A comparative analysis of the metaphase karyotypes of *Aedes excrucians*, *Ae. behningi,* and *Ae. euedes* (Diptera: Culicidae) imaginal discs

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ABSTRACT: Karyotypes of *Aedes* (Culicidae) mosquitoes (*Ae. excrucians*, *Ae. behningi,* and *Ae. euedes*) have been analyzed using the metaphase chromosomes of imaginal discs. Lacto-aceto-orcein, C-banding, and DAPI staining have detected species-specific features in the morphology and lengths of these chromosomes in the examined species. Species-specific features of chromosome 1 in the location of heterochromatin blocks have been shown. Thus, the metaphase chromosomes in the imaginal discs of *Ae. excrucians*, *Ae. behningi,* and *Ae. euedes* are a characteristic for species identification of mosquito species. ***Journal of Vector Ecology* 43: 245-251. 2018.**

Keyword Index: Blood-sucking mosquitoes, Culicidae*, Aedes,* lacto-aceto-orcein, C-banding, DAPI, heterochromatin, mitotic chromosomes, imaginal discs.

INTRODUCTION

The data regarding chromosome morphology, karyotypes, and full-genome sequences of blood-feeding mosquitoes are of great applied value in designing methods for their control (Holt et al. 2002, Nene et al. 2007). The genomes of the most dangerous mosquito vectors, *Anopheles gambiae, Culex quinquefasciatus,* and *Aedes aegypti*, have been sequenced (Holt et al. 2002, Nene et al. 2007). An increase in the occurrence and abundance of many dangerous mosquito-borne diseases has been observed recently on all continents, including dengue fever (Ramchurn et al. 2009), yellow fever (Aitken et al. 1979, Fontenille et al. 1997), chikungunya (de Lamballerie et al. 2008, Vu 2017), and Zika viruses (Marchette et al. 1969, Quereshi 2017). Human cases of West Nile fever (neuroinvasive disease) have been reported annually in Russia since 1999. The highest incidence has been recorded in southern European Russia and the distribution of human cases has expanded northwards considerably (Platonov 2014). The mosquito-borne pathogens are frequently species-specific, which makes it important to have a precise species identification of the corresponding vectors (Halin 2011). There is a risk of "imported" cases of such diseases in Russia as well (Fedorova 2007). So far, at least 15 mosquito species in Russia are potential vectors for West Nile virus (Fedorova 2007) and eight of them, *Ae. cinereus, Ae. vexans, Ae. excrucians, Ae. dorsalis, Ae. cantans, Ae. sticticus, Coq. richiardii,* and *An. messeae,* have been recorded in the city of Tomsk and in the Tomsk region (Andreeva et al. 2017). Climate change can also cause climate-associated adaptive behavior and transmission dynamics of viruses in different areas (Chadee et al. 2016). Consequently, the spread of yellow fever became a global health emergency. Even with knowledge of the typical clinical manifestations, clinical diagnosis can be difficult because yellow fever can mimic multiple febrile illnesses. Laboratory diagnostic tests can detect the virus and specific antibodies in the blood. No specific antiviral drug or immune therapy exists for yellow fever disease (Gostin and Lucey 2016). The 31 mosquito species living in the Tomsk region belong to five genera, namely, *Anopheles, Culex, Culiseta, Coquillettidia,* and *Aedes,* and the genus *Aedes* displays the highest species diversity (Andreeva et al. 2017). *Aedes* mosquitoes are rather well studied with respect to morphological species-specific characteristics of imago and larvae (Gutsevich 1970, Andreeva et al. 2017) as well as to the molecular genetic characteristics, in particular, rDNA genes and COI (cytochrome oxidase *c* subunit 1) gene (Khrabrova et al. 2013a,b).

On the other hand, these methods are not always able to detect cardinal differences between individual species (Khrabrova et al. 2012). Analysis of the polytene chromosomes of *Aedes* and *Culex* species allowing for species-specific identification is complicated because of the methodological difficulties in spreading of the chromosomes (Sutton 1942, French et al. 1962, Chaudhry 1981, Patnaik et al. 1989, Campos et al. 2003). This makes karyotype characterization and analysis of metaphase chromosomes more informative for *Aedes* systematics and species identification (Kabanova and Kartashova 1972, Choochote 2001). A $2n = 6$ karyotype is characteristic of most mosquito species and, in particular, *Aedes* mosquitoes. As a rule, the homologous chromosomes in metaphase either conjugate in the centromeric region or reside nearby (Kabanova and Kartashova 1972, Sharakhova et al. 2011). Earlier, mosquito chromosomes were designated in increasing length; later, the longest metacentric chromosome was defined as chromosome 2 and the shortest metacentric chromosome, chromosome 1; submetacentric chromosome 3 has an intermediate length (McDonald and Rai 1970, Sharakhova et al. 2011). Several *Anopheles* and *Aedes* species display species-specific distinctions in differential C- or G-banding (distribution of heterochromatin blocks), mainly in the sex chromosomes. This demonstrates that the specific C- and G-banding patterns of metaphase chromosomes are

species-specific in several mosquito species (Moosa and Rai 1977, Dev and Rai 1984, Baimai et al. 1995). Correspondingly, a comparative analysis of metaphase karyotypes of different dipteran species, in particular, *Aedes* mosquitoes, is a topical methodical approach when identifying interspecific differences.

Here we examine three mosquito species, *Aedes* (*Ochlerotatus*) *behningi*, *Ae. excrucians,* and *Ae. euedes,* sampled in the Tomsk region, the species-level identification of which present certain difficulties. In particular, the *Ae. behningi* 4th instar larva in its morphology is very similar to *Ae. excrucians* larva, being almost indistinguishable in many cases. Another example is *Ae. euedes* and *Ae. excrucians,* which can be distinguished only according to their larval morphology (Gutsevich et al. 1970). In addition, these species have not been yet karyotyped. The goal of this work was to find species specificity of the *Ae. behningi*, *Ae. excrucians,* and *Ae. euedes* mosquitoes utilizing analysis of their karyotypes.

MATERIALS AND METHODS

The 4th instar larvae of *Ae. behningi*, *Ae. euedes,* and *Ae. excrucians* examined in this work were sampled in water bodies of the Tomsk region. Morphological species-level identification of the sampled larvae was conducted using MBS-12 (Russia) and Stemi 2000-C (Carl Zeiss, Germany) stereo microscopes according to the conventional descriptions and keys (Gutsevich et al. 1970, Gutsevich and Dubitskii 1981, Becker et al. 2010). The nomenclature is given according to the Systematic Catalog of Culicidae (http://mosquitocatalog.org/default.aspx). Larvae were fixed with Carnoy's solution (ethanol to glacial acetic acid, 3: 1).

Metaphase plates of dividing imaginal disc cells of the early 4th instar larvae were examined. The structure of metaphase chromosomes was assayed using lacto-aceto-orcein staining (Kabanova and Kartashova 1972), C-banding, and DAPI staining (Saifitdinova 2008).

Lacto-aceto-orcein staining

Imaginal discs were isolated from *Ae. behningi*, *Ae. euedes,* and *Ae. excrucians* larvae fixed with Carnoy's solution, stained in a drop of lacto-aceto-orcein dye for 15 min, and washed in 45% acetic acid. The stained imaginal discs were covered with a cover glass to get squash preparations by tapping on the cover glass. The squash preparations were examined using a Zeiss Axioimager A1 (Zeiss, Germany) light microscope.

DAPI staining

For this purpose, imaginal discs were isolated from mosquito larvae in a drop of Carnoy's solution, transferred to a drop of 45% acetic acid, covered with a cover glass, and squashed. The cover glass was removed using liquid nitrogen and the preparations were dehydrated by successive treatment with alcohols (50, 70, and 96%; 5 min each). A drop of DAPI (a fluorescent dye) was placed onto air-dried preparations, which were then covered with a cover glass. The resulting slides with DAPI-stained metaphase chromosomes were examined using a Zeiss Axioimager Z1 (Zeiss, Germany) fluorescence microscope.

C-banding

C-banding was performed using pre-staining of chromosome preparations with $Ba(OH)_2$. The air-dried preparations of mosquito imaginal discs were incubated in 0.2 M HCl at room temperature for 1 h and placed in fresh 5% barium hydroxide solution at 50° C for 10 -15 min. Then the preparations were washed and incubated in 2× SSC buffer at 60° C for 1 h. The resulting slides were washed, stained with 4% Giemsa solution for 1.5 h, and examined using a Zeiss Axioimager A1 (Zeiss, Germany) microscope.

Statistical data processing

The chromosomes were identified based on the ratio of their arms and their lengths according to the relevant chromosome classification (McDonald and Rai 1970). The lengths of chromosomes and their arms were measured using the ImageJ program. The centromeric index was calculated as $J^c = p/(p + q)$, where p is the short chromosome arm and q, the long arm; the relative chromosome length was calculated as

$$L^r = \frac{\text{Length of chromosome}}{\text{Total length of all chromosomes}} \times 100\%,$$

where L^r is the relative chromosome length (%).

Over 50 metaphase plates were examined for each species and 30 metaphase plates with the same degrees of condensation were selected for analysis.

RESULTS

The imaginal disc cells of the 4th instar larvae were selected for karyotype analysis of three mosquito species (*Ae. behningi*, *Ae. euedes,* and *Ae. excrucians*) because dividing cells as well as prometaphase and metaphase chromosomes are much more abundant in imaginal discs as compared with ganglion cells. Lacto-aceto-orcein, C-banding, and DAPI staining were used for characterization of karyotypes. The metaphase chromosomes displayed specific and differential patterns for each staining type (Figure 1).

The diploid mitotic chromosome set in the imaginal discs of these species is $2n = 6$, similar to most mosquito species. The chromosomes constituting the karyotype differ in their lengths. According to the classification of chromosomes of the *Aedes* mosquitoes, the chromosomes were distinguished by their lengths, namely, chromosome 1 is the shortest; chromosome 2, the longest; and chromosome 3 is intermediate in its size (McDonald and Rai 1970). The lengths of chromosomes and their arms in three mosquito species were measured using ImageJ and the corresponding mean values were calculated (Figure 2).

Thus, the mean length of *Ae. behningi* chromosome 1 was 2.43 μm; of chromosome 2, 3.82 μm; and of chromosome 3, 3.79 μm; these values for *Ae. excrucians* were 2.7, 7.72, and 7 μm, respectively, and for *Ae. euedes,* 3.93, 6.09, and 5.96 μm, respectively. The corresponding histogram (Figure 2) shows

Table 1. Numerical characteristics of the chromosomes of *Ae. euedes*, *Ae. excrucians,* and *Ae. behningi* mosquitoes (L^r, relative chromosome length, %, and J^c, centromere index, %).

Species	Chromosome					
	1		2		3	
	L^r, %	J^c, %	L^r, %	J^c, %	L^r, %	J^c, %
Ae. behningi	24	50	38	50	37.7	49
Ae. excrucians	26	48	39	45	35	49
Ae. euedes	24	50	37	45	38	51

that the mean values of three pairs of metaphase chromosomes are considerably larger in *Ae. excrucians* as compared with *Ae. behningi* and *Ae. euedes*, while the chromosomes of *Ae. behningi* display the least values. Thus, three mosquito species differ from one another in the mean lengths of three pairs of metaphase chromosomes in imaginal discs.

Calculation of the relative length and centromere index for the metaphase chromosomes of imaginal discs has shown that the all three pairs of chromosomes in the examined species are metacentrics (Table 1). In particular, the relative chromosome lengths amounted to 24–39% and the centromere index, 45–51%, which corresponds to metacentric chromosomes according to the parameters of chromosome nomenclature (Levan et al. 1964).

Examination of the mitotic chromosomes stained with lacto-aceto-orcein has demonstrated a species-specific pattern (distribution of distinctly colored chromatin blocks) of chromosome 1 for all three species (Figure 1a, d, and g). Characteristic of *Ae. excrucians* is an almost totally stained chromosome 1 vs *Ae. euedes* and *Ae. behningi,* distinguishable from one another and *Ae. excrucians* in the number and distribution of stained blocks in chromosome 1. The autosomes of all species were totally stained, interfering with the detection of fine interspecific differences (Figure 1a, d, and g). As is known, differential staining for heterochromatin blocks in animal and plant mitotic chromosomes reveals distinct species specificity (Koryakov and Zhimulev 2009). In particular, C-banding reveals constitutive heterochromatin, which is mainly localized to centromeric and telomeric regions, while DAPI targets AT-rich chromosome regions. That is why we also used C-banding and fluorescent DAPI staining in the comparative analysis of *Ae. behningi, Ae. euedes,* and *Ae. excrucians* karyotypes. The C-banding patterns of *Ae. behningi* mitotic chromosomes displayed staining in the centromeric and several intercalary regions; in particular, chromosome 2 contained a large intercalary C-band. All *Ae. excrucians* chromosomes displayed wide C-bands in peritelomeric regions and narrower bands in pericentromeric regions of chromosomes 2 and 3; in addition, chromosome 2 contained narrower intercalary bands. Characteristic of *Ae. euedes* are centromeric C-bands in all chromosomes. DAPI staining allowed for distinguishing all three mosquito species according to the distribution of bright fluorescent bands in the metaphase chromosomes of imaginal discs. The DAPI bands prevalently resided in intercalary and pericentromeric regions. Wide fluorescent bands in all chromosomes were characteristic of *Ae. excrucians.* The *Ae. behningi* chromosomes displayed rather narrow DAPI bands in pericentromeric and intercalary regions vs *Ae. euedes* that carried one wide band in an arm of chromosome 1 and thinner bands in the intercalary and pericentromeric regions of chromosomes 2 and 3 (Figure 1).

DISCUSSION

The obtained data allowed us to schematize the orcein, C-banding, and DAPI patterns of the metaphase chromosomes of imaginal discs for *Ae. behningi, Ae. euedes,* and *Ae. excrucians* mosquitoes (Figure 3). Evident from Figure 3, the lacto-aceto-orcein pattern of chromosome 1 is distinctly species-specific. The C-banding and DAPI patterns of these mosquito species also display species specificity in the band widths and positions on the chromosomes. In particular, *Ae. excrucians* differs from *Ae. behningi* and *Ae. euedes* by the largest size of its metaphase chromosomes, which carry rather wide C- and DAPI bands. In particular, chromosome 1 q arm is almost totally stained by lacto-aceto-orcein and DAPI, suggesting a high content of heterochromatin. As for chromosomes 2 and 3, they carry rather wide bands differing in their patterns. Lacto-aceto-orcein, C-banding, and DAPI stains also detect pericentromeric and peritelomeric regions of chromosomes 2 and 3. Unlike *Ae. behningi* and *Ae. excrucians,* the *Ae. euedes* chromosomes display different banding patterns for the dyes. In particular, C-banding has been detected only in the centromeric regions and DAPI distinguished between chromosome 1 with its wide bands vs chromosomes 2 and 3, that lack such bands.

Thus, *Ae. excrucians, Ae. behningi,* and *Ae. euedes* have species-specific karyotype morphology, lacto-aceto-orcein pattern of chromosome 1, as well as C-banding and DAPI staining. The observed differences are the most pronounced in chromosome 1. Correspondingly, this chromosome is a candidate marker for species-level identification of these *Aedes* species. Note that the sex-determining chromosomes in this genus are homomorphic and the sex-determining alleles are directly associated with chromosome 1 (McClelland 1962, Rai 1963).

Other *Anopheles* and *Aedes* species also display species-specific differences in the distribution of heterochromatin bands in sex chromosomes, evident in C- or G-stained mitotic chromosomes (Moosa and Rai 1977, Dev and Rai 1984, Baimai et al. 1995). The phenomenon of species specificity of differentially (C- or G-) stained mitotic sex chromosomes has been observed not only in mosquitoes but also in *Lucilia* flies. In

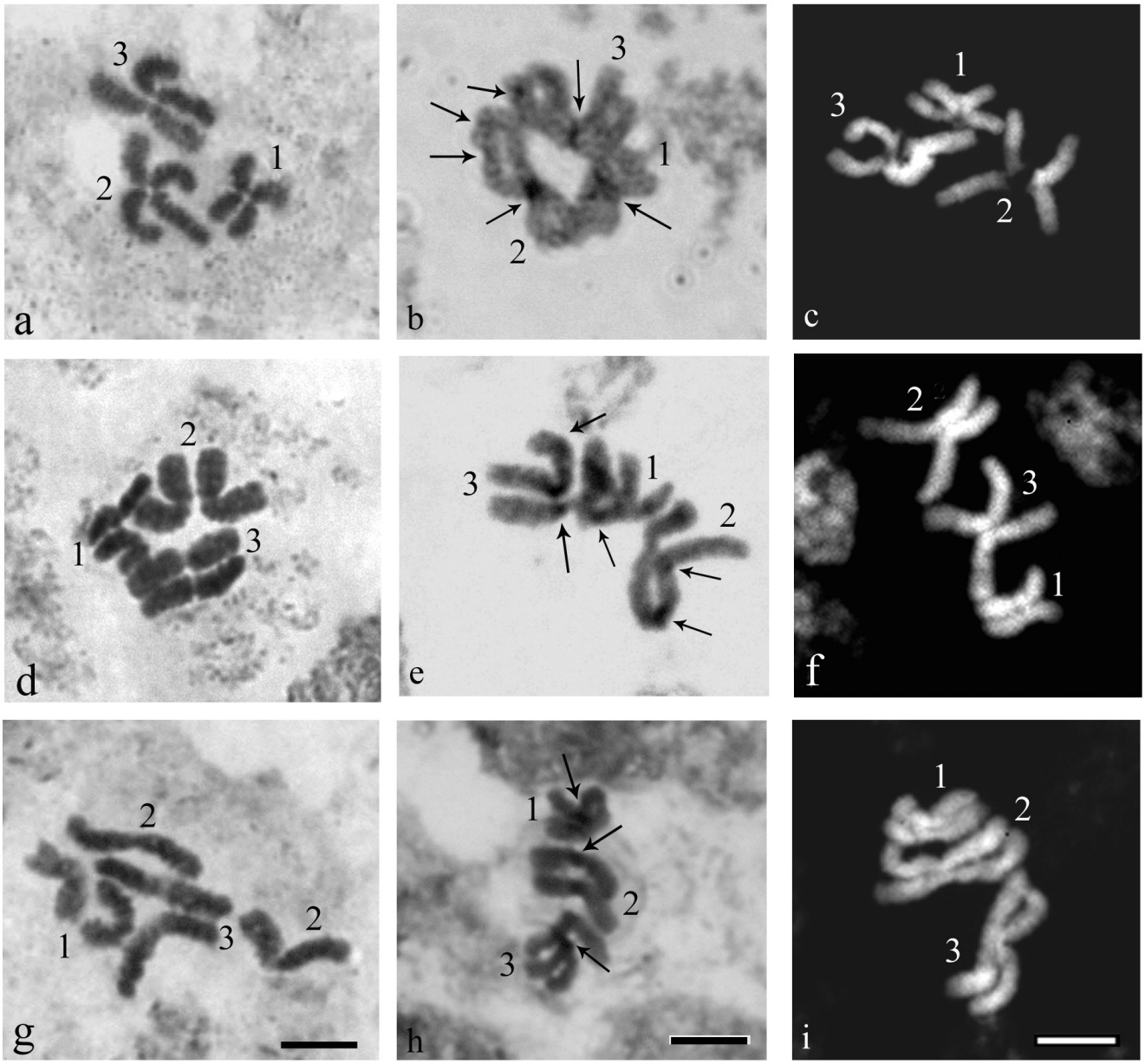

Figure 1. Metaphase chromosomes of imaginal discs of the mosquito species (a–c) *Ae. behningi*, (d–f) *Ae. excrucians*, and (g–i) *Ae. euedes*: (a, d, and g) lacto-aceto-orcein staining; (b, e, and h) C-banding; and (c, f, and i) DAPI staining; 1–3, chromosome designations; arrows denote C-bands; scale bar, 5 μm.

particular, the metaphase karyotypes of *L. cluvia* and *L. sericata* display homology of five autosome pairs and a considerable variation in the sex chromosome in its morphology and size, as well as heterochromatin content and distribution (Chirino et al. 2015). The Calliphoridae autosomes are less variable as compared with the sex chromosomes, changing in their shape and length from one species to another (Boyes and Shewell 1975, Azeredo-Espin and Pavan 1983, Parise-Maltempi and Avancini 2007, Ullerich and Shöttke 2006, Agrawal et al. 2010, Holecová et al. 2012). As is believed, the interspecific variation of the sex X chromosomes is associated with chromosomal rearrangements during speciation as well as with accumulation of a large amount of repetitive DNA sequences, which has contributed considerably to the size of *L. sericata* sex chromosomes (Chirino et al. 2015).

It is noteworthy that the three mosquito species examined here (*Ae. behningi, Ae. euedes,* and *Ae. excrucians*) also display species-specific differences in the size of their chromosomes. *Ae. excrucians* with its longest chromosomes and largest content of heterochromatin blocks displays the most pronounced distinctions from the other two species. Presumably, large chromosomes of this species result from accumulation of considerable heterochromatin amounts during speciation, evidenced by the comparison of large wide heterochromatin bands detected by C-banding in this species vs narrower bands in the remaining species. For example, C-banding in *Ae. euedes* is observable only in the pericentromeric regions. The DAPI patterns of mitotic chromosomes of these mosquito species are also species-specific. Thus, both C-banding and DAPI patterns can be

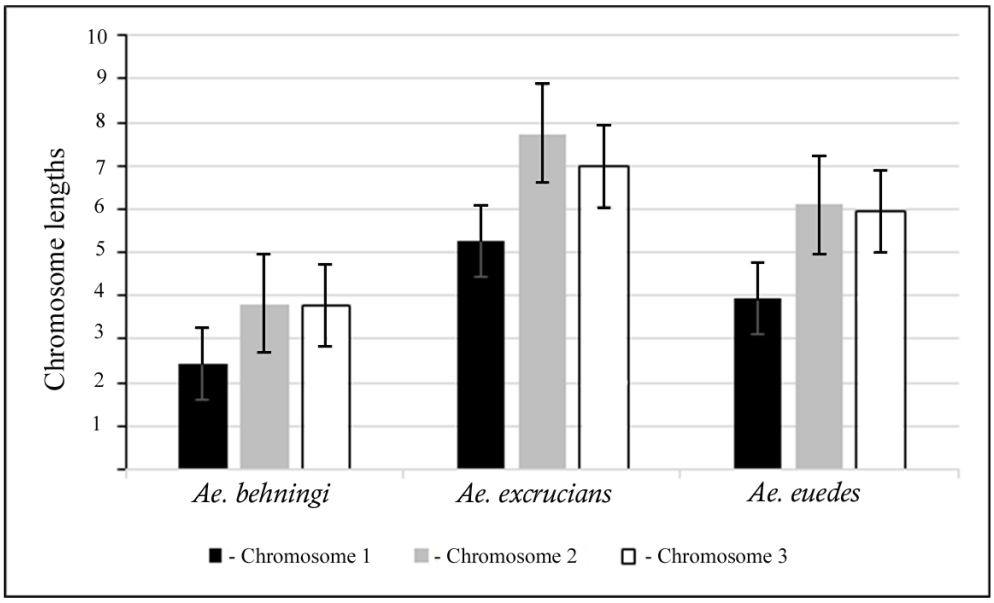

Figure 2. Histogram of the mean lengths of imaginal disc chromosomes constituting the karyotypes of *Ae. behningi*, *Ae. euedes*, and *Ae. excrucians* mosquitoes.

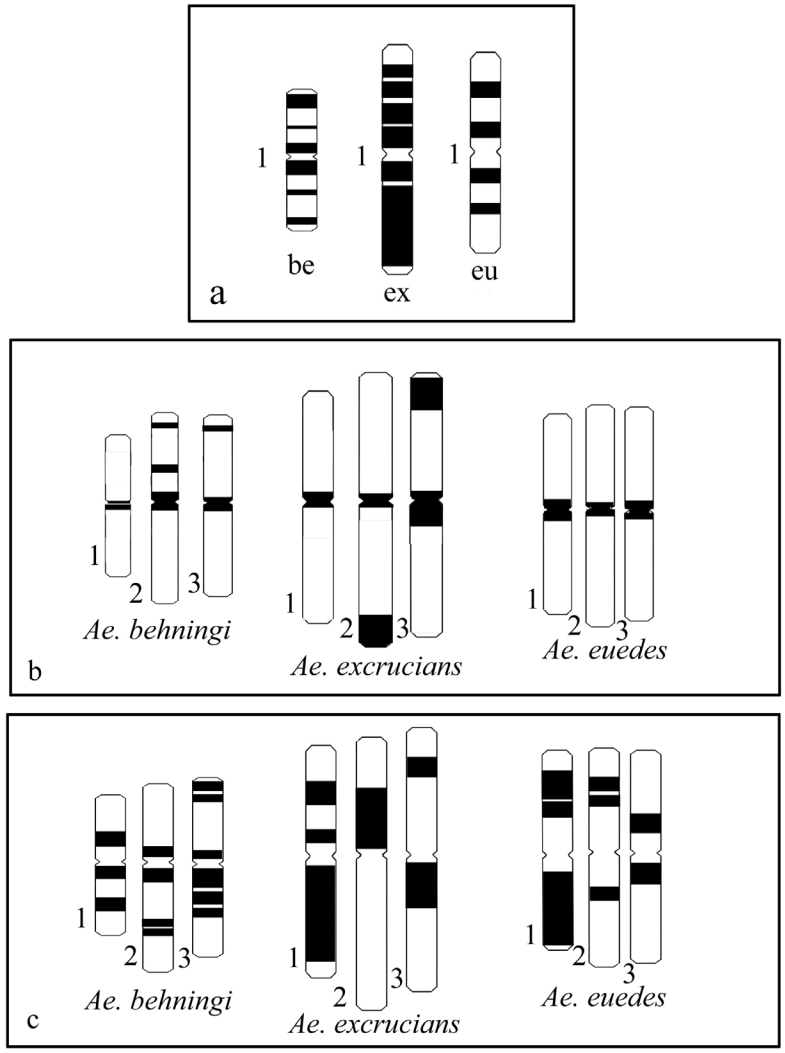

Figure 3. (a) Lacto-aceto-orcein patterns of chromosome 1 of *Ae. behningi, Ae. euedes*, and *Ae. excrucians* (be, *Ae. behningi*; ex, *Ae. excrucians*; and eu, *Ae. euedes*); (b) C-banding; (c) DAPI patterns of metaphase chromosomes of these mosquito species, respectively; 1–3, chromosome designations. be – *Ae. behningi*; ex – *Ae. excrucians*; eu –*Ae. euedes.*

regarded as important cytological markers for comparison of the karyotypes of phylogenetically close species. Such approaches to studying karyotypes are useful to analyze the changes in karyotype associated with evolution and to get a better insight into the relevant taxonomy.

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