Evolutionary history and speciation modes in the cyprinid genus *Barbus*

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Phylogenetic relationships and evolutionary patterns in the genus *Barbus* were examined through the analysis of the complete sequences of three mitochondrial genes: ATPases 8 and 6, which overlap slightly, and cytochrome *b*. This complex genus includes diploid as well as tetraploid and hexaploid species that are distributed throughout the Palaearctic, Ethiopian and Asiatic biogeographical regions. Given that genome duplication is an important evolutionary mechanism in eukaryotes, in the present report we attempt to describe its role in the evolution of the genus *Barbus*, as well as drawing systematic and phylogenetic conclusions. The phylogenetic results indicated the splitting of the current *Barbus* genus into five main mitochondrial lineages corresponding to (i) the genus *Barbus sensu stricto* (tetraploid, which is subdivided into the subgenera *Barbus* and *Luciobarbus*), (ii) the hexaploid species, (iii) the Ethiopian tetraploid species, (iv) the African diploid species, and (v) the Asian diploid species. The branching of 'foreign' genera as sister groups of some of these monophyletic assemblages (such as *Aulopyge* is to *Barbus sensu stricto* or *Varicorhinus* is to the hexaploid barbels) demonstrates the polyphyly of the group. Moreover, the relationships between the proposed lineages also show that genome duplication may be considered as a homoplastic character since it must have occurred over at least three independent periods and/or in three independent areas. In relation to the possible saltational evolutionary model for the polyploid species examined here, it was found that, although feasible at the nuclear level, the mitochondrial markers looked at do not appear to have undergone this type of evolution. Rather, they seem to have experienced more or less constant change through time.

Keywords: mitochondrial DNA; ATP synthase 8 and 6; cytochrome *b*; polyploidy; phylogeny; molecular evolution

1. INTRODUCTION

The 'C-value paradox' of genome size in eukaryotes has prompted the search for an extra or non-genic role for DNA. There is much dispute concerning the increase in the amount of DNA that occurs during evolution. Different mechanisms are invoked in this DNA increase, such as regional increases in genome size, chromosomal duplication or genome duplication (Li & Graur 1991). It seems that genome doubling or large-scale duplications have played a significant role in the evolution of vertebrates and many other eukaryotes (Ohno 1970; Lundin 1993; Pébusque *et al.* 1998; Wendel 2000). Indeed, this increase in DNA could account for some degree of 'acceleration' of evolution. Therefore, in order to explain the evolution of certain taxa, a punctuated equilibrium or saltational model might be considered. It has been previously proposed that duplication of the number of loci could give rise to a higher mutation rate (Ohno 1970) and that new genetic functions could arise through divergence of the structure of the duplicated genes (Stephens 1951; Li 1980). With respect to chromosomal change, even polyploidy can be considered a borderline mechanism of rapid speciation (requiring only one or two generations) (Stebbins & Ayala 1981).

While polyploidy is a frequent phenomenon in plants (ca. 70% of angiosperms and up to 95% of pteridophytes are polyploids; Soltis & Soltis 1999), it is argued that the polyploid condition in animals would be a considerable disadvantage due to alterations in the distribution and

number of sex chromosomes and ensuing hormonal irregularities (Avers 1989). However, some vertebrates, such as reptiles, amphibians and fish, perhaps due to their method of sexual determination (Cataudella *et al.* 1977; Castelli & Philippiart 1993), are known to be polyploid.

Complete families of fish, such as the Castostomidae or Salmonidae, are polyploid or of polyploid origin, even if their meiotic segregation is currently mainly disomic (Ferris & Whitt 1979; May 1980). Other families, such as the Cobitidae or Cyprinidae, show different levels of ploidy. Within the Cobitidae, some species or populations are recognized as triploids or even tetraploids, but most differences in ploidy are usually interpreted as rare phenomena of unisexual reproduction (Osinov *et al.* 1990; Vasil'yev *et al.* 1991). In contrast, the Cyprinidae family includes genera with stable polyploid species, principally of the genus *Barbus*.

The *Barbus* genus is known for its complexity, i.e. its wide distribution, the number of species it comprises, the morphological plasticity of its species, which in some cases hinders identification and also the fact that the genus contains different levels of ploidy. Knowledge of the existence of both diploid and tetraploid species within the genus dates far back (Post 1965; Wolf *et al.* 1969), although some species have recently been recognized as hexaploid (Oellermann & Skelton 1990; Golubtsov & Krysanov 1993; Guégan *et al.* 1995). Indeed, some authors consider this genus to be polyphyletic (Myers 1960; Howes 1987; Berrebi *et al.* 1996).

These features have prompted the analysis of some of the species of this genus and in particular of the so-called *Barbus sensu stricto*. Morphological and allozymic

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Table 1. *Species, location data, numbers of specimens analysed and GenBank accession numbers*

(The numbers that follow the taxa names indicate a different haplotype, which is marked in the phylogenetic tree obtained. Abbreviation: AN, GenBank accession number.)

species	river	locality	<i>n</i>	ATPases AN	cytochrome <i>b</i> AN
<i>Astyanax mexicanus</i>	Frío	Santa Anita, Mexico	1	AF287358	AF287414
<i>Aulopyge huegeli</i>	Busko Lake	Bosnia-Herzegovina	4	AF287359	AF287415
				AF287360	AF287416
<i>Barbus anoplus</i>	Incomati	Ngodwana, South Africa	1	AF287361	AF287417
<i>B. b. macedonicus</i> -1	Axios	Axiopolis, Greece	3	AF287362	—
<i>B. b. macedonicus</i> -2	Aliakmon	Kokinia, Greece	1	AY004721	AY004754
<i>B. b. thessalus</i>	Pinios	Omolio, Greece	1	AF287365	—
<i>Barbus bynni bynni</i>	Nile	Egypt	4	AF287366	AF287420
<i>B. b. occidentalis</i>	Bafing	Sokotoro, Guinea	4	AF287367	AF287421
<i>B. calidus</i> -1	Olifans	Western Cape, South Africa	1	AF287458	AF287422
<i>B. calidus</i> -2	Noordhoeks	Western Cape, South Africa	5	AF287459	AF287423
<i>B. caninus</i>	Judrio	Gorizia, Italy	2	AF287368	AF287424
				AF287369	AF287425
<i>B. canis</i>	David	Bet Shean, Israel	2	AF288484	AF288486
				AF288485	—
<i>B. cyclolepis cholorematicus</i>	Chlororema	Almiros, Greece	2	AF287370	AF287426
				AF287371	AF287427
<i>B. c. cyclolepis</i>	Erithropotamus	Mikro Derio, Greece	1	AF287372	—
<i>B. c. sperchiensis</i> -1	pond	Velestino, Greece	2	AF287373	AF287428
<i>B. c. sperchiensis</i> -2	Sperchios	Lamia, Greece	1	AF287374	—
<i>B. c. strumicae</i>	Agiaki	Kastanies, Greece	2	AF287375	—
	Nestos	Paranesti, Greece	2	AF287376	—
				AF287377	—
<i>B. euboicus</i>	Maniklotiko	Oxilothos, Greece	2	AF287378	—
	Kamaris	Eubolia, Greece	2	AF287379	—
<i>B. fritschii</i> -1	Zamrine	Khouribga, Morocco	1	AF287380	AF287429
<i>B. fritschii</i> -2	Kasab	Essaouira, Morocco	4	AF287381	AF287430
<i>B. gruweli</i>	Samou	Debele, Guinea	2	AF287382	AF287431
<i>B. gurneyi</i>	Mgeni	Kwazulu-Natal, South Africa	2	AF287383	AF287432
<i>B. intermedius</i>	Tana Lake	Ethiopia	4	AF287384	AF287433
<i>B. marequensis</i>	Marico	North West Province, South Africa	1	AF287385	AF287434
<i>B. meridionalis</i>	Tordera	Barcelona, Spain	2	AF287386	—
<i>B. motebensis</i>	Marico	North West Province, South Africa	1	AF287387	AF287435
<i>B. paludinosus</i>	Mooi	North West Province, South Africa	4	AF287388	AF287436
				AF287389	AF287437
<i>B. peloponnesius</i> -1	Thiamis	Parapotamus, Greece	2	AF287390	AF287438
<i>B. peloponnesius</i> -2	Alfios	Karitana, Greece	1	AF287391	—
<i>B. petenyi</i> -1	Aliakmon	Kaloneri, Greece	2	AF287392	AF287439
				AF287393	—
<i>B. petenyi</i> -2	Gallikos	Gallikos, Greece	1	AF287394	AF287440
				AF287395	—
<i>B. petenyi</i> -3	Megalopotami	Gorgopi, Greece	2	AF287396	—
<i>B. petenyi</i> -4	Dimbovitza	Dragomiresti, Romania	2	AF287397	AF287441
				AF287398	AF287442
<i>B. petitjeani</i>	Bafing	Sokotoro, Guinea	5	AF287399	AF287443
<i>B. prespensis</i>	Prespa Lake	Psarades, Greece	2	AF287400	—
<i>B. rebeli</i>	Aoos	Komitsa, Greece	1	AF287401	—
<i>B. reinii</i>	Kasab	Essaouira, Morocco	1	AF287402	AF287444
<i>B. sacratus</i>	Tangala	Kambaya, Guinea	4	AF287403	AF287445
<i>B. serra</i>	Upper Olifants	Western Cape, South Africa	5	AF287460	AF287446
				AF287461	AF287447
<i>B. wurtzi</i>	Kaba	Kouloundela, Guinea	5	AF287404	AF287448
<i>Pseudobarbus afer</i> -1	Blindekloof	Eastern Cape, South Africa	1	AF287405	AF287449
<i>P. afer</i> -2	Swartkops	Eastern Cape, South Africa	1	AF287406	AF287450
<i>P. asper</i>	Vlei	Western Cape, South Africa	1	AF287407	AF287451
<i>P. phlegethon</i>	Noordhoeks	Western Cape, South Africa	1	AF287408	AF287452
<i>P. tenuis</i>	Vlei	Western Cape, South Africa	2	AF287409	AF287453
				AF287410	AF287454
<i>Puntius titteya</i>	Aquarium		2	AF287411	AF287455
<i>Rhamdia laticauda</i>	El Arco	Guelatao de Juárez, Mexico	1	AF287412	AF287456
<i>Varicorhinus maroccanus</i>	Oum Er Rbia	El Borj, Morocco	2	AF287413	AF287457

studies were mainly conducted (e.g. Almaça 1969; Howes 1987; Doadrio 1990; Machordom *et al.* 1995; Slechtová *et al.* 1998), but their large-scale limit of resolution soon became evident. It is proposed that DNA sequencing might clarify the relationships and evolution mechanisms of the genus. Even the existence of a small degree of ploidy complicates the analysis of nuclear genes, since the misidentification of homologous characters can be a source of homoplasy. For this reason, most phylogenetic studies performed on *Barbus* have involved the use of mitochondrial gene sequences (Zardoya & Doadrio 1998, 1999; Tsigenopoulos & Berrebi 2000; Machordom & Doadrio 2001). The established characteristics of this cytoplasmic genome (Brown *et al.* 1982; Boursot & Bonhomme 1986) avoid some of the potential problems arising from the analysis of nuclear genes. Besides this, mitochondrial DNA is preferentially used in phylogenetic studies (some 70% according to Avise (2000)).

The present analysis was therefore based on complete sequencing of the mitochondrial markers ATPases 8 and 6 and cytochrome *b*. These sequences have mainly served to clarify the phylogenetic relationships among the Palaearctic and Ethiopian species. An additional aim was to test the hypotheses predicting phylogenetic relationships among different taxa and the monophyletic condition of the species at each level of ploidy. This information provides insight into the evolution of this genus, the role of polyploidization and the speciation mechanism.

2. MATERIAL AND METHODS

(a) *Samples*

The complete sequences of the cytochrome *b* and ATPase 8 and 6 genes were analysed in 108 specimens belonging to 39 taxa of the genera *Barbus*, *Pseudobarbus*, *Aulopyge*, *Varicorhinus*, *Puntius*, *Astyanax* and *Rhamdia* (table 1). Eighty-six ATPases 8 and 6 and 60 cytochrome *b* sequences (plus 23 sequenced previously; Zardoya & Doadrio 1998, 1999; Zardoya *et al.* 1999) were obtained. Forward and reverse sequences were compared in each gene. Previously published data on 29 taxa of the subgenus *Luciobarbus* (Machordom & Doadrio 2001) were also included in the analysis. *Astyanax mexicanus*, *Rhamdia laticauda* and *Crossostoma lacustre* (GenBank accession number M91245.1) were used as outgroups.

All fish specimens were obtained by electrofishing. In order to avoid taxonomic errors, the specimens were collected from *terrae typicae* whenever possible. Muscle and liver tissues were maintained in alcohol or in liquid nitrogen before storage at -74°C . Voucher specimens were included in collections from the Museo Nacional de Ciencias Naturales (Spain), Tel-Aviv University Zoological Museum (Israel) and the J. L. B. Smith Institute of Ichthyology (South Africa).

(b) *Mitochondrial DNA amplification and sequencing*

Total DNA was extracted from *ca.* 0.1–0.2 g of tissue (preferentially muscle) according to phenol:chloroform extraction procedures (Sambrook *et al.* 1989).

Complete cytochrome *b* and ATPases 8 and 6 gene sequences were amplified by the polymerase chain reaction (PCR). The primers and the PCR conditions were as described in Machordom & Doadrio (2001).

The PCR products were visualized in 1.5% agarose low-melting point gels stained with ethidium bromide and sub-

sequently cut from the gel and extracted with gelase (Epicentre). Five microlitres of this product were used in the cycle sequencing reaction plus 1 or 2 μl of dRhodamine Terminator Cycle Sequencing kit reagents (PE Applied Biosystems), with a final concentration of 1 μM of each primer (in independent reactions) and double-distilled H_2O to yield a final volume of 10 μl . The primers used were the same as for the PCR (all located in flank regions of the target genes). The cycle conditions were 25 cycles at 96°C for 15 s, 50°C for 1 s and 60°C for 4 min. After this amplification, samples were cleaned on G-50 Sephadex columns and loaded onto an automated ABI 377 DNA sequencer (Applied Biosystems).

The sequences obtained (*ca.* 900 bp for each strand) were cleaned at the primer ends (in order to remove flanking fragments not corresponding to the genes analysed), aligned and verified using forward and reverse overlap sequences (Sequencher programme, Gene Code Corporation). Translation to proteins was inferred using this and the MacClade programme (Maddison & Maddison 1992) and base codon positions designated.

(c) *Phylogeny reconstruction*

Analyses were independently performed on complete genes (the overlapping ATPases 8 and 6 with 842 bp and cytochrome *b* with 1141 bp) and on the total number of base pairs sequenced (1983 bp). A partition homogeneity test was performed among the sequence data in order to check for homogeneity of the individual genes. The analyses were performed according to the principles of neighbour joining, maximum parsimony and maximum likelihood. The best model of evolution that fitted our data was obtained using the program Model test 2.1 (Posada & Crandall 1998). Thus, the general time-reversible (GTR) model (Lavane *et al.* 1984; Rodríguez *et al.* 1990) and HKY85 (Hasegawa *et al.* 1985) distances were used first. Due to the fact that the trees were better resolved according to this last model (HKY85), which provides distances corrected for site-to-site rate variation, the results presented for neighbour joining and maximum likelihood were based on this model.

Parsimony analyses were performed by tree-bisection-reconnection branch swapping, the MULPARS option and ten random stepwise additions using the heuristic search algorithm. The maximum-likelihood analysis was performed by quartet puzzling search (with 1000 pseudo-replications) using the PAUP* (Swofford 2000) package. Confidence in the analyses was estimated by bootstrapping (100 repetitions) (Felsenstein 1985) and decay index values (Bremer 1988, 1994) using the AutoDecay program (Eriksson 1998). The transition-to-transversion rate was estimated using a maximum likelihood approach. In order to determine whether a particular tree topology corresponded to a significantly better or worse interpretation of the data than an alternative tree, we used both Wilcoxon signed-ranks (Templeton 1983) and Kishino-Hasegawa (Kishino & Hasegawa 1989) tests, as implemented in PAUP.

3. RESULTS

The stop codon for ATPase 8 was at position 163–165 for the ingroup (including other Cyprinidae species), whereas *Puntius titteya* and the outgroups had one codon more in ATPase 8. The ATPase 6 start codon showed more variability: the majority of the species had two successive methionines (ATGATA) at position 159–164. The second one was absent in the outgroup. Thus, we

assumed the start codon for ATPase 6 was at position 159–161, with the exception of the following species: *Barbus biscarensis*, *Barbus cyclolepis*, *Barbus graecus*, *Barbus petenyi* and *Barbus caninus*. These species had start codons at position 162–164.

Out of the 1983 characters obtained, 855 were constant (43.12%) and 971 (48.97%) were informative (543 cytochrome *b* and 428 ATPase characters). According to codon position, the most informative was the third position (624 informative characters) followed by the first position (247 characters). The empirical percentages of the different nucleotides were A = 29.63%, C = 28.20%, G = 14.37% and T = 27.79%. The transition-to-transversion rate estimate for the ingroup was 6.32. This parameter was taken into account in the maximum-parsimony and maximum-likelihood analyses.

Given that the partition homogeneity test showed no significant differences between individual data corresponding to the ATPases and cytochrome *b* ($P = 0.85$), the entire information corresponding to these genes was analysed and presented together.

The saturation of transition and transversion changes was checked by plotting the absolute number of changes at each codon position against uncorrected percentage divergence values (p). The results of the entire data set were identical for the ATPases and cytochrome *b*. The graph fitted a straight line for all the changes plotted. Nevertheless, there was an indication of transition saturation starting from 20% divergence. This saturation was mainly caused by transitions in the third ($p > 14\%$) and first positions ($p > 20\%$).

The pairwise molecular divergence values could be divided into different ranges. Values up to 4% represented uncorrected divergences between conspecific specimens, the values found among subspecies and some interspecific relationships (e.g. *Barbus rebeli*/*Barbus prespensis*, *B. petenyi*/*B. caninus*, *Barbus wurtzi*/*Barbus gruveli*, *Pseudobarbus tenuis*/*Pseudobarbus asper* and between some *Luciobarbus* species) (see Machordom & Doadrio 2001). Divergences between 5 and 13% were shown by species with the same degree of ploidy, with the exception of the tetraploid species. Within the tetraploids, two lineages were well differentiated, the Palaearctic (including the tetraploid genus *Aulopyge*) and the Ethiopian. The divergences between them were included in the next range. Apart from the divergences between the two tetraploid groups (range of 14–21%), the distances between species of different levels of ploidy (e.g. hexaploids versus tetraploids) and *Puntius*, irrespective of ploidy, are included. Finally, the greatest divergences (higher than 22%) were found between the outgroups and the remaining species.

Thus, the saturation found included the ranges covering relationships between different levels of ploidy or possible clustering with the outgroups.

(a) Intragroup divergences

(i) Palaearctic tetraploids

Since the subgenus *Luciobarbus* was analysed in a previous study (Machordom & Doadrio 2001) the particular features of this group are not detailed here.

The two subspecies of *Barbus barbus* (i.e. *Barbus barbus thessalus* and *Barbus barbus macedonicus*) showed no more than 0.40% divergence. Their sister species is *Barbus plebejus* ($p \approx 3\%$). The population of *B. petenyi* that inhabits the same Greek area as *B. b. macedonicus* also had a taxon from North Italy as a sister species, namely *B. caninus* (figure 1). This Greek population of *B. petenyi* differed highly from Romanian specimens of the same species from areas close to their *terra typica*. *Barbus petenyi* from Romania was the sister taxon of *B. prespensis*/*B. rebeli*, a group clearly differentiated from *B. petenyi* from Greece ($\bar{p} = 9.51\%$, while these Greek populations of *B. petenyi* showed a divergence of 1.46% in relation to *B. caninus* from Italy).

The *B. cyclolepis* subspecies analysed showed two different groups: on the one hand, the northern populations of *Barbus cyclolepis cyclolepis* and *Barbus cyclolepis strumicae* and, on the other hand, the southern populations of *Barbus cyclolepis cholorematicus* and *Barbus cyclolepis sperchiensis*. The divergences within each group were 5.7% for the first pair of taxa, but only 0.55% for the second. The mean divergence between the two *B. cyclolepis* groups was $\bar{p} = 6.72\%$.

The two *Barbus peloponnesius* populations analysed came from two different areas: the Peloponnesus (Alfios River) and the continental Greek region (Thiamis River). The divergence between them was $p = 2.93\%$. However, the species most closely related to *B. peloponnesius* from Peloponnesus (with high bootstrap values of 92% for maximum likelihood, 99% for neighbour joining and 100% for maximum parsimony and decay index of 11) (figure 1) was *Barbus euboicus* from Euboea Island ($p = 1.77\%$, while *B. euboicus*/*B. peloponnesius* from Thiamis showed a divergence of 2.72%).

(ii) Hexaploid barbels

This geographically heterogeneous group showed the lowest degree of molecular divergence between species considering its large distribution area. Thus, species from the Nile River (East Africa), Tana Lake (Central Africa) or the Guinea (West Africa) rivers only showed *ca.* 2.5% divergence (e.g. between *Barbus bynni*, *Barbus intermedius*, *Barbus sacratus* or *Barbus petitjeani*). A second range of

Figure 1. (*Opposite*) Phylogenetic relationships between the taxa analysed based on ATPases 8 and 6 and cytochrome *b* sequences. The tree represented is a bootstrap, majority rule, consensus tree based on maximum-parsimony principles (estimated transition-to-transversion rate 7:1, consistency index = 0.389 and retention index = 0.761). Numbers above branches represent the bootstrap values obtained for 100 replications corresponding to maximum parsimony and neighbour joining (based on HKY85 distances) and numbers below branches indicate those corresponding to maximum likelihood (by quartet puzzling with 1000 replications) and the decay indices. A hyphen replaces the corresponding bootstrap value when a particular branch was not recovered by a certain method. Nodes with bootstrap values below 50% were forced to collapse and yield polytomies. Following the specific names, we indicate the population number as shown in table 1 and the country of origin: Mo, Morocco; Al, Algeria; Tu, Tunisia; Ru, Russia; Gr, Greece; Sp, Spain; Ro, Romania; It, Italy; BH, Bosnia-Herzegovina; GC, Guinea; Eg, Egypt; Et, Ethiopia; SA, South Africa; Is, Israel. (Note: certain species names appear emboldened. This is due to a technical difficulty and such emphasis should be disregarded.)

divergences included *Barbus marequensis* (from South Africa) and *B. gruvelli* and *B. wurtzi* (from Guinea). This group averaged a divergence of $\bar{p} = 5.62\%$ with respect to the first group cited. Finally, the species from Morocco (north-western Africa) (*Barbus fritschii* and *Barbus reinii*) and Israel (Near East) (*Barbus canis*) showed greater divergence ($\bar{p} = 7.68\%$), although this was lower than the divergence shown by *Varicorhinus maroccanus* ($\bar{p} = 9.31\%$) with respect to the two groups defined.

The minimal divergence between *Barbus bynni occidentalis* and *B. petitjeani* ($p = 0.40\%$) or between *B. wurtzi* and *B. gruvelli* ($p = 0.71\%$) within these groups is worthy of note.

(iii) South African tetraploids

This group constitutes the sister group of the Ethiopian diploids analysed here. Within this monophyletic assemblage (with bootstrap values of more than 80% and Bremer or decay index of 16) (figure 1), the genera *Pseudobarbus* and *Barbus* appeared as two different clades. Both are tetraploids and inhabit South African rivers. The mean divergences within species of each genus were $\bar{p} = 5.22\%$ for *Pseudobarbus* and $\bar{p} = 9.12\%$ for *Barbus serra* and *Barbus calidus*. The divergence between the two genera was $\bar{p} = 12.90\%$, taking into account the fact that the data for *B. calidus* and *B. serra* were incomplete since the ATPase 8 genes could not be amplified for these two species (the first 160 nucleotides of the normally sequenced complement of 1983 were missing).

(iv) Diploid barbels

Asian and African diploid barbels showed a polyphyletic origin and appeared as different clades (figure 1). While the phylogenetic relationships of the Asian diploids were not well established, the African diploids constituted a monophyletic group, where the Guinean species was the most differentiated. The distances between the Asiatic and the remaining diploid species were even superior to the mean divergence shown between these Asiatic species and all the species analysed in this study (excluding the outgroups).

Three main branches appeared for these African species: the first, which was formed by *Barbus anoplus*, *Barbus motebensis* and *Barbus gurneyi*, showed a divergence of 5.09–7.67%. The second branch grouped the specimens of *Barbus paludinosus*, the divergence of which was $\bar{p} = 17.85\%$ with respect to the former group and $\bar{p} = 17.26\%$ with respect to the third branch. This third branch was represented by *Barbus bigornei*, the only species of this group from Guinea, West Africa (the rest were South African). *Barbus bigornei* was the basal species of this diploid group, although it showed a degree of divergence with respect to *B. gurneyi* or *B. anoplus* that was less than or equal to that shown between this species and the other South African species *B. paludinosus*. This monophyletic group showed the highest degree of divergence among the taxa analysed.

(b) Phylogenetic relationships

The results indicate multiple phenomena of evolutionary change in each biogeographical region, including duplications or dispersions of different groups of taxa. Although the species mainly grouped according to their

level of ploidy, the relationships between these groups indicated their polyphyletic nature (figure 1).

The hexaploid species were the only ones to cluster in a well-supported monophyletic group (with bootstrap values of 100% for maximum parsimony and neighbour joining and 83% for maximum likelihood and decay index of 41). The basal species within this hexaploid group was *V. maroccanus* (from Morocco), a species included in another genus but with the same chromosome number ($2n = 150$) as the rest of the barbels of this clade. This lineage exhibited the most extensive geographical distribution: northwestern Africa (Maghreb), West Africa (Guinea), East and Central Africa (Egypt and Ethiopia), South Africa and one species from the Near East (*B. canis*, the level of ploidy of which is unknown). The clusterings of these hexaploids did not show an apparent geographical base, since some Guinean species are more related to East African or South African species than to those of the same area. Note the cluster that includes *B. fritschii* (from the Maghreb) and *B. canis* (from Israel).

Two differentiated groups appeared among the tetraploids, the Palaearctic and the Ethiopian. The Wilcoxon signed-ranks test showed significant differences between the tree presented here and another where we forced the monophyletic condition of all the tetraploid species ($p < 0.0001$ for the Templeton tests and $p < 0.002$ for the Kishino–Hasegawa tests).

The Palaearctic clade (including the *Luciobarbus* and *Barbus* subgenera within the *Barbus* genus *sensu stricto*) showed high bootstrap support (100% for maximum parsimony and neighbour joining and 67% for maximum likelihood and decay index of 33) and also contained a species of another genus as its basal species: *Aulopyge huegelii* (with $2n = 100$ chromosomes as the other species in the group). The subgenus *Barbus* formed a monophyletic group represented by North Mediterranean or Central European species. Nevertheless, no strong biogeographical structure was evident, e.g. some Greek species (from one of the areas showing most diversity) clustered with Italian species, others with Romanian species, etc. We only found species distributed in the Circummediterranean area within the other Palaearctic tetraploid subgenus *Luciobarbus*, which appeared as one of the most biogeographically structured groups. The North African taxa constituted the sister group of the taxa from the Caucasus. Two Greek species, which were basal to this group and the Iberian species (including a North African taxon) complete the subgenus (figure 1).

The Ethiopian tetraploids clade (with bootstrap values of 92% for maximum parsimony, 100% for neighbour joining and 80% for maximum likelihood and decay index of 16) only included species from South Africa (such as *Pseudobarbus afer* or *B. serra*). The African diploids formed the sister group of the Ethiopian tetraploids with bootstraps values of 93% (maximum parsimony), 94% (neighbour joining) and 69% (maximum likelihood), although in the heuristic tree the Guinean species *B. bigornei* was basal to the South African diploid and tetraploid species.

Diploid species were not highly represented in our study: we included four South African species, one from West Africa and two Asian species (*P. titteya* and *Puntius conchoniensis*), which are sometimes included in *Barbus sensu*

lato. The former constituted a biogeographically structured, monophyletic group with *B. bigornei* (from Guinea, West Africa) in a basal position. In the neighbour-joining analysis, *P. titteya* and *P. conchoni* appeared in the diploid group in a non-fully resolved basal position. In further analyses (maximum likelihood and maximum parsimony), these two species appeared at the base of the *Barbus* lineage *sensu lato* (figure 1). Thus, the relationships among the diploid barbels were not completely resolved due to the changing position of *B. bigornei* in the different analyses and to the unresolved basal clustering of the Asiatic diploid species of the *Puntius* genus.

4. DISCUSSION

(a) Taxonomic implications

The current taxonomy of the genus *Barbus sensu lato*, which was mainly based on morphological features, maintains the generic *Barbus* denomination for a great number of species (see Eschmeyer 1998). While the molecular markers examined here indicated a monophyletic origin for the ingroup analysed (e.g. ATPase structure related to the stop and start codon positions), the genus *Barbus* appeared as polyphyletic since this ingroup included taxa such as *Pseudobarbus*, *Aulopyge* or *Varicorhinus* that were more closely related to certain *Barbus* species than some species considered as *Barbus* are related among themselves.

Attempts to classify or subdivide *Barbus sensu lato* according to levels of ploidy are clearly inadequate due, for example, to the polyphyly of tetraploids (two different tetraploid clades were demonstrated, one of them sister to the hexaploids and the other to the African diploids). Based mainly on morphological findings, some authors have proposed the subdivision of the genus into different genera or subgenera (see below). However, consensus for these taxa has not yet been reached and clear differences have been shown between certain studies. Nevertheless, some of the proposed divisions are consistent with our phylogenetic results including the splitting of the genus *Barbus sensu stricto* into the subgenera *Barbus* and *Luciobarbus* (Doadrio 1990) or the division of *Barbus sensu lato* into the genera or subgenera *Labeobarbus* (Daget *et al.* 1984), *Varicorhinus* (Banister 1984) or *Pseudobarbus* (Skelton 1993).

Further taxonomic inconsistencies detected at the species level in the present study suggest that in-depth taxonomic revision is required. Some of these doubts are related to species such as *B. barbus* and *B. petenyi* or the supposed relationships between *B. euboicus* and *B. cyclolepis*. Nevertheless, phenomena such as recent diversification or the possible hybrid origin of some of the species analysed should be kept in mind since our phylogenetic analysis was based on mitochondrial genes and therefore reflected a matrilineal phylogeny. Even when clear morphological differences serve to distinguish between species, the homogeneity of mitochondrial DNA could reflect the reticulate evolution of these taxa. Ancestral polymorphism followed by lineage sorting may also explain differentiation of the mitochondrial markers in some lineages.

Barbus euboicus has been considered as being morphologically related to *B. cyclolepis* (Economidis & Banareescu 1991). Our genetic results showed *B. euboicus* to cluster with *B. peloponnesius*. Moreover, the divergences found within *B. cyclolepis* could be consistent with a specific status for at

least three out of the four subspecies described for this species: *B. c. cyclolepis*, *B. c. strumicae* and *B. c. sperchiensis*.

With regard to the putative subspecies of *B. barbus*, i.e. *B. b. thessalus* and *B. b. macedonicus*, these should both be considered as *B. macedonicus* according to the present findings and as already suggested elsewhere (see Kottelat 1997).

The Greek populations of *B. petenyi* analysed may not be included in this species but rather represent an unnamed taxon that is phylogenetically related to the Italian *B. caninus*.

(b) Phylogeny

The proposed phylogenetic relationships derived from complete ATPases 8 and 6 and cytochrome *b* gene sequences and from intergroup divergence indices (figure 1) support the subdivision of the genus *Barbus sensu lato* into nine mitochondrial lineages. The tetraploid subgenera *Barbus* and *Luciobarbus* are two of these. The monospecific genus *Aulopyge* appeared as the sister group of these subgenera. The fourth lineage proposed included all the hexaploid barbels analysed and *Varicorhinus*. The fifth and the sixth groups were formed by South African tetraploids. The African diploid barbels constitute the last three phylogenetic groups. Despite their appearance as a monophyletic cluster, their division into three groups is warranted on the grounds of divergence values. Moreover, the Asiatic diploid *Puntius* was also found to show differentiated clusters.

The Iberian species within the *Luciobarbus* lineage were basal to North African and Near Eastern species (such as *Barbus brachycephalus*). The absence of phylogenetic structure within the North African group lends support to the possibility of explosive radiation at a time of complex geological palaeohistory. Indeed, one Algerian species was included in the Iberian cluster, possibly reflecting Tertiary contacts between Europe and Africa (Machordom & Doadrio 2001). A further relationship within *Luciobarbus* that should be noted is that shown by the Greek taxa, which appeared as paraphyletic. The possibility that the Greek peninsula was colonized more than once has already been postulated based on data from other vertebrate taxa (Beerli *et al.* 1996).

The subgenus *Luciobarbus* appeared as the sister group of the subgenus *Barbus* within the Palearctic tetraploids. Within this last subgenus, two different species from Greece (*B. macedonicus* and *B. petenyi*) appeared as sister species to taxa inhabiting Northern Italy (*B. plebejus* and *B. caninus*, respectively). Given the divergence shown between these species, a more recent contact than that indicated by other groups or a greater capacity for dispersion can be postulated. Thus, contact with large European drainage systems via the Danube (Economidis & Banareescu 1991) and Circummediterranean dispersion after the last glaciations could explain this lack of biogeographic structure. Similar relationships were also suggested for North Mediterranean fauna such as *Leuciscus cephalus* and attributed to relicts of the ice ages (Durand *et al.* 1999).

Aulopyge huegelii was the basal taxon of the genus *Barbus sensu stricto*. Assigned to a similar position through the phylogenetic analysis of barbels from the North Mediterranean by Tsigenopoulos & Berrebi (2000, p. 177), it was concluded that this species might be considered as 'part of

the first migration wave that reached the Mediterranean region'.

The relationships between the Palaearctic tetraploid genus *Barbus* and the hexaploid barbels were not well supported by all the analyses (the bootstrap value for maximum parsimony was only 53%), but their sister-group condition seemed to be most probable (with bootstrap values of 94% for neighbour joining and 74% for maximum likelihood). The hexaploid lineage, which includes species of wide geographical distribution, showed scarcely any biogeographical structure, with Central and South African species differentiated from those of the Maghreb and Near East. As we saw before, this last biogeographical assemblage was also found in the *Luciobarbus* subgenus. With regard to the clustering of the Central and South African species, interconnection between large African basins may have favoured genetic flow between these hexaploid taxa. Once again, a species from a different genus, of the same ploidy level but strongly differentiated, appeared as basal (*V. maroccanus*).

Relationships between two other monophyletic groups, the South African diploids and tetraploids, which showed more intragroup divergence than the other clusters that were recovered, could point to the singularity of these taxa and to a greater degree of isolation. It is possible that the polyploidization event that led to the two South African tetraploid groups occurred in this area. Nevertheless, and in particular with reference to the diploid species, the number of specimens analysed was insufficient for drawing clear conclusions.

(c) *Evolutionary hypotheses*

The mitochondrial DNA genes screened served to recover some monophyletic lineages with a sufficiently high degree of confidence that the relationships between them could be established. Different polyploidization events seem to have occurred independently in different lineages, thus permitting consideration of the ploidy character as homoplastic. The data provided by mitochondrial DNA related to this phenomenon require further investigation, mainly with respect to whether its evolutionary pattern is fitted more to a linear or a saltational model.

Based on the presence of polyploids, two hypotheses might be suggested for the evolution of this vast and complex group. In the first hypothesis, the rate of evolution for polyploids may be faster and the divergences obtained greater than those for the diploids (Ohno 1970). This evolutionary rate will be even faster in allopolyploidy, since reticulate evolution takes place and the different information of both parents would coexist in the new organism (Li 1983). With regard to mitochondrial DNA and mainly in allopolyploids, concerted evolution might be expected, forcing the adaptation of the mitochondrial genome to the 'new' nuclear information (Wendel 2000). In this case, a rapid change in mitochondrial genes would also be expected.

The number of copies of the mitochondrial genome might be greater than in a diploid at a particular moment, but will not be of different origin, as occurs for the nuclear genome in allopolyploids. Thus, in allopolyploidy, the same organism might have intermediate morphological characteristics between both

parents, two differentiated, coexisting nuclear genomes and a single maternal mitochondrial genome.

According to the second hypothesis, the mechanisms of polyploidization might act differently in nuclear and mitochondrial genomes and subsequent evolutionary rates would therefore also be different.

If we compare divergences based on allozymic data (Agnès *et al.* 1990; Karakousis *et al.* 1995; Machordom *et al.* 1995, 1998; Doadrio *et al.* 1998) with the mitochondrial divergences obtained here, nucleotide divergences are up to ten times smaller than allozymic divergences. Thus, even if nuclear–cytoplasmic coadaptation is needed for the survival of the new polyploid organism, this coadaptation does not imply accelerated mitochondrial evolution and, therefore, our polyploid organisms can be considered as evolutive mosaics.

With regard to the origin of these polyploidies and given known karyological data (e.g. Sola *et al.* 1981), the theory lending support to a probable polyploid origin for the Cyprinidae family (Collares-Pereira & Coelho 1989) may be viewed as inconsistent. We believe that the most-parsimonious explanation for the evolution of cyprinids has to consider diploidy as ancestral, with subsequent duplications giving rise to the current polyploid lineages. At least three polyploidization events may have taken place among the taxa analysed here.

One polyploidy event will have involved the tetraploids from South Africa (clade III in figure 1). They are the only tetraploids directly related to diploids, particularly to the diploid African species.

A second tetraploidization event might have occurred in a putative common ancestor of the Palaearctic tetraploid and hexaploid barbels (clades I and II in figure 1). The tetraploidy of this putative ancestor may be discussed in the light of two karyological theories. Guégan *et al.* (1995) considered the autopolyploid origin of African hexaploid species without discussing which type of phenomenon may have caused the autopolyploidy. Oellermann & Skelton (1990) considered an allopolyploid route to hexaploidy to be more plausible. This last hypothesis minimizes the number of duplication events and might be more congruent with our phylogenetic analyses, considering that allozyme studies support allopolyploidy for the Palaearctic tetraploid clade (Machordom 1992). Golubtsov & Krysanov (1993) also suggested the existence of a common tetraploid ancestor for *Varicorhinus* and other African hexaploid barbels.

The third polyploidization event would have occurred in *Varicorhinus* and the African hexaploid barbels. Crossing the tetraploid ancestor of the Palaearctic tetraploid and hexaploid clade with other diploid barbels would have generated an unstable triploid that, after spontaneous chromosome doubling, may have given rise to the stable hexaploid ancestor of the hexaploid lineage, as proposed by Oellermann & Skelton (1990).

To summarize, we propose that the minimum requirement for explaining the evolution of the groups analysed would be at least one common tetraploid ancestor for the South African tetraploid species, another for the genera *Barbus sensu stricto* (Palaearctic tetraploids), *Aulopyge* and *Varicorhinus* and the hexaploid barbels and a subsequent hexaploid ancestor for this last group of hexaploid species (including *Varicorhinus*).

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