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Short note - Nota breve

Preliminary study on phylogeny of *Barbus* genus in the Po River

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RIASSUNTO - *Studio preliminare della filogenesi del genere Barbus nel fiume Po* - Il genere *Barbus* è caratterizzato da una complessa struttura tassonomica. Nel distretto ittiogeografico Padano-Veneto sono state identificate due specie autoctone: *Barbus caninus*, specie prettamente reofila, e *Barbus plebejus*, reofilo anch'esso, ma distribuito più a valle rispetto al primo; entrambi risultano presenti nel Fiume Po. Al fine di valutare le relazioni filogenetiche di queste due specie di barbo è stato effettuato uno studio preliminare tramite il sequenziamento del gene del citocromo b (1103 pb) del DNA mitocondriale. Gli alberi filogenetici ottenuti sono stati integrati e confrontati con i dati molecolari disponibili in GenBank.

Key words: *Barbus*, phylogeny, mtDNA, Po River
Parole chiave: *Barbus*, filogenesi, DNA mitocondriale, fiume Po

1. INTRODUCTION

The genus *Barbus* is characterised by a complex taxonomical structure, due to the high number of species and its morphological plasticity; it counts more than 25 species in Europe, displaying different ecological preferences. Several authors suggest that ecological traits could be of great importance in defining groups within the genus (Tsigenopoulos & Berrebi 2000). In the Padano-Venetian ichthyogeographic district of the Italian peninsula, two autochthonous species are identified: *Barbus plebejus* Bonaparte, 1839 and *Barbus caninus* Bonaparte, 1839 both inhabiting the Po River. Even if they are rheophilic species, *B. plebejus* belongs to the group of large-size barbels, living in the lower parts of rivers, whilst *B. caninus* is a small-size rheophilic barbel that colonizes the upper parts of rivers (Tsigenopoulos *et al*. 1999; Tsigenopoulos & Berrebi 2000).

In this preliminary study, we used mitochondrial DNA sequences of the cytochrome b gene (cyt*b*) (i) to assess their phylogenetic relationships with other European barbels; (ii) to evaluate evolutionary radiation of the genus, considering the autoecology of different species; (iii) to estimate intra and interspecific genetic differentiation among specimens of *B. caninus* and *B. plebejus*, sampled in the Po River. The sequencing of a fragment of cyt*b* gene of some *Barbus sp*. specimens, sampled in the Po River and belonging to an unidentified species, was then performed to establish their taxonomic identity.

2. METHODS

All barbels were collected in the Po River, in particular 11 specimens of *B. caninus* were sampled in Sanfront (CN), 7 specimens of *B. plebejus* in Cardè (CN) and Villafranca Piemonte (TO) and 3 specimens of *Barbus sp*. were collected in Verrua Savoia (TO).

For three specimens of *B. caninus* and *B. plebejus* a 1103 bp fragment of the cyt*b* gene was amplified by PCR (Polymerase Chain Reaction) in two step, using the primer pairs: H15803 + L16461 and L15267 + H15891 (Briolay *et al*. 1998). For the remaining specimens a 487 bp fragment of the cyt*b* gene was amplified using primers L15267 and H15891 (Briolay *et al*. 1998). All PCR amplifications were performed as reported by Tsigenopoulos *et al*. (2002). PCR products were purified, then both strands were sequenced on an automated sequencer using the same PCR primers. Sequences were aligned using the program ClustalX (Thompson *et al*. 1997) and manually checked to solve ambiguous bases.

Phylogenetic analyses on haplotypes of 1103 bp were performed including sequences of *Barbus sp*. available in GenBank (Appendix 1). WinModelTestXP (Posada & Crandall 1998) was used to find the best DNA evolution model that fitted these sequences, then phylogenetic reconstruction was performed using Neighbour-Joining (NJ) and Maximum Parsimony (MP), by PAUP 4.0β10 software (Swofford 2003). Phylogenetic trees were rooted with an haplotype of *Cyprinus carpio* (Acc. N° AB158807) (Tsigenopoulos &

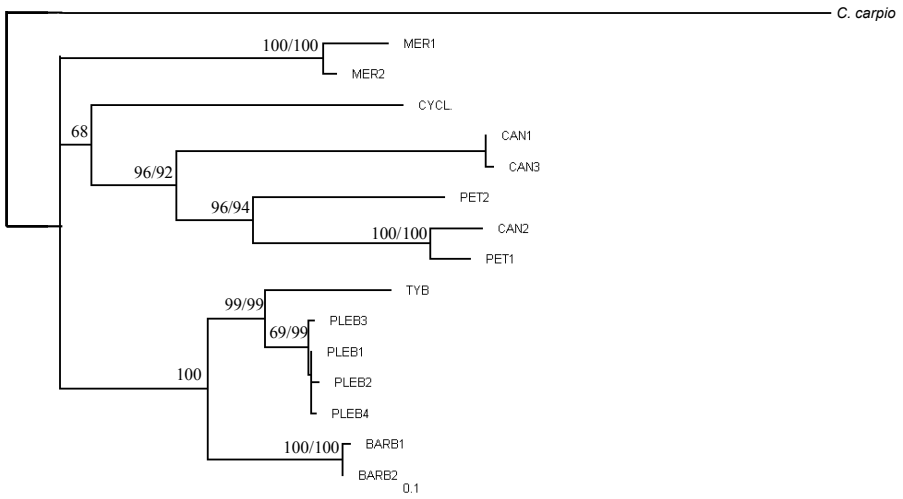

Fig. 1 - Phylogenetic tree of 15 haplotypes of *Barbus* species reconstructed from cyt*b* 1103bp sequences, estimated by NJ method, employing TAMNEI distances. Numbers above branches represent the bootstrap values obtained for 1000 replications for NJ and MP methods, respectively.
Fig. 1 - Albero filogenetico ricostruito tramite metodo NJ, utilizzando il modello TAMNEI, con 15 aplotipi di specie del genere Barbus*, a partire da sequenze di 1103 pb del citocromo* b*. I numeri al di sopra dei rami indicano i valori di bootstrap per i metodi NJ e MP, rispettivamente, ottenuti per 1000 repliche.*

Berrebi 2000). Nonparametric bootstrap test with 1000 replicates provided the reliability of the inferred phylogenies.

Genetic differentiation between sequences of 487 bp was examined using nucleotide diversity (π) and haplotype diversity (*h*) indices calculated by means of DnaSP 3.0 (Rozas *et al*. 2003). Then pairwise genetic distances among our haplotypes and the others from GenBank (Appendix 1) were calculated as p-distances by PAUP 4.0β10 (Swofford 2003).

The taxonomic identity of the three specimens belonging to *Barbus sp*. was inferred sequencing, with primers H15803 and L16641, a fragment of 570 bp, using the same protocol of the previous reactions and aligned with our haplotypes.

3. RESULTS

Extracted sequences of 1103 bp showed three distinct haplotypes for *B. plebejus* (PLEB1, PLEB2 and PLE3) and only one for *B. caninus* (CAN1). The Tamura and Nei (TAMNEI) was the best DNA evolution model that fitted data among the 56 tested. Phylogenetic reconstructions using the NJ, using TAMNEI model, and MP methods showed similar topologies supported by good bootstrap values. Trees displayed two principal traits: the strong partitioning between the groups of the large-size and the small-size barbels, that form well supported groups, and the distance of *B. meridionalis* from the other small rheophilic species, that formed a separated group. Relationships in the group of small-size barbels were not well defined and the trees displayed a close relationship between *B. caninus* and *B. petenyj*. The position of the haplotype CAN2 remained problematic in the two reconstructions (NJ and MP); always it was related with *B. petenyi*, a similar pattern was found also in Tsigenopoulos *et al*. (2002).

Increasing the number of samples, the sequences for the first 487 bp of cyt*b* gene of *B. caninus* (n=8) and *B. plebejus* (n=4) specimens revealed five distinct haplotypes: two for *B. plebejus* (PLEB1 and PLEB56) and three for *B. caninus* (CAN1, CAN23 and CAN25). Haplotype diversity (*h*) showed high values for both the species, but nucleotide diversity (π) was very low, with values close to zero (Appendix 2). In agreement with Tsigenopoulos *et al*. (2002) the mean genetic distance among haplotypes of *B. caninus* and *B. plebejus* was estimated to be 8.6% by p-distance. The mean intraspecific genetic distance among the identified haplotypes of *B. caninus* and *B. plebejus* was 0.2% for both species. Combining data with available haplotypes in GenBank, B. plebjus keeps low genetic distance, while *B. caninus* shows much higher levels of intraspecific genetic variation (3%) (Appendix 3).

For the three *Barbus sp*. specimens it was not possible to obtain the entire cyt*b* sequence; the partial sequence of 570 bp obtained and aligned to the other ones showed concordance with the *B. plebejus* PLEB1 haplotype.

4. DISCUSSION

Three well supported clades come out from phylogenetic reconstruction. This result shows an evident evolutionary dichotomy among barbels that colonize the lower parts of rivers (*B. plebejus*) and those inhabiting the upper parts (*B. caninus*). *B. meridionalis* forms a group very distant from the other ones. This result suggests that ecological traits of each analysed species are supported by molecular data. These preliminary results confirm that *B. caninus* is not a subspecies of *B. meridionalis*, present in France, because it is more related to other small rheophilic barbels as *B. petenyj*. Phylogenetic relationships among small-size barbel species appear to be more related with their geographic distribution rather than their morphological traits.

The mean intraspecific genetic distance of *B. caninus* and *B. plebejus* was calculated comparing haplotypes with others of the North-East Italy available in GenBank. The high divergences found for *B. caninus* haplotypes suggest the autoecology of the species have favoured the isolation of the popuplations. This situation could allow the development of different evolutionary lineages and an high genetic variability, this hypothesis agrees with Tsigenopoulos *et al*. (2002). On the other hand *B. plebejus*, inhabiting the lowland part of rivers, could sustain an higher genetic flow, reducing distance among populations. Strong differences among *h* and π seems to be either ascribable to recent demographic expansion, supporting the growth of haplotypes number (*h*), but characterized by a low genetic differentiation (π) due to a post-glacial colonization of the Po River operated from a low

founders number (Grant & Bowen 1998), or linked to the genetic drift owing to the autochthonous species demographic reduction that is occurring in the Po River.

The taxonomic identification of *Barbus sp.* shows that its sequences are the same of haplotype PLEB1. This result should be studied in detail, cause even if the molecular marker used is a good tool to investigate genetic distances it can't detect possible hybridization processes. In the future it seems necessary to study this matter to identify eventually hybridising cases also with allochthonous species.

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Appendix 1 - Species list of individuals considered in the phylogenetic analyses. Samples ID number and haplotypes available in GenBank with their accession number; primers used for sequencing; haplotypes found for sequences of 1103 bp; haplotypes found for sequences of 487 bp and locality. * N.D.: no data available.
*Appendice 1 - Esemplari utilizzati per le analisi filogenetiche e lista degli aplotipi disponibili in GenBank con relativo numero di acquisizione. Sono riportati anche i primers utilizzati per il sequenziamento; gli aplotipi ottenuti su sequenze di 1103 pb e su sequenze di 487 pb e la provenienza degli esemplari utilizzati. * N.D.: dati non disponibili.*

Species	ID Number or Acc. Num.	Primers used for sequencing	Haplotype (1103 bp)	Haplotype (487 bp)	Locality
B. caninus	04-14	L15267 + H15891 - H15803 + L16461	CAN1	CAN1	Po R. North-West Italy
	04-15	L15267 + H15891 - H15803 + L16461	CAN1	CAN1	Po R. North-West Italy
	04-16	L15267 + H15891 - H15803 + L16461	CAN1	CAN1	Po R. North-West Italy
	04-17	L15267 + H15891		CAN23	Po R. North-West Italy
	04-20	L15267 + H15891		CAN1	Po R. North-West Italy
	04-21	L15267 + H15891		CAN23	Po R. North-West Italy
	04-22	L15267 + H15891		CAN23	Po R. North-West Italy
	04-23	L15267 + H15891		CAN23	Po R. North-West Italy
	04-25	L15267 + H15891		CAN25	Po R. North-West Italy
	04-26	L15267 + H15891		CAN1	Po R. North-West Italy
	04-27	L15267 + H15891		CAN1	Po R. North-West Italy
	AF112124	L15267 + H15891 - H15803 + L16461	CAN2	CAN2	Astico R., Northern Italy
	AF287425	L15267 + H15891 - H15803 + L16461	CAN3	CAN3	Judrio R. Northern Italy
B. petenyi	AF090788	M 13	PET1	PET1	Aliakmon R. Northern Greece
	AF112127	L15267 + H15891 - H15803 + L16461	PET2	PET2	Poprad R., Slovakia
B. plebejus	05-106	L15267 + H15891 - H15803 + L16461	PLEB1	PLEB1	Po R. North-West Italy
	05-107	L15267 + H15891 - H15803 + L16461	PLEB2	PLEB1	Po R. North-West Italy
	05-108	L15267 + H15891 - H15803 + L16461	PLEB3	PLEB1	Po R. North-West Italy
	06-53	L15267 + H15891		PLEB1	Po R. North-West Italy
	06-54	L15267 + H15891		PLEB1	Po R. North-West Italy
	06-55	L15267 + H15891		PLEB1	Po R. North-West Italy
	06-56	L15267 + H15891		PLEB56	Po R. North-West Italy
	AY004750	L15267 + H15891 - H15803 + L16461	PLEB4	PLEB4	Roggia, Udine, Northern Italy
B. meridionalis	AF045977	M 13	MER1	MER1	N.D.*
	AF112130	L15267 + H15891 - H15803 + L16461	MER2	MER2	Aubaygue R., Southern France
B. cyclolepis	AF112134	L15267 + H15891 - H15803 + L16461	CYCL	CYCL	Turkey
B. tyberinus	AF397300	N.D.*	TYB	TYB	Ombrone R., Central Italy
B. barbus	AY331017	L15267 + H15891 - H15803 + L16461	BAR1	BAR1	Danube R., Yugoslavia
	AY331020	L15267 + H15891 - H15803 + L16461	BAR2	BAR2	Danube R., Bulgaria

Appendix 2 - Haplotypes frequency found in the analysed samples of *B. caninus* and *B. plebejus*. Sequences are referred to a 487bp fragment. Values of haplotype diversity (*h*) and nucleotide diversity (π) are recorded for each species.
*Appendice 2 - Elenco degli aplotipi riscontrati in due popolazioni di B. caninus e B. plebejus. Le sequenze sono relative ad un frammento di cty*b *(DNA mitocondriale) lungo 487 pb. Sono specificati anche i valori di haplotype diversity (*h*) e nucleotide diversity (*π*).*

HAPLOTYPES	Haplotype frequencies					N° tot samples	*h* ± dev. st.	π
	CAN23	CAN25	CAN1	PLEB1	PLEB56			
B. caninus	0.36	0.1	0.54			11	0.618±0.104	0.001
B. plebejus				0.9	0.1	7	0.296±0.196	0.0006

Appendix 3 - Genetic distances between 16 *Barbus* haplotypes based on 487 bp, estimated using p-distance.
Appendice 3 - Distanze genetiche di 16 aplotipi di specie del genere Barbus *su sequenze di 487 pb, calcolate usando il metodo p-distance.*

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	MER1															
2	MER2	0.821														
3	CAN1	9.856	9.446													
4	CAN3	9.856	9.446	0.000												
5	CAN23	10.062	9.651	0.205	0.205											
6	CAN25	9.651	9.240	0.205	0.205	0.411										
7	CAN2	8.419	8.419	6.982	6.982	7.187	6.776									
8	PET1	8.830	8.830	6.366	6.366	6.571	6.160	1.232								
9	PET2	7.803	7.803	8.624	8.624	8.830	8.419	5.749	4.928							
10	CYCL.	8.830	8.419	8.624	8.624	8.830	8.419	8.008	8.008	7.598						
11	PLEB56	8.214	7.392	8.624	8.624	8.419	8.830	8.624	9.035	7.803	7.598					
12	PLEB1	8.008	7.187	8.419	8.419	8.624	8.624	8.419	8.830	7.598	7.392	0.205				
13	PLEB4	8.214	7.392	8.419	8.419	8.624	8.624	8.214	9.035	7.803	7.598	0.411	0.205			
14	TYB	9.035	8.214	9.856	9.856	10.062	10.062	9.856	9.856	8.419	9.035	2.875	2.669	2.875		
15	BARB1	8.419	8.008	8.214	8.214	8.419	8.419	9.035	9.035	7.598	6.571	3.901	3.696	3.901	4.928	
16	BARB2	8.419	8.008	8.214	8.214	8.419	8.419	9.035	9.035	7.598	6.571	3.901	3.696	3.901	4.928	0.000