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CICLO XXX

CONSERVATION GENETICS OF MEDITERRANEAN BARBEL SPECIES INSIDE NATURA 2000 SITES:

AN ECOSYSTEM PERSPECTIVE

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Agli amici,

come l'acqua nei fiumi danno vita e significato al paesaggio della vita

“Se il punto in cui ti immergi in un fiume è il presente, pensai, allora il passato è l’acqua che ti ha superato, quella che va verso il basso e dove non c’è più niente per te, mentre il futuro è l’acqua che scende dall’alto”

-Le Otto Montagne- P. Cognetti

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ABSTRACT

Freshwater fishes of the Italian peninsula are, at present, potentially highly threatened by climate change, human activities, increasing demands for water, natural resources fragmentation and loss of habitat. Genus *Barbus* are threatened in Italy by hybridization and introgression caused by allochthonous lineages. This thesis presents a novel dataset including both mitochondrial (255 samples) and nuclear DNA markers (191 samples) in Barbel species inside 15 Natura 2000 sites. Moreover, ecological indices (ISECI, IFF, STAR_iCM) and environmental data (BOD5, TSS , NH_4+, NO_3-,NO_2-, pH, T) were also analysed inside 15 Protected Areas of Natura 2000 sites. Scientific objectives were the following: detecting the evidence of significant population structure in *Barbus plebejus* and *Barbus caninus* and determining introgression and hybridization between native species and other barbel species. Moreover, the last objective was to explain phylogenetic relationships among populations in different hydrogeographic districts and to define whether ecological factors were correlated with genetic factors. Results showed complex scenarios where different species live in the same habitat allowing hybridization and introgression. In addition to these, an altitude gradient between native and allochthonous species was observed. Physical, chemical and ecological data reinforces the existence of an altitudinal zonation in term of barbel species representativeness among sites. These evidences mirrored anthropogenic disturbance, climate change and loss of habitat inside considered Protected Areas. All results will be managed inside European LIFE program NAT/IT/001129 BARBIE for future restocking and spawning activities.

1 INTRODUCTION

1.1 Conservation Status of Freshwater fishes in Italy

Many different species including birds, amphibians and fish underwent 58 per cent decline between 1970 and 2012 with the greatest losses affecting freshwater environments as showed by The Living Planet Index (WWF, 2016). Major threats for freshwater communities are habitat loss and fragmentation (WWF, 2016). Moreover, freshwater fishes comprise the most species-rich group among European vertebrates with 531 native species that described. Almost 80% of these species are endemic to Europe (Freyhof, 2011). Management tools as conservation plans are necessary to cover knowledge gaps related to ecological and environmental status of freshwater and inland waters because population trends still remain unknown for 76% of all freshwater European species (Freyhof, 2011). In Italy 27 freshwater autochthonous fish species are reported in the main global risk categories of IUCN Red List (EX, CR, VU, EN); additional 3 are listed in Near Threatened (NT) and the remaining 16 in Least Concern (LC), besides 6 Data Deficit (DD) (Rondinini, et al., 2013). In the last few years, fish populations decreased in all European countries. Italy was specially affected where the drastically effect of climate changes and human activities linked to agriculture and water request for irrigation led to local ecosystems perturbation causing stress and loss of biodiversity as shown in Fig. 1 (Vorosmarty, et al., 2010; Freyhof, 2011).

Fig. 1: SCI inside Enza water stream (IT4030023) during summer 2015. The picture on the right showes a dead barbel for anoxia (from the analysis of Istituto zooprofilattico del Piemonte, Liguria e Valle d'Aosta).

Another problem related to conservation is species nomenclature. Thanks to genetics and genomics approaches, new taxonomy identities were described: Evolutionary Significant Units (ESUs) and Management Units (MUs) among others (Moritz, 1994; Palsbøll, et al., 2006). These new "species concepts" are not protected by EU government laws, and EU conservation plans do not include all taxonomic identities below species level.

The conservation status of freshwater ecosystem is one of the major priority importance even for United Nations agenda which gives 17 goals for 2030 towards sustainable development. The economic impact of natural disasters caused by ecosystems distribution due to human impact is more than 300 billion US dollars per year. Climate change also has an impact in terms of world costs (Sustainable Development, 2015). One of these important points is Goal 15: "Sustainably management forests, combat desertification, halt and reverse land degradation, halt biodiversity loss" and within this goal the priority is to reduce the impact of invasive alien species on land and water ecosystems through control or eradication. This aim is linked with others like Goal 13: Take urgent action to combat climate change and its impacts". All this points are in line with politics and objectives of LIFE projects that have the priority in management and conservation of ecosystems (Sustainable Development, 2015).

In Italy different taxonomy identities of freshwater fishes have been studied in order to clarify their ecological and evolutionary history through the use of to molecular genetics. In relation to this, Zanetti et al. (2013) proposed to classify the Italian salmonids using an Evolutionary Significant Units approach (Ryder, 1986; Moritz, 1994) and were found different Management Units inside southern pike populations (Gandolfi, et al., 2017). This is an innovative approach in conservation of fishes for this country which is still mainly linked to classical approaches in taxonomy and conservation biology.

Cyprinids are the largest family of freshwater fish and inside this family taxonomical uncertainties still remain and only molecular studies can clarify taxonomic questions.

Hybridization due to habitat alteration

One of the most serious and controversial issues for freshwater fish conservation is hybridization (Allendorf, et al., 2001; Abbott, et al., 2016; Todesco, et al., 2016). Climate change, human activities, increasing water demands, natural resources fragmentation and habitat loss are the major threats for species. Hybridization and local extinction are the direct consequences to these activities (Todesco, et al., 2016). A relevant consequence of barbel species hybridization is the polyploidyzation recorded in several fish orders and families: Cypriniformes (Cyprinidae, Catostomidae, Cobitidae), Siluriformes, Acipenseriformes, Salmoniformes, and in several families of the Ostariophysan (Callichthyidae), Poeciliiformes (Poeciliidae) and Atheriniformes (Klinkhard, et al., 1995; Mable, et al., 2011; Berrebi, et al., 2014). A general character of cyprinids is the capability to hybridize, including intergeneric crossing, but polyploidy may add more plasticity to these animals and for this reason it is necessary to study in depht this phenomenon across barbel species (Berrebi, et al., 2014). These animals are more tolerant to ecological variation through polyploidy because the duplication of their genes provides metabolic advantages similar to a permanent high genomic heterozygosity (Otto & Whitton 2000). Climate changes contribute to increased water tempertures (Foley, et al. 2005; Walther, et al. 2002); as a conseguence of this, hybridization phenonenon increased as showed in the hypotetical scenario which assesed in this thesis (Fig.2).

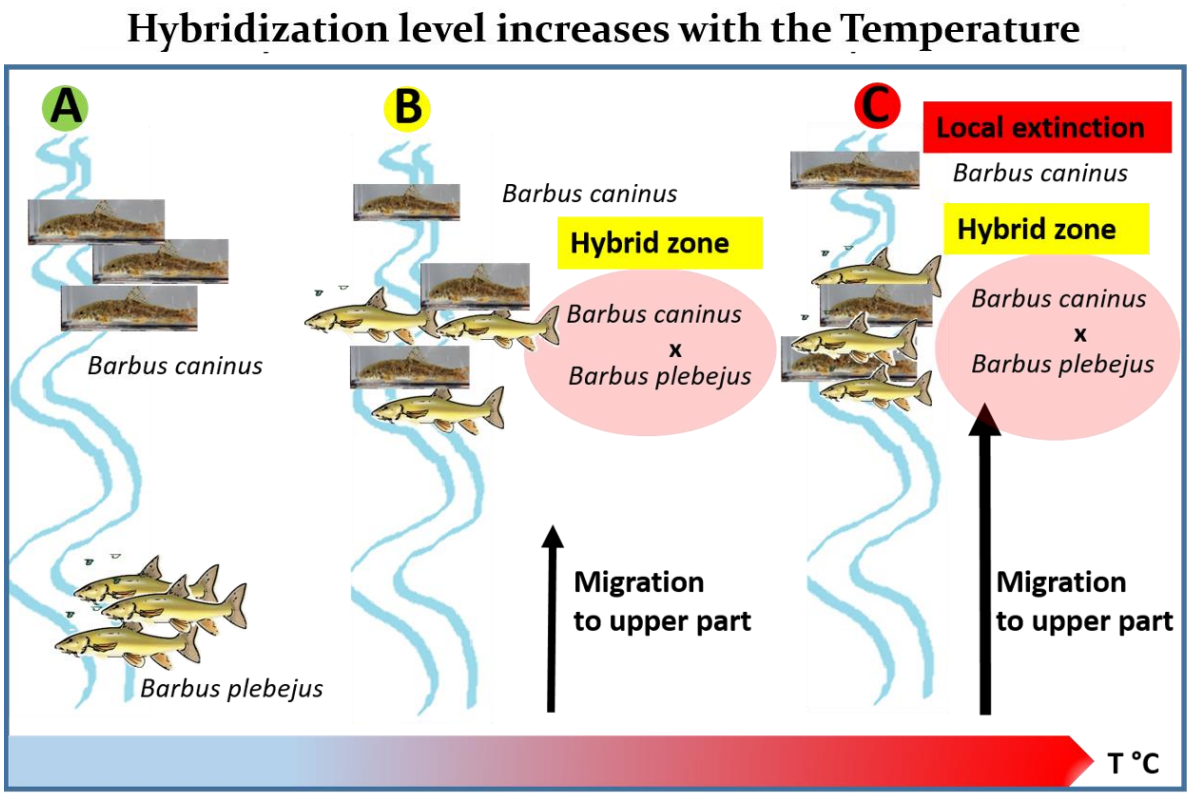

Fig.2: theoretical model of the two population species living inside the same river but in different altitude optimal condition of water temperature (A); as T water increase pushes population's species that lives in the lower part of the river to move in the upper part where interaction with the other species develops a hybrid zone (B). If the T water increases more the hybrid zone increases and leads to local extinction the last pure population of *B. caninus* (C).

1.2 Protected Areas inside Natura 2000 Network

Natura 2000 Network, the largest network of Protected Areas, was established across 28 EU countries and implemented by the Birds and the Habitats Directives of the European Union (EU). This network is a system where all human activities are managed in respect to sustainability and nature conservation and its aim is conservation, irrespective of national boundaries. This program is considered one of the best political chances of success that we can have: EU countries can use these areas to reduce biodiversity loss (Maiorano, et al., 2007) Futhermore, another potential contribution of this network is consist in better covering areas of vertebrate threatened species as quantified by Kukkala et al. (2016).

In Italy protected areas inside Natura 2000 cover more than 19% of the total national area and 4% of marine territory (Ministero dell'Ambiente e del Mare, 2017). As mentioned by Maiorano et al., (2015), Natura 2000 is a unique opportunity to contribute to ecological linkages necessary for the preservation of biodiversity in the EU and can build a bridge between Protected Areas (PAs) as ecological corridors, which are relevant for the preservation of genetic flows between population and reducing habitat fragmentation. If we compare geographical extension of PAs, Natura 2000 Network is the largest net of Protected Areas of European countries with more than 18% of terrestrial territory of the Member States covered as well as significant marine areas (European Commission, 2013).

1.3 LIFE project for conservation

The most important financial instruments supporting nature and environmental conservation, and climate action are European LIFE projects. More than 4500 projects were co-financed by the EU since 1999. The contribution of LIFE projects founding is approximately 3.4 euro billion towards the protection of the environment and climate for the period 2014-2020 (European Commission, 2017).

The "Environment" sub-program involves the priority sectors below:

1. LIFE Environment and Resource Efficiency;
2. LIFE Nature and Biodiversity;
3. LIFE Environmental Governance and Information;

European LIFE projects are relevant tools for conservation practices; not only for financial resources aspect, but also for the impact they have on the local conservation and for stakeholders and citizens involved. In most cases, conservation projects that involved the LIFE program were localized in small geographical areas for different reasons:

1. Administrative and bureaucracy implications: if we want to build a practical project inside a territory we cannot act without the support of local administration and citizens;
2. Stakeholders and ethical implications: many projects have problems related to the actions inside LIFE projects. For example, many fishermen don't agree with the eradications and control of alien and invasive species;
3. LIFE projects are based on practical actions and one of the most important aims of these programs is finding a solution to nature, environment, conservation and climate problems. In most of the case, these achievements are almost impossible to obtain in a big geographical area, even for the budget allocated: the population restocking of all barbel species fish of Italy is almost impossible to do just in one singular LIFE project.

In 2014 the EU LIFE 13 NAT/IT/001129 BARBIE for the reintroduction and reinforcement of native barbel species inside water streams of Po river Basin, was financed in three different provinces of Emilia Romagna: Parma, Piacenza and Reggio Emilia.

The specific objectives of EU LIFE 13 NAT/IT/001129 BARBIE (see seal Fig.3) are listed in 26 specific actions summarized into 4 as follows (Barbie, 2015):

1. Create new populations and/or reinforce existing populations, in connection with environmental suitability and the composition of the fish community, through specific in situ interventions (reduction of biodiversity loss /defragmentation) and ex situ breeding practices);
2. Identify the threats to the survival of the species at a local scale; open up discussion among stakeholders in order to reduce such threats through an interprovincial approach thanks to a lasting governance for the protection of target species and, indirectly, of river biodiversity;
3. Eradicate/control the spread of invasive alien species;
4. Establish guidelines for the conservation and sustainable management of species, that can be also used for the creation of a general European model; to transfer best practices.

Fig.3: Seal of EU LIFE13 NAT/IT/001129 BARBIE, EU LIFE logo and Natura 2000 Network.

1.4 Ecological Indices

EU community set many different parameters for the monitoring of rivers status through different ecological indices. In this study were studied STAR_ICMi, ISECI and IFF as supporting tools to state habitat alterations and potential effects on original populations;

1. STAR: "Standardization of River Classifications: Framework method for calibrating different biological survey results against ecological quality classifications to be developed for the Water Framework Directive" (www.eu-star.at).

2. Intercalibration Common Metric Index (ICMi): combination of the values obtained by ICMs into a single multi-metric index.

3. IFF: Fluvial Functionality Index. The aim of this instrument is the revelation of complex status of river environment and his functional aspects as interaction between biotic and abiotic factors. This instrument is based on 14 questions related to ecological characteristics of rivers and water streams (Siligardi, 2007).
4. ISECI: Ecological Status of Fish Community index. This tool permits to determine the presence of native species expected in particular zoogeographical area (Zeruinian, 2004).

Ecological indices are practical tools useful for detection of threats and pressure inside water streams but they have critical aspects; for example, ISECI index, that gives a general point of view on the zoogeographic status of rivers needs local changes in the analysis as reference populations connected to autochthonous and allochthonous fishes are still debated in many different areas (Zerunian, 2012).

1.5 Conservation genetics as a management tool

Biodiversity strategies recognize the importance of genetics practices for conservation studies and management plans required genetic characterizations of populations. The battle against hybrids and alien species needs molecular approaches as described in the Regulation (EU) No 1143/2014.

EU has investigated distinct types of measures as priority inside Regulation on Invasive Alien Species:

Prevention: early detection and rapid actions against alien species invasions. Different robust measures aimed at preventing invasive alien species of Union concern from entering the EU, either intentionally or unintentionally.

Eradication: to take rapid eradication measures to prevent IAS (Invasive Alien Species) of Union concern from establishing after the identification of their presence of IAS (Invasive Alien Species) of Union concern as early as possible.

Management: not spread any further IAS and minimize the harm they cause.

Conservation genetics try to rescue natural population answering the following questions:

1. Do the populations mix in nature?

2. Which populations can be used as sources for translocations?

3. What is the risk of outbreeding depression?

4. How much gene flow is required to rescue the populations at risk and restore their adaptive potential?

5. How is possible to identify distinct populations and relevant conservation units?

The absence of scientific data on the amount of genetic variation within and among populations has wide implications for other central problems in ecology and evolution.

If we must answer to these questions is required a hierarchical approach with the identification of species in the field, barcoding analysis, phylogenetic studies of the samples with the information of mitochondrial DNA, and investigation of the population structure with the most informative molecular markers described in literature.

In the context of management, the information on genetic 'purity' is often considered (Abbott, et al., 2016). Conservation genetics tries to identify hybrid individuals, if any, inside natural population. Molecular approaches have important management implication aiming at maximizing genetic diversity and minimizing inbreeding (Congiu, et al., 2011).

1 AIMS

This thesis focused the attention on the conservation plans inside 15 Protected Areas of Natura 2000 Network. In particular, barbels were considered as key species to assess biological integrity in the framework of LIFE 13 NAT/IT/001129 BARBIE project based on different conservation actions, among which selection of individuals for reintroduction and restocking was a main topic.

The goals and scientific questions of this PhD dissertation are:

1. Detecting the evidence, if any, of significant population genetic structure in *Barbus plebejus* and *B. caninus*
2. Determining introgression and hybridization between native species and other barbel species
3. Explaining Phylogenetic relationships among populations in different hydrogeographic districts
4. Defining whether ecological and environmental factors correlated to genetic factors

An intense sampling activity was supported inside seven water streams of Po river Basin in three different provinces of Emilia Romagna.

This thesis therefore aims at evaluating the usability of different barbel species genetic makeup as an indicator of water condition and freshwater habitat quality as a whole. The results will help in improving current practice in ecological restoration and water resources preservation at the ecosystem level.

CHAPTER I: ANALYSIS OF ECOLOGICAL STATUS OF FRESHWATER RIVERS INSIDE PROTECTED AREAS

3.1 Introduction

Streams and rivers have become some of the most endangered ecosystems worldwide, there is therefore an urgent demand for comprehensive methodological approaches to evaluate their conservation status and to monitor their rate of change (Li et al., 2010). Mediterranean regions represent key areas for freshwater fish endemism and introductions (Leprieur et al., 2008) as a large portion of European critical catchments for freshwater biodiversity is located primarily in southern Europe (Carrizo et al., 2017). Unfortunately, these areas are severely threatened because of water scarcity and environmental degradation due to high anthropic pressure (e.g., hydropower and agriculture) and climate change (Hermoso and Clavero, 2011; Hermoso et al., 2011).

In inland waters, natural fish stocks are generally over-exploited, and in developed countries, uncontrolled larvae and juveniles' reintroduction have been carried out for recreational fishery purposes (Lewin et al., 2008). Additionally, in last decades the distributions of many exotic fish species have extended worldwide further impairing the integrity of autochtonous fish populations (Carosi et al., 2017). This is especially true for Italy, particularly along the lower stretches of rivers throughout the Apennines (Italian Peninsula) that are characterized by rather warm and slow waters and scarce quality conditions (Carosi et al., 2017). All this translates into less than ideal conditions for the survival of endemic and threatened fish and the conservation of the local population diversity.

Since the beginning of 21st century many actions have been carried out to improve fish populations including mitigation of hydropower development, river fragmentation and hydropeaking (Premstaller et al., 2017). In this context, the Natura 2000 network may represent a strategic tool to preserve both fish genetics and population resources, with the final goal of improving their local and regional survival chances. However, the designation procedures of the Natura 2000 sites often are driven by factors marginally related to ecological criteria, thus reducing their effectiveness (Trochet and Schmeller, 2013). Furthermore, the biotic integrity of biological communities in rivers and lakes is considered a key predictor of colonized water bodies' quality suggesting a possible synergy between the Habitat Directive

(HD, European Union, 1992) and the Water Framework Directive (WFD, European Union, 2000). However, these two fundamental legal issues proceed in isolation with negative effects on their relative efficiency (Bolpagni et al., 2017).

In order to actively and mutually implement the HD and the WFD, and with the aim to improve their effectiveness in terms of threatened fish protection, the Life project BARBIE (LIFE13 NAT/IT/001129) started in 2014, focusing on three congeneric species of the genus *Barbus*. Two of them are of "priority interest" sensu HD: *Barbus caninus* (Bonaparte, 1839) and *B. plebejus* (Bonaparte, 1839). This two native species of *Barbus* are charismatic indicators for the estimation of the conservation status of water bodies (Angelini et al., 2016). The third is an alien species, *Barbus barbus* (Linnaeus, 1758), that showed an exceptionally fast spreading capacity within the Po River basin in the last decades. This invasive species have also hybridized with the endemic *B. plebejus* (Meraner et al., 2013), thus resulting in a widespread genetic introgression in the native *Barbus* species.

In this context, to test the distinct distribution of the different species as per our hypothesis, we described the fish community structure in a representative array of 14 of the watercourses included in the Natura 2000 sites of the Parma, Piacenza and Reggio Emilia provinces and their surroundings (Emilia Romagna Region, Northern Italy). Our main hypothesis is that the exotic taxon (*B. barbus*) may be spatially limited to the lowland sectors, which are those with the highest human disturbance rates. On the contrary, native barbel taxa (*B. caninus* and *B. plebejus*) may be mainly distributed in mountain and/or hill areas. Hence, an altitudinal segregation among these species may be hypothesized. To do this we focused on the presence/absence and representativeness of the *Barbus* species, implementing the current data on local spatial distribution. Therefore, we were also able to assess the contribution of a complex of Natura 2000 sites to support fish communities. Additionally, we collected a set of environmental variables (including physical, chemical, biological, and land-use descriptors) to assess the ecological functionality of sites colonized by the three target species.

3.2 Materials and methods

Study area

This study covered rivers and streams running along 15 sites of the Natura 2000 network of the Parma, Piacenza and Reggio Emilia provinces (Emilia Romagna region, Northern Italy; Fig.1; Tab. 1 of the appendix. The Köppen-Geiger classification includes both humid subtropical (cfa; plains and hill sectors) and oceanic climates (cfb; mountain sector), characterized by few extremes of temperature and pronounced precipitation in all months.

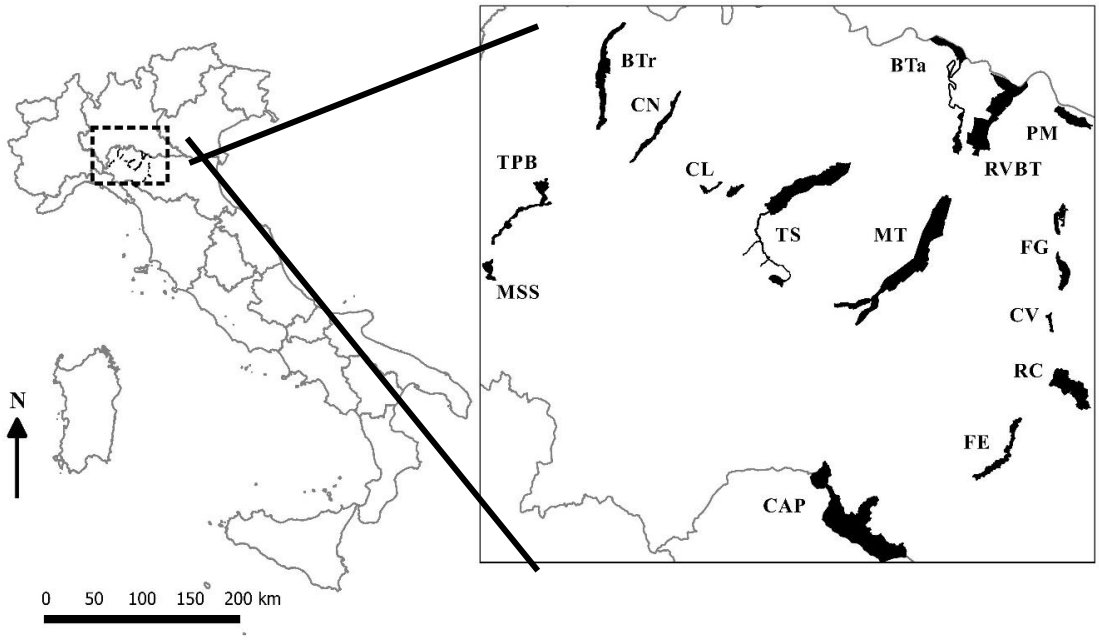

Fig.1: Study area with the indication of the Natura 2000 network: MSS = Meandri di San Salvatore (IT4010006); TPB = Fiume Trebbia da Perino a Bobbio (IT4010011); BTr = Basso Trebbia (IT4010016); CN = Conoide del Nure e Bosco di Fornace Vecchia (IT4010017); CL = Castell'Arquato, Lugagnano Val d'Arda (IT4010008); TS = Torrente Stirone (IT4020003); Bta = Basso Taro (IT4020022); RVBT = Aree delle risorgive di Viarolo, Bacini di Torrile, Fascia golenale del Po (IT4020017); PM = Parma Morta (IT4020025); MT = Medio Taro (IT4020021); FG = Fontanili di Gattatico e Fiume Enza (IT4030023); CV = Cronovilla (IT4020027); RC = Rupe di Campotrera, Rossena (IT4030014); FE = Fiume Enza da La Mora a Compiano (IT4030013); CAP = Crinale dell'Appennino parmense (IT4020020).

The study area includes a complex hydro-system that consists of several streams and rivers across an extended altitudinal range (about 600 m), stretching from the Tuscan-Emilian Apennine ridge to the Po River. Accordingly, the mean annual discharges and the main water chemical and physical conditions are extremely variable. Generally, the investigated water bodies showed a good chemical status, and fall into the sufficient or good quality classes of the ecological status sensu WFD (ARPA Emilia Romagna, 2013).

The study area covers the entire distribution range of the two autochthonous target species in the Emilia Romagna region: *B. caninus* inhabits mountain fast-flowing biological, water quality and morphological characterization between July 2014 and December 2015, a total of 305 barbel individuals were collected by electric fishing from 31 localities of the study area following Cowx and Lamarque (1990). Two localities have been chosen at the border of the Protected Areas because it was not possible to find barbels inside different Natura 2000 sites. Each animal was measured, fin-clipped and subsequently released in the same location. Measurements included total length and weight. Fin fragments were preserved in 70% ethanol until DNA extraction. Starting from the visual based analysis of the fish community, the Index of the Ecological Status of Fish Communities (Italian achronomous is ISECI: Indice stato Ecologico delle Comnità Ittiche,) was also calculated in each of the 31 study sites, in accordance with Zerunian protocol (2009, Tab.1).

Simultaneously to fish characterization, a water sample was collected with a plastic bottle just below the water surface. In situ temperature, pH, conductivity and dissolved oxygen data were collected by a multi-parameter probe (YSI model 556 MPS). Samples for BOD_5 (biochemical oxygen demand) were transferred into glass bottles. Samples for NH_4^+ (ammonium), NO_3^- (nitrate), and NO_2^- (nitrite) determinations were filtered through Whatman GF/F glass fiber filters (Ø 47 mm, porosity 0.45 µm) and transferred to plastic vials. All water samples were kept to 4°C, and transferred to the laboratory. Total suspended solids (TSS) were measured by filtration through a pre-dried and weighed glass fiber filter GF/F (Whatman, UK, Ø 25 mm and 0.45 µm) (APHA, 2012). NH_4^+, NO_3^-, NO_2^-, were determined with

standard spectrophotometric methods APHA (2012), whereas BOD5 was calculated after incubation at 20±1 °C for 5 days according to APHA (2012).

Each sampling sites was characterized by the application of the Fluvial Functionality Index (FFI), that is devoted to investigate the functionality of a river stretch in terms of metabolic capacity (i.e., fine and coarse particulate organic matter retention and cycling) (Siligardi et al., 2000). This method is based on the analysis of riverbank vegetation, physical and morphological structure, the extent of the riparian area, the land use impact, the riverbed structure, and the key biological characteristics of river ecosystem. For further details, see Siligardi et al. (2000).

Statistical analysis

Principal Component Analysis (PCA) was performed on physical and chemical data. The interpretation of PCA ordination was limited to variables with loads higher than the vector representing a variable contributing equally to all the dimensions of the PCA space (Borcard et al., 2011). The relationship between PCA ordination and the structure of *Barbus* populations was assessed by means of the function envfit ("vegan" package In R software) that fits vectors onto a multivariate ordination. Simple regression analysis was used to examine the relationships between IFF and altitude and BOD5 values.

All the analyses and graphs were performed with the statistical software R (R Core Team, 2015), with base version, ggplot2 (Wickham, 2009) and vegan (Oksanen et al., 2016) packages.

Molecular data analysis for molecular taxonomy

For each of the 305 specimens, we analysed a 600 bp long region of the *cytb* mtDNA for species identification. For the phylogenetic analysis, a subset of the samples was chosen according to sequences' quality (see chapter II for further explanation).

Genomic DNA was extracted from fin tissue using Wizard genomic DNA Purification kit (PROMEGA, Madison, WI, USA). DNA quality and concentration were tested by 1% agarose gel electrophoresis in 1% TAE buffer, by visual comparison with a DNA

ladder mix and by spectrophotometry at 260 e 280 nm. The extraction procedure typically yielded no less than 40 ng/ml of HMW (high molecular weight) DNA. Extracted DNA was amplified by polymerase chain reaction (PCR) using primer pair CYTBThr 5'-ACCTCCGATCTTCGGATTACAAGACCG-3' and CYTB-Glu 5'-AACCACCGTTGTATTCAACTACAA - 3' (Zardoya and Doadrio, 1998). A reaction volume of 25 µl containing 1 U of GoTaq Polymerase (PROMEGA, Madison, WI, USA), Mg^{2}+ 1.5 mM and dNTPs 0.2 mM, and 10 pmol of each primer were used. PCR was set as follows: 35 cycles of 45 s at 94°C, 1 min at 47°C, and 2 min at 72°C, after an initial 3 min denaturation step at 94°C and a final extension at 72°C for 10 min. Fragments sequencing was performed by MACROGEN Europe service (Amsterdam, the Netherlands). The obtained sequences were manually corrected using MEGA7.0 and were compared with those available in genomic databases using NCBI BLAST.

3.3 Results

Water quality, morphological and fluvial ecological functionality of sites

Results from water measurements highlight the variability between sites in terms of altitude and human impact gradients. In summer 2015, temperature, pH, dissolved oxygen, and conductivity were in the ranges 14.4–31.0 °C, 7.30–8.91, 37–295% saturation, and 190–825 µS cm-1, respectively. Similarly, the BOD5 and TSS values varied from 0.0 (mountain sites, Trebbia River) to 20.8 mg L-1 (lowland site, Parma Morta), and from 0.0 to 66.2 mg L-1, respectively. At the same time, NH4+ and NO2- concentrations exhibited only small variations, within the range 0.02–0.04 NH4+ mg N L-1, and 0.00–0.02 NO2- mg N L-1. On the contrary, NO3- ranged between 0.08 and 5.00 mg N L-1. Physical, chemical, and morphological (i.e., IFF outputs) data are reported in Tab. 1.

IFF ranged between 102 (poor) and 245 (good). IFF showed a clear spatial arrangement with an almost significant negative correlation with altitude ($r=0.45$, $p=0.07$; $n=17$). A progressive reduction in IFF values was recorded when moving from mountain/hill to lowland sectors. On the contrary, no significant relationship between IFF and BOD5 values was recorded ($r=0.37$, $p>0.1$; $n=17$), although a gradual increase of the

biochemical oxygen demand with the progressive loss of the functionality of riparian belt is generally expected.

Fish community and ISECI assessment

From a general point of view, considering the population size (expressed in terms of number of individuals per sample sites), the observed data were quite low in the range 0-29 individuals. Eight over 31 (26%) study sites showed complete absence of barbel specimens. A structured population of canine barbel constituted of 24 and 25 specimens of different size classes (i.e. juveniles, sub-adults and adults) were retrieved in the Rio Cerezzola (Natura 2000 site IT4030014) and Rio Parmossa streams (neighbouring this Natura 2000 site area), respectively. On the contrary, common barbel peaked at Nure and Enza rivers with 29 and 25 individuals, respectively. The alien European barbel species colonized 7 (23%) study sites belonging to Arda, Trebbia, Taro, Ceno and Enza rivers, (Tab. 1). In particular the allochthonous barbel (*B. barbus*) showed a representative population in Taro River, with 19 individuals, as a result of the only investigated plain river site. The number of individuals detected in each sampling station for the three investigated species is reported in Tab. 1.

ISECI values varied from 0.72 (good ecological quality) at Nure River to 0.20 (poor ecological quality) at lowland site Parma Morta, with a mean value of 0.57 (±0.14) (sufficient ecological quality) (Tab. 1). More specifically, 18 over 31 (58%) sites displayed "good quality" class, 10 (32) "moderate quality", 1 (3%) "poor quality", and 2 (7 %) "bad quality".

Tab. 1: the contents show the qualitiy of ecological indices (High quality= Light blue; Good quality= green; sufficient quality= yellow; scarce quality= orange; bad quality= red). SCIs (Site of Community Interests); ISECI (Freshwater Fish Community Index); STAR ICMi ("Standardization of River Classifications Intercalibration Common Metrix Index); IFF (Functional Fluvial Index).

River	Sampling Site	Natura 2000 sites	ISECI (da 1 a 0)	STAR_ICMi (da 1 a 0)	IFF (da 300 a 14)
ARDA	ARD	IT4010008	0,61	0,545	125
ENZA	NAV	IT4020021	0,68	ND	ND
	CRN	IT4020027	0,72	ND	ND
	MO	IT4030013	0,55	0,653	213
	CN	IT4020021	0,2	ND	ND
	LU	Outside	0,42	ND	ND
	VI	IT4030014	0,5	0,484	158
	CE_1	IT4030014	0,67	ND	ND
	CE_2	IT4030014	0,59	0,456	165
	MT_1	IT4030013	0,6	NA	180
	MT_2	IT4030023	0,53	NA	180
	SI_1	IT4030023	0,64	NA	180
	SI_2	IT4030023	0,63	NA	102
NURE	NU	IT4010017	0,72	NA	180
PARMA	LO	IT4020017	0,66	0,51	181
	MAR_1	Outside	0,4	0,909	210
	MAR_2	Outside	0,52	ND	210
	LAG	IT4020020	0,66	ND	ND
	PM	Outside	0,66	ND	ND
	BA	Outside	0,49	ND	ND
	DD	IT4020025	0,2	NA	123
STIRONE	STR	IT4020003	0,71	0,628	103
TARO	GI	IT4020021	0,63	1,052	195
	CN	IT4020021	0,52	ND	ND
	SS	IT4020022	0,3	0,82	105
TREBBIA	SASA	IT4010006	0,61	0,721	245
	SN	IT4010011	0,64	0.655	215
	TR_1	IT4010016	0,72	0.807	205
	TR2	IT4010016	0,69	0.807	205

High Good Sufficient Scarce Bad

Molecular taxonomy of barbel species

As better described in the next chapter, molecular analyses were carried out to assess the percentage of different species at each sampling site (Tab. 1 of the appendix). From a taxonomy point of view, molecular analyses on mtDNA evidenced the following density values: 72% common barbel (*B. plebejus*), 18% canine barbel (*B. caninus*), and 10% alien European barbel (*B. barbus*)as illustrated in Fig. 2 and 3. See also next chapter for a better description of the phylogenetic methodology and obtained results. Fragments of 600 bps were analyzed and compared to GenBank sequences. Samples displayed 100% identity with deposited sequences according to different species with alignment values E=0.0 and maximum identity in the range 97–100%. ClustalW (using MEGA 7.0) assessment among investigated samples showed a total number of 230 polymorphic sites.

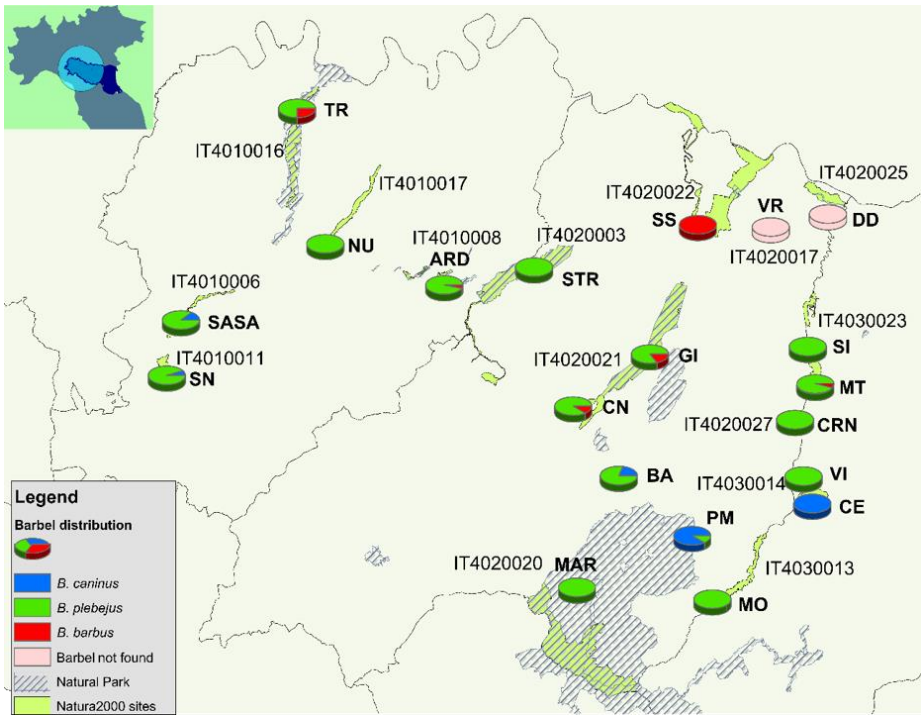

Fig.2: sampling areas with *cytb* mtDNA results. The population codes are: VI (Rio Vico), MO (Mora), CE (Rio Cerezzola), SS (San Secondo), GI (Giarola), ARD (Lugagnano Val d'arda), MT (Montecchio), TR (Taro river), MAR (Marra), STR(Stirone), PM (Parmossa), NU (Nure), SASA (San Salvatore), SN (Pian Casale Gerbini), CRN (Cronovilla), CN (Ceno), VR (Viarolo), DD (Parma Morta), BA (Baganza).

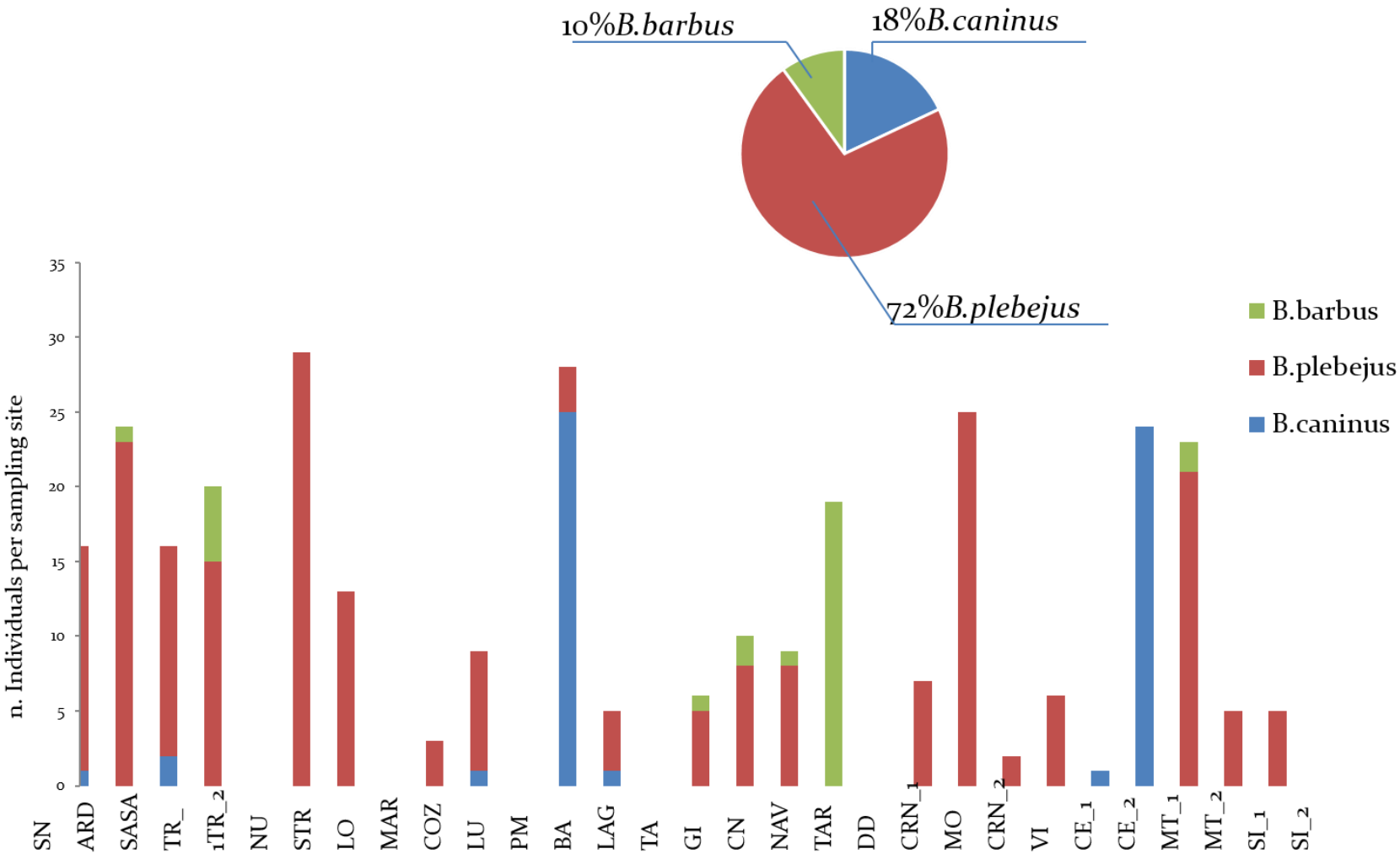

Fig 3. Site scale percentage distribution in pie chart (%) of the three Barbel' species in analysis: alien European Barbel (*Barbus barbus*) in green, Common Barbel (*Barbus plebejus*) in red, and in blue Canine Barbus (*Barbus caninus*).

In terms of environmental drivers of the observed barbel' species spatial distribution, IFF and oxygen saturation were the variables contributing most to respectively PCA axes 1 and 2, explaining the 88% of the total variance. Alien European barbel was significantly related to the PCA ordination ($R2=0.67$, $p<0.05$), while this was not true for the other two barbel species: *B. caninus* ($R2=0.15$, $p>0.05$) and *B. plebejus* ($R2=0.14$, $p>0.05$) (Fig. 4).

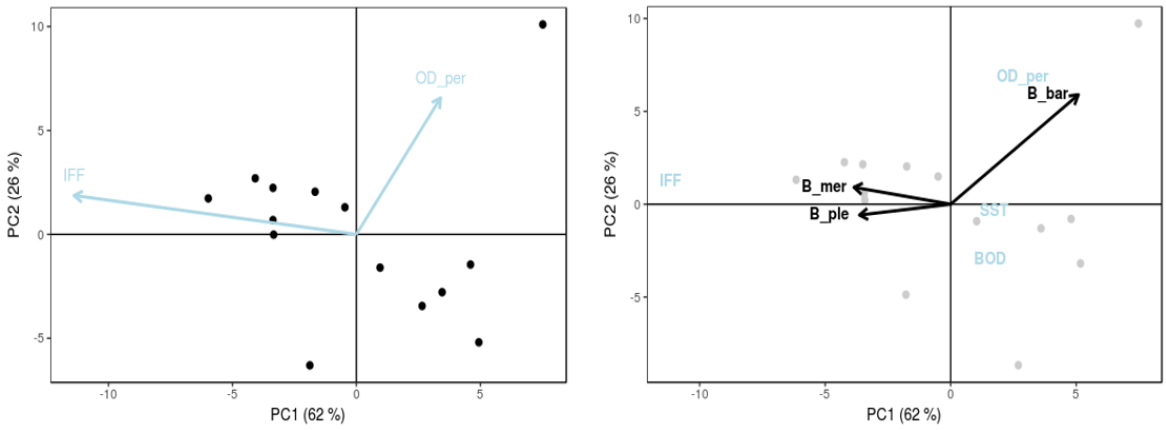

Fig. 4: Results of principal component analysis (left); vectors representing Barbus' population structures superimposed to PCA ordination (right).

3.4 Discussion and Conclusions

Small number of sites under consideration in this work, a quite verified preference of the canine and common barbels for higher IFF values was verified, as a proxy of well-developed and structured riparian contexts and higher altitude values. Marginal vegetated belts are able to efficiently offset the nutrient inputs generated at the basin scale, and to control the main local edaphic factors (Siligardi et al., 2000). Hence, in presence of high IFF values, higher shading values, as well as lower water temperatures and reduced day-night temperature fluctuations are expected. Furthermore, watercourses with high IFF levels should have reduced primary producer rates with rather scarce macrophyte cover values, including algal mats. On the other hand, the alien European barbel was positively and significantly related to high levels of dissolved oxygen, which in turn was associated to higher levels of STT and BOD5. This is not surprising, given that the above-mentioned high dissolved

oxygen levels (up to 295% saturation detected in late morning) were due to the hyper-proliferation of microalgae, and were typical of lowland sites of Po plain where riparian belts/zones have been almost completely lost to land reclamation by agricultural mechanization (Bolpagni and Piotti, 2015; 2016).

All the above clearly remarks the need for more efficiently designed long-term and wide-spatial scales actions to counteract the alien fish expansion, and further focus on the sustainable management of river habitats and water flows. In this context, the reduction of the hydraulic fragmentation of watercourses is an essential paradigm to improve the survival prospects of a very large number of species of conservation interest. In fact, the progressive impairment of the longitudinal river continuity causes significant alterations in river dynamic processes and aquatic vegetation (Bolpagni et al., 2016). However, it is of interest, and possibly of considerable practical importance, taking into account the spatial dynamics of the alien invasive species before operating management actions that could later have an impact to the native fish populations. This critical issue must also be further discussed in the light of the on-going climate change that can affect barbel migration along the altitudinal gradient.

CHAPTER II: MOLECULAR TAXONOMY AND DISTRIBUTION OF BARBEL SPECIES IN 15 NATURA 2000 PROTECTED AREAS OF NOTHERN ITALY SHOW AUTOCHTHONOUS RAREFACTION

4.1 Introduction

Barbels belong to the Cypriniformes order and the Cyprinidae family that includes a large number of species, widespread in Europe, Asia and Africa (Kottelat and Freyhof, 2007)

Among Italian native cyprinids, four species of *Barbus* are recognized: *Barbus caninus*, *Barbus plebejus*, *Barbus tyberinus* and *Barbus balcanicus*, three of which are either endemic or subendemic of the Italian peninsula: *B. plebejus*, *B. caninus*, *B. tyberinus* (Kottelat and Freyhof, 2007; Geiger M.F., 2016). Two barbels species native of Po basin district of Northern Italy deserve particular attention: *Barbus caninus* and *Barbus plebejus*. The Italian IUCN Red List moved *Barbus caninus* from VU (Vulnerable) to EN (Endangered) status and for *Barbus plebeius* (Rondinini, et al., 2013) from LC (Least Concern) to VU (Fig.1).

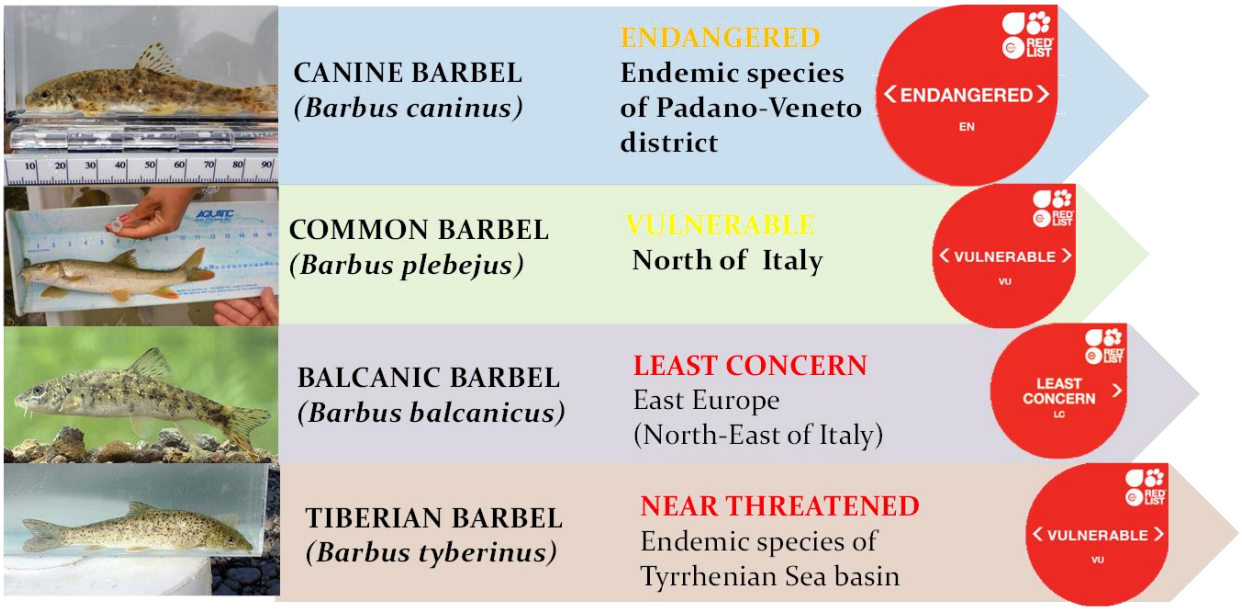

Fig.1: Italian IUCN classification of native barbel species in Italy (Rondinini, et al., 2013).

Both species are strictly affected by environmental conditions. *B. caninus* has the typical morphology of small-sized rheophilic barbels and it is phylogenetically close to *B. meridionalis*, a species living in Southern France and North-Eastern Spain

(Tsigenopoulos, et al., 2002). Taxonomic position of this species has been longly debated. In a review by Bianco (1995), *B. caninus* was considered a subspecies of *B.meridionalis* but many studies based on allozymes (Tsigenopoulos and Berrebi, 2000; Bianco 1998) and mtDNA (Tsigenopoulos & Berrebi, 2000) showed distinct phylogenetic relationships. Although *B. caninus* was considered an independent species from *B. meridionalis*, the Habitats Directive (92/43/EEC) still reports the management name of *B. meridionalis*. As shown in the figure 2, the original distribution of canine barbel was extended from Marecchia and Brenta in the North part of Italy (Padany–Venetian ichthyogeographic district) and was introduced in the Center of Italy (Kottelat and Freyhof, 2007). However, there is a lack of molecular data and species distribution has been mainly hypothesized in relation to limited monitoring based only on external characters. The common *Barbus plebejus* is distributed in all Northern Italy rivers belonging to the Padan-Venetian (Padano-Veneto) district, from the Po River basin up to the Adriatic rivers in northern Croatia (Tsigenopoulos et al., 2002; Kottelat and Freyhof, 2007). This species is ecologically capable to occupy various stretches of a river, while the canine barbel is a typical species of medium-high stretches of water and small tributaries. Even more, historical areas of barbels in Italy have drastically decreased, in particular for *Barbus caninus* that nowadays shows a fragmentary distribution (Buonerba et al. 2015; Geiger et al. 2016).

The major threat to the species and population structure is probably given by habitat alteration, associated to multiple factors, like cumulative effects of anthropogenic pressures and climate changes (Pletterbauer et al., 2015). For this reason, Protected Areas (PAs) and particularly Natura 2000 Network sites are very important to preserve the original distribution of barbel species.

Fig.2: native barbel species distribution in Italy suggested by Kottelat & Freyhof (2007).

Morphological and meristic characters are not enough for the discrimination between barbel species. One of the most powerful and useful methods for the identification of species is molecular barcoding with mitochondrial molecular markers as *COI* (Cytochrome I), *cytb* (Cytochrome b,) and Control Region also stated as D-LOOP. Each one of these markers is suitable to investigate different questions connected to phylogenetics, taxonomy or population genetics.

Even in the genomics era the mitochondrial approach is therefore still useful for many reason:

1. Cost of genomic analysis still too expensive
2. Scientific questions of research studies: if the question is related to identification and phylogeny of specie mitochondrial DNA is a power molecular marker
3. Availability of previous sequence data stored in genetic databanks

In Italy still persist many different knowledge gaps about freshwater fish evolution history. In most of the cases inside freshwater fishes' taxon is difficult to determinate the species level, Cyprinidae family is one of these taxon. After a primary morphological identification in the field is necessary a molecular analysis of samples.

4.2 Materials and Methods

Study Area and Sampling activities

Barbels were collected by electro-fishing as indicated by Cowx and Lamarque (1990) between August 2014 and December 2015 from 20 sample sites inside 15 Pas (Site Community of Importance) and of Emilian tributaries of Po river and 2 sampling sites outside protected areas (Tab.1). Two localities have been chosen at the border of the Protected Areas because barbels were not found inside multiple Natura 2000 sites. In particular, samples were distributed among seven Po river tributaries: Trebbia, Nure, Arda, Taro, Parma, Enza and Stirone. Tissue samples were taken from adults, sub-adults and juveniles (for all species). This was achieved during electro-fishing by accumulating samples from longer river stretches (> 100 running metres) and thus avoiding spot-like sampling. Tissue samples for genetic analyses consisted of small anal fin clips preserved in absolute ethanol. All fish were released unharmed at the sampling site. Specimens were chosen according to Kottelat & Freyhof 2007.

Molecular data

Total genomic DNA was extracted using Wizard genomic DNA Purification kit (PROMEGA, Madison, WI, USA). DNA quality and concentration were tested by 1% agarose gel electrophoresis in 1% TAE buffer, by visual comparison with a DNA ladder mix and by spectrophotometry at 260 and 280 nm. The extraction procedure typically yielded not less than 40 ng/µl of HMW (High Molecular Weight) DNA. For this analysis, a subset of 255 specimens from the entire barbels dataset was selected according to the lengths and quality of sequences; this allowed for more information to be implemented for phylogenetic results. Therefore 728 bp long region of the *cytb mtDNA* was analysed and was amplified using Polymerase Chain Reaction (PCR). Primer pairs and PCR conditions were those previously described by Zardoya and Doadrio, (1998); CYTB-Thr 5' – ACCTCCGATCTTCGGATTACAAGACCG - 3' and CYTB-Glu 5' -AACCACCGTTGTATTCAACTACAA - 3'. A reaction volume of 25 µl containing 1 U of GoTaq Polymerase (PROMEGA, Madison, WI, USA),Mg^{2+} 1.5mM and dNTPs 0.2mM, and 10 pmol of each primer were used. PCR was set as follows: 35 cycles of 45 s at 94 °C, 1 min at 47 °C, and 2 min at 72 °C, after an initial 3 min denaturation step at 94 °C and a final extension at 72 °C for 10 min. Fragments sequencing was performed by MACROGEN Europe service (Amsterdam, the Netherlands). The obtained sequences were compared with those available in genomic databases using Blast and multiple alignments of forward sequences were conducted using Clustal-W (Thompson et al., 1994) implemented in Mega 7.0 (Kumar et al., 2016),

The sequences of unique haplotypes of *cytb* were deposited in Genbank database under the accession numbers (Tab. 2).

Statistical analyses

Sequences were merged to haplotypes (Tab. 2), and phylogenetic relationships were analysed by haplotype networks using TCS 1.21 (Clement et al. 2000).

Maximum likelihood analysis (ML) was performed with PhyML 3.0 (Guindon, et al. 2010) in Smart Model Sostitution SMS v1.8.1 (Lefort, et al. 2017).The best model of nucleotide evolution was chosen using AIC methods (Akaike, 1973) implemented in SMS v1.8.1 software. The starting tree was BioNJ and node support was estimated using a Chi-square branch supports. Output data tree was visualised by FigTree 1.3.1

(Rambaut, 2009). Ratio for transitions and transversions were calculated with MEGA 7.0(Kumar, et al., 2015).

4.3 Results

The *Cytb* gene fragment was successfully sequenced in 255 specimens, previously selected according to morphological characteristics of the three species (Gandolfi et al., 1991).

Alignment among samples was obtained for 728 bp. Starting from overall analyzed samples, results highlighted 192 *B. plebejus*, 43 *B. caninus*, and 20 *B. barbus* according to comparison with previously deposited GenBank sequences.

Fig.3: Sequence profile (A) and alignment of cytochrome b mtDNA fragments of three different samples (B) using MEGA7.0

More specifically, 36 sequences obtained from GenBank database were considered for comparison. The demographic consistency (number of determined individuals belonging to each species) in investigated SCIs, is illustrated in table 2 of the appendix.

TCS analysis showed 36 haplotypes clustered into 3 separate haplogroups defined H1, H2, H3, each one revealing different species (Fig. 4). The haplogroup H1 comprised 15 haplotypes for *B. plebejus*; H2 included 7 haplotypes for *B. caninus*; H3 comprised 13 for *B. barbus* (Fig.4). In the haplogroup H1, 7 new haplotypes were described, in the H2 haplogroup 6 new haplotypes were described, and in H3 no new haplotype was found. Haplotypes were named according to Meraner et al. (2013) and Buonerba et al. (2015) for Italian territory (tab.2. in the appendix) Most common haplotype for *B. plebejus* lineage extrapolated from GenBank was Bp04 (KC465931) which was detected in 48,6% of specimens. It was found in all investigated tributaries of Po river: Arda, Enza, Parma, Nure, Stirone, Taro and Trebbia. The second most common haplotype was PLE1 (A.N. KF923539) which was found in 15,7 % of investigated samples. Besides Bp04 and PLE1, 11 new haplotypes emerged in this work (see tab. 2). A new haplotype named Bp 23 (kX897129) was found in 5,9% of samples, collected along Taro (IT4020021), Enza (IT4030023; IT4030013; Marra), Trebbia (IT4010016) and Parma rivers (Parmossa). Furthermore, among new haplotypes, B24 (kX897130) was found in 4,7% of samples, collected in Enza, Parma and Trebbia rivers. Additional new haplotypes were locally represented: Bp26 (kX897132) found in Nure River, Bp27 (kX897133) and SIo2 were found in Enza stream (IT4030023).

Concerning *B. caninus* haplogroup (7 haplotypes), 6 new haplotypes were found and 1 was already described in GenBank (see Tab. 2). Among these, 3 of them were found in Cerezzola stream (SCI IT4030014), two in Enza river (SCI IT4030023), and one in Trebbia river (SCI IT4010006). It is noteworthy to observe that one specific sequence/haplotype, obtained from GenBank and named *Barbus caninus* (AF112124), turned out to be ambiguously classified; in fact, this haplotype is more similar to *B. meridionalis* sequences. The nucleotide frequencies were 26.69% (A), 28.41% (T/U), 29.64% (C), and 15.26% (G). The transition/transversion rate ratios were k1 = 25.423 (purines) and k2 = 11.098 (pyrimidines). The overall transition/transversion bias was R = 8.09, where R = [A*G*k1 + T*C*k2]/[(A+G)*(T+C)]. Among the sequences investigated, it has shown all sequences showed a total number of 117 polymorphic sites (the outgroup sequences were excluded from this analysis).

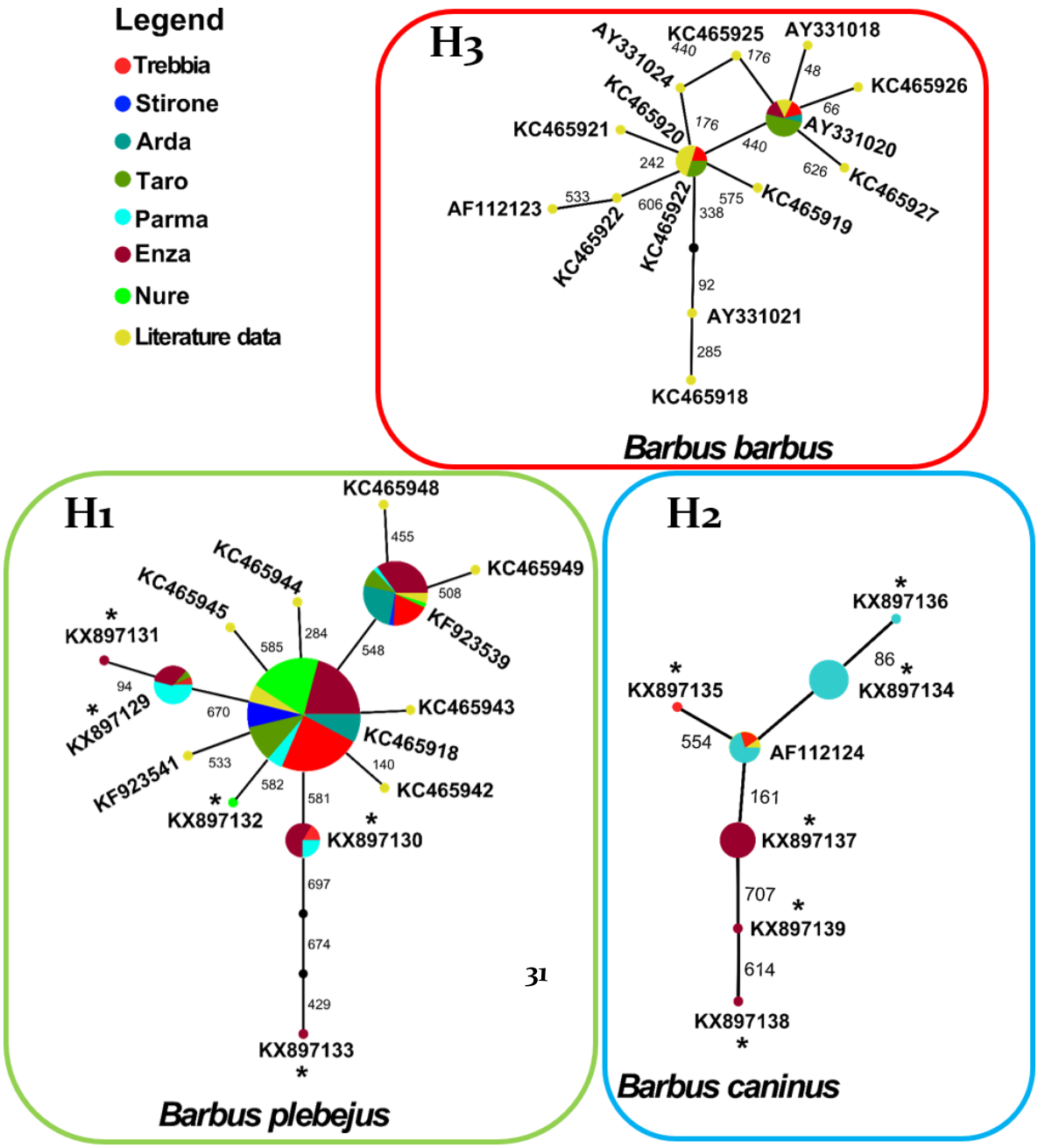

Fig.4: Network analysis implemented by TCS 1.21. Each pie chart represents a specific haplotype, and the size is proportional to haplotype frequency. Different colors represent the proportion of samples from each tributaries Po River. Numbers indicate the position of each SNPs discriminating different haplotypes; black dots represent a possible intermediate haplotype, actually missing in the dataset. Asterisks (*) indicate newly described haplotypes.

For phylogenetic tree analysis was selected GTR +G as the best-fit model by statistical criterial of AIC method.

The ML tree confirmed the phylogenetic relationships assessed by network analysis. In particular, significant differences between the three species were revealed by nodes robustness as evidenced by Approximate Likelihood-Ratio Test for Branches (aLRT) Chi-square (Anisimova and Gascuel, 2006) with a cutoff value to construct strongly supported consensus trees with values of 0,95-1,00. The phylogenetic tree explained the evolution relationships between the three different species; as showed in fig. 5 the cluster of *B. caninus* have a basal position compared with the other two clusters. The division of the other two clusters was supported by aLRT-Chi-squares with value of 1. The length of two branches separated *B. barbus* and *B. plebejus* that showed a more recent split event.

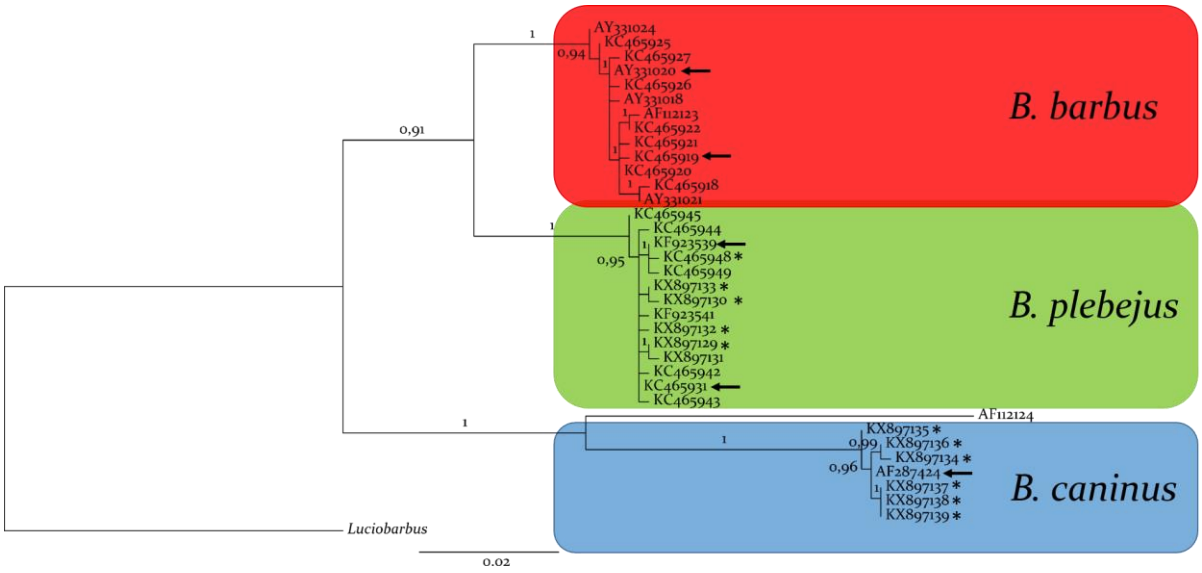

Fig.5 . Maximum Likelihood phylogeny of barbels based on 36 *cyt b* haplotypes from dataset and referee sequences from GenBank implemented in SMS v1.8.1 software. Numbers above and below branches refer to aLRT-Chi-square branch support. Asterisks (*) indicate newly described haplotypes, while arrows (←) indicate all haplotypes found in this study already described in literature.

4.4 Discussion and Conclusions

Molecular taxonomy and distribution of Barbel species

The mitochondrial analysis showed not enough evidence of a distribution pattern of barbels species. The rivers Trebbia, Taro, Arda and Enza were characterized with the presence of *B. barbus,* in SCIs areas: IT4010016, IT4020022, IT4020021, IT4030023. The most critical aspect for Protected Areas was found in the lower part of rivers near to Po River. In particular, the SCIs IT4020017 and IT4020025 showed no evidence of barbels species and in IT4020022 SCI there was a massive introgression of *B. barbus* as showed in Meraner et al. (2013) for additional Italian basins.

B. plebejus was the most common species found in the SCIs areas; it was found in medium stretches of all water tributaries in particular in Nure and Arda rivers. Mitochondrial DNA 'pure' population was found in the northern part of Parma and Enza rivers especially in the sampling sites MO (IT4030013), CRN (IT4020027), SI (IT4030023) and VI (IT4030014) for Enza river and MAR (IT4020020) for Parma river. The 'pure' mtDNA population belonging to the species *B.caninus* was found only in the site SCI IT4030014- Rupe di Campotrera, Rossena where a well preserved habitat seems to positively affect a generalised population welfare; in other SCIs (IT4010006, IT4010011,) and neighbouring sites (PM and BA), an habitat overlap was detected. These results showed a drastically effect of population decrease probably due to water temperature increase for climate changes and human activities (Pletterbauer et al., 2015), plus habitat loss and competition with invasive species. The habitat overlap between native species was probably determinate by human activities, climate changes and in particular habitat fragmentation (Foley, et al. 2005). The consequence of this effect could probably determine hydrid zone as showed in Trebbia, Parma and in rivers where at the same sampling site *B.caninus* and *B.plebejus* were found.

SCIs areas for the conservation of freshwater ecosystems

The species distribution in Trebbia river is very complex; in the highest part of this river *B. caninus* and *B. plebejus* were present while in the lowest part near Po river *B. barbus*

was detected. In relation to this, a gradient effect of allochtonous species introgression was supposed in Trebbia river.
In Enza river, the presence of *B.barbus* was detected in the lowest part; even in the upper part the alien species was found. These results underlined the capacity of the allochthonous species to cross artificial barriers to migration (Meraner et al., 2013). In Parma river, *B. plebejus* was found in all of the tree sampling site and *B caninus* was present in the two sampling sites outside SCIs sites.

Results showed the importance of local sampling scale inside Protected Areas and the importance of the role of molecular taxonomy to detect the species distribution. The historical distribution of *Barbus caninus* has decreased drastically and the introgression of allochtonous *B. barbus* species was confirmed in the Po river tributaries as suggested by Meraner et al. (2013). Moreover, spatial overlap of habitat distribution between native species *B. plebejus* and *B. caninus* was discovered. These results are probably due to climate change, human activities and habitat fragmentation. Conservation efforts within the Po drainage have to focus primarily on native populations. Studies about nuclear markers could help to detect the hybridization level of barbel populations in these Protected Areas.

Regarding management implications, molecular taxonomy is the guide in prioritising conservation project as European Life projects. Thanks to this work, we evidenced critical areas where the native barbel species live. Moreover, no barbel populations were detected in two SCIs (IT4020017 and IT4020025). The future restocking activities should take as principal aspect the genetic information to manage native populations. The focus for conservation plans should take into account preliminary genetic screening and species geographical distribution. Detection of suitable areas for the reintroduction, translocation and selection of spawners must become routine for hatchery activities and conservation plans.

CHAPTER III: HYBRIDIZATION AND INTROGRESSION ALONG AN ALTITUDE GRADIENT

5.1 Introduction

Freshwater ecosystems are one of the most threatened environments on the Earth (Tedesco, et al 2017; Jenkins, et al. 2003; Vörösmarty, et al. 2010). Among various threats, the presence of hybrids and invasive species may play a major role. Different fish species were transferred from a river to another (Gandolfi, et al. 2017; Chiesa et al., 2016), in Italy about 11.9 billion fishes (from 32 species) were traslocated, 16 of which were native (Copp, et al., 2005). The extinction of native species may be due to introgression of non-native ones if they outcompete among them for resources and become invasive (Allendorf, et al. 2010). European endemic fish species are around 80% in freshwater (Freyhof, 2011) and they drastically decreased over the last decade. More than 39% of fish are threatened in Europe as a consequence of indirect and direct human activities and climate changes, loss and fragmentation of habitat (Freyhof, 2011; Taylor, et al., 2015).

Among the major threats in Apennine rivers, water demand is tremendously increased during summer months mainly due to the irrigation request and enhanced population consume. The major subsequent consequence of the increase in water deprivation in rivers is an upstream migration of fish from lower parts of rivers to upper stretches in connection with water warming (Magurran, 2009).

Genetic consequences of all factors can be reflect in the introgression between native and allochthonous species. Negative impact of invasive species could be a deleterious effect with the consequence of introgressive hybridization (Holsbeek, et al. 2008; Aboim, et al.2010; Meraner, et al. 2013). In most of the cases hybridization was due to human activities: when human involvement promoted hybridization, 55% involved husbandry or agriculture, 54% involved invasive species, and 36% involved habitat disturbance and extinction risk was more common in hybridizing vertebrates (69%) (Todesco, et al, 2016).

Moreover, biodiversity level can decrease as a result of disappearance of original endemic species and hybridization that in most of the case inside freshwater fishes beyond the first generation (Gandolfi et al., 2017). The major consequence of introgression for genus *Barbus* is polyploidization as revealed by the evolution history

of this taxon (Meraner et al. 2013; Tsigenopoulos et al. 2002; Chenuil et al. 2004; Lajbner et al. 2009).

Barbus barbus is quickly expanding its range in several Italian waters (Zerunian, 2002; Meraner, et al., 2013; Buonerba, et al, 2015), and widespread anthropogenic hybridization between the invader and the endemic Padanian barbel *B. plebejus* has been hypothesized (Zerunian 2002).

In this work, 191 barbels from 15 SCIs previously selected by means of mtDNA analysis, besides morpho-phenotipic characterization, were studied (see chapter I). Particular attention was focus on cryptic hybridization. For this reason, ten microsatellite nuclear loci were analyzed to identify potentially hybrid individuals. The resulting data would have been a useful tool, setting a management panel for future conservation aquaculture activities. Barbels dataset was chosen inside a small geographical area because freshwater fishes conservation's plans are really complex and depend on different local factors, such as administrations, pollution, human activities and the presence of allochthonous species.

For the first time microsatellite molecular markers were used to identify hybrid species between *B. barbus*, *B. plebejus* and *B. caninus* inside Protected Areas. The sampling area was located inside three different provinces of Emilia Romagna: Reggio Emilia, Parma and Piacenza.

Main objectives for this work were:

1. To identify the population structure of barbel species inside Protected areas and finding hybrid specimen, if any, between *B. barbus*, *B. plebejus* and *B. caninus*
2. Describing hybrid zone and set a conservation plan for the reintroduction and reinforcement of local populations in light of genetic results.
3. Evaluating the presence of possible correlation between molecular data and environmental ones.

5.2 Materials and methods

Sampling collection

In this study were collected 300 individuals by electro-fishing between August 2014 and December 2015 inside 15 SCIs of emilian tributaries of Po river and one sampling site outside the protected areas because others structured populations of canine barbel inside PAs (Tab.1) were not found. Samples were distributed in particular among seven Po river tributaries: Trebbia, Nure, Arda, Taro, Parma, Enza and Stirone. Tissue samples were taken from adults and subadults (SL > 250 mm). This was achieved during electro-fishing by accumulating samples from longer river stretches following Cowx and Lamarque P. (1990). Tissue samples for genetic analyses consisted of small anal fin clips preserved in absolute ethanol. All fish were released unharmed at the sampling site.

Molecular data:

High molecular weight genomic DNA was extracted and purified using the Wizard genomic DNA Purification kit (Promega). DNA quality was visually inspected by 1 % agarose gel electrophoresis in TAE buffer and by spectrophotometry at 260–280 nm. The extraction procedure typically yielded not <40 ng/ml of HMW (High Molecular Weight) DNA.

Ten microsatellite loci were selected from the literature (Tab. 2) as the potentially most informative based on their polymorphic content, repeat motif, annealing temperature (Ta), number of populations and individuals analyzed, sampling sites location, allelic range (bp), number of alleles and null-allele frequency (Table 2). The scarcity of Barbel studies were limited by the tetraploid nature of barbel's genome and interpretation of alleles profiles (Falush, et al, 2007) (Fig.1). The microsatellite markers were amplified for 191 specimens previously genotyped by mtDNA (see chapter I). A reaction volume of 10µl containing 1 U di GoTaq (Promega), Mg^{2+} 2.5 mM, dNTPs 0.2 mM and 10 pmol/µl of each primer was used.

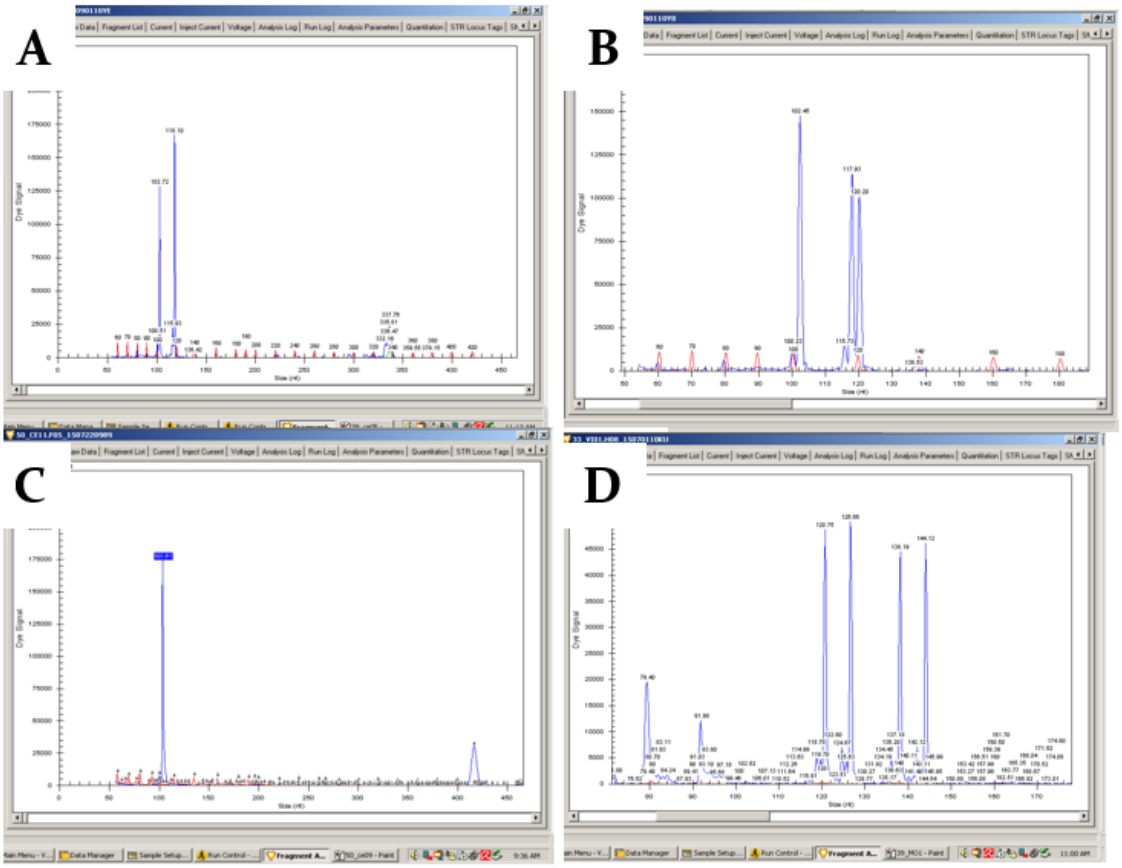

Fig.1: capillary electrophoresis output from CEQTM 8000 DNA Analysis System" (Beckman Coulter) showed four different alleles samples profiles: tow alleles (A), three alleles (B), one allel (C) and four different alleles (D).

The PCR profile was set as follows: 34 cycles of 35 s at 94 °C, a specific Ta for each locus (see Table 2) for 35 s, and 40 s at 72 °C, after an initial 10 min denaturation step at 95°C and a final extension at 72 °C for 10 min. PCR products were purified by Wizard Gel and PCR Clean Up System (Promega), FW and RV fragments sequencing was performed by CEQTM DTCS-Quick Start Kit (Beckman Coulter) and analyzed on "CEQTM 8000 DNA Analysis System" (Beckman Coulter). The entire dataset has been analyzed by Genographer (Vers.1.6.0, Benham J.J., Montana State University 2001). This software allowed the construction of a virtual gel with bands shaped on the basis of peak height, resolution and mobility and a thorough analysis of single fragments. Because of the tetraploid nature of barbels' genome, all microsatellite fragments were scored binarily as 1 or 0 following the "band-based" approach. In

Genetix v. 4.05 (Belkhir et al., 2004) was performed a Factorial Correspondence Analysis (FCA) on the multilocus genotypes dataset to visualize the distribution of genetic variation among individuals.

In addition, a MDS (Multi-Dimensional Scale) was performed using R software (package vegan) toward Jaccard distance that is useful for polyploid dataset (Kosman and Leonard, 2005).

A Bayesian clustering analysis according to a hierarchical approach was applied with the software Structure v. 2.3.3 (Pritchard et al., 2000). Structure is hence based on a Bayesian approach and uses a Monte-Carlo Markov Chain (MCMC) algorithm to assign individuals to K genetic clusters. The uppermost level of genetic structure defining the number of genetic groups (K) most likely representing the entire dataset was initially investigated: ten replicates (10,000 burn-in and 100,000 Markov chain steps; admixture model with independent allele frequencies) of each simulation were performed for K values from 1 to 10. Furthermore, the likelihood ratios associated with each K of Structure's output was compared with the software Structure Harvester v. 0.6 (Earl & vonHoldt, 2012) used to determine the uppermost level of population structure.

5.3 Results

Factorial correspondence analysis (FCA) showed three different population's clusters: *B. barbus*, *B. caninus* and *B. plebejus* explained along the first factor axis (total inertia explained: 6.05%) and with the second axis (total inertia: 4.75%). Multi-Dimensional Scaling (MDS) result present three different clusters (firs axes explained 15.81% and 8.93 for the second axes) allowed to three different barbel species inside PAs. Many individuals showed intermediate positions between the main clusters along both axes 1 and 2 of FCA and MDS analysis.

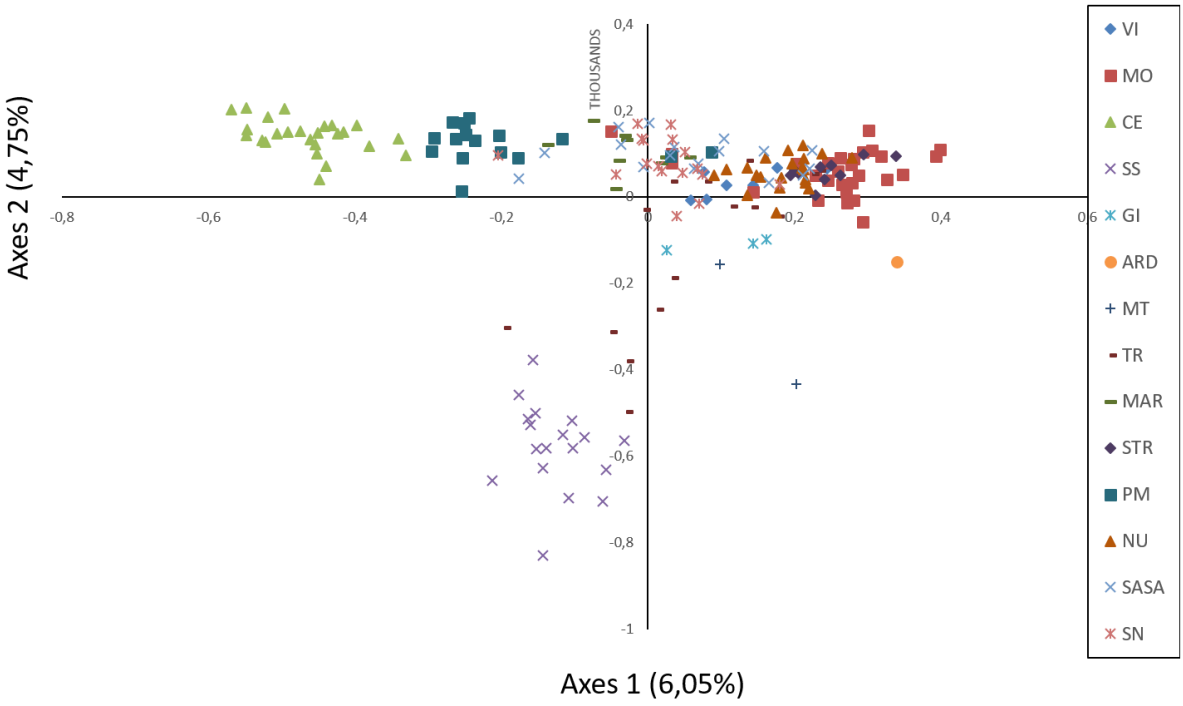

Fig.2: Factorial Correspondence Analysis (FCA) implemented in Genetix 4.05. Scatterplot of factor 1 (F1) and 2 (F2). Samples from each sampling site are labeled with different combination of symbols and colors as indicated by the legend. The population codes are: VI (Rio Vico), MO (Mora), CE (Rio Cerezzola), SS (San Secondo), GI (Giarola), ARD (Arda river), MT (Montecchio), TR (Taro river), MAR (Marra), STR(Stirone), PM (Parmossa), NU (Nure), SASA (San Salvatore), SN (Pian Casale Gerbini).

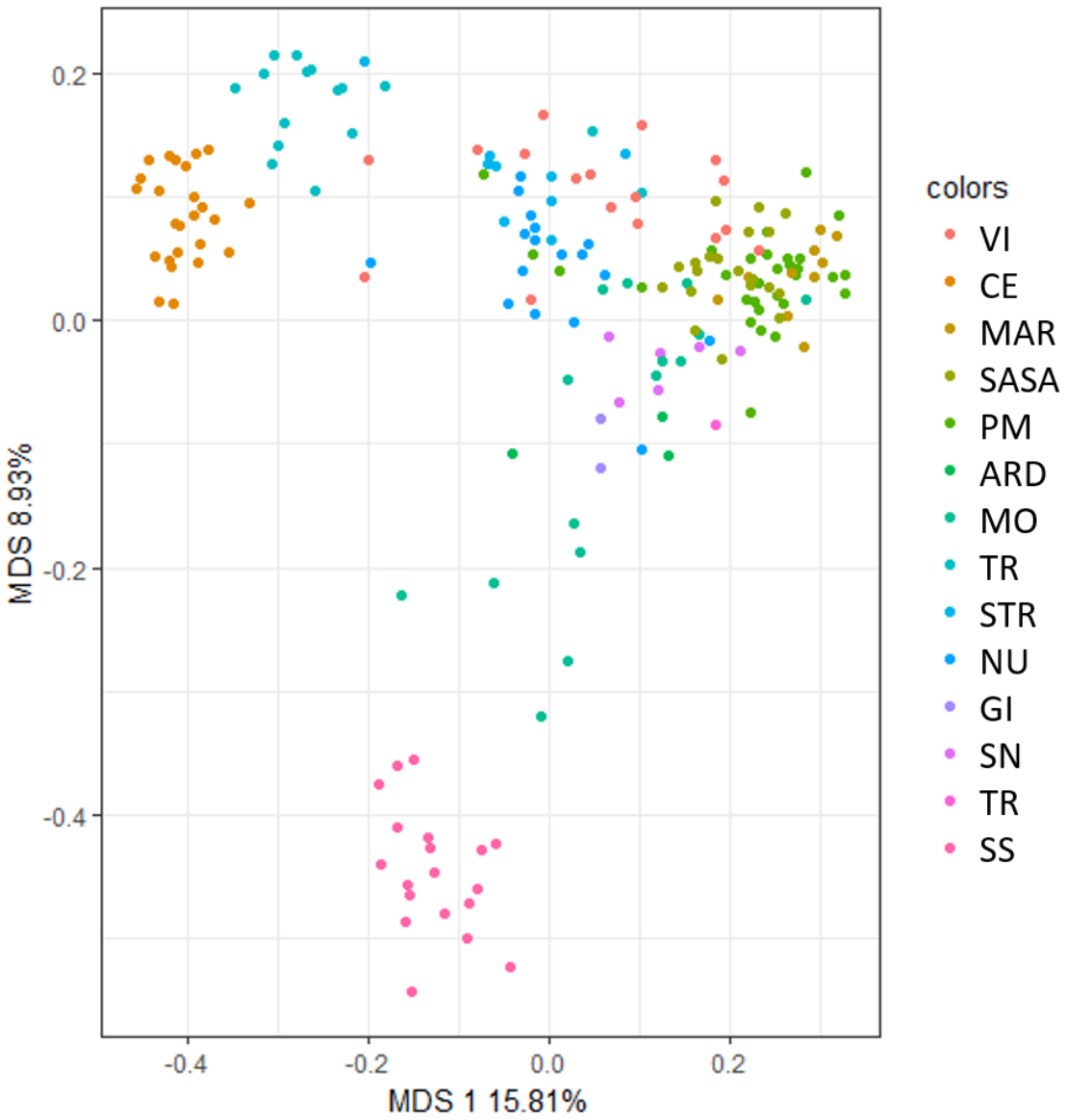

Fig.3: MDS plot analysis. The population's codes are: VI (Rio Vico), MO (Mora), CE (Rio Cerezzola), SS (San Secondo), GI (Giarola), ARD (Arda river), MT (Montecchio), TR (Taro river), MAR (Marra), STR(Stirone), PM (Parmossa), NU (Nure), SASA (San Salvatore), SN (Pian Casale Gerbini).

FCA and MDS analysis results were in accordance with Structure analysis that suggested a cluster with K = 3 based on the DK method (Evanno et al., 2005) which is the most likely solution to represent population structuring of the dataset (Fig. 4). The three clusters clearly correspond to the three species: *B.caninus*, *B. plebejus* and *B. barbus*.

The distribution of barbel species among PAs suggested an altitude pattern starting from lowest part of water stream where 'pure' population of *B. barbus* (SS, San Secondo) were found, and hybrid population between *B. barbus* X *B. plebejus* as showed in the populations of GI (Giarola), ARD (Arda river), MT (Montecchio), TR (Ponte Palladini), STR(Stirone), SASA (San Salvatore), SN (Pian Casale Gerbini). Similarly, the other populations were affected by introgression between *B. plebejus* and *B. caninus* as VI (Rio Vico), MO (Mora), MAR (Marra), PM (Parmossa), SASA (San Salvatore), SN (Pian Casale Gerbini). Only NU (Nure) for *B. plebejus* and CE (Rio Cerezzola) for *B. caninus* were found 'pure' without hybrid individuals.

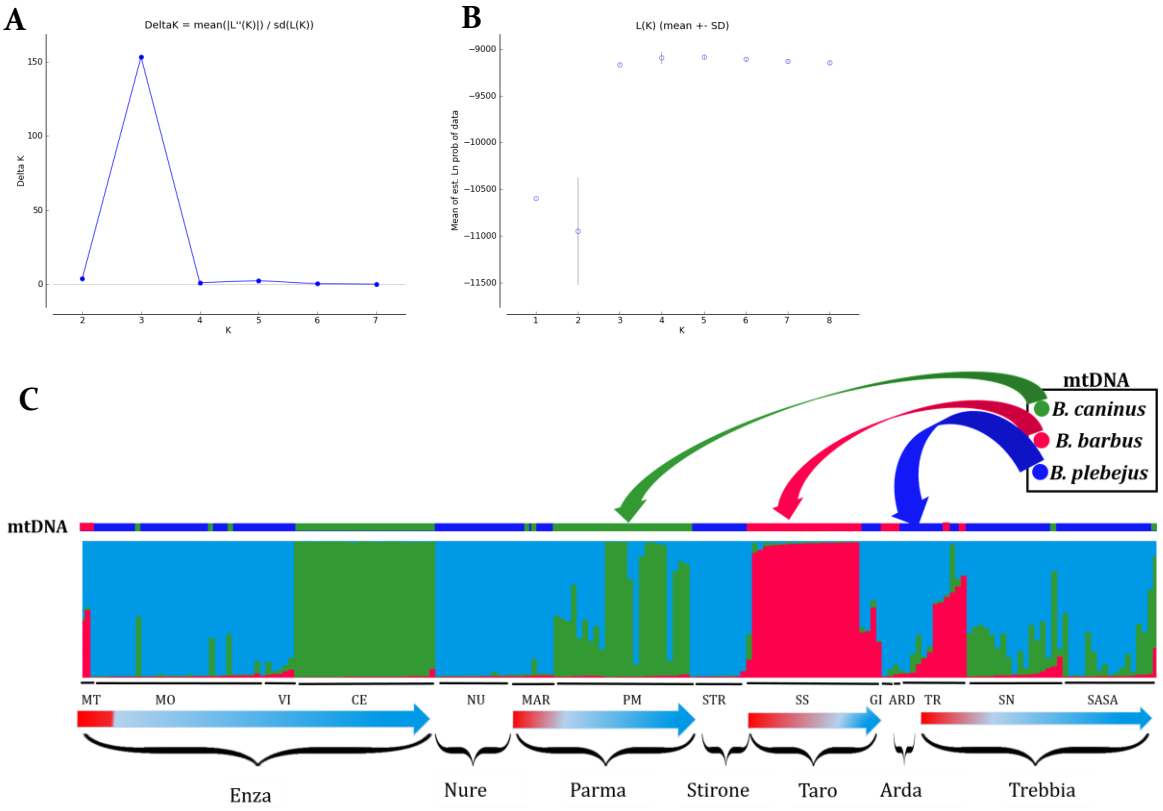

Fig.4: STRUCTURE plot analysis (C) of the most probably K (K=3) by Evanno et al., 2005 (A, B). The line aligned on top of the Structure plot show the individual mitochondrial results according to the separation of the three main clusters: haplotypes included haplogroups of *B. plebejus* (blue), haplogroups of *B. barbus* (red) and haplogroups of *B. caninus* (green). MtDNA haplogroups membership is presented for each individual above mentioned (A). The population codes are: VI (Rio Vico), MO

(Mora), CE (Rio Cerezzola), SS (San Secondo), GI (Giarola), ARD (Arda river), MT (Montecchio), TR (Taro river), MAR (Marra), STR(Stirone), PM (Parmossa), NU (Nure), SASA (San Salvatore), SN (Pian Casale Gerbini). Arrows show altitude gradient from 30 (red) to 700 (blue) m.a.s.l.

The relation between hybrid zone and altitude gradient was estimated with the selection of hybrids with q value of STRUCTURE analysis (Tab. 6 in the appendix of chapter III). The individuals with q values ≥ 0.90 were considered individuals nuclear 'pure' and samples with q values ≤ 0.90 for one cluster were considered 'hybrids'. Genetically 'pure' individuals were 132 and 59 barbels were hybrids. Regression analysis with altitude gradient and hybrids q values revealed no statistical significance for *B. plebejus* as showed in Fig.5 and in tab.3 of appendix. High Positive statistical significance were found between *B. caninus* cluster (p value ≤ 0,05) and altitude as showed in tab. 4. For *B. barbus* an inversely correlation between hybrid forms and altitude were detected (statistical significance p≤ 0,05) as showed in Fig. 5 and in appendix (tab. 5).

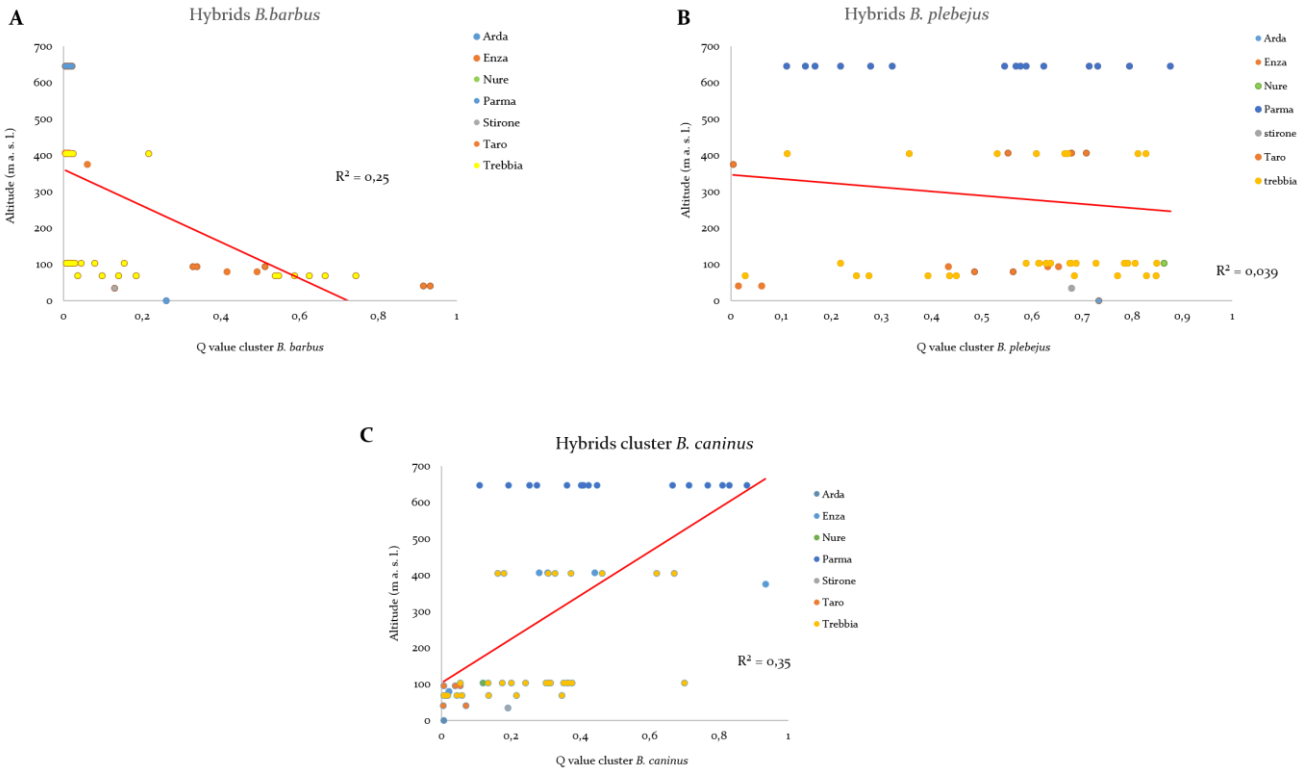

Fig. 5: the three graphics explain the relation between altitude gradient (From 20 to 700 m a.s.l.) and 59 hybrids forms previously selected by q value ≤ 0,90 of STRUCTURE analysis. Altitude gradient of *B. barbus* cluster (A); altitude gradient of *B. plebejus* cluster (B) and altitude gradient of *B. caninus* cluster (C).

5.4 Discussion and Conclusions

The distribution of native species among sampling sites and different water streams suggest an altitude gradient where the nuclear DNA of alien species was more relevant in the lowest part of rivers. *Barbus caninus* nuclear DNA increase with the altitude and these results reflect the ecological distribution of canine barbel.

Our results regarding mtDNA and nDNA could be used for breeders selection in barbel conservation programs.

Other results found that mitochondrial and nuclear introgression is not balanced, due to the predominance of *B. caninus* mtDNA alleles in hybrid specimens. In fact, in PM population's haplotypes *B. caninus* constituted the predominant "mother species" in the *B.caninus* X *B. plebejus* hybridization process. These findings could result from an asymmetrical hybridization between female canine barbel and male common barbel (see fig. 4) as showed in others species (Gandolfi, et al. 2017; Soubrier, et al. 2016). In fact, mean size of sexually mature males is expected to be higher in *B. plebejus* than in *B. caninus*. This difference could generate an important spawning advantage for *B. plebejus* males in situations where both species coexist in sympatry as the both species compete for *B. caninus* females. Hybridization asymmetry was observed in 7 individuals from MAR, SN, SASA and MO populations.

Breeders selection index

A schedule for fish selection for breed activities For future restocking and spawning plans was proposed (Tab.1). Thanks to hierarchical approach, barbels will be selected at first with the use of mtDNA haplotypes while *B. barbus* haplotypes will be discharged. In a second step, thanks to the analysis of microsatellite nuclear markers, pure individual with q value of STRUCTURE ≥ 0,9 will be selected. This table could be

useful for future breeder's selections even for other species. The total number of 'pure' individuals: 33 for canine and 77 for common barbel.

Tab.1: selection of pure individuals for spawning activities considering STRUCTURE Q value ≥ 0,9.

mtDNA	STRUCTURE Q VALUE	VALUE	RESULTS
B. caninus	0-0,10	Different species	114
	0,10-75	High Hybrid	38
	0,75-0,90	Low hybrids	6
	0,90-1	Pure	33
B. plebejus	0-0,10	Different species	55
	0,10-75	High hybrid	43
	0,75-0,90	Low hybrids	16
	0,90-1	Pure	77

All results revealed complex scenarios of introgression and hybridization in the majority of sampling areas of PAs. MtDNA and nDNA have described a dramatic situation of genetic introgression and hybridization of these species; this work also revealed the importance of intense and detailed sampling activities in order to know the real status of natural population in the rivers. In Italy a wide sampling and monitoring activity is necessary to better define native population of freshwater and inland species. Restocking of native population should start from these results, which explain the population structure of different population inside PAs. As showed by Maiorano et al., (2007) Natura 2000 sites were relevant for the preservation of vertebrate species but studies about PAs and their monitoring were not enough. As a consequence of these aspects, conservations plans, such as LIFE programs, determine strong and practical tools for preservation of inland ecosystems. According to LIFE programs and the 17 goals of Agenda 2030 of United Nations, preservation, monitoring and conservation plans are the priority to preserve biodiversity.

6 FINAL DISCUSSION

Our results confirmed a decline of native barbel populations in the study area compared with literature data (Nonnis Marzano et al., 2003; Fish Charts Provinces of Parma, Reggio Emilia, and Piacenza). Barbel populations were historically present in several streams of the three provinces, both in the mid-Apennine and hillsides (Nonnis Marzano et al., 2003). However, a well-structured population of the canine barbel was retrieved only in two sampling stations, highlighting a local high degree of rarity for this species. Additionally, in agreement with our initial hypotheses, the present data confirmed the general existence of a clear zonation pattern among the barbel species analyzed, although a frequent overlapping of populations limited the statistical significance of Principal Component Analysis. A non-negligible altitude segregation between native vs. alien species was detected, with the exotic invasive *B. barbus* mainly limited to lowland watercourses. We also detected invasive *B. barbus* DNA in the native populations of the hill areas as a result of genetic introgression. These observations corroborate recent evidence on the rapid expansion of the European barbel in the Po basin (Meraner et al., 2013), and indicate a higher level of vulnerability for the autochthonous fish populations placed at lower altitudes, where the likely presence of alien barbels is wider. It is noteworthy observing that migration of *B. barbus* mitochondrial haplotypes reaching hill and mid-Apennines catchments could be referred to water heating due to global warming and water deprivation.

More specifically, the species spatial distribution mirrored the anthropogenic disturbance gradients. In fact, all the investigated sites are arranged along an altitudinal gradient stretching from the Tuscan-Emilian Apennine (up to 600 m a.s.l.) to the Po River (~25 m a.s.l.). This gradient distribution overlaps with human pressures, which significantly decrease with altitude increase. Hence, a progressive degradation of all monitored physical, chemical and morphological features was detected with the decreasing of elevation. The strong overlap between physical and chemical data reinforces the existence of an altitudinal zonation in term of barbel species representativeness among sites. With decreasing altitude, the river

functionality (e.g., IFF) varied from good to poor/bad, and BOD5 from 0.0 to 20.8 mg L-1.

The progressive disappearance of well-structured vegetated buffer, the increase of the riverbed incision – that actually encourages the progressive isolation of the watercourse from the local context surrounding – and the intense land use change, are the main reasons of the observed zonation. Consequently, the present results obtained through a multidisciplinary approach support the idea that the native and threatened priority barbel species were found preferentially in moderate to well-preserved watercourses, characterized by more expanded riparian areas and appropriate hydro-morphological characteristics in terms of greater IFF values. Similar results were modelled for two Minnesota watersheds, where substantial changes in agricultural management, including an expansion of the riparian areas, would be expected to significantly improve local brook trout [*Salvelinus fontinalis* (Mitchill, 1814)] populations, by increasing streams shading up to 50% (Blann et al., 2002). Hence, in lowlands and agricultural settings the destruction of riparian communities leads to a rapid physical and chemical deterioration of watercourses with dramatic effects on fish populations, as highlighted by Lorenzoni, et al. (2006) for rivers in Central Italy.

Ecological and genetic factors revealed a strong effect linked to the hybridization and the decrement of native species, especially for *B. caninus*. Results showed complex scenarios, where the presence of different congeneric species, which live in the same habitat, allowed high hybridization and introgression. Moreover, an asymmetrical hybridization between female canine barbel and male common barbel was explained as a consequence of habitat overlap. The ambiguity of hybridization issue is the central aspect of freshwater fishes evolution (Todesco, et al. 2016; Berrebi et al. 2014).

MtDNA results revealed the presence of allochthonous species inside PAs and the decrement of canine barbel distribution. In the past, as revealed by historical data, in Emilia Romagna the habitat of *B. caninus* was extended to almost all high water streams of Po Basin (Bianco & Delmastro, 2004) but now inside western Emilia Romagna in just only one sampling site of PAs a 'pure' genetically canine population was detected. As shown in the three previous chapters, an important correlation

between ecological index and barbel species presence was evidenced. In chapters 2 and 3, that migration of *B. barbus* was observed thanks to mitochondrial and nuclear data; allochthones species reached hill and mid-Apennines as a consequence of global warming and water deprivation. Moreover, the consequence of global warming and temperature increment revealed an intense introgression phenomenon and an increase of hybrid zones across different areas of the same water stream.

The tetraploid genomes of genus *Barbus*, as written by Berrebi et al., (2014), may open many different questions regarding the phylogenetic history of barbel species. Barbel speciation events of different taxa have revealed many hybridization events. In any case anthropic activities generating populations fragmentation, loss of habitat, climate change, modified the historical distribution of inland fishes, and genetic aspects reflect these consequences (Berrebi, et al. 2014). A major attention should be driven to management of hybrid zones where native species habitats are overlapped. Management Units (MUs) and Evolutionary Significant Units (ESUs) are very useful conservation priorities (De Salle and Amato, 2004). Considering ESUs as populations of organisms that are reproductively isolated from other populations of the same species (Moritz 1994; De Salle and Amato, 2004), introgressed barbels inside hybrid zones could be identified as ESUs. On the light of the complex evolutionary history of genus *Barbus*, natural hybridization was one of the major force of *Barbus* speciation. Barbels inside hybrid zones could be managed separately from 'pure' individuals and therefore considered as different Management Units in the absence of further genomic researches regarding this genus.

7 CONCLUSIONS AND FUTURE PROSPECT

The overarching aim of this research thesis was the investigation of genetics of barbel species and the study of the ecological and environmental status inside PAs. Results of this PhD thesis could be useful to define conservation plans inside European LIFE13 NAT/IT/001129 BARBIE, these plans could include future restocking activities and water resource preservation. The issue of freshwater fishes' protection should be included inside priority areas for conservation and preservation of ecosystems and should be one of the objectives of governments and public administrations.

This research highlighted the introgression between different barbel species, and described different hybrid zones inside 15 PAs inside Nature 2000 sites.

Major results of this work were:

- Phylogenetic relationship determined by mtDNA analysis, explained the distribution of species and their relationships between water stream rivers.
- Environmental and ecological indices revealed a strict correlation between environmental data and barbels distribution.
- Mitochondrial and nuclear markers evidenced an altitude gradient distribution of barbel species.
- Selection method of 'pure' fish spawners was set up using mitochondrial and nuclear DNA information. Molecular results could be useful for future restocking and spawning activities.

8 PAPERS PUBLISHED, SUBMITTED OR IN PREPARATION

1. Piccoli F. , Burgazzi G., laini A., **Ferrari C**., Voccia A., Filonzi L., Bolpagni R., Nonnis Marzano F. 2017. Barbel species arrangement in a regional Natura 2000 network (Emilia Romagna, Northern Italy): an altitudinal perspective. ***Journal of Lymnology***. 10.4081/jlimnol.2017.1693.

2. Gandolfi A., **Ferrari C.**, Crestanello C., Girardi M., Lucentini L., Meraner A. 2017. Population genetics of pike, genus *Esox* (Actinopterygii, Esocidae), in Northern Italy: evidence for mosaic distribution of native, exotic and introgressed populations. ***Hydrobiologia***. 794(1): 73–92.

3. Chiesa S., Laura Filonzi L., **Ferrari C**., Vaghi M., Bilò F., Piccinini A., Zuccon G., Wilson R.C., Jørn Ulheim J., Nonnis Marzano F. 2016. Combinations of distinct molecular markers allow to genetically characterize marble trout (*Salmo marmoratus*) breeders and stocks suitable for reintroduction plans. ***Fisheries Research***. 176: 55–64.

4. Chiesa S., Filonzi L., **Ferrari C**., Nonnis Marzano F. 2016. Molecular genetics confirms the existence of different management units belonging to a single phylogenetic lineage within italian populations of *Alosa fallax* (Lacepede, 1803***). Journal of Fisheries Sciences.com***. 10: 14-17.

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E.O. Wilson

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11 APPENDICES

11.1 Appendix Chapter I

Tab. 1. Barbel population size and ISECI results; for each sampled site, the indication of the watercourses (WC) and the Natura 2000 (N2000 site code) site it belongs, the number of individuals (N_ind) and their % distribution value (in brackets) of Canine (*B. caninus*), Common (*B. plebejus*), and alien European (B. *barbus*) Barbel, the values and quality class of ISECI were reported. In light grey the sites without barbels.

Site	**WC**	**code**	**N2000 site**	**N_ind**			**ISECI**	
				Canine	***Common***	***European***	***value***	***quality class***
SASA	Trebbia	MSS	IT4010006	1 (7.5%)	15 (92.5%)	0	0.61	Good
ARD	Arda	CL	IT4010008	0	23 (95.8%)	1 (4.2%)	0.61	Good
SN	Trebbia	TPB	IT4010011	2 (12.5%)	14 (87.5%)	0	0.64	Good
TR	Trebbia	BTr	IT4010016	0	15 (75.0%)	5 (25.0%)	0.72	Good
TR2	Trebbia	BTr	IT4010016	0	0	0	0.69	Good
NU	Nure	CN	IT4010017	0	29 (100.0%)	0	0.72	Good
STR	Stirone	TS	IT4020003	0	13 (100.0%)	0	0.71	Good
LO	Lorno	RVBT	IT4020017	0	0	0	0.66	Good
MAR	Parma		-	0	3 (100.0%)	0	0.40	Poor
mar-02	Parma		-	1 (11.1%)	8 (88.9%)	0	0.52	Moderate
Lagoni	Parma	CAP	IT4020020	0	0	0	0.66	Good
PM	Parmossa		-	25 (89.3%)	3 (10.7%)	0	0.66	Good
FA	Fabiola		-	0	0	0	0.66	Good
MON	Moneglia		-	0	0	0	0.63	Good
BA	Baganza		-	1 (20.0%)	4 (80.0%)	0	0,49	Moderate

GI	Taro	MT	IT4020021	0	5 (83.3%)	1 (16.7%)	0.63	Good
GI	Naviglio	MT	IT4020021	0	8 (80.0%)	2 (20.0%)	0.52	Moderate
CN	Ceno	MT	IT4020021	0	8 (88.9%)	1 (11.1%)	0.68	Good
SS	Taro	BTa	IT4020022	0	0	19 (100.0%)	0.30	Mediocre
DD	Parma morta	PM	IT4020025	0	0	0	0.20	Bad
CRN	Enza	CV	IT4020027	0	7 (100.0%)	0	0.72	Good
MO	Enza	FE	IT4030013	0	25 (100.0%)	0	0.55	Moderate
CED	Cedra		-	0	2 (100.0%)	0	0.42	Moderate
LU	Cedra		-	0	0	0	0.20	Bad
VI	Rio Vico	RC	IT4030014	0	6 (100.0%)	0	0.50	Moderate
CE	Rio Cerezzola	RC	IT4030014	1	0	0	0.67	Good
CE	Rio Cerezzola	RC	IT4030014	24 (100.0%)	0	0	0.59	Moderate
MT1	Enza	FG	IT4030023	0	21 (91.3%)	2 (8.7%)	0.60	Moderate
MT2	Enza	FG	IT4030023	0	5 (100.0%)	0	0.53	Moderate
SI1	Enza	FG	IT4030023	0	5 (100.0%)	0	0.64	Good
SI2	Enza	FG	IT4030023	0	0	0	0.63	Good

Tab. 1a: PhySCIal and chemical features of sampled sites. For each sampled site we reported: the indication of the watercourses (code) and the Natura 2000 site it belongs (site); altitude (Alt; m asl); IFF value (IFF); water temperature (Temp, °C); pH; oxygen dissolved percentage (DO%); conductivity at 25°C (Cond, µS cm-1); biochemical oxygen demand rate (BOD5, mg L-1); total suspended solids (TSS, mg L-1); nitrite (NO2 - , mg L-1), nitrate (NO3 - , mg L-1), and ammonia (NH4 + , mg L-1).

Code	Site	Alt	IFF	Temp	pH	DO%	Cond	BOD5	TSS	NO_2^-	NO_3^-	NH_4^+
SASA	10006	404	245	21.4	7.92	97.0	253	1.8	1.3	0.01	0.99	0.06
ARD	10008	202	158	21.0	7.55	98.5	407	2.9	2.5	0.01	0.94	0.06
TR	10011	68	215	21.9	8.01	109.0	320	0.0	1.7	0.01	0.29	0.03
SN	10016	103	225	25.5	7.86	109.5	337	4.5	1.3	0.01	0.31	0.02
NU	10017	166	180	25.1	7.85	112.5	356	6.1	3.3	0.01	0.35	0.04
STR	20003	34	103	22.7	7.64	87.5	492	4.6	2.8	0.06	0.73	0.04
NU	20017	646	181	14.1	7.62	72.0	495	2.0	6.7	0.02	2.12	0.23
LU	20017	36	158	14.2	7.47	44.5	521	0.9	3.4	0.02	1.50	0.45
MAR	20020	646	210	12.7	7.90	94.5	197	0.8	0.2	0.00	0.32	0.05
GI	20021	94	195	19.9	8.08	113.5	355	4.3	0.6	0.01	0.27	0.10
SS	20022	40	105	20.3	8.40	198.0	499	8.8	14.2	0.01	0.30	0.61
DD	20025	30	102	21.3	7.96	112.0	632	19.1	49.4	0.00	0.14	0.16
MO	30013	406	213	12.2	7.98	99.5	288	7.5	3.0	0.01	0.19	0.08
VI	30014	202	158	21.0	8.18	99.0	507	9.0	24.8	0.11	2.87	0.21
CE	30014	375	165	18.1	8.09	97.0	597	7.0	33.8	0.01	0.61	0.28
MT	30023	79	158	18.2	7.95	83.5	477	164.2	6.5	0.01	0.54	0.15
SI	30023	45	102	16.1	7.20	51.5	822	1.4	20.0	0.03	5.62	0.14

11.2 Appendix Chapter II

Table1. Description of sampling sites inside and outside SCI (Site of Community Importance), SITE CODE= sampling site code; Province (PR=Parma, RE= Reggio Emilia, PC= Piacenza).

SCI	Site code	Water Corse	River Basine	Coordinate	Province	Locality
IT4010006	SASA	Trebbia	Trebbia	44°43'25.54"N 9°23'13.30"E	PC	San Salvatore
IT4010008	ARD	Arda	Arda	44°49'32.95"N 09°50'20.25"E	PC	Lugagnano Val d'arda
IT4010017	NU	Nure	Nure	44°52'28.16"N 09°38'45.64"E	PC	Ponte dell'Olio
IT4010011	SN	Trebbia	Trebbia	44°47'15.37" N 09°24'37.30"E	PR	Pian Casale Gerbidi
IT4010016	TR	Trebbia	Trebbia	45°01'52.85"N 09°36'06.60"E	PR	Ponte Palladini
IT4020003	STR	Stirone	Stirone	44°50'42.67"N 09°59'07.44"E	PR	Parco dello Stirone
IT4020017	VR	Lorno	Parma	44°53'12.5"N 10°16'58.6"E	PR	Tre Casali
IT4020020	MAR	Parma	Parma	44°28'25.9"N 10°02'54"E	PR	Marra centrale
Outside	BA	Parma	Parma	44°31'03.61"N 10°12'45.32"E	PR	Pastorello
IT4020021	GI	Taro	Taro	44°44'34.80"N 10°10'18.6"E	PR	Giarola
	CN	Ceno	Taro	44°55'27.7"N 10°26'38.69"E	PR	Viazzano
IT4020022	SS	Taro	Taro	44°53'27.60"N 10°15'19.70"E	PR	San Secondo Parmense
IT4020025	DD	Parma morta	Parma	44°55'27.19"N 10°26'38.8"E	PR	Riserva Parma morta
IT4020027	CRN	Enza	Enza	44°39'52"N 10°24'19"E	PR	Scornavacca-Cronovilla
Outside	PM	Parmossa	Parma	44°31'59.70"N 10°14'13"E	PR	Agola Fornace
IT4030013	MO	Enza	Enza	44°27'31" N 10° 16'01"E	RE	La Mora
IT4030014	VI	Rio Vico	Enza	44°35'56" N 10° 25' 03" E	RE	Canossa
	CE	Rio Cerezzola	Enza	44°34'48" N 10° 24' 85" E	RE	Rupe di Campotrera
IT4030023	MT	Enza	Enza	44°41'58.5"N 10°25'57.14"E	RE	Montecchio
	SI	Enza	Enza	44°44'55.75"N 10°25'41.70"E	RE	Sant'Ilario

Table 2: GenBank accession number (AN) of haplotypes used in this work. In bolt were showed new haplotypes.

Name Haplotipe	**AN**
Barbus caninus	AF112124
Bcaninus1	AF287424
PK321	AY331018
PK461	AY331020
PK451	AY331021
PK705	AY331024
Bp01	KC465918
Bp02	KC465919
Bp03	KC465920
Bp04	KC465921
Bp05	KC465922
Bp22	KC465922
Bp08	KC465925
Bp09	KC465926
Bp10	KC465927
Bp04	KC465931
Bp15	KC465942
Bp16	KC465943
Bp17	KC465944
Bp18	KC465945
Bp21	KC465948
Bp22	KC465949
PLE1	KF923539
PLE3	KF923541
Bp23	**kX897129**
Bp24	**kX897130**
Bp25	**kX897131**
Bp26	**kX897132**
Bp27	**kX897133**
Bcaninus3	**kX897134**
Bcaninus4	**kX897135**
Bcaninus5	**kX897136**
Bcaninus6	**kX897137**
Bcaninus7	**kX897138**
Bcaninus8	**kX897139**
Luciobarbus	JN049525

11.3 Appendix Chapter III

Tab. 1 : description of sampling sites inside and outside SCIs, , SITE CODE= sampling site code; Province (PR=Parma, RE= Reggio Emilia, PC= Piacenza).

SCI	SITE CODE	WATER CORSE	COORDINATE	PROVINCE	LOCALITY
IT4010006	SASA	Trebbia	44°43'25.54''N 9°23'13.30''E	PC	San Salvatore
IT4010008	ARD	Arda	44°49'32.95''N 09°50'20.25''E	PC	Lugagnano Val d'arda
IT4010017	NU	Nure	44°52'28.16''N 09°38'45.64''E	PC	Ponte dell'Olio
IT4010011	SN	Trebbia	44°47'15.37''N 09°24'37.30''E	PR	Pian Casale Gerbidi
IT4010016	TR	Trebbia	45°01'52.85''N 09°36'06.60''E	PR	Ponte Palladini
IT4020003	STR	Stirone	44°50'42.67''N 09°59'07.44''E	PR	Parco dello Stirone
IT4020020	MAR	Parma	44°28'25.9''N 10°02'54''E	PR	Marra centrale
IT4020021	GI	Taro	44°44'34.80''N 10°10'18.6''E	PR	Giarola
IT4020022	SS	Taro	44°53'27.60''N 10°15'19.70''E	PR	San Secondo Parmense
IT4020025	DD	Parma morta	44°55'27.19''N 10°26'38.8''E	PR	Riserva Parma morta
Outside	PM	Parmossa	44°31'59.70"N 10°14'13"E	PR	Agola Fornace
IT4030013	MO	Enza	44°27'31''N 10° 16'01''E	RE	La Mora
IT4030014	VI	Rio Vico	44°35'56''N 10° 25' 03'' E	RE	Canossa
IT4030014	CE	Rio Cerezzola	44°34'48''N 10° 24' 85'' E	RE	Rupe di Campotrera
IT4030023	MT	Enza	44°41'58.5''N 10°25'57.14''E	RE	Montecchio

Tab. 2.: locus, primer and original references of microsatellites used in this work. Annealing temperature (Ta) were modified in this work.

Locus	Primer	Reference	Ta(°C)
Barbus33	F: TGAATGCATCATGGGCTAGA R: CAGAGCGAATCAAACATGGA	Gettová et al. (2013)	50.5
Barbus39	F: CTGCTGAGACGAGAAAGCAA R: AAAAGGTGCTGGTGTGGAAC	Gettová et al. (2013)	54.5
Barbus50	F: GTTACAGGCCAACGTCAAGG R: GTTAGTCTGCAATCCGCCAT	Gettová et al. (2013)	54.5
Barb59	F: CTGTATCCATCACATAGGCT R: CATGATTTAATAGAACACACAC	Chenuil et al. (199a7)	50.5
Barb79	F: GAGTGAATCATTACATCCCT R: GCTTTTCTTGTATTAGTATTT	Chenuil et al. (1997)	48
MFW1	GTCCAGACTGTCATCAGGAG CA R: GAGGTGTACACTGAGTCACGC	Crooijmans et al. 1997	56
Lid-2	]CCACTCCTCAGCCGACAGA R: AAATGCTGGCGGGGAAATA	Barinova et al. 2004	57.5
LC293	F:TTGCCCTCACCACACTAACA R: CACAGATGCAGATCGAGGAG	Vyskocilova et al. 2007	57.5
Lc04	F:ATCAGGTCAGGGGTGTCACG R: TGTTTATTTGGGGTCTGTGT	Turner et al. 2004	58.5
CypG24	F:CTGCCGCATCAGAGATAAACACTT R:TGGCGGTAAGGGTAGACCAC	Baerwald & May 2004	58.5

Tab.3: Regression analysis between altitude gradient and *B.plebejus* cluster

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0,197450912
R Square	0,038986863
Adjusted R Square	0,022126983
Standard Error	237,5130839
Observations	59

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	130448,4424	130448,4424	2,312404579	0,133873664
Residual	57	3215510,507	56412,46503		
Total	58	3345958,949			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	410,4360941	81,85448528	5,014216297	5,50908E-06	246,5253147	574,3468736	246,5253147	574,3468736
X Variable 1	-205,2940669	135,0033322	-1,520659258	0,133873664	-475,6335764	65,04544265	-475,6335764	65,04544265

Tab.4: Regression analysis between altitude gradient and *B.caninus* cluster

SUMMARY
OUTPUT

Regression Statistics	
Multiple R	0,591891837
R Square	0,350335947
Adjusted R Square	0,338938332
Standard Error	195,2842845
Observations	59

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	1172209,698	1172209,698	30,73765419	7,92033E-07
Residual	57	2173749,251	38135,95178		
Total	58	3345958,949			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	119,4566465	40,63292145	2,939898051	0,004735456	38,09062484	200,8226682	38,09062484	200,8226682
X Variable 1	569,5577192	102,731205	5,544154957	7,92033E-07	363,8420297	775,2734088	363,8420297	775,2734088

Tab.5: Regression analysis between altitude gradient and *B.barbus* cluster

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0,495499452
R Square	0,245519707
Adjusted R Square	0,232283211
Standard Error	210,4489807
Observations	59

ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	821498,862	821498,862	18,54869299	6,60261E-05
Residual	57	2524460,087	44288,77346		
Total	58	3345958,949			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95,0%*	*Upper 95,0%*
Intercept	370,3020196	32,47843026	11,40147528	2,47313E-16	305,2650848	435,3389544	305,2650848	435,3389544
X Variable 1	-577,4658168	134,0817361	-4,306819359	6,60261E-05	-845,9598621	-308,9717715	-845,9598621	-308,9717715

Tab 6: dataset of all samples and STRCUTURE q value of three different clusters (Cluster **B. barbus**, Cluster *B. caninus*, Cluster *B. plebejus*). Population code are: GI (Giarola), ARD (Arda river), MT (Montecchio), TR (Ponte Palladini), STR (Stirone), SASA (San Salvatore), SN (Pian Casale Gerbini).

Population code	**Stream**	**Cluster *B.barbus***	**Cluster *B.caninus***	**Cluster *B.plebejus***	**Altitude (m.a.s.l)**
MT	enza	0,4152	0,022	0,5628	79
MT	enza	0,4925	0,0217	0,4858	79
MO	enza	0,003	0,005	0,992	406
MO	enza	0,004	0,0043	0,9917	406
MO	enza	0,004	0,005	0,991	406
MO	enza	0,0047	0,0053	0,99	406
MO	enza	0,005	0,004	0,991	406
MO	enza	0,005	0,0043	0,9907	406
MO	enza	0,005	0,0047	0,9903	406
MO	enza	0,005	0,006	0,989	406
MO	enza	0,005	0,4423	0,5527	406
MO	enza	0,0053	0,0043	0,9903	406
MO	enza	0,006	0,004	0,99	406
MO	enza	0,006	0,0053	0,9887	406
MO	enza	0,0063	0,0183	0,9753	406

MO	enza	0,0067	0,0053	0,988	406
MO	enza	0,0067	0,0103	0,983	406
MO	enza	0,0077	0,004	0,9883	406
MO	enza	0,0077	0,006	0,9863	406
MO	enza	0,0077	0,0137	0,9787	406
MO	enza	0,0087	0,0073	0,984	406
MO	enza	0,009	0,0047	0,9863	406
MO	enza	0,0093	0,0067	0,984	406
MO	enza	0,0093	0,2822	0,7084	406
MO	enza	0,0103	0,006	0,9837	406
MO	enza	0,0113	0,0057	0,983	406
MO	enza	0,014	0,3068	0,6792	406
MO	enza	0,015	0,0073	0,9777	406
MO	enza	0,0163	0,0053	0,9783	406
MO	enza	0,017	0,0047	0,9783	406
MO	enza	0,0173	0,004	0,9787	406
MO	enza	0,0283	0,0933	0,8783	406
VI	enza	0,0113	0,0093	0,9793	202
VI	enza	0,0197	0,095	0,8853	202
VI	enza	0,025	0,019	0,956	202
VI	enza	0,0267	0,0307	0,9427	202
VI	enza	0,047	0,043	0,91	202
VI	enza	0,057	0,086	0,857	202
CE	enza	0,003	0,9933	0,0037	375
CE	enza	0,0037	0,9903	0,006	375
CE	enza	0,0037	0,9927	0,0037	375
CE	enza	0,004	0,988	0,008	375
CE	enza	0,004	0,992	0,004	375
CE	enza	0,004	0,992	0,004	375
CE	enza	0,004	0,9923	0,0037	375
CE	enza	0,004	0,993	0,003	375
CE	enza	0,0043	0,992	0,0037	375
CE	enza	0,0047	0,9913	0,004	375
CE	enza	0,005	0,982	0,013	375
CE	enza	0,005	0,9903	0,0047	375
CE	enza	0,0053	0,99	0,0047	375
CE	enza	0,0053	0,9907	0,004	375
CE	enza	0,0057	0,9883	0,006	375
CE	enza	0,006	0,9873	0,0067	375
CE	enza	0,006	0,989	0,005	375
CE	enza	0,0063	0,989	0,0047	375
CE	enza	0,0067	0,9883	0,005	375
CE	enza	0,0073	0,9813	0,0113	375

CE	enza	0,008	0,9847	0,0073	375
CE	enza	0,0083	0,9857	0,006	375
CE	enza	0,0087	0,9833	0,008	375
CE	enza	0,009	0,985	0,006	375
CE	enza	0,0607	0,9346	0,0047	375
NU	nure	0,0043	0,006	0,9897	103
NU	nure	0,0053	0,0087	0,986	103
NU	nure	0,0057	0,0063	0,988	103
NU	nure	0,006	0,006	0,988	103
NU	nure	0,006	0,007	0,987	103
NU	nure	0,006	0,008	0,986	103
NU	nure	0,0063	0,0097	0,984	103
NU	nure	0,007	0,0057	0,9873	103
NU	nure	0,008	0,005	0,987	103
NU	nure	0,0083	0,0063	0,9853	103
NU	nure	0,0083	0,034	0,9577	103
NU	nure	0,0087	0,006	0,9853	103
NU	nure	0,0093	0,009	0,9817	103
NU	nure	0,0097	0,0137	0,9767	103
NU	nure	0,0103	0,0083	0,9813	103
NU	nure	0,011	0,0153	0,9737	103
NU	nure	0,0113	0,0117	0,977	103
NU	nure	0,0157	0,12	0,8643	103
NU	nure	0,016	0,0133	0,9707	103
NU	nure	0,0163	0,013	0,9707	103
NU	nure	0,0173	0,0063	0,9763	103
MAR	parma	0,005	0,4487	0,5463	646
MAR	parma	0,0067	0,4249	0,5685	646
MAR	parma	0,0077	0,4033	0,5891	646
MAR	parma	0,0107	0,667	0,3223	646
MAR	parma	0,011	0,275	0,714	646
MAR	parma	0,0127	0,4093	0,578	646
MAR	parma	0,014	0,2542	0,7318	646
MAR	parma	0,014	0,362	0,624	646
MAR	parma	0,0107	0,1939	0,7954	646
PM	parma	0,0043	0,9893	0,0063	646
PM	parma	0,0043	0,9903	0,0053	646
PM	parma	0,005	0,9877	0,0073	646
PM	parma	0,0053	0,9877	0,007	646
PM	parma	0,0063	0,7146	0,2791	646
PM	parma	0,0073	0,086	0,9066	646
PM	parma	0,0073	0,8807	0,112	646
PM	parma	0,009	0,979	0,012	646

PM	parma	0,0093	0,979	0,0117	646
PM	parma	0,013	0,9663	0,0207	646
PM	parma	0,0133	0,9557	0,031	646
PM	parma	0,0137	0,1097	0,8767	646
PM	parma	0,0137	0,7673	0,2191	646
PM	parma	0,0203	0,8306	0,149	646
PM	parma	0,0223	0,81	0,1677	646
STR	stirone	0,0043	0,0043	0,9913	34
STR	stirone	0,0047	0,0043	0,991	34
STR	stirone	0,005	0,005	0,99	34
STR	stirone	0,005	0,0053	0,9897	34
STR	stirone	0,006	0,0057	0,9883	34
STR	stirone	0,006	0,007	0,987	34
STR	stirone	0,0063	0,0077	0,986	34
STR	stirone	0,007	0,0077	0,9853	34
STR	stirone	0,008	0,0043	0,9877	34
STR	stirone	0,0447	0,005	0,9504	34
STR	stirone	0,1297	0,191	0,6793	34
SS	taro	0,915	0,07	0,015	40
SS	taro	0,9326	0,0053	0,062	40
SS	taro	0,9587	0,03	0,0113	40
SS	taro	0,9653	0,0217	0,013	40
SS	taro	0,971	0,0153	0,0137	40
SS	taro	0,9727	0,0087	0,0187	40
SS	taro	0,978	0,0143	0,0077	40
SS	taro	0,9793	0,0077	0,013	40
SS	taro	0,9807	0,01	0,0093	40
SS	taro	0,9827	0,0107	0,0067	40
SS	taro	0,983	0,0067	0,0103	40
SS	taro	0,9833	0,0093	0,0073	40
SS	taro	0,985	0,0063	0,0087	40
SS	taro	0,986	0,0047	0,0093	40
SS	taro	0,986	0,006	0,008	40
SS	taro	0,987	0,0077	0,0053	40
SS	taro	0,9883	0,0043	0,0073	40
SS	taro	0,9897	0,0047	0,0057	40
SS	taro	0,9903	0,005	0,0047	40
GI	taro	0,3296	0,039	0,6315	94
GI	taro	0,3387	0,0073	0,654	94
GI	taro	0,5128	0,0537	0,4335	94
ARD	arda	0,2607	0,006	0,7333	202
TR	trebbia1	0,006	0,0043	0,9897	68
TR	trebbia1	0,011	0,0463	0,9426	68

TR	trebbia1	0,0237	0,0737	0,9026	68
TR	trebbia1	0,0277	0,0227	0,9496	68
TR	trebbia1	0,0297	0,0087	0,9617	68
TR	trebbia1	0,036	0,1357	0,8283	68
TR	trebbia1	0,0987	0,216	0,6853	68
TR	trebbia1	0,1403	0,0117	0,848	68
TR	trebbia1	0,1843	0,0443	0,7714	68
TR	trebbia1	0,5383	0,012	0,4497	68
TR	trebbia1	0,547	0,017	0,436	68
TR	trebbia1	0,5877	0,0187	0,3937	68
TR	trebbia1	0,625	0,3467	0,0283	68
TR	trebbia1	0,666	0,0587	0,2753	68
TR	trebbia1	0,7433	0,006	0,2507	68
SN	trebbia1	0,0073	0,3143	0,6783	103
SN	trebbia1	0,0073	0,3641	0,6285	103
SN	trebbia1	0,0083	0,3763	0,6153	103
SN	trebbia1	0,009	0,3529	0,6381	103
SN	trebbia1	0,0107	0,3013	0,688	103
SN	trebbia1	0,0147	0,2007	0,7847	103
SN	trebbia1	0,0153	0,309	0,6757	103
SN	trebbia1	0,0157	0,1348	0,8496	103
SN	trebbia1	0,0183	0,008	0,9737	103
SN	trebbia1	0,019	0,1747	0,8063	103
SN	trebbia1	0,02	0,0477	0,9323	103
SN	trebbia1	0,0233	0,363	0,6137	103
SN	trebbia1	0,0293	0,2427	0,7279	103
SN	trebbia1	0,045	0,3655	0,5895	103
SN	trebbia1	0,0683	0,0787	0,853	103
SN	trebbia1	0,08	0,7009	0,2191	103
SN	trebbia1	0,154	0,0543	0,7917	103
SASA	trebbia1	0,0053	0,4637	0,531	404
SASA	trebbia1	0,006	0,0063	0,9877	404
SASA	trebbia1	0,006	0,012	0,982	404
SASA	trebbia1	0,0063	0,0073	0,9863	404
SASA	trebbia1	0,0067	0,327	0,6663	404
SASA	trebbia1	0,0077	0,1802	0,8121	404
SASA	trebbia1	0,0087	0,0423	0,949	404
SASA	trebbia1	0,009	0,0897	0,9013	404
SASA	trebbia1	0,0093	0,009	0,9817	404
SASA	trebbia1	0,0097	0,0247	0,9657	404
SASA	trebbia1	0,01	0,0603	0,9297	404
SASA	trebbia1	0,01	0,1623	0,8277	404
SASA	trebbia1	0,013	0,0273	0,9597	404

SASA	trebbia1	0,018	0,3728	0,6093	404
SASA	trebbia1	0,0207	0,3074	0,6719	404
SASA	trebbia1	0,0243	0,6203	0,3553	404
SASA	trebbia1	0,2164	0,6709	0,1127	404