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Phylogeography of Italian barbels (Cyprinidae, *Barbus*) inferred by mitochondrial and nuclear markers

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ABSTRACT

Species of the genus *Barbus*, being primary freshwater fishes intolerant of salt water, are of great value for biogeographic studies since their dispersal strictly depends on geological evolution of the landmasses (i.e. catchments watershed, mountain chains and fluctuations of sea level). In Italian peninsula four specie are formally recognized: *B. caninus, B. balcanicus, B. plebejus* and *B. tyberinus*. Their genetic relationships were assessed using both mitochondrial and nuclear markers. The study was carried out as first developing new nuclear primers for the S7 ribosomal protein and the Growth hormone genes (Gh); then performing a SNPs characterization of these loci on 18 populations (264 specimens in total). Results from nuclear sequences were then compared with those from partial sequences of the Cytochrome *b* mitochondrial gene (733 bp). Recovered phylogenies were congruent with the current morphology-based systematic and taxonomy. Results highlighted the close relationships between species belonging to the fluvio-lacustrine ecological group: *B. plebejus* and *B. tyberinus* and the high genetic distance between species belonging to the riverine group: *B. caninus* and *B. balcanicus*. Moreover findings were congruent with hypotheses of partial permeability of principal biogeographic barriers (Alpine and the Apennine chains) to freshwater fish fauna.

Successively the influence of different ecological preferences on gene flow was tested for *B. caninus* and *B. tyberinus* on 6 and 7 populations respectively. Results pointed out that the riverine *B. caninus* has higher structured populations than *B. tyberinus*, probably due to the different dispersion ability and the different habitat colonized. Moreover, for the first time, molecular evidences were shown about hybridization events occurring between *B. caninus* and *B. plebejus*, *B. tyberinus* and *B. barbus*.

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CHAPTER 1

Introduction

1. Background

The concept of biodiversity was coined by W. G. Rosen in 1985 during the "National Forum on Biodiversity", summarizing the terms "biology" and "diversity", to indicate the variety of life. At nowadays an high number of definitions exists, but following the "Convention on Biological Diversity", biodiversity is defined as: "the variability among living organisms from all sources including, *inter alia*, terrestrial, marine and other aquatic ecosystems and the ecological complexes of which they are part; this includes diversity within species, between species and of ecosystems". This definition describes biodiversity at three levels: genetic diversity, representing the differences among individuals belonging to the same species; (ii) species diversity, referred to the numbers in a particular area; (iii) ecosystems diversity, representing the variety of ecosystems present in a biosphere (Feest *et al.*, 2010).

Biological diversity is of fundamental importance for the functioning of all natural ecosystems, and by extension for the ecosystem services that nature provides free of charge to human society. Living organisms play central roles in the cycles of major elements (carbon, nitrogen, and so on) and water; diversity is important because these cycles require numerous interacting species. General interest in biodiversity has grown rapidly in recent decades, in parallel with the growing concern about nature conservation as a consequence of accelerating rates of natural habitat loss, habitat fragmentation and degradation, and resulting extinctions of species (Frankham *et al.*, 2002).

The geographic distribution of biodiversity is not homogeneous and depends on several factors as climate, altitude, soil composition, the presence of other species. Diversity consistently measures higher in the Equatorial regions and generally tends to decrease moving towards polar regions. Biogeography investigates the geographical distributions of *taxa* or populations on global and regional scales, and the processes that led to actual patterns in the light of evolutionary theory.

Recent advances in genetic field and the development of a growing number of molecular markers, with their easy widespread applications, have provided new approaches to the analysis of biogeographic patterns and underlying evolutionary processes, leading to the rise of a new discipline called phylogeography.

As Avise *et al.* (1987) conceived it, phylogeography is the phylogenetic analysis of geographically contextualized genetic data for testing hypotheses regarding the causal relationship among geographic phenomena, species distributions, and the mechanisms driving speciation (Hickerson *et al.*, 2010).

The formal definition of phylogeography is the end of a gestation begun in the middle ‘70s, with the early applications of restriction sites maps for animal mitochondrial DNA (Brown and Vinograd, 1974; Brown and Wright, 1975; Upholt and Dawid, 1977), the definition of theories on gene genealogies (Watterson, 1975) and the development of statistical analysis to elaborate the new kind of data (Upholt, 1977). In the last decades an exponential growth of phylogeographic studies arose, in which new applications, new theories and new statistical analysis were created or adapted from other disciplines, allowing to identify dispersion routes, geographic isolation among *taxa* or hybrid zones. The phylogeographic inferences contributed to focus into evolutionary pressures that acted active role on speciation events, like hybridization or occurrences of introgression (Gonzales-Rodriguez *et al.*, 2004; Hewitt, 2001). From a geographical point of view four models of natural speciation can be defined, based on the extent to which diverging populations are geographically isolated from one to another: i) allopatric speciation, characterised by a complete geographical separation of a population into two parts by the interposition of extrinsic barrier (Mayr, 1942); the resulting populations, between which the gene flow is interrupted, evolve independently becoming two distinct species. There are many kind of barrier that can separate two populations, classified in ecological, ethological, and geographical. The speciation in allopatry caused by geographical barriers is considered the most common by the majority of evolutionary biologists. ii) Peripatric speciation, characterised by a colonization of a new territory by a group of individuals deriving from a population that remain isolated and successively evolve independently (Provine, 2004). It is similar to allopatric speciation, with two distinctive traits: the colonizing population is smaller than the original population, and the new territory did not host the evolving population before its arrival. iii) Parapatric speciation, characterised by variations in mating frequency within a continuous geographical area (Smith, 1965). In this model there is not an extrinsic barrier that block the gene flow, but intrinsic low dispersal capabilities, that lead individuals to mate with their neighbour, and different evolutionary pressure across the population range could create evolutionary divergence. iv) Sympatric speciation, characterised by the evolution of two different species from a single population without geographical segregation (Poulton, 1903). The interrupted gene flow among two or more groups of individuals that occupy the same area, and that become different species, can be explained by ethological, ecological or genetic reasons, e. g. the duplication of the genome (polyploidy) in some individuals can produce a new species in the same geographic region of the parental population.

Recently, in the phylogeographic studies, speciation hypotheses are more and more tested through the application of genetic markers: polymorphic characters associated in a unequivocal way to a portion of the genome.

Nowadays two big category of genetic markers are used: nuclear and mitochondrial. They posses different and unique features; the former is transmitted biparentally and interlocus recombination should mean that most nuclear markers provide replicate estimates of a common demography, whereas the latter is transmitted maternally as a single nonrecombinig block (Eytan and Hellberg, 2010). Mitochondrial and nuclear markers are able to complement each other. The smaller effective population size of mitochondrial DNA (mtDNA) should allow it to capture signals of demographic events that cannot leave their footprints on the larger effective populations size of nuclear markers. The strength of nuclear DNA (ncDNA) lies in its ability to provide replicate samples of the underlying demographic history affecting the genome of an organism as well as replicate of the coalescent process (Eytan and Hellberg, 2010). The combination and the congruence between both classes of marker allows to identify clades and estimate parameters, such as migration rates and hybridisation events (Lee and Edwards, 2008).

Although the phylogeography offers new tools to reconstruct and shed light on evolutionary relationships among different *taxa*, it is useful reminding that there are many factors that influence the geographic distribution of biodiversity and dispersion of living organisms that can be divided in two categories: abiotic and biotic (Monge-Najera, 2008). All these impediments to movement are referred to as biogeographic barriers (Rahel, 2007). The dominant abiotic factor in determining the composition of regional faunas and in promoting endemism is the presence of geographic (or natural) barriers (Cox and Moore, 1980; Ricklefs and Schluter, 1993). In fact, geographic barrier promotes speciation because it can subdivide a population, principally according two recognized patterns: forming and growing itself inside the areal distribution of the interested population even to completely separate this in two or more parts, or because the areal of population shifts around the geographical barrier becoming disjoined.

The geographical barriers are principally related to geological events ascribable to the movement of tectonic plates, generating volcanic activity, mountain building and oceans formation. Other events, on a reduced scale, can influence the formation and the modification of geographical barriers, as erosion and deposition, that are able to modify the landscape.

Others abiotic factors that played a crucial role in shaping the actual distribution of faunas have been the climate changes. The last important event of climate changing was recorded in Pleistocene, when different glaciations events occurred in the north hemisphere (Hewitt, 2000; Wang, 1999; Šlechtová, 2004). These drastic historical events determined the spreading of the ice cap, provoking a shift and a compression of the biomes toward the equator. The great amount of water, captured by the glaciers and the reduction of the seawater volume due to the low temperature, produced a decrease in the sea level causing the emersion of several land bridges in large parts of the world (Rohling *et al,*. 1998). During this era some species went extinct in large parts of the original range, some other dispersed to new locations.

In the South European continent, that shows high level of biodiversity, several temperate European *taxa* survived through glacial cycles in "glacial refugia", located especially in the three southern peninsulas: Iberian, Balkan and Italian one (Taberlet *et al.* 1998; Hewitt 1999, 2000). These refugia have been traditionally seen as homogeneous sources of colonizers of northern latitudes after glacial periods, even though recently a more complex and heterogeneous picture was depicted evidencing the presence of "refugia within refugia" (Hewitt, 2004; Gomez and Lunt, 2007; Gante, 2009a). The northward expansion following the glacial withdrawals, was strongly influenced by the presence of geographical barriers, like the main European mountain barriers, or because of the deep differences among the glacial refugium in which they survived during the glaciations and the new environment in the making. In this case biotic barriers, in particular the biological features (autoecology) and interactions with other species played a pivotal role in the recolonization ability of *taxa* (Hewitt, 1999).

In this enlarge context valid tools to reconstruct the biogeographic development of a particular region, related to their capability to link their distribution with the historical evolution of the landmass, are the freshwater fishes. In absence of human traslocations, their phylogeographies reflect historical causes more closely than those of terrestrial species (Bernatchetz and Wilson, 1998). The primary freshwater fishes are unable to disperse trough sea water and are restricted to the hydrographical networks of drainage basins (Reyjol *et al.*, 2007). Colonization between basins can only take place on a long temporal scale during the evolution of an hydrographic basin through no more than four modalities: i) river capture (Waters *et al.* 2001, Strange and Burr 1997): occurs when a stream or river drainage system is diverted from its own bed, and flows in the bed of a neighbouring stream; ii) river confluence of downstream courses (Bermingham and Avise 1986, Durand *et al.* 1999b): the sea level

change in time; in particular during glaciations, was lower that the present, and rivers flowing in shallow seas could be in connection through a common mouth; iii) sea dispersal in case of low salinity conditions (Bianco, 1990): a typical example is constituted by the “Lago Mare” phase of the Mediterranean sea, occurred at the and of Messinian age; and iv) proglacial lakes: during the melting phase of glacial cycles, melt water from retreating glaciers formed proglacial lakes; these, dammed and fed at the same time by the glaciers themselves, followed the glacier fronts, changing in size and position and passing sometimes from an hydrogeographic system to another. Aquatic species able to exploit these geographical elements received high opportunities to disperse over wide ranges (Behrmann-Godel *et al.*, 2004, Bernatchez and Wilson, 1998). Such dispersion mechanisms are highly restricted and, as a consequence, relationships among fish lineages also reflect relationships between different areas, minimizing one of the main difficulties (i.e. dispersal) in reconstructing the past biogeographical development of an area (Ronquist, 1997).

2. Hydrographic and ichthyogeographic structure of Italian peninsula

The Alps, the highest mountain chain in Europe with an East-West orientation, isolated Italian peninsula from the main Central European drainage systems such as Danube, Rhine and Rhone, favouring the develop of an high number of endemic freshwater *taxa* (Bianco, 1990). However events of permability among the two sides of Alps are proved by the areal distribution of some species, which are present in more than one region. Permeability of alpine chain is identified for both fish (*Cottus gobio*, Šlechtová *et al.* 2004, *Telestes spp.*, Salzburger *et al.* 2003) and crayfish (Grandjean *et al.*,1998); the distribution of other species as *Lota lota* and *Perca fluviatilis* lead to the hypothesis of a contact among Italian and transalpine districts.

Alpine chain is not the only geographical barrier in the Italian peninsula, also the Apennines, even though present a lesser altitude, can constitute an impediment in dispersal for several species, particularly to those which dispersion is strictly related to the morphology of the landscape, such as aquatic species (Bianco, 1995b). Orientated in a South-North direction, Apennines divide basins drain the Adriatic Sea from those that drain the Tyrrhenian Sea.

Alpine and Apennine chain delimits the Po River basin. Po River is the largest Italian watercourse and drains a large area in northern Italy until the Adriatic sea. In addition a series of smaller rivers, belonging to the same system, drain the Adriatic sea coasts as well (Tsigenopoulos *et al.*, 2002). Among neighbouring regions, the territory of Slovenia is considered to belong to this hydrographic system. According to the intensity of the glaciation events in the Pleistocene, it is known that some of the Adriatic rivers came in confluence allowing an exchange of ichthyofauna among them (Marchetto *et al.*, 2010).

Concerning the Tyrrhenian costs, rivers that drain in this part of Italy are quite different from the ones of the Adriatic slope. These rivers are appreciably shorter, even if there are evidences of contact events, these are likely to have occurred in the upper or middle watercourses, where hydrogeographic structures appear conducive for historical transitory river captures (Marchetto *et al.*, 2010). Because the large distance among main rivers in this region and the bathymetric profile of the Tyrrhenian Sea, a connection via downstream river confluence when the sea level was substantially lower than the present, appear unlikely.

The hydrographic structure of Italian peninsula modulated the allopatric distribution and the diversification of various freshwater *taxa*. The distribution of the primary freshwater fishes allowed the identification of two ichthyogeographic districts on the base of the presence and distribution of endemic cyprinid species (Bianco, 1990) (fig. 1.1; tab. 1.1): i) Padano-

Venetian (PV), including river basins drain the upper part of Adriatic Sea between the River Vomano in Central Italy and the River Krka in Dalmatia. This district corresponds to the basin of Po River during the last glacial maximum; in fact in that period the eustatic level of the Mediterranean Sea was about 100-130 m lower than the present, and the mouth of Po River was near the meso-Adriatic ditch. The Padano-Venetian district confines with the Southern France district in the west and with the Danubian district in the North and in the East, with the alpine chain as border. ii) Tuscano-Latium, including the drainages flowing into the Tyrrhenian Sea between the Serchio River and the Tiber River. Tyrrhenian Sea presents a vertical profile steeper that Adriatic Sea, and the lowering of the sea level didn't influenced the distribution of the freshwater species. The rivers comprised in this district (Serchio, Arno, Ombrone and Tiber) were however repeatedly connected and isolated from the lower Miocene to historic times, so their native freshwater fauna is identical (Bianco 1995b).

Figure 1. 1 Main ichthyogeographic districts of Italian peninsula according to Bianco (1995b)

Table 1. 1 Endemic freshwater fish species in Italy in the two principal ichthyogeographic districts

Padano-Venetian	Tuscano-Latium
Cobitis bilineata	*Barbus tyberinus*
Cobitis conspersa	*Rutilus rubilio*
Barbus caninus	*Padogobius nigricans*
Barbus plebejus	*Scardinius scardafa*
Rutilus aula	*Squalius lucomonis*
Padogobius bonelli	
Knipowitschia punctatissima	
Alosa agone	
Salmo carpio	
Salmo marmoratus	
Cottus ferrugineus	
Sabanejewia larvata	
Lampetra zanandreai	
Chondrostoma genei	
Chondrostoma soetta	

3. Barbels

The genus *Barbus* comprising al least 800 species spread over three continents: Asia, Africa and Europe (Howes, 1987), has been described by Myers (1961) as a monstrous aggregation. Knowledge about taxonomy and systematic of this genus are largely incomplete and should be elaborate in detail. Several species from southern Europe are quite similar in appearance and earlier authors have expressed different views on species limits. Recent molecular data have shown that many southern European *taxa* recognized by earlier authors indeed represent valid species. For instance, in recent years Kotlik *et al.* (2002) formally described two new *Barbus* species belonging to the Danube river system and Markova *et al.* (2010) found that *B. rebeli* could be actually a complex of species due to the high genetic divergences found in its populations.

As said above, primary freshwater fish, defined as physiologically intolerant to marine conditions (Myers, 1938), are particularly suitable in phylogeographic studies due to their limited dispersal ability. Fish genus *Barbus* due to its wide distribution across all Europe, with an high number of species in southern peninsula and a relatively few number in northern region, and its interesting biological characteristics, is an excellent tool to investigate phylogeographic patterns in the Mediterranean region. This group of fishes combines biological features that rarely are included in a single genus: it includes diploid, tetraploid and exaploid species, it is subjected to hybridization and its species could be grouped on the base of ecological preferences.

All species of European barbel are tetraploid (Berrebi, 1995).

From an ecological point of view, the main trait of the distribution of barbels in Europe is the existence of two groups or ecophenotypes. A rheophilic or strictly riverine, which consists of small species (maximum total length L_T=20-25 cm) and a fluvio-lacustrine one which comprises larger species (maximum total length L_T>50 cm) (Tsigenopoulos *et al.*, 1999).

3.1 Small-sized or strictly riverine barbels

Several species in this category have a relative restricted distribution and are allopatric. The rheophilic barbs share many characters such as: body pigmentation formed by dark and irregular marks, long anal fin which extends further back than the root of the caudal fin. In this type of species, based mainly on the structure of the last (fourth) soft dorsal ray, the number and size of the lateral line scales, we can distinguish two groups: the *meridionalis*

with an unserrated ray and the *cyclolepis* which has a moderately serrated ray, and larger number of smaller scales on the lateral line (Karaman, 1971).
Riverine barbels tend to occupy the *Thymallus* mountain region of the basin. In absence of fluvio-lacustrine species they may colonize a bigger portion of river as in the case of *B. peloponnesius*.
In Italian peninsula, at this moment, two species are recognized as strictly riverine barbels: *B. caninus* and *B. balcanicus*

3.1.1 Italian brook barbel: *Barbus caninus* Bonaparte, 1839

Some authors consider the Italian brook barbel a subspecies of *B. meridionalis* (Zerunian, 2002). However genetic studies based on allozyme (Tsigenoppoulos *et al.* 1999) and mtDNA data (Tsigenoppoulos and Barrebi, 2000) showed that Italian rheophilc barbel posses unique genetic markers, and also unique morphological traits (Bianco, 1995a), therefore should be considered a distinct species. *B. caninus* is the biogeographic indicator of the Padano-Venetian ichthyogeographic district, but the southern and eastern limit of its range is uncertain (fig. 1.2). There have been local transfers of this species in central Italy (Bianco, 1994), but there are no documented cases of successful recruitment in these populations.
Barbus caninus has limited degree of ecological adaptability, it occurs in the middle-upper reaches of watercourses and small tributaries, in search of well oxygenated, fast flowing waters, where the riverbed is made up of gravel and stones where which they find refuge. Areas with gravel bottom are necessary for spawning that takes place among April and June. It is a gregarious fish, its total maximum length usually measure 20-22 cm, it has with benthic habits for trophic reasons. It searches actively for macro-invertebrates, using a typical behaviour of overturning pebbles with its mouth and capturing organisms that seek refuge under them. Its diet is composed mainly of insect larvae, crustaceans and annelid worms. It undertakes upstream migrations during spring and summer, and downstream during the cold season. The species is very sensitive to alteration in environmental quality of watercourses. Any kind of intervention on riverbeds seems to be extremely detrimental as well as water pollution and water tapping. Numerous anthropic interventions by man to rivers and minor watercourses have produced local extinctions of *B. caninus*, with the consequent fragmentation of its range. In the past this species was very common, but now it is restricted to about 20-25 reproductive population, located in the western part of Italy. In the IUCN Red

List *B. caninus* is considered "vulnerable", it is in the Annex II in the Directive 92/43/EEC and in the list of protected species of the Bern Convention (Appendix III).

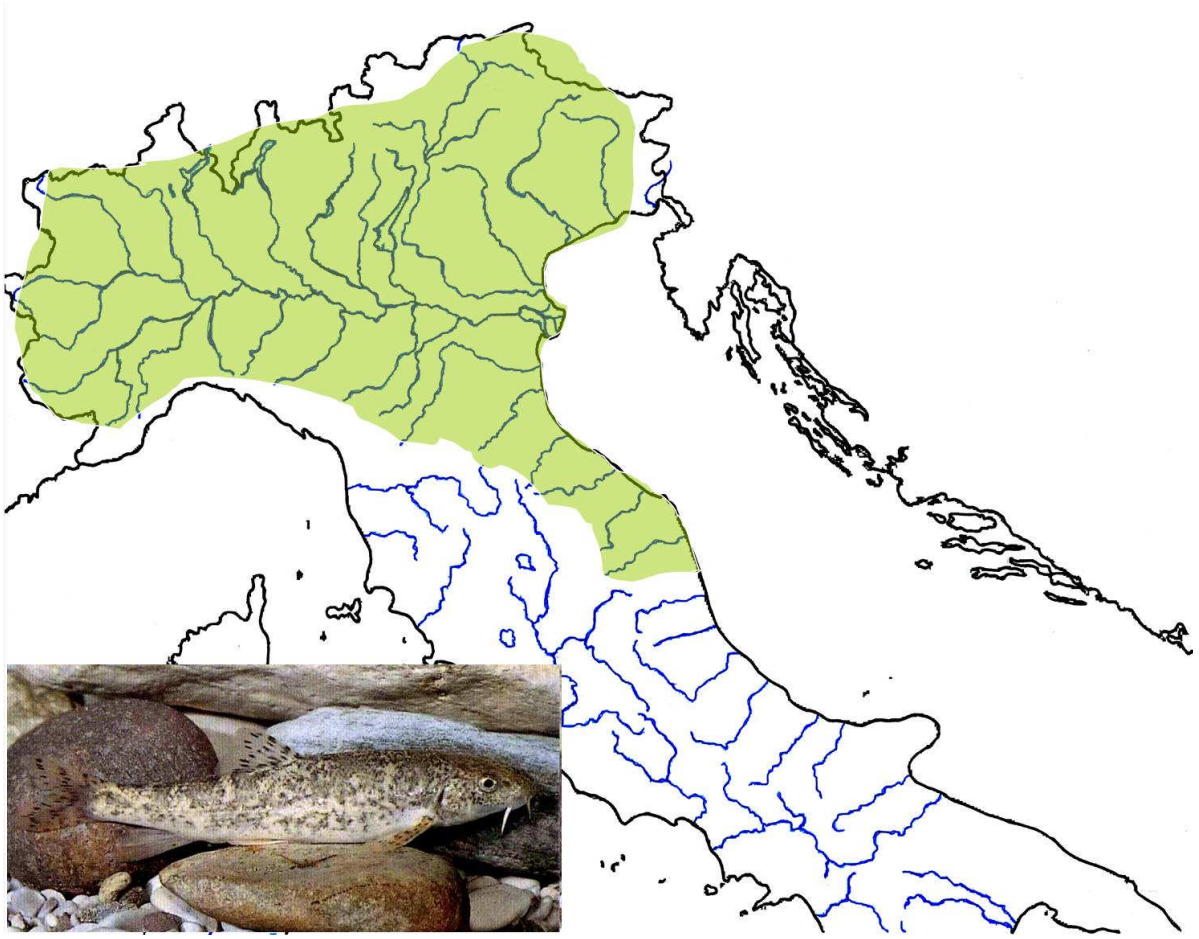

Figure 1. 2 Range distribution of *Barbus caninus* Bonaparte,1839

3.1.2 Large spot barbel: *Barbus balcanicus* Kotlík, Tsigenopoulos, Ráb and Berrebi, 2002

This species was formally described in the 2002, before this date it was classified as *B. petenyi* a small-sized rheophilic barbel widely distributed throughout the mountain regions in the Danube River basin and several adjacent drainages (Tsigenopoulos *et al.*, 2002).

Several studies conducted on nuclear and mitochondrial marker showed that inside *B. petenyi* at least three *taxa*, which have been evolving independently of one another and of all the other *Barbus* species since the Pliocene and are differentiated at the species level (Kotlik and Berrebi, 2002). Inside of these three *taxa B. balcanicus* was formally described also on the basis of morphological characters (fig. 1.3).

B. balcanicus is distributed in mountain and submountain brooks and rivers, and less often lakes and reservoirs, in the Dinaric Mountains on the Balkan Peninsula in Yugoslavia, Slovenia, and most likely also in Bosnia and Herzegovina, and Croatia. Outside the Danube

River basin, populations apparently conspecific with this species are known from rivers of the Aegean Seadrainage in northern Greece. The westernmost known populations of this species are located in the Isonzo River basin of the Adriatic Sea drainage in Italy and Slovenia (Kotlik and Berrebi, 2002; Tsigenopoulos *et al.*, 2002). It has benthic habits and occurs in fast or moderate flowing premontane streams and small rivers with gravel bottom. Usually is most abundant in rapid and riffles during the day. During the spawning period, between May and July, *B. balcanicus* moves to upper reaches to spawn in riffles.

In the IUCN Red List *B. balcanicus* is listed as Least Concern (LC) (Kottelat and Freyhof, 2007).

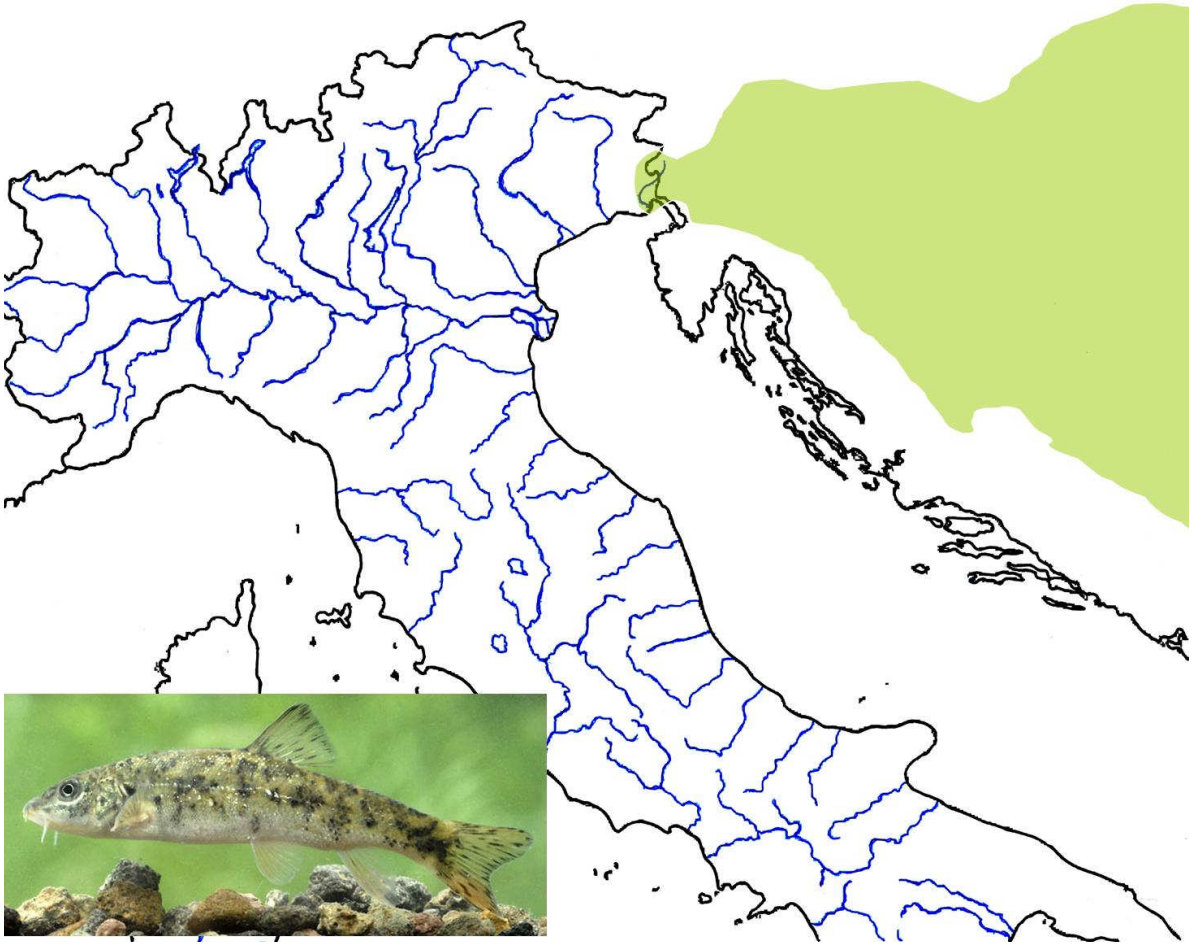

Figure 1. 3 Range distribution of *Barbus balcanicus* Kotlík, Tsigenopoulos, Ráb and Berrebi, 2002

3.2 Large-sized or fluvio-lacustrine barbels

The fluvio-lacustrine species are present in almost all European rivers and are characterized by a spindle shaped body, small scales, short anal fin (which does not reach the root of the caudal fin) and a triangular dorsal fin strengthened with an ossified and generally serrated unbranched ray. Large-sized barbel species, with exception of those of Iberian peninsula, are

allopatric. They prefer wide calm rivers, with a regular current, running through the European plains, and sometimes lakes. Large-sized species tend to form a paired complex with riverine species, but unless there is an ecological and spatial separation between two ecological groups they tend to hybridize in the zone of contact. This can be observed for instance between *B. barbus* and *B. meridionalis* in France (Chenuil *et al*., 2004) and in the North-West Italy, in the upper Po River drainages, between *B. caninus* and *B. plebejus* (Delmastro, pers. comm.). In basins where one of the species in the paired complex is lacking, the species present tend to occupy the entire basin.

3.2.1 The Italian river barbel: *Barbus plebejus* Bonaparte, 1839

It was long debated if Italian population were a good species or just a subspecies of *Barbus barbus* a widely distributed species in Europe. Recent studies showed its high degree of genetic differentiation from *B. barbus* (Tsigenpoulos *et al.*, 1999), which supports the systematic position followed in this study. The range species encompasses the entire Padano-Venetian ichthyogeographic district until the river Zrmanje (Dalmatia) (fig. 1.4). In Tuscano-Latium district it was possibly native, with an original distribution range partially overlapped with the distribution of *B. tyberinus*, as some specimens of this species have been found in historical collections from Tiber River. But for about one century this species has been involved in translocations and its original distribution has been altered (Bianco, 1995a).
The Italian river barbel is a fish with a fair degree of ecological adaptability, capable of occupying several reaches of watercourses, as well as minor ones. However it prefers middle-upper reaches where the current is fast and the riverbed is covered of gravel. This kind of bottom is indispensable for spawning. Spawning takes place when the water temperature reaches 16-17 °C. During this period the Italian river barbel swims upstream until finds good areas with gravel bottom and fast-flowing waters. Probably in this season it comes in contact with *B. caninus*. Its longevity is unknown. It gregarious fish with benthic habits and it reaches total maximum length over 70 cm and 3 kg or more in weight. Its diet consist mainly of macro-invertebrates and occasionally even macrophytes. *B. plebejus* is a relatively resistant species, capable tolerating a certain compromise in water quality, but it feels the negative effects of men's intervention on riverbeds. In some parts of the Po River basin there seems to be a decline in the number of populations due to introduction of *B. barbus*, that tends to substitute *B. plebejus* in virtue of its greater resistance to habitat degradation (Zerunian, 2002).

Some specimens with intermediate phenotype between *B. plebejus* and *B. caninus* have been found in western Italy. These specimens showed intermediate measurements and meristic counts compared these two species, the body pigmentation was also intermediate and the peritoneum as well, therefore they were indicated as hybrids (Bianco, 1995a).

In the IUCN Red List *B. plebejus* is considered "at low risk", it is in the Annex II in the Directive 92/43/EEC and in the list of protected species of the Bern Convention (Appendix III).

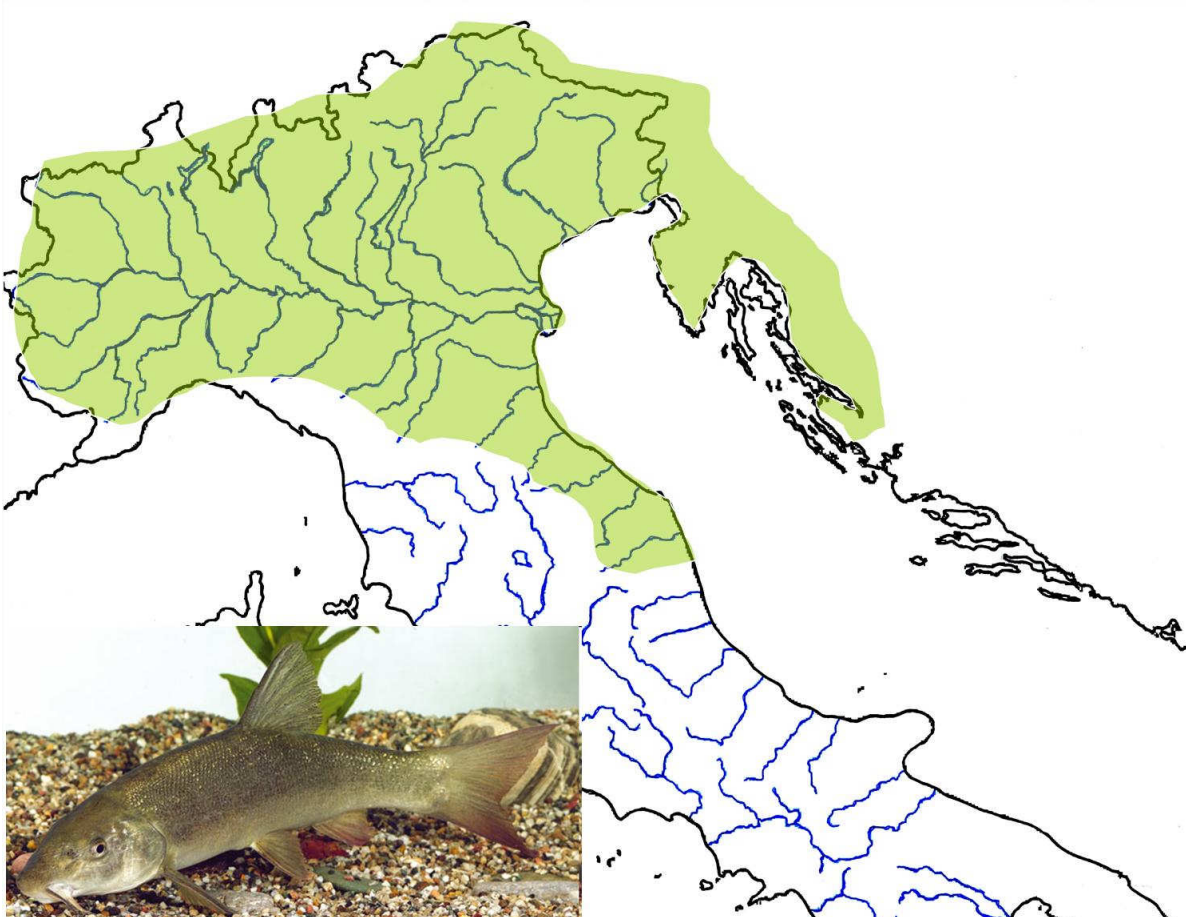

Figure 1. 4 Range distribution of *Barbus plebejus* Bonaparte,1839

3.2.2 *Barbus tyberinus* Bonaparte, 1839

For long time Centro-Italian barbels populations were consider belonging to *B. plebejus*, even if some morphological differences were known from the ones of the North of Italy (Tortonese, 1970). Differences regard bigger scales and the colour of livery and peritoneum. In this work to the Centro-Italian populations it is assigned the status of species according to Bianco (1995a; 2003).

This species is the most widespread in the Tuscano-Latium ichthyogeographic district. The native range was probably not wide as now. Along the Tyrrhenian slope it is native in the

river basins between Magra River and Sele River. On the Adriatic slope it was present in historical time in the Ofanto River, the northern boundary is not traceable (fig. 1.5).

Information on biology and ecology of *B. tyberinus* are not so wide. As the others congeneric species it has benthic habits and it occurs from the middle to the middle upper reaches of watercourses where water is quite deep with good oxygen concentrations. In winter it finds refuge under stones located in zones with deep water. It is considered a pioneer species due to the its low capacity to compete with other cyprinids (Lorenzoni *et al*., 2006). *B. tyberinus* reaches over 50 cm of total length and 4 kg of weight. Presumably specimens of 4 kg may reach 11 or 12 years. The spawning season start at the beginning of summer. It feeds on macrophytes, macroinvertebrates and small fishes.

B. tyberinus is intermediate between *B. plebejus* and *B. caninus* in many features and an hybrid origin it was suggested by Bianco (1995). Morphometric analyses sowed that hybridization phenomena between *B. tyberinus* and *B. barbus* that was introduced in the Tuscano-Latium district are very probable. In some parts of the Tiber River basin there seems to be a decline in the number of populations due to introduction of *B. barbus* (Bianco and Ketmayer, 2001), that tends to substitute *B. tyberinus* in virtue of its greater resistance to habitat degradation and the faster increase in weight and length (Lorenzoni *et al.*, 2006).

B. tyberinus is not listed in the IUCN Red List or in the Directive 92/43/EEC.

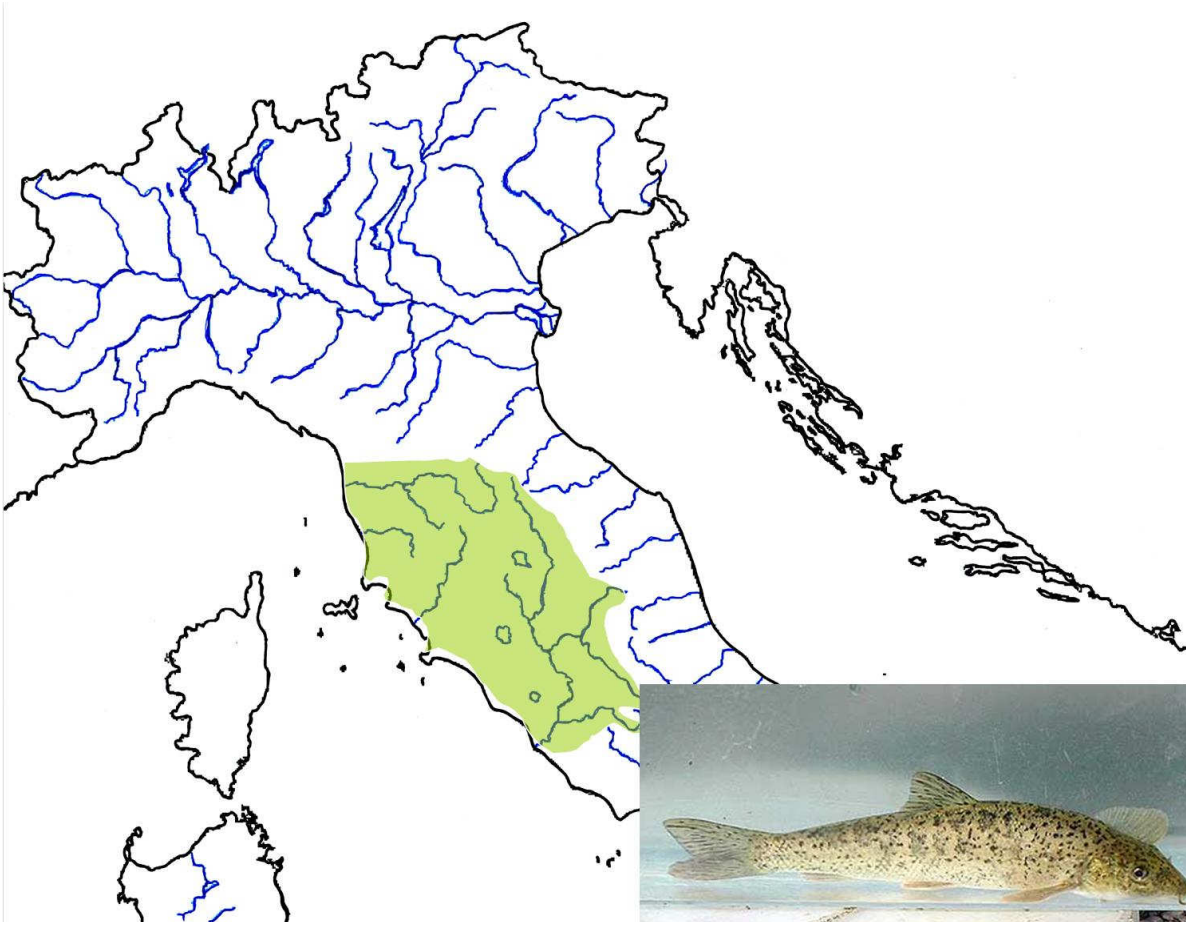

Figure 1. 5 Range distribution of *Barbus tyberinus* Bonaparte,1839

4. Aims

Main goals of this study are:

- Asses phylogenetic relationships and the biogeographic history of Italian barbels.
- Test how differences in the ecological preferences could influence the population genetic structure of the Italian species of the genus *Barbus*.
- Verify the hypothesis that interspecific gene flow occur between *B. caninus* and *B. plebejus* in the transitional habitat where they come in contact, and interspecific gene flow between the sympatric species *B. tyberinus* and *B. barbus.*

CHAPTER 2

Phylogenetic structure of Italian barbels (Cyprinidae, *Barbus*) inferred by mitochondrial and nuclear markers: systematic and biogeographic implications

1. Introduction

The phylogenetic relationships and taxonomy identity of *Barbus* species are the subject of debates since decades. Distributed over most of central Europe, Italian peninsula, partly of Iberian peninsula and partly of Asia (Tsigenopoulos and Berrebi, 2000), the genus *Barbus* was considered for long time as a subgenus of the western Paleartic barbels distinct from the subgenus *Luciobarbus*, which occurs in the Iberian peninsula, southern Greece and North Africa. Subsequently, molecular and morphological studies (Tsigenopoulos and Berrebi, 2000; Machordom and Doadrio, 2001) indicated a deep division among the two subgenera, and they were elevated to full genus status (Kottelat and Freyhof, 2007). The genus *Barbus* counts more than twenty species, all tetraploid, and together with *Luciobarbus* are among the most widespread and diverse primary freshwater fishes in the European continent (Doadrio *et al.*, 2002).

Due to the wide distribution and the interesting distribution pattern, with numerous endemic species in the Mediterranean region and a small number of species in central Europe (Banarescu, 1973), this genus is an ideal evolutionary model for inferring phylogeographic history of the European freshwater fauna. Since the second half of nineties in several molecular studies the relationships among *Barbus* species and populations across all Europe have been investigated (Berrebi, 1995; Tsigenopoulos and Berrebi, 2000; Kotlik and Berrebi, 2001). In particular, the major part of these studies regarded the Iberian peninsula (Zardoya and Doadrio, 1998; Doadrio *et al.*, 2002; Gante *et al.*, 2009a; Gante, 2009b) and the Balkan area (Karakousis *et al.*, 1995; Kotlik *et al.*, 2002; Kotlik and Berrebi, 2002; Kotlik *et al.*, 2004; Markova *et al.*, 2010). Concerning the Italian peninsula, although barbels are one of the most important component of its freshwater fauna, literature records just one work (Tsigenopoulos *et al.*, 2002) that tried to resolve the phylogenetic relationships and taxonomic status of *Barbus* species distributed in this region. Thus, a more exhaustive knowledge seems necessary.

The Italian peninsula, isolated from continental Europe by the Alps, hosts a relatively high number of endemic freshwater *taxa* (Bianco, 1990). Its independence from the main Central European drainage systems Danube, Rhine and Rhone favoured this high degree of endemicity. Moreover, Italian hydrographic structure is largely influenced by the North-South orientated Apennine barrier, which modulated the allopatric distribution and the diversification of various freshwater *taxa* (Marchetto *et al.*, 2010). Based on the distribution of cyprinid fishes, two main ichthyogeographic districts were identified (Bianco, 1990): (i)

the Padano-Venetian district (PV), including basins from the Vomano River to the Krka River, which all drain into the Adriatic Sea; (ii) the Tuscano-Latium district (TL), from the Serchio River to the Tiber River, which drain into the middle Tyrrhenian Sea.

From an ecological point of view, the main trait of the distribution pattern of the genus *Barbus* species in Europe and in Italy as well, is the existence of two main ecophenotypes: riverine or rheophilic, which consists of small species (total length < 25 cm) and fluvio-lacustrine, which comprises larger species (total length > 50 cm) (Tsigenopoulos *et al.*, 1999). Riverine barbels occur in mountain streams and are characterized by speckled large black scales, a long anal fin which extends further back than the root of the anal fin and with weak last dorsal ray (Tsigenopoulos *et al.*, 1999). The fluvio-lacustrine species are present in almost all European rivers with regular flow and are characterized by spindle body, small scales, short anal fin and strong and serrated dorsal ray (Tsigenopoulos *et al.*, 1999). Several authors suggested that these ecological traits can be of great importance in defining groups within the genus (Almaca, 1981; Economidis, 1991).

According to Bianco (1995a) and Kotlik *et al.* (2002), four species of *Barbus* are recognized in Italy: *Barbus caninus*, *Barbus balcanicus*, *Barbus plebejus* and *Barbus tyberinus*.

B. caninus and *B. balcanicus* have the typical morphology of the small-sized barbels. The former species, biogeographic indicator of the PV, is distributed across all Po River and Brenta River basin. *B. balcanicus* is distributed, in Italy, only in the Isonzo River basin.

B. plebejus belongs to the fluvio-lacustrine group and inhabits the Po River basin up to the Adriatic rivers in northern Croatia (Tsigenopoulos *et al.*, 2002). The ecology of *B. tyberinus* is quite similar to *B. plebejus*, therefore it is grouped in the fluvio-lacustrine category as well. It lives in the TL district and it is the only species of *Barbus* present in this region. Bianco (1995a) demonstrated that *B. tyberinus* shows morphological traits intermediate to those of *B. plebejus* and *B. caninus*, namely the body size, the body and peritoneum coloration and the presence of marbling on the body. Therefore he considered this species the result of an ancient hybrid speciation, also because it is found in basins where the other two congeneric species do not occur.

The major part of the studies concerning the phylogeny of the genus *Barbus* were conducted using prevalently a mtDNA marker: the Cytochrome b gene (e.g. Zardoya and Doadrio, 1999; Doadrio *et al.*, 2002; Tsigenopoulos *et al.*, 2003). This is a suitable marker to answer a diversity of systematic questions on a wide geographic range (Faria *et al.*, 2001, Godinho *et al.*, 2008), differently at small geographic scale some interesting information could be lost, keeping populations genetically undifferentiated (Frankham *et al.*, 2002). For these reasons,

in order to have phylogenetic and phylogeographical independent information, the comparison with nuclear markers is essential.

Genes from the nuclear and mitochondrial genomes may produce distinct phylogenies as result of different inheritance pathways, divergent selection pressures, and differential responses to processes such as lineage sorting, gene duplication or deletion, and hybrid speciation. Conversely, congruent phylogenies among these two genomes could strongly suggest that the gene trees are also congruent with the species phylogeny. Therefore, comparison of gene phylogenies of the two genomes will provide an opportunity for robust reconstruction of the phylogenetic relationships (Reyes *et al.*, 2004).

1.1 Aims

I estimated phylogenetic relationships of Italian barbels applying both mtDNA and ncDNA markers, comparing, then, the results in a European context.

Meanwhile two hypotheses were tested: the hybrid origin of *B. tyberinus* and the congruity among groups defined on the bases of ecological features and those defined by genetic analyses.

2. Materials and Methods

2.1 Sampling

According to their morphology, all four previously described species were collected from their *terrae typicae*. Species identification, in some cases uncertain due to the strong morphological plasticity of these fishes, was carried out in the field following the criteria proposed by Kottelat and Freyhof (2007). Samples were collected by electrofishing across the Padano-Venetian and the Tuscano-Latium ichthyogeographic districts. In particular six populations of *B. caninus*, three populations of *B. plebejus*, two populations of *B. balcanicus* and seven populations of *B. tyberinus* (fig. 2.1; tab. 2.1) were sampled for a total of 264 specimens.

Each population was represented at least from 5 individuals per site with an average of 15 specimens. After the identification, a clip of the anal fin was stored in 100% ethanol and kept refrigerated at 4 °C. Samples of *B. barbus*, *B. prespensis* and *B. carpathicus* from central and eastern Europe, kindly provided by H. Gante, were included, while three other species, belonging to the subgenus *Luciobarbus* from Iberian peninsula were used as outgroup (tab. 2.1).

Once in laboratory, total genomic DNA was extracted using a proteinase K digestion followed by sodium chloride extraction and ethanol precipitation (Aljanabi and Martinez, 1997).

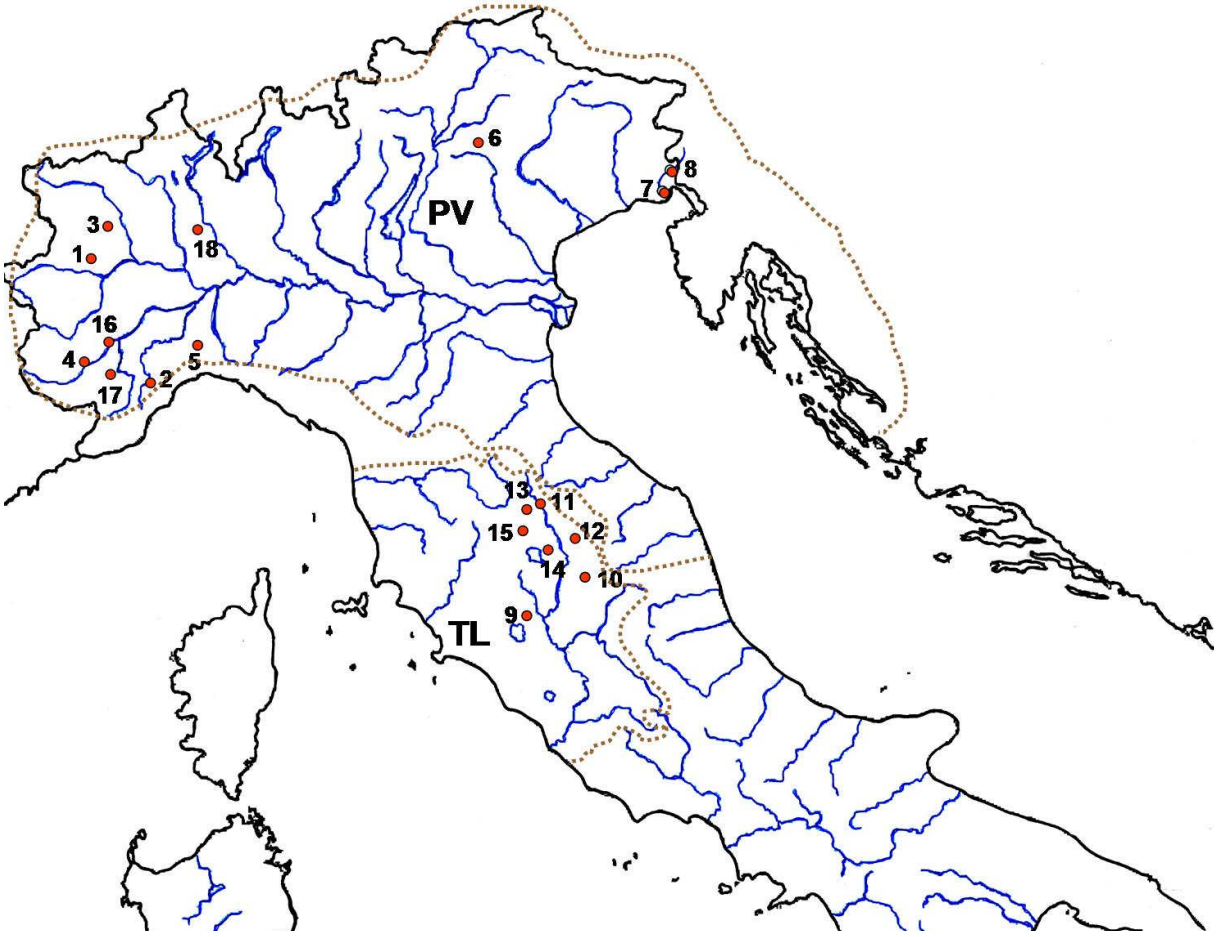

Figure 2. 1 Map of northern and central Italy showing the main river system, the ichthyogeographic districts and the sampling sites. PV: Padano-Venetian ichthyogeographic district; TL: Tuscano-Latium ichthyogeographic district

Table 2. 1 Sampling location of *Barbus spp.* populations analysed in this study

Population	Localities	River	Main river basin	Ichthyogeographic district	N° of specimens	Species
1	Trana	Sangone	Po river	PV	15	*B. caninus*
2	Priola	Tanaro	Po river	PV	15	*B. caninus*
3	Varisella	Ceronda	Po river	PV	30	*B. caninus*
4	Sanfront	Po	Po river	PV	11	*B. caninus*
5	Voltaggio	Lemme	Po river	PV	17	*B.caninus*
6	Fonzaso	Cismon	Brenta river	PV	14	*B. caninus*
7	Costa Bona	Piumizza	Isonzo river	PV	20	*B. balcanicus*
8	Grojna	Groina	Isonzo river	PV	15	*B. balcanicus*
9	Albergo la Nona	Paglia	Paglia river	LT	21	*B. tyberinus*
10	Scanzano	Topino	Tyber river	LT	8	*B. tyberinus*
11	San Giustino	Tyber	Tyber river	LT	13	*B. tyberinus*
12	Valfabbrica	Chiascio	Tyber river	LT	20	*B. tyberinus*
13	Passano	Lama	Tyber river	LT	6	*B. tyberinus*
14	Soara	Soara	Tyber river	LT	8	*B. tyberinus*
15	Lupo	Cerfone	Tyber river	LT	20	*B. tyberinus*
16	Cardè	Po	Po river	PV	9	*B. plebejus*
17	Savigliano	Maira	Po river	PV	15	*B. plebejus*
18	Novara	Terdoppio	Ticino river	PV	12	*B. plebejus*
			European samples			
NA	NA	NA	Danube river	D	2	*B. barbus*
NA	NA	NA	Danube river	D	2	*B. carpathicus*
NA	NA	NA	Prespa Lake	B	2	*B. prespensis*
NA	NA	NA	Ebro river	IBP	1	*B. haasi*
NA	NA	NA	Duoro river	IBP	1	*Luciobarbus bocagei*
NA	NA	NA	Gaudiana river	IBP	1	*Luciobarbus comizo*
NA	NA	NA	Gaudiana river	IBP	1	*Luciobarbus microcephalus*

Population number, locality, river, drainage system, ichthyogeographic district, number of specimens, species. NA: not available; PV: Padano-Venetian; TL: Tuscano-Latium; D: Danubian; B: Balkan; IBP: Iberian peninsula

2.2 Amplification and data analyses of mtDNA

The entire cytb gene (1141 bp) was amplified by polymerase chain reaction (PCR) using primer pair L15267 (5' –AAT GAC TTG AAG AAC CAC CGT- 3') and H16461 (5' –CTT CGG ATT ACA AGA CC- 3') (Briolay *et al.*, 1998). All PCR amplifications were performed using Multiplex PCR kit (Qiagen) in 10µL reaction volume containing approximately 10ng of template DNA and 0,25µM of each primer. Thermal cycling was performed as follow: denaturation of 15 min at 95 °C, followed by 30 cycles of 94 °C for 30 sec, 90 sec at 56 °C of annealing temperature and the extension step at 72 °C for 90 sec, the final elongation was at 72 °C for 10 min. Negative PCR controls with no template DNA were used in each experiment. A 1.5µl aliquot of each PCR product was electrophoresed in a 1.5% agarose gel stained with ethidium bromide for visualization. From the remaining PCR products 1.5µl were took and purified using Exo-Sap, subsequently sequenced on an ABI 3130xl Genetic Analyzer using Big Dye 3.1 terminator (Applied Biosystem).

A fragment of 733 bp long, from generated sequences, was used for data analyses. The alignment of sequences was carried out manually to eliminate ambiguities and to check polymorphic sites.

To determine the level of genetic variation within populations, several measures of polymorphism were calculated using DnaSP, version 5 (Librado and Rozas, 2009). For each locus, the number of haplotypes (h), number of polymorphic sites (S), haplotype diversity (Hd), mean number of nucleotide differences (π), and two commonly statistics D (Tajima, 1989) and R_2 (Ramos-Onsins and Rozas, 2002) were estimated to test for non-neutral evolution of the analysed data set. Parameter estimates "Hd" and "π" and their variances were calculated according to formulae given in Nei (1987). The significance of the D, and R_2 statistics was tested by generating random samples under constant population size using a coalescent simulation (Ramirez-Soriano *et al.*, 2008). For neutral markers significant low D and R_2 values can be expected in cases of population expansion. Net between-group mean distances among all major mtDNA lineages were determined according to Nei and Li (1979). Distances and standard error, using 500 bootstrap replicates, were calculated using the Kimura 2-parameter (K2P) model in MEGA4 (Tamura *et al.*, 2007).

Phylogenetic relationships were inferred using two different analytical approaches: maximum parsimony (MP) performed with PAUP 4.0b10 (Swofford, 2002) and maximum likelihood (ML) using PhyML (Guindon and Gascuel, 2003). The optimal model of molecular evolution for ML analysis was determined using MODELTEST 3.7 (Posada and Crandall, 1998), using the corrected Akaike Information Criteria (AIC). The selected molecular evolution model was TrNef+I+G with the equal base frequency, base transition probabilities of 23.4765 for rAG, 13.243 for rCT and 1.00 for the remaining categories, the gamma distribution shape parameter α equalling to 0.7626 and 0.4575 the proportion of invariable sites. ML and MP analyses were performed using an heuristic search algorithm. Then 1,000 of the non parametric bootstrap test replicates were performed to asses internodes robustness. Phylogenetic trees were rooted using cytb sequences of 3 *Luciobarbus* species: *L. bocagei*, *L. comizo* and *L. microcephalus*.

Then, a minimum spanning network, coupled with statistical parsimony analysis was constructed for each of the *taxa* investigated . For the construction of the network was used the computer program TCS (Clement *et al.*, 2000).

2.3 Amplification and data analyses of ncDNA

Nuclear sequences are becoming a widespread tool to resolve phylogenetic relationships of several organisms (Pinho *et al.*,2008; Eytan and Hellberg, 2010) and many PCR primers have become available. Primers for the Growth Hormone gene (Gh) and the S7 Ribosomal Protein (S7), developed and successfully used in phylogenetic studies of species belonging to the family of Cyprinidae (Moyer *et al.*, 2009; Gante, 2009b, Kotlik *et al.*, 2008), have been selected.

Since species from the genus *Barbus* are tetraploid nuclear loci cannot be sequenced directly, but specific primer pairs have to be used to amplify selectively a single paralog locus. Gante (2009b) developed, for some of the European *Barbus* species, forward and reverse specific primers that bind to single copies of these two different nuclear genes (fig. 2.2). I tested this set of primers on the Italian *Barbus* species. From here onward I will refer to the two different copies of the genes with the acronym S7_1 and S7_2 for the ribosomal protein and Gh1 and Gh2 for the Growth hormone gene.

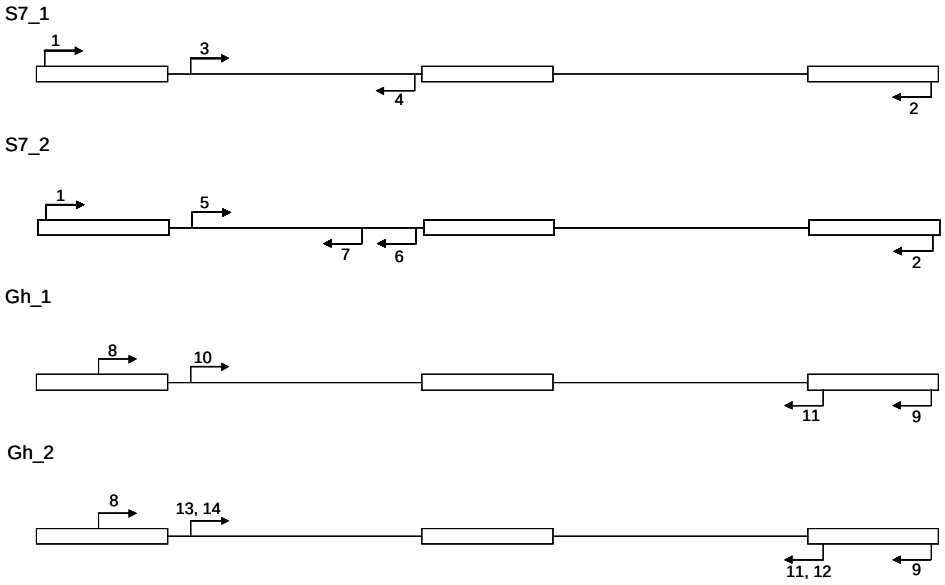

Figure 2. 2 Schematic representation of the studied loci and annealing sites of primers used for amplifications and sequencing. Numbers refer to table 2.2.

S7_1, S7_2 and Gh1 were successfully amplified with available primers (tab. 2.2); regarding Gh2 reliable amplicons were obtained only for *B. tyberinus* and *B. plebejus*, therefore new primer pairs were developed for *B. caninus* and *B. balcanicus* as follow.

Once amplified both paralog Gh gene copies with general primer pair (Unmack, unpub. data), PCR products were visualized on a 2% agarose gel stained with GelRed® (Phoenix, Research Products). The single paralog copy of interest was isolated by gel excision and DNA was eluted from the gel using MinElute Gel Extraction Kit (Qiagen). Then individual band was

sequenced as above. Generated sequences were manually aligned and used to develop new paralog-specific primers.

Table 2. 2 Nucleotide sequences of primers used to amply nuclear loci S7_1, S7_2, Gh1 and Gh2.

Primer name	No	Sequence (5'-3')	Annealing site	Source
S7RPEX1F	1	TGGCCTCTTCCTTGGCCGTC	S7 exon I	Chow & Hazama, 1998
S7RPEX3R	2	GCCTTCAGGTCAGAGTTCAT	S7 exon III	Chow & Hazama, 1998
S7BL1F	3	CCCAGCTAAAGAGTTTTCAAATG	S7_1intron I	Gante pers. comm.
S7BH1cR	4	GCACATGGGGCCCAGTAAT	S7_1 intron I	Gante pers. comm.
S7BL2F	5	CCCAGCTAAAGAGTTATCAAGTT	S7_2 intron I	Gante pers. comm.
BS72i1R	6	AACTCCAAGCATGTTCTTAGCTTATCG	S7_2 intron I	This study
S7BH2cR	7	GAAACTGATTTATTAACTCCCAAA	S7_2 intron I	Gante pers. comm.
Ghe3.3F	8	GACAACCTGTTGCCTGAGGAACGC	Gh exon III	Unmack unpubl. data
Ghe5.183R	9	CTACAGGGTGCAGTTGGAATC	Gh exon V	Unmack unpubl. data
BGh1.i3.79f	10	GGGGTCTGTGGAAAAGTTTGG	Gh intron III	Gante pers. comm.
BGh2.E532SR	11	AGTGGCAGGGAGTCATTG	Gh exon V	Gante pers. comm.
BGh2.E532SRb	12	AGTGGSAGGGAGTCGTTY	Gh exon V	This study
BGh2.i3.226F	13	GTACTATAGTAAGCAGAAATGG	Gh intron III	Gante pers. comm.
BGh2.i3.226Fb	14	GTACTAKAGTRRGCAGAAATGG	Gh intron III	This study

No: number corresponds to fig. 2.2; F and R refer to forward and reverse respectively

Once developed and tested all necessary primer pairs, PCR assays were performed using Multiplex PCR kit (Qiagen) in 10µL reaction volume containing approximately 10ng of template DNA and 0,25µM of each primer pairs. Thermal cycling was performed as follow: denaturation of 15 min at 95 °C, followed by 30 up to 40 cycles (depending on the primer pair used) of 94 °C for 30 sec, 90 sec at the appropriate annealing temperature (tab. 2.3) and the extension step at 72 °C for 90 sec, the final elongation was at 72 °C for 10 min. PCR products (2.0µl) were purified using Exo-Sap and sequenced in both directions on an ABI 3130xl Genetic Analyzer using Big Dye 3.1 terminator (Applied Biosystem).

Table 2. 3 Number of PCR cycles and annealing temperature of each primers pair used for amplification of nuclear loci

Locus	Primers pair	N° cycles	T° annealing
S7_1	S7BL1F S7BH1cR	40	56°C
S7_2	S7BL2F S7BH2cR	35	62°C
	S7BL2F BS72i1R	35	62°C
Gh_1	BGH1.i3.79f BGH2.E532SR	30	59,5°C
Gh_2	BGH2.i3.226F BGH2.E532SR	35	55°C
	BGH2.i3.226Fb BGH2.E532SRb	35	55°C

F and R refer to forward and reverse respectively

Heterozygous specimens for insertions or deletions (indels) were manually phased analyzing the complementary information carried by the forward and the reverse sequences (Flot *et al.*, 2006). To verify the right application of the Flot's method to decode the superimposed traces produced by direct sequencing, I cloned PCR products of four of these heterozygous samples for two different loci (Gh2 and S7_2). Cloning was carried out using TOPO® TA cloning kit (Invitrogen) according to manufacturer's instructions. Each sequence of samples at each locus was found to be very similar (less 0.5% difference) or identical to one or the other sequences obtained from direct sequencing; no more than 5 differences in nucleotide composition were found. Appendix I reported site of variation among phased and cloned sequences.

Haplotypes with known phases were subsequently used to phase the remaining single nucleotide polymorphism heterozygotes haplotypes with PHASE (Stephen *et al.*, 2001). PHASE input files were generated using seqPHASE (Flot, 2010). Consistency of the inferred haplotypes was assessed in five independent PHASE runs as recommended by the author.

The level of genetic variation within *taxa* was estimated using the same program and the same indices calculated for the cytb (see above). In order to test if intragenic recombination may have affected the patterns of variation in the 4 nuclear loci, I used the four-gamete test, which estimates the minimum number of recombination events (Rm) in the history of each samples.

Nuclear gene genealogies were inferred using two different analytical approaches: maximum parsimony (MP) performed with PAUP 4.0b10 (Swofford, 2002) and maximum likelihood (ML) using GARLI v0.96 (Zwickl unpublished, available at http://www.nescent.org/wg_garli/).

For each data set, two replicates were run for 20,000 generations with a threshold score of 0.05 and a log-likelihood threshold value of 0.01, allowing the sequences to evolve under a GTR+I+G model with parameters estimated from the data by MODELTEST 3.7 (Posada and Crandall, 1998), using the corrected Akaike and Bayesian Information Criteria (AIC). ML and MP analyses were performed using an heuristic search algorithm . Then 1,000 (for MP analisys) and 1,000 (for ML analysis) of the non parametric bootstrap test replicates were performed to asses internodes robustness. Since nuclear genes showed different levels of recombination, relationships among haplotypes were illustrated also with a median-joining network using the program NETWORK (available at http://www.fluxus-technology.com/). Data are transformed into a set of compatible bipartitions, that are presented by a split network, where reticulations can be interpreted as evidences of conflicting phylogenies (Bryant and Moulton, 2004). Multiple base insertions or deletions are likely to have resulted from a single evolutionary step, therefore indels were cut from the data in order to leave the first base of them. Nevertheless, some segregating positions located within the indels were removed, reducing the numbers of polymorphic sites.
Phylogenetic trees were rooted using nuclear sequences of 3 *Luciobarbus* species: *L. bocagei*, *L. comizo* and *L. microcephalus*.

2.4 Bayesian clustering analysis of nuclear data

Each unique allele was identified using the NRDB program (written by Warren Gish, Washington University, unpublished data) available at http://pubmlst.org. Due to the complexity of the total nuclear data set and the high levels of polymorphism of nuclear sequences, I verified the reliability of results obtained with NRDB also using the program MacClade v.4.03 (Maddison and Maddison, 2002); before to start the analysis data set was subdivided in smaller data set as recommended by Pritchard and Wen (2002).
Then a Bayesian clustering analysis of nuclear DNA data was assessed using the program STRUCTURE v2.2 (Prithchard *et al.*, 2000). to demonstrate the presence of distinct genetic populations, to assign individuals to populations, to identify migrants and admixed individuals. It exploits the Bayes' theorem to assign a posterior probability for every individuals to belong a population. STRUCTURE identifies clusters by assigning individuals to K populations in the way to maximize linkage disequilibrium between them.
To asses reliability of solutions, 10 iterations were run for each *K*. Each run was made of 20,000 MCMC (Markov Chain Monte Carlo) generations as burn-in, followed by 50,000

MCMC replicates to estimate the posterior sample distribution, using the admixture and correlated allele frequency models. Three different methods were used to determine the number of groups (*K*) identified by STRUCTURE of each data set. The first identifies the most likely value of *K* by comparing changes in LnP(D) values of consecutive *K* (Prithchard *et al.*, 2000). The second method, developed by Evanno *et al.* (2005), finds the ad hoc quantity based on the second order rate of change of the likelihood function with respect to *K*(ΔK).

3. Results

3.1 Sequences variation and *taxa* polymorphism

For each specimens (N=264) a nucleotide sequence of 733 bp long, corresponding to a partial region of the cytb gene, was analyzed. Combination of variable sites defined 27 different haplotypes. Of these 27 haplotypes two were already deposited in GenBank with the following accession numbers: AF112124 corresponding to Bc6 (*B. caninus*) and AY331019 corresponding to Bb10 (*B. barbus*). Table 2.4 shows the distribution of the detected haplotypes across *taxa* analysed.

Table 2. 4 Haplotypes distributions across sampled populations.

Taxa/haplotype	Bc1	Bc2	Bc3	Bc4	Bc5	Bc6*	Bc7	Bc8	Bc9	Bb10*	Btyb11	Bp12	Bp13	Bp14
B. caninus	61	1	1	4	1	3	1	3	1	7	1	3	15	
B. balcanicus														
B. tyberinus										20	7	2	16	
B. plebejus										5		2	25	1

Taxa/haplotype	Bbal15	Bbal16	Bb17	Bb18	Bp19	Btyb20	Btyb21	Btyb22	Btyb23	Btyb24	Btyb25	Btyb2	Btyb27
B. caninus													
B. balcanicus	21	14											
B. tyberinus						18	1	21	1	1	2	2	1
B. plebejus			1	1	1								

* Haplotypes already deposited in GenBank

Levels of sequence polymorphism were summarized in table 2.5. Concerning the cytb, the highest level of haplotype diversity (0.831) was found in *B. tyberinus*, the lowest (0.494) in *B. balcanicus* that showed just two haplotypes. *B. caninus* showed the highest value of π (0.033) with the highest number of polymorphic sites (S=86). In *B. balcanicus* was detected the lowest value of π (0.002) with just 4 polymorphic sites. Overall nucleotide diversity among 264 samples was 0.053. For Pop. n°8 Tajima's D test gave a significant result and R_2 test gave a significant results for Pop. n°15 (Appendix II), suggesting that these two populations might had experienced a bottleneck; while the hypothesis of neutral evolution could not be rejected for haplotypes of other *taxa*. Within each species group p-values of all statistical test were not significant.

Sequences analysis of four nuclear genes yielded 2662 aligned sites (S7_1: 373 bp; S7_2: 598 bp; Gh1: 588 bp; Gh2: 1103 bp). Several indels were assumed in the alignments to maximise base pair identity in conserved sequenced blocks flanking the indels. Indels ranged from 1 bp

up to 95 bp (found in Gh2). Growth hormone genes (1 and 2) were the most variable markers, Italian *Barbus* specimens analysed exhibited 42 and 68 alleles for Gh1 and Gh2 respectively. Conversely ribosomal protein S7 (1 and 2) genes were less variables; from the alignments were recognised 27 and 32 alleles for S7_1 and S7_2 respectively.

Polymorphism levels calculated from the dataset excluding sites with gaps were reported in table 2.5. If Hd showed similar values among nuclear and mitochondrial markers, the same it was not true for π values. Nuclear loci, despite being introns, showed polymorphism levels considerably lower than those observed in the mtDNA fragment analysed (π_{Gh1}=0.011; π_{Gh2}=0.016; π_{S7_1}=0.021; π_{S7_2}=0.010; π_{cytb}=0.053). As for cytb, in general, *B. caninus* showed the highest values of polymorphism, meanwhile *B. balcanicus* the lowest; *B. plebejus* and *B. tyberinus* showed values of polymorphism similar between each other.

Nuclear genes displayed significantly values of Tajima's D and R_2 test indicating non neutral evolution in *B. balcanicus*, *B. tyberinus* and *B. plebejus* (tab. 2.5). Nuclear genes failed to pass the four-gamete test, supporting the hypothesis to have suffered several recombination events at least 3 for Gh1, 6 for Gh2, 2 for S7_1 and 3 for S7_2 (tab. 2.5).

Table 2. 5 Summary of polymorphisms for each locus and each species.

Gene	Species	Lenght (bp)	Indels (bp)	N	Polymorphism				S	D	R_2	Rm
					h	*k*	*Hd* ± SD	π ± SD				
Cytb	All	733	-	264	27	38.93	0.868 ± 0.010	0.053 ± 0.001	111	3.523*	0.175	-
	B. caninus	733	-	102	13	24.209	0.617 ± 0.051	0.033 ± 0.003	86	1.405	0.139	-
	B. balcanicus	733	-	35	2	1.976	0.494 ± 0.039	0.002 ± 0.000	4	2.552	0.247	-
	B. tyberinus	733	-	91	12	13.704	0.831 ± 0.016	0.018 ± 0.001	39	2.476	0.175	-
	B. plebejus	733	-	36	7	8.854	0.506 ± 0.009	0.012 ± 0.002	30	0.012	0.145	-
GH1	All	533-587	6 (54, 12, 9, 14, 36, 3)	528	42	4.966	0.876 ± 0.008	0.011 ± 0.001	32	0.440	0.077	3
	B. caninus	542-587	4 (12, 9, 36, 10)	204	28	4.276	0.914 ± 0.000	0.008 ± 0.000	21	0.538	0.538	3
	B. balcanicus	555-569	1 (14)	68	2	2.094	0.349 ± 0.057	0.003 ± 0.001	6	1.611	0.174	-
	B. tyberinus	533-587	2 (54, 3)	182	9	1.81	0.663 ± 0.032	0.003 ± 0.001	14	-0.641*	0.064*	1
	B. plebejus	533-587	2 (54 , 9)	68	7	2.035	0.296 ± 0.072	0.003 ± 0.001	18	-1.377*	0.055*	-
GH2	All	898-1041	8 (3, 1, 13, 20, 8, 95)	516	68	14.153	0.887 ± 0.009	0.016 ± 0.000	59	1.783	0.119	6
	B. caninus	898-1024	9 (5, 6, 3, 95, 22, 1, 1)	198	42	8.491	0.957 ± 0.004	0.009 ± 0.000	49	0.047	0.086	5
	B. balcanicus	917-1041	8 (3, 1, 13, 20, 8, 95)	70	8	6.111	0.720 ± 0.047	0.006 ± 0.000	14	3.173	0.210	1
	B. tyberinus	898-1029	6 (6, 12, 95, 22, 1, 1)	180	7	6.699	0.496 ± 0.039	0.007 ± 0.000	32	0.596	0.104	2
	B. plebejus	898-1029	6 (5, 13, 3, 95, 22, 1)	68	8	1.543	0.518 ± 0.003	0.001 ± 0.000	30	-2.240*	0.052*	-
S7_1	All	329-352	7 (1, 1, 2, 4, 6, 3, 7)	516	27	6.526	0.882 ± 0.006	0.021 ± 0.000	31	1.144	0.105	2
	B. caninus	341-354	5 (1, 1, 3, 7, 1)	200	11	3.992	0.772 ± 0.021	0.011 ± 0.001	17	0.550	0.117	2
	B. balcanicus	329-343	4 (1, 1, 28, 12)	68	5	1.556	0.561 ± 0.041	0.004 ± 0.001	12	-1.065*	0.064*	-
	B. tyberinus	329-344	7 (1, 1, 2, 4, 6, 3, 7)	180	9	1.768	0.702 ± 0.026	0.005 ± 0.000	18	-1.153*	0.049	-
	B. plebejus	341-352	5 (1, 4, 6, 3, 7)	68	10	2.801	0.678 ± 0.048	0.008 ± 0.001	23	-1.291*	0.059*	2
S7_2	All	535-562	7 (5, 2, 2, 2, 3, 27, 6)	520	32	5.31	0.883 ± 0.006	0.010 ± 0.000	28	0.650	0.094	3
	B. caninus	535-562	7 (5, 2, 2, 2, 3, 6, 27)	202	15	3.639	0.804 ± 0.021	0.006 ± 0.000	17	0.675	0.106	2
	B. balcanicus	567	-	70	3	0.292	0.188 ± 0.061	0.000 ± 0.000	2	-0.490*	0.073*	-
	B. tyberinus	558-562	4 (5, 2, 3, 6)	178	14	1.396	0.641 ± 0.037	0.002 ± 0.000	14	-1.084*	0.049*	-
	B. plebejus	535-562	7 (5, 2, 2, 2, 3, 27, 6)	70	7	2.393	0.638 ± 0.042	0.004 ± 0.000	15	-0.673*	0.079*	-

N: number of sequences; *h*: number of haplotypes; k: mean number of nucleotide differences among sequences; Hd: haplotype diversity; π: nucleotide diversity; S: number of segregating sites; Rm: minimum recombination events. * Statistically significant values for D and R_2 statistics $p<0.05$.

Net between-group mean sequence divergences for cytb are provided in table 2.6. Genetic distances among species varied, excluding outgroup species, from 1.2%, between *B. tyberinus* and *B. plebejus*, and 9.4% between *B. barbus* and *B. balcanicus*.

Table 2. 6 Average distance between pairs of species; all estimates are expressed as percentage.

Gene	Species	*B. caninus*	*B. balcanicus*	*B. plebejus*	*B. tyberinus*	*B. barbus*	*B. carpathicus*	*B. prespensis*	*B. haasi*	*L. comizo*	*L. bocagei*
Cytb	*B. caninus*										
	B. balcanicus	6.1									
	B. plebejus	8.7	8.3								
	B. tyberinus	9	8.8	**1.2**							
	B. barbus	8.6	**9.4**	3.9	3.7						
	B. carpathicus	8.1	5.3	7.6	7.1	7.8					
	B. prespensis	7.1	7.9	6.6	6.7	6.4	7.5				
	B. haasi	8.9	8.1	6.2	6.1	6.4	6.8	7.5			
	L. comizo	9.3	11.9	8.5	9.3	9.1	11.3	8.7	10.9		
	L. bocagei	8.7	10.8	8	8.9	8.5	10.7	8.1	9.9	1.7	
	L. microcephalus	10	12.3	10.1	10.1	9.1	11.3	9.1	11.1	4.9	4.9

Bold values record maximum and minimum distance values between species.

Net between-group mean sequence divergences for nuclear loci are provided in table 2.7. Genetic distances among species varied, excluding outgroup species, from 0.00% (S7_1) between *B. tyberinus* and *B. plebejus*, and 3.8% between *B. caninus* and *B. balcanicus* (S7_1). In general *B. tyberinus* and *B. plebejus* showed the lowest mean distance values except in locus Gh2. Distances among single haplotypes were not calculated due to the low genetic distances found among groups of species.

Table 2. 7 Average distance between pairs of species calculated for the different nuclear loci; all estimates are expressed as percentage.

Gene	Species	*B. caninus*	*B. balcanicus*	*B. plebejus*	*B. tyberinus*	*B. barbus*	*B. carpathicus*	*B. prespensis*	*B. haasi*	*L. comizo*	*L. bocagei*
GH1											
	B. caninus										
	B. balcanicus	1.5									
	B. plebejus	1.1	1								
	B. tyberinus	1.3	1	**0.1**							
	B. barbus	**2.1**	1.2	1.5	1.8						
	B. carpathicus	1.9	0.8	1.4	1.3	1.9					
	B. prespensis	N/A	N/A	N/A	N/A	N/A	N/A				
	B. haasi	1.4	0.5	0.7	0.7	1.6	N/A	0.9			
	L. comizo	2.4	1.6	1.9	2.1	1.8	N/A	1.8	1.5		
	L. bocagei	2.6	1.7	2.1	2.3	2	N/A	2	1.7	0.2	
	L. microcephalus	2.6	1.7	2.1	2.3	2	N/A	2	1.5	0.2	0.3
GH2											
	B. caninus										
	B. balcanicus	1.7									
	B. plebejus	2.2	1.7								
	B. tyberinus	**2.3**	2	1.9							
	B. barbus	1.3	1	1.2	1.4						
	B. carpathicus	2	1.7	1.4	1.8	1.2					
	B. prespensis	1.6	1.5	1.9	1.8	**0.9**	1.7				
	B. haasi	**2.3**	2	1.9	2.5	1.7	1.9	2.1			
	L. comizo	4	3.4	3.8	3.4	2.7	3.8	3.4	4.4		
	L. bocagei	4	3.4	3.8	3.4	2.7	3.8	3.4	4.4	0.3	
	L. microcephalus	3.8	3.3	3.7	3.4	2.7	3.7	3.3	3.9	1.3	1.2
S7 1											
	B. caninus										
	B. balcanicus	**3.8**									
	B. plebejus	2.8	2.7								
	B. tyberinus	2.9	2.9	**0,00**							
	B. barbus	3.5	2	3.2	3.5						
	B. carpathicus	N/A	N/A	N/A	N/A	N/A	N/A				
	B. prespensis	2.8	1.1	2.6	2.8	1.6	N/A				
	B. haasi	1.9	2.1	1.8	1.9	2.1	N/A	1.8			
	L. comizo	2.3	2.8	3	3.1	3.3	N/A	3	2.4		
	L. bocagei	2.3	2.8	3	3.1	3.3	N/A	2.9	2.3		
	L. microcephalus	3.2	3.5	3.6	3.8	4.3	N/A	3.9	3.3	1.7	1.7
S7 2											
	B. caninus										
	B. balcanicus	**2.2**									
	B. plebejus	1.7	1.5								
	B. tyberinus	1.5	1.3	**0.2**							
	B. barbus	1.6	1.6	1	0.8						
	B. carpathicus	**2.2**	**0.2**	1.3	1.1	1.6					
	B. prespensis	2	2	1.5	1.3	0.6	2				
	B. haasi	1	1.7	1.3	1.1	1	1.5	1.3			
	L. comizo	2.9	3.1	2.8	2.6	3.8	3.2	3.9	2.1		
	L. bocagei	2.6	3.1	2.6	2.4	3.7	3.2	3.8	1.9	0.7	
	L. microcephalus	2.5	3.2	2.6	2.4	3.4	3	3.5	1.8	1.1	1.1

N/A: value not assessed. Bold values record maximum and minimum distance values between species.

3.2 Phylogenetic Analyses

3.2.1 Cytochrome b

The phylogenetic trees were built on sequences of 733bp long using an enlarged dataset in which sequences available in GenBank have been included (Appendix III). In this dataset

have been detected 220 polymorphic sites, 173 of which were phylogenetically informative. Phylogenetic relationships of most representative European *Barbus* species reconstructed with MP and ML methods recovered well resolved trees that displayed no differences in their topology (fig. 2.3).

Five monophyletic clusters were recognised. The clade A grouped all species belonging to the lineage of the fluvio-lacustrine barbels (*B. barbus*, *B. plebejus* and *B. tyberinus*); Italian species appear to be sisters of the wide distributed central European *B. barbus*. A little incongruence was present in the cluster of *B. tyberinus* and *B. plebejus*. It was ascribable to two haplotypes of *B. tyberinus* downloaded from GenBank (acc. n°: AF274354; AF274355). that possessed an insufficient number of characters (594bp instead of 733bp) to generate well resolved clades (Gante, 2009b). Conversely all new *B. tyberinus* haplotypes detected in this study (Btyb11, 20, 21, 22, 23, 24, 25, 26, 27) clustered in a well supported clade different to that of *B. plebejus*. MP analysis, performed excluding sequences of GenBank with less of 733 bp, led to well resolved groups, with high values of bootstrap supporting nodes (data not shown).

Riverine barbels did not form an unique monophyletic group as the fluvio-lacustrine ones. They were clustered in four different monophyletic groups (B, C, D, E) according to geographic distribution of the species, in agreement to Kotlik and Berrebi (2002). The clade B comprised species from southern France and the northern Iberian peninsula: *B. meridionalis* and *B. haasi*, this latter species is the only one belonging to the genus *Barbus* inhabiting Iberian peninsula. In clade C were grouped species afferent to the Danubian district: *B. balcanicus* and *B. carpathicus*.

The clade D was the most heterogeneous and contained species from Balkan peninsula inhabiting rivers that drain the Adriatic sea with the exception of *B. strumicae* that is distributed across the Aegean sea basin from Greece to Bulgaria. A little incongruence within this clade was the presence of one haplotype of *B. rebeli* grouped together to *B. peloponnessius*. These two species cannot be easily distinguish morphologically (Markova *et al*., 2010) and thus the *B. rebeli* haplotypes might be a misclassified specimens of *B. peloponnesius*. The clade E comprises all haplotypes of *B. caninus*. No appreciable divergences were present among the 9 haplotypes detected.

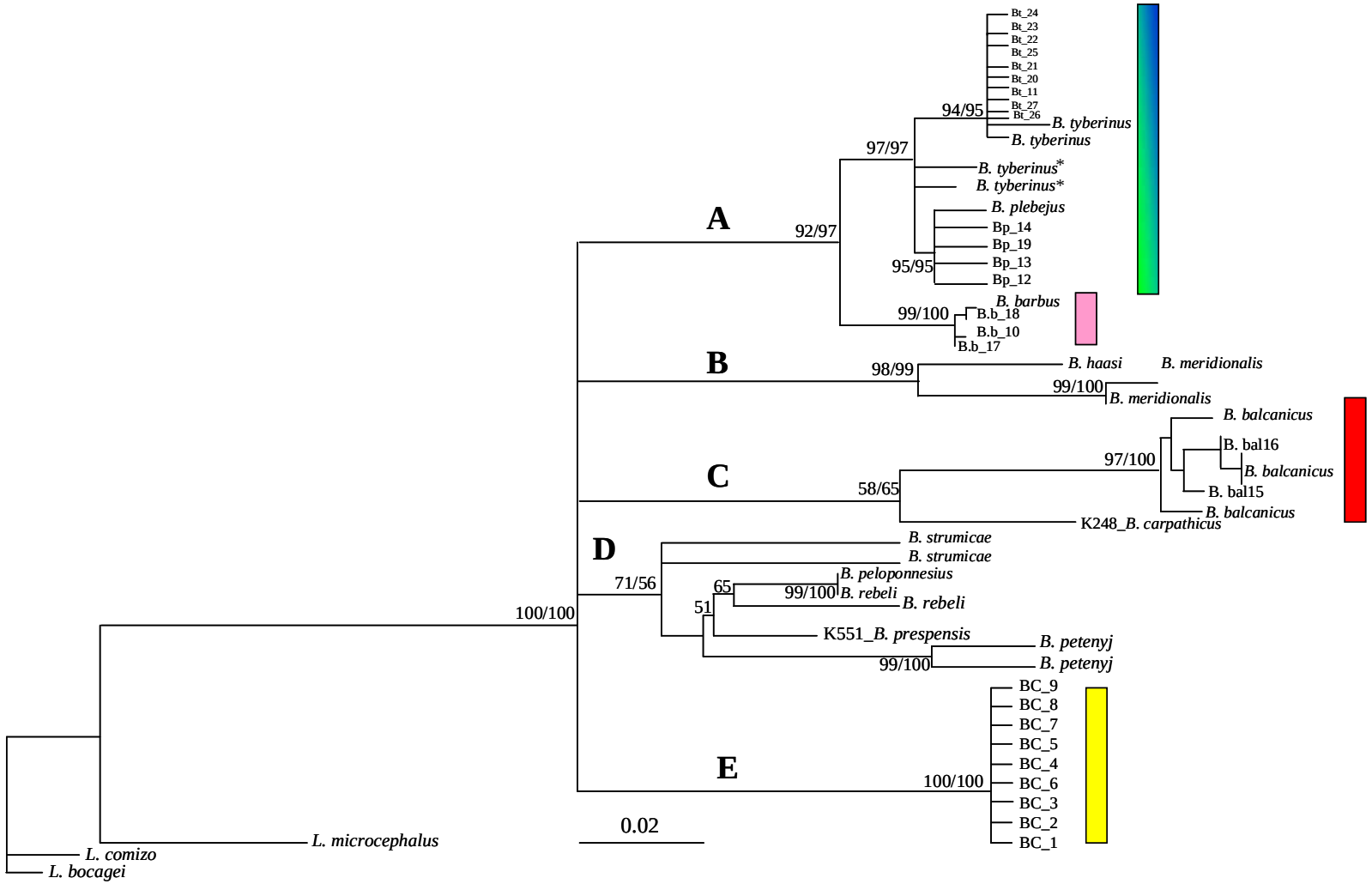

Figure 2. 3 Maximum likelihood phylogeny for cytb haplotypes (tab. 2.4) for all individual sampled. Statistical support for clades is expressed as percentage of posterior probability of 1000 bootstrap replicates and as percentage of bootstrap support. Blue-green bar: *B. tyberinus* and *B. plebejus* haplotypes; purple bar: *B. barbus* haplotypes; red bar: *B. balcanicus* haplotypes; yellow bar: *B. caninus* haplotypes. Asterisks showed haplotypes from GenBank with the following acc. num.: AF274354 and AF274355.

Successively, the minimum-spanning networks based on the Italian dataset, produced in this study, confirmed a clear separation of *B. plebejus* and *B. tyberinus*, clustering their haplotypes in two different networks (fig. 2.4). Moreover minimum-spanning networks recovered the presence of a fifth clade confirming the presence of *B. barbus* as allocthonous species in the Italian watercourses. Within the cluster of each *taxa*, TCS programm showed a general low level of divergence.

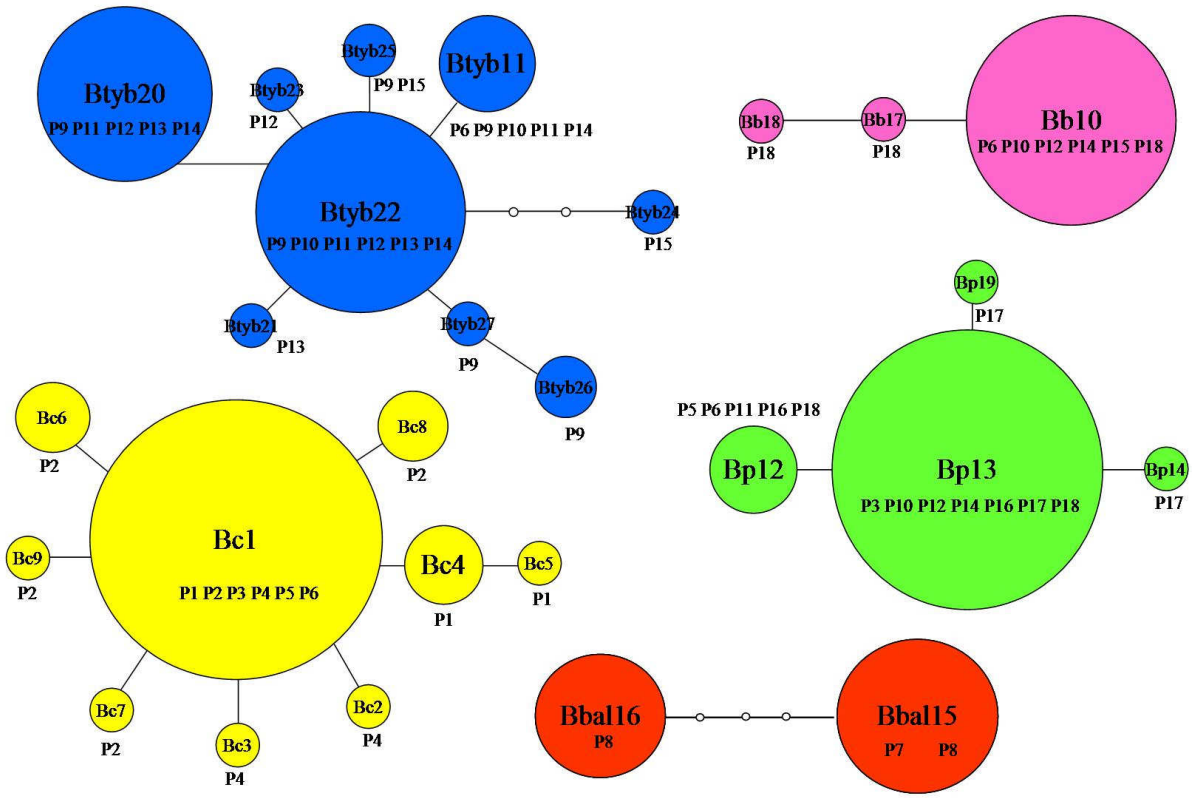

Figure 2. 4 Minimum-spanning network for *Barbus spp.* cytb haplotypes according to statistical parsimony criterion. Solid lines between haplotypes represent single mutation step. Haplotypes not detected in the samples are represented by small white circles. Dimensions of each circle is indicative of the haplotype absolute frequency. Different colours refer to haplotypes of different species: blue *B. tyberinus*; green *B. plebejus*; purple *B. barbus*; red *B. balcanicus*; yellow *B. caninus* . Symbols for locations and haplotype numbers refer to fig.2.1 and table 2.4.

3.2.2 Nuclear loci

Phylogenetic trees based on ncDNA resulted in some cases different from that recovered by the mtDNA. In general the main feature of nuclear gene phylogenies was the presence of several monophyletic groups. Gh1 and Gh2 recorded a similar phylogeny between each other, and the same was for reconstructions with S7_1 and S7_2. But differently, phylogenies of the two genes were not completely congruent. Gh1and Gh2: topologies constructed by MP and ML were similar.

For Gh1, analyses recovered two monophyletic clusters, one composed of *B. barbus* samples and the other comprising the remaining *taxa*. However the node of this latter clade had low bootstrap support (63/64 btp) for both ML and MP analyses (fig. 2.5). For Gh2, the two reconstructions, conversely, recovered the monophyletic origin of all species analyzed (fig. 2.6).

Focusing at the species level, in Gh1, all *taxa* had clades with well supported bootstrap values with the exception of *B. balcanicus* (54/53 btp). In this cluster was present also an allele recovered from *B. plebejus* samples. This allele was a rare one found in my samples with a frequency lower than 0.05%. A MP tree built without this allele recovered a well resolved clade for *B. balcanicus* (data not shown). In Gh2 the only clade with low bootstrap values (51 btp) was the one of *B. barbus*.

In Gh1 *B. plebejus* and *B. tyberinus* clade was not resolved with a mix of alleles belonging to the different *taxa* (fig. 2.5). Gh2, instead, was the only nuclear marker able to resolve better phylogenetic relationships between *B. plebejus* and *B. tyberinus*, clustering alleles of some populations of the latter species in a different monophyletic group (fig. 2.6).

Both markers recovered *B. caninus* in a unique monophyletic group, within which was not possible to evidence any genetic or geographic structure, despite the high number of alleles detected.

No relationships were recovered between *B. balcanicus* and *B. carpathicus*, and *B. haasi* formed a monophyletic group as well.

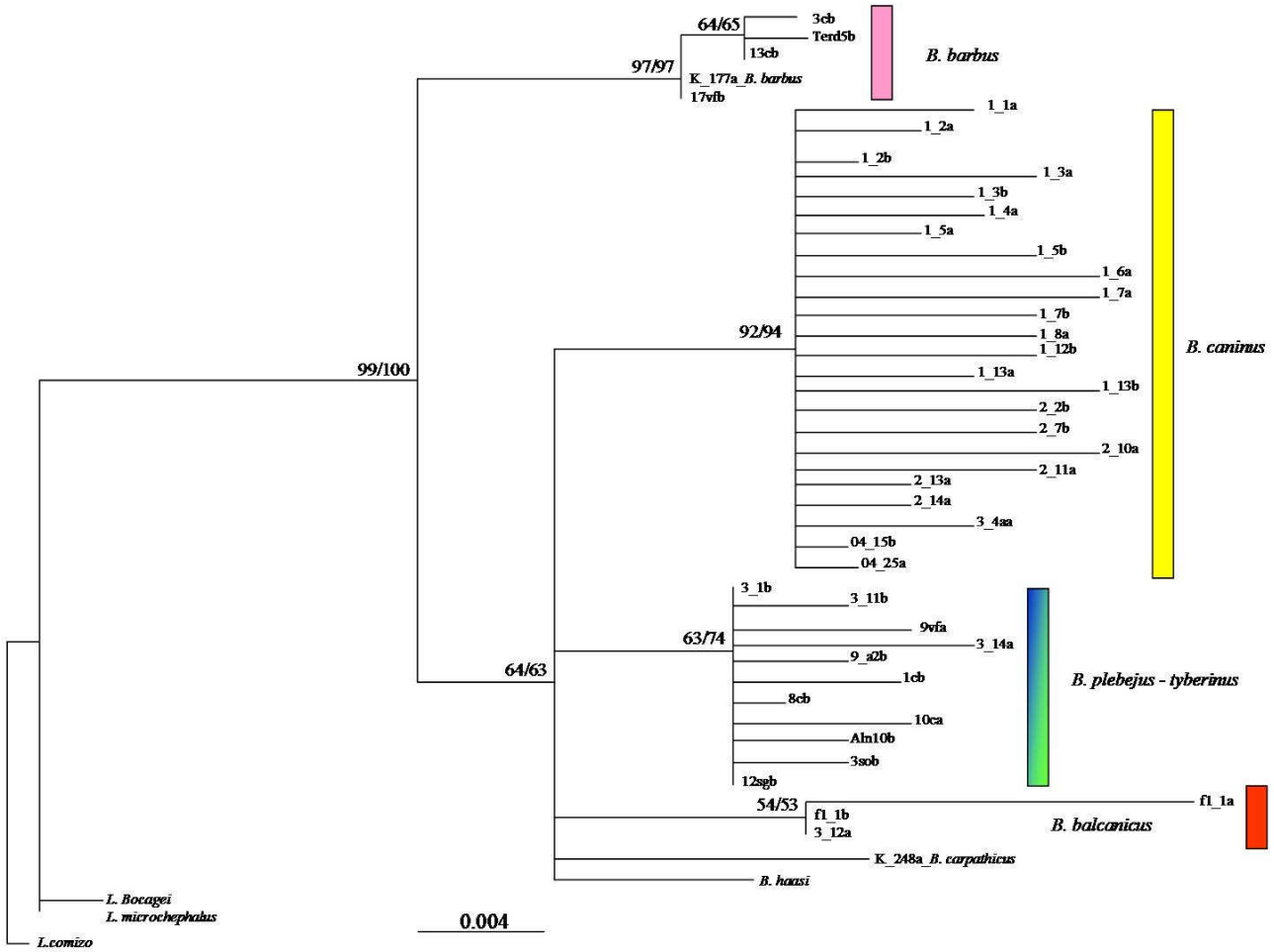

Figure 2. 5 Maximum likelihood phylogeny of Gh1 for all individual sampled. Statistical support for clades is expressed as percentage of posterior probability of 1000 bootstrap replicates and as percentage of bootstrap support. Blue-green bar: *B. tyberinus* and *B. plebejus* clade; purple bar: *B. barbus* clade; red bar: *B. balcanicus* clade; yellow bar: *B. caninus* clade.

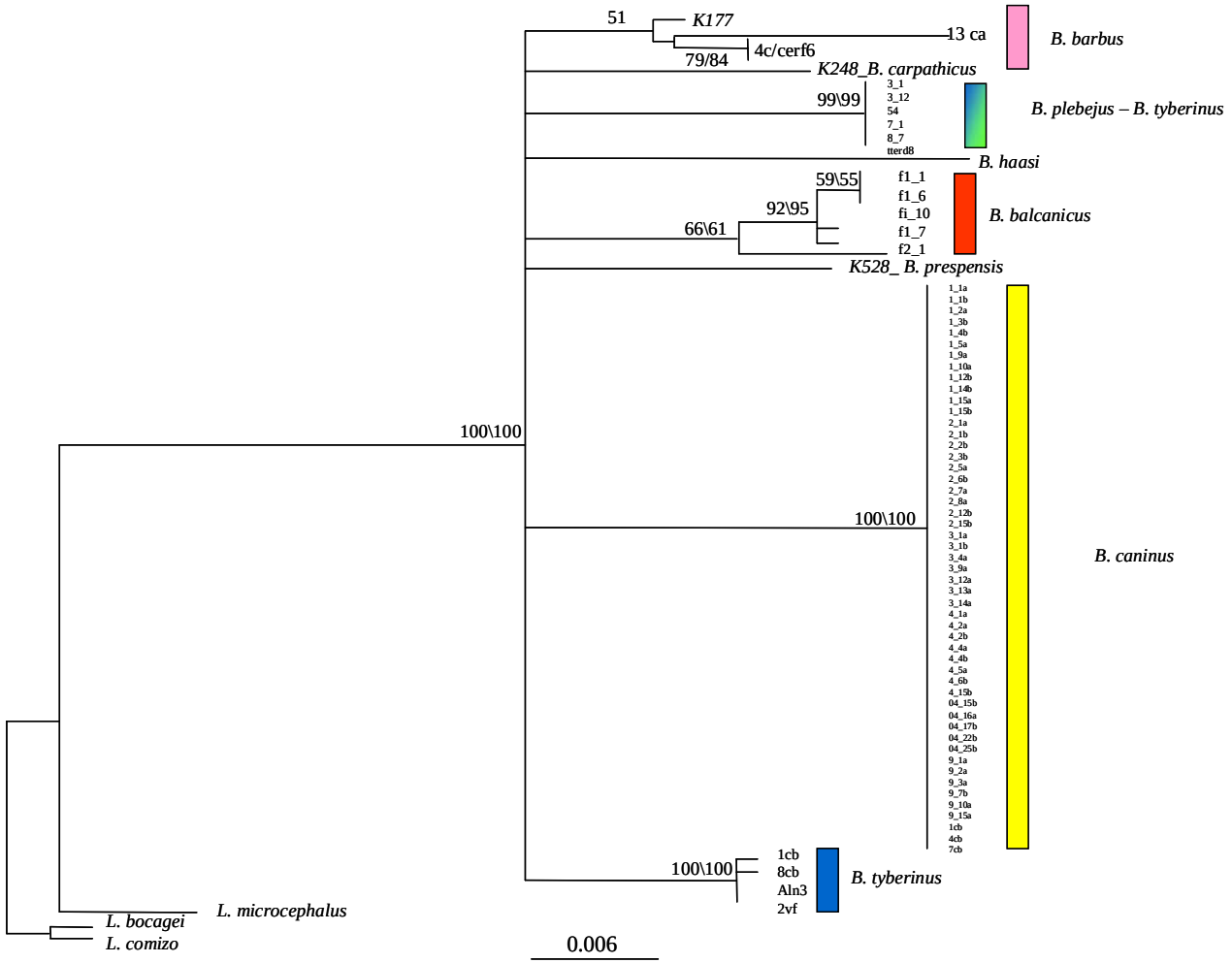

Figure 2. 6 Maximum likelihood phylogeny of Gh2 for all individual sampled. Statistical support for clades is expressed as percentage of posterior probability of 1000 bootstrap replicates and as percentage of bootstrap support. Blue-green bar: *B. tyberinus* and *B. plebejus* clade; purple bar: *B. barbus* clade; red bar: *B. balcanicus* clade; yellow bar: *B. caninus* clade.

Alleles network reconstructed evidenced similar pattern of ML and MP analyses. The principal point of conflict was the monophyly of *B. caninus* clade that in Gh1 network appeared to be closely related to the *B. tyberinus* and *B. plebejus* clade. Conversely phylogenetic reconstruction, networks seemed to define better the differences among *B. plebejus* and *B. tyberinus* alleles (fig. 2.7). In Gh2 network some uncertainties were present in defining relationships among *B. barbus*, *B. plebejus* and *B. tyberinus* (fig. 2.8). It was evident that in the two reconstructions the high number of *B. caninus* alleles generated a tangled reticulation due to conflicting phylogenies (fig. 2.7; fig. 2.8).

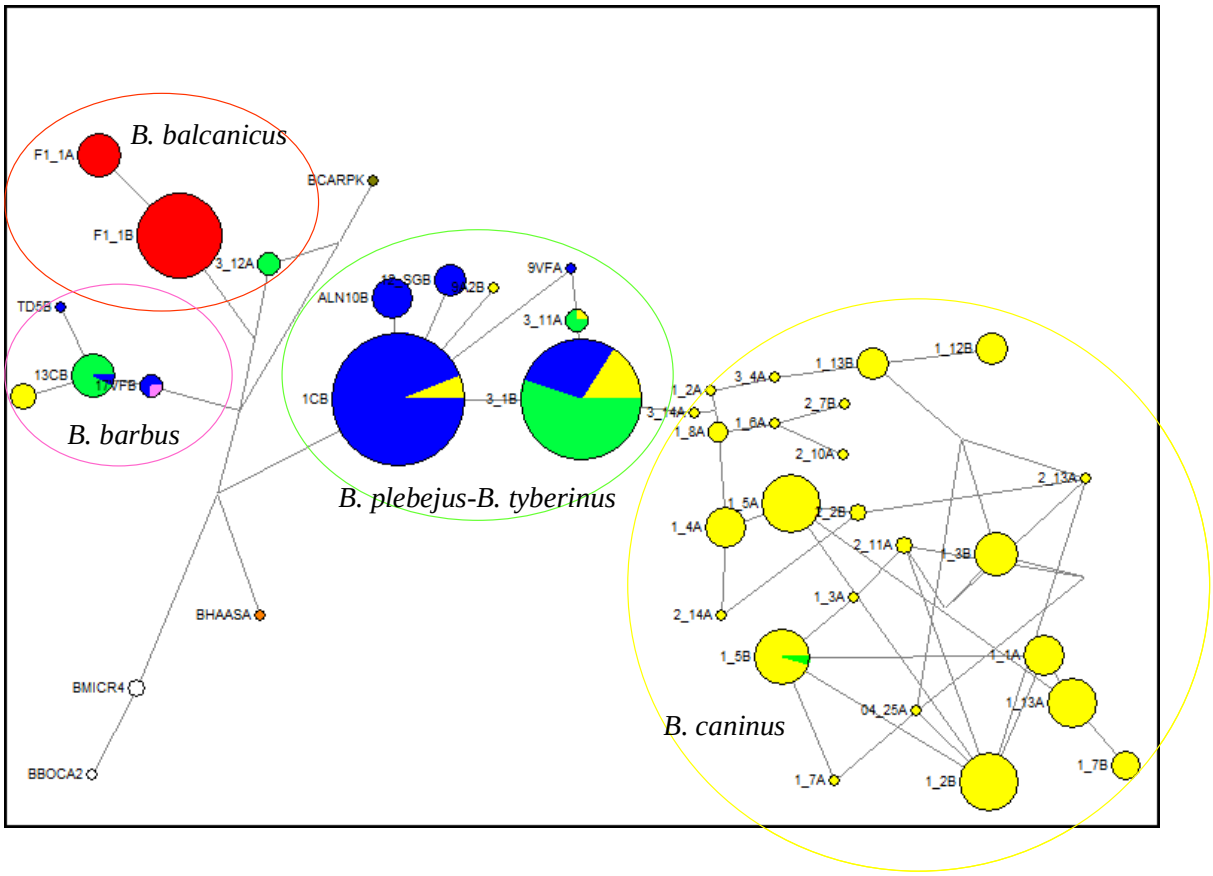

Figure 2. 7 Gene genealogies for Gh1 alleles. Colours refer to alleles recovered in different species: yellow *B. caninus;* green *B. plebejus*; blue *B. tyberinus*; purple *B. barbus*; orange *B. haasi*; dark green *B. carpathicus*; white outgroup species. Dimensions of each circle is indicative of the alleles absolute frequency. Draw ellipses refer to the different clades found with ML and MP analyses in fig. 2.5.

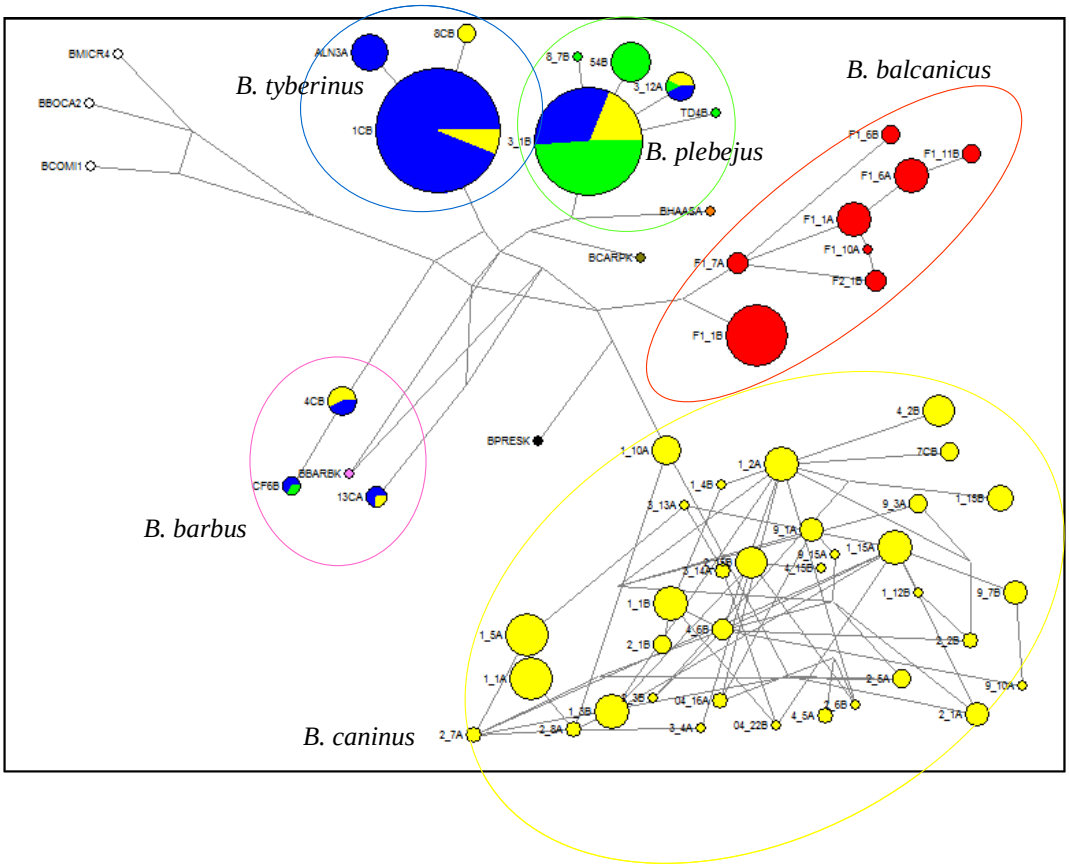

Figure 2. 8 Gene genealogies for Gh2 alleles. Colours refer to alleles recovered in different species: yellow *B. caninus;* green *B. plebejus*; blue *B. tyberinus*; purple *B. barbus*; orange *B. haasi*; dark green *B. carpathicus*; white outgroup species. Dimensions of each circle is indicative of the alleles absolute frequency. Draw ellipses refer to the different clades found with ML and MP analyses in fig. 2.6.

Phylogenetic reconstructions among *Barbus* species with S7 genes highlighted a more complex pattern than those of Gh genes. In S7_1 and S7_2 topologies constructed by MP and ML were similar. As in Gh genes, S7_1 recovered almost all species as monophyletic groups all showing high bootstrap values. In the clade of *B. plebejus* was present an additional cluster, with quite good bootstrap values, that recovered some alleles of *B. tyberinus*, but in general few differences were present (fig. 2.9). S7_2 recovered the presence of three monophyletic groups: one composed of *B. caninus*, the second of *B. haasi* and the third composed of the remaining species. However this last cluster was less supported (fig. 2.10). A second feature in S7_2 was the presence of the undifferentiated collection of *B. plebejus* and *B. tyberinus* alleles with no evidence of monophyly (fig. 2.10).

The main difference with Gh genes was the presence, in S7_1, of a monophyletic group composed of *B. barbus, B. balcanicus* and *B. prespensis*, even if with not very high bootstrap values (fig. 2.9). *B. barbus* clustered together with *B. prespensis* also in S7_2, but *B. balcanicus* formed, with *B. carpathicus*, a different monophyletic group, supported by high

bootstrap values, as found for the cytb reconstruction (fig. 2.10). *B. haasi* continued to be a monophyletic *taxa* very different from the other species in both genes (fig. 2.9; 2.10).

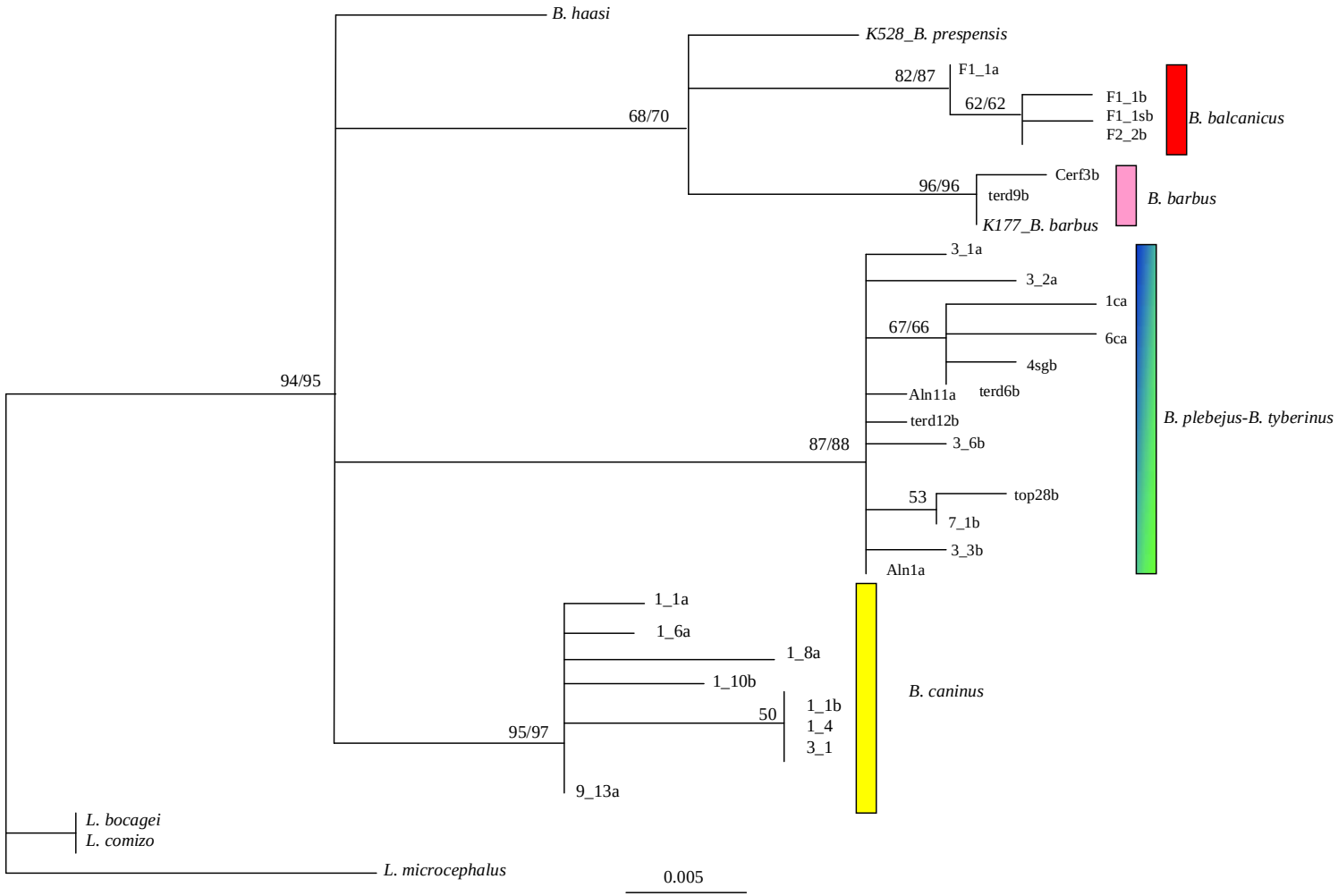

Figure 2. 9 Maximum likelihood phylogeny of S7_1 for all individual sampled. Statistical support for clades is expressed as percentage of posterior probability of 1000 bootstrap replicates and as percentage of bootstrap support. Blue-green bar: *B. tyberinus* and *B. plebejus* clade; purple bar: *B. barbus* clade; red bar: *B. balcanicus* clade; yellow bar: *B. caninus* clade.

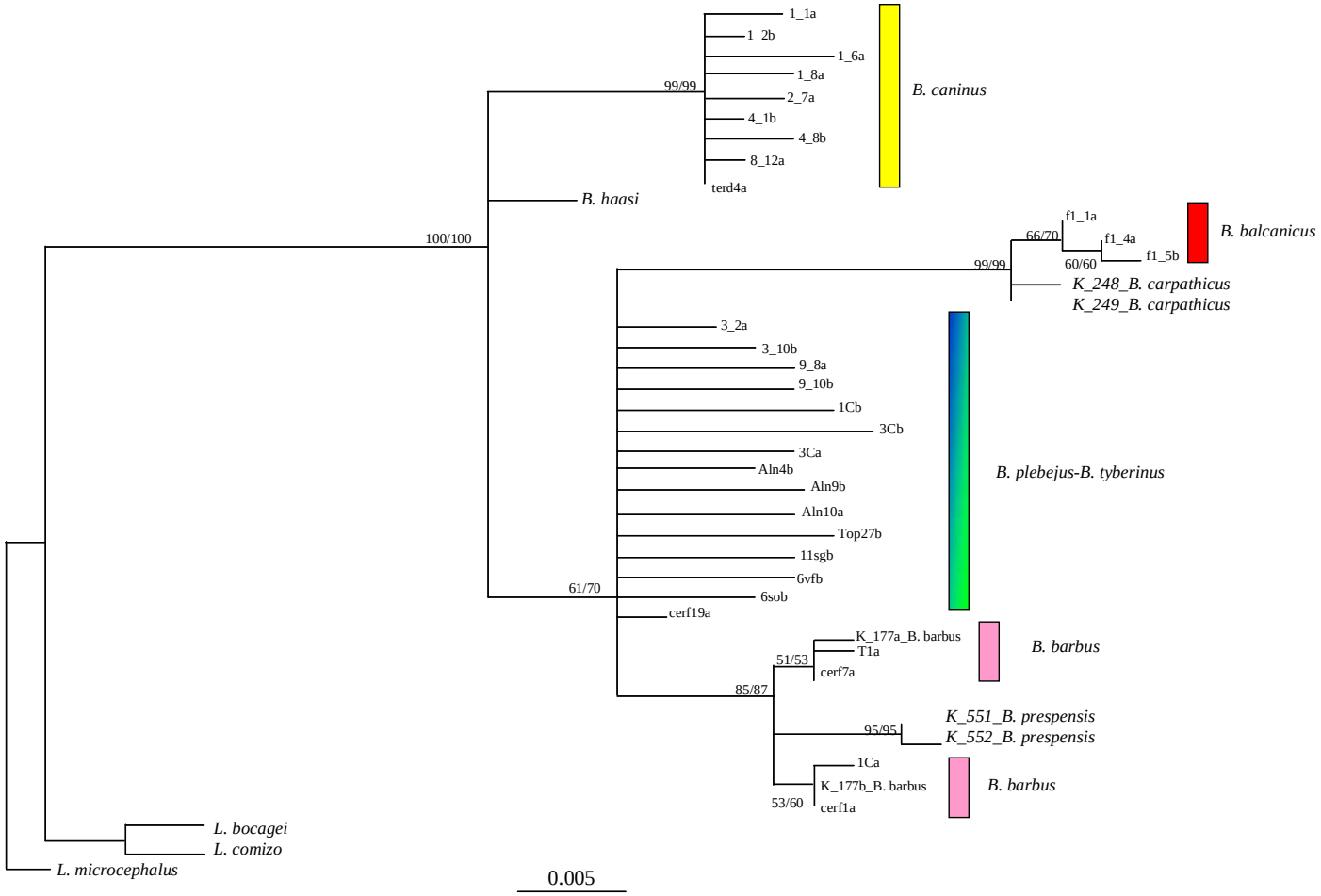

Figure 2. 10 Maximum likelihood phylogeny of S7_2 for all individual sampled. Statistical support for clades is expressed as percentage of posterior probability of 1000 bootstrap replicates and as percentage of bootstrap support. Blue-green bar: *B. tyberinus* and *B. plebejus* clade; purple bar: *B. barbus* clade; red bar: *B. balcanicus* clade; yellow bar: *B. caninus* clade.

The network reconstructions described in general the same relationships of the trees. In S7_1 network, the monophyletic assemblage of *B. barbus*, *B. balcanicus* and *B. prespensis* was less marked; S7_2 network retrieved a close relationship among *B. barbus*, *B. tyberinus* and *B. plebejus*, showing similar relationships as for the cytb (fig. 2.12). Differences were recovered for *B. tyberinus* and *B. plebejus* alleles in both networks. *B. caninus* and *B. haasi* groups were as usual well differentiated from the other species (fig. 2.11; fig. 2.12). Relation between *B. carpathicus* and *B. balcanicus* and those between *B. barbus* and *B. prespensis* were retained in S7_2 network (fig. 2.12).

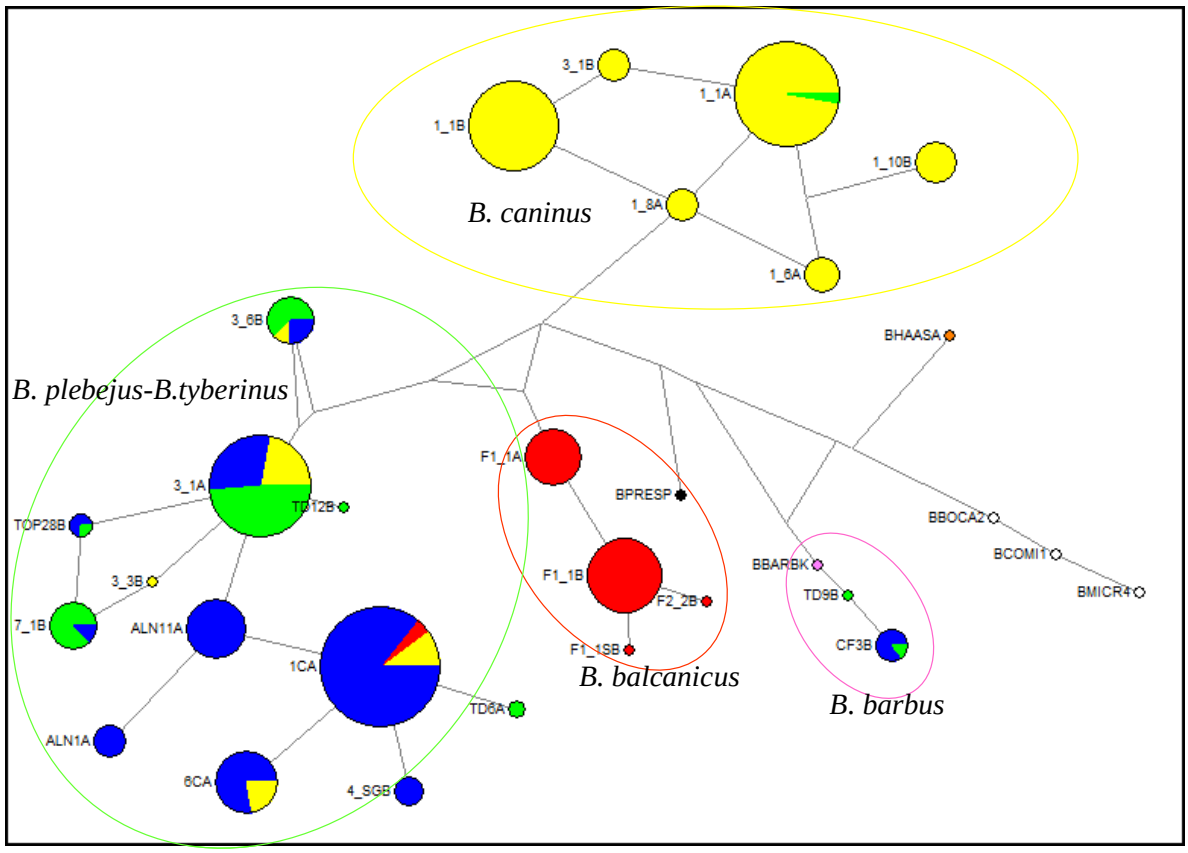

Figure 2. 11 Gene genealogies for S7_1 alleles. Colours refer to alleles recovered in different species: yellow *B. caninus;* green *B. plebejus*; blue *B. tyberinus*; purple *B. barbus*; orange *B. haasi*; dark green *B. carpathicus*; white outgroup species. Dimensions of each circle is indicative of the alleles absolute frequency. Draw ellipses refer to the different clades found with ML and MP analyses in fig. 2.9.

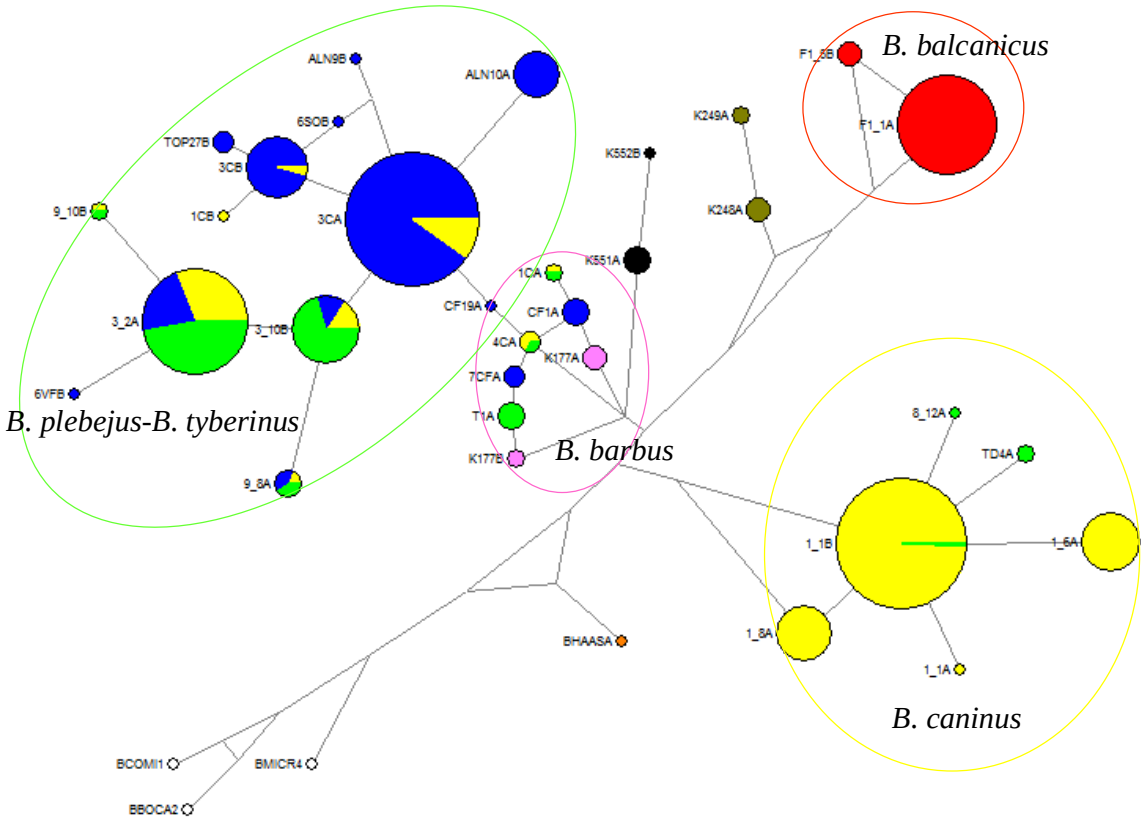

Figure 2. 12 Gene genealogies for S7_2 alleles. Colours refer to alleles recovered in different species: yellow *B. caninus;* green *B. plebejus*; blue *B. tyberinus*; purple *B. barbus*; orange *B. haasi*; dark green *B. carpathicus*; white outgroup species. Dimensions of each circle is indicative of the alleles absolute frequency. Draw ellipses refer to the different clades found with ML and MP analyses in fig. 2.10.

3.3 Population differentiation and relationships among populations

More than one population displayed the presence of haplotypes and alleles belonging to different species, suggesting likely the presence of introgressed genomes or misclassified individuals.

Concerning mtDNA, surprisingly in one specimen of *B. caninus* from the Brenta River (pop.6), belonging to the PV district, an haplotype of *B. tyberinus* was identified (tab. 2.8). Moreover three populations of *B. tyberinus* recorded, in total, 18 specimens carrying three different haplotypes of *B. plebejus*. Strong was also the presence of haplotypes belonging to the allochthonous species *B. barbus*; among the total of 264 specimens, 34 displayed haplotypes ascribable to this latter species. Table 2.8 shows the distribution of the detected haplotypes across sampling localities.

All samples from the Brenta River basin, ascribable to the *B. caninus* species, carried its most frequent haplotype found in the westernmost tributaries of the Po River (Bc1).

B. caninus, *B. tyberinus* and *B. plebejus* were characterized by abundant and widespread haplotypes distributed in many of the sampled watercourses: Bc1 relative frequency 60% present in all *B. caninus* populations; Btyb20 and Btyb22 total relative frequency >30% presents in six population; Bp13 relative frequency 71% present in all *B. plebejus* populations. Many others haplotypes were similar to the most distributed ones, but present in single copies. This pattern is typical for widespread species originated from a small number of founding specimens (Avise, 2000), as showed by the "star phylogeny" of the minimum-spanning network in figure 2.4.

Conversely, *B. balcanicus* showed two haplotypes each one characterizing a different population (tab. 2.8). These two haplotypes were quite different each other, since they differed by 3 mutational step (fig. 2.4). This was an important different, since the sampled populations of *B. balcanicus* were separated by just few kilometres. This divergence could be related with the hypothesis of bottleneck which population 8 underwent according to the significant value of the Tajima's D test (Appendix II).

Table 2. 8 Haplotypes distribution across sampled populations.

Population/Haplotype	Bc1	Bc2	Bc3	Bc4	Bc5	Bc6	Bc7	Bc8	Bc9	Bb10	Btyb11	Bp12	Bp13	Bp14	Bbal15	Bbal16	Bb17	Bb18	Bp19	Btyb20	Btyb21	Btyb22	Btyb23	Btyb24	Btyb25	Btyb26	Btyb27	Total	Ichthyogeographic Districtc
1	10			4	1																							15	
2	7					3	1	3	1																			15	
3	15												15															30	
4	9	1	1																									11	PV
5	15											2																17	
6	5									10	1	1																14	
7															20													20	
8															1	14												15	
9																				10		5			1	2	1	19	
10										1	1		4									2						8	
11											2									2		9						13	
12										1	2	2	10							2		2	1					20	TL
13																				3	1	2						6	
14										2			2							1		1						6	
15										16	1													1	1			19	
16												1	8															9	
17													13	1					1									15	PV
18										5		1	4				1	1										12	
Total	61	1	1	4	1	3	1	3	1	32	7	7	56	1	21	14	1	1	1	18	1	21	1	1	2	2	1	264	

BC: *B. caninus*; Bp: *B. plebejus*; Bbal: *B. balcanicus*; Bb: *B. barbus*; Btyb: *B. tyberinus.* Number of populations refer to Table 2.1. PV: Padano-Venetian; TL: Tuscano-Latium.

For ncDNA, population differentiation analysis was carried out with Bayesian cluster analysis performed by STRUCTURE. The program indicated that the most likely number of genetically differentiated groups in the entire data set was K= 4 and the two statistics used to infer the number of clusters, LnP(D) and ΔK (fig. 13), were consistent for this result (fig. 2.14).

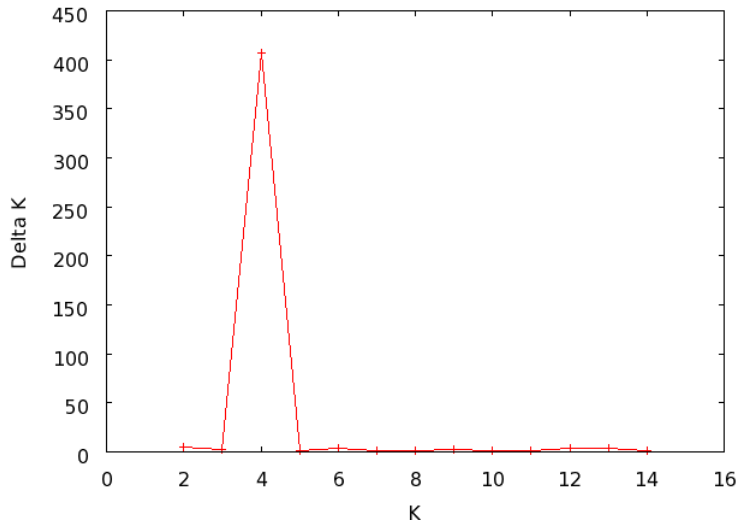

Figure 2. 13 Estimate of ΔK for each possible value of K using data obtained from STRUCTURE.

Groups identified with nuclear data correspond to the 4 *taxa* studied. Conversely phylogenetic analyses, but in agreement with mtDNA results, the Bayesian clustering of ncDNA alleles supported a strong differentiation among *B. plebejus* and *B. tyberinus*, even if alleles of the former *taxa* ware present in the cluster of *B. tyberinus* (tab. 2.9).

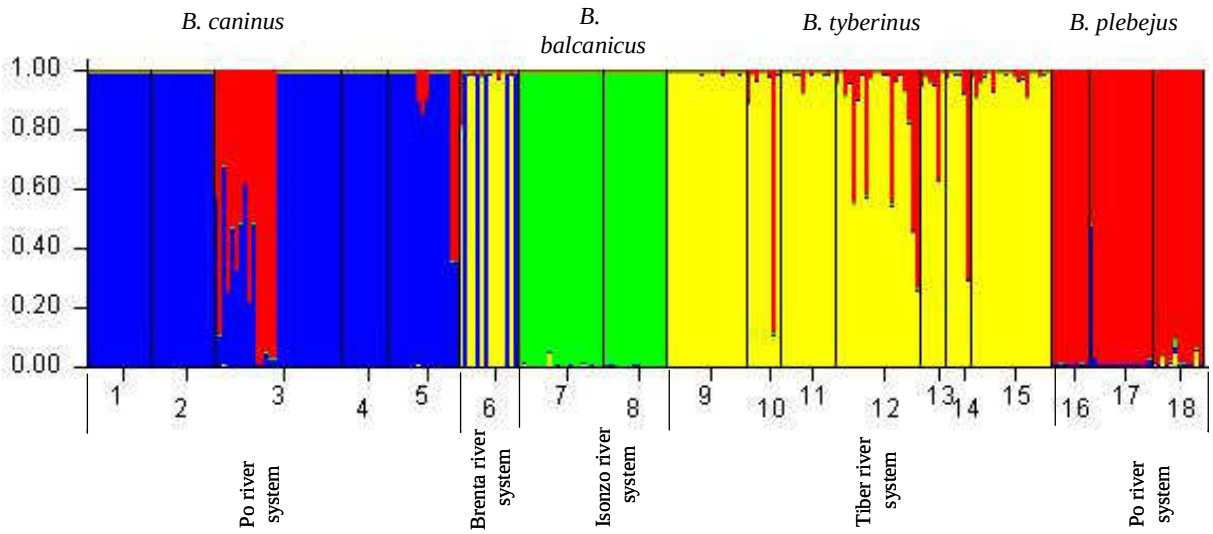

Figure 2. 14 STRUCTURE analysis of all *Barbus spp.* samples for the population assignment test (K=4) of using all four nuclear loci. Each individuals is represented by a vertical line; different colours refer to assignment to different groups.

Table 2. 9 Average population inferred ancestry (*Q*) for *K*=4 calculated by STRUCTURE across 10 iterations.

Population/ Species	n°	*B. caninus*	*B. tyberinus*	*B. balcaninus*	*B. plebejus*	Specimens
Sangone/ *B. can*	1	0.987	0.004	0.004	0.004	15
Tanaro/*B. can*	2	0.988	0.004	0.004	0.004	15
Ceronda/*B. can*	3	0.637	0.005	0.004	0.354	30
Po/*B. can*	4	0.987	0.004	0.004	0.005	11
Lemme/*B. can*	5	0.892	0.005	0.004	0.099	17
Cismon/*B. can*	6	0.356	0.62	0.004	0.02	14
Piumizza/*B. balc*	7	0.004	0.007	0.985	0.004	20
Groina/*B. balc*	8	0.004	0.004	0.988	0.004	15
Paglia/ *B. tyb*	9	0.004	0.988	0.004	0.004	19
Topino/ *B. tyb*	10	0.004	0.86	0.004	0.132	8
Tyber/ *B. tyb*	11	0.004	0.981	0.004	0.011	13
Chiascio/ *B. tyb*	12	0.004	0.833	0.004	0.159	20
Lama/ *B. tyb*	13	0.004	0.903	0.004	0.089	6
Soara/ *B. tyb*	14	0.005	0.862	0.004	0.129	6
Cerfone/ *B. tyb*	15	0.004	0.967	0.004	0.024	19
Po/ *B. pleb*	16	0.004	0.005	0.004	0.987	9
Maira/ *B. pleb*	17	0.038	0.004	0.004	0.954	15
Terdoppio/ *B. pleb*	18	0.007	0.017	0.006	0.97	12

B. can: B. caninus; B. balc: B. balcanicus; B. tyb: B. tyberinus; B. pleb: B. plebejus; n°= number assigned to each population; Specimens= total number of individuals per each population.

At the same time the plot of STRUCTURE shows the presence of shared alleles between *B. plebejus* and two populations of *B. caninus*, but this topic will be treated in detail in the next chapter of this dissertation.

Clustering analysis confirmed also the presence of sympatric specimens of *B. caninus* and *B. tyberinus* in the population n°6 (tab. 2.9; fig. 2.14) sampled in the Brenta River basin, as already retrieved from mtDNA.

4 Discussion

The phylogenetic history revealed by a single locus might not reflect the true species phylogeny (Moyer *et al.*, 2009), for this reason evolutionary relationships among the Italian *Barbus* species were reconstructed using both mitochondrial and nuclear genome. Mitochondrial and nuclear data recovered well resolved trees at the species level (except for *B. plebejus* and *B. tyberinus*), adding molecular evidences to the current systematic classification of *Barbus* species. However these ncDNA data revealed also points of conflict with cytb results concerning phylogenetic relationships among fluvio-lacustrine barbels and those between *B. balcanicus* and *B. carpathicus* (fig. 2.3; fig. 2.5; fig. 2.6). Using Gh and S7 nuclear genes, similar incongruence in phylogenetic reconstructions were recovered also by Moyer *et al.* (2009) for fishes of the genus *Hybognatus*. These topological disagreements prevented from estimating relationships by a full combined nuclear and mitochondrial dataset and could be due to a complex species histories. Moreover it appeared that the extant molecular information available for the genus *Barbus*, might not be sufficient to draw definitive conclusions about its molecular phylogeny, at least with respect to some of its species.

Many potential problems could arise when estimating phylogenetic relationships from multiple markers, and several discrepancies between data inferred from mtDNA and ncDNA were reported for many organisms, e.g. Sota and Vogler (2001), Shan and Gras (2010), Palandacic *et al.* (in press) and many others (Palumbi *et al.*, 2001). In such cases the support of morpho-cladistic analyses could be useful to interpret disaccording results (Pinho *et al.*, 2008).

At the moment it was not possible to exclude that nuclear loci used might no be suitable markers for European barbels as well.

The following discussions will be carried on using information derived from previous morphological and molecular studies and the congruent information shared by the different markers.

4.1 Italian barbels phylogeny

This work was the first attempt to study in detail, with an extended sampling, the phylogenetic relationships among *Barbus* species inhabiting Italian peninsula.

mtDNA analyses showed that most of specimens possessed haplotypes concordant with their morphological traits; some discordances arose typically in zone of contact, regarding *B. caninus* and *B. plebejus*, in agreement with Tsigenopoulos *et al.* (2002), and areas of sympatry with respect to *B. plebejus*, *B. barbus* and *B. tyberinus* (tab. 2.8). This was not surprising for both the high morphological plasticity of *Barbus* species (Doadrio *et al.*, 2002) and because maternally cytoplasmic genomes easily pass trough species boundaries. Therefore attention must be applied when using mtDNA in delimiting species (Chan and Levin, 2005; see Chapter 3).

Concerning Italian species, both dataset suggested monophyly of *B. caninus,* less clear, instead, were the origin of *B. balcanicus* and relationships between *B. plebejus* and *B. tyberinus*; any comparison was not possible for the nuclear dataset, because the only data available in literature were principally referred to the Iberian barbels (Gante 2009b).

General phylogenetic relationships retrieved from ML and MP analyses of 733bp of the cytb were concordant with previous studies (Tsigenopoulos and Berrebi, 2000; Tsigenopoulos *et al.*, 2002). But in this work, because the higher number of populations, samples (N=264) and characters analysed, resulting trees were better resolved (fig. 2.3).

With respect to Italian barbels, the major point of conflict among different authors were the relationships between fluvio-lacustrine species. If authors as Bianco (1995a) and Lorenzoni *et al.* (2006) considered *B. tyberinus,* on the base of the morphological characters, a good species, some others (Tortonese, 1970; Gandolfi *et al.*, 1991; Zerunian, 2002) asserted that Centro-Italian populations of barbels are constituted by *B. plebejus*. Tsigenopoulos *et al.* (2002) attempted to clarified the taxonomic status of these *taxa* using both nuclear and mitochondrial markers (allozyme mobility and cytb sequences), but also for these authors was impossible to drawn any definitive conclusion. On the contrary, results presented in this work pointed out interesting differences.

All haplotypes and alleles of *B. tyberinus* and *B. plebejus* detected in this study were genetically very close to each others (tab. 2.6; 2.7) being their genetic distances the lowest found among different species analysed and in some case this distance was even less than 0.2%. Nevertheless, the phylogenetic hypothesis recovered in the present work identified two different lineages for *B. plebejus* and *B. tyberinus.*

The cytb gene tree presented a clear spilt between these two *taxa* supported by diagnostic haplotypes. Incongruence found in figure 2.3 were due to the presence of *B. tyberinus* sequences (acc. num. AF274354; AF274355) that possessed an insufficient number of characters.

The inclusion of nuclear gene data allowed to test the validity of the two mitochondrial lineages identified. Three of four nuclear gene genealogies failed to detect distinct lineages. Alleles of the two *taxa* clustered in a single big group where it was impossible to detect any structure within it (Pinho *et al.*, 2008). At the same time, networks, even if with a better resolution, did not retrieve a clear subdivision and many alleles were shared between *B. plebejus* and *B. tyberinus*. This discordance could suggest that cytb and Gh2 defined *taxa* that didn't correspond to true evolutionary entities (fig. 2.5; 2.9; 2.10) and that this differentiation was the result of stochastic effects acting on these two markers. Indeed it was demonstrated that quite deep phylogeographic breaks in a single gene genealogies might appear in absence of historical barriers to gene flow (Ballard *et al.*, 2002; Irwin, 2002).

However, there was a remarkable congruence between units defined based on cytb and Gh2 and those observed by morphological analyses and geographic distribution (Bianco, 1995a). Moreover, Bayesian cluster analysis, that took in account all the information carried from all nuclear markers, was able to discriminate a sharp differences between *B. plebejus* and *B. tyberinus*.

Monophyly or exclusivity at majority of nuclear genes was not necessarily a reasonable assumption in recent and/or rapidly radiating lineages (Gamble *et al.*, 2008). Lacking of resolution of the single nuclear marker could be due to incomplete lineage sorting of ancestral polymorphism. This is a likely scenario because nuclear genes take on average four-times as much time to reach monophyly than mtDNA (Gamble *et al.*, 2008; Phino *et al.* 2008; Gante, 2009b; Markova *et al.*, 2010). Moreover gene flow between *taxa* could influence the undifferentiated pattern shown in nuclear markers and Bayesian cluster analysis highlighted the presence, in some specimens, of admixed genomes of *B. plebejus* and *B. tyberinus* (see Chapter 3).

4.2 Hybrid origin of *B. tyberinus*

From the results any evidence of an hybrid origin of *B. tyberinus* was not found. According to Salzburger *et al.* (2002), hybrids species should show a mixture of parental genome both in nuclear and mitochondrial markers. In *B. tyberinus* specimens (N=92) either both the phylogenetic inferences and Bayesian cluster analysis showed a mixture of *B. caninus* and *B. plebejus* genome, that according to Bianco (1995a) should be the parental species of *B. tyberinus*.

4.3 Biographic scenario for Italian barbels

All molecular markers showed a deep divergence between the small-sized barbel species from the Po and Brenta River basins (*B. caninus*) and those of the Isonzo River (*B. balcanicus*) (tab. 2.6, 2.7). Nuclear markers revealed that these species didn't share any ancestral polymorphism (figs. 2.5; 2.6; 2.9; 2.10), thus assuming that ncDNA evolved slower than mtDNA, divergence among *B. caninus* and *B. balcanicus* should be older than the one between *B. tyberinus* and *B. plebejus*. Rheophilic barbels formed two different monophyletic groups, confirming the existence of significant evolutionary divergence between the two lineages. The high genetic distance between *B. caninus* and *B. balcanicus* was comparable with the data published for *Telestes* (Ketmaier *et al.*, 2004; Salzburger *et al.*, 2003), which dated the split among the *Telestes* lineages in correspondence of the Messinian salinity crisis occurred 5 Myra.

B. balcanicus is a widespread species afferent to the Danubian ichthyogeographic district. mtDNA showed that population from the Isonzo River were very close to population of rivers draining in the Danube basin (fig. 2.3; Appendix IV). Results, in according with Tsigenopoulos *et al*. (2002), were consistent for the hypothesis of a recent dispersion from Danubian district to the South. Therefore the presence of *B. balcanicus* in the Italian peninsula was a clear evidence that eastern part of Italian peninsula in recent time (Quaternary) was an exchanging zone among PV ichthyogeographic district and the Danubian one. Past connections, but with an opposite gene flow in this area, were also documented in Danubian populations of bullhead that recovered haplotypes belonging the Adriatic basin (Sletchtova *et al.*, 2004). Similar results were also retrieved for *Telestes* as well, by Salzburger *et al.* (2003). But the presence of Danubian *Telestes* haplotypes in the Adriatic basin could be easily explained by human translocations. Italian vairone, indeed, is frequently used as "live bait" by Slovenian anglers, stocks of these baits are often taken in river draining the Danube basin, therefore the release of baits in rivers draining the Isonzo basin its very likely (Weiss 2002; Sušnik *et al.* 2001; Moro, pers. comm.). The presence of *B. balcanicus* in the Adriatic basin highlighted the permeability of an important geographic barrier as the Alpine chain. It reasonable to suppose that connections between the two side of the Alps were favoured by the peculiar geological characteristics of this Alpine sector, that is composed principally by calcareous stones (Semeraro, 2000). Rivers in karstic region undergo easily changing their flowing direction (Semeraro, 2000), this could allow that different

watercourses of different regions went in contact, allowing the dispersion of species in new territories.

According to Bianco (1989), the present distribution of freshwater fishes in southern Europe was greatly influenced by geological and hydrological events in the Pleistocene and Holocene. In particular the drop of sea level and the subsequent extension of freshwater networks at the glacial maxima allowed a local dispersal via river confluence of freshwater organisms. As previously discussed, the level of genetic differentiation between the fluvio-lacustrine species *B. plebejus* and *B. tyberinus* was relatively shallow (no more than 1.2%); this value was lower than the average level of divergence found among of the other *Barbus* species included in this study. Results were, however, comparable with those obtained by Ketmaier *et al.*, (2009) and Kotlik *et al.*, (2004) among taxonomically distinct lineage of *Alburnus* and *Barbus*, respectively, of recent origin (Middle to Late Pleistocene). It was possible to suppose that divergence between these two *Barbus taxa* started in this period with isolation processes of the PV and TL ichthyogeographic districts supported by restricted gene flow. It was possible that colonization events of TL by *B. plebejus* populations took place also after the separation of the *B. tyberinus* lineage. In fact mitochondrial and nuclear data showed introgression of *B. plebejus* haplotypes in *B. tyberinus* populations (fig. 2.3; 2.14). To really support this hypothesis a more extended sampling should be necessary. From the data it was no possible to exclude that this introgression was men-mediated. Identical haplotypes and alleles of *B. plebejus* were found in wide geographical range from North-West to North-East of Italy, documenting recent connection within these populations. Indeed it was proposed that the fluvio-lacustrine species could rapidly disperse via river confluence during the lowering of the sea level (Tsigenopoulos *et al.*, 2002).

The high number of haplotypes and alleles found in *B. tyberinus*, moreover, could reflect a rapid expansion of its populations in a recent time, data confirmed by the general high haplotypes diversity and the low level of nucleotide diversity (tab. 2.5) (Zaccara *et al.*, 2007).

The strong presence of *B. tyberinus* genome in the *B. caninus* population of the Brenta River basin remained somewhat elusive. Two hypotheses could be proposed: the first one which proposes the easiest scenario, is an anthropic transfaunation, but this is also very improbable. *Barbus* species translocations are known just for the exotic species as *B. barbus*, no information were available to confirm *B. tyberinus* moving in PV district. The second scenario regards past and documented connections between TL and PV district along the Apennine ridge (Cattuto *et al.*, 1988). These limited events of river captures with trans-Apennine connections could have favoured the dispersion of populations of *B. tyberinus* in

the PV district as documented for *Telestes muticellus* (Marchetto *et al.*, 2010) and the genus *Austropotamobius* (Fratini *et al.*, 2005). In this case seems very unlikely that nobody, up today, recorded the presence of *B. tyberinus* in the PV district. However due to the intermediate morphology of *B. tyberinus* and the lacking of an extended genetic study of Italian *Barbus* populations, this last hypothesis could not be excluded.

The high number of haplotype (h=9), with their typical "star phylogeny" (fig. 2.4), and alleles found in a restricted area (the western part of the Po River basin) led to suppose, also in this case, a recent and rapid expansion of *B. caninus* along side the courses of Po River tributaries, probably due to the habitat changes which took place during the last glaciation. The hypothesis that habitat changed up or down along the courses of the rivers during glaciation cycles was supported also for grayling (Sunsik *et al.*, 2001), bullhead (Slechtova *et al.*, 2004) and Italian vairone (Zaccara *et al.*, 2007), all species that shared with *B. caninus* similar ecologies. Moreover the existence of shared haplotypes and alleles between very distant populations, e.g. haplotype Bc1 in localities 1, 2, 3, 4, 5, 6 suggested a relatively recent connection between Po and Brenta Rivers, that should representing the eastern limit of *B. caninus* distribution areal (Kottelat and Freyhof, 2007; Moro pers. comm.). The estuaries of Po and Brenta River are distant at the present sea level, which makes it impossible for *B. caninus* to cross these distances through the open sea. However, sea level was considerably lower during glacial maxima. For the last glacial maximum, between 22,000 and 19,000 years ago, sea level was about 120 m lower than today (Yokoyama *et al.*, 2000). Such a lowstand associated with changes in the course of rivers and possibly their confluence might have permitted exchanges between river systems and thus explain the observed haplotype distribution in the southern populations of *B. caninus*. Another explanation for postglacial contact could be the exchanges of individuals via periglacial rivers or proglacial lakes during deglaciation (Salzburger *et al.*, 2003). A human-induced faunal translocation seems highly unlikely, given the large distances between populations with shared haplotypes, the low economic interest of this species, and the relative rarity of *B. caninus*.

Banarescu (1998) and Tsigenopoulos *et al.* (2002) proposed that rheophilic species, living upstream, might not easily disperse via the lower part of the basin. The low ability of rheophilic species to disperse through the plain open part of the rivers in the presence of large-sized fluvio-lacustrine species would isolate these populations in their mountainous biotope in each respective tributary (or group of tributaries). Found evidences supported that not only fluvio-lacustrine species, as *B. plebejus*, could efficiently disperse during glaciation (Tsigenopoulos *et al.*, 2002), but this is possible for riverine species too.

CHAPTER 3

Population genetic diversity in *Barbus caninus* and *Barbus tyberinus*: ecological preferences or hybridization events?

1. Introduction

Understanding how ecological traits of the species could affect genetic variability and population genetic structure is an important issue in the field of molecular ecology and biological conservation. It is well know that for freshwater fishes physical barriers are important factors limiting the gene flow and promoting populations subdivision (De Woody and Avise, 2000; Youngson *et al*., 2003). On the other hand it was not well established if ecological characteristics in freshwater fishes could modulate gene flow and thus their population genetic structure (Hanfling and Brandl, 1998; Blanchet *et al*., 2010).

Trying to answer to this question a comparative population genetic structures of two endemic Italian barbels (*Barbus caninus* and *Barbus tyberinus*) has been carried out in this thesis. This species are good candidate for this purpose since they share many characteristics: i) belong to the same genus, ii) inhabit the same biogeographical region, iii) have limited distribution, iv) have the same feeding, behavioural and reproductive habits (Zerunian, 2002). The main differences regard the body size and the different ecological preferences. In fact *B. caninus* belongs to the ecological group of the small-sized riverine barbels (total length < 20 cm) (Tsigenopoulos *et al*., 1999). It is moderately cold-water adapted occurring in mountain streams, autochthonous specie of the Padano-Venetian (PV) district (see Chapter 1). In the past was very common, nowadays, *B. caninus* has a severely fragmented distribution with just 20-25 reproductive populations in tributaries of the Po River drainage (Bianco 2003a; Salviati *et al*. 2004). Although there is an ecological separation in the hill zones of rivers *B. caninus* could live in sympatry with its congeneric *B. plebejus* (see Chapter1) creating zones of contact where it possible to find several barbels with intermediate phenotype (Betti, 1993) suggesting that hybridization events might occur between these species.

Barbus tyberinus belongs, instead, to the ecological group of the large-sized fluvio-lacustrine barbels (total length > 50 cm). It prefers wide calm rivers, with regular current. It is one of the most widespread autochthonous species of the Tuscano-Latium (TL) district (see Chapter 1) and it colonises with continuity the middle part of the principal watercourses. Until some years ago *B. tyberinus* was the only barbel species present in the TL district, but nowadays, due to the translocation of fish stocks, lives in sympatry with the allochthonous *B. barbus* (Bianco, 2001). As previously reported for *B. caninus*, also in this case several specimens with intermediate phenotype were found in rivers where these species are known to live in sympatry (Lorenzoni *et al*., 2006).

1.1 Aims

The aims of this study is to depict and to compare genetic structure of *B. caninus* and *B. tyberinus* populations in the light of their different ecological traits. Moreover it is tested the hypothesis that hybridization events could occur between sympatric barbel species and if the presence of hybrids specimens could influence population structure of these fishes.

The information presented in this Chapter are just preliminary explanation on the role that ecology of the species could have in shaping the genetic structure of populations. In order to have a robust validation of this hypothesis a more detailed study should be necessary.

2. Materials and methods

2.1 Sampling

Six populations of *B. caninus* (N=93), seven of *B. tyberinus* (N=91) and three of *B. plebejus* (N=36) were collected by electrofishing across the Padano-Venetian and the Tuscano-Latium ichthyogeographic districts. After the identification a fin clip was sampled and stored in 100% ethanol; after that fishes were immediately released (fig. 3.1 tab. 3.1; see also Chapter 2).

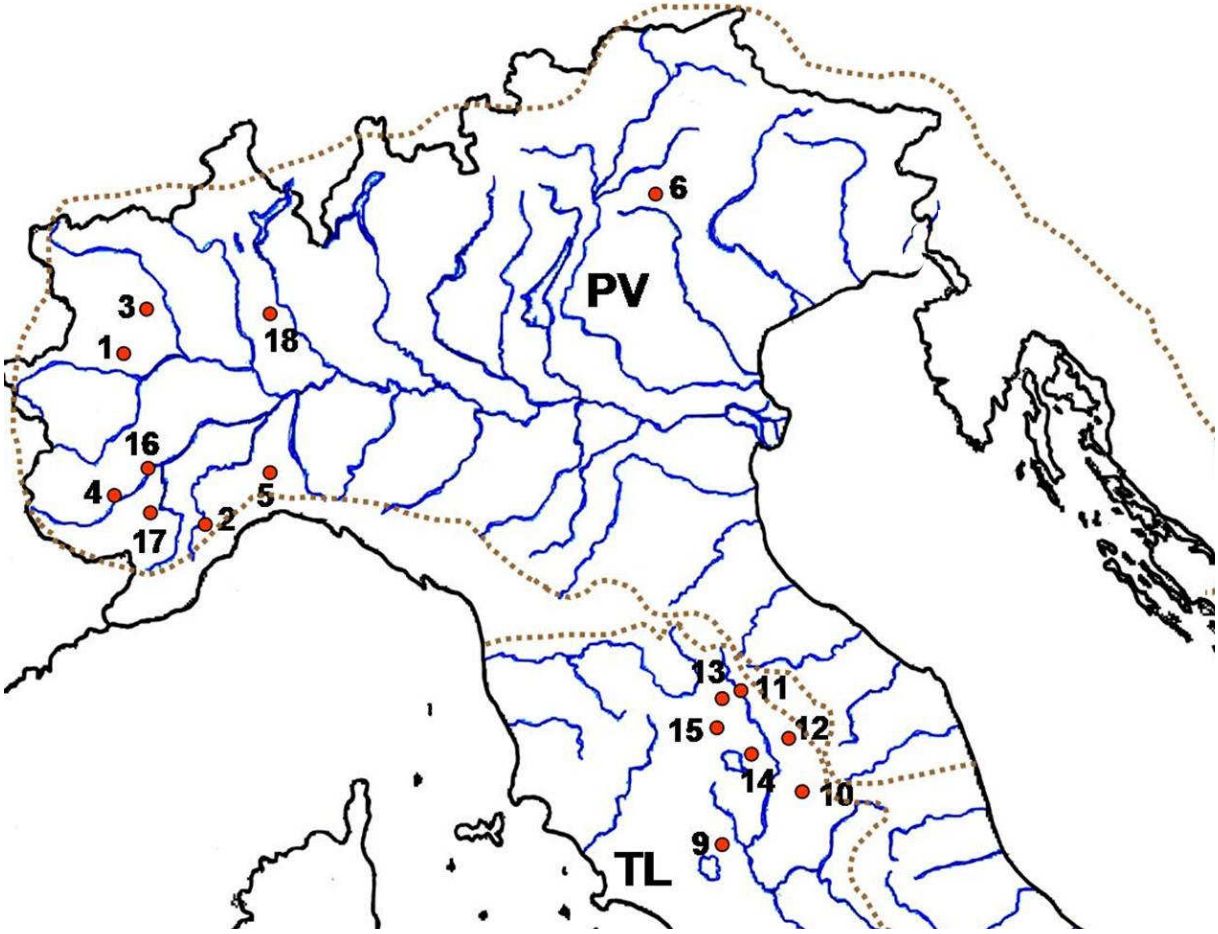

Figure 3.1 Map of northern and central Italy showing the main river system, the ichthyogeographic districts and the sampling sites. PV: Padano-Venetian ichthyogeographic district; TL: Tuscano-Latium ichthyogeographic district.

Table 3.1 Sampling location of *Barbus spp*. populations analysed in this study

Population	Localities	River	Main river basin	Ichthyogeographic district	N° of specimens	Species
1	Trana	Sangone	Po river	PV	15	*B. caninus*
2	Priola	Tanaro	Po river	PV	15	*B. caninus*
3	Varisella	Ceronda	Po river	PV	30	*B.caninus*
4	Sanfront	Po	Po river	PV	11	*B. caninus*
5	Voltaggio	Lemme	Po river	PV	17	*B.caninus*
6	Fonzaso	Cismon	Brenta river	PV	5	*B. caninus*
9	Albergo la Nona	Paglia	Paglia river	TL	21	*B. tyberinus*
10	Scanzano	Topino	Tyber river	TL	8	*B. tyberinus*
11	San Giustino	Vertola	Tyber river	TL	13	*B. tyberinus*
12	Valfabbrica	Chaiscio	Tyber river	TL	20	*B. tyberinus*
13	Passano	Lama	Tyber river	TL	6	*B. tyberinus*
14	Soara	Soara	Tyber river	TL	8	*B. tyberinus*
15	Lupo	Cerfone	Tyber river	TL	20	*B. tyberinus*
16	Cardè	Po	Po river	PV	9	*B. plebejus*
17	Savigliano	Maira	Po river	PV	15	*B. plebejus*
18	Novara	Terdoppio	Ticino river	PV	12	*B. plebejus*
NA	NA	NA	Danube river	D	3	*B. barbus*

Population number, locality, river, drainage system, ichthyogeographic district, number of specimens, species. NA: not available; PV: Padano-Venetian; TL: Tuscano-Latium; D: Danubian

2.2 DNA extraction, amplification of nuclear loci and alleles scoring

Total genomic DNA was extracted in laboratory using a proteinase K digestion followed by sodium chloride extraction and ethanol precipitation (Aljanabi and Martinez, 1997). Then genetic variation was assayed in all the above populations using SNPs at 4 nuclear loci. Details of PCR conditions and references for all loci are given in Chapter 2 (fig. 2.2; tab. 2.2; tab. 2.3).

Each unique allele was identified using the NRDB program (written by Warren Gish, Washington University, unpublished data) available at http://pubmlst.org. Due to the complexity of the total nuclear data set and the high levels of polymorphism of nuclear sequences, I verified the reliability of results obtained with NRDB also using the program MacClade v.4.03 (Maddison and Maddison, 2002); before to start the analysis data set was subdivided in smaller data set as recommended by Pritchard and Wen (2002) (see Chapter 2 for details).

2.3 Data analysis

The software MSA (Dieringer and Schlötterer, 2002) was used to determine mean allele number (Na), observed heterozygosity (Ho), expected heterozygosity (He) and the Shannon index (I) within the analyzed populations.

Genepop version 3.2a (Rousset, 2008) was employed to estimate deviations from Hardy-Weinberg Equilibrium (HWE) across populations (within loci) and across loci (within populations) using the probability test, with 10,000 dememorization steps, 100 batches and 5,000 iterations per batch based on the approach by Guo and Thompson (1992).

Computation of pairwise multilocus F_{st} values (Weir, 1996) among populations was performed using the software Genetix v. 4.02 (Belkhir *et al*., 2001) with 1,000 permutation and an allowed level of missing data of 0.05. Genetix v. 4.02 was also used to infer, by Mantel test, the significance of the relationships between geographical distance and F_{st} values for all pair of populations.

Factorial Correspondence Analysis (FCA) (Benzécri, 1973), which displays the genetic similarity among samples in a three-dimensional graphical space and an assessment of the genetic variability in each population was performed using the software Genetix v. 4.02 (Belkhir *et al*., 2001).

STRUCTURE version 2.2 (Pritchard *et al*,. 2000) was used to determine the population structure, to identify migrant and admixed individual by Bayesian clustering analysis. To asses reliability of solutions, 10 iterations were run for each *K* tested. Each run was made of 20,000 MCMC (Markov Chain Monte Carlo) generations as burn-in, followed by 50,000 MCMC replicates to estimate the posterior sample distribution, using the admixture and correlated allele frequency models. The most likely true K was evaluated both by "L(K)" method, suggested by Evanno *et al*. (2005) and by the lnPr(X|K) suggested by Pritchard *et al*. (2000), varying *K* from a minimum of 1 to a maximum of 10.

Analysis of the molecular variance (AMOVA), performed by GENALEX (Peakall and Smouse, 2006), was used to examine hierarchical partitioning of genetic variation. Differentiation was examined among individuals, among populations and among groups of populations.

The number of segregating sites of each population was calculated by DNA DnaSP, version 5 (Librado and Rozas, 2009).

3. Results

3.1 *B. caninus*

Within populations the lowest mean number of alleles per locus (2.50) was observed in the Brenta River (pop.6) and the highest (12.75) in Ceronda River (pop.3) (tab. 3.2). Observed heterozigosity was higher than 0.50 for each sampling sites. In general differences between total expected and observed heterozigosity resulted quite limited, ranging from -0.144 up to 0.166. Population 3 recovered the highest value of Ho (0.749) and population 6 the lowest (0.588) (tab. 3.2).

Table 3.2 Estimates of genetic diversity in the six populations of *B. caninus* across four loci

Pop	N	Na	Ne	I	Ho	He	He mean - Ho mean
Pop1	14.750	9.250	6.257	1.872	0.630	0.796	0.166
Pop2	15.000	9.250	6.894	1.830	0.700	0.768	0.068
Pop3	29.750	12.750	6.465	2.032	0.749	0.810	0.061
Pop4	10.750	8.000	5.743	1.639	0.659	0.697	0.038
Pop5	16.750	7.750	4.689	1.679	0.601	0.758	0.156
Pop6	4.750	2.500	2.058	0.734	0.588	0.444	-0.144

N: mean number of samples at each population; Na: mean number of alleles per population; Ne: mean number of alleles effective per population; I: Shannon index; Ho: observed heterozigosity; He: expected heterozigosity.

A global test conducted for all loci and for each population showed no significant deviations from Hardy-Weinberg equilibrium. On the other hand, Hardy-Weinberg tests conducted by single locus showed significant deviations. Locus S7_2 recovered significant values in overall 6 populations showing a deficit of heterozygotes too; also S7_1 recorded a significant deviation from HWE in the pop. 6 (data not shown).

Test for genotypic differentiation among populations showed significant differences ($p<0.05$) in all comparisons with the exception of Trana and Sanfront populations (pop.1 and pop.4). The estimator of population differentiation F_{st} ranged from 0.026 to 0.293. This test suggested that at least 5 of the 6 groups analysed represented genetically definable populations (tab. 3.3).

Table 3.3 Estimates of Fst comparisons among populations across all loci

Fst	Pop 2	Pop 3	Pop 4	Pop 5	Pop 6
Pop 1	0.044*	0.057*	0.026	0.086*	0.221*
Pop 2	-	0.079*	0.072*	0.092*	0.276*
Pop 3		-	0.056*	0.081*	0.255*
Pop 4			-	0.081*	0.267*
Pop 5				-	0.293*

* Asterisks highlight statistically significant Fst values ($p<0.05$)

The Mantel test revealed significant correlation ($p<0.05$) between F_{st} values and the logarithm of the geographic distance. The Mantel test failed to retrieve a significant structuring, due to geographic distance, when the dataset took in account only the populations of the Po River basin ($p>0.05$).

FCA analysis allowed to identify four principal groups according, in part, to results from F_{st} calculation: indeed it was possible to group separately all populations with the exception of Sangone, Trana and Sanfront populations (respectively pop. 1, 2, 4) (fig. 3.2). The first 3 axes explained the 76.07% of the total genetic variation. The separation among populations afferent to different river basins was clear; less marked was the separation among populations of the Po River basin (fig. 3.2).

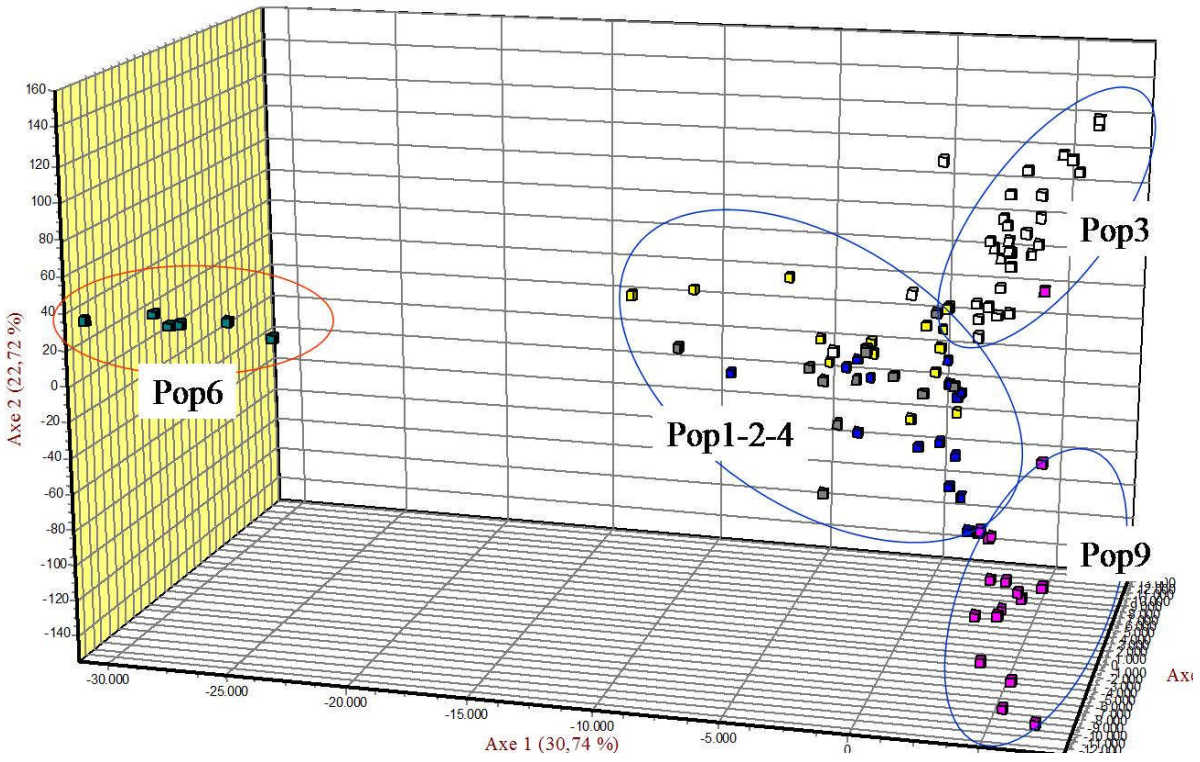

Figure 3.2 Genetic differentiation of *B. caninus* populations based on Factorial Correspondence analysis (FCA). Blue ellipses represent different populations from Po River basin, red ellipse refers to Brenta River population.

Bayesian cluster analysis performed by STRUCTURE indicated that the most probable number of genetic distinct populations was 4, as in the FCA analyses, for both ΔK and the mean estimated logarithm lnPr(X|K) (fig. 3.3). Individual assignment at each cluster was summarized in table 3.4.

Figure 3.3 STRUCTURE analysis of 93 *B. caninus* samples for the population assignment test (*K*=4) using all four nuclear loci. Each individuals is represented by a vertical line; different colours refer to assignment to different groups. Numbers of populations refers to table 3.1.

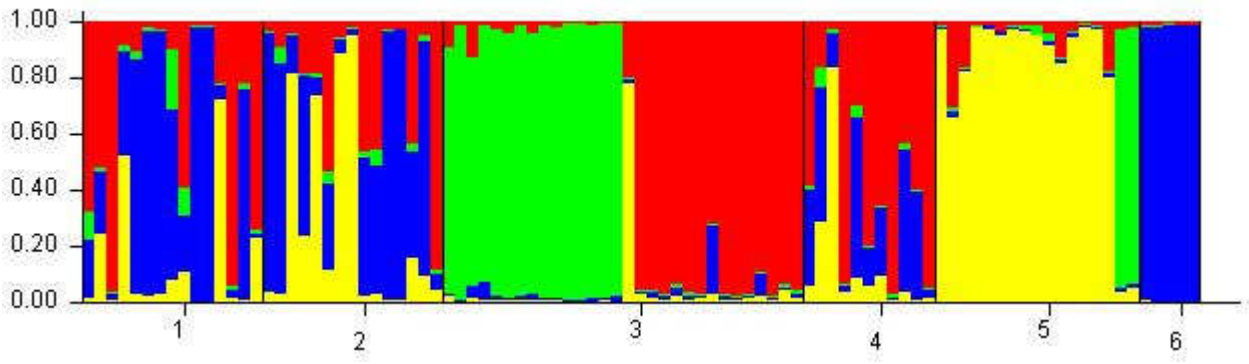

Table 3.4 Average population inferred ancestry (*Q*) for *K*=4 calculated by STRUCTURE across 10 iterations

	Inferred cluster				
Population	1	2	3	4	Specimens
1	0.347	0.038	0.477	0.138	15
2	0.23	0.021	0.467	0.281	15
3	0.458	0.477	0.025	0.04	30
4	0.581	0.02	0.258	0.141	11
5	0.057	0.119	0.01	0.814	17
6	0.009	0.006	0.979	0.007	5

In order to quantify population genetic structuring within and among populations the AMOVA was performed grouping populations according to the subpopulations suggested by STRUCTURE. This analysis revealed that almost all of the variation in the data 86% ($p<0.001$) was due to individuals within populations. Genetic variation among groups was 7% ($p<0.001$), among populations within groups 7% ($p<0.001$).

3.2 *B. tyberinus*

Population of the Paglia River (pop.9) recovered the lowest mean number of alleles per locus (3.75) and the lowest mean-observed heterozigosity (0.375); instead population of the Cerfone stream (pop.15) recovered the highest mean number of alleles per locus (7.25) and

the highest mean observed heterozigosity (0.738) (tab. 3.5). In general differences between total expected and total observed heterozigosity resulted limited, ranging from -0.058 up to 0.211. Hardy-Weinberg tests conducted for all locus and all populations showed no significant values (tab. 3.5).

Table 3.5 Estimates of genetic diversity in the seven populations of *B. tyberinus* across four loci

Pop	N	Na	Ne	I	Ho	He	He mean - Ho mean
Pop9	18.750	3.750	1.905	0.840	0.414	0.461	0.047
Pop10	8	4.250	2.840	1.116	0.375	0.586	0.211
Pop11	13	4.750	2.399	1.081	0.519	0.547	0.028
Pop12	20	6	3.469	1.398	0.663	0.689	0.026
Pop13	20	6	3.469	1.398	0.663	0.689	0.026
Pop14	6	5	4.096	1.401	0.583	0.691	0.108
Pop15	18.250	7.250	4.223	1.608	0.738	0.733	-0.005

N: mean number of samples at each population; Na: mean number of alleles per population; Ne: mean number of alleles effective per population; I: Shannon index; Ho: observed heterozigosity; He: expected heterozigosity.

A global test conducted for all loci and for each population recovered no deviations from HWE; at single locus, significant deviations were detected just for locus Gh1 in the Topino River population and for locus S7_2 in the Cerfone stream population (data not shown).
Genetic differentiation was modest among all populations. The highest and significant ($p<0.05$) value was between pop.1 and pop.5 (0.156) the lowest and significant between pop.4 and pop.5 (0.030) (tab. 3.6). Structuring in *B. tyberinus* populations was not due to the geographic distances. The Mantel test revealed no significant correlation ($p>0.05$) between F_{st} values and the logarithm of the geographic distance.

Table 3.6 Estimate of Fst comparison among populations across all loci

Fst	Pop 10	Pop 11	Pop 12	Pop 13	Pop 14	Pop 15
Pop 9	0.099*	0.028	0.111*	0.156*	0.154*	0.091*
Pop 10	-	0.037	0.035	0.046	0.010	0.038*
Pop 11		-	0.064*	0.081*	0.062*	0.036*
Pop 12			-	0.030	0.029	0.034*
Pop 13				-	0.028	0.046*
Pop 14					-	0.013

* Asterisks highlight statistically significant Fst values ($p<0.05$)

Due to the low values of genetic differentiation, FCA analysis failed to identify clear differences among populations as retrieved from F_{st} calculation. Just two clusters could be identified: one including specimens from the Paglia River (pop.9) and the second, more

heterogeneous including remaining individuals. The first 3 axes explained the 70.49% of the total genetic variation (fig. 3.4).

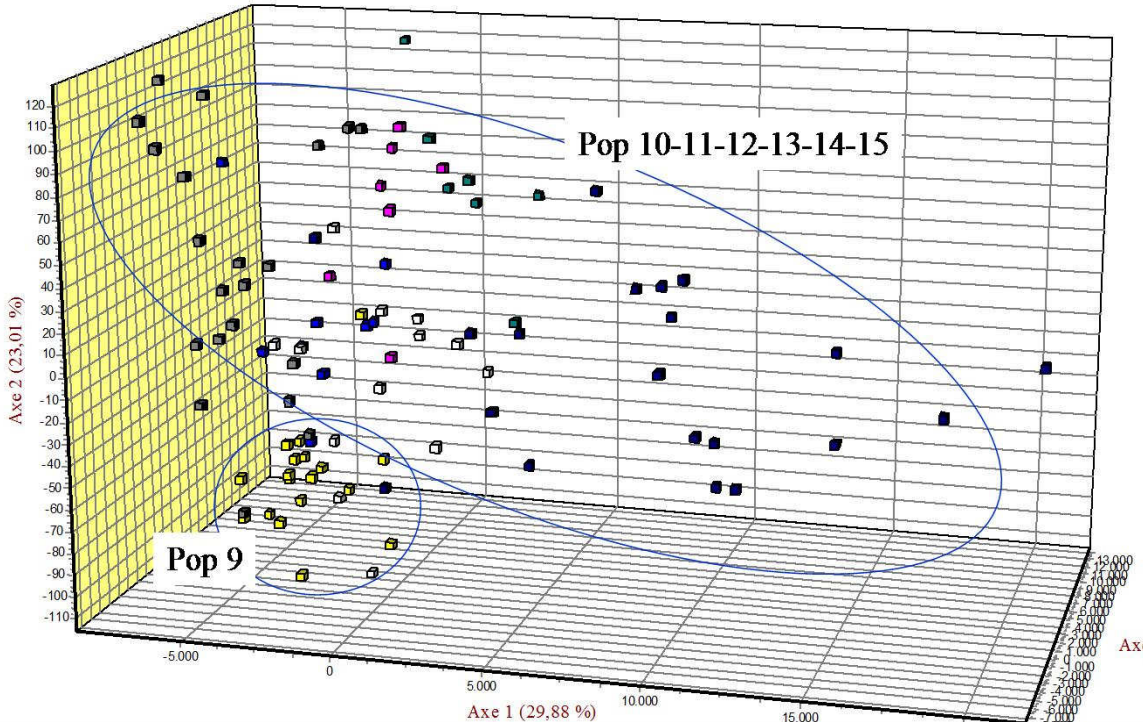

Figure 3.4 Genetic differentiation of *B. tyberinus* populations based on Factorial Correspondence analysis (FCA).

Bayesian cluster analysis performed by STRUCTURE indicated that the most probable number of genetic distinct populations was 2 according to Δ*K* and the mean estimated logarithm lnPr(X|K) (fig. 3.5). Individual assignment were summarized in table 3.7.

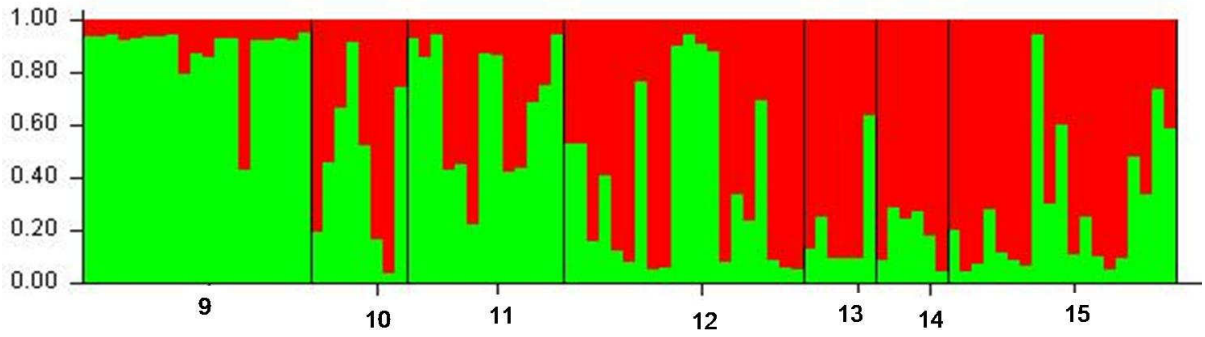

Figure 3.5 STRUCTURE analysis of all *B. tyberinus* samples for the population assignment test (*K*=2) using all four nuclear loci. Each individuals is represented by a vertical line; different colours refer to assignment to different groups. Numbers of populations refers to table 3.1

Table 3.7 Average population inferred ancestry (*Q*) for *K*=2 calculated by STRUCTURE across 10 iterations

	Inferred cluster		
Population	1	2	Specimens
9	0.114	0.886	19
10	0.572	0.428	8
11	0.329	0.671	13
12	0.603	0.397	20
13	0.759	0.241	6
14	0.793	0.207	6
15	0.666	0.334	19

In order to quantify population genetic structuring within and among populations an AMOVA was performed grouping populations according to the subpopulations suggested by STRUCTURE. Analysis revealed that almost all of the variance in the data 92% ($p<0.05$) was by individuals within populations. Genetic variance among groups was 4% ($p<0.05$), among populations within groups 4% ($p<0.05$).

3.3 Hybridization

The presence of hybrids specimens, that could explain the genetic differentiation retrieved among *B. caninus* populations, was tested performing a Bayesian cluster analysis with STRUCTURE enlarging the dataset by 36 specimens of *B. plebejus* coming from 3 different populations (fig. 3.1).

For *B. caninus* Bayesian cluster analysis performed indicated that the most probable number of genetic distinct populations was $K=2$ according to ΔK; and $K=4$ for the mean estimated logarithm lnPr(X|K).

Figure 3.6 showed a clear admixed genomes in half specimens (N=15) of Ceronda River (pop.3), in two individuals of Lemme population (pop.5) and in one individual of pop.8 (*B. plebejus*).

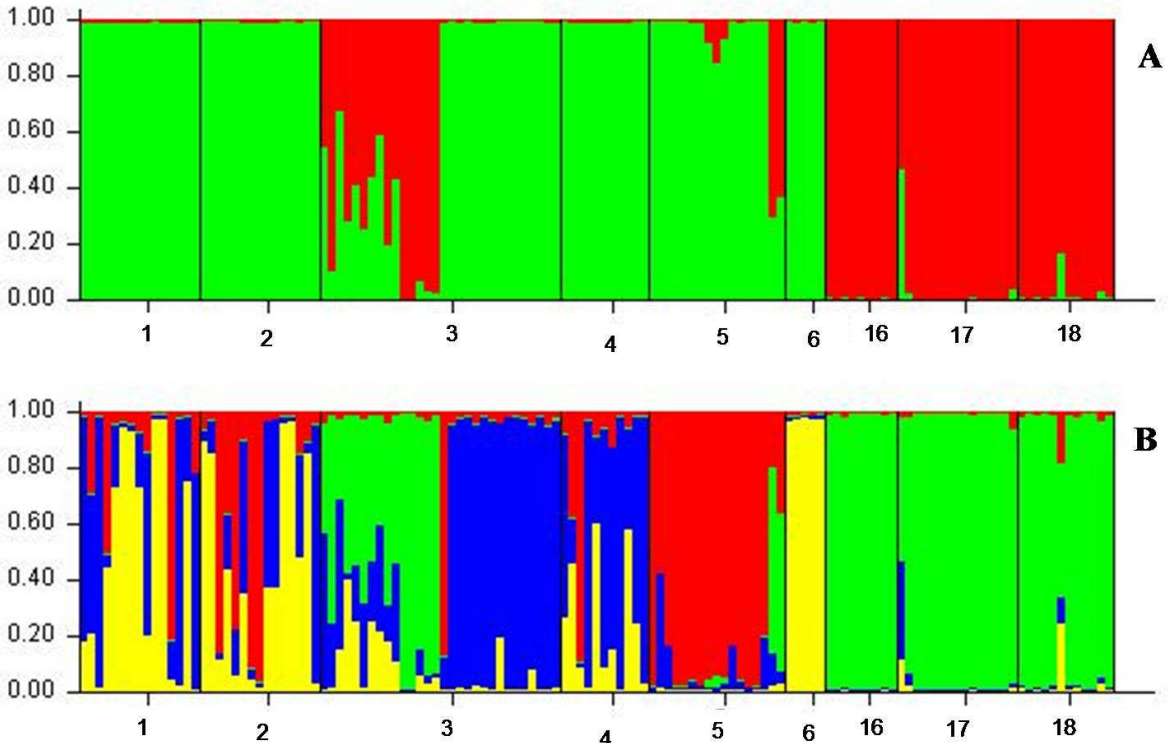

Figure 3.6 STRUCTURE analysis of all *B. caninus* samples for the population assignment test using all four nuclear loci. Each individuals is represented by a vertical line; different colours refer to assignment to different groups. A) *K*=2; B) *K*=4. Numbers of populations refers to table 3.1

In order to quantify which *K* explained the major percentage of variation an AMOVA was performed grouping populations according to the subpopulations suggested by STRUCTURE: *K*=4 explained the major percentage among groups (17%; $p<0.05$).

Another FCA performed without samples from the Brenta River basin and samples with admixed genome revealed that populations of *B. caninus* continue to show some differences in their genetic composition even if less sharp, confirming the value of *K*=4 found by lnPr(X|K). The first three axes explained the 83.34% of the total genetic variation (fig. 3.7).

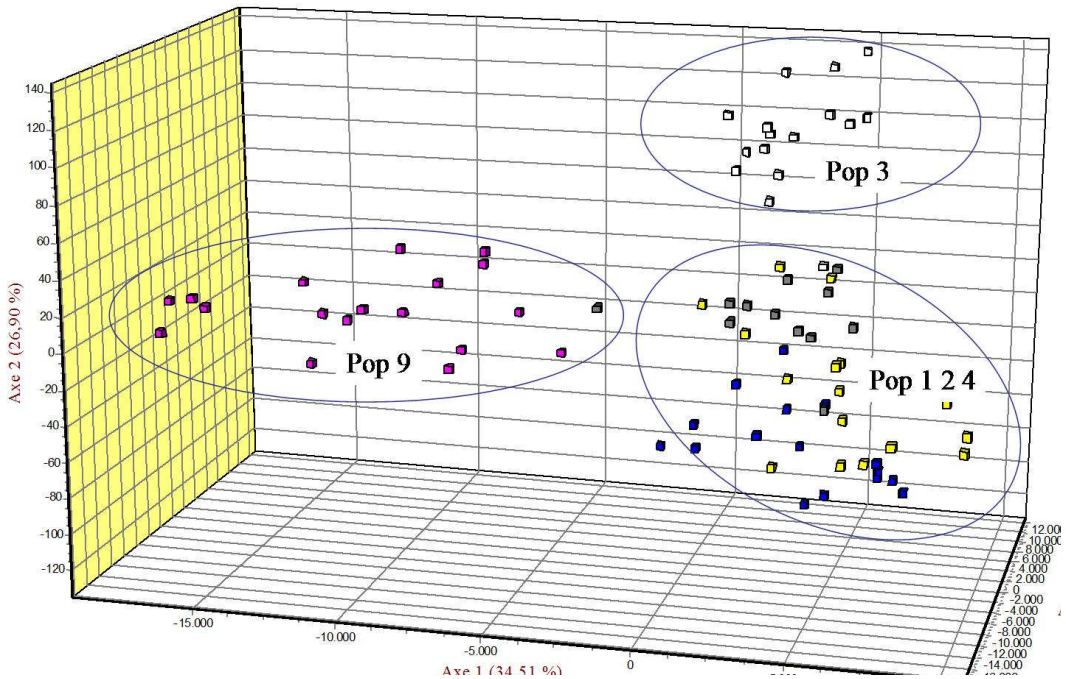

Figure 3.7 Genetic differentiation of *B. caninus* populations based on Factorial Correspondence analysis (FCA). From the analysis were deleted hybrids specimens.

The same populations of *B. plebejus* plus 3 samples of *B. barbus* (used in the phylogenetic study, see Chapter 2) were used to test if it was possible to detect admixed individuals also in *B. tyberinus*, as retrieved for *B. caninus*. Bayesian cluster analysis performed by STRUCTURE indicated that the most probable number of genetic distinct populations was K=2 according to ΔK and K=3 according to lnPr(X|K). Also in this case lnPr(X|K) seems to retrieve the most probable value of K, since dataset was composed of 3 different *taxa*, and previous analysis with just *B. tyberinus* samples retrieved for both lnPr(X|K) and ΔK, K=2. In *B. tyberinus* populations were recorded three distinct genetic pools and was evident how the genome of *B. barbus* was introgressed in almost all populations sampled. Only population from Paglia Rivers seemed too maintain its genetic integrity (fig. 3.8). The few traces of the *B. plebejus* genome in *B. tyberinus* population seems due to the retaining of a common and shared ancestral polymorphisms between *taxa* (see Chapter 2) rather than hybridization events. The presence of *B. barbus* introgressed genome seemed to be present also in some specimens of *B. plebejus* (fig. 3.8) coming from Terdoppio River, where since many years the Italian barbel and the allochthonous one are known to live in sympatry.

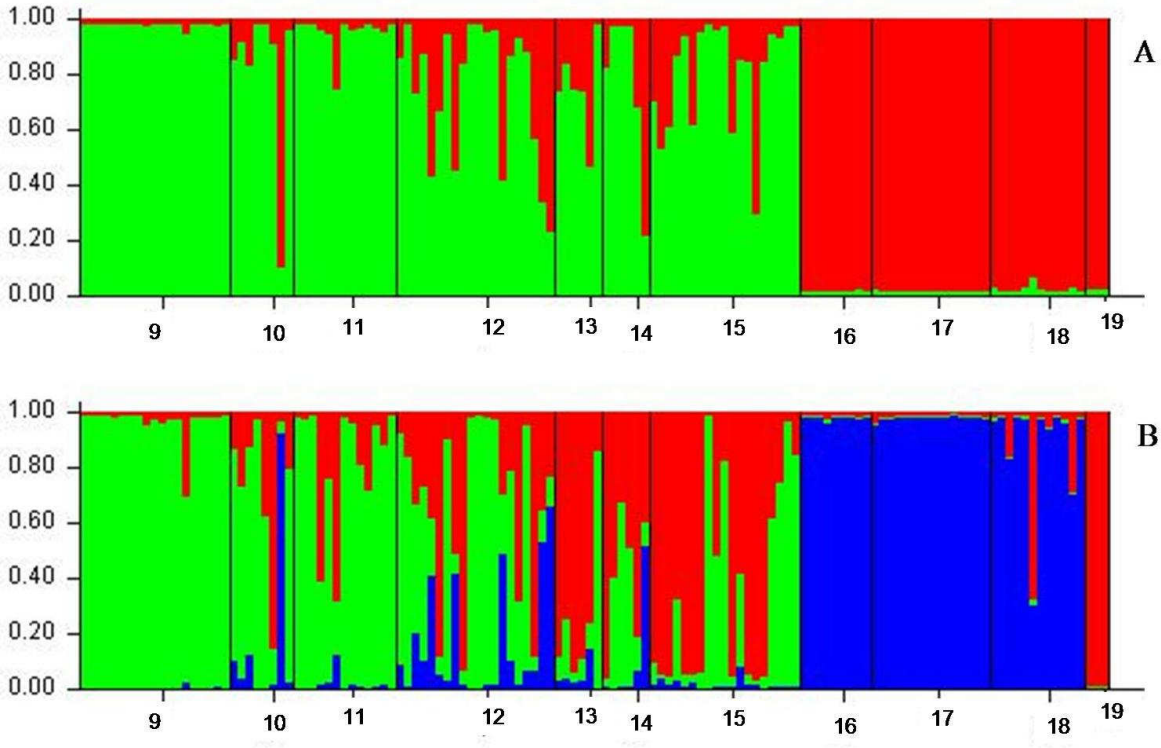

Figure 3.8: STRUCTURE analysis of all *B. tyberinus* samples for the population assignment test using all four nuclear loci. Each individuals is represented by a vertical line; different colours refer to assignment to different groups. A) *K*=2; B) *K*=3. Numbers of populations refers to table 3.1

In order to quantify which *K* explained the major percentage of variation an AMOVA was performed grouping populations according to the subpopulations suggested by STRUCTURE; *K*=2 explained the 4% ($p < 0.01$) of the total variation and *K*=3 explained the 21% ($p < 0.01$). Number of segregating sites (S) (see Chapter 2 and Appendix II) was another parameter to estimate the genetic variation within population and could confirm the presence of hybrids specimens as recovered from STRUCTURE. In fact, populations supposed to be hybrids showed, in general, a number of polymorphic sites extremely higher than populations composed of "pure" specimens. Table 3.8 reports nuclear polymorphism across all loci and populations.

Table 3.8 Nuclear polymorphism across all loci and populations

N	Species/Population	S
1	*B. caninus* /Trana	27
2	*B. caninus* /Priola	24
3	*B. caninus* /Ceronda	73
4	*B. caninus* /Po	27
5	*B. caninus* /Lemme	60
6	*B. caninus* /Brenta	9
9	*B. tyberinus* /Paglia	10
10	*B. tyberinus* /Topino	28
11	*B. tyberinus* /Vertola	40
12	*B. tyberinus* /Chiascio	40
13	*B. tyberinus* /Lama	27
14	*B. tyberinus* /Soara	42
15	*B. tyberinus* /Cerfone	70
16	*B. plebejus* /Po	10
17	*B. plebejus* /Maira	63
18	*B. plebejus* /Terdoppio	61

N: number of populations refers to table 3.1; S: segregating sites

4. Discussion

In this study *B. caninus* showed high values of genetic variability comparable with other small cyprinids, as *Cottus gobio, Gobio gobio, Anaecypris ispanica* and *Telestes muticellus*, that share, with *B. caninus*, similar habitat and the same ecological features (Hanfling *et al.*, 2002; Salguerio *et al.*, 2003; Blanchet *et al.*, 2010; Marchetto *et al.*, 2010, Muenzel *et al.*, 2010). *B. tyberinus* showed, instead, a little lower degree of genetic variability than *B. caninus*. Usually organisms that colonize a large part of the river network, as *B. tyberinus*, are characterized by higher levels of genetic variability (Hanfling and Brandl, 1998; Hanfling *et al.*, 2004; Bergek and Bjorklund, 2009). However this conclusion did not hold across all species. For example, low levels of genetic variability were reported also for *Barbus barbus* and *Sander lucioperca*, common fishes in the European waters (Bjorklund *et al.*, 2007; Schreiber, 2009).

All comparisons for genetic differentiation (F_{st}) among *B. caninus* populations were statistically significant, with the exception of pop.1 and pop.4 (tab. 3.3). Similar values of F_{st}, were found for *Telestes muticellus* populations sampled in the Po River system (Marchetto *et al.*, 2010).

Despite the similar values of F_{st} recovered for *B. caninus*, almost an half of the comparisons among *B. tyberinus* populations resulted not significant. Comparable values of F_{st}, analysing different populations, within a single river drainage, were found for *Leuciscus cephalus* (Hanfling and Brandl, 1998) and for *Barbus barbus* (Schreiber, 2009), ecological vicariant of *B. tyberinus* in central European watercourses.

Differentiation among *B. caninus* populations were recovered also from the FCA plot and from the Bayesian cluster analysis (fig. 3.3); these different methods showed in *B. caninus*, clearly, four clusters with distinct genetic features. By contrast FCA and Bayesian analysis showed no more of two groups for *B. tyberinus* (fig. 3.5).

As found in the previous comparative studies on *Cottus gobio*, *Gobio gobio* and *Leuciscus cephalus* (Hanfling and Brandl, 1998; Blanchet *et al.*, 2010), all divergences encountered between *B. caninus* and *B. tyberinus* could be explained with differences in their ecological characteristics.

In absence of gene flow, stochastic factors within each population will separate them genetically in an unpredictable manner by genetic drift (Bjorklund *et al.*, 2007). It is reasonable to suppose that *B. caninus* populations, that are strictly bound to the small mountain brooks habitat (Bianco, 2003a), could not sustain an high gene flow, leading to their

genetic differentiation. Moreover fragmentation of populations might be influenced by the presence in the lower part of the watercourses of the sympatric species and potential competitor *B. plebejus* (Tsigenopoulos *et al*., 2002). The reduction of the *B. caninus* populations, due to the habitat alteration (Bianco and Delmastro, 2004) might have influenced the fragmentation as well.

On the other hand *B. tyberinus*, distributed along all the Tiber River basin (Carosi *et al*., 2006), having the high dispersal ability of the fluvio-lacustrine barbel species (Ovidio *et al*., 2007; Schreiber, 2009) could lead to genetic homogenization of its populations within of the Tiber River basin. The same findings were found for another fluvio-lacustrine species: *B. barbus* that showed just a moderate tendency to evolve genetically distinctive local populations (Schreiber, 2009). It was also demonstrate that in general large-bodies species, as *B. tyberinus*, are better disperser and therefore less sensitive to fragmentation than smaller one, as *B. caninus* (Ewer and Didham, 2006; Blanchet *et al*., 2010). The idea, that species with intermediate dispersal ability were used to develop genetically differentiated populations than species with higher dispersal ability, was already predicted theoretically (Fahrig, 1998) and empirically demonstrated in a butterfly community (Thomas, 2000).

Genetic differences found among populations might be explained also by the presence of hybrid specimens in the samples, because it is known that interspecific gene flow increases genetic diversity (Gante, 2009b). Indeed *Barbus* species are known for the easiness with which can hybridise each other, and several studies were conducted about this topic (Machordom *et al*., 1990; Persat and Berrebi, 1990; Crespin and Berrebi, 1999; Chenuil *et al*., 2004; Gante, 2009b; Lajbner *et al.*, 2009). For the Italian barbels just one case was reported by Tsigenopoulos *et al*. (2002), concerning hybridization between *B. caninus* and *B. plebejus*.

Concerning the nuclear markers used in this study, all the four revealed the existence of diagnostic alleles that can easily distinguish pure specimens of *B. caninus* and *B. plebejus* (see Chapter 2). Using altogether information carried by nuclear markers it was possible to evidence the presence of admixed genomes within individuals (Gante, 2009b).

Hybridization could occur when *B. caninus* and *B. plebejus*, representing two different ecophenotypes, meet along a transitional habitat, typically between upstream and downstream part of the rivers (Lajbner *et al.*, 2009). This was the case of populations from Ceronda River (pop.3) and Lemme River (pop.5), that were sampled in a transitory habitat between the ecological niche of *B. plebejus* and the one of *B. caninus.* Analysis recovered in several *B. caninus* specimens (N=17) the presence of admixed genomes (fig. 3.6 A and B). Levels of nucleotide polymorphism could be also informative about gene flow between different

species. Data presented showed that populations with presumable hybrid specimens had a number of polymorphic sites extremely higher than "pure" populations (tab 3.8). All these evidences were consistent with ongoing gene flow between *B. caninus* and *B. plebejus* in their zone of contact. Analogous findings were found by Gante (2009b) analysing Iberian barbels. Successive analyses, taking into account just pure *B. caninus* specimens from the Po River basin, continued to recover genetically distinct populations (fig. 3.7), confirming that hybrids specimens did not influenced previous analyses on genetic differentiation.

Since a previous morphological study highlighted the possibility of hybridization events between *B. tyberinus* and the allochthonous *B. barbus* might occur (Lorenzoni *et al.*, 2006), the presence of hybrids was also tested for *B. tyberinus* and *B. barbus* (samples from the Danube basin, see Chapter 2). As in the case of *B. caninus* and *B. plebejus*, nuclear loci used in this study presented diagnostic alleles to distinguish pure specimens of *B. tyberinus* and *B. barbus* (Chapter 2). Therefore, despite the paucity of *B. barbus* samples, findings were well supported. Analyses retrieved a massive presence of *B. barbus* genome within all populations of *B. tyberinus*, with exception of the one from the Paglia River (fig. 3.8 A and B). Also the level of nuclear polymorphism for these population confirmed the results (tab. 3.8). These findings were in agreement with data concerning the distribution of *B. barbus* in Tiber River basin (Lorenzoni *et al*., 2006; ARPA Umbria, 2008). In fact this species is wide distributed in all the main tributaries of the Tiber River, but it is absent from the upper part of the Paglia River, where pop.9 came from. On the other hand, remaining populations were sampled in watercourses where the presence of *B. barbus* was known at least since the 1996 (ARPA Umbria, 2008).

Conversely to the previous hybridization case, *B. tyberinus* and *B. barbus* are two species belonging to the ecological group of the fluvio-lacustrine barbels. This means that they share the same habitat and therefore the probability of interactions and hybridization, how showed by results, is very high. Bayesian cluster analysis recovered also the presence of *B. plebejus* genome in *B. tyberinus* populations. In this case was difficult to asses if there was a retention of ancestral polymorphism or introgressive hybridization, because the previous study (see Chapter 2) showed a close relationship between the two species. To disentangle this topic a more specific sampling should be necessary.

In conclusion, differently from *B. caninus*, divergences among population found in *B. tyberinus* were not due to a real genetic differentiation but more probably to the presence of an high number of specimens with admixed genome.

CHAPTER 4

Conclusions

The overarching theme of this research was to investigate the hierarchical levels of relatedness in natural populations of Italian barbels (genus *Barbus*), a widespread and important group the Italian freshwater fauna. Formerly, this was the first detailed study on Italian species belonging to this genus. In order to conduct these analyses, new molecular tools useful for phylogeographic inferences were developed.

Phylogenetic analyses of mitochondrial and nuclear markers clarified the systematic status of *B. plebejus* and *B. tyberinus*, that were recovered as two clear different *taxa*. Phylogenetic analyses confirmed current morphology-based systematic and the monophyly for the remaining species *B. caninus* and *B. balcanicus* as well. The study highlighted the importance in using combinations of nuclear markers in a complex genus as the *Barbus* one, and in general in all phylogeographic studies as well.

New evidences confirmed the hypothesis of permeability to the freshwater fish of the main biogeographic barriers as the Alpine and the Apennine chains. Moreover this was the first time, at knowledge of the author, that was recorded the presence of *B. tyberinus* in the Brenta River.

The second branch of this thesis was to depict the genetic diversity of *B. caninus* and *B. tyberinus* in order to estimate the importance of the ecological preferences in shaping the structure of their populations. Results indicated that actually ecology of the species could play an important role in modulating gene flow.

The exploitation of new ncDNA markers, developed in this research, allowed to show evidences that Italian barbels species easily hybridize when they come in contact or live in sympatry as found for other European *Barbus* species.

The study of phylogenetic relationships of barbels group is not concluded, it will be interesting to extend the use of these new markers to the remaining European *Barbus* species to estimate the biogeographic patterns across the Mediterranean region, that shows a complex geological and climatic history.

Further analyses could confirm the role of ecological preferences in shaping the genetic structure of Italian *Barbus* species. In this contest, extending the sampling on a wider geographical range, it will be possible, also, to highlight the presence of ESUs (Evolutionary Significant Units) in *B. caninus*, endangered species listed in the IUCN Red List that needs of management plans.

Deeper researches should be addressed to depict better the hybridization events between Italian barbels in order to evidence the direction of the gene flow, the extension of the phenomenon and its ecological consequences. Moreover a broader study seems necessary to

prevent genetic pollution of *B. tyberinus* populations endangered by the presence of the allochthonous species *B. barbus*.

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APPENDICES

I-IV

Appendix I, Table1 : Variable nucleotide position in bp found in Gh2 among direct phased samples and their cloned sequences

Growth hormone 2 (Gh2) - short sequence						
samples/clones	Nucleotide position					
	31	79	125	143	366	395
3_4	T	gap	T	A	T	T
clone1	T	gap	T	A	T	**G**
clone5	T	**G**	**C**	**G**	T	T
clone6	T	gap	T	A	T	T
clone7	T	gap	T	A	**A**	T
3_5	T	gap	T	A	T	T
clone1	T	gap	T	A	T	T
clone3	T	gap	T	A	T	T
clone5	T	gap	T	A	T	T
clone6	**C**	gap	T	A	T	T

Samples cloned; position in bp of the differences found in nucleotide sequence. Bold character highlight differences amomg cloned and direct phased sequences

Appendix I Table 2: Variable nucleotide position in bp found in S7_2 (long fragment) among direct phased samples and their cloned sequences

Ribosomal Protein S7 (S7_2) - long sequence			
samples/clones	Nucleotide position		
	61	62	494
cerf7	C	G	T
clone_1	**G**	**C**	**A**
clone_3	C	G	T
9c	C	G	T
clone_3	C	G	**A**

Samples cloned; position in bp of the differences found in nucleotide sequence. Bold character highlight differences amomg cloned and direct phased sequences

Appendix I, Table 3: Variable nucleotide position in bp found in S7_2 (long fragment) among direct phased samples and their cloned sequences

Ribosomal Protein S7 (S7_2) - short sequence				
samples/clones	Nucleotide position			
	36	268	415	416
cerf7	G	A	A	T
clone_1	G	**G**	**T**	T
clone_3	G	A	**T**	**A**
clone_4	**A**	A	**T**	**A**

Samples cloned; position in bp of the differences found in nucleotide sequence. Bold character highlight differences amomg cloned and direct phased sequences

Appendix II: Summary of polymorphism for each locus and each population

Gene	Population	Lenght (bp)	Indels (bp)	N	Polymorphism				S	D	R_2	Rm
					h	*k*	*Hd* ± SD	π ± SD				
Cyt	Pop.1	733	-	15	3	0.612	0.514 ± 0.029	0.000 ± 0.000	2	-0.002	0.187	-
	Pop.2	733	-	15	5	0.952	0.743 ± 0.014	0.001 ± 0.000	4	-0.045	0.160	-
	Pop.3	733	-	30	2	31.034	0.517 ± 0.054	0.042 ± 0.002	60	3.942	0.110	-
	Pop.4	733	-	11	3	0.364	0.345 ± 0.000	0.001 ± 0.000	2	-0.005	0.200	-
	Pop.5	733	-	17	2	13.456	0.221 ± 0.001	0.018 ± 0.000	61	0.093	0.135	-
	Pop.6	733	-	14	4	34.516	0.659 ± 0.012	0.047 ± 0.004	80	-0.082	0.141	-
	Pop.7	733	-	20	1	0	0.000 ± 0.000	0.000 ± 0.000	0	NA	NA	-
	Pop.8	733	-	15	2	0.533	0.133 ± 0.034	0.001 ± 0.000	4	-0.080*	0.160	-
	Pop.9	733	-	19	5	1.111	0.673 ± 0.119	0.002 ± 0.000	4	-0.085	0.1305	-
	Pop.10	733	-	8	4	13.214	0.750 ± 0.145	0.018 ± 0.002	36	-0.257	0.219	-
	Pop.11	733	-	13	3	0.564	0.513 ± 0.041	0.001 ± 0.000	2	0.350	0.141	-
	Pop.12	733	-	20	7	9.463	0.742 ± 0.014	0.011 ± 0.001	38	0.013	0.013	-
	Pop.13	733	-	6	3	0.933	0.733 ± 0.124	0.001 ± 0.000	2	0.310	0.130	-
	Pop.14	733	-	6	4	19.000	0.867 ± 0.078	0.020 ± 0.000	36	0.026	0.026	-
	Pop.15	733	-	19	4	8.105	0.298 ± 0.110	0.010 ± 0.001	31	-0.342	0.130*	-
	Pop.16	733	-	9	2	0.222	0.222 ± 0.006	0.000 ± 0.000	1	-1.088	0.314	-
	Pop.17	733	-	15	3	0.267	0.257 ± 0.056	0.000 ± 0.000	2	-1.490	0.170	-
	Pop.18	733	-	12	5	14.439	0.758 ± 0.008	0.020 ± 0.003	28	2.498	0.255	-

N: number of sequences; *h*: number of haplotypes; k: mean nuber of nucleotide differences among sequences; Hd: haplotype diversity; π: nucleotide diversity; S: number of segregating sites; Rm: minimum recombination events. * Statistically significant values for D and R_2 statistics $p<0.05$.

Appendix II: Continued

Gene	Population	Lenght (bp)	Indels (bp)	N	Polymorphism				S	D	R_2	Rm
					h	*k*	*Hd* ± SD	π ± SD				
Gh_1	Pop.1	542-578	1 (36)	30	14	2.379	0.931 ± 0.000	0.004 ± 0.000	8	0.541	0.143	2
	Pop.2	542-579	1 (36)	30	12	2.110	0.910 ± 0.029	0.003 ± 0.000	5	0.163	0.211	-
	Pop.3	542-587	4 (12, 9, 36, 10)	60	11	5.277	0.865 ± 0.000	0.010 ± 0.000	17	1.358	0.154	2
	Pop.4	542-578	1 (36)	22	8	2.364	0.874 ± 0.001	0.004 ± 0.000	6	1.351	0.077	1
	Pop.5	542-587	2 (9, 36)	34	5	1.529	0.622 ± 0.002	0.002 ± 0.000	10	-1.154*	0.074*	-
	Pop.6	533-587	3 (54, 9, 36)	28	6	7.124	0.788 ± 0.001	0.014 ± 0.001	17	2.188	0.209	-
	Pop.7	555-569	1 (14)	40	2	2.146	0.358 ± 0.070	0.003 ± 0.000	6	1.391	0.178	-
	Pop.8	555-569	1 (14)	28	2	2.095	0.349 ± 0.090	0.003 ± 0.000	6	1.040	0.174	-
	Pop.9	587	-	38	4	0.549	0.514 ± 0.062	0.000 ± 0.000	3	-0.510*	0.105*	-
	Pop.10	587	-	16	4	0.575	0.517 ± 0.132	0.000 ± 0.000	3	-1.055*	0.122*	-
	Pop.11	587	-	26	5	0.643	0.557 ± 0.104	0.001 ± 0.000	4	-1.032*	0.085*	-
	Pop.12	533-587	1 (54)	40	6	1.140	0.641 ± 0.003	0.002 ± 0.000	12	-1.835*	0.108*	-
	Pop.13	587	-	12	3	0.818	0.682 ± 0.008	0.001 ± 0.000	2	0.687	0.204	-
	Pop.14	533-587	2 (54, 3)	12	6	2.803	0.879 ± 0.060	0.005 ± 0.000	12	-1.237*	0.214	1
	Pop.15	533-587	1 (54)	38	6	4.183	0.748 ± 0.047	0.007 ± 0.001	13	1.109	0.159	-
	Pop.16	587	-	18	2	0.222	0.111 ± 0.096	0.000 ± 0.000	2	-1.507*	0.229	-
	Pop.17	578-587	1 (9)	30	4	1.294	0.193 ± 0.095	0.002 ± 0.001	11	-1.718*	0.060*	-
	Pop.18	533-587	1 (54)	20	5	4.074	0.568 ± 0.119	0.007 ± 0.007	11	1.113	0.178	-

N: number of sequences; *h*: number of haplotypes; k: mean nuber of nucleotide differences among sequences; Hd: haplotype diversity; π: nucleotide diversity; S: number of segregating sites; Rm: minimum recombination events. * Statistically significant values for D and R_2 statistics $p<0.05$.

Appendix II: Continued

Gene	Population	Lenght (bp)	Indels (bp)	N	Polymorphism				S	D	R_2	Rm
					h	*k*	*Hd* ± SD	π ± SD				
Gh_2	Pop.1	1021	-	28	12	3.521	0.894 ± 0.035	0.004 ± 0.000	13	0.181	0.129	2
	Pop.2	1020-1021	1 (1)	30	15	4.159	0.940 ± 0.022	0.004 ± 0.000	14	0.589	0.148	2
	Pop.3	898-1021	6 (5,1, 13, 3, 95, 22)	60	20	9.972	0.907 ± 0.023	0.011 ± 0.000	28	2.128*	0.177	2
	Pop.4	1020-1021	1 (1)	20	14	3.753	0.968 ± 0.022	0.004 ± 0.000	15	-0.414*	0.114*	1
	Pop.5	898-1021	6 (5,1, 13, 3, 95, 22)	32	11	6.018	0.897 ± 0.026	0.006 ± 0.001	26	-0.239*	0.115*	2
	Pop.6	898-1023	8 (5, 6, 3, 95, 22, 1, 1)	28	8	13.188	0.862 ± 0.036	0.014 ± 0.000	39	1.176	0.166	3
	Pop.7	918-1040	6 (3, 13, 20, 8, 95)	40	7	6.181	0.721 ± 0.057	0.006 ± 0.000	14	2.770	0.219	1
	Pop.8	917-1040	7 (3, 1, 13, 20, 8, 95)	30	6	6.179	0.721 ± 0.074	0.006 ± 0.001	14	2.498	0.220	1
	Pop.9	1023	-	38	2	0.309	0.309 ± 0.080	0.000 ± 0.000	1	0.430	0.154*	-
	Pop.10	898-1023	5 (6, 12, 95, 20, 1)	16	2	6.800	0.400 ± 0.114	0.007 ± 0.002	17	1.297	0.200	-
	Pop.11	898-1023	5 (6, 12, 95, 20, 1)	26	3	2.723	0.342 ± 0.110	0.003 ± 0.001	18	-1.498*	0.075*	-
	Pop.12	898-1023	5 (6, 12, 95, 20, 1)	40	3	8.076	0.555 ± 0.020	0.009 ± 0.000	18	2.966	0.224	-
	Pop.13	898-1023	5 (6, 12, 95, 20, 1)	12	3	9.439	0.621 ± 0.087	0.010 ± 0.001	18	2.544	0.258	1
	Pop.14	898-1023	5 (6, 12, 95, 20, 1)	12	3	9.439	0.621 ± 0.087	0.010 ± 0.001	18	2.544	0.258	1
	Pop.15	898-1029	6 (6, 12, 95, 22, 1, 1)	36	6	10.025	0.638 ± 0.082	0.011 ± 0.001	31	1.197	0.160	2
	Pop.16	898	-	18	2	0.320	0.307 ± 0.132	0.000 ± 0.000	2	-1.096*	0.125*	-
	Pop.17	898-1021	5 (5, 13, 3, 95, 22)	30	5	2.090	0.593 ± 0.006	0.002 ± 0.001	25	-2.381*	0.158	-
	Pop.18	898-1029	4 (13, 95, 22,1)	20	4	1.942	0.574 ± 0.009	0.002 ± 0.002	16	-2.116*	0.178	-

N: number of sequences; *h*: number of haplotypes; k: mean nuber of nucleotide differences among sequences; Hd: haplotype diversity; π: nucleotide diversity; S: number of segregating sites; Rm: minimum recombination events. * Statistically significant values for D and R_2 statistics $p<0.05$.

Appendix II: Continued

Gene	Population	Lenght (bp)	Indels (bp)	N	Polymorphism				S	D	R_2	Rm
					h	*k*	*Hd* ± SD	π ± SD				
S7_1	Pop.1	345-352	1 (7)	30	5	1.218	0.713 ± 0.059	0.003 ± 0.000	3	1.433	0.203*	-
	Pop.2	345-353	1 (7, 1)	30	3	1.202	0.605 ± 0.045	0.003 ± 0.000	3	1.383	0.200	-
	Pop.3	341-354	4 (1, 3, 7, 1)	58	9	4.912	0.786 ± 0.033	0.014 ± 0.001	15	1.544	0.162	2
	Pop.4	352	0	22	4	1.000	0.541 ± 0.094	0.002 ± 0.000	3	0.556	0.166	-
	Pop.5	344-352	2 (7, 1)	34	4	2.012	0.576 ± 0.056	0.005 ± 0.001	11	-0.789*	0.091*	1
	Pop.6	343-353	4 (1, 1, 7, 1)	26	4	6.228	0.726 ± 0.042	0.018 ± 0.001	13	2.820	0.239	-
	Pop.7	329-343	4 (1, 1, 28, 12)	38	4	2.306	0.619 ± 0.047	0.007 ± 0.002	11	-0.365*	0.103*	-
	Pop.8	329	0	30	3	0.501	0.480 ± 0.073	0.001 ± 0.000	2	-0.014*	0.140	-
	Pop.9	343	0	38	4	0.778	0.508 ± 0.086	0.002 ± 0.000	0.778	0.198	0.129	-
	Pop.10	343-344	1 (1)	16	5	1.250	0.775 ± 0.068	0.003 ± 0.000	4	0.115	0.156	-
	Pop.11	343-344	1 (1)	26	5	1.105	0.711 ± 0.062	0.003 ± 0.000	5	-0.444*	0.109	-
	Pop.12	340-344	2 (1, 2)	40	4	0.932	0.614 ± 0.045	0.002 ± 0.000	4	-0.021	0.117	-
	Pop.13	340-344	2 (1, 2)	12	4	1.03	0.711 ± 0.085	0.003 ± 0.000	4	-0.781*	0.162	-
	Pop.14	340-344	2 (1, 2)	12	4	1.303	0.712 ± 0.105	0.003 ± 0.000	4	-0.057	0.158*	-
	Pop.15	341-343	5 (1, 4, 6, 3, 7)	36	5	4.067	0.741 ± 0.044	0.012 ± 0.002	15	0.403	0.133	-
	Pop.16	344	0	18	4	1.392	0.647 ± 0.095	0.004 ± 0.000	4	0.591*	0.174	-
	Pop.17	341-352	3 (1, 3, 7)	30	5	3.021	0.639 ± 0.080	0.008 ± 0.002	15	-0.681*	0.099*	-
	Pop.18	341-344	5 (1, 4, 6, 3, 7)	20	7	3.705	0.768 ± 0.069	0.011 ± 0.003	17	-0.848*	0.104*	-

N: number of sequences; *h*: number of haplotypes; k: mean nuber of nucleotide differences among sequences; Hd: haplotype diversity; π: nucleotide diversity; S: number of segregating sites; Rm: minimum recombination events. * Statistically significant values for D and R_2 statistics $p<0.05$.

Appendix II: Continued

Gene	Population	Lenght (bp)	Indels (bp)	N	Polymorphism				S	D	R_2	Rm
					h	*k*	*Hd* ± SD	π ± SD				
S7_2	Pop.1	556	-	30	4	1.179	0.733 ± 0.040	0.002 ± 0.000	3	1.311	0.196	-
	Pop.2	556	-	30	4	0.864	0.662 ± 0.070	0.001 ± 0.000	2	1.458	0.216	-
	Pop.3	556-562	5 (5, 2, 2, 2, 3)	60	6	5.090	0.684 ± 0.040	0.009 ± 0.000	13	2.402	0.195	-
	Pop.4	556	-	22	5	1.030	0.532 ± 0.120	0.001 ± 0.000	1.030	-0.171*	0.128*	-
	Pop.5	535-556	5 (5, 2, 2, 2, 3, 27)	34	7	4.480	0.800 ± 0.040	0.008 ± 0.001	13	1.316	0.170	-
	Pop.6	558-562	6 (5, 2, 2, 2, 3, 6)	26	9	5.332	0.803 ± 0.070	0.009 ± 0.001	18	0.462	0.145	1
	Pop.7	567	-	40	3	0.404	0.273 ± 0.088	0.000 ± 0.000	2	-0.266*	0,101*	-
	Pop.8	567	-	30	2	0.133	0.067 ± 0.061	0.000 ± 0.000	2	-1.507*	0.179	-
	Pop.9	562	-	36	5	0.708	0.563 ± 0.085	0.001 ± 0.000	5	-1,076*	0.088*	-
	Pop.10	562	-	16	5	1.175	0.767 ± 0.066	0.002 ± 0.000	4	-0.079*	0.142*	-
	Pop.11	562	-	26	5	0.855	0.655 ± 0.077	0.001 ± 0.000	3	0.216	0.144	-
	Pop.12	562	-	40	8	1.606	0.832 ± 0.028	0.002 ± 0.000	6	0.370	0.133	1
	Pop.13	562	-	12	3	0.985	0.530 ± 0.126	0.001 ± 0.000	3	-0.028*	0.164*	-
	Pop.14	558-562	4 (5, 2, 3, 6)	12	5	1.652	0.727 ± 0.113	0.002 ± 0.001	8	-1.503*	0.151	-
	Pop.15	558-562	4 (5, 2, 3, 6)	36	8	2.662	0.743 ± 0.064	0.004 ± 0.000	11	0.010	0.117	-
	Pop.16	535-562	1 (27)	18	3	0.680	0.523 ± 0.112	0.001 ± 0.000	2	0.412	0.169	-
	Pop.17	556-562	5 (5, 2, 2, 2, 3)	30	4	1.871	0.579 ± 0.047	0.003 ± 0.001	12	-1.247*	0.076*	-
	Pop.18	558-562	4 (5, 2, 3, 6)	22	5	5.052	0.753 ± 0.057	0.009 ± 0.001	17	0.304	0.146	-

N: number of sequences; *h*: number of haplotypes; k: mean nuber of nucleotide differences among sequences; Hd: haplotype diversity; π: nucleotide diversity; S: number of segregating sites; Rm: minimum recombination events. * Statistically significant values for D and R_2 statistics $p<0.05$.

Appendix III: Accession number of haplotypes available in GeneBank and used for cytb MP and ML analyses

Species	Acc. Num
B. caninus	AF287424
	AF287425
B. plebejus	AY004750
B. tyberinus	AF274356
	AF274355
	AF274354
	AF397300
B. balcanicus	GQ302790
	GQ302792
B. petenyi	GQ302804
	GQ302805
B. barbus	AF112123
B. meridionalis	AF112130
	AF045977
B.strumicae	AF112134
	AF112128
B. rebeli	GQ302784
	GQ302785
B. haasi	AF334101
B. peloponnesius	AF112131
L. bocagei	AF334064
L. microcephalus	AF334085
L. comizo	AF334050

Appendix IV: P distance among haplotypes used to reconstruct molecular cytb phylogeny of the genus *Barbus*

		1	2	3	4	5	6	7	8	9	10	11	12
1	Bc1	-											
2	Bc2	0.00136	-										
3	Bc3	0.00136	0.00273	-									
4	Bc4	0.00136	0.00273	0.00273	-								
5	Bc5	0.00273	0.00409	0.00409	0.00136	-							
6	Bc6	0.00136	0.00273	0.00273	0.00273	0.00409	-						
7	Bc7	0.00136	0.00273	0.00273	0.00273	0.00409	0.00273	-					
8	Bc8	0.00136	0.00273	0.00273	0.00273	0.00409	0.00273	0.00273	-				
9	Bc9	0.00136	0.00273	0.00273	0.00273	0.00409	0.00273	0.00273	0.00273	-			
10	B.balcanicus	0.06821	0.06685	0.06821	0.06958	0.07094	0.06958	0.06958	0.06958	0.06958	-		
11	B.balcanicus	0.06821	0.06685	0.06821	0.06958	0.07094	0.06958	0.06958	0.06958	0.06958	0.00000	-	
12	Bp19	0.08322	0.08458	0.08322	0.08458	0.08595	0.08458	0.08458	0.08458	0.08458	0.07776	0.07776	-
13	Bp12	0.08322	0.08458	0.08322	0.08458	0.08595	0.08458	0.08458	0.08458	0.08458	0.08049	0.08049	0.00273
14	Bp13	0.08186	0.08322	0.08186	0.08322	0.08458	0.08322	0.08322	0.08322	0.08322	0.07913	0.07913	0.00136
15	Bp14	0.08322	0.08458	0.08322	0.08458	0.08595	0.08458	0.08458	0.08458	0.08458	0.08049	0.08049	0.00273
16	B.plebejus	0.08186	0.08322	0.08186	0.08322	0.08458	0.08322	0.08322	0.08322	0.08322	0.07776	0.07776	0.00273
17	Bbal15	0.06958	0.06821	0.06958	0.07094	0.07231	0.07094	0.07094	0.07094	0.07094	0.00819	0.00819	0.07503
18	Bbal16	0.06958	0.06821	0.06958	0.07094	0.07231	0.07094	0.07094	0.07094	0.07094	0.00273	0.00273	0.07776
19	B.balcanicus	0.06276	0.06139	0.06276	0.06412	0.06548	0.06412	0.06412	0.06412	0.06412	0.01228	0.01228	0.07094
20	B.balcanicus	0.06548	0.06412	0.06548	0.06685	0.06821	0.06685	0.06685	0.06685	0.06685	0.01228	0.01228	0.07913
21	Bb17	0.08049	0.08186	0.08049	0.08186	0.08322	0.08186	0.08186	0.08186	0.08186	0.08595	0.08595	0.03683
22	Bb10	0.08186	0.08322	0.08186	0.08322	0.08458	0.08322	0.08322	0.08322	0.08322	0.08731	0.08731	0.03820
23	Bb18	0.08186	0.08322	0.08186	0.08322	0.08458	0.08322	0.08322	0.08322	0.08322	0.08731	0.08731	0.03820
24	B.barbus	0.08049	0.08186	0.08049	0.08186	0.08322	0.08186	0.08186	0.08186	0.08186	0.08868	0.08868	0.03956
25	Bt20	0.09004	0.09141	0.09004	0.09141	0.09277	0.09141	0.09141	0.09141	0.08868	0.08458	0.08458	0.02183
26	Bt11	0.09004	0.09141	0.09004	0.09141	0.09277	0.09141	0.09141	0.09141	0.08868	0.08731	0.08731	0.02183
27	Bt21	0.09004	0.09141	0.09004	0.09141	0.09277	0.09141	0.09141	0.09141	0.08868	0.08731	0.08731	0.02183
28	Bt22	0.09141	0.09277	0.09141	0.09277	0.09413	0.09277	0.09277	0.09277	0.09004	0.08595	0.08595	0.02046
29	Bt23	0.09277	0.09413	0.09277	0.09413	0.09550	0.09413	0.09413	0.09413	0.09141	0.08731	0.08731	0.02183
30	Bt24	0.08731	0.08868	0.08731	0.08868	0.09004	0.08868	0.08868	0.08868	0.08868	0.08458	0.08458	0.01910
31	Bt25	0.09004	0.09141	0.09004	0.09141	0.09277	0.09141	0.09141	0.09141	0.08868	0.08458	0.08458	0.02183
32	Bt26	0.08868	0.09004	0.08868	0.09004	0.09141	0.09004	0.09004	0.09004	0.08731	0.08322	0.08322	0.01774
33	Bt27	0.09004	0.09141	0.09004	0.09141	0.09277	0.09141	0.09141	0.09141	0.08868	0.08458	0.08458	0.01910
34	B.tyberinus	0.08884	0.09058	0.08874	0.09044	0.09033	0.08879	0.09052	0.08893	0.08879	0.09240	0.09240	0.01876
35	B.tyberinus	0.07856	0.08032	0.07845	0.08016	0.08004	0.07855	0.08025	0.07867	0.07855	0.08536	0.08536	0.01514
36	B.tyberinus	0.07861	0.08037	0.07849	0.08021	0.08010	0.07861	0.08030	0.07871	0.07861	0.08875	0.08875	0.01180
37	B.tyberinus	0.09823	0.09959	0.09823	0.09959	0.10095	0.09959	0.09686	0.09959	0.09959	0.09277	0.09277	0.03274
38	B.merionalis	0.09141	0.09004	0.09141	0.09277	0.09413	0.09277	0.09277	0.09277	0.09277	0.07913	0.07913	0.07094
39	B.merionalis	0.09550	0.09413	0.09550	0.09686	0.09823	0.09686	0.09686	0.09686	0.09686	0.08049	0.08049	0.07776
40	B.strumicae	0.08322	0.08186	0.08458	0.08458	0.08595	0.08458	0.08458	0.08458	0.08458	0.08186	0.08186	0.07231
41	B.strumicae	0.07640	0.07503	0.07776	0.07776	0.07913	0.07776	0.07776	0.07776	0.07776	0.08595	0.08595	0.06821
42	B.rebeli	0.06958	0.06821	0.07094	0.07094	0.07231	0.07094	0.07094	0.07094	0.07094	0.08186	0.08186	0.07503
43	B.rebeli	0.06821	0.06685	0.06821	0.06958	0.07094	0.06958	0.06958	0.06958	0.06958	0.08458	0.08458	0.07367
44	B.peteny	0.08186	0.08049	0.08322	0.08322	0.08458	0.08322	0.08322	0.08322	0.08322	0.09277	0.09277	0.07503
45	B.peteny	0.08868	0.08731	0.09004	0.09004	0.09141	0.09004	0.09004	0.09004	0.09004	0.09686	0.09686	0.07640
46	B.carpathicus	0.07913	0.07776	0.07913	0.08049	0.08186	0.08049	0.08049	0.07776	0.08049	0.05593	0.05593	0.07367
47	B.prespensis	0.07231	0.07094	0.07367	0.07367	0.07503	0.07367	0.07367	0.07367	0.07367	0.08049	0.08049	0.06685
48	B.peloponnesius	0.07094	0.06958	0.07231	0.07231	0.07367	0.07231	0.07231	0.07231	0.07231	0.08322	0.08322	0.07640
49	L.bocagei	0.08595	0.08458	0.08731	0.08731	0.08868	0.08731	0.08731	0.08731	0.08731	0.10641	0.10641	0.08049
50	L.microcephalus	0.09686	0.09550	0.09686	0.09823	0.09959	0.09823	0.09823	0.09823	0.09823	0.11460	0.11460	0.09141
51	B.haasi	0.09277	0.09141	0.09277	0.09413	0.09550	0.09413	0.09413	0.09413	0.09413	0.07913	0.07913	0.06412
52	L.comizo	0.09277	0.09141	0.09277	0.09413	0.09550	0.09413	0.09413	0.09413	0.09413	0.11323	0.11323	0.08458

Appendix IV: continued

		13	14	15	16	17	18	19	20	21	22	23	24
13	Bp12	-											
14	Bp13	0.00136	-										
15	Bp14	0.00273	0.00136	-									
16	B.plebejus	0.00273	0.00136	0.00273	-								
17	Bbal15	0.07776	0.07640	0.07776	0.07776	-							
18	Bbal16	0.08049	0.07913	0.08049	0.08049	0.00546	-						
19	B.balcanicus	0.07367	0.07231	0.07367	0.07367	0.01228	0.01228	-					
20	B.balcanicus	0.08186	0.08049	0.08186	0.08186	0.01228	0.01228	0.01364	-				
21	Bb17	0.03411	0.03547	0.03683	0.03683	0.08322	0.08595	0.08186	0.08458	-			
22	Bb10	0.03547	0.03683	0.03820	0.03820	0.08458	0.08731	0.08322	0.08595	0.00136	-		
23	Bb18	0.03547	0.03683	0.03820	0.03820	0.08458	0.08731	0.08322	0.08595	0.00136	0.00273	-	
24	B.barbus	0.03683	0.03820	0.03956	0.03956	0.08595	0.08868	0.08458	0.08731	0.00273	0.00409	0.00136	-
25	Bt20	0.01910	0.02046	0.02183	0.02183	0.08186	0.08458	0.08049	0.08322	0.03956	0.04093	0.04093	0.04229
26	Bt11	0.01910	0.02046	0.02183	0.02183	0.08458	0.08731	0.08322	0.08595	0.03956	0.04093	0.04093	0.04229
27	Bt21	0.01910	0.02046	0.02183	0.02183	0.08458	0.08731	0.08322	0.08595	0.03683	0.03820	0.03820	0.03956
28	Bt22	0.01774	0.01910	0.02046	0.02046	0.08322	0.08595	0.08186	0.08458	0.03820	0.03956	0.03956	0.04093
29	Bt23	0.01910	0.02046	0.02183	0.02183	0.08458	0.08731	0.08322	0.08595	0.03956	0.04093	0.04093	0.04229
30	Bt24	0.01637	0.01774	0.01910	0.01910	0.08186	0.08458	0.08049	0.08322	0.03411	0.03547	0.03547	0.03683
31	Bt25	0.01910	0.02046	0.02183	0.02183	0.08186	0.08458	0.08049	0.08322	0.03956	0.04093	0.04093	0.04229
32	Bt26	0.01774	0.01637	0.01774	0.01774	0.08049	0.08322	0.07913	0.08186	0.03820	0.03956	0.03956	0.04093
33	Bt27	0.01910	0.01774	0.01910	0.01910	0.08186	0.08458	0.08049	0.08322	0.03956	0.04093	0.04093	0.04229
34	B.tyberinus	0.01712	0.01711	0.01886	0.01881	0.08895	0.09229	0.08759	0.09080	0.04067	0.04226	0.04065	0.04062
35	B.tyberinus	0.01349	0.01351	0.01526	0.01521	0.08191	0.08525	0.07725	0.08378	0.03701	0.03859	0.03701	0.03699
36	B.tyberinus	0.01017	0.01017	0.01192	0.01187	0.08529	0.08864	0.07726	0.08716	0.03371	0.03530	0.03370	0.03369
37	B.tyberinus	0.03274	0.03138	0.03274	0.03274	0.09004	0.09277	0.08868	0.09141	0.05457	0.05593	0.05593	0.05730
38	B.merionalis	0.06821	0.06958	0.07094	0.07094	0.07640	0.07913	0.07776	0.08049	0.07231	0.07367	0.07367	0.07503
39	B.merionalis	0.07503	0.07640	0.07776	0.07776	0.07776	0.08049	0.07913	0.08186	0.07640	0.07776	0.07776	0.07913
40	B.strumicae	0.07231	0.07367	0.07503	0.07503	0.07913	0.08186	0.08049	0.08322	0.06276	0.06412	0.06412	0.06548
41	B.strumicae	0.06821	0.06685	0.06821	0.06821	0.08322	0.08595	0.07913	0.08186	0.06412	0.06548	0.06548	0.06685
42	B.rebeli	0.07231	0.07367	0.07503	0.07503	0.07913	0.08186	0.07776	0.07503	0.06139	0.06276	0.06276	0.06412
43	B.rebeli	0.07094	0.07231	0.07367	0.07367	0.08186	0.08458	0.07776	0.08322	0.06139	0.06276	0.06276	0.06139
44	B.peteny	0.07231	0.07367	0.07503	0.07503	0.09277	0.09277	0.08595	0.09141	0.06821	0.06958	0.06958	0.07094
45	B.peteny	0.07367	0.07503	0.07640	0.07640	0.09550	0.09686	0.08731	0.09277	0.06958	0.07094	0.07094	0.07231
46	B.carpathicus	0.07094	0.07231	0.07367	0.07367	0.05457	0.05593	0.05457	0.05184	0.07094	0.07231	0.07231	0.07367
47	B.prespensis	0.06412	0.06548	0.06685	0.06685	0.07776	0.08049	0.07367	0.07913	0.06139	0.06276	0.06276	0.06412
48	B.peloponnesius	0.07367	0.07503	0.07640	0.07640	0.08049	0.08322	0.07913	0.07640	0.06276	0.06412	0.06412	0.06548
49	L.bocagei	0.08049	0.07913	0.08049	0.08049	0.10368	0.10641	0.09959	0.10232	0.08322	0.08458	0.08458	0.08595
50	L.microcephalus	0.09141	0.09004	0.09141	0.09141	0.11187	0.11460	0.11050	0.11187	0.08322	0.08458	0.08458	0.08595
51	B.haasi	0.06139	0.06276	0.06412	0.06412	0.07640	0.07913	0.07776	0.08049	0.06139	0.06276	0.06276	0.06412
52	L.comizo	0.08458	0.08322	0.08458	0.08458	0.11050	0.11323	0.10641	0.10914	0.08731	0.08868	0.08868	0.09004

Appendix IV: continued

		25	26	27	28	29	30	31	32	33	34	35	36
25	Bt20	-											
26	Bt11	0.00273	-										
27	Bt21	0.00273	0.00273	-									
28	Bt22	0.00136	0.00136	0.00136	-								
29	Bt23	0.00273	0.00273	0.00273	0.00136	-							
30	Bt24	0.00546	0.00546	0.00546	0.00409	0.00546	-						
31	Bt25	0.00273	0.00273	0.00273	0.00136	0.00273	0.00546	-					
32	Bt26	0.00409	0.00409	0.00409	0.00273	0.00409	0.00682	0.00409	-				
33	Bt27	0.00273	0.00273	0.00273	0.00136	0.00273	0.00546	0.00273	0.00136	-			
34	B.tyberinus	0.00353	0.00177	0.00347	0.00177	0.00353	0.00177	0.00353	0.00351	0.00176	-		
35	B.tyberinus	0.01663	0.01825	0.01993	0.01822	0.01999	0.01816	0.01999	0.01664	0.01825	0.02020	-	
36	B.tyberinus	0.02004	0.01831	0.01664	0.01827	0.02004	0.01822	0.02004	0.01668	0.01829	0.02020	0.01347	-
37	B.tyberinus	0.02319	0.02319	0.02319	0.02183	0.02319	0.02319	0.02319	0.02183	0.02046	0.01365	0.03013	0.03013
38	B.merionalis	0.07231	0.07231	0.07231	0.07094	0.07231	0.06958	0.06958	0.07094	0.07231	0.07218	0.07192	0.07198
39	B.merionalis	0.07913	0.07913	0.07913	0.07776	0.07913	0.07640	0.07640	0.07776	0.07913	0.08051	0.08026	0.08032
40	B.strumicae	0.07776	0.08049	0.07776	0.07913	0.08049	0.07503	0.07776	0.07913	0.08049	0.07723	0.07030	0.06696
41	B.strumicae	0.07367	0.07367	0.07094	0.07231	0.07367	0.07094	0.07094	0.06958	0.07094	0.07765	0.06909	0.06401
42	B.rebeli	0.07640	0.07913	0.07640	0.07776	0.07913	0.07367	0.07640	0.08049	0.07913	0.08077	0.07711	0.07044
43	B.rebeli	0.07913	0.08186	0.07913	0.08049	0.08186	0.07640	0.08186	0.08049	0.08186	0.08226	0.06524	0.06531
44	B.peteny	0.08322	0.08322	0.08049	0.08186	0.08322	0.08049	0.08322	0.08186	0.08322	0.08072	0.07385	0.06376
45	B.peteny	0.08186	0.08458	0.08186	0.08322	0.08458	0.08186	0.08458	0.08322	0.08458	0.08262	0.06907	0.06571
46	B.carpathicus	0.07094	0.07094	0.07367	0.07231	0.07367	0.06821	0.07367	0.07231	0.07367	0.07052	0.06689	0.07029
47	B.prespensis	0.06958	0.07231	0.06958	0.07094	0.07231	0.06685	0.06958	0.07094	0.07231	0.06923	0.06228	0.05562
48	B.peloponnesius	0.07776	0.08049	0.07776	0.07913	0.08049	0.07503	0.07776	0.08186	0.08049	0.08237	0.07870	0.07204
49	L.bocagei	0.09277	0.09550	0.09277	0.09413	0.09550	0.09004	0.09277	0.09141	0.09277	0.08988	0.07952	0.07282
50	L.microcephalus	0.09550	0.09823	0.09550	0.09686	0.09823	0.09277	0.09550	0.09413	0.09550	0.09815	0.09792	0.09458
51	B.haasi	0.06139	0.06412	0.06412	0.06276	0.06412	0.06139	0.06139	0.06276	0.06412	0.06216	0.05864	0.05867
52	L.comizo	0.09413	0.09686	0.09413	0.09550	0.09686	0.09141	0.09413	0.09550	0.09413	0.09159	0.08791	0.08122

		37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
		-														
37	B.tyberinus	0.07913	-													
38	B.merionalis	0.08595	0.00682	-												
39	B.merionalis	0.09141	0.07640	0.08049	-											
40	B.strumicae	0.08049	0.07231	0.07640	0.06139	-										
41	B.strumicae	0.08595	0.07913	0.08322	0.05184	0.05321	-									
42	B.rebeli	0.09141	0.07913	0.08322	0.05866	0.06003	0.03820	-								
43	B.rebeli	0.09413	0.09141	0.09550	0.07503	0.06139	0.06003	0.05593	-							
44	B.peteny	0.09686	0.09550	0.09959	0.07367	0.06548	0.05866	0.05457	0.03001	-						
45	B.peteny	0.08186	0.06958	0.07094	0.07503	0.07776	0.07367	0.07231	0.08731	0.09277	-					
46	B.carpathicus	0.08186	0.08049	0.08458	0.04911	0.04638	0.03411	0.03820	0.05730	0.04638	0.07094	-				
47	B.prespensis	0.08731	0.08049	0.08458	0.05321	0.05457	0.00136	0.03956	0.06139	0.06003	0.07503	0.03547	-			
48	B.peloponnesius	0.08868	0.09959	0.10232	0.08458	0.08868	0.08186	0.09004	0.09959	0.09277	0.10095	0.07913	0.08322	-		
49	L.bocagei	0.09413	0.10368	0.10641	0.09823	0.09686	0.09004	0.09823	0.10505	0.09823	0.10368	0.09004	0.09141	0.04229	-	
50	L.microcephalus	0.07913	0.04366	0.05048	0.07094	0.08049	0.08049	0.08049	0.09004	0.09141	0.06685	0.07640	0.07913	0.10232	0.10641	-
51	B.haasi	0.08868	0.10914	0.11187	0.09686	0.09823	0.08595	0.09413	0.10095	0.09686	0.10505	0.08595	0.08731	0.01637	0.04229	0.10914