
<th>Species</th>
<th>Differential Proximal C-bands in chromosome(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 to 7</td>
</tr>
<tr>
<td><em>Oxya hyla intricata</em></td>
<td>G and QM</td>
</tr>
<tr>
<td><em>O. japonica japonica</em></td>
<td>G and QM</td>
</tr>
<tr>
<td><em>O. chinensis formosana</em></td>
<td>G and QM</td>
</tr>
<tr>
<td><em>O. yezoensis</em></td>
<td>G and QM</td>
</tr>
</tbody>
</table>

ing 3 species since no such differential staining of proximal bands was detected on chromosomes 5–7. This is consistent with the finding in mitochondrial DNA sequences (Aikawa et al. 2003) and results of random amplified polymorphic DNA (Zhu et al. 2001). Taken together, the various results indicate that differential staining can yield a great deal of information about the karyosystematic relationships among acridid and catantopid grasshoppers.

**Ag-NOR banding**

In most cases of vertebrates, SCs contain silver-binding proteins and are sites of genes for ribosomal RNAs, and they are strongly stained by Ag-NOR technique (Howell 1982, Sumner 1990, Suja and Hernandez-Vedun 1996). These observations are also applicable to the NORs of acridid grasshopper chromosomes, as verified by Fox and Santos (1985) in *Schistocerca gregaria* and *Locusta migratoria*. However, in *Oxya* species we examined, the SC of chromosome 8 did not correspond to NORs at all (Fig. 5). As seen in human chromosomes 1, 9 and 16, SCs are not always related with NORs (Funaki et al. 1975, Verma et al. 1982). King (1980) noted 5 different types of SC in Australian hylid frogs by sequential staining with conventional Giemsa, C-banding and silver NOR technique. Suja et al. (1986), studying an acridid species *Psophus stridulus*, showed the sixth type of SC that corresponded to potential NOR. The ‘type 6’ was inconstant constriction, while the SC of chromosome 8 in *Oxya* always appeared. Therefore, it corresponds, in all likelihood, to the ‘type 3’ defined by King (1980) that is positive for C-banding and negative for silver staining.

All 4 species of *Oxya* had small silver-stained segments at the centromere of all chromosomes (Fig. 5), differing in this regard from most of the grasshopper species reported to date, in which only a few chromosome pairs, 5 pairs at the most, are involved in nucleolar organization (Rufas and Gosálvez 1982, Fox and Santos 1985, Cabrero et al. 1986, 1987, Yadav and Yadav 1987, Camacho et al. 1991). It is generally accepted that the maximum number of nucleoli per nucleus corresponds to the number of NORs (Thode et al. 1983). Numerous silver deposits were found in silver-stained interphase nucleus of rice-field grasshoppers (Fig. 5a). Thus, it is likely that the silver deposits on all the centromeres in *Oxya* reflect the presence of active NORs. However, none of the centromeres of pachytene chromosomes had silver deposit when silver staining was performed using male meiotic cells of *O. chinensis formosana* (Yoshimura et al. unpublished). Such inconsistency in the NOR distribution between mitotic and meiotic chromosomes was reported in an acridid species *Pamphagus ortolanii* (Mansueto and Vitturi 1989), and Mansueto and Vitturi (1989) interpreted as being due to difference in transcriptional activity of ribosomal DNA. On the other hand, it is also known that the AgNO₃ treatment is capable of depositing silver grains on the kinetochores of the highly condensed grasshopper chromosomes, depending on the treatment condition (Rufas et al. 1994). Therefore, some of the silver deposits on the centromeres of *Oxya* chromosomes might be unrelated to the presence of active NORs rather reflecting silver-stained kinetochores. FISH analysis with ribosomal DNA probes is necessary to solve the question whether the centromeric Ag-NORs function as NORs in the genus *Oxya*, and to show that the SC of chromosome 8 is not the site of ribosomal RNA gene clusters.

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