Study on siphonal measurements and usefulness in delimitation of “rural” and “urban” ecotypes of Culex pipiens

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Abstract

Mosquitoes of the Culex pipiens complex (Diptera Culicidae) are of great medical and veterinary importance as vectors for various bacterial, filarial and viral diseases. Taxonomic status, biology and ecology of members of the C. pipiens complex are still matter of study and discussion and their morphological, physiological, genetic and ecological characteristics are imprecise and often controversial. The aim of this study was to compare the usefulness of different siphonal measurements in delimitation of “rural” and “urban” ecotypes of C. pipiens. In this study, three samples from different biotopes (underground - ecotype I; aboveground - ecotypes II and III) were analysed for morphological variation using nine siphonal indices - SI (as a ratio of the length to the width measurements) and one width index - WI (representing the ratio of the width of the base and top of the siphon). Highly significant differences among the three ecotypes in all siphonal indices (S11-S19) were observed. It was revealed significant differences (Tukey post hoc test) between ecotype III versus I and II. Within each ecotype CV values for all nine siphon indices were similar, with the exception of WI. Therefore, usefulness and sensitivity of nine SI studied are equal.

Key words: Culex pipiens, Culicidae, ecotype differentiation, siphonal index, siphonal variability.

Introduction

The members of the Culex pipiens complex are of medical and veterinary importance as vectors for various bacterial, filarial and viral diseases (Vinogradova and Shaiekevich, 2007; Becker et al., 2010). Understanding the population structure and gene flow among populations of mosquito species is critical for public health issues such as local dispersion patterns, evolution and spread of insecticide resistance alleles, epidemiology of mosquito-borne pathogens and developing and testing management strategies and control of vector borne diseases (Tabachnick and Black, 1995; Smith et al., 2005; Rasgon et al., 2006).

The members of the C. pipiens complex represent one of the outstanding problems in the current mosquito taxonomy, with opinions on Culex pipiens L. taxonomy ranging from distinct species to physiological forms with considerable genetic introgression (Cornel et al., 2003). Taxonomic status, biology and ecology of members of the complex are still matter of study and discussion and their morphological, physiological, genetic and ecological characteristics are imprecise and often controversial (Harbach et al., 1985; Chevillon et al., 1995, 1998; Vinogradova, 2003; Fonseca et al., 2004; Kent et al., 2007; Gomes et al., 2009; Weitzel et al., 2009; Kothera et al., 2010). Members of C. pipiens occur in two forms: biotype pippins (“rural”) and biotype molestus (“urban”), separated based on physiological, behavioral characteristics and differences of structural genes. The form pippins is anautogenous and eurygame, it has a reproductive diapause and feeds mostly on birds, even though the number of its anthropophilic populations has been increasing recently (Spielman, 1967, 2001; Harbach et al., 1985; Byrne and Nichols, 1999; Hamer et al., 2008). The form molestus is autogenous and stenogame, it cannot enter diapause and prefers to feed on humans (Spielman, 1967; Harbach et al., 1984; Byrne and Nichols, 1999; Vinogradova and Ivnitsky, 2009). The anautogenous biotype occurs mainly in aboveground and rural habitats, while the autogenous biotype occurs in the underground and also aboveground urban habitats (Ribeiro et al., 1983; Harbach et al., 1984; Gomes et al., 2009; Reusken et al., 2010). Besides on biological and behavioral diagnostic characters, molecular markers provide useful tools to identify molestus, pippins and their hybrids. For example, based on fixed allelic differences and significant differences in the allele frequencies at the allozyme loci Aat, Hbd, Mpi, Pgm, Ak-1 and Ik (Chevillon et al., 1998; Becker et al., 1999; Weitzel et al., 2009) two C. pipiens forms were distinguished. In addition, one microsatellite locus was proposed as promising diagnostic marker (Bahnck and Fonseca, 2006; Kent et al., 2007; Gomes et al., 2009).

Morphological characters have long been in use in solving the taxonomic status of members of the C. pipiens complex. The respiratory siphon, a process of the eighth abdominal segment, is a valuable diagnostic character for identification and distinguishing genera and species within the family Culicidae (Becker et al., 2010). One of the oldest and commonly used taxonomic characters is the siphonal index - SI (the ratio of the length of the siphon to its width). Although the great variations in the values of this character have caused doubts about its diagnostic validity in the determination of members of the complex (McMillan, 1958; Harbach et al., 1984) this parameter, with certain modifications, continues to be used in the separation of species C. pipiens and Culex quinquefasciatus Say (Ishii, 1980; Brogdon, 1981, 1984; Kruppa, 1987; Cornel et al., 2003), as well as C. pipiens and Culex torrentium Martini (Service, 1968; Dahl, 1988; Vinogradova et al., 1996; Vinogradova and Ivnitsky, 2009) and biotypes pippins and molestus (Vinogradova, 2003). However,
consistent measures used for SI is lacking, which caused difficulties in comparison of published results. For example, measures in use for siphon width were: the middle of siphon (Belkin, 1962; Harbach and Knight, 1980) or widest part of the siphon at its base not counting acus (Gutsevich et al., 1974; Jupp, 1978; Cranston et al., 1987; Eritja and Aranda, 1995; Vinogradova et al., 1996). For siphon length, from base to tip excluding siphon valve, in use is: measurement along the middle of siphon (Brogdon, 1981; Cranston et al., 1987), along the posterior side (Gutsevich et al., 1974; Vinogradova et al., 1996) or not clearly defined (Eritja and Aranda, 1995; Cornel et al., 2003).

The study presented herewith is a part of a larger research project to quantify the spatial and temporal distribution of genetic and phenotypic variation of “urban” and “rural” ecotypes of *C. pipiens*. The current study was undertaken to test usefulness of different measures of length and width of the siphon as a standard measure of SI. Such different siphonal indices were used in delimitation of simpatrically occurring “rural” and “urban” ecotypes of *C. pipiens*.

**Materials and methods**

Larvae of *C. pipiens* were collected from a broader area of the city of Novi Sad (45°15’N; 19°50’E) (Autonomous Province of Vojvodina, northern part of Serbia) during September 2009, from three different biotopes: 1. street manhole (the underground habitat of urban type; ecotype I), 2. draining ditch and pond in the city (aboveground habitat of urban type; ecotype II) and 3. pond (swamp) outside urban area (aboveground habitat of rural type; ecotype III). Larvae of EIII were collected in Special Nature Reserve “Koviljsko-Petrovaradinski rit” with a complex of marshes and forest ecosystems (in 2004 it was included in the list of important water-related protected areas in the Danube basin - ICPDR, since 2005 this wetland is IPA - Important Plant area, and also a Ramsar Site candidate). Specimens of each biotype were collected at the same breeding site and time. Numerous adults and larvae were present on each locality at the time of sampling, which imply that sampled larvae had been obtained from many females. From each breeding site, around 300 larvae were collected and reared in the laboratory in their original water without feeding. After adult eclosion, exuviae of 4th instar larvae were preserved in 96% ethanol. Since exuviae (not larvae) were used for the study, all individuals were at the same point of development and that allowed using adults for sex determination and for further genetic and morphological analyses. In this study, a total of randomly chosen 144 exuviae (72 female and 72 male specimens) were analyzed. Due to lack of statistically significant differences between sexes found in a preliminary study of siphonal character variability, in further analysis genders were not analyzed separately. Specimens were identified based on the morphological characters of adults, defined for *C. pipiens* (Gutsevich et al., 1974; Božić, 1985).

Each larval exuvia was placed in watch glass in a drop of water in the lateral position, and then photographed with a digital camera Leica DFC320 associated with a stereo microscope Leica MZ12.5, and the images were used in further morphometric analysis. Exuviae were not mounted in order to avoid being flattened.

Siphonal index (SI) variation was observed from 54, 48 and 42 specimens from ecotype I, II and III, respectively. Using Leica Application Suite Measurement Module (ver. 2.4.0) three length values (L1, L2, L3) and three width values (W1, W2, W3) on each siphon were measured (figure 1). Ten indices were calculated as a ratio of the measured linear distances - nine indices named siphonal indices (SI1-SI9) representing the ratio of the length to the width measurements (SI1 = L1/W1; SI2 = L1/W2; SI3 = L1/W3; SI4 = L2/W1; SI5 = L2/W2; SI6 = L2/W3; SI7 = L3/W1; SI8 = L3/W2; SI9 = L3/W3) and one index named width index (W1) representing the ratio of the width of the base and top of the siphon (W1 = W1/W3) (figure 1).

Descriptive statistics (mean, standard deviation, minimal and maximal values, coefficient of variation) were calculated for each of 10 indices and differences among ecotypes were tested using one-way analysis of variance (ANOVA with the post hoc Tukey HSD test). All statistical analyses were done using Statistica for Windows (version 9.1).

![Figure 1.](image.png) Length (L1, L2, L3) and width (W1, W2, W3) measurements of siphon of *C. pipiens*. 


Table 1. Siphonal (SI1-SI9) and width (WI) indices for “urban” (I and II) and “rural” (III) ecotypes of *C. pipiens* from Novi Sad. For SI1-SI9 Tukey post hoc test revealed significant differences (p < 0.001) between ecotype pairs I/III and II/III.

<table>
<thead>
<tr>
<th>Index</th>
<th>Ecotype I (n = 54)</th>
<th>Ecotype II (n = 48)</th>
<th>Ecotype III (n = 42)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SD)</td>
<td>Min</td>
<td>Max</td>
<td>Mean (SD)</td>
</tr>
<tr>
<td>SI1</td>
<td>4.735 (0.457)</td>
<td>3.867</td>
<td>5.829</td>
<td>4.851 (0.493)</td>
</tr>
<tr>
<td>SI2</td>
<td>4.624 (0.454)</td>
<td>3.991</td>
<td>5.702</td>
<td>4.752 (0.490)</td>
</tr>
<tr>
<td>SI3</td>
<td>4.853 (0.457)</td>
<td>5.199</td>
<td>6.196</td>
<td>4.959 (0.496)</td>
</tr>
<tr>
<td>SI4</td>
<td>5.510 (0.531)</td>
<td>4.408</td>
<td>6.619</td>
<td>5.774 (0.679)</td>
</tr>
<tr>
<td>SI5</td>
<td>5.382 (0.525)</td>
<td>4.279</td>
<td>6.453</td>
<td>5.655 (0.670)</td>
</tr>
<tr>
<td>SI6</td>
<td>5.648 (0.534)</td>
<td>4.554</td>
<td>6.785</td>
<td>5.902 (0.688)</td>
</tr>
<tr>
<td>SI7</td>
<td>8.937 (0.723)</td>
<td>7.229</td>
<td>11.267</td>
<td>9.207 (1.071)</td>
</tr>
<tr>
<td>SI8</td>
<td>8.729 (0.716)</td>
<td>7.088</td>
<td>10.984</td>
<td>9.018 (1.053)</td>
</tr>
<tr>
<td>SI9</td>
<td>9.161 (0.728)</td>
<td>7.394</td>
<td>11.549</td>
<td>9.413 (1.090)</td>
</tr>
<tr>
<td>WI</td>
<td>1.893 (0.103)</td>
<td>1.656</td>
<td>2.206</td>
<td>1.898 (0.116)</td>
</tr>
</tbody>
</table>

Results and discussion

Descriptive statistics for ten analyzed indices were given in table 1. For each siphonal index (SI1-SI9) it was shown that ecotype III specimens had the highest mean index value comparing to specimens of ecotypes I and II (table 1). The ANOVA showed highly significant differences (p < 0.001) among the three ecotypes in all siphonal indices (SI1-SI9). Tukey post hoc test revealed significant differences (p < 0.001) between ecotype III versus I and II. Contrary to siphonal indices, width index (WI) among ecotypes revealed no significant differences (F(2,14) = 0.96; p = 0.39), although mean value for ecotype III was slightly higher than values for ecotypes I and II (table 1).

To study usefulness and sensitivity in quantifying siphonal variability, for each ecotype 10 indices coefficient of variation (CV) were calculated. Thus, within each ecotype CV values were compared, revealing that CV values for all nine siphonal indices were similar (ranged from 7.95% to 9.81%, 10.00% to 11.85% and 7.11% to 9.90% for I, II and III ecotypes, respectively), with the exception of WI. Lower values of CV for WI were obtained; there were 5.47%, 6.10% and 5.08% for I, II and III ecotypes, respectively (figure 2). Since, all siphonal indices (SI1-SI9) have equivalent sensitivity and usefulness, we decided to use SI1 for further comparison and discussion. In our study mean value of SI1 ranges from 4.73 (EI), 4.85 (EII) to 5.49 (EIII), which was slightly higher than previously published data, with SI values from 3.08 to 5.14 recorded for biotype *molestus* and from 4.4 to 6.4 for biotype *pipiens* (Jobling, 1938; Eritja and Aranda, 1995; Vinogradova et al., 1996; Vinogradova and Ivnitsky, 2009), or about 4.5 or less for *molestus* and about 5.0 or more for *pipiens* (Marshall, 1944; Gutsevich et al., 1974). Discordance of SI values may be due to the influence of different factors, primarily the use of exuviae, not the larvae, the measurement without permanent mounting and environmental conditions. Although the use of exuviae can affect the results because of the flexibility in the shape, there is no evidence that this happened here, especially as they not permanently mounted. This method has the advantage over the use of larvae, because it allows the application of different methods for larvae and adult individuals and thus allows obtaining different types of data for a particular individual.
It is important to highlight that in many mosquito species factors influencing differentiation of populations and species act at the larval stage, following morphological, physiological and behavioural adaptations of the adult stage (Ivnitsky, 1994; Vinogradova and Ivnitsky, 2009). In addition, it was suggested that niche-specific selection, related to the specificity of larval feeding, determined the exploration of different trophic niches and types of aquatic biotopes (Ivnitsky, 1994). Therefore, morphological constraints imposed by a highly specialized ecological niche, such as urban habitats, influence the divergence of certain morphological characters between “urban” and “rural” forms (Vinogradova and Ivnitsky, 2009). Although the siphon is not directly involved in the feeding process, siphon characters, such as SI, show divergence among populations of C. pipiens and can be used for their separation (Vinogradova et al., 1996). Results presented herein showing that differences between “urban” (I and II) and “rural” (III) ecotypes are in concordance with published data (Petrarca et al., 1980; Sabatinielli and Petrarca, 1980; Eritja and Aranda, 1995). Since both C. pipiens forms feed on avian and mammalian hosts (including humans) they are considered a bridge-vector for the transmission of arboviruses (such as West Nile virus) from hosts to humans (Kilpatrick et al., 2007; Reusken et al., 2010). Therefore, precise identification of such taxa of epidemiological importance is of great significance for designing vector control strategies. Beyond of this study, differences between biotypes will be fully tested by other larval (number of pectin spines and number of siphonal setae) and adult (wing measurements) morphometric parameters and genetic data using more discriminating methods (geometric morphometrics of siphon and wings and protein electrophoresis) (Krtinić B., Ludoški J., Milankov V., unpublished).

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