Folia biologica (Kraków), vol. **58** (2010), No 3-4
doi:10.3409/fb58_3-4.245-249

Use of the Chromosomal Co-location of the Minor 5S and the Major 28S rDNA as a Cytogenetic Marker within the Genus *Leuciscus* (Pisces, Cyprinidae)*

Lech KIRTIKLIS, Katarzyna PORYCKA, Alicja BOROŃ, Jean-Pierre COUTANCEAU, and Agnes DETTAI

Accepted May 25, 2010

KIRTIKLIS L., PORYCKA K., BOROŃ A., COUTANCEAU J-P., DETTAI A. 2010. Use of the chromosomal co-location of the minor 5S and the major 28S rDNA as a cytogenetic marker within the genus *Leuciscus* (Pisces, Cyprinidae). Folia biol. (Kraków) **58**: 245-249.

The chromosomes of three species from the genus *Leuciscus* (the ide *L. idus*, the European chub *L. cephalus* and the common dace *L. leuciscus*) were examined with the FISH technique for 5S and 28S rDNA probes. The analysis showed that among the three examined species, 5S rDNA signals were located on two large and four small subtelocentric chromosomes in *L. leuciscus*, on one large and five small subtelocentric chromosomes in *L. idus*, while in *L. cephalus* the probe signals were found on two metacentric chromosomes and one large and one small subtelocentric chromosome pairs. In all analysed species, the 28S rDNA probe signals were placed on only one chromosome pair, subtelocentric in the common dace and the European chub, and submetacentric in the ide. The three species differed in the number of sites in which both probe signals were present. In conclusion, the co-location of the 5S and 28S rDNA proved to be a useful cytogenetic marker among the studied fishes. Moreover, this marker could be adapted to other cyprinids.

Key words: Chromosome, FISH, rDNA, *Leuciscus*, gene mapping, species identification.

Lech KIRTIKLIS, Katarzyna PORYCKA, Alicja BOROŃ, Department of Zoology, Faculty of Biology, University of Warmia and Mazury in Olsztyn, Oczapowskiego 5, 10-718 Olsztyn, Poland.
E-mail: leo@uwm.edu.pl
katarzyna.woroniecka@uwm.edu.pl
alibo@uwm.edu.pl
Jean-Pierre COUTANCEAU, Agnes DETTAI, Département Systématique et Evolution, Museum National d'Histoire Naturelle, 43, rue Cuvier, 75005 Paris, France.
E-mail: coutance@mnhn.fr)
adettai@mnhn.fr

The species of the sub-family Leuciscinae, including the fishes mentioned above, have many karyological similarities. Most are characterized by 2n = 50 chromosomes with a relatively low number of uniarmed elements (RÁB & COLLARES-PEREIRA 1995). Proper identification of the chromosome set for each of these species is problematic using classic karyological methods. However, recent results based on cytogenetic molecular markers have revealed that the karyotypes of the species from some genera could be easily identified (GROMICHO & COLLARES-PEREIRA 2006; BOROŃ *et al.* 2008). Despite this, there is no useful method for karyotypic identification of *Leuciscus* species. On the other hand, there is a need of having such a tool for distinguishing chromosome sets in this group of species and their putative hybrids.

Three species from the genus *Leuciscus* (the ide *L. idus*, the common dace *L. leuciscus* and the European chub *L. cephalus*) are broadly distributed in Europe (BANARESCU 1992). *L. leuciscus* is used as a bio-indicator in degraded rivers with an altered composition of their ichthyofauna (WOJDA *et al.* 1993), while *L. idus* is also bred in polyculture with other cyprinids used in freshwater aqua-culture (WITKOWSKI & BŁACHUTA 1989). Moreover, the ide and the European chub are of interest to anglers. Proper identification of the spe-

*This study was financially supported by the European Commission's Research Infrastructure Action via the SYNTHESYS Project, the research grant No. 2 P04C 082 29 from the State Committee for Scientific Research, Poland and the research grants No. 0208.805 and No. 0208.211 from the University of Warmia and Mazury in Olsztyn, Poland.

cies, especially during their early stages, is required for their effective management.

Molecular taxonomy seemed to be a highly efficient tool for providing species-specific markers especially among species that are difficult to distinguish based solely on morphology. However, these methods cannot be applied to investigations at the chromosome level. Thus, the possibility of karyotype identification could support both chromosome research and species identification. Moreover, the comprehensive use of cytogenetic and molecular methods should increase the level of certainty in proper identification.

We decided to use the FISH (fluorescence *in situ* hybridization) technique for mapping known sequences on chromosomes (PHILLIPS 2006) to look for species-specific markers for the karyotypes of these three *Leuciscus* species. Ribosomal genes in fishes occur in multiple copies in the genome and are divided into two classes: major (18S, 5,8S and 28S) and minor (5S). These two gene families often have separate locations on the chromosomes. The mapping of the 5S rDNA sequence on a single chromosome pair appears to be common in vertebrates (MARTINS & GALETTI 2001), but this pattern of 5S rDNA distribution may correspond to the ancestral rDNA gene organization among Teleostei (SOLA *et al.* 2003). The distribution of 5S ribosomal gene sequences on two or more chromosome pairs is considered to be a derived condition (INAFUKU *et al.* 2000; MARTINS & WASCO 2004; BOROŃ *et al*. 2006; GROMICHO *et al*. 2006). More practically, ribosomal RNA genes have an alternating occurrence of highly conserved and more variable regions (SONNENBERG *et al*. 2007), where the most conserved regions can be used as probes across a wide range of species. The high number of copies also ensures easier visualisation by the FISH techniques. The loci of major rDNA are located on different chromosomes than those bearing 5S rDNA clusters in the genomes of most fishes (non-syntenic location). Such a distribution would represent the primitive condition in chromosomal evolution (MARTINEZ *et al*. 1996) and, contrary to the syntenic location, could participate in preventing undesirable translocation among major and minor rDNA sequences (MARTINS & GALLETI-Jr 1999). Some major rDNA locations in fish could correspond to the Ag-NORs sites (regions with silver-positive signals previously thought to correspond to nucleolar ribosomal regions) on chromosomes, but it is not a solid cytogenetic rule (GROMICHO *et al*. 2005).

Material and Methods

All specimens of *L. idus*, *L. cephalus* and *L. leuciscus* were caught with nets in the Bug and Odra Rivers (Poland). Two females and two males (20 metaphases from each individuals at least) of the three species were examined. Metaphase chromosome preparations were made according to the method described by RÁB and ROTH (1988) with modification (BOROŃ 2006).

The FISH protocol with 5S and 28S rDNA probes was used as proposed by ALMEIDA-TOLEDO *et al*. (2002) with modifications. Briefly, chromosomes were denatured for 1 min. in a mixture of 70% formamide and 2xSSC at 70°C. Both probes were mixed together in a hybridization buffer and denatured 5 min at 74°C. The probes were obtained by PCR from two reference clones in our collection. Sequence-specific amplifications were performed by PCR in a final 25 μl volume with 0.3 μM of Taq DNA Pol (QBioGen), 2.5 μl of buffer (QBioGen), 0.001 μg of DNA, 5% DMSO, 300 μM of each dNTP and 1.7 pM of each of the two standard primers M13R and M13 l. After denaturation for 2 min, the PCR ran for 30 cycles of (30 s, 94 C°; 30 s, 54 C°; 1 min, 72 C°), with a terminal elongation of 3 min. The result was visualized on ethidium bromide-stained agarose gels, and marked with the Nick translation Mix and Digoxigenin 11 dUTP or Biotin 16 dUTP from Roche Diagnostics according to the instructions of the manufacturer. The marked probe mixture (15 μl on each slide) was hybridized overnight at 37°C. Post-hybridization washes were performed with 5 min wash in 2XSSC, 70°C and one 2 min wash in phosphate buffer detergent. Slides were mounted with DAPI/Antifade solution and analysed using a Zeiss Axioplan fluorescence microscope equipped with the FISH imaging 'Genus' software from Applied Imaging.

Results

The chromosome number in all analysed species is 2n=50. The results of FISH using the two rDNA probes are presented in Fig. 1. 5S rDNA signals presented some small variation within and among individuals of some of the species. 5S rDNA signals were located on two large and four to six small subtelocentric chromosomes (numbers of hybridization signals ranged from 6 to 8, mode: 6) in *L. leuciscus*, on one large and five small subtelocentric chromosomes (ranged from 5 to 6, mode: 6) in *L. idus*, while in *L. cephalus* the probe signals were found on two metacentric chromosomes and one large and one small subtelocentric chromosome pairs (Fig. 1a, b, c). In all the species and specimens, the 28S rDNA probe was always located on a single chromosome pair, subtelocentric in the common dace and the European chub, and submetacentric in the ide (Fig.1d, e, f), and presented no variability. Two biarmed chromosomes

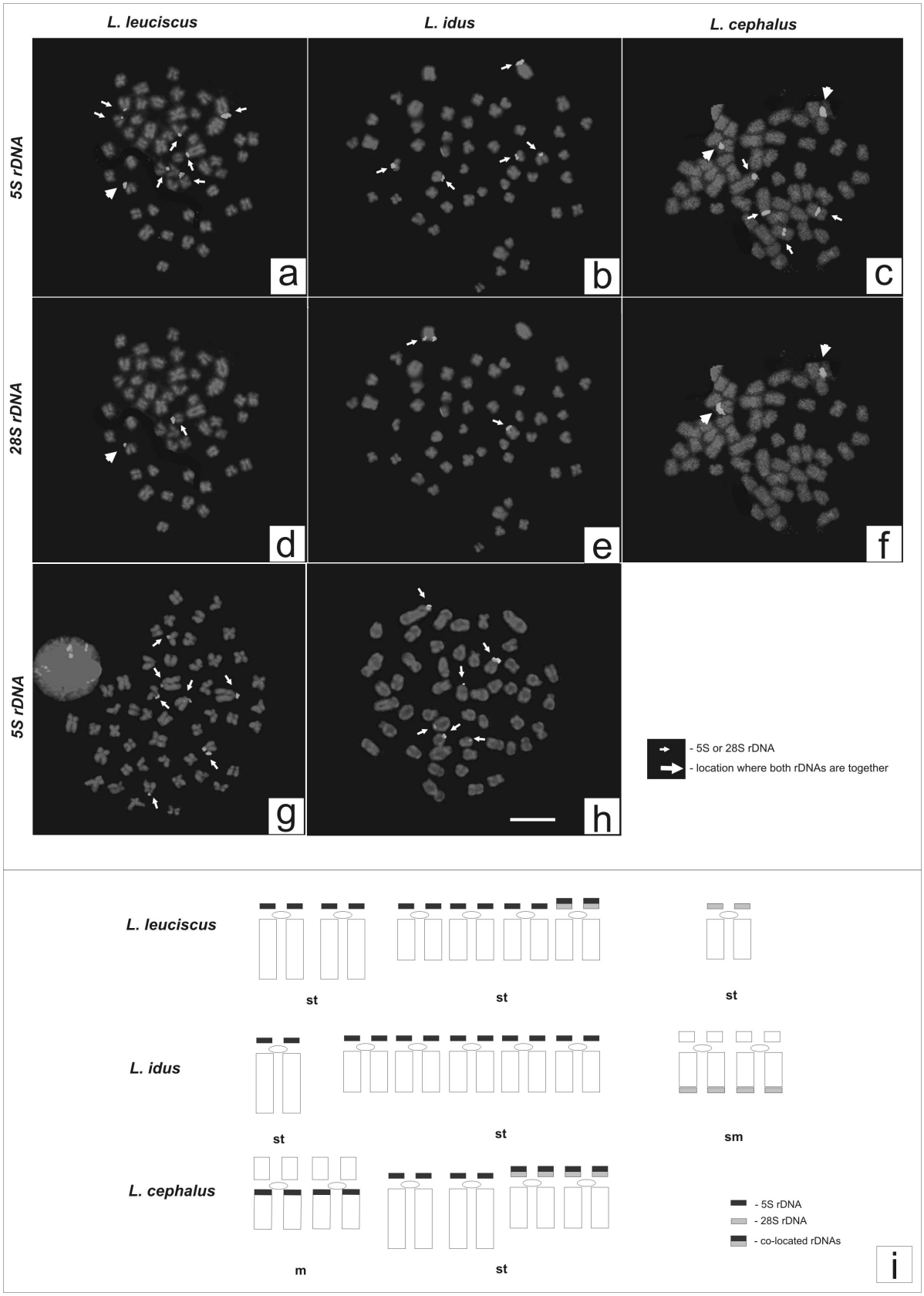

Fig 1 a-i. Distribution of 5S and 28S rDNA on the chromosomes of *Leuciscus leuciscus* (a, d accordingly; g – another variant of 5S rDNA distribution), *L. idus* (b, e accordingly; h – another variant of 5S rDNA distribution) and *L. cephalus* (c, f accordingly) (arrows indicate rDNA sites after hybridization with probes); schematic diagrams (i). Bar = 10 μm.

in *L. cephalus* and one uniarmed chromosome in *L. leuciscus* had signals for both rDNA probes, whereas no co-location was detectable on the chromosomes of *L. idus* (Fig. 1i). There was no variability in the co-location of the signals.

Discussion

The location of the 5S rDNA sequence exclusively on several uniarmed chromosomes shows that the size of this rDNA fragment and its distribution is similar in *L. idus* and *L. leuciscus* (Fig. 1a, b). However, there are some differences regarding the big subtelocentric chromosomes, on which the 5S rDNA probe hybridized with only one in *L. idus,* but with two in *L. leuciscus*. One way to explain this situation is that the number of ribosomal gene copies on ide chromosomes is sometimes too low to be detected with cytogenetic techniques (ALMEIDA-TOLEDO *et al*. 2002). On the other hand, a lack of probe signal may be a direct consequence of sequence elimination, which is often associated with the activities of repetitive DNA fragments or transposon elements (MA & GUSTAFSON 2005). These also explain the variability of hybridization signals observed for the 5S rDNA probe (Fig.1a, b, g, h). In the case of the European chub, the 5S rDNA sequence was found on uniarmed and on bi-armed chromosomes. Signals in the pericentromeric regions of meta- submetacentric chromosomes seem to be species-specific (Fig.1c), but the within and between individual variability observed here makes their use for karyotype identification difficult.

The location of the 28S rDNA, contrary to the above-mentioned similarities of 5S rDNA distribution, shows different patterns in *L.idus* and *L.leuciscus*. In all the specimens we found at most one pair of chromosomes with hybridization signals after FISH with 28S rDNA probe (Fig. 1d-f). The signals were observed in the distal part of "q" arms of the submetacentric chromosomes in the ide, and small "p" arms of the subtelocentric chromosomes in the common dace and the European chub. Generally, we have not seen any variation in the number of chromosomes bearing 28S rDNA sites. BOROŃ *et al*. (2009) have described two more 28S rDNA sites located on the metacentric chromosomes in *L. leuciscus*. We could not detect these loci in the present study, but these extra signals of 28S rDNA would not change the pattern of co-location regarding 5S and 28S rDNA in that species.

Most of the previous cytogenetic data published on these three species presented classical analyses and discussed features such as diploid chromosome number (2n), arm number (NF), C-band and NOR location (COLLARES-PEREIRA *et al*. 1998). The two papers on molecular chromosome analysis of *Leuciscus* species (BIANCO *et al*. 2004; BOROŃ *et al.* 2009) did not present any data on the co-location of 5S and 28S rDNA sequences. The location of the 5S rDNA revealed some polymorphism, and its pattern was very similar only for *L. idu*s and *L. leuciscus* (different in *L. cephalus*). The 28S rDNA distribution pattern is stable within species, but cannot be distinguished between *L. leuciscus* and *L. cephalus* even if *L.idus* presents some differences. Neither the 5S nor the 28S rDNA probe used separately allows for the identification of the chromosome sets of all three species. Moreover, the chromosomes in sub-family Leuciscinae (as in the whole cyprinid group) are small and have a high level of chromatin density. This is an important problem in describing their morphology, particularly in uni-armed chromosomes. The character of the co-location described in this paper is different for each species, and could be relatively easy to observe and analyse in microscopic photographs. The observer only has to count the number of chromosomes with 5S and 28S rDNA overlapping hybridization signals (zero in *L. idus,* two in *L. cephalus* and one in *L. leuciscus*) (Fig. 1i); (the term "signal number" means the number of chromosomes on which the rDNA sequence was mapped). Nonetheless, this remains to be tested by a wider sampling.

In conclusion, FISH using simultaneously 5S and 28S rDNA probes allows the detection of overlapping hybridization signals. The number of these signals seems to be species-specific for these three *Leuciscus* species. This cytotaxonomical method could represent a useful and accurate tool for karyotype identification and also for tracking chromosome re-arrangements regarding some rDNA fragments within the species and their interspecific hybrids. Moreover, the described method could be applied for supporting the process of species determination in this fish group.

Acknowledgements

We thank Dr. C. OZOUF-COSTAZ for her help in organizing this study and her input on the manuscript, and Dr. S. BOROŃ for kindly providing fish for examination.

References

ALMEIDA-TOLEDO L. F., OZOUF-COSTAZ C., FORESTI F., BONILLO C., PORTO-FORESTI F., DANIEL-SILVA M. F. Z. 2002. Conservation of 5S-bearing chromosome pair and colocalization with major rDNA clusters in five species of *Astyanax* (Pisces, Characidae). Cytogenet. Genome Res. **97**: 229-233.

BANARESCU P. 1992. Distribution and dispersal of fresh water animals in North America and Eurasia. (In: Zoogeography of Fresh Waters, vol. 2. P. Banarescu ed. Aula Verlag, Wiesbaden): 524-1087.

BIANCO P., APREA G., BALLETTO B., CAPRIGLIONE E., FULGIONE T., ODIEMA D. G. 2004. The karyology of the cyprinid genera *Scardinius* and *Rutilus* in southern Europe. Ichthyol. Res. **51**: 274-278.

BOROŃ A., OZOUF-COSTAZ C., COUTANCEAU J-P., WORONIECKA K. 2006. Gene mapping of 28S and 5S rDNA sites in the spined loach *Cobitis taenia* (Pisces, Cobitidae) from a diploid population and a diploid-tetraploid population. Genetica **128**: 71-79.

BOROŃ A., PORYCKA K., ITO D., ABE S., KIRTIKLIS L. 2009. Comparative molecular cytogenetic analysis of three *Leuciscus* species (Pisces, Cyprinidae) using chromosome banding and FISH with rDNA. Genetica **135**: 199-207.

COLLARES-PEREIRA M. J., PROSPERO M. I., BILÉU R. I., RODRIGUES E. M. 1998. *Leuciscus* (Pisces, Cyprinidae) karyotypes: Transect of Portuguese populations. Genet. Mol. Biol. **21**: 1-13.

GROMICHO M., COELHO M. M., ALVES M. J., COLLARES-PEREIRA M. J. 2006. Cytogenetic analysis of *Anaecypris hispanica* and its relationship with the paternal ancestor of the diploid-polyploid *Squalius alburnoides* complex. Genome **49**: 1621-1627.

GROMICHO M., COLLARES-PEREIRA M. J. 2006. The Evolutionary Role of Hybridization and Polyploidy in an Iberian Cyprinid Fish – A Cytogenetic Review. (In: Fish Cytogenetics, E. Pisano, C. Ozouf-Costaz, F. Foresti, B. G. Kapoor eds, Science Publishers, Enfield, NH): 41-67.

GROMICHO M., OZOUF-COSTAZ C., COLLARES-PEREIRA M. J. 2005. Lack of correspondence between CMA_3, Ag-positive signals and 28S rDNA loci in two Iberian minnows (Teleostei, Cyprinidae) evidenced by sequential banding. Cytogenet. Genome Res. **109**: 507-511.

INAFUKU J., NABEYAMA M., KIKUMA Y., SAITOH J., KUBOTA S., KOHNO S. 2000. Chromosomal location and nucleotide sequence of 5S ribosomal DNA of two cyprinid species (Osteichthyes, Pisces). Chromosome Res. **8**: 193-199.

MA X. F., GUSTAFSON J. P. 2005. Genome evolution of allopolyploids: a process of cytological and genetic diploidization. Cytogenet. Genome Res. **109**: 236-249.

MARTINEZ J. L., MORAN P., GARCIA-VAZQUEZ E., PENDAS A. M. 1996. Chromosomal localization of the major and 5S rRNA genes in the European eel (*Anguilla anguilla*). Cytogenet. Cell Genet. **73**: 149-152.

MARTINS C., GALETTI P. M. 2001. Two 5S rDNA arrays in Neotropical fish species: is it a general rule for fishes? Genetica **111**: 439-446.

MARTINS C., GALETTI-Jr. P. M. 1999. Chromosomal localization of 5S rDNA genes in *Leporinus* fish (Anostomidae, Characiformes). Chromosome Res. **7**: 363-367.

MARTINS C., WASCO A. P. 2004. Organization and evolution of 5S ribosomal DNA in the fish genome. (In: Focus on Genome Research. C. R. Williams ed., Nova Science Publishers, Hauppauge, NY): 289-318.

PHILLIPS R. 2006. Application of Fluorescence in situ Hybridization (FISH) to Genome Mapping in Fishes. (In: Fish Cytogenetics, E. Pisano, C. Ozouf-Costaz, F. Foresti, B. G. Kapoor eds, Science Publishers, Enfield, NH): 445-471.

RÁB P., COLLARES-PEREIRA M. J. 1995. Chromosomes of European cyprinid fishes (Cyprinidae, Cypriniformes): a review. Folia Zool. **44**: 193-214.

RÁB P., ROTH P. 1988. Cold-blooded vertebrates. (In: Methods of Chromosome Analysis, P. Balicek, J. Forejt, J. Rubes eds, Cytogenetic Section of the Czech Biological Society, Brno): 115-124.

SOLA L., ROSSI A. R, ANESSI F., GORNUNG E. 2003. Cytogenetic studies in *Sparus auratus* (Pisces, Perciformes): Molecular organization of 5S rDNA and chromosomal mapping of 5S and 45S ribosomal genes and of telomeric repeats. Hereditas. **139**: 232-236.

SONNENBERG R., NOLTE A. W., TAUTZ D. 2007. An evaluation of LSU rDNA D1-D2 sequences for their use in species identification. Front. Zool. **4**: 6.

WITKOWSKI A., BŁACHUTA J. 1989. A natural hybrid *Leuciscus idu*s (L.) x *Leuciscus cephalus* (L.) from the Odra River, Poland. Zool. Abhand. Staatl. Mus. Tierk. (Dresden) **45**: 1-10.