

Karyotypes of some Central European mayflies (Ephemeroptera) and their contribution to phylogeny of the order

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Abstract. Based on study of larval spermatocytes the karyotypes of 24 species of 16 genera and 9 families of Central European Ephemeroptera have been described in addition to 8 species previously mentioned in literature. Karyotypes of the families Siphonuridae, Oligoneuriidae, Leptophlebiidae and Potamanthidae are mentioned for the first time. The species studied represent all major recent phylogenetic lineages within the order. In general, the Ephemeroptera show karyotypes with a range of $n = 4-10$ chromosomes with unclear modal number and two types of sex determination (XY/XX and X/XX, males heterogametic). Anagenetic trends forming karyotypes in individual families are suggested. Plesiomorphic line (Siphonuridae-Bactidae) is characterized mainly by still relatively high number of acrocentric chromosomes and XY/XX sex determination. Derived lines show various transitions to submetacentric and metacentric chromosomes, changes in chromosome numbers (mostly reduction) and, finally, the emergence of X/XX sex determination type in the advanced Ephemeridae and Caenidae. Parsimonial hypothetical scheme of karyotype anagenesis suggested here mostly corresponds to results obtained by study of external and internal morphological structures. Relations of the mayfly karyotype to that of the Odonata, a phylogenetically most allied order, are discussed. Quite different Ephemeroptera karyotype supports the idea of the Ephemeroptera and Odonata + Neoptera sister grouping of pterygote insects.

Chromosome numbers, sex determination, chromosome anagenesis, phylogeny, pterygote insect clades, higher classification, Ephemeroptera

INTRODUCTION

Study of karyotypes and cytogenetical study in general may bring a new insight on insect taxonomy and classification. This becomes apparent e. g., in Lepidoptera and Diptera where quite new opinions concerning higher classification are being currently formulated. On the other hand, the order Ephemeroptera is largely omitted in this respect and our present knowledge is very scarce and rather fragmentary.

Of the 25 families and more than 4,000 species described so far (Hubbard 1991), the chromosome numbers are known only in 8 species of 4 families. Bernhard (1907) first studied the karyotype of *Cloeon dipterum* (Linné, 1761) (Baetidae). He found a haploid chromosome number of $n=5$ in oocytes of this species. The same chromosome number was found in oogenetic and cleavage stages of the same species by Wolf (1960) who first mentioned the sex determination in mayflies (XY/XX, males heterogametic). Identical chromosome numbers and type of sex chromosome determination were observed in both oocytes and spermatocytes of *Baetis rhodani* Pictet, 1843 and *B. vernus* Curtis, 1834 (Baetidae) by Bohle (1969). Katayama (1939) published the karyotype of *Ameletus costalis* (Matsumura, 1931) (Ameletidae) ($n=9$) and found the same type of sex determination as in Baetidae (XY/XX, males heterogametic). However, Wolf (1946) and Mol (1978) observed a derived

sex chromosome system, X/XX in *Ephemera danica* Müller, 1764 (missing Y chromosome in males), ($2n=11$ in males, $2n=12$ in females). The system X/XX was also found in *Caenis horaria* (Linné, 1758) (Caenidae), whereas *Serralla ignita* (Poda, 1761) (Ephemerellidae) and *Ecdyonurus dispar* (Curtis, 1834) (Heptageniidae) possessed the XY/XX system like the family Baetidae (Mol 1978). Kiauta & Mol (1978) described the whole development of spermatocyte chromosomes and suggested a very simple phylogenetic relationships within the order comprising two basic lineages defined mainly by the sex determining system. According to the karyotype arrangement they distinguished the heptagenioid line comprising the families Ameletidae and Baetidae (higher chromosome number, XY/XX sex determination) on one side and ephemeroid line (lower chromosome number, X/XX sex determination) on the other. Updated knowledge on karyotypes of 7 species occurring in Austria was summarized by Humpesch & Elliott (1984).

The objective of this study is to describe, on the basis of Central European species, chromosome numbers and type of sex determination of all major stem groups within the order and reconstruct possible anagenesis of the Ephemeroptera karyotype. In addition, we have compared the Ephemeroptera karyotype with that of Odonata in order to contribute to the discussion on definition of major clades of pterygote insects.

MATERIAL AND METHODS

Karyotypes of the following species have been studied (all the material collected by senior author in the Labe basin, Czech Republic, coordinates of localities according to the uniform grid system) (see e. g., Buchar 1982):

Siphonuridae: *Siphonurus lacustris* (Eaton, 1870): Modravský brook, Modrava (7046), September 7, 1995.

Baetidae: *Baetis alpinus* (Pictet, 1843): Krásetínský brook, Krásetín, (7151), April 6, 1996; *B. rhodani* (Pictet, 1843): Klíčava brook, Zbočno (5949), August 14, 1995; *B. vernus* Curtis, 1834: Malše river, Kaplice (7252), August 1, 1995; *Centroptilum luteolum* (Müller, 1776): Malše river, Kaplice (7252), August 1, 1995; *Nigrobaetis niger* (Linné, 1761): Vltava river, Větrní (7251), April 17, 1996.

Heptageniidae: *Ecdyonurus austriacus* Kimmins, 1958: Zhůřský brook (tributary of the Vydra river), Turnerova chata (6947), June 15, 1995; *E. subalpinus* Klapálek, 1905: small unnamed brook, Rakovník (5848), March 23, 1996; *E. submontanus* Landa 1970: Blanice river, Blažejovice (7049), August 1, 1996; *E. torrentis* Kimmins, 1942: Vltava river, Třisov (7152), August 6, 1996; *Electrogena fasciocolata* (Sowa, 1974): Oupošský brook, Skryje (6048), August 15, 1995; *Epeorus sylvicola* (Pictet, 1865): Vltava river, Větrní (7251), April 17, 1996; *Heptagenia flava* Rostock, 1877: Malše river, Kaplice (7252), August 1, 1995; *H. sulphurea* (Müller, 1776): Malše river, Kaplice (7252), August 1, 1995; *Rhithrogena savoiensis* Alba-Tercedor et Sowa, 1987: Malše river, Pořešín, August 8, 1995; *R. semicolorata* (Curtis, 1834): Kleštínský brook, Čertova stěna (7351), April 17, 1996.

Oligoneuriidae: *Oligoneuriella rhenana* (Imhoff, 1852): Malše river, Kaplice (7252), July 10, 1995.

Leptophlebiidae: *Habroleptoides confusa* Sartori et Jacob, 1986: Klíčava brook, Lány (5849), March 25, 1996; *Paraleptophlebia submarginata* (Stephens, 1835): Křemžský brook, Holubov (7051), April 6, 1996.

Caenidae: *Caenis macrura* Stephens, 1835: Křemžský brook, Holubov (7051), August 10, 1995.

Ephemerellidae: *Ephemerella mucronata* Bengtsson, 1909: Krasetínský brook, Krasetín (7151), April 10, 1996.

Ephemeridae: *Ephemera danica* Müller, 1764: Pšovka river, Kokořín (5553), April 1, 1996; *E. lineata* Eaton, 1870: Nová řeka river, Mláka (6955), June 10, 1995; *E. vulgata* Linné, 1758: Pšovka brook, Kokořín (5553), April 1, 1996.

Potamanthidae: *Potamanthus luteus* (Linné, 1767): Berounka river, Skryje (6048), August 15, 1996.

Larvae of species studied were collected by usual hydrobiological techniques (kicking techniques, sweeping of submerged vegetation, individual collecting from larger stones etc.). Since the number of larval instars cannot be properly determined in mayflies and this number varies even within the same population we used so called "older larvae", i. e. those with fully developed larval characters, several instars before the subimaginal moulting. This appears to be an optimal stage of larval development since both spermatocytes and oocytes undergo meiotic division just in these instars. Later (in the last and last but one instars) mostly spermatids and mature spermatozoa occur in male larvae and vitellogenesis is nearly finished in female ones (Soldán 1979a, 1979b).

To study chromosome numbers, fresh generative tissues of gonads were used. Larvae of both sexes were studied but we preferred to determine spermatocyte chromosome numbers since oocyte chromosomes are mostly superimposed by yolk granules and their counting is more difficult. We followed essentially the procedure described in

Traut (1976). Specimens were dissected in a physiological saline, gonadal tissues were fixed for 5 minutes with the Carnoy fixative then disintegrated with wolfram needles on microscopic glasses and macerated for several minutes with 60 % acetic acid at 45 °C. After drying up preparations were stained with lacto-acetic-orcein, embedded and observed under Jenalumar (Karl Zeiss Jena) and Leitz Orthoplan microscope. Photographs were taken by the Expomat apparatus under oil immersion 100x objective. In addition, squash preparations were made as well and the fluorescent dye DAPI was used for staining chromosomes of unfertilized eggs (e. g., in *Baetis alpinus* and *Ecdyonurus subalpinus*). In the latter case, the fluorescence microscope Jenalumar was used.

RESULTS

In *Siphonurus lacustris*, the only representative of the Siphonuridae studied, the karyotype of 2n (male) = 16+XY was found. At spermatogonial metaphase, a rosette of 18 relatively small acrocentric chromosomes of nearly the same length was observed (Fig. 1A).

Within the family Baetidae, a uniform chromosome number, 2n (male) = 8+XY and 2n (female) = 8+XX, was found in all 4 genera studied. Correspondingly, 10 chromosomes observed in *Baetis alpinus* spermatogonia, one of couplets being apparently smaller (Fig. 1B). The chromosomes of the same species are well separated and much longer in late mitotic prophase of the same species. Chromosomes of *B. vernus* and *B. rhodani* seems to be microscopically identical with those of *B. alpinus* and *Centroptilum luteolum*. Contrary to *B. alpinus*, 10 acrocentric chromosomes of approximately the same length were observed in *Nigrobaetis niger* (Fig. 1C).

All the material of the Heptageniidae studied (9 species, 5 genera) shows the same chromosome numbers and sex determination type, 2n (male) = 18+XY and 2n (female) = 18+XX. Chromosomes of the Heptageniidae are very small in comparison with those of the Baetidae, equal in size and length but relatively short. At late diakinesis, ten elements can be found in the male, one of them being slightly asymmetrical. Their length in metaphase is only about 2–3 times longer than width as seen e. g., in 20 approximately identical chromosomes in metaphase of spermatogonia of *Rhithrogena semicolorata* or *Epeorus sylvicola*. Chromosomes of this group are submetacentric, in contrast to Siphonuridae and Baetidae. Submetacentric chromosomes and the same type of sex determination were found also in the family Oligoneuriidae. Chromosomes are very small and equal in length as in Heptageniidae but their number is by two chromosome pairs lower, 2n (male) = 14+XY.

Although we studied only a single representative of the Ephemerellidae, *Ephemerella mucronata*, with 2n (male) = 6+XY and 2n (female) = 6+XX, karyotype of this family seems to show some peculiar characters. Mitotic metaphase clearly shows 2 large metacentric chromosomes and 6 smaller acrocentric ones (Fig. 1D). Large chromosomes are V-shaped and approximately double-sized, others are more or less rod-shaped.

As to the family Leptophlebiidae, we failed to determine proper chromosome number and type of sex determination. Size of chromosomes in both species studied, *Habroleptoides confusa* and *Paraleptophlebia submarginata* is extremely small (probably due to minute spermatozoa size) not permitting either to count their number or to find heterochromosome under the light microscope. Although we cannot define it with certainty, we estimate the chromosome number of both these species as follows: 2n (male) = 12–14.

The genus *Ephemera* Linné, 1758 was the only representative of the Ephemeridae studied. Surprisingly, different karyotypes have been found within this genus: 2n (male) = 10+X (*E. danica* and *E. lineata*) and 2n (male) = 12+X (*E. vulgata*). Thirteen chromosomes, two of them with apparent negative heteropycnosis were observed in the latter species (Fig. 1E).

Like in the Ephemeridae, also in the Potamanthidae (*Potamanthus luteus*) and Caenidae (*Caenis horaria*) a different type of sex determination, 2n+X (male heterogametic) has been found. The same autosome number (2n = 6) occurs in both these species studied. In *P. luteus*, there are 3 bivalents in pachytene and unclear, positively stained pycnotic formation most probably repre-

senting the X chromosome (Fig. 1F). Apparently differentiated 7 metaphase chromosomes were observed in *C. macrura*.

DISCUSSION AND CONCLUSIONS

Comparison of results with literature data

Our results generally agree with literature data. We would like only to emphasize, contrary to e. g., Mol (1978), that the last larval instar ("ultimate stage of larvae") can hardly be used to study mayfly karyotypes, at least in males. In this instar, all generative cells are in the stage of spermatids, sperms or even mature spermatozoa, meiotic division is completely finished and chromosomes mostly not discernible at all. Both spermatogenesis and oogenesis in mayflies are realized in only a single way and are completely finished before larval-subimaginal ecdysis, perhaps except the species with relatively long-term subimaginal stages like e. g., *Ephemera danica* (Soldán 1979a). Based on a description of meiotic spermatocytes (Kiauta & Mol 1977) probably last but one or earlier instars were used anyway. However, this study can be conducted in females since meiosis is mostly not finished before fertilization (cf. Soldán 1979c) although counting chromosomes is very difficult due to high content of yolk granules.

The Ephemeroptera karyotypes seem to be very similar each other at least at the family level. For instance, within the family Baetidae, we found the same chromosome numbers $2n=10$ not only in the genera *Baetis* Leach, 1815 (cf. Bohle 1969) and *Cloeon* Leach, 1815 (cf. Bernhard 1907, Wolf 1960, Kiauta & Mol 1977) but also in *Nigrobaetis* Novikova & Kluge, 1987 and *Centroptilum* Eaton, 1869. Moreover, these genera represent relatively remote phylogenetic lines (different subfamilies) within this group. The same applies to the Heptageniidae, identical karyotype occurs at least in 5 genera mentioned in this study (same karyotype found also by Mol 1978 for *Ecdyonurus dispar*). Within the superfamily range, the karyotype seems to be similar in related families as seen in Siphonuridae and Ameletidae (same chromosome number in the latter described by Katayama 1939 for *Ameletus costalis*). However, different chromosome numbers evidently occur in different lines at this hierarchic level (Siphonuridae vs. Baetidae or Heptageniidae vs. Oligoneuriidae).

The only exception of this rule known so far is genus *Ephemera* (Ephemeridae). While the karyotype $2n = 10+X$ was found in *E. danica* (cf. Wolf 1946, Mol 1978) and also in *E. lineata*, *E. vulgata*, although closely related, apparently showed the male karyotype of $2n = 12+X$. Although we have nearly no data concerning the same genus in advanced families, this might indicate some more detailed karyotype differentiation in this group of mayflies. On the other hand the karyotypes of *Caenis horaria* and *C. macrura* are identical (cf. Mol 1978).

General features of the Ephemeroptera karyotype and comparison with the order Odonata

Generally, there are three theoretical possibilities of sister grouping of the Pterygota: (1) monophyly of Paleoptera, (2) monophyly of Ephemeroptera + Neoptera, and (3) monophyly of Odonata + Neoptera (see e. g., Kukalova-Peck 1991, Kristensen 1981 and Soldán 1997 for details). However, the proper reconstruction of phylogeny and higher classification of pterygote insects remains still open due to numerous contradictions and disputable circumstances concerning extant representatives. Many authors (e. g., Matsuda 1981, Kukalova-Peck 1983, Riek & Kukalova-Peck 1984 and Pfau 1986) believe that the views of recent dissenters cannot be reconciled with modern systematic arguments. Consequently, all major pterygote clades can be treated as independent groups, three paleopterous (ephemerids, odonatoids and the paleozoic plant-sucking paleodictyopteroids) and one neopterous.

Within the frame of this considerations, the comparison of the Ephemeroptera on one hand and the Odonata on the other hand seems to be the main source of knowledge. Although only 24

species of 9 families in Ephemeroptera contrary to about 500 species of 22 families of Odonata (Kiauta 1972) are known from the cytogenetic point of view genotypes of these to orders exhibit very remote similarities. The Ephemeroptera possess monocentric chromosomes, whereas chromosomes of Odonata are holokinetic (cf. Kiauta & Mol, 1977). Chiasmatic meiosis occurs in both sexes in both Ephemeroptera and Odonata but the recombination indices (RI) are different. In Ephemeroptera

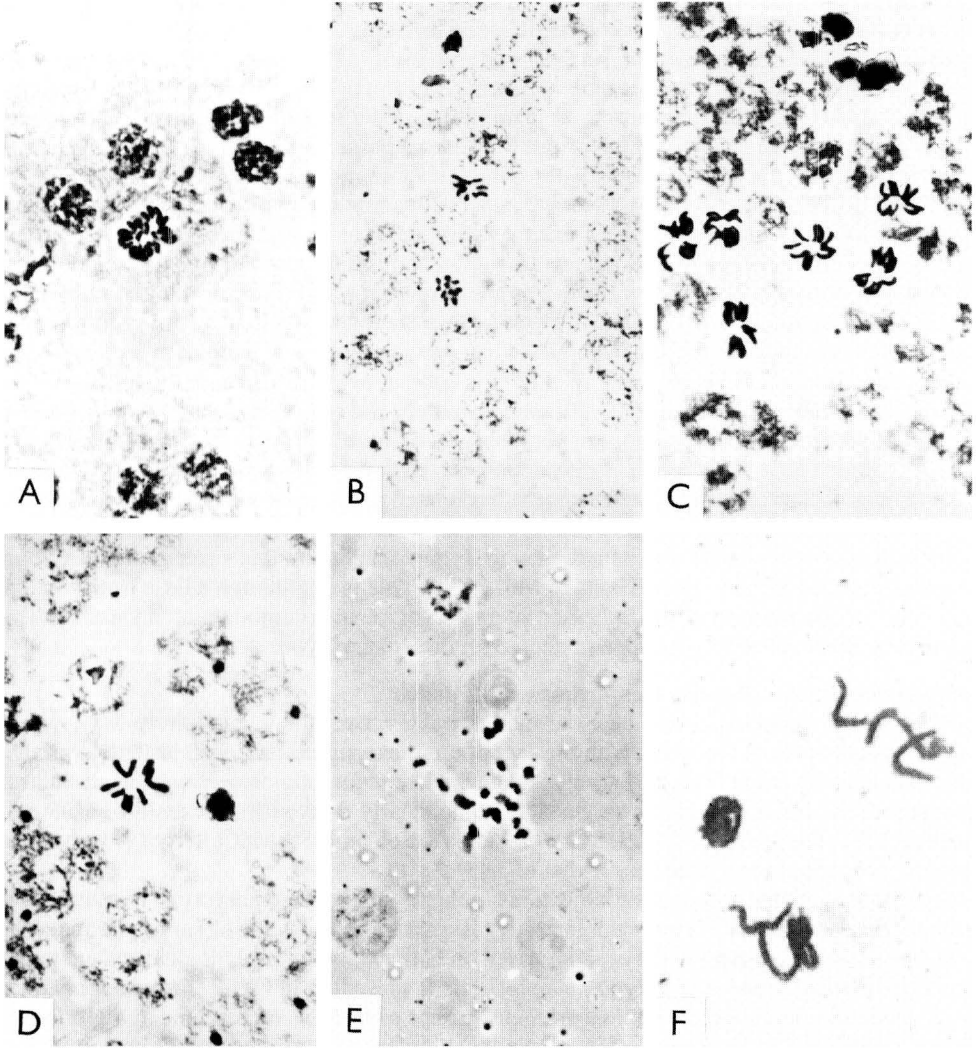


Fig. 1. Chromosomes of generative testicular cell of some species of Central European Ephemeroptera. A – spermatogonial metaphase of *Siphonurus lacustris* (Eaton). B – late mitotic prophase of *Baetis alpinus* Pictet spermatocyte. C – spermatogonial metaphase of *Nigrobaetis niger* (Linné). D – mitotic metaphase of *Ephemerella mucronata* (Bengtsson) spermatocyte. E – metaphase of *Ephemerella vulgata* Linné male generative cell. F – pachytene in spermatocytes of *Potamanthus luteus* (Linné).

tera, the are probably $2n < RI < 3n$, $RI \geq 2n$ in female of XY/XX sex determination species while $RI = 2n - 1$ in females of Odonata (in primary complements only). Chromosomal numbers widely vary in different families of Ephemeroptera, in the range of $n = 3-9$ in so far known species. Hence, their type number is uncertain. In Odonata, the type number is $n = 13$ with range of 9-14 (in primary complements only). Sex determination also differs in Odonata and Ephemeroptera. While the X/XX system exclusively was found in the former, the Ephemeroptera possess either XY/XX or X/XX systems. Males are heterogametic in both Ephemeroptera and Odonata although the sex element(s) are prereduced in the former but postreduced in the latter (Kiauta & Mol 1977). Moreover, karyotypes in Ephemeroptera differ at the family or even higher level (in Neoptera usually at generic or lower level) and chromosome numbers represent at most 2.5 multiplication of lowest number observed. Provided that chromosome multiplication by mutations mostly occur in derived taxa, the Ephemeroptera can be ranged, from the general point of view, to very primitive groups. This well agree with the general absence of prominent apomorphies in this order.

To conclude, the above arguments seem to support the monophyly of Odonata + Neoptera and their respective sister-grouping to the Ephemeroptera seems to be parsimonial although morphological autapomorphic of mayflies (sliding articulation of mandibles, arrangement of tentorial muscles, presence of true hypopharyngeal superliguae, prominent basal subcostal brace and terminal filament in larvae, retention of subimaginal stage etc.) are not so obvious, contrary to prominent ordinal autapomorphies of the Odonata. This sister-grouping is also supported by some recent ultrastructural findings, like presence of the telotrophic type of ovarioles (Gottanka & Büning 1992), spermatozoa with a unique 9+9+0 axoneme pattern and only a single mitochondrial derivative (Baccetti et al. 1969) and presence of very derived, extraordinarily small spermatozoa without flagellum (Soldán 1979b). Wheeler (1989) demonstrated that sequences of bases in the ribosomal DNA are quite different in the Ephemeroptera but related in Odonata + Neoptera. Moreover, the karyotype of Ephemeroptera seems to be more related to that of Archaeognatha, at least as far as the chromosome numbers are concerned, than to Zygentoma, a possible sister-group of ectognathous, true insects (Argilas 1941, Kiauta & Mol 1977). This fact, although a little irrelevant, also stresses the unique position of the Ephemeroptera karyotype in relation to that of Odonata which is apparently more related to the general neopteran insect karyotype.

Karotype constitution of major stem-groups of the order

In spite of only 9 families studied here our model species cover the whole family-level range of extant representatives of the order, with the exception of extralimital tricorythid-leptohyphid and prosopistomatid-baetiscid lines the karyotypes of which remains unknown. However, all studies of phylogeny of the order suppose these lineages to be clearly derived from caenid-like ancestors (Edmunds 1972, Edmunds & McCafferty 1979, Landa & Soldán 1989, McCafferty 1991) the idea of karyotype similarity seems to be very realistic.

Taking into account, with certain objections, general apomorphic karyotype constitution defined by Kiauta & Mol (1977) we are able to suggest parsimonial scheme of karyotype anagenesis and to discuss it with respect to the opinion based on both external morphological and anatomical study. Hypothetical scheme of karyotype anagenesis is presented in Fig. 2. Based simply on the type of sex determination, there are two principal groups of families represented by superfamilies Siphonuroidea (and/or Heptagenioidea + Leptophebioidea) on one hand and Ephemeroidea and Caenoidea (possibly plus Prosopistomatoidea) on the other. We suggest the following anagenetic trend to form the karyotype of the above main stem groups: (1) Provided that metacentric chromosomes arose from acrocentric ones by Robertsian fusion, mayfly acrocentric chromosomes (plesiomorphic) gradually changed to submetacentric and then to metacentric ones (apomorphic), (2) autosome numbers are gradually reduced (highest numbers in the Ameletidae and Siphonuridae

plesiomorphic), at first in a lesser degree that substantially and their size gradually increases (apomorphic), and (3) reduction of the non-homological chromosome (presence plesiomorphic) gradually takes place, XY X (missing heterochromosome apomorphic).

The plesiomorphic line of the order (classified as the suborder Pisciformia by McCafferty 1991) and generally including schistonotan families (for definition see McCafferty & Edmunds 1979) with fish-like larvae is characterized by still high number of acrocentric chromosomes as seen in the Ameletidae (Katayama 1939) and Siphonuridae (Fig. 2). Smaller number of chromosomes in extremely diversified world-wide family Baetidae (identical in both major subfamilies, Baetinae and Cloeoninae) can be explained by possible secondary reduction, taking into consideration half chromosome number and double size in comparison with the Siphonuridae/Ameletidae. First of all, some speculations on permanent blocking of homeotypic meiotic division or endomitosis might be taken into account.

Evident sister groups, the Heptageniidae and Oligoneuriidae (superfamily Heptagenioidea in higher classification by Edmunds & McCafferty 1979 and Landa & Soldán 1989, suborder Setisura in that by McCafferty 1991) are characterized by evident plesiomorphy in the chromosome number and apomorphy in the centromere position (chromosomes submetacentric). The latter character seems to be important since a secondary multiplication of originally reduced chromosome number (see evidently smaller chromosome size in comparison e. g., with Siphonuridae) might occur.

From the karyotype arrangement point of view, clearly derived (apomorphic) line of the order is represented by the families Ephemeridae and Caenidae within our model groups (Fig. 2). This line is characterized, first of all, by different type of sex determination (XX/X, males heterogametic). Especially the largely accepted superfamily Ephemeroidea containing, besides the Ephemeridae and Potamanthidae studied here further 4 families characterized by specialized burrowing larvae

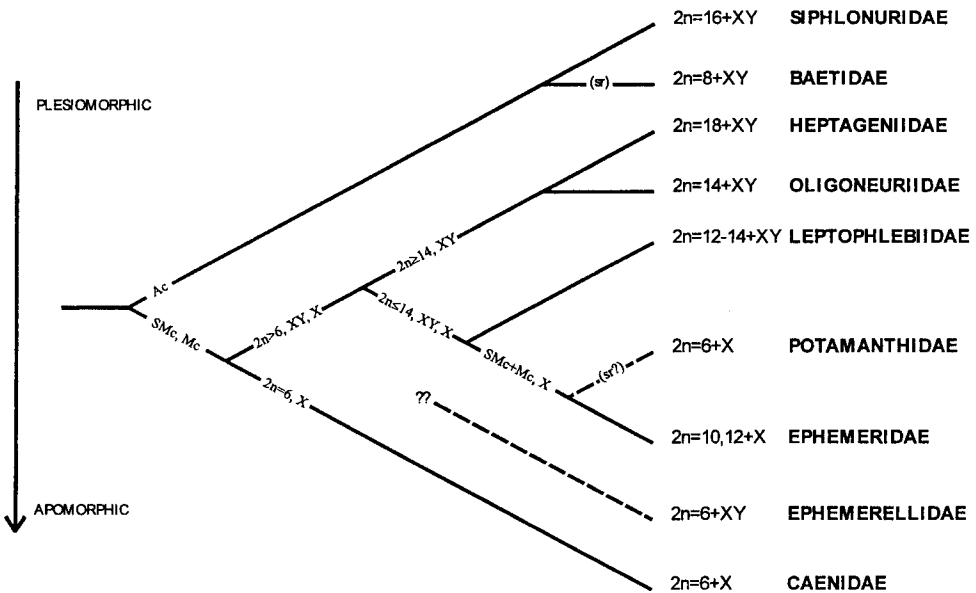


Fig. 2. Hypothetical scheme of karyotype anagenesis of the Ephemeroptera. Ac – acrocentric, SMc – submetacentric, Mc – metacentric chromosomes, (sr) – possible somatic reduction of chromosomes.

and other characters (Landa & Soldán 1989, McCafferty 1991) seems to be very well defined by its karyotype. However, the genus *Ephemer* is the only known case showing different karyotype at generic level (see above). Although we were not able to determine the chromosome number of the family Leptophlebiidae perfectly, this family belongs to this line as well. Despite probably XX/XY sex determination type, its chromosomes are still metacentric and due to their extremely small size seem to be very derived. Their size can be documented e. g. by an extremely small size of spermatozoa reaching only 2–4 µm in diameter (Soldán 1979b), the smallest size within the whole animal kingdom. Consequently, we classify this family within the ephemeroid line as a transitory type from the cytogenetic point of view. McCafferty (1991) classifies the Leptophlebiidae and Ephemeroidea in the suborder Lanceolata. However, differentiation of karyotypes seems to be very old, taking into account different type of sex determination and differences in position of centromere.

The only family of those studied here, Ephemerellidae, does not fit to conventional classification as far as its karyotype is concerned. According to recent opinions (Edmund & McCafferty 1979, Landa & Soldán 1989, McCafferty 1991) the Ephemerellidae constitute a sister-group to the Caenidae with respect to derived characters of thoracic nota and abdominal segments, arrangement of gills, centralization of ventral nerve cord, reduction of visceral tracheae etc. Consequently, the Ephemerellidae are currently classified within the suborder (or infraorder) Pannota. This does not concern the chromosome number itself – in this case it is lower than in the ephemeroid lineage and higher than in the Caenidae. However, both acrocentric and metacentric chromosomes have been found in the genus *Ephemerella* and XX/XY sex determination type apparently separates this group out from the clearly pannotan, caenioid lineage. Being strictly formal in construction of our scheme (Fig. 2) we are not able to properly root the family Ephemerellidae with the pannotan stem-groups. Although more detailed cytogenetic study of other genera is urgently needed, this contradiction might be explained by some type of retention of rather primitive karyotype. This hypothesis is supported by the fact that this family represent the most primitive lineage of caenoid group showing evident relationships to schistonotan groups.

A c k n o w l e d g e m e n t

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