

Comparative cytogenetics of two endangered leuciscine fish, *Squalius aradensis* and *S. torgalensis* (Teleostei, Cyprinidae), from the Iberian Peninsula

Catarina Nabais¹, Massimiliano Rampin^{1,2}, Maria João Collares-Pereira¹

1 Universidade de Lisboa, Faculdade de Ciências, Centro de Biologia Ambiental, Campo Grande, Lisboa, Portugal **2** Present address: University of South Bohemia in České Budějovice, Faculty of Fisheries and Protection of Waters, Research Institute of Fish Culture and Hydrobiology, Vodňany, Czech Republic

Corresponding author: Maria João Collares-Pereira (mjpereira@fc.ul.pt)

Academic editor: Petr Rab | Received 12 January 2013 | Accepted 14 February 2013 | Published 18 March 2013

Citation: Nabais C, Rampin M, Collares-Pereira MJ (2013) Comparative cytogenetics of two endangered leuciscine fish, *Squalius aradensis* and *S. torgalensis* (Teleostei, Cyprinidae), from the Iberian Peninsula. Comparative Cytogenetics 7(1): 33–42. doi: 10.3897/CompCytogen.v7i1.4672

Abstract

In this study, the description of the karyotypes of the endangered chubs *Squalius aradensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998) and *Squalius torgalensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998) is presented by means of conventional (Giemsa-staining, Chromomycin A₃ (CMA₃)-fluorescence, Silver-impregnation (Ag-NORs)) and molecular (fluorescence *in situ* hybridization (FISH) with 18S rDNA probe) protocols. These endemic sister-species have an allopatric but adjacent distribution in the most southwestern part of the Iberian Peninsula. Diploid chromosome number was invariably 2n = 50 and karyotypes of both species were grossly similar, composed of metacentric and sub-metacentric elements with a reduced number of acrocentric pairs. Sequential staining using FISH with an 18S rDNA probe, CMA₃ and Ag-NORs treatments revealed consistent positive signals located at the end of the short arms of a submetacentric chromosome pair, likely homologous in both species. While providing useful cytogenetic comparative data against other members of the genus *Squalius* Bonaparte, 1837, the work aimed to draw attention towards the conservation of two narrow-range and highly confined fish species.

Keywords

Leuciscinae, Cytotaxonomy, FISH with rDNA, NOR-phenotype, silver staining, chromomycin A₃

Introduction

The genus *Squalius* Bonaparte, 1837 belongs to the subfamily Leuciscinae, the major element of the Iberian cyprinid fauna. In Portuguese inland waters, four bisexual species with an allopatric distribution (Leunda et al. 2009) are recognized: *S. pyrenaicus* (Günther, 1868), in most drainages from the Tejo River basin southwards; *S. carolitertii* (Doadrio, 1988), from the most northern smaller drainages of the Atlantic slope to the Mondego River basin; *S. aradensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998), in the Seixe, Aljezur, Alvor, Arade and Quarteira River basins; and *S. torgalensis* (Coelho, Bogutskaya, Rodrigues and Collares-Pereira, 1998), only present in the southern Mira River basin.

Most chubs of the genus *Squalius* were formerly included in the genus *Leuciscus* Cuvier, 1816 (see Sanjur et al. 2003). The two species with a wider geographic distribution, *S. carolitertii* and *S. pyrenaicus* (also present in Spain), were the first to be recognized as different taxonomic units. However, some genetic studies (Coelho et al. 1995, Brito et al. 1997) hypothesized the existence of a more complex differentiation pattern in the most south-western Iberian populations. Two new species, with a restricted geographic range, were then described by Coelho et al. (1998), *S. aradensis* and *S. torgalensis*, mainly based on meristic, morphometric and osteological traits. Further studies using in-depth mitochondrial and nuclear markers have confirmed that *S. aradensis* and *S. torgalensis* are separated, but sister-taxa (see Doadrio and Carmona 2003, Sanjur et al. 2003, Mesquita et al. 2005, 2007, Almada and Sousa-Santos 2010, Perea et al. 2010, Waap et al. 2011).

Some cytogenetic data were recorded for populations from Arade, Aljezur and Bordeira drainages (currently assigned to *S. aradensis*) and Mira (currently assigned to *S. torgalensis*) (Collares Pereira et al. 1998), but previously to the acknowledgement that they should be considered distinct taxa from *S. pyrenaicus* (Coelho et al. 1998). Besides, Collares-Pereira et al. (1998) reported higher karyotype variability in chubs belonging to southern populations, when compared to the northern drainages. Therefore, the present work aimed to describe the karyotypes of *S. aradensis* and *S. torgalensis* using specimens recently collected. Conventional (Giemsa staining, GC-specific CMA₃ fluorescence and Ag impregnation) and molecular (rDNA major complex by FISH) cytogenetic techniques were used to characterize both species chromosome sets. Both endemics are “Critically Endangered” (Cabral et al. 2005) and besides an inherent heuristic nature, this work might draw attention towards the conservation of two narrow-range and highly confined fish species, while withdrawing useful cytogenetic comparative data against other members of the genus *Squalius*.

Material and methods

Adult specimens were captured by electrofishing in two distinct southern populations. Six *S. aradensis* (four males and two females) were collected in the Arade basin (Ode-

louca River) and two *S. torgalensis* (unknown sex) in the Mira basin (Torgal River). All procedures were developed in accordance to the recommended ethic guidelines (ASAB 2006). Some old chromosome images from material collected in the same drainages and studied by Collares-Pereira et al. (1998) were also reanalyzed (2 specimens of *S. aradensis* and 3 specimens of *S. torgalensis*) in order to confirm the results obtained with the new material.

Chromosome spreads were obtained for one specimen using standard kidney protocol and for the remaining using fin fibroblast cultures (Rodrigues and Collares-Pereira 1996) to avoid fish sacrifice. Chromosome spreads were obtained by conventional splashing and selected for further cytogenetic analysis.

Chromosomes were stained with a solution of 4% Giemsa (pH=6.8). CMA₃ fluorescence staining was performed according to Sola et al. (1992), with a slide pre-wash in McIlvaine/MgCl₂ buffer, one hour incubation with CMA₃ (Calbiochem) and Methyl green counterstaining. Ag-NORs detection followed Howell and Black (1980) with modifications (Gold and Ellison 1983), using Giemsa counterstaining. Whenever possible, slides were destained and used in sequential treatments.

The physical mapping of major rDNA gene cluster on the chromosomes was accomplished by FISH with rDNA probe. An rDNA clone containing 18S-5.8S-28S genes plus the intergenic spacers and untranscribed sequences from the genome of *Drosophila melanogaster* Meigen, 1830 (clone pDm 238, Rohia et al. 1981) was used as probe and labelled by nick translation with digoxigenin-11-dUTP according to the manufacturer's specifications (Roche Applied Science). The probe was resuspended in hybridization mix composed of 50% ultra-pure formamide pH=7.5 (Sigma-Aldrich), 2× SSC and 10% (w/v) dextran sulfate powder (Sigma-Aldrich). Slides were denatured in 70% formamide in 2× SSC at 65°C for 3 minutes, and immediately dehydrated in an ice-cold ethanol series (70%-90%-100%) for 7 minutes each and air-dried. The probe mixture was denatured at 75°C for 10 minutes, immediately placed on ice for another 10 minutes and added to the chromosome preparation. Hybridization was performed overnight in a dark moist chamber at 37°C. Post-hybridization washes were performed at room temperature (RT), for 7 minutes each: twice in 2× SSC and once in 2× SSC/0.1% Tween₂₀. Slides were incubated with 3% bovine serum albumin (BSA) for 30 minutes at 37°C, in a dark moist chamber. Anti-digoxigenin antibody conjugated with fluorescein isothiocyanate (FITC) (Roche Applied Science) was used to detect the probe for an hour and a half incubation at 37°C in a moist chamber. The slides were washed twice in 1x Phosphate Buffered Saline solution (PBS) at RT for 7 minutes and counterstained with DAPI in antifade solution.

Slides were screened in an Olympus BX 60 epifluorescence microscope equipped with a DP50 Olympus CCD camera. All images were processed using Adobe Photoshop CS4 software. Chromosomes were arranged in a decreasing size order and classified according to their arm ratios (Levan et al. 1964) in three categories: metacentric (m), submetacentric (sm) and sub-telocentric to acrocentric (st/a). To determine the fundamental number (NF value), chromosomes of the m and sm groups were considered biarmed and those of group st/a as uniarmed.

Results

All *S. aradensis* and *S. torgalensis* karyotypes revealed a diploid number of $2n = 50$ chromosomes.

Karyotypes of both *S. aradensis* and *S. torgalensis* are composed of five pairs of metacentric (m), eighteen pairs of submetacentric (sm) and two pairs of subtelo/acrocentric (st/a) chromosomes. As the general karyotypes are the same for both species, only the results for *S. aradensis* using giemsa staining were included in Fig. 1. This genome composition leads to a high fundamental number (NF=96). As regards the eventual occurrence of heteromorphic sex chromosomes, no clear distinction between male and female karyotypes was observed in *S. aradensis*, the species where the specimens' sex could be accurately assessed.

The NORs' phenotype was constant throughout all treatments, consistently positively labeling only one NORs-bearing chromosome pair likely homologous in both *S. aradensis* and *S. torgalensis* species. Their rDNA-positive signals were co-localized to CMA₃- and Ag-positive signals: in the short arm of a middle-size sm chromosome pair in both species as documented by sequential staining (Figs 2 and 3), indicative of being GC-rich and transcriptional active. No evidences of multi-chromosomal positive NOR's signals were registered.

Discussion

S. aradensis and *S. torgalensis* karyotypes display the general pattern described for most Leuciscinae: a diploid chromosome number of $2n = 50$, a chromosome set with mainly bi-armed elements (dominated by submetacentrics with some metacentrics), and only one NOR-bearing chromosome pair. In cyprinids this is assumed to correspond to the ancestral character state and most likely represents also the synapomorphy for the genus *Squalius* (Ráb and Collares-Pereira 1995). A large subtelocentric/acrocentric

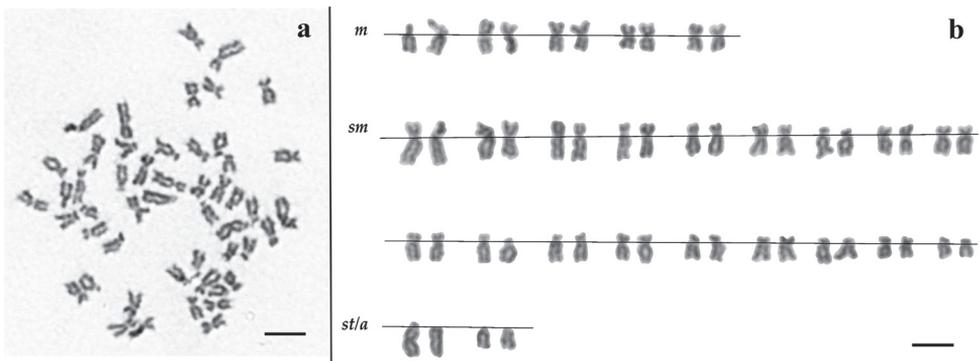


Figure 1. a Giemsa stained metaphase and **b** corresponding karyotype of a *S. aradensis* male from Odelouca River (Arade drainage). Scale bar = 10 μ m.

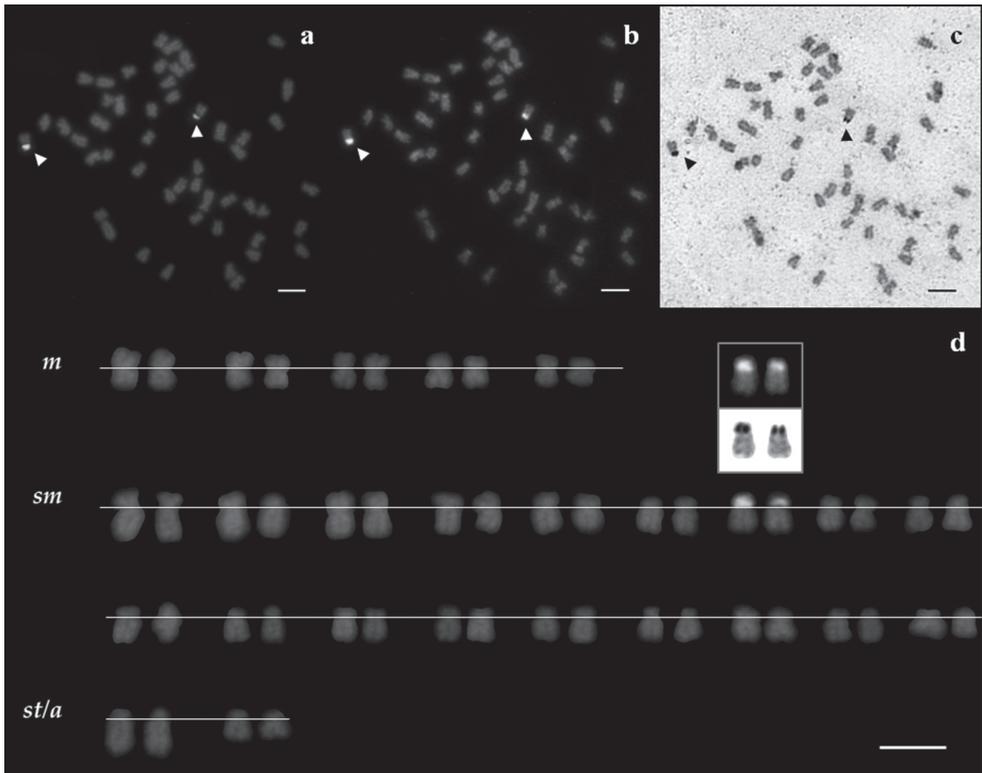


Figure 2. Metaphase spreads of a *S. aradensis* male, stained with sequential treatments **a** rDNA FISH **b** CMA₃-NOR, and **c** Ag-NOR treatments **d** corresponding karyotype of the same individual after rDNA FISH, with CMA₃- and Ag-NOR signals inset. NORs are indicated by arrowheads. Scale bar = 10µm.

pair previously considered a chromosome marker for the subfamily (Ráb and Collares Pereira 1995, Ráb et al. 2008) is also present in both species. The most common situation found in European Leuciscinae of a slightly higher number of *st/a* chromosomes might be due to the difficulty in obtaining high quality images for an accurate classification of chromosomes (Ráb and Collares-Pereira 1995, Collares-Pereira et al. 1998, Boron et al. 2009).

The number and location of rDNA gene clusters have been used as chromosome markers in fish cytotaxonomy (e.g. Rábová et al. 2003, Boron et al. 2009, Kirtiklis et al. 2010, Pereira et al. 2012, Rossi et al. 2012). Conversely to what was reported in *S. pyrenaicus* (Gromicho et al. 2005), no NORs polymorphism was observed in this study. Besides, Collares-Pereira et al. (1998) did not report any variation in NORs' phenotype in all the populations analyzed. Karyotypes of these species conserved the plesiomorphic condition, where leuciscines have, in general, only one NOR-bearing chromosome pair (Ráb and Collares-Pereira 1995, Bianco et al. 2004, Luca et al. 2010, Rossi et al. 2012). Multiple NORs sites were detected in some *S. pyrenaicus* individuals using 28S rDNA FISH mapping, presenting intra-individual variation and also failure in Ag-NOR in detecting most rDNA copies (Gromicho et al. 2005). However, those

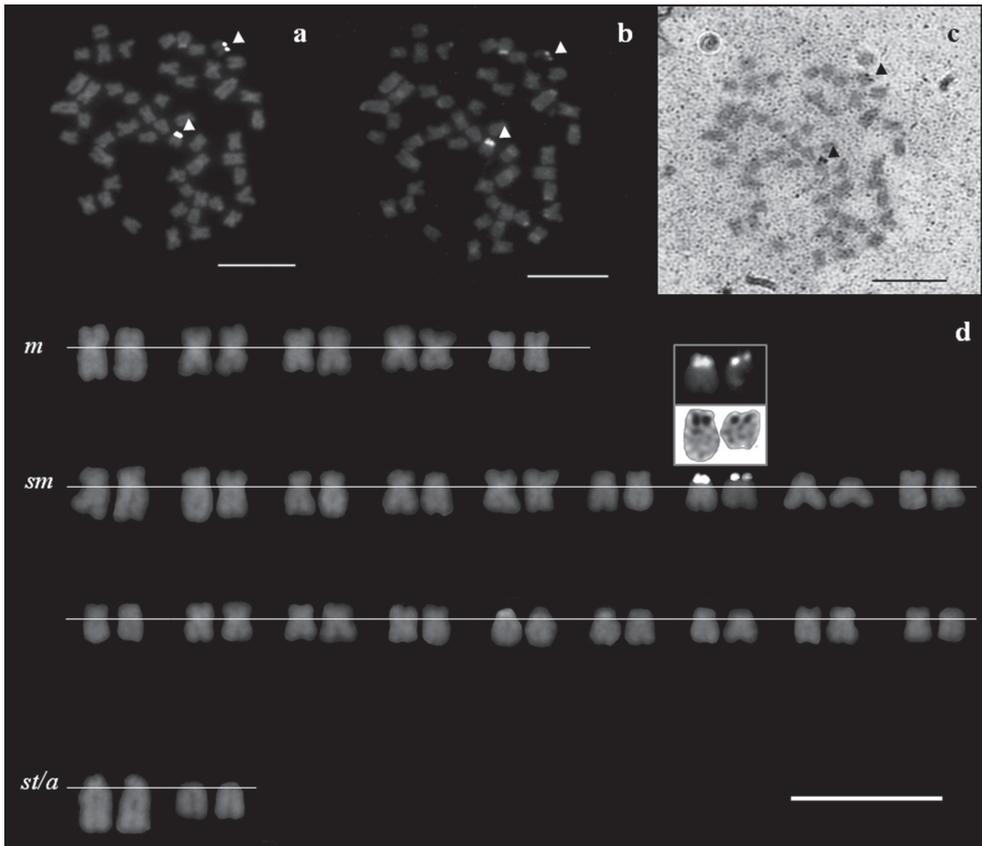


Figure 3. Metaphase spreads of a *S. torgalensis*, stained with sequential treatments **a** rDNA FISH **b** CMA₃-NOR, and **c** Ag-NOR treatments **d** corresponding karyotype of the same individual after rDNA FISH, with CMA₃- and Ag-NOR signals inset. NORs are indicated by arrowheads. Scale bar = 10µm.

observations were made in specimens living in sympatry with the *S. alburnoides* hybridogenetic complex (reviewed in Collares-Pereira and Coelho 2010) where hybridization is a recurrent process and might thus potentiate such variation in NORs.

The karyotypes of the two species proved to be grossly similar at a macrostructural level. They are sister-taxa strongly clustered in many phylogenetic analyses, however consistently reciprocally monophyletic (Almada and Sousa-Santos 2010, Waap et al. 2011). Given their differentiation estimated at 7-8 MY ago (Doadrio and Carmona 2003, Sousa-Santos et al. 2007, Almada and Sousa-Santos 2010), perhaps more subtle chromosome differences will be found when new cytogenetic tools with a higher resolution will be operating in cyprinid's chromosomes. The clear segregation of their lineage to the one including *S. pyrenaicus* and *S. carolitertii* is also consensual in phylogenetic analyses, and tree topology strongly supports them as basal members of the south-western Iberian *Squalius* lineage. Taking that into consideration, it is not surprising the conservation of the plesiomorphic state of the karyotype in both *S. aradensis*

and *S. torgalensis* but their differentiation to the other two species living in Portuguese inland waters, respectively with 10–12m + 30–32sm + 8st/a for *S. carolitertii*, and 12m + 32sm + 6st/a for *S. pyrenaicus* (Collares-Pereira et al. 1998).

Despite the apparent conservative pattern found in the two species here addressed by conventional cytogenetic tools, the karyotype variability present in the Iberian species of the genus *Squalius* so far analysed (Collares-Pereira et al. 1998, Gromicho et al. 2005), supports the occurrence of speciation processes favored by drastic changes in hydrological regimes (namely drought events), hence the difference between northern and southern populations. Whenever isolation and population bottlenecks occur, the sporadic mass reductions of population size might contribute to the stochastic fixation of chromosomal and genome mutations (Pereira et al. 2012). In particular, *S. torgalensis* is geographically confined to a single intermittent river system and characterized by a very low level of genetic diversity (see also Almada and Sousa-Santos 2010, Henriques et al. 2010). Thus specific conservation measures have to be adopted if the option will be to preserve the genome integrity of this highly vulnerable species.

Acknowledgements

We thank Carla Pereira for her help during lab work and the remaining CBA Evolutionary Genetics Group in particular Luis Moreira da Costa for assistance in the field, as well as AFN and ICNB for fishing permits. This research was funded by Centro de Biologia Ambiental [PEst-OE/BIA/UI0329/2011]. MR was supported by the FCT Post-Doctoral grant SFRH/BPD/48428/2008, and by projects RPP JU No. CZ.1.07/2.3.00/30.0049 and CENAKVA No. CZ.1.05/2.1.00/01.0024 since November 2012.

References

- Almada V, Sousa-Santos C (2010) Comparisons of the genetic structure of *Squalius* populations (Teleostei, Cyprinidae) from rivers with contrasting histories, drainage areas and climatic conditions based on two molecular markers. *Molecular Phylogenetics and Evolution* 57: 924–931. doi: 10.1016/j.ympev.2010.08.015
- ASAB (The Association for the Study of Animal Behaviour) (2006) Guidelines for the treatment of animals in behavioral research and teaching. *Animal Behaviour* 71: 245–253. doi: 10.1016/j.anbehav.2005.10.001
- Bianco PG, Aprea G, Balletto E, Capriglione T, Fulgione D, Odierna DG (2004) The karyology of the cyprinid genera *Scardinius* and *Rutilus* in southern Europe. *Ichthyological Research* 51: 274–278. doi: 10.1007/s10228-004-0221-y
- Boron 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. doi: 10.1007/s10709-008-9269-3

- Brito RM, Briolay J, Galtier N, Bouvet Y, Coelho MM (1997) Phylogenetic relationships within genus *Leuciscus* (Pisces, Cyprinidae) in Portuguese freshwaters, based on mitochondrial DNA cytochrome *b* sequences. *Molecular Phylogenetics and Evolution* 8: 435–442. doi: 10.1006/mpev.1997.0429
- Cabral MJ, Almeida J, Almeida PR, Dellinger T, Ferrand de Almeida N, Oliveira ME, Palmeirim JM, Queiroz AI, Rogado L, Santos-Reis M (2005) Livro Vermelho dos Vertebrados de Portugal. Instituto da Conservação da Natureza, Lisboa. [in Portuguese]
- Coelho MM, Bogutskaya N, Rodrigues JA, Collares-Pereira MJ (1998) *Leuciscus torgalensis* and *L. aradensis*, two new cyprinids for Portuguese freshwaters. *Journal of Fish Biology* 52: 937–950. doi: 10.1006/jfbi.1998.0642
- Coelho MM, Brito RM, Pacheco TR, Figueiredo D, Pires AM (1995) Genetic variation and divergence of *Leuciscus pyrenaicus* and *L. carolitertii* (Pisces, Cyprinidae). *Journal of Fish Biology* 47: 243–258. doi: 10.1111/j.1095-8649.1995.tb06059.x
- Collares-Pereira MJ, Coelho MM (2010) Reconfirming the hybrid origin and generic status of the Iberian cyprinid complex *Squalius alburnoides*. *Journal of Fish Biology* 76: 707–715. doi: 10.1111/j.1095-8649.2009.02460.x
- Collares-Pereira MJ, Prospero MI, Bileu RI, Rodrigues EM (1998) *Leuciscus* (Pisces, Cyprinidae) karyotypes: transect of Portuguese populations. *Genetics and Molecular Biology* 21: 63–69. doi: 10.1590/S1415-47571998000100011
- Doadrio I, Carmona JA (2003) Testing freshwater Lago Mare dispersal theory on the phylogeny relationships of Iberian cyprinid genera *Chondrostoma* and *Squalius* (Cypriniformes, Cyprinidae). *Graellsia* 59: 457–473. doi: 10.3989/graelisia.2003.v59.i2-3.260
- Gold JR, Ellison JR (1983) Silver staining for nucleolar organizing regions of vertebrate chromosomes. *Stain Technology* 58: 51–55.
- Gromicho M, Ozouf-Costaz C, Collares-Pereira MJ (2005) Lack of correspondence between CMA₃-, Ag-positive signals and 28S rDNA loci in two Iberian minnows (Teleostei, Cyprinidae) evidenced by sequential banding. *Cytogenetics and Genome Research* 109: 507–511. doi: 10.1159/000084211
- Henriques R, Sousa V, Coelho MM (2010) Migration patterns counteract seasonal isolation of *Squalius torgalensis*, a critically endangered freshwater fish inhabiting a typical Circum-Mediterranean small drainage. *Conservation Genetics* 11: 1859–1870. doi: 10.1007/s10592-010-0078-8
- Howell WM, Black DA (1980) Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. *Experientia* 36: 1014–1015. doi: 10.1007/BF01953855
- Kirtiklis L, Porycka K, Boroń A, Coutanceau JP, 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 Biologica (Krakow)* 58: 245–249. doi: 10.3409/fb58_3-4.245-249
- Leunda P, Elvira B, Ribeiro F, Miranda R, Oscoz J, Alves MJ, Collares-Pereira MJ (2009) International Standardization of Common Names for Iberian Endemic Freshwater Fishes. *Limnetica* 28: 189–202.

- Levan A, Fredga K, Sandberg AA (1964) Nomenclature for Centromeric Position on Chromosomes. *Hereditas* 52: 201–220. doi: 10.1111/j.1601-5223.1964.tb01953.x
- Luca C, Suciú R, Costache M (2010) Comparative karyotype in different lineages of cyprinid fish (Teleostei: Cypriniformes: Cyprinidae). *Studia Universitatis “Vasile Goldiș”, Seria Științele Vieții* 20: 37–41.
- Mesquita N, Cunha C, Carvalho GR, Coelho MM (2007) Comparative phylogeography of endemic cyprinids in the south-west Iberian Peninsula: evidence for a new ichthyogeographic area. *Journal of Fish Biology* 71: 45–75. doi: 10.1111/j.1095-8649.2007.01518.x
- Mesquita N, Hanfling B, Carvalho GR, Coelho MM (2005) Phylogeography of the cyprinid *Squalius aradensis* and implications for conservation of the endemic freshwater fauna of southern Portugal. *Molecular Ecology* 14: 1939–1954. doi: 10.1111/j.1365-294X.2005.02569.x
- Perea S, Böhme M, Zupančič P, Freyhof J, Šanda R, Özuluğ M, Abdoli A, Doadrio I (2010) Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. *BMC Evolutionary Biology* 10: 265. doi: 10.1186/1471-2148-10-265
- Pereira CS, Ráb P, Collares-Pereira MJ (2012) Chromosomes of European cyprinid fishes: comparative cytogenetics and chromosomal characteristics of ribosomal DNAs in nine Iberian chondrostomine species (Leuciscinae). *Genetica* 140: 485–495. doi: 10.1007/s10709-013-9697-6
- Ráb P, Collares-Pereira MJ (1995) Chromosomes of European cyprinid fishes (Cyprinidae, Cypriniformes): a review. *Folia Zoologica* 44: 193–214.
- Ráb P, Rábová M, Pereira CS, Collares-Pereira MJ, Pelikanová S (2008) Chromosome studies of European cyprinid fishes: interspecific homology of leuciscine cytotoxic marker, the largest subtelocentric chromosome pair, as revealed by cross-species painting. *Chromosome Research* 16: 863–873. doi: 10.1007/s10577-008-1245-3
- Rábová M, Ráb P, Ozouf-Costaz C, Ene C, Wanzebock J (2003) Comparative cytogenetics and chromosomal characteristics of ribosomal DNA in the fish genus *Vimba* (Cyprinidae). *Genetica* 118: 83–91. doi: 10.1023/A:1022965302614
- Rodrigues E, Collares-Pereira MJ (1996) NOR polymorphism in the Iberian species *Chondrostoma lusitanicum* (Pisces: Cyprinidae). *Genetica* 98: 56–63. doi: 10.1007/BF00120219
- Rohia H, Miller JR, Woods LC, Glover DM (1981) Arrangements and rearrangements of sequences flanking the two types of rDNA insertion in *D. melanogaster*. *Nature* 290: 749–753. doi: 10.1038/290749a0
- Rossi AR, Milana V, Hett AK, Tancioni L (2012) Molecular cytogenetic analysis of the Apennine endemic cyprinid fish *Squalius lucumonis* and three other Italian leuciscines using chromosome banding and FISH with rDNA probes. *Genetica* 140: 469–476. doi: 10.1007/s10709-012-9695-0
- Sanjur OI, Carmona JA, Doadrio I (2003) Molecular phylogeny of Iberian chub (genus *Squalius*, Cyprinidae), inferred from molecular data. *Molecular Phylogenetics and Evolution* 29: 20–30. doi: 10.1016/S1055-7903(03)00088-5
- Sola L, Rossi AR, Iaselli V, Rasch EM, Monaco PJ (1992) Cytogenetics of bisexual/unisexual species of *Poecilia*. II. Analysis of heterochromatin and nucleolar organizer regions in *Po-*

- ecilia mexicana mexicana* by C-banding and DAPI, quinacrine, chromomycin A3, and silver staining. Cytogenetics and Cell Genetics 60: 229–235. doi: 10.1159/000133346
- Sousa-Santos C, Collares-Pereira MJ, Almada V (2007) Reading the history of a hybrid fish complex from its molecular record. Molecular Phylogenetics and Evolution 45: 981–996. doi: 10.1016/j.ympev.2007.05.011
- Waap S, Amaral AR, Gomes B, Coelho MM (2011) Multi-locus species tree of the chub genus *Squalius* (Leuciscinae: Cyprinidae) from western Iberia: new insights into its evolutionary history. Genetica 139: 1009–1018. doi: 10.10107/s10709-011-9602-0