

## KARYOTYPE CHARACTERIZATION OF THE ENDEMIC PISCINE LEECH FROM LAKE BAIKAL WITH VINDICATION OF *BAICALOBDELLA COTTIDARUM* DOGIEL, 1957 (PISCICOLIDAE, HIRUDINEA)

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**Abstract:** In this paper, we present for the first time data on karyotype analysis of leeches of the genus *Baicalobdella* (Piscicolidae) parasitizing Lake Baikal endemic cottoid fishes. Both mitotic and meiotic chromosomes are described. Leech testisacs were processed by a “shaking-blotting” technique, and chromosomal preparations were stained with water-based fuchsin. Diploid and haploid chromosome sets demonstrated  $2n=34$  and  $n=17$ , respectively, with maximal chromosome length of 1.5-3.0  $\mu\text{m}$ . Comparative karyotype analysis of two ecological forms of *Baicalobdella* leeches revealed differences in chromosome numbers and its morphology. Previously studied *Baicalobdella torquata* (Grube, 1871) parasitizing Baikal amphipods had smaller diploid and haploid sets ( $2n=32$ ,  $n=16$ ). In addition to numerical superiority, differing patterns of chromosome size gradation and presence of satellite elements were found in the karyotype of piscine *Baicalobdella* leeches. This confirms the systematic position of the Baikal cottoid leech parasite as a separate species, validating the original name *Baicalobdella cottidarum* sensu Dogiel, 1957.

**Key words:** Karyotype; chromosomes; piscine leech; *Baicalobdella cottidarum*; Lake Baikal

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### INTRODUCTION

Leeches (Hirudinea) are highly specialized annelids widely distributed worldwide, except Antarctica. Lake Baikal (Eastern Siberia, Russia), as a recognized center of megadiversity, also demonstrates a high level of leech diversity (27 species from 13 genera) and endemism, both at the genus (31%) and species (50%) levels (Kaygorodova, 2012; Kaygorodova, 2013; Kaygorodova and Pronin, 2013). Understanding of the genetic and species diversity of leeches remains incomplete and the cytogenetic data for this group are poor. To date, only 23 of 680 world species have been studied, in spite of the fact that chromosome set analyses were regarded as a source of useful information for solving taxonomic problems (Cichocka and Bielecki, 2008; Utevsky et al., 2009; Kaygorodova and Natyaganova, 2012). Lack of data is partly due to technical problems, primarily the comparatively small size of leech chromosomes.

The leeches studied in this paper belong to the *Baicalobdella* Dogiel, 1957, an endemic to Lake Baikal genus from the family Piscicolidae. All representatives of this genus are parasites of minor size (up to 10 mm). Initially, two species belonging to the genus were described – *Baicalobdella torquata* (Grube, 1871) and *Baicalobdella cottidarum* Dogiel, 1957, feeding on amphipods and cottoid fish, respectively. *B. cottidarum* was discovered by Prof. V. Dogiel, a renowned Russian zoologist, and its description was published after his death (Dogiel and Bogolepova, 1957). Later on, this species was regarded as an ambiguous taxon, since the young zoologist V. Epstein abolished the autonomy of *B. cottidarum* and considered only *B. torquata* as a valid species parasitizing both amphipods and sculpins (Epstein, 1959, 1973). Then he admitted his mistake and retrieved the Dogiel's species (Epstein, 1987). However, there are still controversial viewpoints on the species status of this

leech, since the taxonomic importance of morphological differences between “torquata” and “cottidarum” was differently estimated (Lukin, 1976; Kaygorodova, 2013; Kaygorodova and Sorokovikova, 2014).

In the present paper, *Baicalobdella* leeches parasitizing cottoid fishes have been karyologically studied, providing the first data on their diploid and haploid chromosome sets. A comparison of chromosome data will help us uncover the systematic position of the two ecological forms inhabiting Lake Baikal.

## MATERIALS AND METHODS

### Identification of leeches

*Baicalobdella* leeches (Fig. 1) were collected by hand directly from the host fish *Cottocomephorus grewinkii* in the southern part of Lake Baikal (Listvennichny Bay: N51°52′05”, E104°49′50”), in March of 2012. Morphological identification was conducted by Dr. Irina Kaygorodova using a WILD M4C-61 stereomicroscope and an AxioStar plus binocular microscope (Carl Zeiss Microimaging GmbH.). The species was identified in accordance with its original description (Dogiel and Bogolepova, 1957) and further with Epstein’s identification key (1987). Images of the specimens were taken by a NIKON D700 camera. All slides and voucher specimens were deposited at the Laboratory of Molecular Systematics, Limnological Institute, Russia.

### Chromosome preparation

Chromosome preparations were usually made immediately after collection of animals. In some cases, leeches were kept alive in aquaria with cooled aerated Baikal water for some days before investigation. Live specimens were incubated in the Baikal water containing mitotic inhibitor 0.05% colchicine for 3 h in the cold store (at 4°C) to arrest cells at the required metaphase stages. Immediately before fixation, the animals were placed in hypotonic trisodium citrate solution, their suckers were quickly cut, and after a few lengthwise incisions, the tissue was left for 40–60 min at room temperature. The tissue was then fixed

in fresh and cold Carnoy’s fluid (ethanol-acetic or methanol-acetic mixture, 3:1 v/v). After 24-h fixation, the gonads were isolated and transferred to macerating mixture (60% lactic acid/ glacial acetic acid, 1:15 v/v) for 2–3 min. Air-dried cytological preparations were made following the “shaking-blotting” technique (Baranov, 1989; Natyaganova and Sitnikova, 2012). Glass slides of fixed cells were stained in 1% solution of water-based fuchsin for 2–3 min.

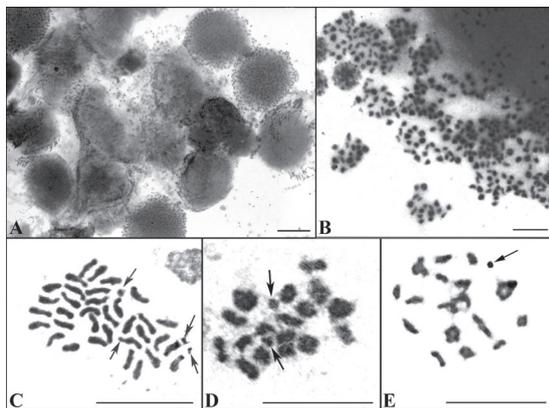
Well-spread metaphase plates were observed under the binocular microscope and X100 oil immersion objective (Zeiss, AxioStar). The chromosomes were photographed using a digital camera system (Pixera Penguin 600CL) and recorded with the AxioVS 40 v.4.7.1.0 Program (Zeiss, Axiovision). The grayscale chromosome images were inverted, visualized and subsequently saved as TIFF files using the software Adobe Photoshop CS4 ver. 11.0. Chromosome lengths were measured with the Image-Pro Plus 6.0 Program.

## RESULTS

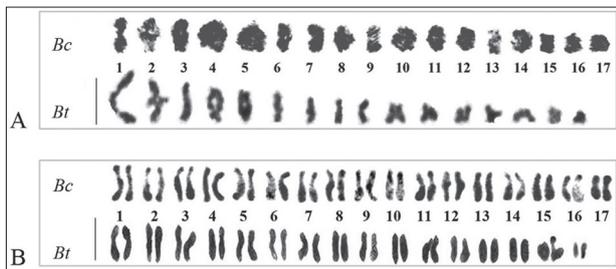
Cytogenetic preparations were obtained from ten individuals of piscine *Baicalobdella* leeches. Since the testes were used as actively proliferating tissues, numerous cytophores as scattered spheres were observed in specimens at low magnification (Fig. 2A). Each cytophore was a carrier of certain spermatogenesis stages and usually was coated with a cell population (for instance, a spermatocyte layer) that developed synchronously. Meiotic stages of adjacent cytophores can be quite different, and the countable squashes needed were found infrequently. Therefore, many cells were obtained at the meiosis stage. The vast majority of the well-spread metaphase plates were represented at diakinesis-metaphase I of the meiotic stage. Cytophores with the cells at this stage were especially promising, since multiple metaphase plates in one field of view could be analyzed at the same time (Fig. 2B). Like other authors (e.g., Singhal et al., 1986), during the analysis of chromosome preparations we encountered difficulties associated with the small size of chromosomes and their tendency to clump at metaphase cell divisions. Nevertheless, we succeeded to identify 84



**Fig. 1.** Leech *Baicalobdella* parasitizing cottoid fish *Cottocomephorus grewinkii* from Lake Baikal. Scale bar=1 mm.



**Fig. 2.** Air-dried chromosome preparations obtained by the “shaking-blotting” technique from the testes of Baikal endemic leech *Baicalobdella* parasitizing cottoid fish. A – a scattering of cytophores with cells at different stages of spermatogenesis; B – a segment of cytophore with spermatocytes in diakinesis-metaphase I stage; C – a mitotic metaphase plate with 34 chromosomes; D, E – meiotic metaphase plate with 17 chromosome bivalents. Arrows point to satellite elements in C and D, and a supernumerary B-chromosome in E. Scale bars in (A–E)=10  $\mu$ m.



**Fig. 3.** Comparison of chromosome sets of two closely related leech species of the genus *Baicalobdella*. A – karyograms of meiotic (haploid) chromosome sets; B – karyograms of mitotic (diploid) chromosome sets; Bc and Bt – abbreviations of *B. cottidarum* and *B. torquata* (correspondingly). Scale bar=3  $\mu$ m.

cell nuclei with countable chromosomes, 83 of them proved suitable for analysis of meiotic chromosomes and only one of mitotic chromosomes.

The chromosomes of the Baikal piscine leech were rather small (1.5-3.0  $\mu$ m maximum) at both mitotic and meiotic metaphases (Fig. 2). In the karyogram, where homologous pairs lined up in size, chromosomes formed gradually decreasing series (Fig. 3A<sub>Bc</sub> and B<sub>Bc</sub>). The analysis of mitotic metaphase revealed a diploid set of 2n=34 (Fig. 2C and 3B<sub>Bc</sub>). This value was confirmed by the number of bivalents in meiosis. The majority of meiotic metaphase plates (Fig. 2B, D, E and Fig. 3A<sub>Bc</sub>) contained 17 bivalents, and only 5 out of 83 examined plates showed a variance from this value. A centromere position in mitotic chromosomes was not always clear; therefore, it was impossible to assess their complete morphology. However, most of the chromosomes could be discerned as submetacentric (unequal-arm). At the stage of diakinesis-metaphase I of meiosis, chromosomes formed morphologically different bivalents – annular, rod-like and cruciform, which indicated the presence of various numbers of chiasmata (Fig. 2D, E).

The presence of satellites at some chromosome pairs is a special feature of the chromosome set of this leech species. The satellites were clearly visible on the mitotic and some meiotic metaphase plates (Fig. 2C, D). Genetic elements that carried satellites corresponded to the 6<sup>th</sup> and 16<sup>th</sup> pairs of homologs in the karyogram of experimental species (Fig. 2C and 3A<sub>Bc</sub>). Another cytogenetic characteristic of the chromosome set was supernumerary B-chromosomes, which were sometimes observed on the metaphase plates. Most often, they appeared as intensely colored punctiform elements (Fig. 2E).

**DISCUSSION**

As a whole, chromosome sets have been determined for 23 leech species from over 680 currently known Hirudinea (Perez, 1907; Jørgensen, 1908, 1913; Wendrowsky, 1928; Macino and Puccinelli, 1964; Puccinelli and Macino, 1966, 1968; Singhal et al., 1986; Davies

and Singhal, 1987; Vitturi et al., 2002; Utevsky et al., 2009; Kaygorodova and Natyaganova, 2012). Eight species have been studied within the family Glossiphoniidae with six chromosome diploid numbers ranging from 14 to 32; four species of Piscicolidae from 12 to 32; seven species of Erpobdelliformes from 16 to 22, and four species of Hirudiniformes from 24 to 28. The diploid set of the piscicolid *B. cottidarum* consisting of 34 chromosomes has been identified for the first time in the present study. This was the highest chromosome number within the karyologically studied leech species. Thus, the range of variation of chromosome numbers in Hirudinea increased up to 12-34.

Earlier, high chromosome numbers were regarded as an evolutionarily advanced character among Hirudinea and the whole Annelida group (Wendrowsky, 1928). The initial number of chromosomes in Hirudinea was proposed to be  $2n=16$ , since the parasitic leech-like annelid worms *Branchiobdella astaci* (Branchiobdellida) and relict species *Acanthobdella peledina* (Acanthobdellida) had this diploid number of chromosomes. On the contrary, Mann (1961) supposed that the family Glossiphoniidae within Rhynchobdellida was regarded as primitive in relation to Piscicolidae, whereas among Arhynchobdellida, Hirudinidae gave rise to terrestrial jawed leeches Haemadipsidae, carnivorous leeches Haemopidae and macrophagous leeches Erpobdellidae. A recent review of available karyological data concluded that the jawed forms ( $2n=24-28$ ) developed from macrophagous forms ( $2n=16-22$ ), and consequently, Erpobdelliformes were a primitive group compared to Hirudiniformes (Singhal et al., 1987; Cichočka and Bielecki, 2008). This reaffirms the initial Wendrowsky hypothesis. Moreover, it was assumed that aquatic “proto-leech” laid hard-shell cocoons on a substrate (smooth stones, aquatic vegetation, etc.); this type of behavior was evident in aquatic oligochaetes (an ancestral group in relation to the leeches) and was shown in the “primitive leech” *Acanthobdella peledina*, as well in “true” leeches such as erpobdellids (Sawyer, 1986; Kutschera and Wirtz, 2001). A more complicated behavioral pattern (e.g., maternal care) observed only in snail leeches was undoubtedly an advanced evolutionary trait, which indirectly confirmed

the leech evolutionary trend from Arhynchobdellida to Rhynchobdellida with Glossiphoniidae at the top as the most probable. Contradictory evolutionary hypotheses were applied in numerous molecular studies for inferring phylogenetic schemes according to which the ancestral leech was related to the existing erpobdellids or piscicolids (or even glossiphoniids!).

Contemporary cytological data are more consistent with the purposes of systematics within this group. However, the relatively small amount of available karyological data still makes it difficult to reveal the phylogenetic trend of chromosomal evolution within the Hirudinea. Despite the deficiency of data, it was possible to notice that the chromosome numbers and chromosome morphology vary significantly among and within different taxonomic groups. This fact suggests that speciation in leeches is accompanied by intensive karyotypic rearrangements. Consequently, the use of cytogenetic methods is reasonable for successful solutions to taxonomic problems, even in closely related taxa within the Hirudinea.

We applied a special method for obtaining chromosomal preparations in our cytological study of Baikal leeches. A shaking-blotting technique (Baranov, 1989) seemed relevant to get a monolayer from gonadal cells or other tissues and organs. To realize this method, a small set of reagents and tools was required; therefore, chromosomal preparations could be prepared even in the field. Furthermore, such cytological preparations are stable over a long time and they can be stained and re-stained with different dyes. This technique has been successfully applied to different groups of Baikal invertebrates – mollusks (Poberezhny, 1989), isopods (Natyaganova et al., 1996), amphipods (Natyaganova and Sitnikova, 2012) and leeches (Kaygorodova and Natyaganova, 2012). We understood that with the appropriate pretreatment of the samples (colchicine treatment, hypotension and maceration), this technique yielded better chromosomal preparations for such a difficult object as leeches in a cytogenetic approach.

In order to clarify the actual taxonomic status of two *Baicalobdella*, we conducted a comparative

analysis of their karyological data, as the chromosome set of the incontestable *B. torquata* parasitizing Baikal amphipods is already known (Kaygorodova and Natyaganova, 2012). These data showed differing chromosome numbers in leeches parasitizing Baikal cottoid fish and in its congener infecting Baikal amphipods. The diploid sets of these *Baicalobdella* were 34 and 32, respectively (Fig. 3). Moreover, a comparison of these two species revealed a visual difference in chromosomal lengths. As seen in the diploid and haploid karyograms, there were various patterns of chromosome size gradation (Fig. 3). Hence, there is a smooth variation (chromosomes of nearly the same length) in chromosome lengths of the cottoid parasite set, whereas *B. torquata* possesses a constant excess of small- and medium-sized chromosomes. This can be seen more clearly in the haploid set of the latter (Fig. 3A<sub>B1</sub>). Another distinctive feature of the “cottidarum” form is the presence of two chromosome pairs with satellite elements (Fig. 2C, D), which were not detected in the *B. torquata* karyotype (Kaygorodova and Natyaganova, 2012). Secondary chromosomal strangulations that separate satellites are loci of ribosomal genes (Bostock and Sumner, 1978; Branco and Pombo, 2007), serving as cytological markers for the identification of homologs (paired chromosomes) in a set. It is likely that the tiny pairs mentioned by Wendrowsky (1928) in the karyotypes of some glosiphoniids (*G. complanata*, *A. heteroclita* and *A. papillosa*) referred to these chromosomal structures. He considered their presence as “fragmentation of chromosomes, leading to a change in diploid set and, ultimately, formation of new species”.

In chromosome complexes of piscine *Baicalobdella*, the so-called tiny supernumerary or B-chromosomes were encountered on rare occasions and were solitary (Fig. 2E). These genetic elements are found in animals and plants. They vary in number (from 1 to 45) and size (from a point to the size of normal chromosomes), structurally differ from the core set of chromosomes (autosomes, A-chromosomes), are not paired with them in meiosis, and therefore are inherited by chance (non-Mendelian inheritance).

Since these genetic elements are heterochromatic and contain repetitive DNA sequences, their presence was thought to have no effect on the phenotype and viability of the organism. To date, it is known that B-chromosomes may possess transcriptionally active genes causing resistance to adverse environmental conditions (Camacho et al., 2000). Supernumerary chromosomes may be identified both in sporadic individuals and in most of a population. In some species, there are populations without B-chromosomes. Thus, the presence of B-chromosomes is taxonomically insignificant. Nevertheless, such chromosomal elements have not been detected in the karyotype of the sister species *B. torquata*, dwelling under the same conditions of Lake Baikal.

Thus, we can conclude that there are explicit qualitative and quantitative differences in two ecological forms of *Baicalobdella* leech karyotypes. Comparative karyotype analysis revealed differences in chromosome number and its morphology. As shown in our previous study, *Baicalobdella torquata* parasitizing Baikal amphipods had smaller diploid and haploid sets ( $2n=32$ ,  $n=16$  versus  $2n=34$ ,  $n=17$ ) as well as differing patterns of chromosome size gradation (Kaygorodova and Natyaganova, 2012). A karyotype of piscine *Baicalobdella* leeches, on the contrary, had satellite and supernumerary elements. The revealed complex of karyotypic differences suggests genetic isolation of two *Baicalobdella* that, in turn, is convincing proof of freestanding species. Hence, we can assert that the *Baicalobdella* parasitizing Baikal cottoid fishes is an independent species, as originally stated by Dogiel (1957). Therefore, the species name *B. cottidarum* should be valid again.

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## REFERENCES

- Baranov, V. S. (1989). The "Shaking-Blotting" technique as a new reliable approach to direct chromosome preparations from the chorion villus sampling. *Tsitologiya*. **31**, 251-253.
- Bostock, C. J. and A. T. Sumner (1978). The eukaryotic chromosome. North Holland Publishing Co., Amsterdam.
- Branco, M. R. and A. Pombo (2007). Chromosome organization: new facts, new models. *Trends Cell Biol.* **17**(3), 127-134.
- Camacho J. P. M., Sharbel T. F. and L. W. Beukeboom (2000). B chromosome evolution. *Phil. Trans. R. Soc. Lond.* **355**, 163-178.
- Cichocka, J. and A. Bielecki (2008). Biological diversity of leeches (Clitellata: Hirudinida) based on characteristics of the karyotype. *Wiadomoosci Parazytologiczne*. **54**(4), 309-314.
- Davies, R. W. and R. N. Singhal (1987). The chromosome numbers of five North American and European leech species. *Can. J. Zool.* **65**, 681-684.
- Dogiel, V. A. and I. Bogolepova (1957). Parasite fauna of Lake Baikal fishes. *Proceedings of the Baikal Limnological Station*. **15**, 449-452.
- Epstein, V. M. (1959). On systematic position, life style and origin of endemic Baikal leech *Trachelobdella torquata* (Grube). *Doklady AN SSSR*. **125**(4), 935-937.
- Epstein, V. M. (1973). Diagnoses of genera *Calliobdella*, *Tracheobdella*, *Limnotracheobdella* and *Baicalobdella* (Hirudinea, Piscicolidae) and evaluation of taxonomic value of used characteristics. *Russ. J. Zool.* **42**(3), 332-341.
- Epstein, V. M. (1987). Leeches. In: Key of the freshwater fish parasites of the USSR fauna, 3(2) (Eds. O.A. Skarlato), 340-372. Nauka, Leningrad.
- Jørgensen, M. (1913). Untersuchungen über die Eibildung bei *Nephele vulgaris* Maquin Tandon (*Herpobdella atomaria* Carena). *Archiv für Zellforsch. ng.* **2**, 279-347.
- Kaygorodova, I. A. (2012). A revised checklist of the Lake Baikal leech fauna. *Lauterbornia*. **75**, 49-62.
- Kaygorodova, I. A. (2013). An illustrated checklist of leech species from Lake Baikal (Eastern Siberia, Russia). *Dataset Papers Sci.* **4**, 261521.
- Kaygorodova, I. A. and A. V. Natyaganova (2012). Chromosome characterization of endemic *Baicalobdella torquata* (Grube, 1871) (Piscicolidae: Hirudinidae) from Lake Baikal, Eastern Siberia, Russia. *Lauterbornia*. **75**, 63-70.
- Kaygorodova, I. A. and N. M. Pronin. (2013). Species Composition and Distribution of Leech Fauna in Chivyrkuy Gulf (Lake Baikal, Russia). *ScientificWorldJournal*. **10**, 206590.
- Kaygorodova, I. A. and N. V. Sorokovikova (2014). Mass leech infestation of sculpin fish in Lake Baikal, with clarification of disease-prone species and parasite taxonomy. *Parasitol. Int.* **63**, 654-757.
- Kutschera U. and P. Wirtz (2001). The Evolution of Parental Care in Freshwater Leeches. *Theory Biosci.* **120**, 115-137.
- Lukin, E. I. (1976). Leeches of fresh and saline waters (Fauna of the USSR. Leeches). Nauka, Leningrad.
- Macino, G. and I. Puccinelli (1964). Corredo cromosomico e meiosi di *Pontobdella muricata* (L.) (Hirudinea, Piscicolidae). *Bollettino de Zoololia*. **31**, 1311-1320.
- Mann, K.H. (1961). Leeches (Hirudinea): Their Structure, Physiology, Ecology and Embryology. Rergamon Press, New York.
- Natyaganova A. V., Kamaltynov R. M. and D. Y. Sherbakov (1996). The chromosomes of the *Baicalasellus angarensis* (Isopoda, Asellidae). *Crustaceana*. **69**(6), 696-702.
- Natyaganova, A. V. and T. Y. Sitnikova (2012). Karyotype of the Baikal amphipod *Polyacanthisca calceolata* Bazikalova, 1937, (Crustacea, Amphipoda). *Chromosome Science*. **15**, 43-48.
- Perez, N. (1907). Chromosome number in *Branchellion torpedinis*. *Arch Russ.* **1**, 108-117.
- Poberezhnyi, E. S. (1989). Baikalskie endemichnie molluski kak obyekt gidro-biologicheskogo monitoringa. Abstract of Doctoral Thesis, Irkutsk.
- Puccinelli, I. and G. Mancino (1966). Il cariotipo e la linea germinale femminile di *Erpobdella testacea* (Hirudinea, Erpobdellidae). *Atti Soc. Toscana Sci. Nat. Pisa Mem. Ser. A* **73**, 106-112.
- Puccinelli, I. and G. Mancino (1968). Osservazioni carilogiche sul genere *Trocheta* (Hirudinea, Erpobdellidae). *Atti Accad. Naz. Lincei Cl. Sci. Fis. Mat. Nat.* **45**, 597-605.
- Sawyer, R. T. (1986). Leech Biology and Behaviour, Clarendon Press, Oxford, UK.
- Singhal, R. N., Davies, R. W. and C. C. Chinnappa (1986). Karyology of *Erpobdella punctata* and *Nepheleopsis obscura* (Annelida: Hirudinoidea). *Caryologia*. **39**, 115-121.
- Utevsky, S., Kovalenko, N., Doroshenko, K., Petrauskien, L. and V. Klymenko (2009). Chromosome numbers for three species of medicinal leeches (*Hirudo* spp.). *Syst. Parasitol.* **74**, 95-102.
- Vitturi, R., Libertini, A., Armetta, F., Sparacino, L. and M. Colomba (2002). Chromosome analysis and FISH mapping of ribosomal DNA (rDNA), telomeric (TTAGGG)*n* and (GATA)*n* repeats in the leech *Haemopsis sanguisuga* (L.) (Annelida: Hirudinea). *Genetica*. **115**, 189-194.
- Wendrowsky, V. (1928). Über die Chromosomencomplexe der Hirudinen. *Mitteilungen aus der Zoologischen Station zu Neapel*. **8**, 153-232.