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Classical and molecular cytogenetic characterization of allochthonous European bitterling *Rhodeus amarus* (Cyprinidae, Acheilognathinae) from Northern Italy

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A cytogenetical study was carried out on 34 specimens of the European bitterling *Rhodeus amarus* (Teleostei: Cyprinidae, Acheilognathinae) from four rivers of the Venice district (NE Italy). This allochthonous fish species was accidentally introduced in the North-East of Italy about 20 years ago and is now rapidly spreading all over the rivers of the Northern part of the country. All the studied specimens are characterised by the same karyotype (2n = 48: 8M + 20SM + 20ST), i.e., the typical one of the native populations of the species. However, a polymorphism in the number of NOR bearing chromosomes has been found. In fact, in addition to the main species-specific NORs, on the short arms of chromosome pair 7, two to five additional 18S rDNA sites have been revealed by FISH in different specimens. Sequential staining with silver nitrate, chromomycin A_3 and DAPI revealed that most of the additional sites are inactive and CMA_3-positive.

Data herein reported confirm that in spite of an overall morphological karyological conservativeness, significant differences for the finer cytogenetic features can be found within the Acheilognathinae with the 2n = 48 and NF = 76 karyotype.

Key words: freshwater fish conservation, karyotype polymorphism, fluorescence *in situ* hybridization, rDNA

INTRODUCTION

Bitterlings are freshwater fish species ascribed to the sub-family Acheilognathinae (Cyprinidae). The taxonomy of this group has long been debated, a fact reflected in the variation over time of the number of valid genera, from one to six, and the synonymy of many species. Recently, three valid genera, *Acheilognathus*, *Rhodeus,* and *Tanakia* (Nelson, 2006; see Okazaki et al., 2001), grouping approximately 60 species or subspecies (Froese and Pauly, 2008), have been recognized. All bitterlings are characterized by peculiar reproductive behavior which involves egg and sperm deposition in the mantle cavity of unionoid freshwater mussels. Acheilognathinae are distributed throughout Eurasia, and more widely in East Asia.

Rhodeus is the only bitterling genus distributed in Europe, from the Neva basin, in Russia, to the Rhone basin, in France, and southward to the Black Sea. The European populations have a long and controversial taxonomic history (revised in Holčík, 1999). In fact, they have been considered as belonging either to a valid species, *Rhodeus amarus* (Bloch, 1782) and distinct from the morphologically similar eastern Asian *R. sericeus* (Pallas, 1776), inhabiting the Amur basin, or to a western subspecies of this latter, *R. sericeus amarus.* According to Holčík (1999), based on morphological, morphometric and karyological data, as well as on their similar ecology, "ssp *amarus* should be synonymized with the nomynotypic form, *sericeus*". More recently, molecular data, based on the mitochondrial cytochrome b gene sequence (Bohlen et al., 2006), identified five clades, corresponding to four species. The east Asian lineage corresponds to *R. sericeus* and is the sister clade to the four remaining

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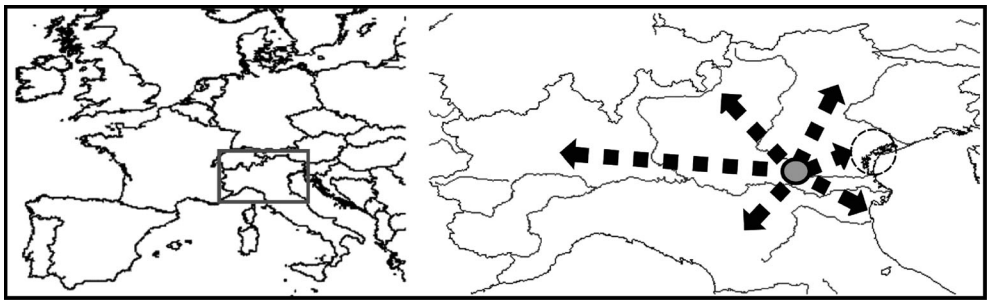

Fig. 1. Site (grey circle) of the first record of *Rhodeus amarus* in Italy and invasion routes through the whole of Northern Italy. The white circle includes all the five collecting sites of the specimens analyzed in this study.

ones, represented by all bitterlings from the Euro-Mediterranean zoogeographic sub-region. These include two *R. amarus* clades, one from Central and one from Eastern Europe, and *R. meridionalis* and *R. colchicus,* respectively from Greece and the Caucasus. However, the taxonomy of these latter, and of other putative bitterling species, has yet to be elucidated. Therefore, Van Damme et al. (2007) suggested considering bitterlings from Europe and Asia Minor as representative of the single taxon *R. amarus*, which is the nomenclature adopted in this study.

R. amarus is native over much of its present range in Europe (Van Damme et al., 2007), although in western Europe its presence is due to the increase in carp cultivation during the middle ages. In Italy, this species has been accidentally introduced as a consequence of mixed cyprinid stock importation, for restocking purposes, from Eastern European countries (Confortini, 1990; Bianco and Ketmaier, 2001; Confortini et al., 2005). The first record in Italian waters dates to 1990 (Confortini, 1990) in the Verona District, Northeast of Italy. In less than 20 years, from the original area of introduction, the invasion has continued westward to the whole Po River Basin, and northward to the South Tyrol alpine lakes (Ruffo and Stoch, 2003) (Fig. 1).

In the present study, a cytogenetic survey of Italian samples of *R. amarus* from the Venice district was carried out, aimed at investigating some karyological features. In fact, very few and limited cytogenetic data (Sofradzija et al., 1975; Bozhko et al., 1976; Hafez et al., 1976; Meszaros and Kato, 1976) are available for European bitterlings from presumptive native sites. There are absolutely no data on the heterochromatin distribution and composition and on the major ribosomal gene locations, both for native and introduced populations. The identification of possible chromosomal markers could be helpful in the comparison of the two lineages of the species from Central and Eastern Europe (Bohlen et al., 2006) and might be helpful in tracing the origin of the Italian populations. Moreover, as the phylogeny based on 12S sequences (Okazaki et al., 2001) is congruent with chromosome numbers in identifying two groups of species within the *Rhodeus* genus, the use of chromosome markers other than the standard Giemsa karyotype could cast more light on the relationships among the congeneric species and clarify the phyletic diversification within the subfamily.

MATERIALS AND METHODS

A total of 34 adult fishes, 14 males and 20 females were collected from the rivers Rio-Cimetto (N = 8), Marzenego (N = 3), Dese (N = 20) and Braganziolo (N = 3) in the Venice district (Fig. 1, white circle). Chromosome preparations were obtained using conventional air-drying techniques from cephalic kidney cells. Metaphases were analysed by sequential staining with chromomycin A_3 (CMA_3) (Schweizer, 1976), 4',6-diamidino-2-phenylindole

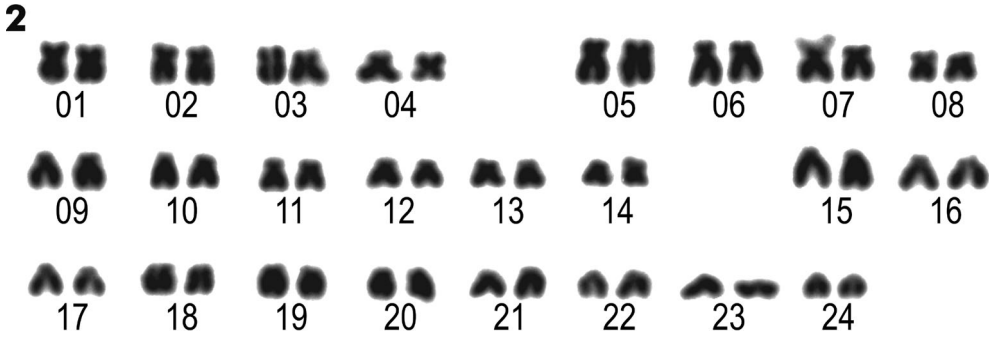

Fig. 2. Giemsa stained karyotype of a female *R. amarus*.

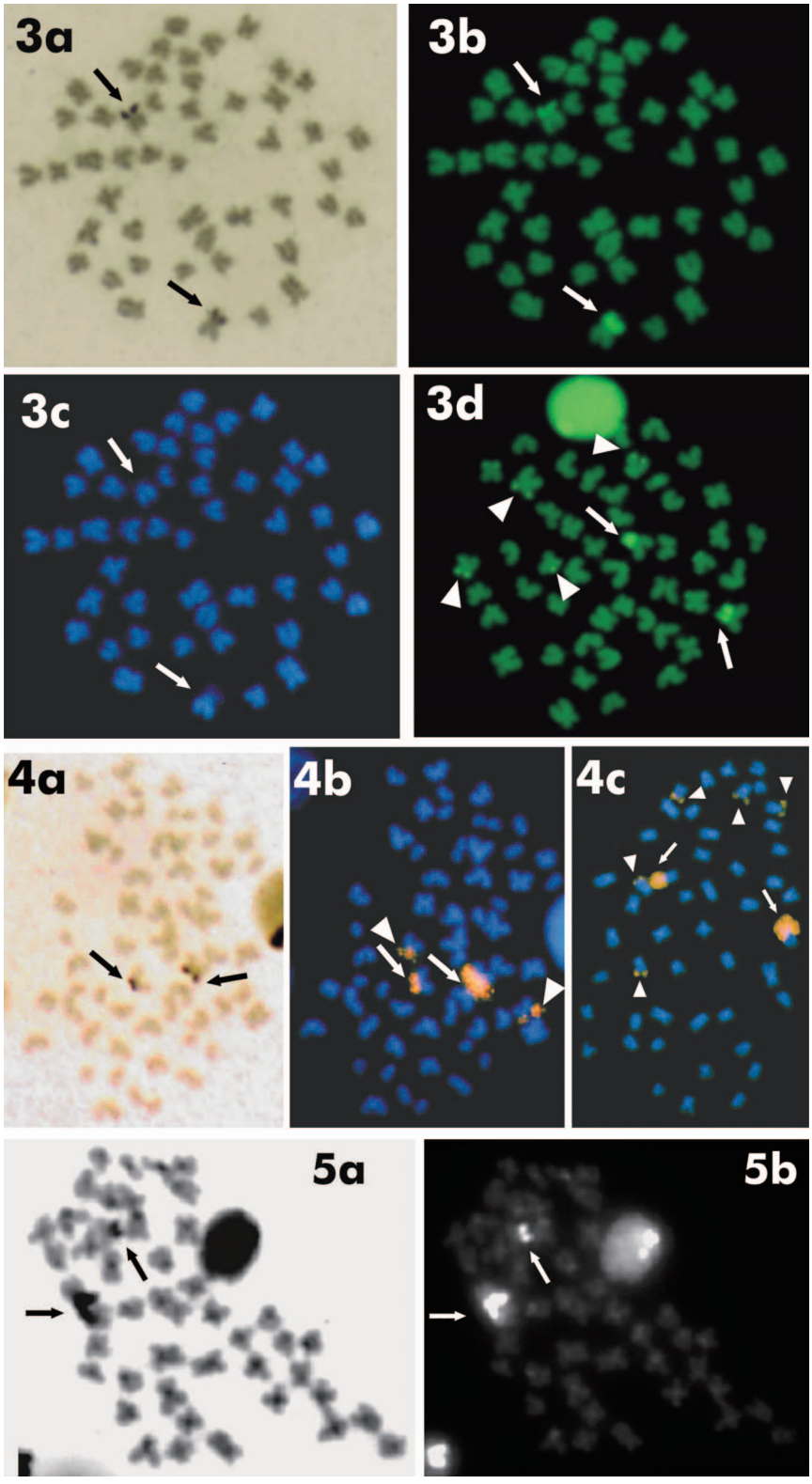

Fig. 3. Metaphase plate sequentially stained with (a) silver nitrate, (b) chromomycin A_3, (c) DAPI. Ag-NORs correspond to CMA_3-positive sites, negatively stained with DAPI (arrows); (d) metaphase plate stained with CMA_3 showing 2 main (arrows) and 4 additional positive sites (arrowheads). **Fig. 4.** Metaphase plate after sequential (a) Ag-staining and (b) FISH with a 18S rDNA probe; (c) metaphase plate after FISH with a 18S rDNA probe showing 7 positive sites. The two main NORs are indicated by arrows, smaller additional NORs by arrowheads. **Fig. 5.** Metaphase plate after sequential (a) C-banding and (b) CMA_3 staining. Arrows indicate the large C- and CMA_3-positive regions, corresponding to the two main NORs.

(DAPI) (Schweizer, 1976), and silver nitrate (Howell and Black, 1980). C-banding of chromosomes was obtained following the method of Sumner (1972). Staining with CMA_3 was sequentially applied to C-banded plates.

Mapping of 18S ribosomal genes was performed by means of fluorescence *in situ* hybridization (FISH) with a probe containing 18S-5.8S-28S genes plus an intergenic spacer of the fruit fly *Drosophila melanogaster* (pDm238) (Roiha et al., 1981). The probe was labelled by nick translation with digoxigenin-11-dUTP or biotin-14-dATP (Roche Molecular Biochemicals or Invitrogen). Chromosomes were denatured for 4 min in 70% formamide/2X SSC at 69°C. Denaturation of the probe was performed for 10 min at 75°C. Hybridization was allowed to proceed overnight at 37°C. Slides were washed three times in 50% formamide/2X SSC at 40°C (5 min each), twice (5 min) in 2X SSC at room temperature (RT), once (5 min) in 2X SSC/0.1% Tween20 at room temperature (RT) and finally, once (5 min) in PBS/0.1% Tween/0.5% skimmed milk powder at RT. Hybridization signals were detected with FITC-conjugated-antidigoxigenin (Roche Molecular Biochemicals) or Cy3-conjugated extravidin (Sigma). Slides were mounted in AF1 antifade solution (Citifluor) containing 2 ìg/ml DAPI (4,6-diamidino-2-phenylindole).

Observations were made with a JenaMed2 fluorescence microscope (Carl Zeiss Jena, Germany) equipped with the 410/450 and the 510/570 filter sets. Normal light and fluorescence images were taken with a Canon EOS 10D digital camera, and elaborated and merged with Adobe Photoshop Elements 2.0.

RESULTS

All the analyzed specimens, regardless of sex and collecting site, share the same 2n = 48 karyotype (Fig. 2), composed of 8 metacentric (M, pairs 1–4), 20 submetacentric (SM, pairs 5–14) and 20 subtelocentric (ST, pairs 15–24) chromosomes. The short arms of the submetacentric chromosome pair number 7 appear often decondensed and heteromorphic in size.

As would be expected from Giemsa staining, after silver staining (Fig. 3a) Ag-NORs were found to be located on the short arms of chromosome pair number 7. All individuals showed a maximum of two Ag-NORs, with the exception of two individuals which showed additional Ag-positive signals on the short arms of a single subtelocentric chromosome (data not shown).

Sequential staining with CMA_3 (Fig. 3b) produced overlapping bright signals in the same Ag-NORs location. The CMA_3-positive chromosome regions were negatively stained with DAPI (Fig. 3c). In 11 out of 22 specimens investigated with this technique, additional, but smaller, CMA_3 positive signals, for a maximum of six positive sites, generally located on the short arms (but one, in a telomeric position) of submeta/subtelocentric chromosomes were also observed (Fig. 3d). Apart from these sites, the GC-specific CMA_3 produced a uniform staining pattern along chromosomal arms, as did the AT-specific DAPI (Fig. 3c).

In situ hybridization with the 18S rDNA probe evidenced a variable number of fluorescent signals. In all the individuals, two large signals (Fig. 4b), corresponding to the two Ag-NORs (Fig. 4a), were always present and often heteromorphic, on the short arms of chromosome pair number 7. Additional smaller sites for the major ribosomal genes were also detected, in varying numbers among different individuals, probably corresponding to the additional CMA_3-positive sites, so that up to four (Fig. 4b) or seven hybridization signals (Fig. 4c) were observed.

C-banding showed a pericentromeric distribution of the constitutive heterochromatin in all chromosomes as well as on the short arms of some submeta-subtelocentric chromosomes. Two very large, often heteromorphic, heterochromatic blocks (Fig. 5a) can be observed on the short arms of two submetacentrics, corresponding to the Ag-NOR bearing chromosome pair, as confirmed by sequential staining with CMA_3 (Fig. 5b).

DISCUSSION

The analyzed specimens of *Rhodeus amarus* show the general cyprinid karyotype, characterized by a relatively high number of biarmed (meta- and submetacentrics) compared to uniarmed (subtelo- and acrocentrics) chromosomes (Klinkhardt et al., 1995). The chromosome number of 2n = 48 observed in the allochthonous Italian samples corresponds to data reported in literature (Bozhko et al., 1976; Meszaros and Kato, 1976; Sofradzija et al., 1975; Hafez et al., 1976) for specimens collected in the native distribution range, though the reported fundamental number is different in different studies, ranging from NF = 76 (present study) to NF = 86 (Hafez et al., 1976). However, these discrepancies are probably due to a different chromosome classification in different studies, more than to real differences in the chromosome complement, as the borderline between metacentric or submetacentric or subtelocentric chromosomes is influenced by both the subjectivity of the observer and by the degree of condensation of the chromosomes.

The karyotype of *R. amarus* displays, therefore, the general morphology shown by all the species of *Rhodeus* with the 2n = 48 karyotype, which has been suggested as the basal karyotype in Acheilognathinae (Arai and Akai, 1988), from which the different chromosome formulae observed in other species of this taxon (*Rhodeus* species with 2n = 46 and FN = 50; *Acheilognathus* species with 2n from 42 to 44, and FN from 70 to 74) derived, mainly through pericentric inversions and centric fusions (Ueda et al., 2001). Consistent with this picture, molecular data based on the nucleotide sequences of the mitochon-

drial 12S ribosomal RNA gene, by Okazaki et al. (2001), identified two main clades, the monophyletic *Acheilognathus* and the *Tanakia-Rhodeus* clade. Within this latter, a monophyletic group, corresponding to the group of *Rhodeus* species with the diploid chromosome number of 46, was also identified.

By comparing the cytogenetic features of *R. amarus* to those shown by the other *Rhodeus* species with the same chromosome complement, literature data on the NORs number and location are available mainly from Ag-staining. Nevertheless, through the use of this staining technique, a great amount of variation in the number and location of the Ag-positive sites has been observed in the five species investigated, delineating structural chromosome interspecific differences which certainly merit further investigation.

Indeed, the maximum number of the Ag-positive sites is 2 in *R. ocellatus ocellatus* (Ueda et al., 2001) and in *R. ocellatus kurumeus* (Sola et al., 2003), 3 in *R. amarus* (present study), 5 in *R. lighti* (Ueda et al., 1997) but can reach 15 in *R. sinensis* (Ueda et al., 2001). *R. ocellatus kurumeus* (Sola et al., 2003) and *R. amarus* (present study) were investigated for NORs also through CMA_3-staining and FISH with 18S rDNA, and while in the first species, the unique location of NORs on a single chromosome pair was confirmed, in the latter additional sites, up to six, though of smaller sizes, were detected. Thus, though recent evidence in some species, including fish (Gromicho et al., 2005), have cast doubt upon the accuracy of Ag (and CMA_3) in NORs detection, data obtained with all staining techniques for NORs in *R. amarus*, along with the whole data on the Ag-NORs in the *Rhodeus* species with 2n = 48, seem to indicate that NORs variability is a common feature in these species. Such variability is also shared by the other species of the *Rhodeus-Tanakia* clade (Sola et al., 2003; Takai and Ojima, 1986; Ueda, 2007; Ueda et al., 1996, 2001, 2006), suggesting that these chromosome complements may have intrinsic and structural bases that make them prone to ribosomal gene rearrangements.

In the same five *Rhodeus* species with 2n = 48, differences were also detected in the NOR location. In fact, the large NORs present on the short arms of chromosome pair 7 in all specimens of *R. amarus* (present study) identify this pair as the species-specific one, and this pair is certainly different from the only NOR-bearing chromosome pair observed in *R. ocellatus kurumeus* (Sola et al., 2003) and in *R. ocellatus ocellatus* (Ueda et al., 2001). Indeed, in these latter species NORs are located on the short arms of two subtelocentrics which constitute the smallest chromosome pair in the complement. However, the NOR bearing chromosome pair observed in *R. amarus* might be homeologous to one of the two chromosome pairs showing Ag-positive signals in *R. lighti* (Ueda et al., 1997), as well as to one pair among the 15 chromosomes showing Ag-positive signals in *R. sinensis* (Ueda et al., 2001).

The constitutive heterochromatin distribution, as identified by C-banding in *R. amarus*, shows a pattern corresponding to the general one reported for the other species of the genus studied with this banding technique, *R. ocellatus kurumeus* (Sola et al., 2003), *R. sinensis* and *R. ocellatus ocellatus* (Ueda et al., 2001), though a population from South Korea of this latter species shows additional interstitial C-bands in three subtelocentric chromosome pairs. In *R. amarus,* the pericentromeric heterochromatin appears to be present in a more limited amount, compared to the large heterochromatic centromeric blocks detected on some metacentric chromosomes in other Acheilognathinae species, such as *Tanakia koreensis* and *T. signifer* (Ueda et al., 2001). In *R. amarus*, as well as in *R. ocellatus kurumeus* (Sola et al., 2003) constitutive GC-rich heterochromatin appears to be interspersed with ribosomal genes, as demonstrated by the positive signals obtained after C-banding and CMA_3-staining, on the NOR location of the main NOR-bearing chromosome pair. As far as the constitutive heterochromatin composition is concerned, differentially GC- or AT-enriched repetitive DNA cannot be identified along the chromosomes of *R. amarus*. Similar results have also been obtained in *R. ocellatus kurumeus* (Sola et al., 2003). In spite of the overall similarity of the constitutive heterochromatin distribution and composition, one must consider that rapid changes in copy number of repetitive elements occurred during the diversification of *Rhodeus* bitterlings (Saitoh et al., 2000).

In summary, the data here reported for the introduced European bitterling *R. amarus* confirms that in spite of an overall morphological karyological conservativeness, significant differences for the finer cytogenetic features can be found within the Acheilognathinae with the 2n = 48 and NF = 76 karyotype. The results obtained in *R. amarus* are therefore of considerable interest and further karyological studies should be carried out on other populations of the wide species range as well as on the remaining undescribed species of bitterlings, in order to provide a more general picture of karyoevolutive trends in Acheilognathinae.

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