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Two cytotypes of *Kiefferulus tendipediformis* (Goetghebuer, 1921) (Diptera, Chironomidae)

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Abstract — The material identified as *Kiefferulus tendipediformis* on the basis of the larval morphology consisted of two cytogenetically distinct species, which we called cytotype 1 (2n=8) (Bulgaria) and cytotype 2 (2n=6) (Poland). The karyotype and external morphology (larva, pupa and imago - males) of *Kiefferulus tendipediformis* from pond on mine spoils in the Olkusz region with Zn-Pb ore deposits (Southern Poland) are described. The karyotype of cytotype 2 (2n =6) (Poland) consists of two metacentric chromosomes (AB and CD) and one submetacentric GEF. The chromosomes AB, CD of both cytotypes are very similar, the chromosome GEF is formed through tandem fusion of two acrocentric chromosomes of cytotype 1 (chromosomes EF and G). The external morphology of the species from Poland is compared with materials of the same species from Bulgaria and Austria. The larvae of Poland and Bulgaria are differed in a number of ultrastructure characters. Pupae and imagoes of *K.tendipediformis* from Poland are distinguished from those of Austria. The current taxonomic position of the *K.tendipediformis* in Europe is discussed.

Key words: Chironomidae, cytotype, karyotype, *Kiefferulus tendipediformis,* polytene chromosomes.

INTRODUCTION

Genus *Kiefferulus* is known as monotypic in Europe, while in Australia it is represented by 3 species. At least 3 species are known from Holarctic and one from Afrotropical region (Pinder and Reiss 1983, Martin 1963). The karyotype of *Kiefferulus tendipediformis* from Bulgaria and Hungary has been studies by Michailova (1995, 1996) who established 2n = 8 and found the larvae of the species in mud and sediments of the water bodies.

In the present paper the cytogenetic characteristics of *Kiefferulus tendipediformis* from pond on mine spoil at Olkusz industrial region (Zn-Pb ore deposits) (Southern Poland) is described. Egg masses of the species are successfully reared through to the adults, which allow an examination of external morphology during all stage of the life cycle and making a comparative external morphological analysis of the species from different populations.

MATERIAL AND METHODS

Larvae and egg masses were collected during the spring of 2001 and 2002 from small pond on „young" mine spoil at Bolesław (Olkusz industrial region, near Krakow). There is a small pond, shallow (+/– 1 m. depth), overgrown by *Typha*, with sand-muddy bottom. Larvae were miners of leaves of different macrophytes. From stamps of the water plants egg masses were collected that reared in laboratory conditions by modified method (Michailova 1985). Forth instar larvae from reared egg masses were fixed in alcohol - acetic acid (3:1) and used for karyological analysis. Cytogenetically were analyzed 31 larvae applying well-known acet-orcein method (Michailova 1989). Chromosome arms were designated: Ist-AB, IInd- CD, and IIIrd - GEF. For the arm comparison we used Michailova (1995). The heterochromatin localization was analyzed after applying "C" banding method (Michailova 1987).

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The specimens from which the salivary glands were squashed were treated in 10% KOH and mounted on the slides. From the reared materials pupae and imago (males) were analyzed. The reared material was identified according to Pinder and Reiss (1983, 1986) and Cranston *et al.* (1989). Also, the comparative analysis of external morphology was done with materials (larvae, pupae and imago-males) from Natural History Museum (Vienna) and materials (larva and pupa) from Bulgaria (Table 1).

Table 1 — Investigated materials of *Kiefferulus tendipediformis*

	Larvae		Pupae	Imago
	karyotype	morphology	morphology	morphology
Poland	+	+	+	+
Bulgaria	+	+	+	–
Austria	–	+	+	+

For analyzing the ventromental plates of larvae we used scanning electron microscope, type Philips, SEM 515 following the method described by Michailova (2004). The Saether (1980) terminology was used for analyzing the external morphology of the species.

The samples of water for chemical analyses was taken from one site about 20 cm below the surface, samples of the upper layer (0–5 cm) of the bottom sediment were collected at three sites. In the samples of the water chemical and physico-chemical parameters (pH, conductivity, dissolved oxygen, $N\text{-}NO_3$, PO_4, SO_4 and total hardness and alkalinity) were determined by methods given in Apha (1992). Sediment samples used to total metal contents were dried at 105 °C for ≥ 24 hr, and then homogenized using the planetary mill (Pulverisette 5) with teflon grinding balls. The measurements were made using a Perkin-Elmer 403 atomic absorption spectrophotometer equipped with a Graphite Furnace (HGA–74).

RESULTS

1. Physical-chemical properties of the water and sediment in the studied pond - The water from the Bolesław pond was hard (294.4 mg $CaCO_3$ L^{-1}) and had an alkaline pH 8.4, in which general trace elements occurred in forms less available and toxic for organisms (Table 2). The oxygen conditions in the water were good. The quantities of nutrients i.e. nitrates were relatively low, whereas those of phosphates elevated in comparison with those most frequently encountered in the other water bodies. The salt concentration was high (high conductivity) with a small content of chlorides and a high level of sulphates. Elevated amount of sulphates in the water was connected with the weathering of surrounding rocks. Metal sulphids and oxidized mineral components: sphalerite (ZnS), galena (PbS), marcasite and pyrite (FeS_2), cerusite ($PbCO_3$), smithsonite ($ZnCO_3$), and limonite (Fe_2O_3) dominated in Zn-Pb ore deposits in the area of Bolesław.

Table 2 — Contents (minimum, maximum, mean) of trace elements in the bottom sediment of the pond in Bolesław.

Contents	Cd $\mu g\ g^{-1}$	Pb $\mu g\ g^{-1}$	Cu $\mu g\ g^{-1}$	Zn $\mu g\ g^{-1}$	Mn $\mu g\ g^{-1}$
Min	1,6	390,0	78,1	1080,1	142,9
Max	3,0	757,7	222,8	4129,6	694,6
Mean	2,4	528,4	128,1	2164,9	336,5

In general, the content of Mn in the sediment of the pond was rather low, Cd and Cu elevated, whereas Pb and Zn were extremely high (Table 2) in comparison with unpolluted water bodies (Klavins *et al.* 1998, Drabl 1991, Sobczynski *et al.* 1997, Solecki and Chibowski 2000). Received contents of Pb were 26 times and Zn 23 times higher when comparing to those given by Turiekien and Wedepohl (1961) as a background for shale. The content of lead and zinc in the sediment revealed the same range as polluted river sediments from other metal mining areas (Schintu *et al.* 1991, Sanchez *et al.* 1998).

2. The Karyotype - Material identifies as *Kiefferulus tendipediformis* on the basis of the larval morphology consists of two cytogenetically distinct forms, which we called cytotype 1 with chromosome set - 2n = 8 (Bulgaria) and cytotype 2 with chromosome set - 2n = 6 (Poland).

Michailova (1995, 1996) has described the karyotype of **cytotype 1** (2n = 8). The chromosome arm combination is AB, CD, EF G. Chromosome AB and CD are metacentric, while EF and G - acrocentric.

The **cytotype 2** (2n = 6) contains three long salivary gland chromosomes. Chromosome AB and CD are metacentric while chromosome GEF is submetacentric (Figs.1a, b, c, b). All chromosomes have well expressed centromere regions. There are two nucleoli: one at the telomere region of chromosome arm A and other nucleolus is in

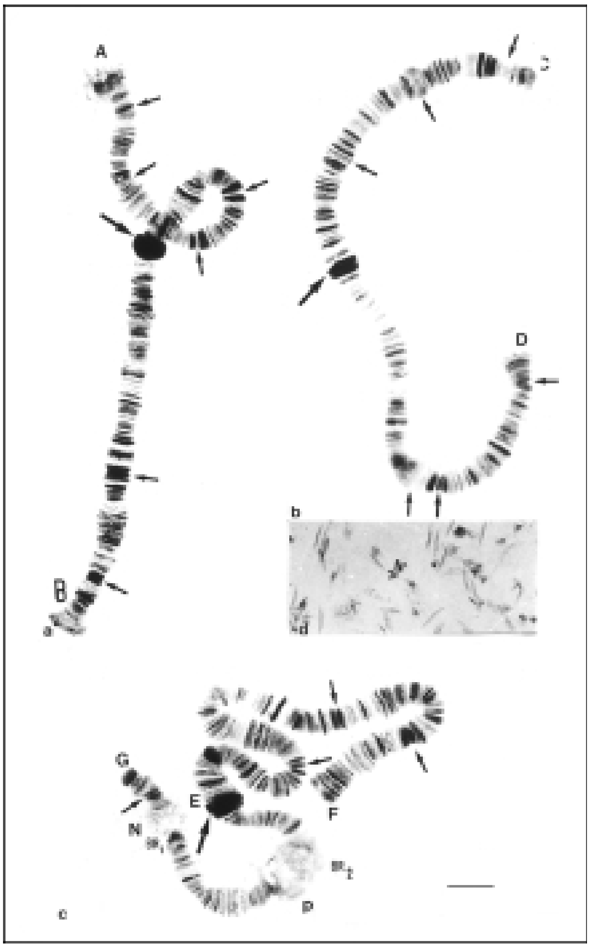

Fig. 1 — Polytene chromosomes of *Kiefferulus tendipediformis*. a. Chromosome AB. b. Chromosome CD. c. Chromosome GEF. d. Meiotic chromosomes. N - nucleolar organizer; BR - Balbiani ring. Large arrows indicate the centromere regions of the chromosomes; Small arrows indicate the markers of the chromosomes. Bar - 10 µm.

chromosome arm G that fused with arm E. Two Balbiani rings are found in chromosome arm G.

Chromosome AB has a number of constrictions and obvious bands, which can be used in its identification (Fig. 1a). One heterozygous pericentric inversion was found with frequency 25, 80%.

Chromosome CD is readily recognized by the consriction near to the telomere of arm C as well as by the several swelling along the chromosome (Fig. 1b).

Chromosome GEF has been formed by the fusion of arm E and arm G (Fig.1 c). The chromosome EF can be recognized by the group of bands located in the middle of arm E and arm F (Fig.1c). Arm G has a nucleolus and two Balbiani rings (BR1, BR2). One heterozygous inversion with frequency 12, 90 % is noticed in chromosome arm G (Fig.2a). Very often the centromere region of chromosome AB appears in decondensed state (Fig.2a). All chromosomes have well seen centromere heterochromatin (Fig. 2b).

3. Morphology - **Larval morphology**. Lateral tubules of both cytotypes are absent, one pair of ventral tubules present. In both cytotypes mentum has well separated trifid middle tooth, and six pairs lateral teeth grated in size (Figs. 3a, 4a). Their ventromental plates have smooth anterior margin. However, the both cytotypes are distinguished by the striations of ventromental plates well seen under SEM (Figs 3b, c and 4b, c). In both cytotypes the striates terminated in different way to the anterior margin of the plate. Near to the anterior margined of the plate of cytotype 1 there is well formed rounded structure (Figs. 4 b, c), while at the anterior margin of the plate of the cytotype 2 such rounded structure is not observed, the striates are serrated (Figs. 3b, c).

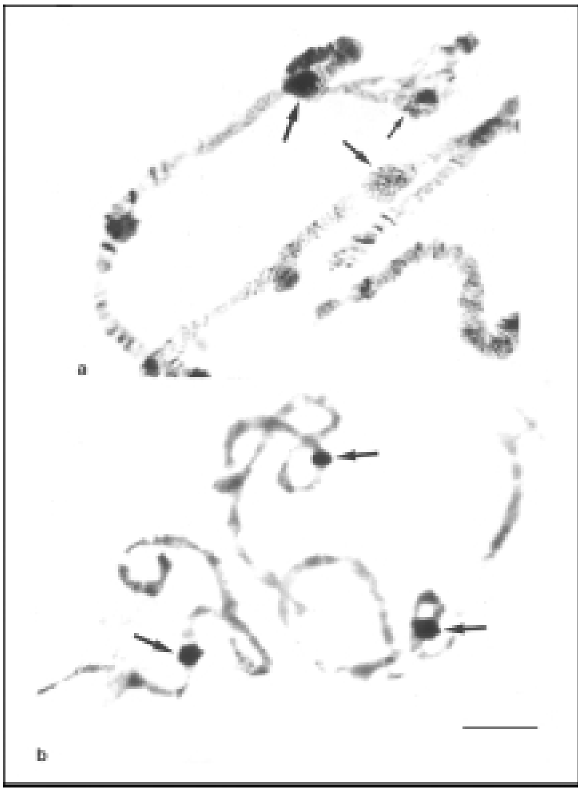

Fig. 2 — Chromosomes GEF and AB. a. Heterozygous inversion in chromosome arm G and decondenzation of centromere region of chromosome AB. b. Constitutive heterochromatin in chromosomes of *Kiefferulus tendipediformis*. Bar - 10 µm.

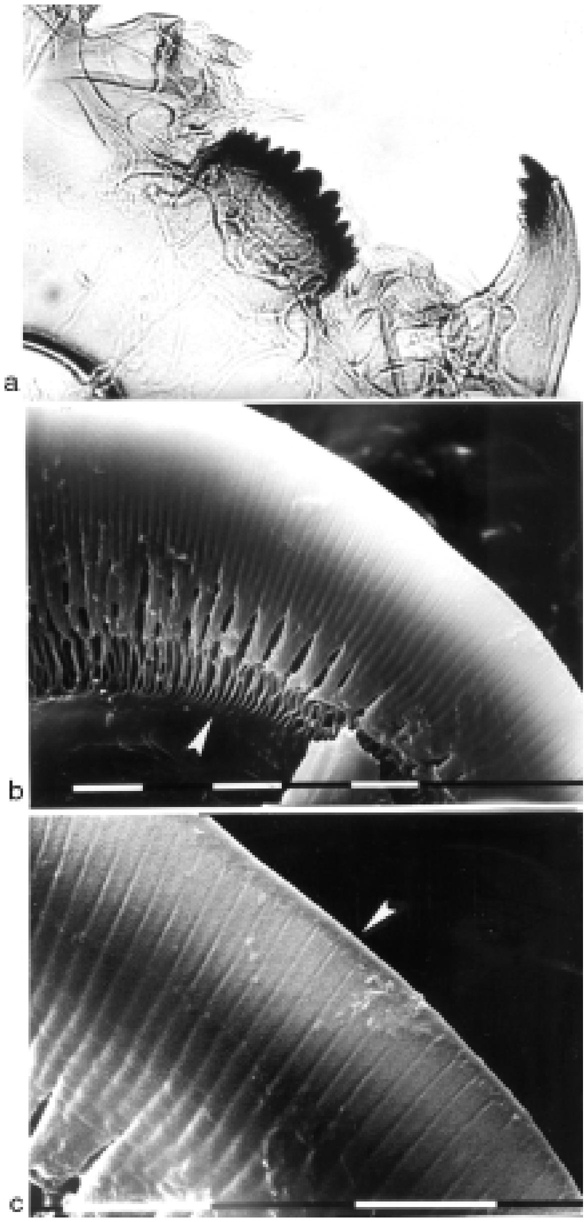

Fig. 3 — Cytotype 2 - larval morphology. a. Mentum of the larva; b. Vetromental plate (SEM) – a general view; c. Ventromental plate (SEM) – a view of anterior inner surface. The arrow indicates the anterior surface of vetromental plate, which is serrated. Bar - 10 µm.

Premandibles of both cytotypes are with six pointed teeth. Antenna consists of 5 segments; RO (Ring organ) is about in the proximal part of the first third of the first segment. Antennal blade reaches to the middle of IVth segment of the antenna. Mandibles with three dark teeth, last one is pale. The apical inner dendicales light brown.

Pupa. The morphology of the pupa of both cytotypes can be seen in Figs. 5a, b and 6a, b. The both cytotypes are differed by the morphology of the frontal apotema and the morphology of the spura of the 8th segment (Figs.5a, b and 6a, b). Also, by these features they are distinguished from the larval morphology of the species from Vienna (Figs 5a, b; 6a, b and 7a, b). However, the morphology of the pupa of species from Poland is not distinguished from those described by Pinder and Reiss (1986).

Imago. The studied adult specimens from Poland are distinguished by the morphology of their hypopygium from those of Vienna (Figs.8a and b). However, they are not differed by the morphology of the hypopygium from the imago described by Cranston *et al.* (1989).

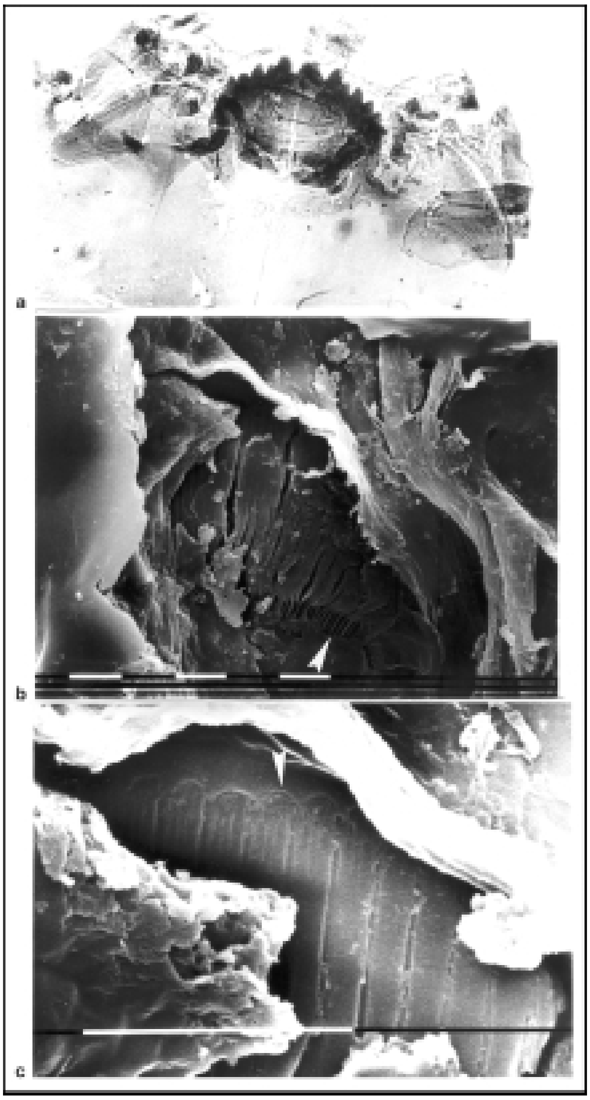

Fig. 4 — Cytotype 1 - larval morphology. a. Mentum of the larva; b. Vetromental plate (SEM) – a general view; c. Ventromental plate (SEM) – a view of anterior inner surface. The arrow indicates the anterior surface of vetromental plate, which is rounded. Bar - 10 µm.

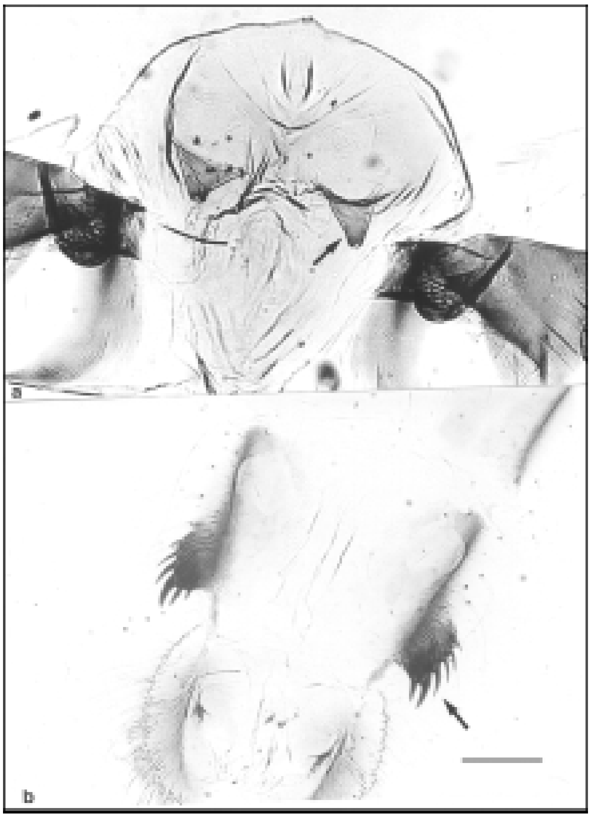

Fig. 5 — Cytotype 2 - Pupa morphology. a. Frontale apoteme. b. VIIIth segment with a spura. Bar - 10 µm.

DISCUSSION

According to Michailova (1995, 1996) *Kiefferulus tendipediformis* has 2n=8, chromosome G is separated from chromosome EF. The same chromosome set has been announced for three different Australian species (Martin 1963). However, in the present study we describe the occurrence of the other chromosome set of *Kiefferulus tendipediformis* - 2n = 6 - the chromosome G is preferably fused with arm E. So, it is found that material identified as *Kiefferulus tendipediformis* on the basis of the larval morphology consisted of two cytologically distinct species, which we called cytotype 1 (2n=8) and cytotype 2 (2n=6). The chromosomes of AB and CD of both cytotypes are very similar (Figs1.a and b, Michailova 1995). The third chomosome of cytotype 2 (GEF) is a product of tandem fusion of two acrocentric chromosomes of cytotype 1 (chromosome EF and chromosome G) (Figs. 9a - c). This is corroborated by the homology in the banding pattern between chromosome GEF of cytotype 2 with chromosome EF and chromosome G of cytotype 1 (Figs.9a - c). Due to the permanent association of both acrocentric chromosomes a new gene linkage groups is created and the process of reducing the chromosome number is observed. According to King (1993) there are two paths of the formation of this fused chromosome:

1. It is involved the loss of a centromere from one of the acrocentric chromosome after rearrangements.

2. Resulting from the fusion of the chromosomes, one of the centromere region is inactivated and producing a new submetacentric chromosome.

Our cytogenetic analysis of cytotype 2 allow us to consider that the formation of submetacentric chromosome GEF is due to the inactivation of one of centromere region and the formation of a new chromosome (only one heterochromatinized centromere region is established after C banding staining) which has been stabilized during the

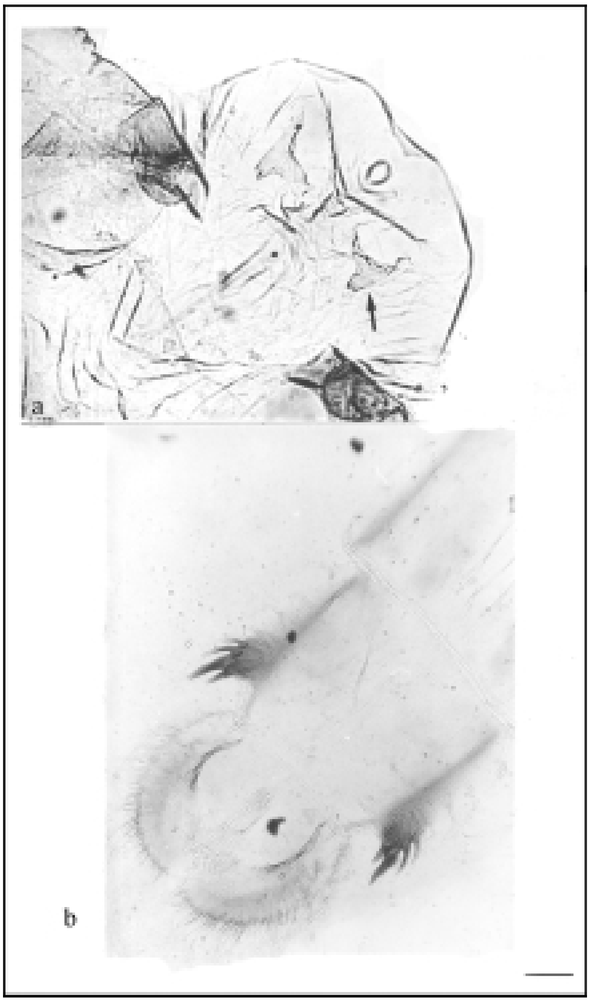

Fig. 6 — Cytotype 1 - Pupa morphology. a. Frontale apoteme. b. VIIIth segment with a spura. Bar - 10 µm.

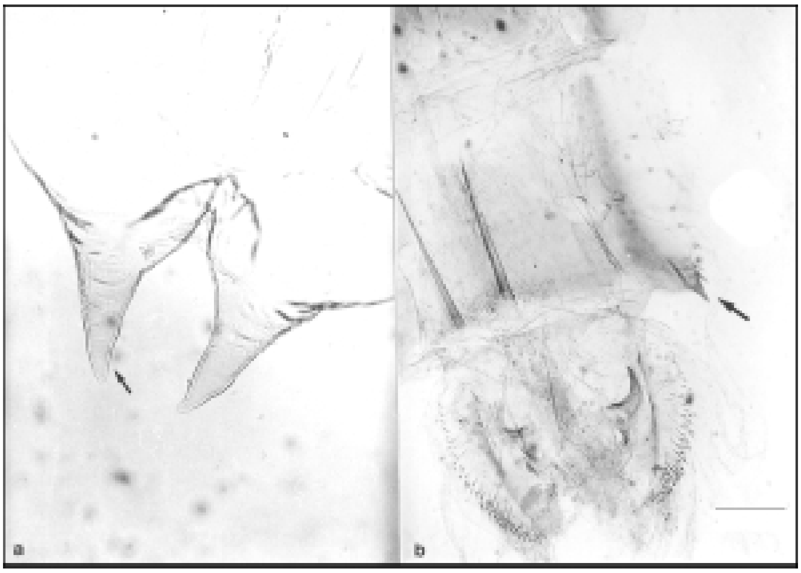

Fig. 7 — Pupa morphology of *Kiefferulus tendipediformis* from Vienna Museum. a. Frontale apoteme. b. VIIIth segment with a spura. Bar - 10 µm.

Fig. 8 — Imago - hypopygium of *Kiefferulus tendipediformis*. a. Imago of cytotype 2. b. Imago of Vienna Museum. Bar - 10 µm.

evolution process. So, our results showed that the tandem fusion plays a significant role in chromosome evolution of the studied species.

Our study also provides evidence for the exceptional role of chromosome rearrangements (fusion) in gene activation. It was demonstrated that the fused chromosome G possesses an inactivation capacity. For instance, BR located in the middle of arm G in cytotype 1 is not expressed in cytotype 2, where chromosome G is fused with arm E. Also, there is a small homozygous inversion in the middle of arm G that distinguished the both cytotypes (Figs. 9a, b). Also, BR2 is very slightly expressed.

The larva morphology of both cytotypes is differed by the month structures. The comparative SEM analysis of larval morphology of both cytotypes indicated the differences of ventromental

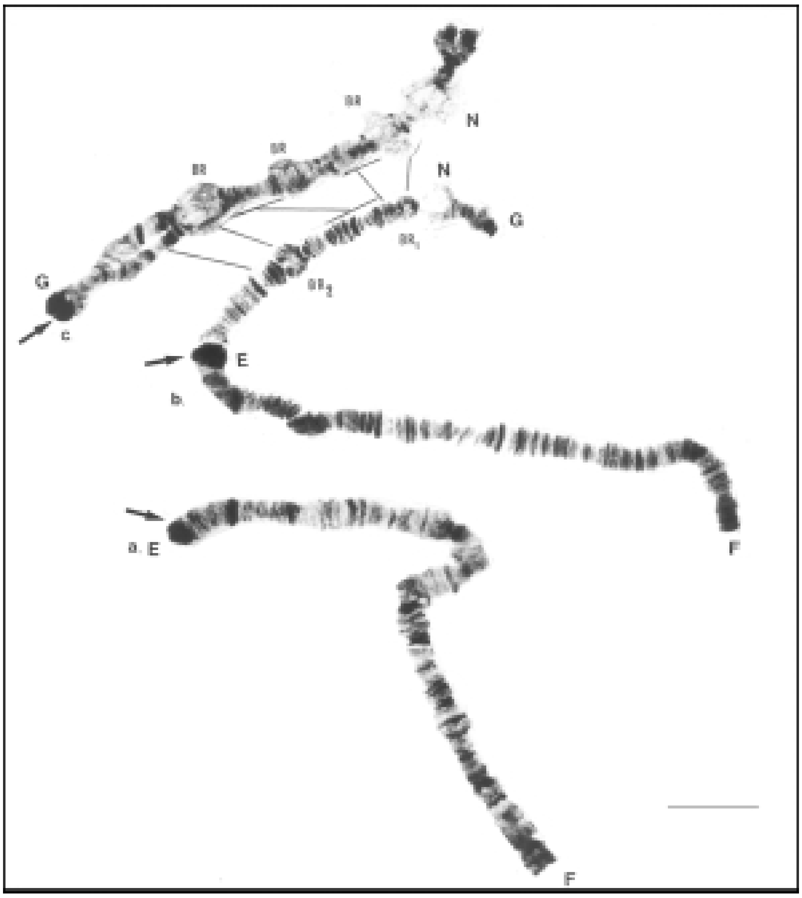

Fig. 9 — Cytotype 1 - a. Chromosome EF; c. Chromosome G. Cytotype 2 - b.Chromosomes GEF. Bar - 10 µm. The lines indicate the similar banding patterns of the polytene chromosomes of both cytotypes.

plates of mentum of both cytotypes. Such differences in the ventromental plates of genus *Kiefferulus* have been used for taxonomic characterization of the Australian species (Webb *et al.* 1987). The larval morphology of both cytotypes is almost not distinguished from the individuals of Vienna (there are slight differences in the length to the RO (Ring organ)). Apart from that the pupa and imagos (hypopygium) from Vienna species do seem to have specific structure (Figs. 7a, b; 8a, b). So, it is quite possible in Europe the genus Kiefferulus to be presented by several species. Our results indicate that the current taxonomic position of *Kiefferulus tendipediformis* should therefore be revised, although such a revision would additionally require an examination of all life history stages of the material from Bulgaria as well as cytogenetic analysis of the Viennese specimens. Nevertheless, on the basis of the work presented here we propose that the two cytotypes belong to two separate species. Thus our results change the current opinion that the genus *Kiefferulus* is monotypic in Europe.

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