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Multiple origins of polyploidy in the phylogeny of southern African barbs (Cyprinidae) as inferred from mtDNA markers

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The cyprinid genus *Barbus*, with more than 800 nominal species, is an apparently polyphyletic assemblage to which a number of unrelated species, groups and/or assemblages have been assigned. It includes species that exhibit three different ploidy levels: diploid, tetraploid and hexaploid. Several lineages of the family Cyprinidae constitute a major component of the African freshwater ichthyofauna, having about 500 species, and fishes assigned to the genus *'Barbus'* have the most species on the continent. We used complete sequences of the mitochondrial cytochrome *b* gene in order to infer phylogenetic relationships between diploid, tetraploid and hexaploid species of *'Barbus'* occurring in southern Africa, the only region where representatives of all of the three ploidy levels occur. The results indicate that most of the lineages are incorrectly classified in the genus *'Barbus'*. The southern African tetraploids probably originated from southern African diploids. They constitute a monophyletic group distinct from tetraploids occurring in the Euro-Mediterranean region (*Barbus sensu stricto*). The 'small' African diploid species seem to be paraphyletic, while the 'large' African hexaploid barbs species are of a single, recent origin and form a monophyletic group. The evidence of multiple, independent origins of polyploidy occurring in the African cyprinine cyprinids thus provides a significant contribution to the knowledge on the systematic diversity of these fishes, and warrants a thorough taxonomic reorganization of the genus.
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Introduction

Polyploidy is considered as an important evolutionary mechanism for diversification and speciation in eukaryotes (Ohno, 1970; Soltis and Soltis, 1999). While polyploidy by means of hybridization and/or genome duplication has been documented to be an extremely significant evolutionary mechanism by botanists, zoologists have not seriously considered its importance as an evolutionary process, mostly due to the lack of relevant data (Dowling and Secor, 1997; Otto and Whitton, 2000). The relative paucity of polyploidy in animals has been observed and first explained by its interference in sex determination (Müller, 1925) and more precisely in sex ratio (Basolo, 1994). For a review of additional factors contributing to the rarity of polyploidy in animals see Otto and Whitton (2000).

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Nevertheless polyploidy, when established in the past (generally several million years ago), obviously gives the concerned lineage an added impetus in terms of speciation and dispersion. In fish in particular, the evolutionary success of groups such as catostomids (Buth, 1983), salmonids (Allendorf and Thorgaard, 1984) and *Barbus* (Berrebi *et al*, 1996) can be understood as a consequence of ancestral polyploidization. Polyploids are considered to have more tolerance to ecological variation because the duplication of their genes provides metabolic flexibility (Uyeno and Smith, 1972; Otto and Whitton, 2000).

In fish, published data-sets have shown, however, that bisexual and/or unisexual polyploids occur in representatives of several fish orders: Acipenseriformes, Salmoniformes, several families of ostariophysan orders Cypriniformes (Cyprinidae, Catostomidae, Cobitidae) Siluriformes (Callichthyidae), Poeciliiformes (Poeciliidae) and Atheriniformes (Vasil'ev, 1985; Klinkhardt *et al*, 1995). One of the vertebrate groups where polyploidy mechanisms have undoubtedly played a conspicuously significant role during its evolution is the family Cyprinidae (Ohno *et al*, 1967).

The family Cyprinidae with more than 2000 species (Nelson, 1994) is the most abundant and widespread of all primary freshwater fish families across Europe, Asia, Africa and North America. The cyprinid genus *Barbus*, with more than 800 nominal species, is an apparently polyphyletic assemblage to which a number of unrelated

species, groups and/or assemblages have been assigned (Myers, 1960). In Africa, the fishes classified into the genus '*Barbus*' are ubiquitous with more than 300 species (Lévêque and Daget, 1984; Skelton, 1988; Skelton *et al*, 1991), distributed in all the African ichthyologic provinces (Roberts, 1975). The taxonomy of these fishes, especially in Africa is still muddled (Skelton, 1988; Berrebi *et al*, 1996) because the morphological characteristics that have been used to group the species have very low systematic value. For example, the number of pairs of barbels and the presence/absence of ossified and/or serrated rays in the dorsal fin were considered as discriminant characters, but in fact, they do not allow these species to be classified into proper, systematically well-defined genera (Howes, 1987, 1991; Banarescu, 1992; Berrebi *et al*, 1996).

Recently, ploidy level has been proposed as a new key character for a reorganization of such a large group (Berrebi *et al*, 1996). Until now, three ploidy levels were recognized in African species of cyprinine cyprinids (sensu Howes, 1991): diploid (about 50 chromosomes), tetraploid (about 100 chromosomes), and hexaploid (about 150 chromosomes) (Agnèse *et al*, 1990; Berrebi *et al*, 1990, 1996; Oellerman and Skelton, 1990; Golubtsov and Krysanov, 1993; Guégan *et al*, 1995; Ráb *et al*, 1995; Naran, 1997).

The only non-Mediterranean African tetraploid species with the chromosome numbers $2n = 100$ were recently identified through analyses of karyotypes among a number of endemic barb species, also classified into the genus '*Barbus*' (Naran, 1997) in the southern African region. This discovery naturally poses the question of the mutual phylogenetic relationships with another tetraploid group of barbs inhabiting the Euro-Mediterranean region. The tetraploid status of the most widely distributed European species *B. barbus* was first reported by Ohno *et al* (1967). Recent studies (Ráb and Collares-Pereira, 1995; Ráb *et al*, 1996) have suggested that this species is a member of a monophyletic lineage distributed mainly in Europe and in adjacent regions of North-western Africa, the Middle East and Caspian/Aral Seas (Berrebi, 1995; Berrebi *et al*, 1996). This hypothesis, initially based on morphological characters (see Howes, 1987; Berrebi *et al*, 1996), was then supported using allozyme studies (El Gharbi, 1994; Machordom *et al*, 1995; Tsigenopoulos *et al*, 1999), karyotype structures (Ráb and Collares-Pereira, 1995; Ráb *et al*, 1996) and mtDNA sequences (Zardoya and Doadrio, 1999; Zardoya *et al*, 1999; Tsigenopoulos and Berrebi, 2000; Kotlik and Berrebi, 2001). However, the phylogenetic relationship between these two tetraploid groups (peri-Mediterranean and south-African) presently still classified in the genus *Barbus* is not resolved.

The large geographical gap in the distribution of both tetraploid groups of barbs may be the result of (1) two independent tetraploidization events, (2) a zone of extinction of phylogenetically linking forms in a broad geographical area suggesting one common tetraploidization event, or (3) simply the absence of relevant data for species occurring between the two regions. In the present paper, we use mitochondrial DNA (mtDNA) sequences to test the above hypotheses as a means of inferring phylogenetic relationships of selected species classified in the genus *Barbus* with different ploidy levels.

Materials and methods

Taxa examined

Table 1 shows the taxa used in the analysis, the collection sites and the chromosome numbers (when known). The fish were collected by electrofishing or nets. Figure 1 shows the localities of a total of 47 selected cyprinid species from Europe, Africa and Asia. The complete cytochrome *b* sequences of already karyotyped Euro–Mediterranean species of *Barbus* (Tsigenopoulos and Berrebi, 2000) as well as other cyprinid species recovered from the GenBank/EMBL (see accession numbers in brackets in Table 1) were included. In order to check variability in each species and to test for misidentifications due to PCR-generated errors, at least three individuals per taxon were analyzed when available. Phylogenetic trees were rooted using sequences of three species from the closely related cypriniform family Catostomidae.

According to Berrebi *et al* (1996), only the species belonging to the genus *Barbus sensu stricto* are true *Barbus*, including the European and peri-Mediterranean tetraploid species. All the *Barbus sensu lato* species still currently called *Barbus* have to be called '*Barbus*' (in single quotation marks) until a taxonomic analysis elucidates their systematic position.

DNA extraction, polymerase chain reaction (PCR) amplification and sequencing

Samples were either muscle preserved in 70% ethanol or frozen (at –80°C) protein extracts, which were already available in the Montpellier laboratory from previous allozyme studies (Agnèse *et al*, 1990; Berrebi *et al*, 1990).

DNA extraction followed the Sambrook *et al* (1989) method adapted in Tsigenopoulos and Berrebi (2000).

For PCR amplifications we used primers L15267 (5′-AAT GAC TTG AAG AAC CAC CGT-3′), L15803 (5′-TGA GGG GGA TTT TCA GTA GA-3′), H15891 (5′-GTT TGA TCC CGT TTC GTG TA-3′) and H16461 (5′-CTT CGG ATT ACA AGA CC-34) (Briolay *et al*, 1998). The L15803 primer was slightly modified from the original definition. Numbers refer to the position of the 3′ end of the primers in the complete mitochondrial DNA sequence of the carp *Cyprinus carpio* (GenBank accession no. X61010). The use of L15267 and H15891 in the PCR reactions resulted in the amplification of a 664 base pair (bp) fragment of the cytochrome *b* gene. Primers L15803 and H16461 were used for amplification and sequencing of the second half of the gene corresponding to 693 bp and were undertaken to recover complete cytochrome *b* sequences (1140 bp in total) only for the dominant haplotype found in each population or species.

The volume of each PCR was 50 μl and consisted of 1.8 mM MgCl2, 1 μM of each primer, 0.18 mM of each dNTP, 1 unit of Taq polymerase (Promega) and 1 × of amplification buffer (Promega).

Amplification conditions (for both pairs of primers) involved a first denaturation at 94°C for 2 min, annealing at 50°C for 1 min, extension at 71°C for 1 min, and then 30 cycles of denaturation at 94°C, annealing at 50°C for 30 sec and extension at 71°C for 40 sec (followed by a final extension at 71°C for 1 min). For visualization, 5 μl of each PCR product were electrophoresed on a 0.8% agarose gel and stained with ethidium bromide. The remaining amplified product was purified using Glassmilk silica matrix of the Gene-Clean III kit (following the manu-

Table 1 Species included in the analysis, their chromosome number (when known), sampling localities, and GenBank accession numbers (between brackets = already published sequences)

Species	*Cromosome No.*	*Locality*	*Map code*	*GenBank No.*
'barbus' anoplus	2*n* = 48–50	Buffalo R, South Africa	1	AF112405
'B'. trimaculatus	2*n* = 48	Letaba R, S Africa	2	AF180839
'B'. mattozi	*2*n* = 50	Zambesi R, Mozambique	3	AF180838
'B'. macrops	2*n* = 50	Tinkisso R (Niger Basin), Guinea	4	AF180832
'B'. guineensis	*2*n* = 50	Konkoure R, Guinea	5	AF180833
'B'. cadenati	*2*n* = 50	Pampana (Jong) R, Sierra Leone	6	AF180834
'B'. ablabes	2*n* = 50	Agnebi R, Ivory Coast	7	AF180835
'B'. sublineatus	*2*n* = 50	Mazan R, Comoé, Ivory Coast	8	AF180837
'B'. kerstenii	*2*n* = 50	Sagana R, Kenya	9	AF180840
'B'. nyanzae	*2*n* = 50	Oloibortoto R, Nyiro System, Kenya	9	AF180841
'B'. sp	*2*n* = 50	Baringo Lake System, Kenya	9	AF180842
'B'. andrewi	2*n* = 100	Breede R, S Africa	10	AF180843
'B'. serra	2*n* = 100	Stellenbosch, S Africa	10	AF180844
'B'. erubescens	2*n* = 100	Twee R, S Africa	10	AF180845
'B'. calidus	2*n* = 100	Noordhoeks R, S Africa	10	AF180846
'B'. trevelyani	2*n* = 100	Buffalo R, S Africa	1	AF180847
Pseudobarbus burchelli	2*n* = 100	Breede R, S Africa	10	AF180848
P. afer	2*n* = 100	Blindekloof R, S Africa	11	AF180851
P. asper	2*n* = 100	Groot R, S Africa	12	AF180850
P. burgi	2*n* = 100	Berg R, Tulbagh, S Africa	13	AF180849
Barbus bocagei	2*n* = 100	Jerte R, Spain	14	(AF112125)
B. meridionalis	2*n* = 100	Aubaygue R, France	15	(AF112130)
B. barbus	2*n* = 100	Drôme R, France	15	(AF112123)
B. cyclolepis cyclolepis	2*n* = 100	Evros (Maritza) R, Greece	16	(AF237579)
Aulopyge huegelii	2*n* = 100	Krka R, Croatia	17	(AF112133)
'B'. marequensis	2*n* = 150	Tzanee Lake, S Africa	2	AF180830
'B'. polylepis	2*n* = 150	Incomati R, S Africa	2	AF180877
'B'. capensis	2*n* = 150	Olifants R, S Africa	10	AF180831
'B'. bynni occidentalis	2*n* = 148	Bafing R (Senegal Basin), Guinea	5	AF180829
'B'. ethiopicus	2*n* = 150	Tana Lake, Ethiopia	18	AF180828
'B'. intermedius	2*n* = 150	Tana Lake, Ethiopia	18	AF145948
'B'. reinii	–	Tensift R, Morocco	19	AF145946
'B'. luteus	–	Tigris R, Diyarbakir, Turkey	20	AF145944
Schizothorax prenanti	2*n* = 148	GenBank/EMBL	–	(AF051880)
S. pseudaksiensis	*2*n* = 98–100	Chue R, Kazakhstan	–	AF180827
Cyrpinus carpio	2*n* = 100	GenBank/EMBL	–	(X61010)
Carassius auratus	2*n* = 100	GenBank/EMBL	–	(AF045966)
C. auratus langsdorfi	2*n* = 150	GenBank/EMBL	–	(AB006953)
Puntius ('Barbus') gonionotus	2*n* = 50	Depok Station, Sumatra	–	AF180822
P. ('Barbus') schwanenfeldii	2*n* = 50	Thailand, domestic strain	–	AF180823
Cyclocheilichthys. sp.	2*n* = 50	Sumatra	–	AF180824
Garra variabilis	*2*n* = 50–52	Savur Stream, Mardin, Turkey	20	AF180825
Cyprinion macrostomus	–	Tigris R, Diyarbakir, Turkey	20	AF180826
Tinca tinca	2*n* = 48	GenBank/EMBL (Tincinae)	–	(Y10451)
Leuciscus leuciscus	2*n* = 50	GenBank/EMBL (Leucisicinae)	–	(Y10449)
Gobio gobio	2*n* = 50	GenBank/EMBL (Gobioninae)	–	(Y10452)
Rhodeus sericeus	2*n* = 48	GenBank/EMBL (Acheilognathinae)	–	(Y10454)
Myxocyrpinus asiaticus	2*n* = 100	GenBank/EMBL (Myxocyprininae, Catostomidae)	–	(AF036176)
Scartomyzon congestus	–	Canada (Catostominae, Catostomidae)	–	AF1808209
Moxostoma breviceps	*2*n* = 96–100	Canada (Catostominae, Catostomidae)	–	AF180821

The asterisks (*) stand for cases where the chromosome number is not known, but expected according to close species karyotypes. Underlined: species names of southern African barbs. Data are from Magtoon and Arai, 1989; Golubtsov and Krysanov, 1989; Collares-Pereira, 1994; Guégan *et al*, 1995; Ráb and Collares-Pereira, 1995; Naran, 1997 and Collares-Pereira and Moreira Da Costa, 1999.

facturer's protocol, BIO 101, Inc). The sequencing reaction consisted of denaturation at 95°C for 4 min 30 sec and 25 cycles at 95°C for 30 sec and 60°C for 30 sec. Products were run out on a 6% denaturing acrylamide gel (Biorad) and visualized on a Pharmacia automated sequencer following the manufacturer's instructions. In order to control sequence accuracy and to resolve any ambiguous bases, both strands were sequenced using each one of the two initial PCR primers.

All mtDNA sequences determined in this study are deposited in the GenBank database under the accession numbers shown in Table 1.

Phylogenetic inference

The set of unique mtDNA haplotypes was analyzed with the MEGA program (version 1.01; Kumar *et al*, 1993), in order to determine the nucleotide composition separately for each codon position and for the entire gene, as well as the number of variable and parsimony informative sites. The levels of saturation of each codon position

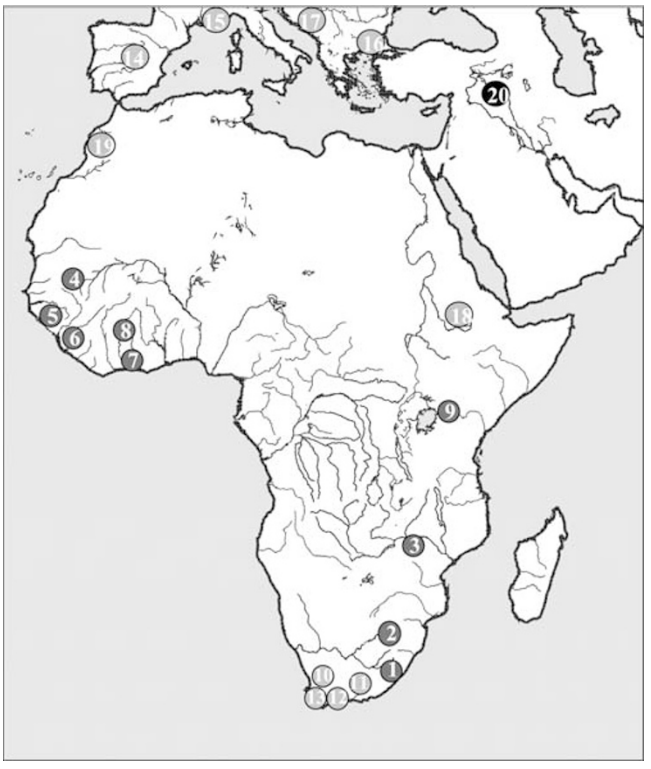

Figure 1 Map showing sampling sites of analyzed cyprinid taxa. Numbers refer to species detailed in Table 1. Sampling sites of Southeast Asian and North American taxa are not shown.

separately and of the entire data set were assessed by pairwise sequence comparison and by plotting the number of transitions versus transversions.

Phylogenetic analysis was performed using the neighbor-joining (NJ) method (Saitou and Nei, 1987) and maximum parsimony methods, both using PAUP (version 4.0, Swofford, 1999). The NJ tree was reconstructed using Tamura and Nei's (1993) distance. The distance estimate takes into account nucleotide composition biases and different substitution rates between purines and pyrimidines. Maximum-parsimony (MP) trees were generated with different weighting schemes, in which weighting varied across codon position to reflect differences in transition: transversion (Ts : Tv) rates across sites. We performed (1) equal weighting for both substitution types at all sites, (2) equal weighting for both substitution types in the first and second codon positions, while transitions in the third codon position were not considered, (3) down-weighting transitions in all codon positions by the Ts : Tv ratio estimated for the complete cytochrome *b* gene sequence, and (4) down-weighting transitions in third codon position by the Ts : Tv ratio estimated for this position. Tree topologies were evaluated by their consistency index (CI). In all cases, the heuristic search option with TBR branch swapping and 100 stepwise random additions of taxa was employed. The reliability of the inferred phylogenies was evaluated using bootstrap (Felsenstein, 1985) with 1000 and 100 replicates for NJ and MP, respectively. In all cases, trees were drawn using TreeView (Page, 1996). Finally, we used the Wilcoxon signed ranks test (Templeton, 1983) to determine whether alternative topologies were significantly worse than those supported by the data.

Results

The complete cytochrome *b* sequences of 1140 bp of 45 cyprinid species were aligned, and only two species (*'B'. trimaculatus* and *'B'. sp* from Lake Baringo) showed alignment problems with a stop codon two amino acids before the normal end of the gene. The cytochrome *b* gene of these two species seems to code for 378 instead of 380 amino acids.

Cytochrome *b* gene variation

Among the sequenced 1140-nucleotide sites, 594 sites were variable and 519 sites were parsimony-informative across all taxa. The third codon positions were found to be almost all variable (99.4%) and substitutions at these sites were responsible for 63.6% of variable sites and 70.5% of parsimony-informative characters used. The second and particularly the third codon position had an under-representation of G (13% and 6.1%, respectively), while first codon position showed no base composition bias.

Uncorrected sequence divergences (*p* distances) between cyprinids involved in this study varied from 2.7% to 25.7%. Sequence divergences among the 'small' African species varied from 11.6% (*'B'. ablabes – 'B'. macrops*) to 20.8% (*'B'. trimaculatus – 'B'. anoplus*) with a mean value of 16.3%.

In Figure 2 we plotted the absolute number of transitions and transversions in each codon position. Transitions showed particular saturation in the third codon and to a lesser extent in the first codon position.

Phylogenetic relationships among African and Euro-Mediterranean barbs

The phylogenetic tree obtained with the NJ method using Tamura and Nei's (1993) genetic distance, with three catostomid species as outgroups, is shown in Figure 3. Three lineages of *Barbus* were observed, each with high bootstrap values: (1) the Euro-Mediterranean tetraploids (*Barbus s. str.*), (2) the 'large' African hexaploids, and (3) African barbs including the 'small' diploid species and the southern African tetraploids. The latter clade is further subdivided, tetraploids are monophyletic (74% of bootstraps) and the 'small' diploid species are unresolved. The 'large' African hexaploid barbs form a strongly supported monophyletic clade with the 'large' species from Morocco (*'B'. reinii*) and the Middle East (*'B'. luteus*) at a basal position.

Southern African tetraploid *'Barbus'* are clearly separated from the Euro-Mediterranean tetraploid species of *Barbus s. str.* (Figure 3). Among 'small' African barbs, only four associations are supported by more than 50% bootstrap values, those between *'B'. ablabes* and *'B'. macrops* and among *'B'. sp*, *'B'. trimaculatus*, *'B'. nyanzae* and *'B'. sublineatus*.

Maximum-parsimony analysis with equal weighting of Ts and Tv sites resulted in 10 parsimonious trees of 4207 steps (CI = 0.243). Topologies generated with or without weighting substitution types had lower consistency indices (results not shown). A more consistent topology is found with the second weighting model, where transitions at third codon positions are excluded (CI = 0.311). However, because transitions seem to be saturated in the first and particularly in the third codon position (Figure 2), only the 50% majority-rule consensus tree using a Ts : Tv ratio of 2:1 in all positions is shown in Figure 4.

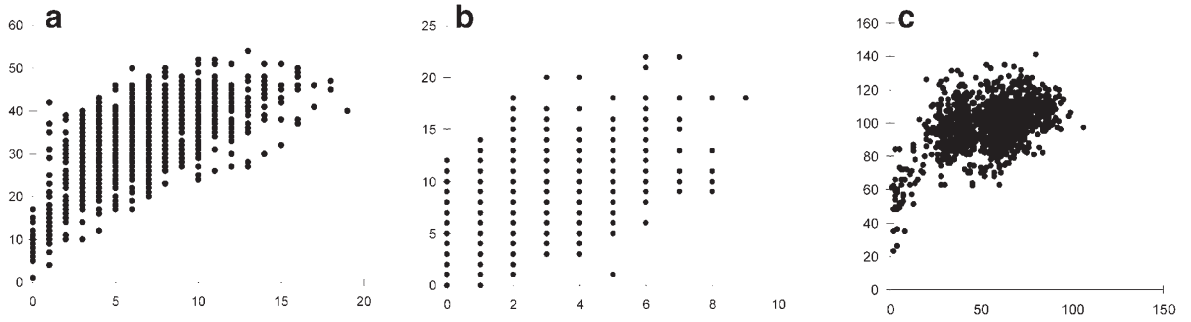

Figure 2 Saturation analysis of cytochrome *b* sequences. The absolute number of transitions was plotted against the absolute number of transversions for (**a**) the first, (**b**) the second, and (**c**) the third codon position.

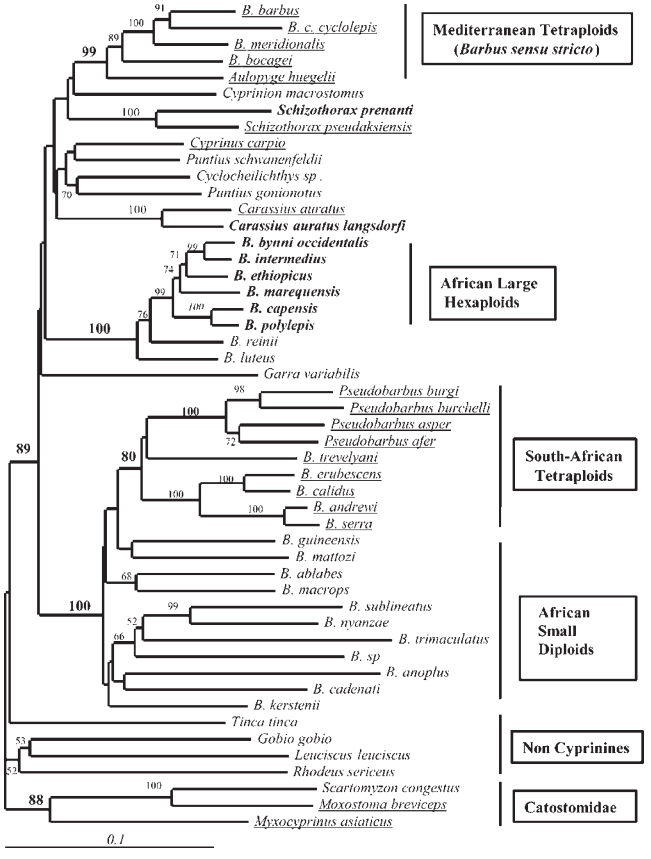

Figure 3 Phylogenetic relationships of 47 cyprinids based on cytochrome *b* sequence data. A neighbor-joining tree constructed using Tamura and Nei's (1993) distance method is shown. Branch lengths are proportional to the mean number of substitutions per site (see scale bar). Numbers above nodes indicate bootstrap values higher than 50%. Sequences of three catostomid taxa were used as an outgroup. Tetraploid taxa are underlined, and hexaploids are in bold.

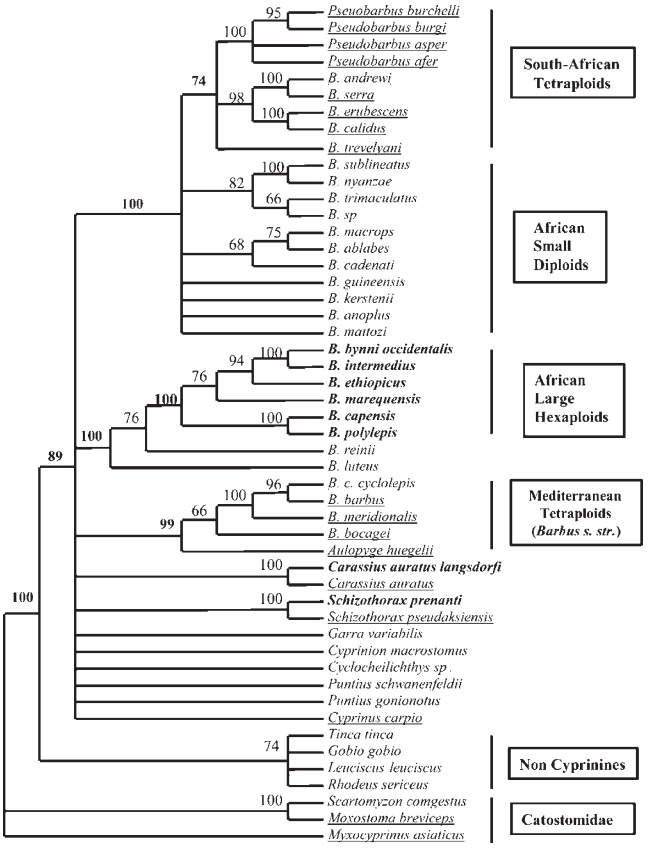

Figure 4 Phylogenetic relationships among cyprinid polyploids based on cytochrome *b* sequence data. The 50% majority-rule consensus tree was obtained using maximum parsimony with a Ts : Tv ratio of 2 : 1 for all positions (consistency index = 0.242, 5528 steps). Numbers above nodes indicate bootstrap values after 100 replicates. Nodes with bootstrap values below 50% were forced to collapse and yield polytomies. Sequences of three catostomid taxa were used as an outgroup. Tetraploid taxa are underlined, and hexaploids are in bold.

This topology is similar to that obtained with the NJ method (Figure 3). Parsimony analyses excluding third position transitions (six MP trees of 1654 steps), and a Ts : Tv ratio of 2:1 only in the third position (two MP trees of 5026 steps) recovered congruent topologies, with differences only for deeper phylogenetic relationships.

Using the Wilcoxon signed ranks test (Templeton, 1983), the only alternative topology not significantly different to that presented in Figure 4, is one grouping the common carp (*C. carpio*) with the Mediterranean tetraploid *Barbus s. str.* (data not shown).

Discussion

There is no support for the monophyly of the genus *Barbus*, which seems, as expected, polyphyletic with numerous species interspersed with representatives of other genera. 'Small' African diploids and southern African tetraploids form a strongly supported monophyletic group (100% in both NJ and MP analyses). The Euro-Mediterranean clade of *Barbus* tetraploids is associated

with *Aulopyge huegelii*, a monotypic genus endemic in Croatia as expected by Howes (1987). African diploid *'Barbus'* are polyphyletic, and genetically distant from their Asian counterparts (genus *Puntius*). The 'large' hexaploid barbs form a highly supported monophyletic assemblage, with 'large' species of undetermined ploidy level, from Morocco and the Middle East (*'B'. reinii* and *'B'. luteus*, respectively). The Wilcoxon signed ranks test (Templeton, 1983) clearly indicates recurrent origins of polyploid species in the Cyprininae subfamily, with multiple origins for tetraploids. The following detailed interpretation will address the taxa at each ploidy level and/or apparent monophyletic lineage separately.

Evolution of the diploid lineages

Collares-Pereira and Coelho (1989) proposed that tetraploidy (100 chromosomes) is the ancestral (plesiomorphic) state and that diploidy (48–50 chromosomes) is derived (apomorphic), attained by successive reduction in chromosome number through processes such as chromosome fusions and deletions. In our results, the tree topologies in Figures 3 and 4 clearly indicate that diploid cyprinids are basal to the polyploid species and rather support the hypothesis of Ohno (1970) of a diploid-tetraploid evolution.

On a basis of karyological data, Ráb (1981), Golubtsov and Krysanov (1993) and Ráb *et al* (1995), suggested that the 'small' African barb species may be close to some Asian diploid taxa assigned to the genus *Puntius*. In our phylogenetic analysis this proposal is not supported, at least for the African species and representatives of *Puntius* species that have been analyzed (Figures 3 and 4). This confirms that the symplesiomorphic character (evolutionary diploid state) is phylogenetically uninformative. The African taxa appear to be polyphyletic with few well-supported nodes. Bootstrap values, however, become higher when weighted parsimony is employed (Figure 4).

Evolution of the tetraploid lineages

The phylogenetic analyses based on complete cytochrome *b* sequence data (Figures 3 and 4) suggest that the tetraploids present in southern African and Euro-Mediterranean barbs (*Barbus s. str.*) are of independent origin. Tetraploidy in the genus *Aulopyge* and in the species of the genus *Barbus s. str.* proved to be of the same origin (Tsigenopoulos and Berrebi, 2000). The tetraploidy of the common carp *Cyprinus carpio* seems to stem from the same polyploidization event, but this interpretation needs further confirmation.

Analysis of the mtDNA markers clearly supports the multiple origins of tetraploid species of barbs. However, such markers are uninformative as to the mode of polyploidization, ie either via autopolyploidy (genome duplication of a single species) or allopolyploidy (genome combination after species hybridization). Among fishes, only salmoniformes are thought to have originated through actual autopolyploidy (Allendorf and Thorgaard, 1984), while the origins of polyploids in catostomids, cobitids, and cyprinids are still unclear. For both Euro-Mediterranean and southern African lineages of barbs, tetraploidy seems to be a very ancient phenomenon that arose probably once. The absence of chromosome quadrivalents in meiotic nuclei of *C. carpio* (Ohno *et al*, 1967) and *B. barbus* (see references in Ohno, 1970; Ráb and Collares-Pereira, 1995) is consistent with an allotetraploid origin. Moreover, new evidence from the analysis of microsatellite data in polyploid barbs (Chenuil *et al*, 1999) suggests interspecific hybridization as a more plausible mode of polyploidization. In Euro-Mediterranean tetraploid *Barbus s. str.*, hybridization is a rather common phenomenon (Machordom *et al*, 1990; Berrebi *et al*, 1993; Slechtova *et al*, 1993). Moreover, hybridization is suspected to occur between tetraploid and hexaploid taxa in Middle East barbs (Mir, 1988). Frequent hybridization among diploid barbs associated with an altered mode of reproduction and/or increase in ploidy level could be the origin of several independent tetraploid lineages via so-called reticulate speciation (Vasil'ev *et al*, 1989).

The origin of European tetraploid barbs cannot currently be resolved (but can probably be if several additional Asian diploid and polyploid species were analyzed in a similar way); this is not the case of the southern African tetraploid barbs. The latter species form a strongly supported monophyletic clade along with the 'small' African *'Barbus'* species, a part of which has been shown to be diploid (Figures 3 and 4). The origin of tetraploidization in southern African barbs appears to be very ancient given the high genetic divergence between the two subgroups (*'Barbus'* and *Pseudobarbus*) in the assemblage. Undoubtedly, they most likely originated from diploid species of the same region. Besides, there are dozens of 'small' *'Barbus'* species in Africa (Lévêque and Daget, 1984), which appear to be more numerous in the east and south than in the west (Skelton, 1988). Moreover, southern African tetraploids do not have any particular morphological relationship with other African cyprinine cyprinid lineages (Skelton, 1980). All this information suggests that the polyploidization event probably occurred in the southern African region, before the divergence of *'Barbus'* and *Pseudobarbus*.

Evolution of the hexaploid lineages

Oellermann and Skelton (1990) pointed out that the most plausible way toward hexaploidy involves a combination of both allopolyploidy and autopolyploidy. In this scenario, hexaploidy may arise after spontaneous chromosome doubling in an unstable triploid fish from a diploid-tetraploid hybridization. Golubtsov and Krysanov (1993) suggested that if the most probable pathway for the origin of hexaploids is via a tetraploid stage, an independent origin of Ethiopian and southern African hexaploid forms seems unlikely. Therefore, the African hexaploids might originate either from undefined African tetraploid *'Barbus' s. l.*, from Euro-Mediterranean *Barbus s. str.* or from an Asian genus like *Tor*, ie a genus containing tetraploid taxa. The authors have also postulated that the formation of the hexaploid lineage preceded its dispersal on the African continent, although there were no data about cyprinine hexaploids outside of Africa.

The results of the present study demonstrate that African hexaploids originating from geographically distant parts of Africa are genetically very close and are part of the same lineage (Figures 3 and 4). The hexaploid species are not associated with any particular diploid and/or tetraploid African lineages of barbs. This could therefore suggest that this lineage originated either from non-African polyploid *'Barbus'*, before its dispersal in Africa, or from other African cyprinines not analyzed in this study. In addition, karyological

data for Anatolian (*'B'. luteus*) and Moroccan species (*'B'. reinii*), which are strongly grouped with hexaploid barbs are not available. This information is necessary for choosing among the African or non-African origin hypotheses. Based on allozyme data, El Gharbi (1994) suggested that *'B'. reinii* might belong to the hexaploid *'Barbus'* lineage (see also in Doadrio, 1994; Berrebi, 1995). Recently, Krysanov (1999) reported the first hexaploid barb species in the Middle East region, therefore a new hypothesis of an 'out of Africa hexaploid' origin for African hexaploids can be proposed.

Systematic implications

(1) Diploid barbs: An important result is that probably no diploid lineage is shared between Africa and Asia. The diploid level being the ancestral one, it is clear that the divergence between the numerous diploid lineages (300 species in Africa, probably more in Asia) happened mostly before the appearance of the polyploid lineages among the barbines. Berrebi *et al* (1996) proposed a close phylogenetic relationship among the diploid barbs of the two continents. The present new data clearly indicate that 'small' African barbs do not belong to the Euro-Mediterranean genus *Barbus* s. str. nor to those Asian diploid barbs genera such as *Puntius* and other related genera that have been analyzed. A new taxonomic solution is therefore necessary for small-sized African barbs, Figures 3 and 4 indicating that several genera will be necessary. Levêque and Daget (1984) proposed more than 10 different generic and subgeneric names, on the basis of morphological characters, but they have not been used.

(2) Tetraploid barbs: As previously suggested (Howes, 1987; Berrebi *et al*, 1996), the genus *Barbus* s. str. must be limited to the monophyletic lineage which contains the species *B. barbus*. It corresponds to the tetraploid species of barbs occurring around the Mediterranean region (including North Africa) and to the Caspian Sea and the Middle East.

On the other hand, the southern African tetraploid barbs form an independent lineage, stemming undoubtedly from local diploid species. The monophyletic genus *Pseudobarbus* is justified but several local tetraploid genera must be erected (at least two, Figure 3), and more taxa need to be analyzed. Present results indicate that other independent tetraploid lineages may be discovered among tetraploid cyprinine cyprinids.

(3) Hexaploid barbs: The lineage of African hexaploid barbs is clearly monophyletic. This homogeneous group is widespread in Africa and treated as a single genus. Golubtsov and Krysanov (1993) and Berrebi *et al* (1996) proposed erecting the name of the subgenus to which they are classified (*Labeobarbus*) to generic rank (Doadrio, 1994; Berrebi, 1995).

However, hexaploid species of barbs do undoubtedly occur outside of Africa. The possible hexaploidy of *'B'. luteus* (see above) and recent karyological (Krysanov, 1999; Demirok, 2000) analyses have shown that hexaploid barbs occur in eastern Europe and the Middle East. A more detailed phylogenetic study of these taxa is necessary to understand if they belong to the *Labeobarbus* genus or to distinct genera and/or lineages.

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