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ECOLOGICAL IMPACTS OF THE INVASIVE AND CRYPTIC *Barbus barbus* (L.) (PISCES: CYPRINIDAE)

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This research was carried out at the University of Insubria (Como and Varese-Italy) where the PhD school is based and at Bournemouth University (UK) where I went as a visiting postgraduate researcher during an exchange program between the two institutions. It was also realised in collaboration with the University of Perugia (Italy).

It is said that before entering the sea
a river trembles with fear.

She looks back at the path she has traveled,
from the peaks of the mountains,
the long winding road crossing forests and villages.

And in front of her,
she sees an ocean so vast,
that to enter
there seems nothing more than to disappear forever.

But there is no other way.
The river can not go back.

Nobody can go back.
To go back is impossible in existence.

The river needs to take the risk
of entering the ocean
because only then will fear disappear,
because that's where the river will know
it's not about disappearing into the ocean,
but of becoming the ocean.

(Fear by Kahlil Gibran)

Abstract

Invasive species are among the principal causes of community and ecosystem integrity loss worldwide and freshwaters fishes are among the most threatened and introduced species. The invasive riverine fish *Barbus barbus* was used in this thesis as a model to study the ecological consequences deriving by two key mechanisms: interspecific trophic interactions and introgressive hybridisation. *B. barbus* is a large bodied cyprinid native to central Europe that has been introduced outside its native range in western England and Italy. The consequences of interspecific competition with functionally analogous fishes were tested in a serious of experimental conditions at different scales (from tank aquaria to mesocosms) with impacts measured on trophic niches and fish growth rates. Trophic ecology of *B. barbus* was also investigated in 11 wild populations of the UK also in relation to the use of angler's baits (pelletized meal) that can act as trophic subsidies and facilitate *B. barbus* integration into the invaded communities. Introgressive hybridization consequences on functional traits (i.e. trophic ecology, morphology and life traits) was instead tested in wild Italian populations where *B. barbus* readily hybridize with native co-generic analogous *B. plebejus* and *B. tyberinus*. Finally, a further aspect that was considered in this study was the cryptic diversity of *Barbus* fluvio-lacustrine

species in Italy that can lead to an underestimation of the extinction risk faced by barbels also in relation to *B. barbus* invasion.

The experimental approaches demonstrated that competitive interaction among *B. barbus* and other analogous cyprinids (i.e. *Leuciscus idus* and *Squalius cephalus*) can result in suppressed growth rate but trophic niche segregation and constriction (i.e. diet diversification and specialisation) allow fish to co-occur and avoid out-competition. Compared to intra-specific competition, the effects on fish growth rate were similar (i.e. reduced in both cases), but contrastingly, intraspecific competition produces an increase in niche size (i.e. generalization of diets). This provided experimental evidence for the niche variation hypothesis and explains the strong niche partitioning observed in previous studies on invasive *B. barbus* populations in English rivers. Moreover, although *B. barbus* appeared as a weaker competitor than the invasive *L. idus*, its introduction can result in isotopic niche reorganizations that can scale out to other community members with this requiring further elucidations.

In agreement with previous studies, we found that some adult individuals in 11 UK wild *B. barbus* populations specialized their diets on allochthonous anglers' baits as shown by their carbon isotope ratio ($\delta^{13}C$) strongly differentiated from that of

freshwater macroinvertebrates. However, this varied considerably over space also according to angling pressure and it is unlikely that it helped to ease the interspecific competition of the barbel with native species that is instead more likely to be driven by niche variation processes.

Introgressive hybridization with Italian native barbel populations resulted in hybrid populations, with mitochondrial DNA skewed toward *B. barbus* genotype and only 23% to 4% purebred native genotypes remaining in nuclear DNA. Significant alterations in morphology, enhanced growth rate, different diet and trophic position were detected in one hybrid population highlighting as introgressive hybridization is not only eroding the genetic integrity of native barbel species, but it has the potential to alter the functional role of barbel with consequent impacts that may influence also non-barbel members of the receiving community. Conversely, the detection of hybrid vigour underlined the adaptive role of introgression with hybrids that may be able to persist in areas where native barbel are disfavoured thus raising contrasting conservation perspectives. Purebred native species are likely to be confined to locations where barriers prevent *B. barbus* expansion and therefore there is a need to reconcile conservation needs to restore fluvial connectivity with the important role of isolated river stretches in offering refuge to native species.

Geometric morphometrics and molecular analyses revealed the presence of two previously undetected barbel lineages in southern Italian basins for which a new description (*B. samniticus* sp. nov.) and a re-establishment (*B. fucini* Costa 1853) are proposed. Evolutionary history of these lineages may reveal some new insights into the evolution of the southern Italian basins and are therefore of great conservation interest. However, like *B. plebejus* and *B. tyberinus* species, the southern Italian lineages are already threatened especially by fish translocations and *B. barbus* and other exotic species invasions and they urgently require adequate protection.

In conclusion, this thesis enhanced our understanding of the complex mechanisms governing the ecological and evolutionary consequences associated with biological invasions and brought new insights into *Barbus* genus diversity in Italy with important conservation implications.

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It seems like three years have gone in the blink of an eye but actually, these three years have changed my life. It has been hard and frustrating sometimes, but it has been amazing. Anyway, it would not have been so amazing without all the people I came across during this trip. Conscious or not all these people have helped me to go ahead and achieve my objectives. As I have so many people I wish to thank, I have decided to start from the very beginning: my stay as a visiting post graduate researcher at Bournemouth University (UK).

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List of Publications and authors contribution

This thesis is based on the following research papers, published (or prepared) in collaboration with J. Robert Britton (JRB), Catherine Gutman Roberts (CGR), Fatima Amat Trigo (FAT), Emma T. Nolan (ETN), Serena Zaccara (SZ), Silvia Quadroni (SQ), Isabella Vanetti (IV), Antonella Carosi (AC), Massimo Lorenzoni (ML) and Giuseppe Crosa (GC). Contribution of co-authors in each paper-led chapter is outlined below (Table 1).

Chapter III. Britton JR, Gutmann Roberts C, Fatima Amat-Trigo F, Nolan ET & De Santis V. 2019. Predicting the ecological impacts of an alien invader: experimental approaches reveal the trophic consequences of competition. Journal of Animal Ecology 88: 1066-1078. https://doi.org/10.1111/1365-2656.12996.

Chapter IV. De Santis V, Gutmann Roberts C, Britton JR. Trophic consequences of competitive interactions in freshwater fish: density dependent effects and impacts of interspecific versus intraspecific competition. Freshwater Biology. https://doi.org/10.1111/fwb.13643.

Chapter V. De Santis V, Gutmann Roberts C, Britton JR. 2019. Influences of angler subsidies on the trophic ecology of European barbel *Barbus barbus.* Fisheries Research 214: 35-44. https://doi.org/10.1016/j.fishres.2019.01.028.

Chapter VI. Zaccara S, Quadroni S, De Santis V, Vanetti I, Carosi A, Crosa G, Britton JR, Lorenzoni M. 2020. Genetic and phenotypic displacement of an endemic *Barbus* complex by invasive European barbel *Barbus barbus* in central Italy. Biological Invasions. https://doi.org/10.1007/s10530-020-02379-2.

Chapter VII. De Santis V, Quadroni S, Carosi A, Gutmann Roberts C, Lorenzoni M, Crosa G, Britton R, Zaccara S. Biological and trophic consequences of genetic introgression between endemic and invasive *Barbus* fishes. (Submitted to Biological Invasions).

Chapter VIII. Zaccara S, Quadroni S, De Santis V, Vanetti I, Carosi A, Britton JR, Lorenzoni M. 2019. Genetic and morphological analyses reveal a complex biogeographic pattern in the endemic barbel populations of the southern Italian peninsula. Ecology and Evolution 9: 10185-10197. https://doi.org/10.1002/ece3.5521.

Chapter IX. Lorenzoni M, Carosi A, Quadroni S, De Santis V, Vanetti I, Delmastro GB, Zaccara S. Cryptic diversity within endemic Italian barbels: revalidation and description of new *Barbus* species (Teleostei: Cyprinidae). (Accepted for publication in the Journal of Fish Biology).

Table1. Authors contributions to each data-lead chapter composing this thesis

	Idea conception	Methodology designation	Data collection or Sampling	Data analysis	Manuscript leadership	Draft revision	Submission approval
Chapter III	JRB	JRB	JRB	JRB, FAT, CGR, ETN, **VDS**	JRB	**VDS**, FAT, CGR, ETN, JRB	JRB, FAT, CGR, ETN & **VDS**
Chapter IV	JRB	JRB	**VDS**, JRB	**VDS**, CGR & JRB	JRB	**VDS**, CGR & JRB	**VDS**, CGR & JRB
Chapter V	JRB	JRB	JRB	**VDS**, CGR & JRB	JRB	**VDS**, CGR & JRB	**VDS**, CGR & JRB
Chapter VI	**VDS**, JRB, SZ, GC	SZ, **VDS**, JRB, ML	**VDS**, SQ, AC, ML	**VDS**, SQ, IV, AC	SZ	**VDS**, SQ, JRB, ML & SZ	SZ, SQ, **VDS**, IV, AC, JRB, GC, ML
Chapter VII	**VDS**, JRB, SZ, GC	**VDS**, JRB, SZ, ML	**VDS**, SQ, AC, ML, CGR	**VDS**, SQ, AC, ML	**VDS**	JRB, SZ, CGR, ML, SQ & **VDS**	JRB, SZ, CGR, ML, SQ, **VDS**
Chapter VIII	SZ, ML, SQ, AC	SZ, ML, SQ, AC	ML, AC	SQ, **VDS**, IV	SZ	SQ, **VDS**, ML, AC, JRB, IV & SZ	SZ, SQ, **VDS**, IV, AC, JRB, ML
Chapter IX	SZ, ML, SQ, **VDS**, AC	SZ, SQ, **VDS**	ML, AC, **VDS**, SQ	SQ, **VDS**, AC, IV	AC	SQ, **VDS**, ML, AC, SZ, IV	SQ, **VDS**, ML, AC, SZ, IV

Ethical statement

This thesis is based on a series of experimental and field activities that involved handling and sacrifice of fish. I hereby declare that all the experiments conducted in this study (Chapter III and Chapter IV) followed ethical review and were performed under the UK Home Office project licence 70/8063. Field activities (Chapter VI, VII, VIII and IX) involved sampling protocols that have been established in compliance with the ethical standards, ensuring that all necessary precautions, required by Italian legislation, have been taken and the welfare of the fish have been respected. I thank the fisheries departments for the local authorization to promote research activities in the field.

CHAPTER I

1. Introduction

1.1 Invasive Species: what and why?

Considered one of the main threats to biodiversity conservation (Clavero & Garciaberthou, 2005; Mollot et al., 2017), invasive aline species (IAS) are those that once introduced in a new ecosystem by humans, successfully colonize it, giving birth to self-sustained populations. In addition, the term "invasive" indicates the ability of these species to modify some aspects of the receiving system that is IAS can generate impacts at different level of biological organisation, from the smallest scale (genes) up to the entire ecosystem (Cucherousset & Olden, 2011). Introduced species can also cause severe economic impacts (e.g. Cuthbert et al. 2020; Diagne et al., 2020). The economic costs can be due to damages caused to ecosystem services (Charles & Dukes, 2008) that have consequences on human health (Schindler et al., 2015; Young et al., 2017), agriculture (Paini et al., 2016), and other productivity sectors (e.g. Diagne et al., 2020) as well as by the costs arising from IAS control and management (e.g. removal and eradication actions; Gallardo et al., 2019). Consequently, national and international agreements, regulations and conservation plans have been instituted around the world

including in Europe (e.g. EU Regulation 1143/2014) to control, eradicate and manage existing invasive species and prevent further introductions. Nonetheless, new introductions continue and are likely to increase (Seebens et al., 2017).

Invasive aline species offer the opportunity to study evolutionary and ecological processes at a smaller temporal scale than previously possible (Blackburn, 2004; Bock et al., 2015). Therefore, studies of the mechanisms by which IAS successfully adapt to new ecosystems and generate impacts are not only of interest for a conservation and ecosystem management perspective but also to other disciplines including macroecology, biogeography, evolutionary biology and disciplines related to human society such as politics, economy and sociology (Hobbs & Richardson, 2010; Richardson, 2010).

1.2 IAS ecological impacts

The term "ecological impacts" associated to IAS refers to "*any measurable change to the property of an ecosystem*" by an alien invasive species (Ricciardi et al., 2013). The most apparent impact that IAS may have is the local extinction of native taxa (Mollot et al., 2017). This can result either by direct interactions such as antagonistic competition (for food or reproductive/refuge sites) or predation and indirect interactions, which are those mediated by another factor like the spread of

new pathogens or exploitative competition (i.e. indirect competition for a limited resources).

However, species extinction is not always the endpoint of IAS introductions. In many cases the alien species integrate into the receiving community (Ricciardi et al., 2013; Jackson et al., 2017), though causing other impacts that can affect more than one biological level and that can result in cascading effects (both bottom-up or top-down) (Cucherousset & Olden, 2011; Jackson et al., 2017).

Phenotpic changes (in behaviour (e.g. Blanchet et al., 2008) and morphology (e.g. Bourke et al., 1999)) and vital traits alterations (i.e. growth and reproduction; Cucherousset & Olden, 2011) can manifest at the individual level and can result in disruption in the ecology of native populations (e.g. altered demographic structure (Pope, 2008); altered abundances (Alcaraz et al., 2008)). At the population level, impacts can also occur via interspecific hybridisation (discussed further below) or alterated genetic variability (e.g. Wittmann et al., 2013). These, in turn, can cause changes in community composition (e.g. Leuprieur et al., 2008) and alteration in community (local extinctions, e.g. Witte et al., 1992) and food web (e.g. Vaner Zanden et al., 1999) structures. Invaded communities can eventually modify biogeochemical cycles (Figueredo &Giani,

2005), energy fluxes (e.g. Syväranta et al., 2009) and physical habitat (Rowe, 2007), affecting the entire ecosystem (Fig. 1.1).

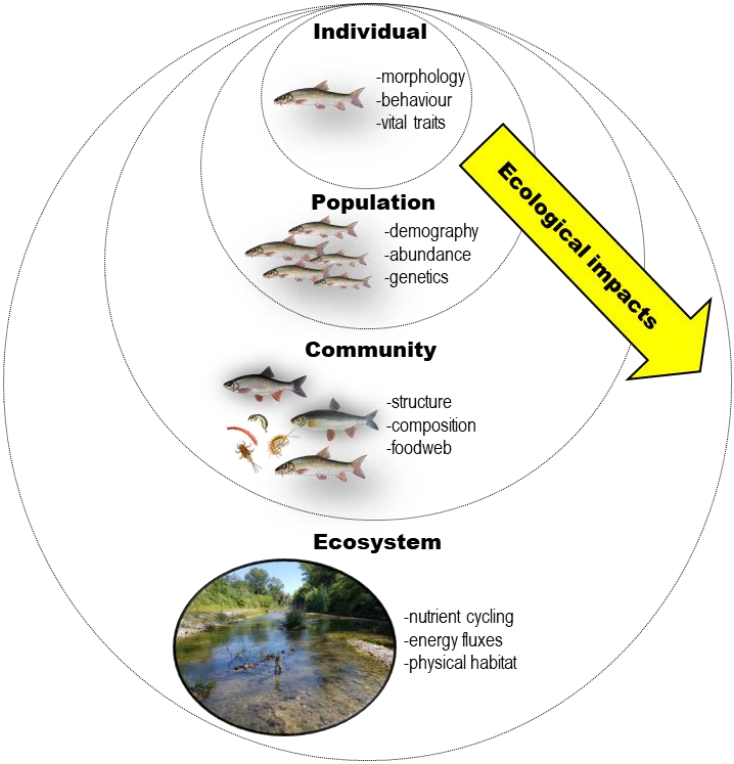

Figure 1.1 *Summary of the potential ecological impacts across different biological levels. The arrow indicates these effects may be not restricted to a single level, but they can spread across them (i.e. cascading effects).*

Predicting impacts of IAS can be very difficult because they are strongly context dependent and vary considerably among ecosystems and species (Kumschick et al., 2015). Among the factors that might complicate predictions there is the relation

between the invader abundance (i.e. density) and the impact (Sofaer et al., 2018), which can be linear as well as non-linear (Ricciardi et al., 2013) or even density-independent (Jarić et al., 2019). This is because the impacts of a species can vary considerably in space and time. The invasion history of the receiving ecosystem for instance can be responsible for non-linear density-impact relationships. The presence of other exotic taxa can either facilitate the integration of new invaders (i.e. invasional meltdown hypothesis; Lanzoni et al., 2018) or even hinder it (biotic resistance, Britton, 2012). As a component of global environmental change, IAS impacts can also interact with other anthropogenic perturbations such as river regulation and inter-basins transfers (Gallardo & Aldridge, 2018; Ruhi et al., 2019) and land use change (Didham et al., 2007).

Due to these complexities, to better characterise the risk of an invader and also to be able to predict future responses, more data on impacts and their mechanisms are required (Lenzner et al., 2019), possibly combining different approaches to produce conclusions (Ricciardi et al., 2013).

1.2.1 *Interspecific trophic interactions*

The most severe impacts of the introduction of invasive alien species are a consequence of changes in interspecific trophic interactions (Jackson et al., 2017) that arise via predator-prey

links or interspecific competitions. As food webs are a result of interspecific interactions and the influence of abiotic and biotic factors, understanding the alteration of trophic interactions resulting from biological invasions allows important effects to be identified that otherwise could go unnoticed while they are still reversible (Jackson et al., 2017).

Ecological niche theory can then be used to predict the consequences of disrupted trophic interactions while invasive species consequently serve as good models to test empirical hypotheses on food web dynamics (Catford et al., 2009; Britton, 2019). For instance, ecological theory predicts that the strength of interspecific competition is a function of resource availability and community structure. If an invader enters an impoverished community in which resources are not fully exploited, then interspecific competition would be avoided easing the integration of the invader in the receiving systems (Mason et al., 2008; Juncos et al., 2015). On the contrary, should the receiving community be highly structured and characterised by limiting resources, the strength of competition would be higher. This could result in both the niches of the invader and the native being reduced and divergent (i.e. the species will differentiate their diets; Bolnick et al., 2010; Tran et al., 2015; Jackson et al., 2016) or their niches can expand as a consequence of the species using a wider range of resources to maintain energetic

requirments (Svanbäck & Bolnick, 2007). Finally, if the competition is asymmetrical that is one competitor (i.e. the invader) acquire resources more efficiently extinction by out-competition (i.e. competitive exclusion) can occur, if resources are limited (Bøhn et al., 2008).

1.2.2. *Interspecific hybridization*

Introductions of IAS can result in contact between previously isolated species that, if interspecific breeding has not been selected against, will hybridize. Then introgression of one species genome into another can follow, with this being more readily possible among closely related species (Mallet, 2005). Natural genetic admixture has been proven to be ubiquitous and common among different taxa (Baack & Rieseberg, 2007). It is also recognised as a strong adaptative force that has guided the speciation of different organisms (Seehausen, 2004; Selz & Seehausen, 2019; Svardal et al., 2019), including modern humans (Racimo et al., 2015). Anthropogenic hybridisation, however, has the potential to cause conservation issues (Rhymer & Simberloff, 1996; Allendorf et al., 2001; Brennan et al., 2014). Hybridisation can lead to species extinction and genetic homogenisation through swamping of species genotypes and/or outbreeding selection (i.e. lowered fitness; Rhymet & Simberloff, 1996). There are cases where hybrids

have similar or enhanced fitness compared to parental species (i.e. hybrid vigour; Pfenning et al., 2007). These include cases where hybrids have similar traits to one of the parental species or cases of transgressive hybridisation (Reisenberg et al., 1999) where hybrids display extreme traits compared to the parental ones. Hybrids can also be more invasive than the parental exotic species (Hovick & Whitney, 2014) and generate indirect impacts forming new trophic interactions or altering existing ones (Ryan et al., 2009). Hybrids can be advantaged in degraded environments (Best et al., 2017) thanks to their potential ability to exploit alternative trophic niches (e.g. Selz & Seehausen, 2019) deriving from a usually higher standing genetic and/or phenotypic variation (Baack and Rieseberg, 2007).

The evolutionary implications of hybridizations are still debated and the ecological consequences derived from variation in functional traits (e.g. trophic niches and morphological traits) have been rarely tested (Rosenfield et al., 2004; Matsuzaki et al., 2010). This is despite the importance of this information in assessing the impacts of invasive species and the valuable implications in evolution.

1.3 Cryptic species

Many plant and animal species descriptions have been based on morphological traits for years following Mayr (1963) outlined

his biological species concept. However, speciation it is not always associated with morphological differentiation and this have led to the erroneous attribution of different, often closely related (i.e. sibling) species to a unique one (Bickford et al., 2007). There are different mechanisms that may be responsible of the lack of morphological distinctiveness (Bickford et al., 2007; Fišer et al., 2018). Morphological traits may have not been already fixed as the species have diverged recently, additionally biological constraints (i.e. adaptation to a specific ecological niche or a particular environment) may prevent phenotypic differentiation, a phenomenon known as morphological stasis (Bickford et al., 2007) or lastly, similar morphologies may be a result of an adaptative convergence.

Although identifications of cryptic taxa are rapidly increasing, many of these remain undescribed and therefore are still not included in biodiversity studies (Fiser et al., 2018). Nevertheless, the existence of undescribed species is a biodiversity conservation challenge given the high rate of extinctions driven by anthropogenic activities. Indeed, inaccurate species diversity assessments may have several consequences (Bickford et al., 2007). These include the potential underestimation of the extinction risk resulting from an overestimation of species distribution when a species complex was previously ascribed to one species with a wider

distribution and actually results in numerous distinct species with a narrower distribution. In addition, species that form cryptic complexes can have different ecological requirements resulting in negative impacts for biodiversity conservation (i.e. inadequate conservation actions) and even human health (i.e. environmental quality indicators).

Furthermore, morphological similarities can mask the invasion of exotic lineages (Morais & Reichard, 2018). This can cause several issues for the management of invasive alien species, and also makes predicting subsequent impacts more diffucult (Jarić et al., 2019). Cryptic features may extend to functional traits as well as species morphology (e.g. trophic and non-trophic interactions), either due to a lack of recognition of such traits or because they are novel (Jarić et al., 2019). Hybridization can play a significant role in generating cryptic shifts in species function.

Therefore, species and functional crypticism is an important and widespread process with conservation importance that must be accounted for in the context of biological invasion to better address impacts following a successful introduction of an invasive alien species.

1.4 Freshwater fish invasions in Italy

Freshwaters are among the most altered ecosystems by human activities and are especially prone to biological invasions also thanks to the aquatic connections that allow species to spread (Gherardi et al., 2009; Gozlan et al., 2010; Hermoso & Clavero, 2011; Gallardo et al., 2016). They are more susceptible to invasion impacts due to the strong trophic links that characterize aquatic organisms (Gallardo et al., 2016).

Freshwater fish are among the most introduced vertebrate worldwide (Gozlan et al., 2010) and among the most threatened with extinction (Darwall et al., 2008). This is particularly true for biodiversity hot spots like the Mediterranean region (Hermoso and Clavero, 2011) where more than 70% of inland fish are threatened with extinction (Darwall et al., 2008; Hermoso and Clavero, 2011) and where invasive fish account for more than a quarter of the total number of species found in the region's freshwaters (Leprieur et al., 2008). In this region, Italy has one of the highest number of fish introductions, together with Spain and Israel (Hermoso and Clavero, 2011; Bianco, 2014). Indeed, in some Italian catchments, the number of non-native species exceeds that of the indigenous ones. To date, in Italy there are 57 established freshwater alien fishes (Table 1.1; Bianco, 2014; Nocita et al., 2017; Lorenzoni et al., 2019) against 55 native species (Table 1.2), with at least 15

additionally species that have yet be established (Bianco, 2014; Nocita et al., 2017; Lorenzoni et al., 2019). The 57 alien fish species belong to 10 orders and 18 families (Table 1.1). The most represented family is the leuciscid family (16 species) followed by salmonid and poeciliid (7 species each) that together account for 53 % of the total number of species (Table 1.1). The potential presence of cryptic lineages coupled with the unresolved taxonomical status of some species can enhance these numbers further (Bianco, 2014; Nocita et al., 2017; Lorenzoni et al., 2019; De Santis et al., 2020).

Twenty-nine out of the 57 alien fish species established in Italy, are native to central Europe or Eurasia (Table 1.1), indicating as central Europe (and the Danube River catchment (Lanzoni et al., 2018)) is the principal source of many of the introduced species in the last decades (Lanzoni et al., 2018). In major river catchments (Po River in Northern Italy (Lanzoni et al., 2018) and Tiber and Arno rivers in central Italy (Nocita et al., 2017)), the situation is such that the fish community resembles that of the Danube River, especially so for the Po River (Bianco, 2014).

Fish introductions in Italy have seen a marked increase since the 60s, particulalrly between 1981 and 2000 (Table 1.1; Nocita et al., 2017). This is probably attributable to restocking programs that have been performed widely during the 60s and the 70s around Europe, including Italy, to enhance and sustain angling

(Bianco, 1995; Gherardi et al., 2009; Gozlan et al., 2010a; Bianco, 2014; Nocita et al., 2017). Translocation has been a major driver altering Italian inland fish communities (Table 1.2), where fish are stocked from one biogeographic district to another within the same country. These practices continue, with the benign aim to preserve species, for example, to counter the impacts of summer droughts (Meraner et al., 2013; Geiger et al., 2016; Nocita et al., 2017; Zaccara et al., 2019).

Apart from their (unfortunately) high introduction rate, fish are also good models in biological invasions for two main reasons. At the experimental level, fish are relatively easy organisms to maintain in experimental conditions, they are adaptable and their indeterminate growth enables correlation with competitive interactions (Ward et al., 2006). Moreover, freshwaters fish and, in particular, primary fishes (i.e. stenohaline, halophobic), are well known to be good biogeographic models, as they are unable to pass physical barriers. As such, their distribution reflects river network connections and their evolution (Buonerba et al., 2015). For a similar reason, anthropogenic species introductions among inland fish communities are relatively more trackable than with other organisms (Leprieur et al., 2008).

Table 1.1 *List of the 57 alien freshwater fish species (Bianco, 2014; Nocita et al., 2017; Lorenzoni et al., 2019) divided per order and family with updated nomenclature (Fricke et al., 2020).Origin and period of introduction/first detection is reported. * Indicate tropical species that have been introduced in thermal streams of Italy where they found suitable condition for their establishment.*

Taxon	Common name	Origin	Introduction
Order Centrarchiformes			
Family Centrarchidae			
Lepomis gibbosus (L.)	Pumpkinseed, common sunfish	N. America	1901-1920
Micropterus salmoides (Lacepède 1802)	Largemouth bass	N. E. America	1901-1920
Order Atheriniformes			
Family Atherinopsidae			
Odontesthes bonariensis (Valenciennes 1835)	Argentinian silverside	S. W. Atlantic	1961-1980
Order Cichliformes			
Family Cichlidae			
Amatitlania nigrofasciata (Günther 1867)*	Convict cichlid	Central America	2001-2020
Hemichromis Peters 1857 *sp.**	Jewel fish	W. Africa	2001-2020
Oreochromis niloticus (L.)	Nile tilapia	N. and E. Africa	2001-2020
Order Cypriniformes			

Table 1.1 (Continue)

Taxon	Common name	Origin	Introduction
Family Acheilognathidae			
Rhodeus amarus (Bloch 1782)	European bitterling	Eurasia	1981-2000
Family Cobitidae			
Misgurnus anguillicaudatus (Cantor 1842)	Oriental weatherfish	E. Asia	1981-2000
Family Cyprinidae			
Barbus barbus (L.)	European barbel	Central Europe	1981-2000
Carassius auratus (L.) complex	Goldfish	E. Asia: China and Japan	<1800
Cyprinus carpio L.	Common carp	Eurasia	<1800
Luciobarbus graellsii (Steindachner 1866)	Ebro barbel	Spain	2001-2020
Family Gobionidae			
Gobio gobio L.	Gudgeon	Eurasia	1981-2000
Pseudorasbora parva (Temminck & Schlegel 1846)	Topmouth-gudgeon	N. E. Asia: Japan and China	1981-2000
Family Leuciscidae			
Abramis brama (L.)	Common bream	Eurasia	1981-2000

Table 1.1 (Continued)

Taxon	Common name	Origin	Introduction
Alburnoides bipunctatus (Bloch 1782)	Spirlin	Eurasia	unknown
Alburnus alburnus (L.)	Bleak	Eurasia	unknown
Ballerus ballerus (L.)	Blue bream	Eurasia	unknown
Blicca bjoerkna (L.)	White bream	Eurasia	1981-2000
Chondrostoma nasus (L.)	Common nase	Eurasia	1961-1980
Leuciscus aspius (L.)	Asp	Eurasia	1981-2000
Leuciscus idus (L.)	Ide	Eurasia	1901-1920
Leuciscus leuciscus (L.)	Common dace	Eurasia	unknown
Pachychilon pictum (Heckel & Kner 1857)	Albanian roach	S. E. Europe	1981-2000
Phoxinus phoxinus (L.) complex	European minnows	Eurasia	unknown
Rutilus rutilus (L.)	European roach	Eurasia	1981-2000
Scardinius erythrophthalmus (L.)	Common rudd	Europasia	unknown

Table 1.1 (Continued)

Taxon	Common name	Origin	Introduction
Squalius cephalus (L.)	European chub	Eurasia	unknown
Squalius vardarensis Karaman 1928	Vardar chub	S. E. Europe: Greece and Macedonia	2001-2020
Vimba vimba (L.)	Vimba bream	Eurasia	unknown
Family Tincidae			
Tinca tinca (L.)	Tench	Europe	unknown
Family Xenocyprinidae			
Ctenopharyngodon idella (Valenciennes 1844)	Grass carp	E. Asia: China and Russia	1961-1980
Cyprinodontiformes			
Family Poeciliidae			
Poecilia reticulata Peters 1859 *	Guppy	N. S.America	1981-2000
Gambusia holbrooki Girard 1859	Eastern mosquitofish	N. America (E. S. U.S.A.)	1921-1940
Poecilia latipinna (Lesueur 1821) *	Sailfin molly	S. U.S.A.	1981-2000

Table 1.1 (Continued)

Taxon	Common name	Origin	Introduction
Poecilia sphenops Valenciennes 1846 *	Black molly	Central America	1981-2000
Poecilia velifera (Regan 1914) *	Sailfin molly	Central America	1981-2000
Xiphophorus hellerii Heckel 1848 *	Green swordtale	Central America	1981-2000
Xiphophorus maculatus (Günther 1866) *	Southern platyfish	Central America	1981-2000
Order Esociformes			
Family Esocidae			
Esox lucius L.	Northern pike	Paleartic	unknown
Order Gobiiformes			
Family Gobiidae			
Neogobius melanostomus (Pallas 1814)	Round goby	Eurasia	2001-2020
Order Perciformes			
Family Percidae			
Gymnocephalus cernus (L.)	Ruffe	Europe	1981-2000
Perca fluviatilis L.	European perch	Europe	<1800
Sander lucioperca (L.)	Pikeperch	Eurasia	1901-1920

Table 1.1 (Continued)

Taxon	Common name	Origin	Introduction
Order Salmoniformes			
Family Salmonidae			
Coregonus lavaretus (L.) complex	European whitefish (pelagic and litoral morphs)	Eurasia	1901-1920
Oncorhynchus kisatch (Walbaum 1792)	Coho salmon	N. Pacific and Arctic	1961-1980
Oncorhynchus mykiss (Walbaum 1792)	Rainbow trout	N. America	1901-1920
Salmo trutta L.	Domestic strain of Atlantic brown trout	Atlantic	1901-1920
Salvelinus alpinus (L.)	Artic charr	Circumpolar	<1800
Salvelinus fontinalis (Mitchill 1814)	American brook charr/trout	Atlantic slope of N. America	1901-1920
Thymallus thymallus (L.)	European grayling	Eurasia	1961-1980
Order Siluriformes			
Family Ictaluridae			
Ameiurus melas (Rafinesque 1820)	Black bullhead	N. America	1941-1960
Ameiurus nebulosus (Lesueur 1819)	Brown bullhead	N. America	1941-1960

Table 1.1 (Continued)

Taxon	Common name	Origin	Introduction
Ictalurus furcatus (Valenciennes 1840)	Blue catfish	N. America (Central U.S.A.)	1981-2000
Ictalurus punctatus (Rafinesque 1818)	Channel catfish	E. N. America	1981-2000
Family Loricaridae			
*Pterygoplichthys pardalis (Castelnau 1855)**	Sailfin catfish	S. America	2001-2020
Family Siluridae			
Silurus glani L.	European catfish	Eurasia	1961-1980

Table 1.2 *List of the native Italian freshwater fish species (Bianco, 2014, Nocita et al., 2017, Lorenzoni et al., 2019) with updated nomenclature (Fricke et al., 2020) divided per order and family. Common name, ecology and distribution in Italy are aslo reported. Taxa in bold are those that have been translocated across different geographic districts in Italy. PV: Padano- Venetian district (N. Adriatic basin), TL: Tuscano-Latium district (central Thyrrenian basin).*

Species	Common name	Ecology	Distribution in Italy
Order Acipenseriformes			
Family Acipenseridae			
Acipenser naccarii Bonaparte 1836	Adriatic sturgeon	Euryhaline	Adriatic Sea, PV
Order Anguilliformes			
Family Angullidae			
Anguilla anguilla (L.)	European eel	Euryhaline	Mediterranean Sea
Atherina boyeri Risso 1810	Big-scale sand smelt	Euryhaline	Mediterranean Sea
Blenniiformes			
Blenniidae			
Salaria fluviatilis (Asso y del Rio 1801)	Freshwater blenny	Euryhaline	Mediterranean Sea

Table 1.2 (Continued)

Species	Common name	Ecology	Distribution in Italy
Carangiformes			
Pleuronectidae			
Platichthys flesus (L.)	European flounder	Euryhaline	Mediterranean brakish and coastal waters
Clupeiformes			
Clupeidae			
Alosa agone (Scopoli 1786)	Agone (Lake shad)	Stenohaline	N. Italy
Alosa fallax (Lacepède 1803)	Twait shad	Euryhaline	Mediterranean Sea
Cypriniformes			
Cobitidae			
Sabanejewia larvata (De Filippi 1859)	Italian loach	Stenohaline	PV
Cobitis bilineata Canestrini 1865	Common loach	Stenohaline	PV
Cobitis zanandreai Cavicchioli 1965	Volturno loach	Stenohaline	S. Italy
Cyprinidae			
Barbus balcanicus Kotlík, Tsigenopoulos, Ráb & Berrebi, 2002	Danube barbel	Stenohaline	N.E. Italy
Barbus caninus Bonaparte 1839	Brook barbel	Stenohaline	PV

Table 1.2 (Continued)

Species	Common name	Ecology	Distribution in Italy
Barbus plebejus Bonaparte 1839	Padanian barbel	Stenohaline	PV
Barbus tyberinus Bonaparte 1839	Tiber barbel	Stenohaline	TL
Gobionidae			
Romanogobio benacensis (Pollini 1816)	Italian gudgeon	Stenohaline	PV
Leuciscidae			
Alburnus albidus (Costa 1838)	Italian or Southern bleak	Stenohaline	S. Italy
Alburnus arborella (Bonaparte 1841)	Italian bleak	Stenohaline	PV
Chondrostoma soetta Bonaparte 1840	Italian nase	Stenohaline	PV
Phoxinus lumaireul (Schinz 1840)	Italian minnow	Stenohaline	PV
Protochondrostoma genei (Bonaparte 1839)	South European nase	Stenohaline	PV
Rutilus aula (Bonaparte 1841)	Triotto	Stenohaline	PV
Rutilus pigus (Lacepède 1803)	Italian roach	Euryhaline	PV
Sarmarutilus rubilio (Bonaparte 1837)	Southern Europe roach	Stenohaline	TL
Scardinius hesperidicus Bonaparte 1845	Italian rudd	Stenohaline	PV
Scardinius scardafa (Bonaparte 1837)	Tiber rudd	Stenohaline	TL
Squalius lucumonis (Bianco 1983)	Etruscan chub	Stenohaline	TL
Squalius ruffoi Bianco & Recchia 1983	Chub	Stenohaline	S. Italy

Table 1.2 (Continued)

Species	Common name	Ecology	Distribution in Italy
Squalius squalus (Bonaparte 1837)	Italian chub	Stenohaline	PV
Telestes comes (Costa 1838)	Vairone	Stenohaline	S. Italy
Telestes muticellus Bonaparte 1837	Vairone	Stenohaline	PV
Telestes souffia (Risso 1827)	Vairone	Stenohaline	N. E. Italy
Nemacheilidae			
Barbatula barbatula (Linnaeus 1758)	Stone loach	Stenohaline	PV
Cyprinodontiformes			
Aphaniidae			
Aphanius fasciatus (Valenciennes 1821)	Mediterranean banded killfish	Euryhaline	Mediterranean Sea
Esociformes			
Esocidae			
Esox cisalpinus Bianco & Delmastro 2011	Italian pike	Stenohaline	PV
Gadiformes			
Lotidae			
Lota lota (L.)	Burbot	Euryhaline	PV
Gobiiformes			

Table 1.2 (Continued)

Species	Common name	Ecology	Distribution in Italy
Gobiidae			
Knipowitschia panizzae (Verga 1841)	Adriatic dwarf goby	Euryhaline	Adriatic Sea
Orsinigobius punctatissimus (Canestrini 1864)	Italian spring goby	Stenohaline	North-eastern Italy
Padogobius bonelli (Bonaparte 1846)	Common goby	Stenohaline	PV
Padogobius nigricans (Canestrini 1867)	Arno goby	Stenohaline	TL
Ninnigobius canestrinii (Ninni 1883)	Canestrini's goby	Euryhaline	PV
Mugiliformes			
Mugillidae			
Chelon ramada (Risso 1827)	Thinlip mullet	Euryhaline	Mediterranean brakish and coastal waters
Mugil cephalus L.	Striped mullet	Euryhaline	Mediterranean brakish and coastal waters
Perciformes			
Gasterosteidae			
Gasterosteus aculeatus L.	Three-spined stickleback	Euryhaline	Mediteannean Sea

Table 1.2 (Continued)

Species	Common name	Ecology	Distribution in Italy
Cottidae			
Cottus gobio L.	Bullhead	Euryhaline	Italy
Cottus scaturigo Freyhof, Kottelat & Nolte 2005	Timavo sculpin	Stenohaline	PV
Moronidae			
Dicentrarchus labrax (L.)	European seabass	Euryhaline	Mediterranean brakish and coastal waters
Petromyzontiformes			
Petromyzontidae			
Lampetra fluviatilis (L.)	European river lamprey	Euryhaline	TL
Lampetra planeri (Bloch 1784)	European brook lamprey	Stenohaline	S. Italy
Lampetra zanandreai Vladykov 1955	Po brook lamprey	Stenohaline	PV
Petromyzon marinus L.	Sea lamprey	Euryhaline	Mediterranean Sea
Salmoniformes			
Salmonidae			

Table 1.2 (Continued)

Species	Common name	Ecology	Distribution in Italy
Salmo carpio L.	Carpione	Stenohaline - Lake population	Lake Garda (N. Italy)
Salmo cettii Rafinesque 1810	Mediterranean trout	Stenohaline	Italian islands
Salmo fibreni Zerunian & Gandolfi 1990	Fibreno trout	Stenohaline - Lake population	Lake Fibreno (central Italy)
Salmo ghigii Pomini 1941	Abruzzi trout	Stenohaline	S. Italy
Salmo marmoratus Cuvier 1829	Marble trout	Stenohaline	PV
Salmo trutta L.	Adriatic brown trout strain	Euryhaline	Central Italy
Thymallus aeliani Valenciennes 1848	Italian grayling	Stenohaline	PV
Syngnathiformes			
Syngnathidae			
Syngnathus abaster Risso 1827	Black-striped pipefish	Euryhaline	Mediterranean brakish and coastal waters

CHAPTER II

2. Thesis objectives and structure

2.1 The model species *Barbus barbus* (L.)

The European barbel is a benthic cyprinid: it is considered a primary rheophilic fish that populates fast flowing waters of the middle/lower European river reaches (i.e. "barbel zone" (Huet, 1949)). Its natural distribution is wide and extends from southeastern England to the Black Sea. The southern boundary of its distribution is formed by the main mountain chains like the Pyrenees and the Alps (Britton & Pegg, 2011)(Fig. 2.1a).

In its native range, it is considered an indicator of river quality given to its habitat requirements (Britton & Pegg, 2011) and response to river chemical pollution and fragmentation (dams and weirs construction) that have caused the decline in the most degraded habitats during the 20th century (e.g. Bašić et al., 2017).

Where undisturbed, *B. barbus* occurs in aggregative groups and can live up to 18 years, with individuals reaching sizes exceeding 8 kg (Amat Trigo et al., 2017). These characteristics have made the European barbel a valuable resource for angling, leading to the introduction of the species outside its native range for example in northern and central Italy and in western flowing

rivers of Britain (west England and Wales) (Zaccara et al., 2014; Buonerba et al., 2015a; Antognazza et al., 2016) (Fig. 2.1 b).

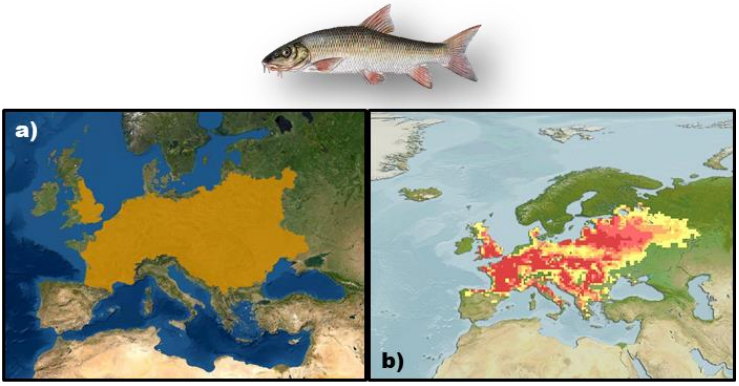

Figure 2.1 B. barbus *native range obtained from IUCN website (a) (Freyhof, 2011) and extended range (b) comprising where the species has been introduced with colours indicating densities; red=higher density; yellow=lower density. (b) Map retrieved from https://mare.istc.cnr.it/.*

In western England, the species tends to occur with other cyprinids typical of the "barbel" zone such as the chub *Squalius cephalus* (L) that displays several similar functional traits (i.e. body size, lifespan) to European barbel, while no co-generic species are present (Antognazza et al., 2016; Gutmann Roberts & Britton, 2018a). In these rivers, barbel have established successful populations that co-exist with the other species with apparently limited competitive interactions, allegedly facilitated by a high trophic niche partitioning between barbel

and the other resident species (Gutmann Roberts & Britton, 2018a, 2018b).

However, ecological impacts may arise from non-trophic interactions by, for instance, the zoogeomorphic activity of the species (Gutmann Roberts et al., 2019) that, together with its benthivorous trophic ecology, may impact the reproductive success of other fishes and/or the macroinvertebrate community, potentially generating indirect trophic effects (i.e. cascade effects).

B. barbus was first introduced to Italy in 1994, specifically in the Po River (Meraner et al., 2013), located in the north of the country. In 1998 it was also introduced to the Tiber basin, a main river catchment in central Italy (Carosi et al., 2017). In both basins the exotic barbel underwent rapid expansion and thanks to its high dispersal ability (some individuals have an home range >20 km, Britton and Pegg, 2011) it was able to colonise all main tributaries (Meraner et al., 2013; Zaccara et al., 2014; Carosi et al., 2017; Zaccara et al., 2019a). Here, the species occurs within the epipotamal zone of rivers where two co-generic and ecologically equivalent species (i.e. fluvio-lacustrine) are present, and populate two different icthyogeographic districts (Buonerba et al., 2015). The Tiber barbel *B. tyberinus* Buonaparte 1839 is endemic to the Tuscany-Latium district, which comprise the Tiber and the Arno basins

and all the catchments that drain into the middle Tyrrhenian Sea (Fig. 2.2 a). The common barbel *B. plebejus* Buonaparte 1839 is endemic to the Padano-Venetian district, formed by all the river basins that drain into the North and the middle Adriatic Sea, including the Po River (Fig. 2.2 b).

The two Italian endemics are of important conservation value. Both are listed in annexes II and V of the European Habitat Directive 92/43/CEE and in appendix III of Bern Convention. *B. plebejus* has been listed as of least concern (LC) in the last update of the International Union for Nature Conservation (IUCN) red list (Freyhof, 2011). *B. tyberinus* has been listed as near threatened (NR; Freyhof, 2011) and its population in decline (Fig. 2). Nevertheless, more recent studies highlight a strong decline also for *B. plebejus* that was mainly attributed to the invasion by *B. barbus* (Meraner, et al., 2013) although habitat destruction is also likely contributing (Piccoli et al., 2017).

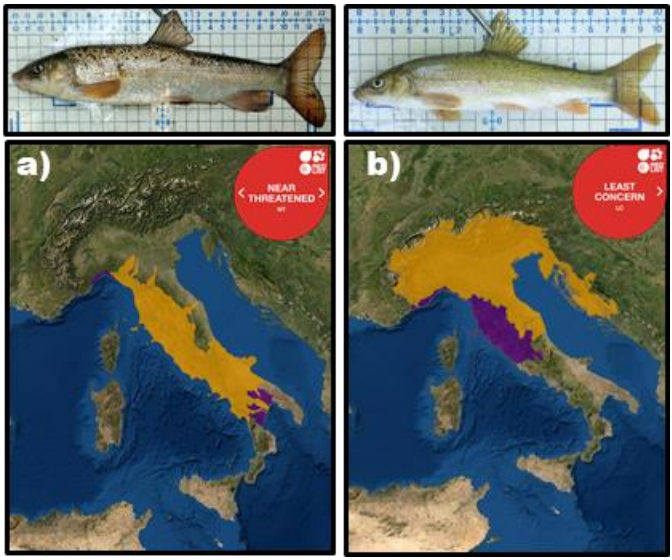

Figure 2.2 *Distribution range of* B. tyberinus *(a) and* B. plebejus *(b) obtained from IUCN website (Freyhof, 2011) with relative threat designation (near threatened for* B. tyberinus *and least concern for* B. plebejus*) and pictures of live specimens taken during sampling campaigns. Yellow are as highlight the natural ranges of the species. Purple areas show where the species have been translocated.*

The main impact of *B. barbus* invasion in Italy has been the interspecific hybridization followed by introgression, a process shown to occur in very short time (i.e. only 5 generations; Meraner et al., 2013; see also Zaccara et al., 2014 and Geiger et al., 2016). These studies focused however only on the detection and description of the introgression process and did not examine potential ecological consequences. Carosi et al. (2017) and Piccoli et al. (2017) examined instead the ecological impacts of *B. barbus* on native Italian fluviolacustrine species. The first

study found that in the presence of *B. barbus*, the endemic *B. tyberinus* had a reduced body condition as a result of resource competition, while the second study found that *B. barbus* is able to take advantage of degraded habitats avoided by the native *B. plebejus*. These studies relied on morphological traits or a single mitochondrial marker to distinguish between species. However, in cryptic species like the fluvio-lacustrine barbels, this is not efficient, especially when hybrid forms are present (Geiger et al., 2016). Moreover, trophic interactions between *B. barbus* and the endemic Italian species have been speculated based on their functional similarity but never directly tested. In the early phase of the *B. barbus* invasion, the species seemed to be limited to the lowland parts of the rivers (Piccoli et al., 2017), however upstream expansions favoured by river restoration projects that aim to remove migration barriers have been detected (e.g. Zaccara et al., 2014; Carosi et al., 2017). As a consequence, native populations that have not yet been impacted and found refuge in the headwaters are potentially at risk and require imminent conservation actions.

2.2 Thesis aims and outline

Despite the rapid expansion of *B. barbus* in Italian rivers, the decline in Italian native barbel species and the detected introgressive hybridisation, little information is available regarding the mechanisms of invasion by European barbel and the associated ecological consequences, especially in Italy. The broad aim of this study was therefore to clarify the evolutionary and trophic consequences arising from *B. barbus* invasion. Trophic interactions are key drivers of the invasion process and their study is therefore essential to understand the resulting impacts. Moreover, genetic introgression is increasingly recognised as an important evolutionary force whose ecological consequences are still unclear. Although hybridisation between endemic and exotic barbels in Italy was detected previously in some populations (e.g. Po River basin), hybrid ecology was not characterised. Hybrids may display new or intermediate traits compared to the two parental species that, under certain circumstances may favour them as documented in several vertebrate taxa, including fish (Best et al., 2017).

Such knowledge is not only important to better allocate conservation efforts but also provides valuable information on the evolutionary mechanisms that control species range expansions. Such understanding is important for our

understanding of how life on earth has been shaped and how it will respond to global change.

Barbels are cryptic species (Geiger et al., 2016; Zaccara et al., 2019a). Only a few characters differ between spcies, making distinction based on their morphologies difficult. *B. barbus* introductions and translocations (Bianco, 1995; Meraner et al., 2013; Zaccara et al., 2019b) has made tracking species (and hybrids) distribution based on their phenotypic characters even more difficult and molecular tools are required to distinguish between species (and their hybrids). Nevertheless, to date, molecular studies have not yet been performed in Southern Italy. Considering that, being in the Mediterranean region, Italy is a biodiversity hot spot, the possibility that barbel diversity was underestimated is likely. Filling this gap is fundamental for an appropriate management of species that are often subject to restocking plans.

Given these premises, the specific aims of this thesis (Fig. 2.3) were to:

I) Investigate the trophic ecology of purebred *B. barbus* in order to characterise its ability to acquire food resources and evaluate the strength and consequences of competitive interactions with non-barbel fishes;

II) Characterise the hybrid forms beteween the European barbel and the native Italian barbels from a morphological and ecological point of view;

III) Provide evidence for cryptic diversity patterns of *Barbus* genus in Italy to better define conservation strategies;

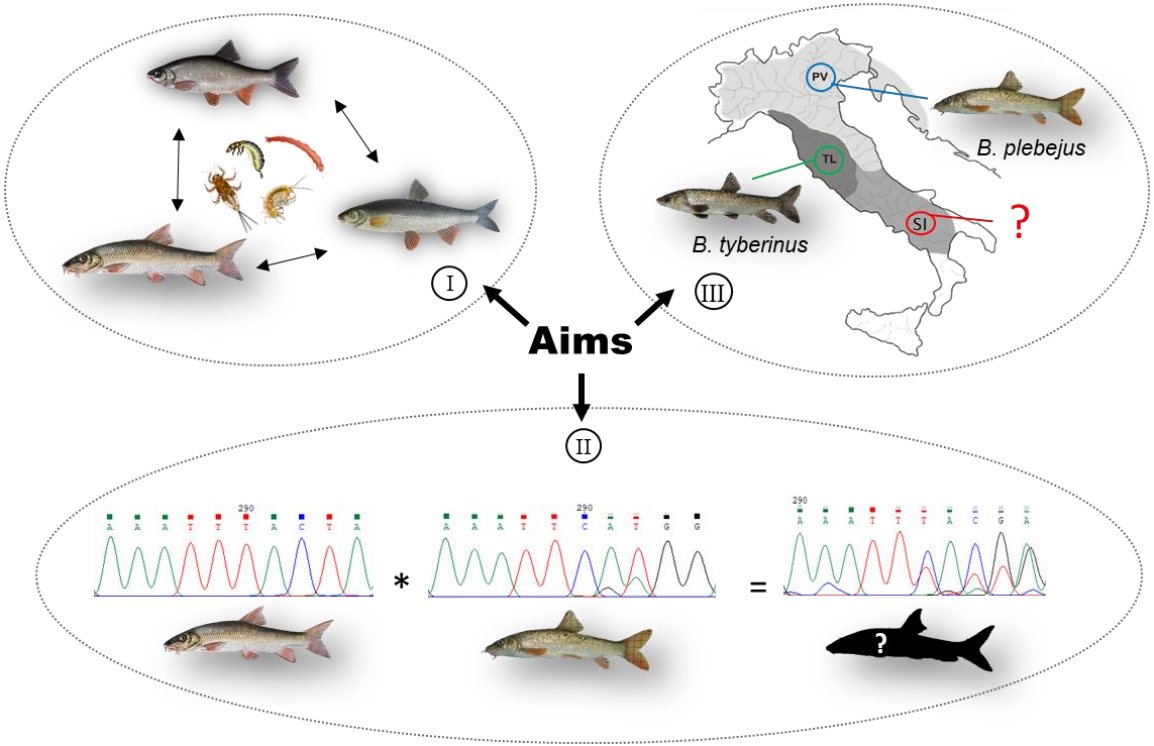

Figure 2.3 *Graphical outline of the three specific aims of this thesis*

2.2.1 B. barbus *trophic ecology*

Rivers are among systems the most impacted by human activities. As such, decoupling between the effects of biological invasions from other confounding factors can be difficult (Corse et al., 2015). Experimental approaches allow researchers to isolate and simulate trophic interactions between selected species (e.g. native *vs.* invasive) under controlled conditions (Britton, 2019). In spite of their utility, empirical experiments may lack realism. However, experiments at different spatial scales that are characterised by a growing degree of ecological complexity (e.g. from tank aquaria to pond mesocosms) can be used to overcome this. Such empirical approaches can be useful both to predict the impacts of a selected exotic species or to clarify the mechanisms behind the observed impacts (Britton et al., 2019). A combination of experimental settings was used in **Chapter III** to study potential competitive interactions between *B. barbus* and other two cyprinid species, *Squalius cephalus* (L.) and *Leuciscus idus* (L.).

Increased interspecific competition is one of the main drivers of the impacts deriving by IAS. However, as there is a growing evidence of non-linearity between invader abundance and impact, it is important to understand how the strength of competition varies with invader biomass (Sofaer et al., 2018).

While considering the severity of the impacts of increased competition that follows the invasion of a community, it is also important to account for the effect of increased intra-specific competitions (Buoro et al., 2016). In **Chapter IV**, a mesocosm experiment study was conducted to assess how the trophic impacts of *B. barbus* on *S. cephalus* trophic niche and somatic growth varies across different abundances and how intraspecific competition was related to these impacts.

A possible alteration of the trophic interactions predicted in experimental approaches can be represented by the contribution to fish diet of allochthonous resources (Bašić et al., 2015; Gutmann Roberts et al., 2017). Energy inputs in rivers from terrestrial ecosystems can be mediated by insects, while anadromous fishes like salmon transfer nutrients from marine to freshwaters during their spawning migrations. Similarly, pelletized energy-rich fishmeal that originate from aquaculture and is increasingly used within European recreational anglers can constitute alternative food resources for freshwater fishes. In **Chapter V** we tested how the trophic ecology of barbel varied spatially, with fish size and in relation to the use of pelletized fishmeal in 11 populations in the UK. Some individuals may specialise on these alternative trophic resources, easing the co-existence with indigenous fishes and limiting the impact of an invader accordingly.

2.2.2 *Morphological and ecological traits of* B. barbus *hybrids*

Extensive introgressive hybridisation between *B. barbus* and the endemic *B. plebejus* was already detected in the Po River catchment and in the Arno basin (Meraner et al., 2013; Zaccara et al., 2014; Geiger et al., 2016). In the Po basin, this has result in unimodal hybrid populations (Meraner et al., 2013) in which hybrids, constituted by several backcrosses and with genotype skewed toward *B. barbus,* tend to dominate until forming hybrid swarms in some populations. This suggest that hybrids are vital, fertile and have a fitness that is presumably higher (or at least equal) than that of the parental species. However, any study to date have addressed further the ecological consequences of the European barbel introgression with the endemic Italian species. It is often assumed that hybrids have intermediate phenotypes to that of the parental species (e.g. Hayden et al., 2011), and at present putative barbel hybrid populations are identified according to their morphologies. However, fluvio-lacustrine barbels are characterised by little appreciable morphological traits (i.e. cryptic) that may result in erroneous "purebred" status attributions, especially in recently introgressed populations. Indeed, phenotypic traits may evolve at a slower rate than genotypes, especially at neutral or under divergent selection loci (Ward et al., 2012a). Moreover, hybrids may have instead

phenotypic traits that resembles one of the parental species (Pfenning et al., 2007; Ward et al., 2012), thus leading to wrong conclusions on the invasion impacts. If hybrids tend to resemble phenotypic traits of the exotic lineage, species displacement due to competitive interactions and species erosion derived by the introgression process may be difficult to distinguish if not addressed properly. Therefore, given the importance of genetic introgression in driving the invasion of *B. barbus,* the study of the functional responses of its hybrids (e.g. morphology, trophic ecology and biological traits) is fundamental to better define *B. barbus* impacts' mechanism and to guide conservation and management programs accordingly.

To fill these knowledge gaps, in **Chapter VI,** phenotypes of two putatively pure populations of the two endemic barbel species (*B. plebejus* and *B. tyberinus* respectively) were compared to the phenotypes of two putative hybrid populations where mitochondrial alleles of *B. barbus* were previously found (Zaccara et al., 2019b). Phenotypic and genotypic variations were analysed and hypotheses on the consequences and mechanisms of *B. barbus* introgression have been discussed. In **Chapter VII** the ecological consequences of *B. barbus* introgression were examined, comparing the trophic ecology (trophic niche width, trophic position and diet composition) and life traits (i.e. somatic growth, demographic structure and body

condition) between the introgressed and the purebred barbel populations identified in Chapter VI.

2.2.3 *Cryptic diversity patterns of the* Barbus *genus in Italy*

Identification of cryptic lineages is fundamental for a correct management and conservation of biodiversity. If a cryptic species complex is attributed to a single species, an inaccurate reconstruction of the distribution range of the latter would lead to an erroneous assessment of its risk status. Moreover, a wrong management would take place in case cryptic species within the same complex would require differentiate conservation actions.

Barbus genus have been widely employed as model in biogeographic studies thanks to it being composed of primary fish species (Buonerba et al., 2015). Phylogenetic relations have been mainly solved in the past 20 years however, new species are continuously described (e.g. Levin et al., 2019). Cryptic species are characterised by very little morphological differences and as such require the use of more sophisticated tools that comprises for instance the use of molecular analysis and geometric morphometry (Geiger et al., 2016; Zaccara et al., 2019). There are still areas in which these surveys have yet to be carried such as the south of Italy. In **Chapter VIII** the

presence of previously undetected lineages was tested in basins of southern Italy along the Adriatic and Tyrrhenian slopes. As the recognition of lineages as species is a useful approach for the conservation and management of endemic fish, in **Chapter IX** the description of the lineages detected in Chapter VIII is proposed.

Finally, in **Chapter X**, the results obtained are discussed and future strategies for the conservation of Italian endemic *Barbus* species are proposed.

CHAPTER III

Predicting the ecological impacts of an alien invader: experimental approaches reveal the trophic consequences of competition

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Key words: Comparative functional response; inter-specific competition, invasive species, non-native, predator-prey.

Abstract

1. Ecological theory on the trophic impacts of invasive fauna on native competitors is equivocal. While increased inter-specific competition can result in coexisting species having constricted and diverged trophic niches, the competing species might instead increase their niche sizes to maintain energy intakes. Empirical experiments can test invasion theory on competitive interactions and niche sizes across different spatial scales and complexity.

2. The consequences of increased inter-specific competition from a model alien fish *Leuciscus idus* were tested on two taxonomically and trophically similar native fishes, *Squalius cephalus* and *Barbus barbus*. Competitive interactions were tested in tank aquaria using comparative functional responses (CFRs) and cohabitation trials. The consequences of these competitive interactions for the trophic niche sizes and positions of the fishes were tested in pond mesocosms.

3. CFRs revealed that compared to *B. barbus, L. idus* had significantly higher attack and consumption rates; cohabitation trials revealed *B. barbus* growth rates were depressed in sympatry with *L. idus*. For *L. idus* and *S. cephalus,* differences in their functional response parameters and growth rates were not significant.

4. In pond mesocosms, used stable isotope were used to quantify shifts in the trophic niche sizes of the fishes between allopatry and sympatry using a substitutive experimental design. Isotopic niches were smaller and more divergent in sympatric paired species than predicted by their allopatric treatments, suggesting trophic impacts from inter-specific competition. However, an all-species sympatric treatment revealed similar niche sizes with allopatry. This maintenance of niche sizes in the presence of all species potentially resulted from the buffering of direct competitive effects of the species-pairs by indirect effects.

5. Experimental predictions from tank aquaria assisted the interpretation of the constricted and diverged trophic niches detected in the paired-species sympatric treatments of the pond mesocosms. However, the all-species sympatric treatment of this experiment revealed greater complexity in the outcomes of the competitive interactions within and between the species. These results have important implications for understanding how alien species integrate into food webs and influence the trophic relationships between native species.

3.1 Introduction

The ecological impacts of biological invasions are wide ranging and include habitat disruption and genetic introgression with native species (Gozlan et al. 2010). Ecological impacts can also develop through the trophic interactions of the invader with native species, including via predator-prey relationships (Dick et al. 2013; Alexander et al. 2014) and competitive interactions with other consumers (Britton et al. 2018). The intensity of competitive interactions and so the severity of their impacts are predicted to be stronger and more intense when the invader and native species are taxonomically and/ or trophically similar due to their likelihood of exploiting similar prey resources (Dick et al. 2017).

Ecological theory can help predict the trophic consequences of biological invasions (Britton et al. 2018). Hypotheses on trophic niche theory suggest how alien and native species can coexist in food webs (Catford, Jansson & Nilsson 2009). If the alien species utilises resources that are unlimited or unexploited by native species, there will be little change in the competitive pressures of the invaded system, enabling the co-existence of species (Mason et al. 2008; Juncos et al. 2015). Should competitive interactions be more intense due to the alien species exploiting similar and limited prey resources to native species, their niches could constrict in size as the diets of each species

becomes more specialized (e.g. Tran et al. 2015; Jackson et al. 2016). These smaller niches might also be divergent if the species exploit alternative resources to minimize their competitive interactions (Busst & Britton 2017; Britton et al. 2018). Competitive exclusion of native species from their original niche could occur if the inter-specific competitive interactions are particularly intense and asymmetric (Bøhn, Amundsen & Sparrow 2008). Conversely, if species diversify their diet in response to increased competition then their niches might increase in size (Britton et al. 2018). The intensity of intra-specific competition can also have considerable influences on trophic niche sizes, with optimal foraging theory predicting that as it intensifies, niche breadths will increase as individuals diversify their diet in response to resource depletion (Svanbäck & Bolnick 2006). Moreover, as competitive interactions are important for structuring the populations of many taxa then understanding how alien species compete with native biota and integrate into native food webs is integral to understanding their ecological impacts (Riccardi et al. 2013; Gallardo et al. 2016).

Across taxa, it remains equivocal as to how these potential shifts in the trophic niches of native species manifest following an invasion (Britton et al. 2018) and so can be investigated further using empirical experiments. Manipulating the abundances of alien and native species enables the outcomes of the altered

strength of their competitive interactions to be measured (Britton 2018). For example, cohabitation pond mesocosm experiments can compare the results of inter-specific competition between sympatric alien and native fishes versus allopatric treatments involving only intra-specific competition (Britton 2018). Alterations in niche sizes and trophic positions between allopatry and sympatry can be quantified by stable isotope metrics (Tran et al. 2015; Britton et al. 2018). The competitive relationships between the species can then be informed by aquaria experiments (Britton 2018). Cohabitation aquaria experiments can utilise the same species as pond experiments, but under controlled conditions (Busst & Britton, 2016), where higher growth rates within species indicates higher resource acquisition and greater competitive ability (Ward, Webster & Hart 2006). Comparative functional response experiments (CFRs) compare consumption rates as a function of prey density between the alien and native species (Dick et al. 2013, 2014, 2017). A species with a significantly higher consumption rate than a comparator species has the ability to acquire more resources, i.e. their inter-specific interactions will be asymmetric.

The aim here was to use these experimental approaches to empirically predict the trophic impacts of an invasion by a model alien freshwater fish on two trophically and

taxonomically similar native fishes. The model area was Great Britain, a temperate region where the model alien fish, ide *Leuciscus idus*, is non-native. The species is, however, present in many lentic environments due to introductions of hatchery reared fish for angling, despite risk assessment suggesting their invasion risk is high in Britain (Britton et al. 2010). It has yet to disperse widely in lotic environments. The species is also taxonomically similar to chub *Squalius cephalus* (synonym: *Leuciscus cephalus*), a native riverine species that tends to coexist with the trophically similar European barbel *Barbus barbus* (Gutmann Roberts & Britton 2018). Consequently, *S. cephalus* and *B. barbus* were the model native fishes. As CFRs tend to predict that high-risk alien species have significantly higher consumption rates than native analogues (Dick et al. 2013), it was predicted that: (i) inter-specific competition between the alien and native fishes would be asymmetric, with *L. idus* the superior competitor; and (ii) this asymmetric competition would result in the native fishes having reduced niche sizes and growth rates when in sympatry compared to allopatry, but with *L. idus* having niche sizes and growth rates similar between allopatry and sympatry.

3.2 Materials and Methods

3.2.1 *Model fishes*

The three model fishes are all species in the Cyprinidae family that are either benthic or bentho-pelagic foragers. Although primarily lotic fishes, they are all also present in a range of lentic habitats (e.g. Jurajda, Ondračková & Reichard 2004; Taylor et al. 2004). Whilst their diets typically comprise of macroinvertebrates, plant material can also be an important food source (Brabrand 1985; Balestrieri et al. 2006; Caffrey et al. 2008). In all experiments, *L. idus*, *S. cephalus* and *B. barbus* were sourced from an aquaculture site in Southern England, with all fish of age 1+years and 65 to 80 mm starting length (individuals of different lengths were randomly distributed across the experiments). All fish were tagged with 7 mm passive integrated transponder tags (approximate weight: 0.03 g) to enable individual identification. Fish were weighed post-tagging (to 0.1 g). These fish had been pond-reared on a diet of natural and formulated feeds. For aquaria-based experiments, the fish were allowed to acclimate to the aquaria conditions for 28 days at 20 ^{o}C before use. In the aquaria, fish were held in 45 L tanks where water filtration was provided via flow-through systems. When not being used experimentally, the fish were fed a formulated feed based on plant material to standardize prior experience. As different batches of fish were used in each

experiment, the fish used in the experimental treatments and replicates were all of similar length and mass to eliminate experimental confounds based on differences in body sizes.

3.2.2 *Comparative functional responses (CFRs)*

The prey species used in the CFRs were *Gammarus pulex* and chironomid larvae. In the experiments, individual fish were randomly selected 24 h prior to use and allocated to 10 L experimental tanks at 20 ^{o}C supplied with oxygen to provide constant conditions. They were kept without food in this period to standardize hunger levels. Individual fish were then presented with a prey species at one of six densities (2, 4, 8, 16, 32 and 64), with a minimum of three replicates generated per density and prey species. Prey exposure was for one hour. The fish were then removed from the tank, the number of prey remaining counted, and the number of prey consumed determined by subtracting this number from the original prey density.

In the CFRs, the comparisons were between the non-native *L. idus* versus the two native fishes. For *B. barbus* and *S. cephalus*, consumption rate data were as per Guo et al. (2017). The *L. idus* consumption rate data were generated at the same time as *B. barbus* and *S. cephalus*, but these data have not been used previously. Analyses of CFRs of all fishes were assessed using

the integrated package for functional response analysis in R ('Frair') (Pritchard et al. 2017). Logistic regressions of prey density versus the proportion of prey consumed were performed per fish species, with type II functional responses indicated by significant negative first-order terms (Pritchard et al. 2017). Values of the attack rate (a) and handling time (h) were then obtained using maximum likelihood estimation (MLE) in the Random Predator Equation (Rogers 1972), which assumes a Type II response and non-replacement of prey:

$$N_e = N_0 (1 - \exp(a(N_e h\text{-}T))) \qquad \text{(Equation 1)}$$

where N_e is the number of prey eaten, N_0 is the initial density of prey, a is the attack rate, h is the handling time and T is the total time available. Finally, to visualise the uncertainty around the fitted functional responses, bootstrapping (n = 1500) was used to construct empirical 95% confidence intervals of the fitted functional responses (Paterson et al. 2015). These bootstrapped data provided the CFR plots between the species; where there was overlap in their 95 % confidence limits, differences in the functional response curves were considered as not significant (Paterson et al. 2015).

3.2.3 *Co-habitation aquaria experiments*

The cohabitation experiments in tank aquaria were completed in 45 L tanks arranged on shelving with three tiers (top, middle and bottom shelves) and completed at 18 ºC on 16:8 h light:dark regime. Each species was used in allopatry (N = 10) and then in each two-species sympatric combination (n = 5 + 5), with three replicates per treatment. Feeding was once per day using a sinking, fishmeal based pellet (1.0 mm diameter; 45 % protein, 20 % oil) at a fixed ration of 2 % mean starting body mass per day. Prior to their release into the tanks, the starting weight of each species per treatment was measured. The experiment ran for 30 days.

At the end of the experimental period, the fish were removed from the tanks and re-weighed. The increase in mass per species and treatment during the experimental period was determined by the 'specific growth rate' (SGR):

$$([(\ln W_{t+1}) \ln W_t) / t]/n) \times 100 \qquad \text{(Equation 2)}$$

where W_t = total starting weight of the species in the tank, W_{t+1} = total finishing weight, n = number of fish, and t = number of days between W_t and W_{t+1}. Differences in SGR between treatments and species were tested in a linear mixed effects model. This tested the effect of the interaction of species x treatment on SGR, where tank position (i.e. whether it was on

the top, middle or bottom shelf) was used as the random variable and fish starting weight was used initially as a covariate. However, starting weight per species was removed from the final model as its effect was not significant ($P > 0.05$). Model outputs were the overall significance of the model and the mean SGR values (± 95 % confidence intervals) according to species and treatment.

3.2.4 *Co-habitation pond mesocosms*

The experimental design was based on substitutive treatments using allopatric and sympatric contexts. There were three allopatric treatments, where each species was used individually (N = 12) and three sympatric treatments using paired species (*L. idus*/ *B. barbus*; *L. idus*/ *S. cephalus*; *B. barbus*/ *S. cephalus*; n = 6+6). A final sympatric treatment then used the three fishes together (n = 4+4+4). All treatments were replicated three times.

The experiment was completed using the treatments within enclosures as per Britton et al. (2018), with the enclosures sitting within a larger, man-made pond (30 x 30 m; 1 m consistent depth) that was located in Southern England. The enclosures comprised of an aluminium frame (length 1.66 m; width: 1.05 m; height: 1.2 m) within a net (mesh: 7 x 7 mm) that prevented fish ingress and egress, but allowed both movements

of invertebrates and the growth of macrophytes into the enclosure. The enclosures were placed randomly across the pond, other than in shallow, littoral areas, with approximately 0.5 m between each enclosure. They remained *in-situ* throughout the experimental period. Their placement on top of the substrate enabled macrophytes to grow through them (*Elodea* spp.); all enclosures had similar areal macrophyte coverage during the experiment. Netting (15 x 15 mm mesh) over the enclosures prevented bird predation. The experiment ran for 150 days from April 2017. This provided time for approximately four stable isotope half-lives in the fish dorsal muscle (i.e. at least 94 % isotopic turnover) (Thomas & Crowther 2015). Temperature loggers (TinyTag TGP-4017) in the larger pond revealed the mean water temperature was 17.3 ± 0.8 ºC during the experiment.

On day 150, all the fish were recovered from the enclosures, euthanized (anaesthetic overdose, MS-222) and taken to the laboratory. Samples of putative food resources were taken from the larger pond for stable isotope analysis (SIA) using a sweep net. These focused on the two major macroinvertebrate putative prey species sampled, *Gammarus pulex* and Chironomid larvae (that also ensured consistency with the CFRs). The presence of these macro-invertebrates was checked in each enclosure at the conclusion of the experiment, although their abundances were

not accurately quantified. No other macro-invertebrates were detected in sufficient abundances inside or outside of enclosures to warrant their analysis; as their abundances were low outside of enclosures then their low abundance inside enclosures was not considered to be due to fish predation pressure. The other major food resource was plant material ('macrophyte') that was highly abundant in all enclosures, and was also sampled for SIA. All putative food resources were sorted into samples (one sample = 3 to 9 individuals per species for macroinvertebrates), with triplicate samples analysed for each group.

In the laboratory, individuals were identified by their PIT tag and re-weighed, enabling calculation of their SGR (Equation 2). A dorsal muscle sample was taken for SIA. SI sample sizes were a minimum of 12 fish per species per treatment, with a minimum of four fish taken randomly per replicate (Britton et al. 2018). All samples were dried at 60 °C to constant mass before SIA ($\delta^{13}C$, $\delta^{15}N$) at the Cornell University Stable Isotope Laboratory, New York, USA. Prior to analysis, samples were ground to powder and weighed (approximately 1000 µg, but with precise measures taken) in tin capsules. They were then analysed on a Thermo Delta V isotope ratio mass spectrometer (Thermo Scientific, USA) interfaced to a NC2500 elemental analyser (CE Elantach Inc., USA). Analytical precision associated with the $\delta^{15}N$ and $\delta^{13}C$ sample runs was estimated at

0.42 and 0.15 ‰ respectively. Data outputs were in delta (δ) isotope ratios (‰). The C:N ratios of the analysed samples were between 3.15 and 3.61, indicating relatively low lipid levels (Post et al. 2007). These ratios did not differ significantly between experimental treatments (Supplementary material; Fig. S3.1). Comparison of original versus lipid-normalised data (Kiljunen et al. 2006) revealed a very strong and significant relationship, indicating that the variability in the original $\delta^{13}C$ data was not an artefact of differences in lipid levels (Fig. S3.2). The shift between the mean original and mean normalised $\delta^{13}C$ data per species and treatment was 0.61 to 0.69 ‰ (Table S3.1), thus had a negligible effect on the relative positions in isotopic space of the species per treatment. In addition, the lipid concentrations of the analysed fish tissues were not a significant predictor of their growth rates, i.e. faster growing fish did not have higher lipid concentrations (Fig. S3.3). Thus, the original $\delta^{13}C$ data were used throughout all analyses, as lipid levels were not a confound in the experiment.

The SI data were used to calculate the trophic niche size of each fish species per treatment using the isotopic niche (Jackson et al. 2011). Whilst closely related to the trophic niche, the isotopic niche is also influenced by factors including growth rate and metabolism, and thus represents a close approximation of the trophic niche (Jackson et al. 2011). The isotopic niche

was calculated using standard ellipse areas (SEA) in SIBER (Jackson et al. 2011; Jackson et al. 2012). This is a bivariate measure of the distribution of individuals in isotopic space, with the ellipses enclosing the core 40 % of data, so indicates the typical resource use of the analysed population (Jackson et al. 2011). A Bayesian estimate of SEA (SEA_B) tested differences in niche sizes between treatments per species, calculated using a Markov chain Monte Carlo simulation (10^4 iterations per group) (Jackson et al. 2011; Jackson et al. 2012). Differences in the size of isotopic niches (as SEA_B) were evaluated by calculating the probability that the relative posterior distributions of the niche size of the allopatric treatment were significantly smaller or larger than those of each of their sympatric niches ($\alpha = 0.05$) in SIBER. The SI data were then used to calculate isotopic niche overlap (%) between the species using SEA_C also calculated in SIBER, where subscript '$_C$' indicates a small sample size correction was used (Jackson et al. 2012). Use of SEA_c was mainly to get a representation of the extent of niche overlap between species, as it is more strongly affected by small sample sizes (< 30) than SEA_B (Jackson et al. 2012).

The SI data were then applied to a Bayesian mixing model to predict the relative proportions of the three putative food resources to fish diet per treatment within the package 'Mixing

Models for Stable Isotope Analysis in R' (MixSIAR; Stock et al. 2018) Stock & Semmens 2016). The model ran using 'short' run length (chain length: 50,000 iterations with burn-in of 25,000, with posterior thinning (thin: 25) and 3 chains). Model diagnostics were based on Gelman-Rubin and Geweke, with sufficient convergence to accept the results (Stock & Semmens, 2013). The isotopic fractionation values between the prey resources and fish were $\delta^{15}N$: 5.10 ± 0.25 ‰; $\delta^{13}C$: 3.8 ± 0.25 ‰, based on the fractionation factors derived for *B. barbus* and *S. cephalus* values on controlled diets based on plant and invertebrate protein sources (Busst & Britton 2016). Mixing model results were reported as means of all feasible solutions, with 5 to 95th percentiles of the distribution ranges.

To assist evaluation of the competition strength within and between species in the treatments, the mean intra- and inter-specific isotopic dissimilarities were calculated (Calizza et al. 2017). For the mean intra-specific isotopic dissimilarity (MND_{ii}), the first step was to calculate intraspecific isotopic dissimilarity (ND_{ii}) for each individual fish per species and treatment, determined as the mean isotopic (Euclidean) distance between each individual and their conspecifics in the treatment. The mean intraspecific isotopic dissimilarity for each species per treatment was then taken as the mean ND_{ii} value of all specimens in that treatment; higher values indicate increased

dissimilarity. The same process was followed to determine the mean inter-specific isotopic dissimilarity (MND_{ij}) per species and treatment, except the first step was to calculate the mean isotopic distance of each individual fish from their sympatric species (ND_{ij}) (Calizza et al. 2017).

The SI, predicted diet, isotopic dissimilarity and SGR data were then tested for differences between treatments. Differences in $\delta^{13}C$, $\delta^{15}N$ and SGR were tested in linear mixed effects models (LMEM). Enclosure was used as a random effect on the intercept to avoid inflating the degrees of freedom that would occur if individual fish were used as true replicates (Tran et al. 2015). Total starting mass of fish in each enclosure was initially used as a covariate, but was removed from final models as it was not significant ($P > 0.05$). Outputs from the models were the mean $\delta^{13}C$, $\delta^{15}N$ and SGR per species and treatment. The mean $\delta^{13}C$, $\delta^{15}N$ and SGR data from the models were then used to determine the extent of the change in each species between their allopatric treatment and each sympatric treatment. The extent of the change between allopatry and sympatry was then also determined for isotopic niche size (as SEAc) and the relative assimilation of each food resource from the mixing model outputs. These data were then tested for the significance of their relationships using linear regression. The relationships of MND_{ii} and MND_{ij} with SGR were also tested using linear

regression to determine if changes in intra- and/ or inter-specific isotopic dissimilarity were significantly related to growth rates. Initially, multiple regression was used, where the mean isotopic dissimilarity that explained most of the SGR variability was indicated by the highest standardised ß coefficient value; univariate linear regression was then used on both dissimilarity indices. Note that in these tests, only data from sympatric treatments were used, as MND_{ij} could only be determined for treatments involving at least two fish species.

Statistical analyses were performed in R (Version 3.5.2; R Development Core Team 2018). In all results, error around the mean represents 95 % confidence limits. All experiments were completed following ethical review and under the UK Home Office project licence 70/8063.

3.3 Results

3.3.1 *Comparative functional responses*

In the functional response experiments, the first order linear coefficient from logistic regressions revealed the functional responses of all species were Type II and significant (first order linear coefficients from logistic regressions: *G. pulex*: -0.02, -0.04, and -0.06, Chironomid larvae: -0.02, -0.01 and -0.06, for *B. barbus*, *S. cephalus* and *L. idus* respectively; $P < 0.01$ in all cases). For *B. barbus* versus *L. idus* using *G. pulex* as prey, *B.*

barbus had a significantly lower attack rate (*a*) and higher handling time (*h*) than *L. idus* (a: 1.18 vs. 3.23, z = -2.64, P < 0.01; h: 0.12 vs. 0.06, z = 2.58, P < 0.01). On Chironomid larvae, *h* was also significantly higher for *B. barbus* (0.03 vs. 0.04, z = 3.93, P < 0.01), but the difference in *a* was not significant (3.38 vs. 4.79, z = -1.42, P = 0.15). In the functional response curves, *L. idus* had higher consumption rates compared with *B. barbus*, with their 95 % confidence limits having minimal overlap (Fig. S3.4, S3.5).

For S. *cephalus* versus *L. idus*, differences in *a* were not significant for *G. pulex* (2.09 vs. 3.23, z = -1.65, P = 0.10), but were significantly higher for *L. idus* on Chironomid larvae (1.37 vs. 4.79, z = -4.18, P < 0.01). Handling times were significantly lower in *S. cephalus* on both *G. pulex* (0.03 vs. 0.06, z = -3.84, P < 0.01) and Chironomid larvae (0.01 vs. 0.03, z = -4.16, P < 0.01). For both prey species, the functional response curves revealed high overlap in the 95 % confidence limits of their consumption rates (Fig. S3.4, S3.5).

3.3.2 *Co-habitation aquaria experiment*

Across the three species, there was considerable variation in their specific growth rates, varying between 0.39 ± 0.21 (*B. barbus* in sympatry with *L. idus*) and 1.07 ± 0.21 (*S. cephalus* in sympatry with *B. barbus*). The LMEM testing differences

across the treatments was significant ($P < 0.01$). For *S. cephalus* and *L. idus*, differences in SGR between treatments were low, with substantial overlaps in their 95 % confidence limits (Fig. 3.1A). However, for *B. barbus*, there was a substantial reduction in SGR in sympatry with *L. idus* compared with their SGR in allopatry (Fig 3.1A).

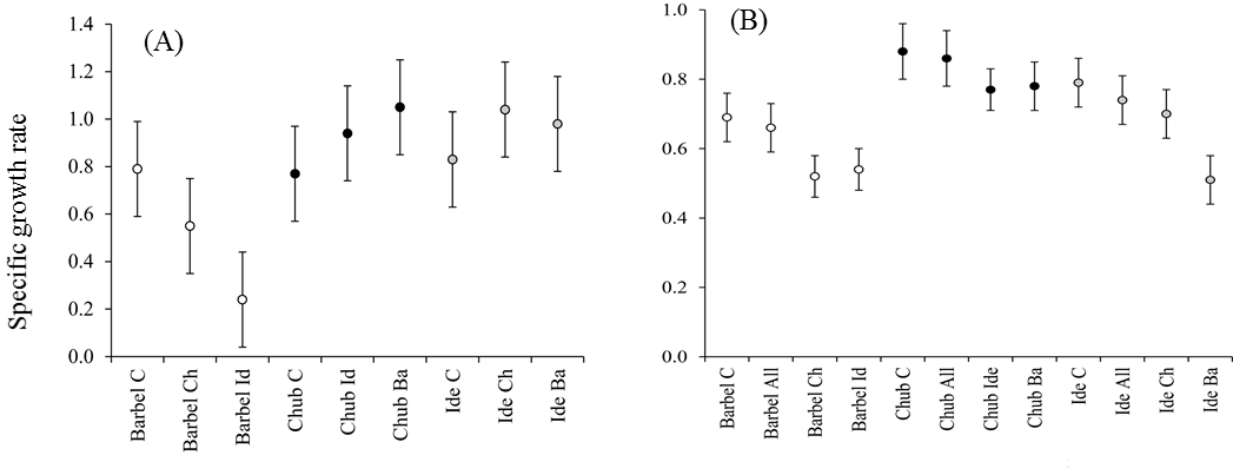

Figure 3.1 *Mean specific growth rates of cohabitation experiments completed in (A) tank aquaria, and (B) pond enclosures, where C = control (i.e. each species in allopatry), Ch = sympatry with chub* Squalius cephalus*, Id = sympatry with ide* Leuciscus idus*, Ba = sympatry with barbel* Barbus barbus*, and All = all species in sympatry. Clear circles: barbel, black circles: chub, grey circles: ide. Note differences in axes values between (A) and (B).*

3.3.3 *Cohabitation pond mesocosms*

The largest ranges of $\delta^{13}C$ and $\delta^{15}N$ across the experiment were measured in the allopatric treatments and the sympatric treatment where all the species were together (Table 3.1; Fig. S3.6). When two fishes were sympatric, the SI ranges reduced, especially in the *B. barbus*/ *L. idus* treatment (Table 3.1; Fig. S3.6). These reduced SI ranges were concomitant with changes in the positions of the isotopic niches between allopatry and sympatry (Fig. 3.2). The predicted isotopic niche overlap between the species in allopatry was 31 to 39 % (Fig. 3.2A). When all the fish were in sympatry, these overlaps were reduced to 3 % for *L. idus* versus *B. barbus*, 11 % for *S. cephalus* versus *L. idus*, and 12 % for *S. cephalus* versus *L. idus* (Fig. 3.2B). This reduction in niche overlap when in sympatry was also apparent in treatments involving two sympatric fishes, where the extent of overlap varied from 5 % for *S. cephalus* versus *B. barbus* (Fig. 3.2D) to 15 % for *S. cephalus* versus *L. idus* (Fig. 3.2E). Concomitantly, isotopic niche sizes (as SEAc) reduced, with the posterior distributions of SEA_B revealing these reductions were significant for both native species in sympatry with *L. idus* (Table 3.2).

Table 3.1 *Minimum, maximum and ranges of $\delta^{13}C$ and $\delta^{15}N$ per treatment in the pond mesocosm experiment. Note data are combined for all species*

	$\delta^{13}C$ (‰)			$\delta^{15}N$ (‰)		
Treatment	**Minimum**	**Maximum**	**Range**	**Minimum**	**Maximum**	**Range**
Allopatric *B. barbus*	-26.3	-23.2	3.1	9.1	9.8	0.7
Allopatric *S. cephalus*	-26.1	-23.4	2.7	9.0	9.6	0.7
Allopatric *L. idus*	-26.1	-23.3	2.8	9.0	9.9	0.9
Sympatric *B. barbus*/ *S. cephalus*	-25.4	-22.9	2.5	9.1	10.2	1.1
Sympatric *S. cephalus/ L. idus*	-25.5	-23.2	2.3	9.2	10.2	0.9
Sympatric *B. barbus*/ *L. idus*	-24.4	-22.8	1.6	9.2	9.8	0.6
All species in sympatry	-26.1	-23.2	2.8	8.9	9.9	1.0

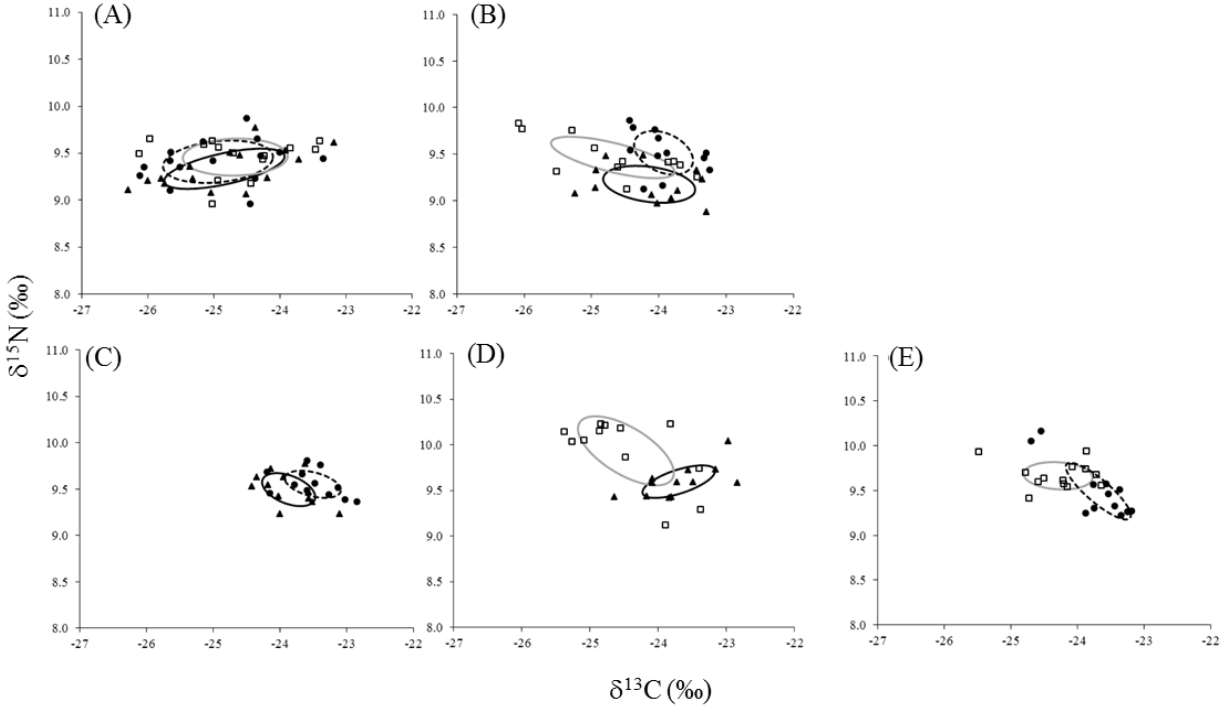
(A)
(B)
(C)
(D)
(E)
δ15N (‰)
δ13C (‰)

Figure 3.2 *Stable isotope bi-plots comparing the standard ellipse area (SEA_C) the fishes in allopatry and sympatry, where A) SEA_C of each species in allopatry, B) the species all in sympatry, C) sympatric* L. idus *and* B. barbus*, D) sympatric* B. barbus *and* S. cephalus*, and E) sympatric* L. idus *and* S. cephalus*. Filled circles/ black dashed line:* L. idus*, filled triangles and black solid lines:* B. barbus*; clear squares, and grey solid lines:* S. cephalus*. The mean SI data for the fish putative food resources were chironomid larvae: $\delta^{13}C$: -31.4 ± 1.5 ‰, $\delta^{15}N$: 5.3 ± 1.5 ‰;* G. pulex*: $\delta^{13}C$: -26.2 ± 0.7 ‰, $\delta^{15}N$: 7.4 ± 0.4 ‰; macrophyte: $\delta^{13}C$: -27.8 ± 0.7 ‰, $\delta^{15}N$: 1.5 ± 0.6 ‰.*

Table 3.2 *Mean stable isotope values, isotopic niche size (as standard ellipse areas, SEA_c (c = correction for small sample size) and SEA_B (Bayesian estimate of SEA) of the macroinvertebrate and macrophytes food resources, and for each fish species by treatment in pond mesocosms. For SEA_B, the mean and standard error at a credible interval of 95% (in parentheses) are presented. *Difference in niche size as SEA_B between the treatment and allopatry is significantly different (P < 0.05).*

Spp.	Treatment	N	Mean $\delta^{13}C$ (‰)	Mean $\delta^{15}N$ (‰)	SEA_c ($‰^2$)	SEA_B $‰^2$ (CI 95%)
Gammarus pulex		3	-26.2 ± 0.7	7.4 ± 0.4		
Chironomid larvae		3	-31.4 ± 1.5	5.3 ± 1.5		
Macrophyte		3	-27.8 ± 0.7	1.5 ± 0.6		
L. idus	Allopatry	15	-24.9 ± 0.2	9.4 ± 0.1	0.61	0.51 (0.31-0.93)
	B. barbus	12	-23.5 ± 0.1	9.5 ± 0.1	0.19	0.19 (0.10-0.34)*
	S. cephalus	12	-23.7 ± 0.1	9.5 ± 0.1	0.27	0.32 (0.15-0.51)
	All species	12	-23.9 ± 0.1	9.5 ± 0.1	0.33	0.33 (0.14-0.53)
B. barbus	Allopatry	15	-24.8 ± 0.2	9.34 ± 0.05	0.51	0.64 (0.26-0.81)
	L. idus	12	-23.9 ± 0.1	9.49 ±0.05	0.21	0.22 (0.08-0.27)*
	S. cephalus	12	-23.7 ± 0.1	9.60 ± 0.05	0.24	0.26 (0.12-0.41)*
	All species	12	-24.1 ± 0.2	9.18 ± 0.06	0.49	0.35 (0.22-0.71)

Table 3.2 (Continued)

Spp.	**Treatment**	**N**	**Mean $\delta^{13}C$ (‰)**	**Mean $\delta^{15}N$ (‰)**	**SEA_c (‰^{2})**	**SEA_B ‰^{2} (CI 95%)**
S. cephalus	Allopatry	15	-24.7 ± 0.2	9.5 ± 0.1	0.52	0.50 (0.27-0.80)
	L. idus	13	-24.3 ± 0.1	9.7 ± 0.1	0.26	0.26 (0.13-0.42)*
	B. barbus	12	-24.5 ± 0.2	9.9 ± 0.1	0.70	0.73 (0.33-1.16)
	All species	12	-24.7 ± 0.3	9.5 ± 0.1	0.50	0.65 (0.25-0.85)

The LMEM testing differences in SGR between treatments was significant ($P < 0.01$). Compared to allopatry, *B. barbus* and *L. idus* growth rates were significantly reduced in their sympatric treatments involving paired species. This was, however, not apparent in *S. cephalus* (Fig. 3.1B), where differences in $\delta^{13}C$ and $\delta^{15}N$ between the species per treatment were also significant ($P < 0.01$). Differences in metrics between allopatry and sympatry per species and treatment revealed that as niche size reduced, $\delta^{13}C$ was significantly more positive ($R^2 = 0.55$, $F_{1,7} = 8.39$, $P = 0.02$; Fig. 3.3A). This was not apparent for $\delta^{15}N$ ($R^2 = 0.01$, $F_{1,7} = 0.74$, $P = 0.79$). The stable isotope mixing model predicted this shift to enriched ^{13}C was through a significant dietary shift away from chironomid larvae and towards macrophyte and *G. pulex* (Chironomid: $R^2 = 0.92$, $F_{1,7} = 65.54$, $P < 0.01$; *G. pulex*: $R^2 = 0.93$, $F_{1,7} = 79.99$, $P < 0.01$; macrophyte: $R^2 = 0.59$, $F_{1,7} = 8.79$, $P = 0.03$; Fig. 3B). The 5 - 95 % percentiles of the mixing model dietary predictions suggested, however, that these dietary shifts were only significant in sympatric treatments involving *B. barbus* and *L. idus*, but not *S. cephalus* (Table 3.3).

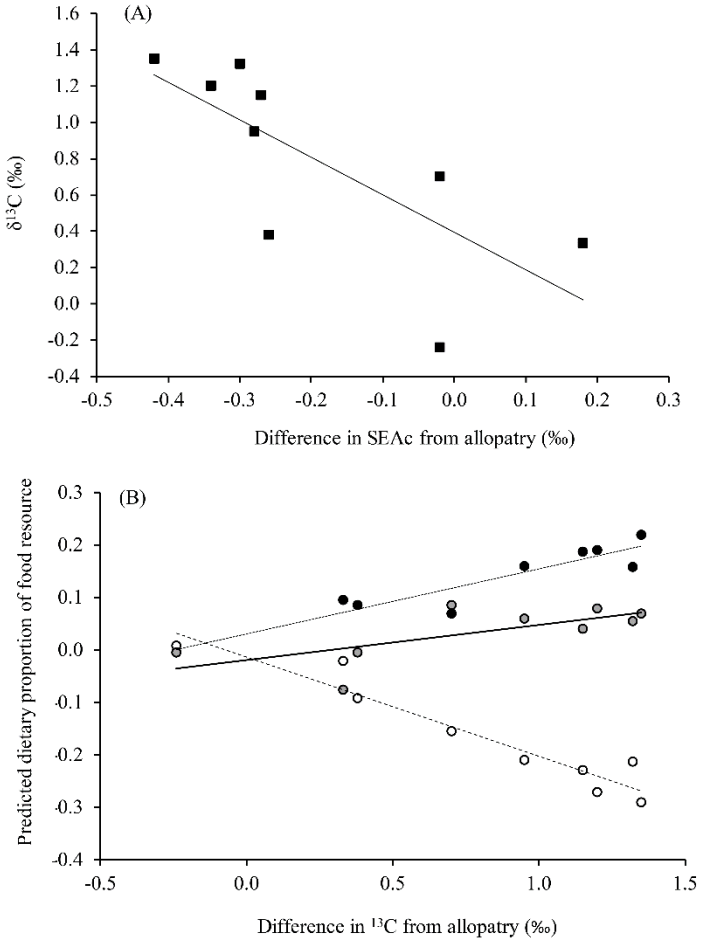

Figure 3.3 *(A) Relationships of differences in isotopic niche size (as SEA_c) between allopatric and sympatric treatments versus their differences in $\delta^{13}C$; and (B) Relationships of differences in mean $\delta^{13}C$ between allopatric and sympatric treatments per species versus differences in their predicted dietary proportions per food resource (Chironomid larvae: clear circles, dashed line; Gammarus pulex: filled circles, small dashed line; macrophytes: grey circles, solid line).*

All straight lines represent the significant linear relationship between the variables (linear regression: $P < 0.03$).

The multiple regression testing the influence of MND_{ij} and MND_{ii} on SGR was not significant ($R^2 = 0.52$; $F_{2,6} = 3.22$, $P = 0.11$), but with MND_{ii} explaining more of the variability in SGR (standardised ß = 0.69, P = 0.09) than MND_{ij} (standardised ß = 0.04, P = 0.93). Univariate linear regression revealed the relationship between MND_{ii} and SGR was significant ($R^2 = 0.47$; $F_{1,7} = 6.32$, $P = 0.04$; Fig. 4A), but was not significant for MND_{ij} ($R^2 = 0.28$; $F_{1,7} = 2.65$, $P = 0.14$; Fig. 3.4B).

Table 3.3 *Predicted dietary proportions of the three putative food resources for the three fishes by treatment in the pond mesocosms.*

		Mean predicted dietary proportion (5-95th percentile of distribution range)		
Spp.	**Treatment**	**Chironomidae**	***Gammarus pulex***	**Macrophyte**
B. barbus	Allopatry	0.33 (0.22-0.44)	0.25 (0.18-0.33)	0.42 (0.35-0.48)
	All species	0.18 (0.09-0.27)	0.32 (0.25-0.39)	0.50 (0.44-0.56)
	S. cephalus	0.10 (0.03-0.19)	0.44 (0.38-0.50)	0.46 (0.40-0.51)
	L. idus	0.12 (0.05-0.21)	0.41 (0.35-0.47)	0.47 (0.41-0.52)
S. cephalus	Allopatry	0.31 (0.21-0.42)	0.28 (0.21-0.36)	0.41 (0.34-0.47)
	All species	0.32 (0.21-0.46)	0.28 (0.19-0.36)	0.40 (0.33-0.47)
	L. idus	0.22 (0.13-0.32)	0.37 (0.31-0.44)	0.40 (0.34-0.46)
	B. barbus	0.29 (0.18-0.42)	0.38 (0.29-0.46)	0.33 (0.26-0.39)
L. idus	Allopatry	0.36 (0.24-0.49)	0.24 (0.16-0.33)	0.40 (0.32-0.47)
	All species	0.15 (0.07-0.23)	0.40 (0.34-0.46)	0.46 (0.40-0.51)
	S. cephalus	0.09 (0.03-0.18)	0.43 (0.37-0.48)	0.48 (0.42-0.53)
	B. barbus	0.07 (0.01-0.14)	0.46 (0.40-0.51)	0.47 (0.43-0.53)

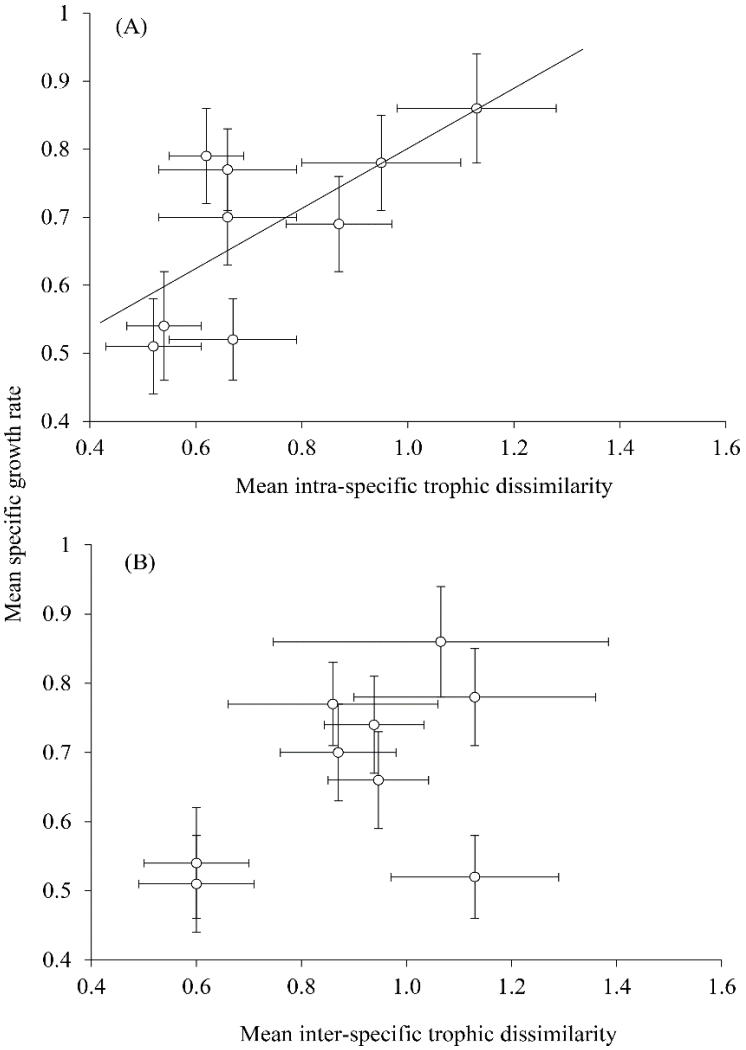

Figure 3.4 *Relationships of the mean intra-specific (A) and intra-specific (B) trophic dissimilarity versus specific growth rate for fishes in sympatric treatments in the pond mesocosm experiment. The solid line represents the significant relationship between the variables according to linear regression.*

3.4 Discussion

In general, CFRs predict that ecologically damaging invaders have higher consumption rates than native species (e.g. Dick et al. 2013; Alexander et al. 2014). Here, they predicted that alien *L. idus* had higher attack rates and lower handling times than native *B. barbus*, resulting in significantly higher consumption rates in *L. idus*. In the cohabitation experiments in aquaria, the growth rates of *B. barbus* were significantly depressed in the presence of *L. idus* compared to allopatry. In contrast, the consumption rates of the taxonomically similar *S. cephalus* and *L. idus* were not significantly different and their growth rates did not differ significantly between treatments in the cohabitation experiment. In combination, these results suggest that competitive interactions between *L. idus* and *B. barbus* were asymmetric, as per the prediction. The superior competitor was *L. idus* due to their greater ability to access prey. This asymmetry in inter-specific competition was not, however, apparent between *L. idus* and *S. cephalus*, contrary to the prediction.

A criticism of CFRs for assessing the ecological impacts of alien species is that they do not adequately represent the ecological complexity inherent within more natural systems, where species can utilise multiple prey resources and are competing within a community of species of varying population

abundances (e.g. Vonesh et al. 2017). They also cannot easily measure the competitive interactions within and between species directly (Guo et al. 2017). This is despite the potential importance of intra- and inter-specific competition in driving invasion-mediated changes in food web structure (David et al. 2017). Notwithstanding, the CFRs here did provide information on the comparative consumption rates of the fishes on the two major macroinvertebrate prey species used in the SIA of the pond experiment. Correspondingly, their predictions provided a basis for evaluating the competitive interactions of the fish in pond mesocosms.

In the pond mesocosms, there were some significant shifts in the size and position of the isotopic niches of the fishes across the treatments. Comparison of the niche sizes of the species in allopatry versus their paired sympatric treatments revealed some important differences. For *L. idus* and *S. cephalus*, the aquaria experiments predicted their competitive interactions would be symmetric and in the pond experiment, their isotopic niche sizes were both reduced compared to allopatry (significantly so for *S. cephalus*). Whilst both species increased their dietary proportions of *G. pulex* and reduced their proportion of chironomid larvae, there were sufficient dietary differences to result in their increased niche divergence in sympatry versus allopatry. This result was consistent with other

studies that suggest trophic niche constriction and divergence occurs when an invader and competing native species exploit similar food resources (Tran et al. 2015; Jackson et al. 2016). The growth rates of both species in sympatry were, however, similar to allopatry. For *L. idus and B. barbus*, the aquaria experimental predictions of asymmetric competition favouring *L. idus* were not evident in the pond mesocosms. When paired, there were significant reductions in niche sizes in both species, with increased niche divergence, when compared to allopatry. These changes were accompanied by significantly reduced growth rates. These results were, however, also consistent with other studies suggesting increased inter-specific competition is an important determinant of invasion-mediated trophic impacts (e.g. Bøhn et al. 2008; Tran et al. 2015).

The results of the sympatric treatment involving all species in the pond mesocosm experiment revealed that compared with allopatry, there were no significant changes in isotopic niche sizes or growth rates of any species. Also, across the entire experiment, there was a significant relationship between reduced growth rates and reduced mean intra-specific isotopic dissimilarity, but not between growth and mean inter-specific trophic dissimilarity. In combination, these results suggest that inter-specific competition was not the only mechanism responsible for the measured changes in isotopic niche sizes and

position across the experiment, with differences in the intensity of intra-specific competition also potentially important. Theory predicts that as intra-specific competition intensifies, individuals should become increasingly opportunistic and thus have greater niche variation (Svanbäck & Bolnick 2006; Rossi et al. 2015). The relatively large niches apparent in all allopatric treatments were consistent with this, where the intensity of intra-specific competitive interactions was assumed to be highest. In the sympatric treatments, however, the smallest isotopic niche sizes occurred when conspecifics were at n = 6, not at n = 4, contrary to theory (Svanbäck & Bolnick 2006). Correspondingly, the interaction of reduced intra- and inter-specific competition in the all-species treatment might have been positively interacting to facilitate the niche expansions (Nelson et al. 2017). Alternatively, in the all-species treatment, the species-pair direct effects that were apparent in the species-pair sympatric treatments might have been buffered by indirect effects (Calizza et al. 2017; David et al. 2017). However, further work is needed to decouple these competition processes to more fully understand why the species-pair direct effects did not scale up and influence niche sizes in the all-species treatment.

The changes in the fish isotopic niche sizes and positions in the pond mesocosms highlight how aquatic invasive species can influence food web structure. In a meta-analysis on the impacts

of aquatic invaders, Gallardo et al. (2016) revealed that competition and predation are the key processes driving ecological impacts in aquatic ecosystems, with indirect competitive effects from alien consumers often adversely affecting native species, leading to substantial modifications in food web structure (David et al. 2017). Invasions of alien fishes including *Carassius auratus*, *Cyprinus carpio*, *Pseudorasbora parva* and *Lepomis gibbosus* have all been shown to result in major re-organisations of the isotopic structure of the food web (e.g. Jackson & Britton 2014; Tran et al. 2015; Copp et al. 2017; Britton et al. 2018). Here, the alien *L. idus* also resulted in some food web re-structuring, with the effects involving both direct and indirect competitive effects depending on the number of fishes in the treatments.

Predicting the trophic consequences of invasive species remains an important theoretical and applied research area. Predictions from CFRs are that high-risk alien species tend to have significantly higher consumption rates than native analogues (Dick et al. 2013), with this consistent across fish (Alexander et al. 2014), amphipods (Laverty et al. 2015), snails (Xu et al. 2016) and decapods (Howard et al. 2018). Here, CFRs were used to predict the symmetry of inter-specific competition between species according to comparisons of their consumer-resource dynamics under standardised conditions. The results

of the pond mesocosms between allopatry and species-pair treatments then revealed some consistency with the CFR results, especially *S. cephalus* versus *L. idus*. In the all-species treatment, however, there was greater complexity apparent in the results, and this complexity was beyond what the CFRs could measure and predict. Thus, whilst CFRs have substantially increased understandings of the trophic impacts of invasive species (e.g. Alexander et al. 2014; Howard et al. 2018), their utility for predicting impacts is more limited in complex environments that involve a number of competing consumers. This is important, as competitive processes are important for structuring populations over a wide range of taxa, including snakes (e.g. Luiselli 2006), lizards (e.g. Mitchell 1979) and birds (e.g. Shochat et al. 2004). Moreover, studies across taxa suggest that the outcomes of competitive interactions are also influenced by a range of traits (e.g. body size and foraging behaviours) that then determine the diet of individuals, with food web structure being the sum of these individual diets (Petchey et al. 2008). The experiment here thus makes an important contribution to understanding how alterations in competition strength within and between species can impact the trophic niche sizes and positions of populations, and thus food web structure, whilst controlling for the effects of body size. The results also highlight how alien species integrate

into food webs and alter the trophic relationships between native species.

A potential confounding effect within the experiments was the use of hatchery-reared fishes, rather than fish collected from the wild. Hatchery-reared fishes were used due to the difficulty of obtaining sufficient numbers of wild fish to satisfy the experimental designs whilst controlling for size. There were also no wild *L. idus* British populations of sufficient abundance to provide the sample sizes. Literature suggests that there can be differences in the behaviours of hatchery-reared versus wild fish. For example, the movement behaviour and habitat use differed between wild and hatchery reared *S. cephalus* (Bolland et al. 2008), although the hatchery fish could cope with elevated flows and remained close to their stocking locations, as per wild fish (Bolland et al. 2009). Moreover, hatchery-reared fishes that are conditioned with natural stimuli and exposed to natural foods tend to have elevated post-release survival and more natural behaviours (e.g. Brown et al. 2003). The hatchery-reared fishes used in the experiments were all pond-reared, feeding on a mix of natural and supplementary foods. Consequently, as their husbandry used similar conditions to those in the enclosure experiment, and involved pond habitats and natural foods, the fish were considered a strong proxy for testing the interactions of wild fishes.

In summary, three experimental approaches tested the trophic consequences of an alien fish on two native fishes. Aspects of the shifts in isotopic niches and growth rates of fish in relatively complex environments were interpreted using the results of two relatively simple experiments completed in controlled conditions. However, the greater complexity of the pond systems when all the species were present resulted in more complex interactions and less predictable outcomes, and highlighted the direct and indirect interactions that enable alien species to integrate into native food webs.

3.5 Supplementary materials

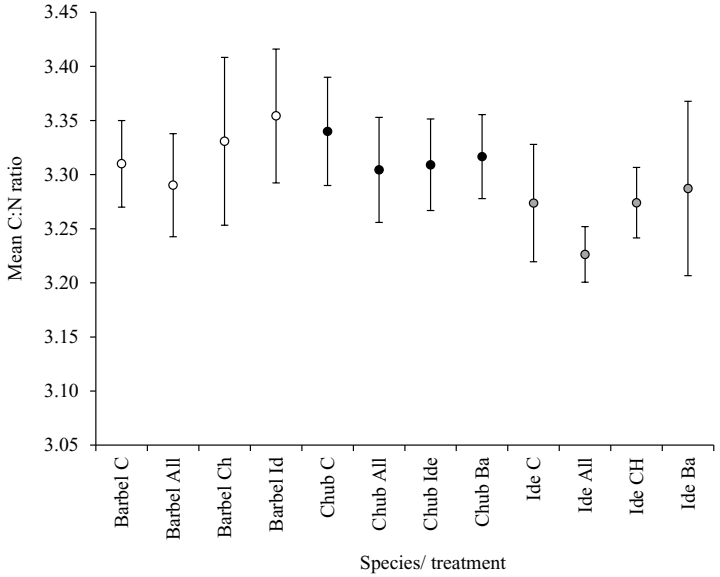

Figure S3.1 *Mean C:N per species and treatment in the pond enclosures, where C = control, Ch = sympatry with chub* Squalius cephalus*, Id = sympatry with ide* Leuciscus idus*, Ba = sympatry with barbel* Barbus barbus*, and All = all species in sympatry. Clear circles: barbel, black circles: chub, grey circles: ide. Note differences in axes values between (A) and (B). Differences in C:N ratios between the species per treatment were not significant ($F_{1,152}$ = 1.74, P = 0.10).*

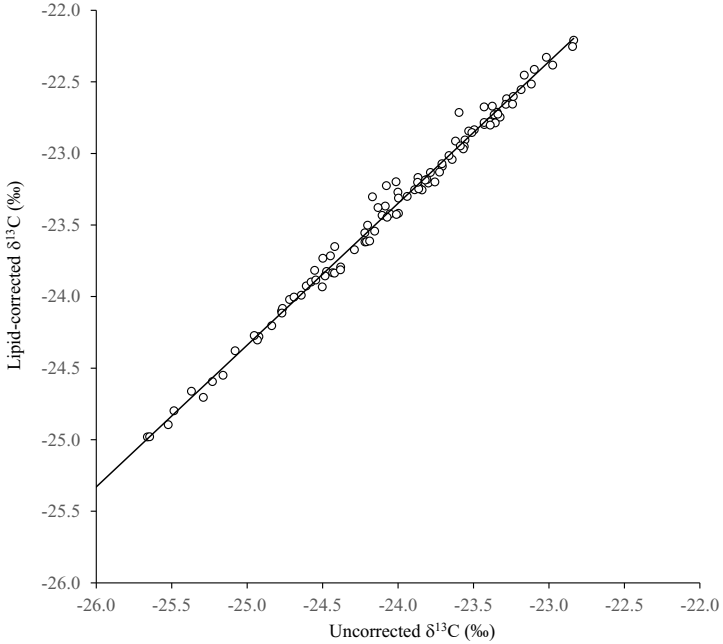

Figure S3.2 *Relationship of uncorrected versus lipid corrected $\delta^{13}C$ for all fish samples (Kiljunen et al. 2006), where the solid line is the significant relationship according to linear regression ($R^2 > 0.99$, $F_{1, 152} = 15066.9$, $P < 0.001$).*

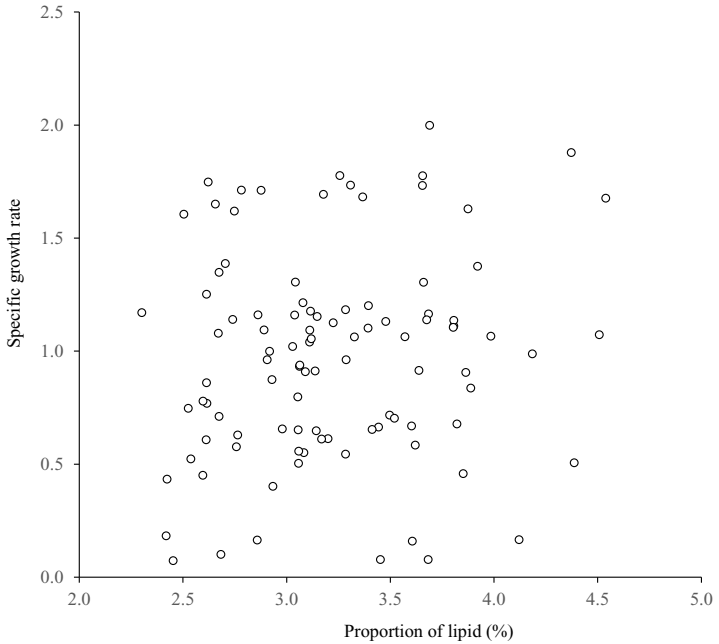

Figure S3.3 *Relationship of proportion of lipid in the analysed dorsal muscle samples of each individual fish, as calculated $\delta^{13}C$ and C:N ratios (Post et al. 2007), versus their specific growth rates. The relationship was not significant according to linear regression (R^2 = 0.02, $F_{1, 152}$ = 2.18, P = 0.14).*

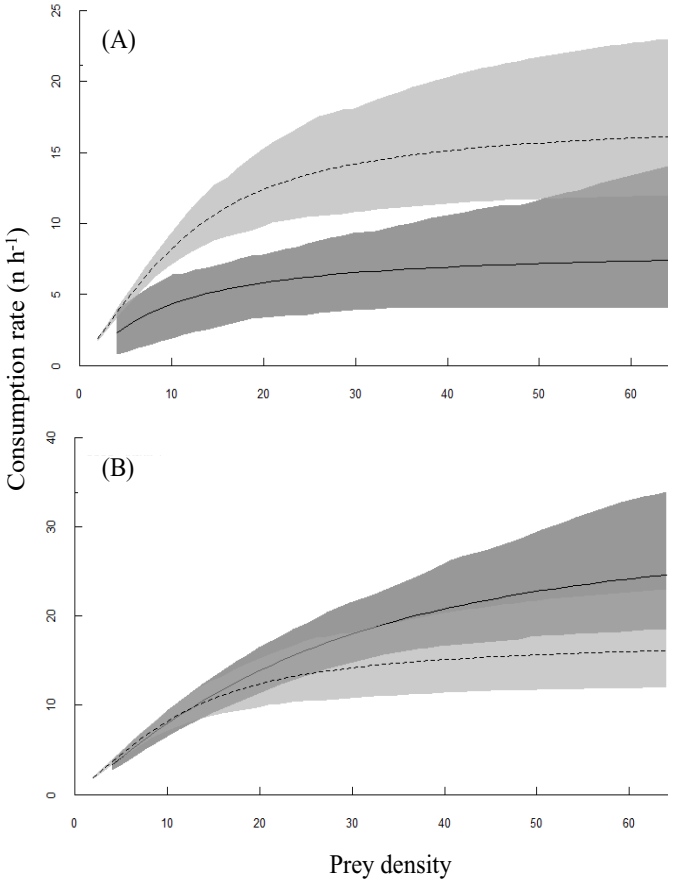

Figure S3.4 *Comparative functional response curves for* Gammarus pulex *as prey, comparing* Leuciscus idus *(dashed line) versus (A)* Barbus barbus *(solid line) and (B)* Squalius cephalus *(solid line). Shaded areas around the curves represent 95 % confidence intervals generated by boot-strapping. Note differences in values on the Y axis.*

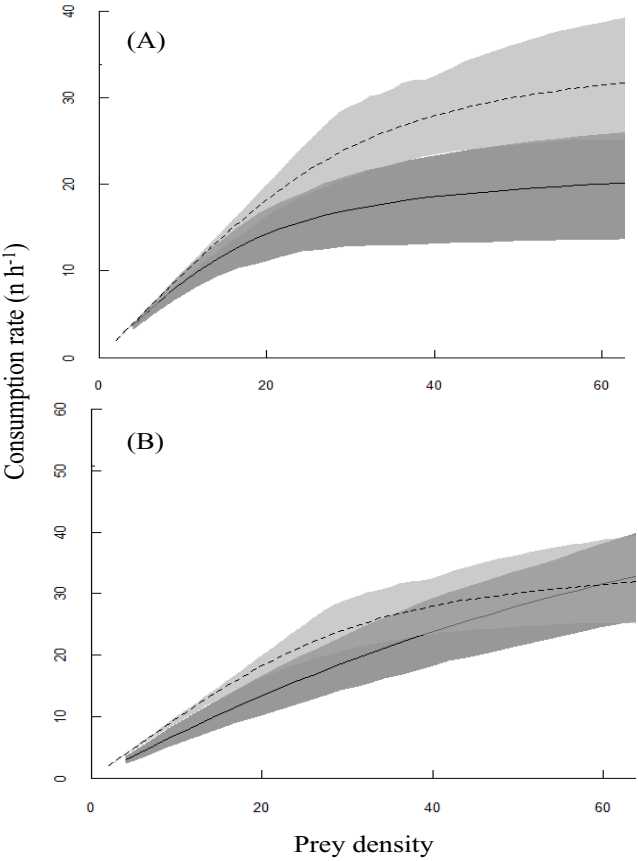

Figure S3.5 *Comparative functional response curves for Chironomid larvae as prey, comparing* Leuciscus idus *(dashed line) versus* Barbus barbus *(solid line) (A) and (B)* Squalius cephalus *(solid line). Shaded areas around the curves represent 95 % confidence intervals generated by boot-strapping. Note differences in values on the Y axis.*

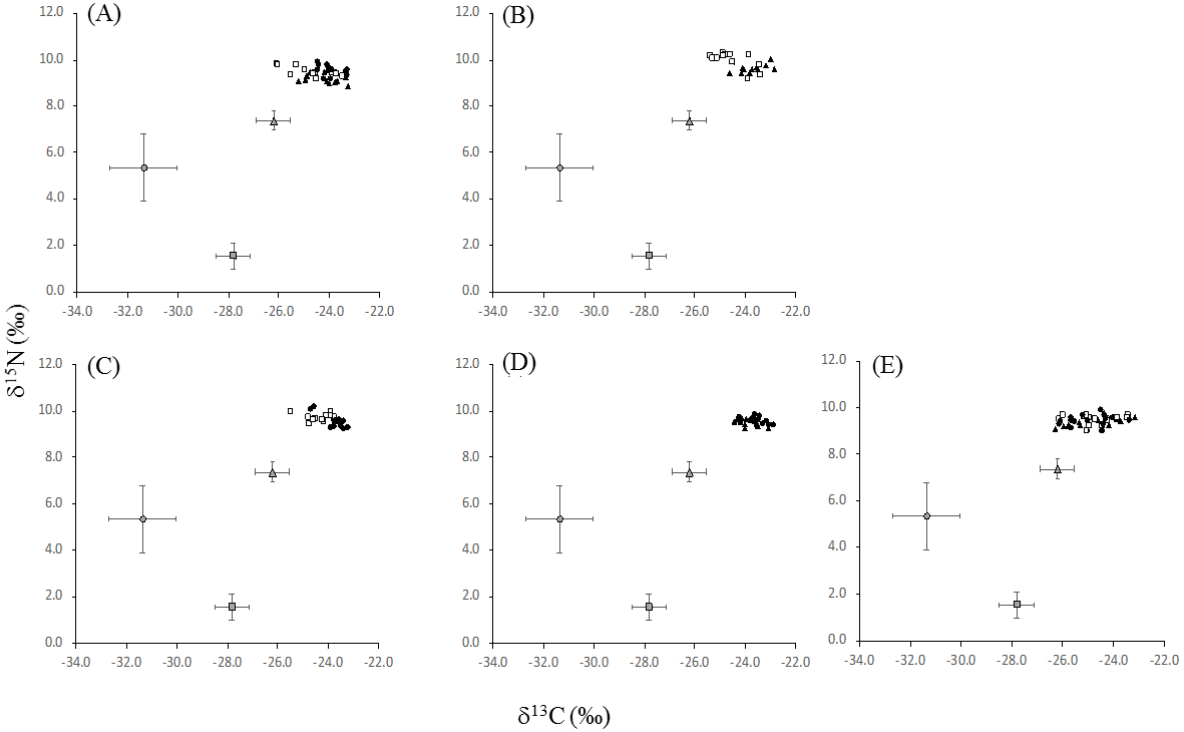
(A)
(B)
(C)
(D)
(E)
12.0
10.0
8.0
6.0
4.0
2.0
0.0
-34.0
-32.0
-30.0
-28.0
-26.0
-24.0
-22.0
δ15N (‰)
δ13C (‰)

Figure S3.6 *Stable isotope biplots for (A) All species sympatric treatment; (B)* Barbus barbus*/* Squalius cephalus *species pair treatment; (C)* S. cephalus*/* Leuciscus idus *species pair treatment; (D)* B. barbus*/* L. idus *species pair treatment; and (E) All species in allopatry. For fish, filled circles:* L. idus*; filled triangles:* B. barbus*; clear squares:* S. cephalus*. For putative prey used in the stable isotope mixing models to predict fish diet, grey circle = Chironomid larvae; grey triangle =* Gammarus pulex*; grey square = macrophyte. Error bars represent 95 % confidence limits.*

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CHAPTER IV

Trophic consequences of competitive interactions in freshwater fish: density dependent effects and impacts of interspecific versus intraspecific competition

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Abstract

1. Determining the comparative impacts of increased intra- versus inter-specific competition is important in freshwater ecosystems for understanding the ecological changes can result from activities such as fish stocking events (using alien and/ or native fish species), as well as from natural processes that elevate population abundances (e.g increased annual recruitment success). While increased inter-specific competition can result in slower growth rates and/ or reduced population density in the weaker or less abundant competitor, it is important that this is assessed in relation to the impacts of increased intra-specific competition.

2. We tested how the strength of inter-specific competition from a co-existing species varies with abundance, and how this compares with increased intra-specific competition. Fish were the model taxa, as their growth rates strongly correlate with competitive success. Replicated pond mesocosms (150 days) used chub *Squalius cephalus* in an allopatric control (n=5; C5) and allopatric treatment (n=10; C10), and in sympatric treatments (n=5) with European barbel *Barbus barbus* (n=5 (T1), 10 (T2) and 15 (T3)). Treatment effects were tested on fish specific growth rates (SGR), and the size and position of the trophic and isotopic niche (stomach contents and stable isotope analyses (SIA) respectively).

3. Chub SGRs were significantly higher in C5 versus all other treatments but did not differ among the other allopatric and sympatric treatments. Chub trophic niche sizes in T1 to T3 were significantly smaller than C5, indicating more specialised diets in the presence of barbel. Chub trophic niche size in C10 was, however, larger than C5 and T1, indicating a shift to a more generalised diet as intra-specific competition increased.

4. As SGRs reduced in treatments, so did the predicted extent of fish stable isotope turnover, with SI data in T1 to T3 not at isotopic equilibrium with their diet in the mesocosms at the experiment's end. Following conversion of fish SI data to represent values at 95% isotopic turnover, chub isotopic niches also revealed shifts to a more general diet as intra-specific competition increased, but to more specialised diets as inter-specific competition increased.

5. Increased intra- and inter-specific competition impacts on the trophic and isotopic niches were contrasting; both metrics indicated niche constrictions in sympatry but niche expansions in allopatry. Impacts on fish growth were evident from both. These results have important implications in evaluating the ecological significance of competitive impacts resulting from intra- and inter-specific competition.

4.1 Introduction

Anthropogenic activities in freshwater ecosystems frequently manipulate the fish assemblage to either diversify the species present and/ or increase their abundance (Piria et al. 2018; Vitule et al. 2019). This often involves the release of alien species that can ultimately result in an invasion that could have detrimental impacts on native biodiversity (Simerloff et al., 2013; Dominguez Almela et al., 2020). However, it also often involves the release of native species, either translocated from other water or through use of hatchery-reared fish (Cowx and Gerdeaux 2004). Irrespective of whether the released fish are of alien or native origin, they have the potential to impact native species through increased inter-specific competitive interactions (Gozlan et al., 2010; Britton et al., 2018). The intensity of trophic impacts resulting from these interactions can be more severe when the released and native species are taxonomically similar (Ricciardi & Atkinson, 2004; Li et al., 2015) or functionally analogous (Dick et al., 2016, 2017), as it is more probable that the species will share the same prey resources (Buoro et al., 2016). However, increases in the abundance of fish populations can also occur naturally, especially in temperate lowland rivers where the main drivers of recruitment success are abiotic factors such as water

temperature and river discharge that fluctuate annually (Nunn et al. 2007).

The ability of fishes to co-exist within communities is at least partially related to the extent of partitioning of the prey resources between the species, which then relates to how the trophic niche of each species is modified between their allopatric and sympatric contexts (Britton et al., 2018). There are a number of hypotheses regarding how the trophic niches of co-existing species respond to changes in the intensity of their inter-specific competitive interactions (Ricciardi et al., 2013). If the species co-exist in an ecosystem where some prey resources are either unexploited or under-utilised, then the increased exploitation of these resources by at least one of the species should reduce their inter-specific competitive interactions (Okabe & Agetsuma, 2007; Mason et al., 2008; Juncos et al., 2015). Where the resources are either fully exploited or less abundant in the new ecosystem, niche theory suggests that through increased inter-specific competitive interactions, the trophic niche sizes of all species will be reduced compared with their allopatric contexts (Bolnick et al., 2010; Tran et al., 2015; Jackson et al., 2016). Alternatively, this increased inter-specific competition can result in larger niche sizes through the populations exploiting a wider range of prey items (Svanbäck & Bolnick, 2007). If the inter-specific

competitive interactions are asymmetric between the coexisting species, the weaker species might be competitively excluded (Tran et al., 2015), leading to trophic niche displacement that potentially results in reduced food intake, slower growth rates and/ or reduced population density (Bøhn, Amundsen & Sparrow, 2008).

Given this apparent importance of inter-specific interactions in driving how competition alters the trophic ecology of populations, it is then important to understand how population density modifies the strength of inter-specific competition (Jackson et al., 2014). In invasion biology, impacts are often assumed to increase in proportion with invader abundance (e.g. Yokomizo et al., 2009, Elgersma & Ehrenfeld, 2011), with studies having a tendency to only compare scenarios of high invader density versus situations where the invader is absent (e.g. Britton et al., 2010). There is increasing evidence that many ecological impacts actually increase non-linearly with fish abundance (Elgersma & Ehrenfeld, 2011), with Jackson et al. (2014) revealing that across a range of population densities of the Asian invasive fish, topmouth gudgeon *Pseudorasbora parva*, impacts were both linear (e.g. on phytoplankton standing stock) and non-linear (e.g. on benthic invertebrate abundance). While testing the extent of alien versus native species can be important, it should also be considered in the context of the

strength of increased intra-specific competition, as Buoro et al. (2016) suggested that increased numbers of conspecific fish (e.g. from fish stocking exercises) can have greater ecological consequences than releasing alien fishes, due to the released conspecifics having virtually identical traits to the extant fish that can result in a greater extent of resource sharing.

Therefore, the aim of this study was to test how the trophic ecology (e.g. trophic niche size and position) of a model species is altered by the increased abundance of a co-existing species, and how these impacts relate to those from increased intra-specific competition. The model animals were freshwater fishes, as these are adaptable and tractable animals that provide excellent model systems for experimental competitive studies with, for example, their indeterminate nature of growth enabling correlation with competitive success (Ward et al., 2006; Britton et al., 2019). The model species was chub *Squalius cephalus*, a fish of the Cyprinidae family that is found throughout much of Northwest Europe. Although generally considered a lotic species, it is also encountered in lentic environments. The coexisting species was European barbel *Barbus barbus*, which has been introduced widely outside of its natural range to enhance angling in both lentic and lotic habitats (Taylor et al., 2004; Britton & Pegg, 2011). Alien barbel in rivers in western England usually co-exist with native chub

Squalius cephalus, where the two fishes tend to be the largest cypriniform fishes present (Gutmann Roberts & Britton, 2018a,b). In rivers in Eastern England, they coexist as native species, as barbel is considered indigenous in these areas due to its post-Pleistocene colonisation of eastern flowing rivers that had connection with the Rhine and Danube (Wheeler & Jordan, 1990; Antognazza et al. 2016). The relatively large body sizes and omnivory of both species suggest they will also share similar prey resources, especially in the absence of recreational angling that can otherwise result in some barbel feeding mainly on angler bait (De Santis et al., 2019). Correspondingly, using a pond mesocosm experiment with chub as the model species and barbel as the co-existing released species, the experiment tested the relative strength of increased inter- and intra-specific competition on chub somatic growth rates, and their trophic and isotopic niche sizes.

4.2 Materials and Methods

4.2.1 *Experimental design*

The experimental design (hereafter referred to as the 'experiment') used 5 additive and substitutive treatments across a combination of allopatric and sympatric contexts, with each treatment replicated three times (Table 4.1). Two control treatments used native chub in allopatry ('Allopatry'; N = 5, 10;

Table 4.1). Three substitutive treatments then paired the native chub and non-native barbel in the three different sympatric combinations (Table 4.1). All the fish used in the treatments were juveniles (starting mass 2.5 to 3.8 g) and sourced from a hatchery in southern England where they had been pond-reared for at least six months prior to the experiment and so were expected to demonstrate natural behaviours. The experiment ran for 150 days between March and July 2018, providing time for the fish to potentially be at isotopic equilibrium with their new diet, given that for fish of starting weight < 10 g, the mean estimate stable isotope half-life of dorsal muscle is 36 and 38 days for $\delta^{13}C$ and $\delta^{15}N$ respectively (Thomas & Crowther 2015).

Table 4.1 *Overview of the experimental design, including the name used for each treatment in analyses, where Chub n = number of chub per replicate, Barbel n = number of barbel per replicate, and N = total fish number of fish per replicate. Each treatment was replicated three times.*

	Code	Chub n	Barbel n	N
Allopatric control (5)	C5	5	0	5
Allopatric control (10)	C10	10	0	10
Sympatric treatment 1	T1	5	5	10
Sympatric treatment 2	T2	5	10	15
Sympatric treatment 3	T3	5	15	20

The experiment was completed using treatments within enclosures that were located within a larger, man-made pond (30 x 30 m; 1 m consistent depth), located in Southern England. Following Britton et al., (2018), the enclosures that were constructed of an aluminium frame (length 1.7 m; width: 1.1 m; height: 1.2 m) within a net (mesh: 7 mm^2) that prevented fish in- and egress but allowed movements of invertebrates. The enclosures were placed randomly across the pond, with at least 0.5 m between them; they were sufficiently heavy that they remained *in-situ* throughout the experimental period without movement and they sat on the substrate, with macrophytes (primarily *Elodea* spp.) able to grow within each of them (Britton et al., 2018). The enclosures were covered by netting (15 mm mesh) to prevent bird predation. The total mass of fish per species was weighed (nearest 0.1g) prior to release into each

replicate per treatment. Temperature loggers (TinyTag TGP-4017) in the larger pond (located in the lower third of the water column) revealed the mean water temperature was 15.6 ± 0.2 ºC (range 8.9 to 18.4 ºC) during the experiment. On day 150, all the fish were recovered from the enclosures, euthanized (anaesthetic overdose, MS-222) and taken to the laboratory on ice. For the purpose of stable isotope analysis (SIA), putative prey samples of the fish were collected from the larger pond, comprising of aquatic macroinvertebrates, terrestrial invertebrates and macrophyte samples. These were sorted into samples (one sample = 3 to 9 invertebrate individuals per species), with triplicate samples taken.

In the laboratory, the fish were measured and weighed, and a dorsal muscle sample taken for SIA. Along with the putative prey resources, all samples were dried at 60°C to constant mass before analysis of $\delta^{13}C$ and $\delta^{15}N$ at the Cornell University Stable Isotope Laboratory, New York, USA, where they were ground to powder and weighed precisely to ~1000 µg in tin capsules and analysed on a Thermo Delta V isotope ratio mass spectrometer (Thermo Scientific, USA) interfaced to a NC2500 elemental analyser (CE Elantach Inc., USA). Data outputs were in the format of delta (δ) isotope ratios expressed per mille (‰). As the C:N ratios indicated very low lipid content (≤ 3.5) (Post et al., 2007), data were analysed without lipid corrections.

4.2.2 *Data analysis*

To determine fish growth rates in the experiment, the mean specific growth rate (SGR) in mass per replicate and species was calculated using: $[(\ln W_{t+1}) - (\ln W_t))]/t$, where W_t = mean starting weight of the species in the replicate, W_{t+1} = mean end weight of the species in the replicate, and t = the duration of the experiment (days). A generalized linear model (GLM) tested the differences in SGR between treatments for each species, where SGR was the dependent variable, treatment was the independent variable, and total fish starting mass in each replicate being the covariate. Model outputs were mean SGR per treatment (adjusted for the effect of the covariate) and the significance of differences in SGR between treatments according to pairwise comparisons.

Fish stomach contents analyses were completed by examining the contents of the entire intestine of each fish under a dissecting microscope (×5 to × 50 magnification). During the analyses, the number of empty intestines was noted and converted to the vacuity index ([number of empty stomachs/ number of stomachs]x100), and the prey items identified to the lowest taxonomic group possible before being grouped into the appropriate categories. The initial analyses were for prey specific abundance (%*Pi*), calculated from $100(\Sigma S_i \times \Sigma S_{ti}^{-1})$, where S_i = the stomach content (number) composed of prey $_i$

and S_{ti} is the total number of prey items in stomachs that contained that item (Leunda et al., 2008). For estimating the trophic niche size, the dietary data were square-root transformed and a Bray Curtis similarity matrix built to enable calculation of the 40% standard deviation ellipses through a non-metric multidimensional scaling (nMDS) approach within the R package '*vegan*' within R 3.4.2 (R Core Team 2017) (Oksanen et al., 2019), where the Bray-Curtis dissimilarity index and 30 maximum numbers of random starts were used to identify a stable solution. Then, to assess whether the experimental treatments were having significant effects on these niche sizes, permutational ANOVA was performed for each species within the treatments using the *adonis* function available in the *vegan* R package. To control for any effect of pond mesocosm position in the model, pond number was used as a covariate. Pairwise comparisons were then used to determine the significance of differences between the treatments.

As the treatments were completed within the same larger pond, all the fish had the same isotopic baseline and thus their SI data and niche data were able to be compared between species and treatments without any baseline corrections. Data per species were combined from replicates for each treatment to provide representative sample sizes sufficient for subsequent analyses.

A minimum of four randomly chosen individuals were sampled from each replicate to provide a balanced dataset across the experiment.

The initial analyses using the SI data tested the $\delta^{13}C$ and $\delta^{15}N$ data per replicate versus their SGR. This relationship was significant, with the fish of lower SGRs having significantly higher $\delta^{13}C$ and $\delta^{15}N$ (see Results). This suggested that in some replicates and treatments, the fish had yet to reach isotopic equilibrium with their new diet, which is generally considered to be when the extent of isotopic turnover in tissues is at 95 % (Vander Zanden et al. 2015; Winter et al. 2019). Therefore, the fish SI data were converted to values that represented isotopic equilibrium with their new diet. This required the application of a conversion factor to the SI data that was determined from the relationship between the rate of change in the SI data with the rate of stable isotope turnover as the fish approached dietary equilibrium. This was completed in a three-step process: (i) for each species per replicate, determine the mean SI value by species and predict their mean extent of isotopic turnover during the experiment; (ii) calculate the stable isotope conversion factors each species per replicate; and (iii) apply the replicate- and species-specific conversion factors to each fish in that replicate. These steps were completed as follows:

(i) Following determination of mean $\delta^{13}C$ and $\delta^{15}N$ per species in each replicate ($SI_{\bar{x}}$), the extent of their isotopic turnover in the experiment was then predicted (G_{actual}) using their change in mean mass ($W_{\bar{x}}$) between the start (W_t) and end of the experiment (W_{t+1}). Rates of isotopic turnover can be expressed as a function of change in mass ('G', where $G_{0.5}$ = increase in mass for 50 % isotopic turnover and $G_{0.95}$ = increase in mass for 95 % turnover (Winter et al., 2019). For $\delta^{15}N$ of barbel dorsal muscle, one half-life of isotopic turnover equals 1.39 x body mass ($G_{0.5}$) (Busst & Britton 2018). As equivalent data were unavailable for barbel $\delta^{13}C$, and for $\delta^{13}C$ and $\delta^{15}N$ of chub, then this value of $G_{0.5}$ was applied to both species and isotopes to convert values of $W_{\bar{x}}$ to predicted isotopic turnover rates during the experiment (G_{actual}). This was completed by interpolating to find mass at 95% isotopic turnover ($G_{0.95}$) which we considered isotopic equilibrium with the new diet. For example, using $G_{0.5}$ = 1.39 x body mass (Busst & Britton 2018), a fish of starting mass 3.0 g is predicted to be 11.20 g at 93.75% isotopic turnover (4 half-lives), 15.6 g at 96.9% turnover (5 half-lives), and thus 13.0 at $G_{0.95}$.

(ii) To calculate the conversion factors for each isotope, species and replicate, the initial step was to determine the mean ratio of $SI_{\bar{x}}$ and G_{actual} per species across all replicates. This was taken as the mean value of all of the ratios ($SIG_{\bar{x}}$) calculated for each

replicate ('rep') combination, determined from: [($SI_{\bar{x}rep}$- $SI_{\bar{x}rep+1}$)/ ($G_{actualrep}$- $G_{actualrep+1}$)]. The conversion factor (CF) for each isotope, species and replicate was then determined from [($G_{0.95}$ – G_{actual}) x $SIG_{\bar{x}}$].

(iii) The predicted SI data for each fish ($SI_{G0.95}$) was determined from CF x SI_{actual}, where SI_{actual} was the original value of $\delta^{13}C$ or $\delta^{15}N$ of that fish.

The SI data were then used to calculate the trophic niche size of each species per treatment using the isotopic niche (Jackson et al., 2011). Both SI_{actual} and $SI_{G0.95}$ data were used to assess their niche positions and sizes, where they represented these metrics at the end of the experiment (SI_{actual}) and when the fish would have been at isotopic equilibrium had the experiment continued ($SI_{G0.95}$). Whilst closely related to the trophic niche, the isotopic niche is also influenced by factors including growth rate and metabolism (due to their respective effects on stable isotope turnover rates; Busst & Britton 2018), and thus represents a close approximation of the trophic niche (Jackson et al., 2011). It was calculated using standard ellipse areas (SEA) in SIBER (Jackson et al., 2011), a bivariate measure of the distribution of individuals in isotopic space; as each ellipse encloses ≈ 40% of data, they reveal the population's typical resource use (Jackson et al., 2012). Due to the small samples in the experiment (i.e. <30) a Bayesian estimate of SEA (SEA_B) was used to test

differences in niche sizes between species, calculated using a Markov chain Monte Carlo simulation (10^4 iterations per group) (Jackson et al., 2011). Where 95% confidence intervals of SEA_B overlapped between comparator species, the isotopic niches were interpreted as not being significantly different in size. The stable isotope data were then used to calculate isotopic niche overlap (%) between the species in each treatment and across treatments using SEA_C calculated in SIBER, where subscript '$_C$' indicates a small sample size correction was used (Jackson et al., 2012). Use of SEA_C was only to get a representation of the extent of niche overlap between species, as it is more strongly affected by small sample sizes <30 than SEA_B (Jackson et al., 2012; Syväranta et al., 2013).

4.3 Results

4.3.1 *Fish recovery at the end of the experiment*

At the conclusion of the experiment, the recovery rate of chub from across the mesocosms was 83.3 %, with the main loss being one replicate of C5 (all fish lost) and one replicate of T1 (1 of 5 fish recovered). Both losses were assumed to be due to netting failure due to a storm the day before the experiment's conclusion, with these replicates removed from subsequent analyses. This resulted in the number of chub being analysed

for their stable isotopes in C5 and T1 being constrained to n = 10 and n = 8 respectively (Table 4.2, 4.3).

4.3.2 *Specific growth rates and gut contents data*

The GLM testing the effect of the experimental treatments on chub SGR revealed significant differences between the treatments (GLM: Wald χ^2 = 81.56, P < 0.01), although the effect of initial fish mass was not significant (P = 0.65). SGR was significantly higher in C5 than in all other treatments (P < 0.01 in all cases), whereas differences between C10 versus T1 to T3 were not significant (P = 1.00) (Fig. 4.1).

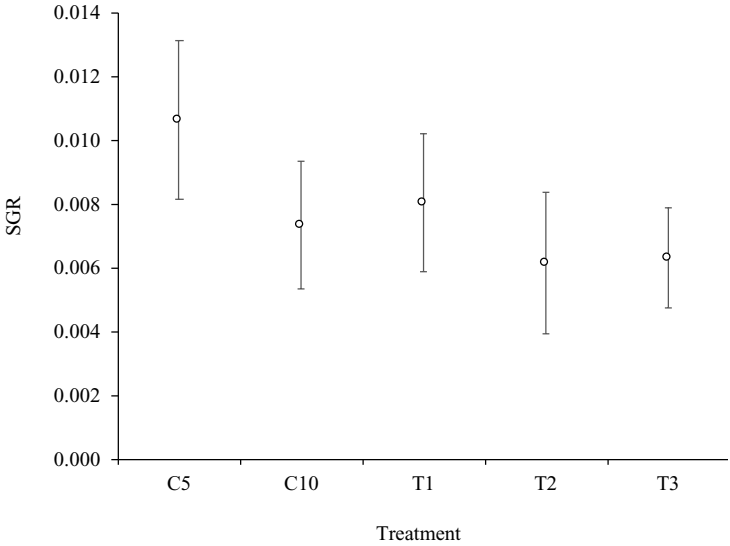

Figure 4.1 *Mean specific growth rates of chub (as estimated marginal means with the effect of fish starting weight controlled as a covariate) per treatment, where the error bars represent 95 % confidence limits.*

The vacuity indices of the fish guts were 0 % for barbel and 1.3 % for chub. The main prey of both species were aquatic insects and macrophytes (Table 4.2). Prey specific abundances varied between species and treatments that translated into considerable differences in trophic niche sizes between the chub treatments with the smallest niche being in T3 and largest in C10 (Table 4.2). These differences in chub niche size were significant (PERMANOVA: $F = 8.02$, $P < 0.01$), with pairwise comparisons revealing the niche size in C5 was significantly larger than those in T1, T2 and T3 (Bonferroni adjusted $P = 0.05, 0.01, 0.02$, respectively). The nMDS plot also revealed some inter-specific differences in trophic niche positions, with intra-specific differences also evident in chub, where niche overlap was apparent between C5 with C10 and T1, but with no overlap in C5 versus T2 and T3 (Fig. 4.2).

Table 4.2 *Prey-specific abundance (% P_i) of principal prey items in fish diet and the associated trophic niche size ('Niche'; as 40% standard deviation ellipses) in barbel and chub between the treatments (C5, C10, T1 to T3), where n = number of fish analysed per treatment and 'Insects' are unidentified aquatic insects.*

				%P_i								
		n	**Niche**	**Insect**	**Macrophyte**	**Corixid**	**Diptera**	**Cladocera**	**Chironomid**	**Hydracarina**	**Chaoboridae**	**Gastropoda**
Barbel	T1	12	0.640	48	32	5	5	8	17	18	5	15
	T2	29	0.498	36	29	8	8	21	29	18	0	2
	T3	41	0.623	48	45	9	5	10	23	10	20	5
Chub	C5	10	0.539	37	23	8	3	5	40	8	22	13
	C10	26	0.744	55	12	28	12	0	11	30	18	12
	T1	8	0.490	59	37	20	20	2	7	20	13	6
	T2	14	0.381	70	35	0	5	0	12	21	15	5
	T3	13	0.215	74	22	5	0	6	9	11	20	0

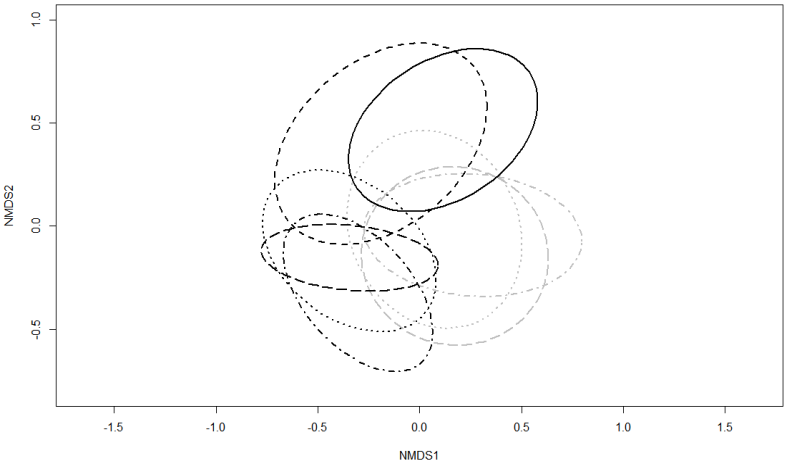

Figure 4.2 *Non-metric multidimensional scaling (NMDS) plot showing the trophic niches as 40% standard deviation ellipses of chub (black) and barbel (grey) per treatment, where lines represent: solid = C5; dashed = C10; dotted = T1; dot-dashed = T2; and long-dashed = T3.*

4.3.3 *Stable isotope analyses*

The relationships of SGR versus both $\delta^{13}C$ and $\delta^{15}N$ (as SI_{actual}) were both negative and significant, with enriched values of both isotopes as SGR decreased ($\delta^{13}C$: $R^2 = 0.88$, $F_{1,12} = 84.08$, $P < 0.01$; $\delta^{15}N$: $R^2 = 0.82$, $F_{1,12} = 54.66$, $P < 0.01$; Fig. 4.3A). Conversion of SGR to the predicted isotopic turnover rate (G_{actual}) revealed the number of half-lives (according to the change in fish mass over the experiment; $G_{0.5}$) varied between 3.4 and 4.7, with this also significantly related to both $\delta^{13}C$ and $\delta^{15}N$, where more enriched isotope values were associated with

lower $G_{0.5}$ values ($\delta^{13}C$: $R^2 = 0.86$, $F_{1,12} = 78.28$, $P < 0.01$; $\delta^{15}N$: $R^2 = 0.68$, $F_{1,12} = 25.85$, $P < 0.01$; Fig. 4.3B). The relationship between SGR and $G_{0.5}$ was also significant, best described by polynomial regression ($R^2 = 0.97$, $F_{2,11} = 88.21$, $P < 0.01$; Fig. 4.3C). Due to these significant relationships of SGR, $G_{0.5}$ and the SI_{actual} data (Fig. 4.3), values of SI_{actual} were converted to their predicted values at $G_{0.95}$ ($SI_{G0.95}$). The conversion had the effect of depleting the $\delta^{13}C$ and $\delta^{15}N$ values of the fish in T1 and T3 (Fig. 4.4A,B) and brought the fractionation factors of the experimental fish with their putative prey resources across the experiment to values generally within the range of those expected in both species (Busst & Britton 2016) (Table 4.3; Fig. 4.4 A, B).

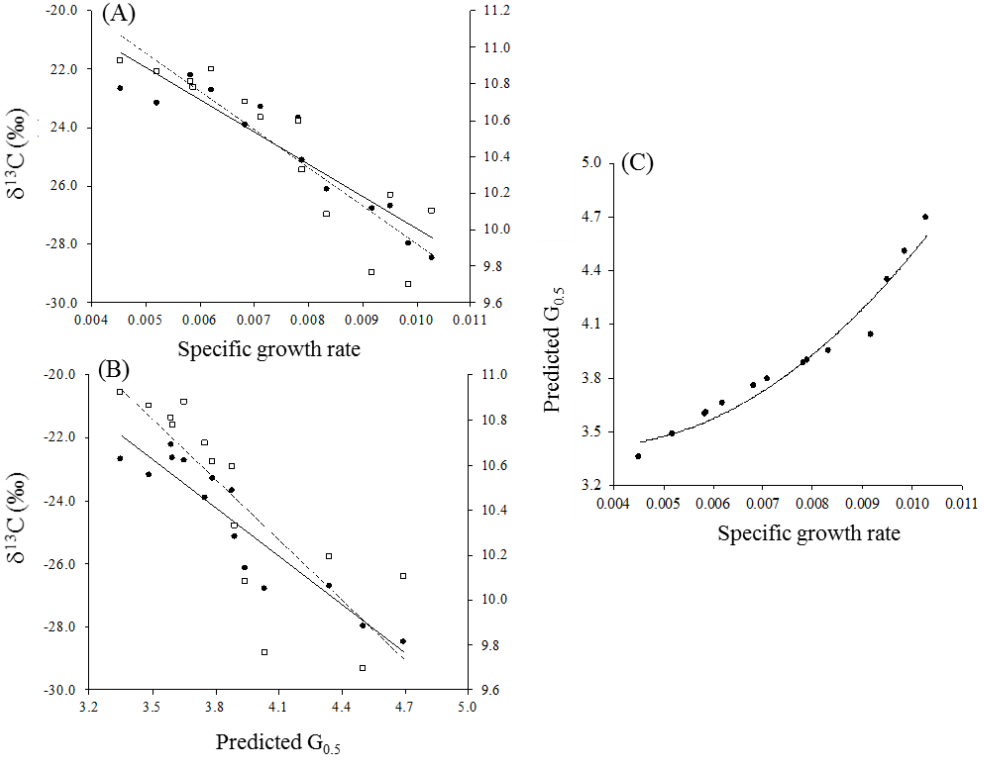
(A)
δ13C (‰)
Specific growth rate
(B)
δ13C (‰)
Predicted G0.5
(C)
Predicted G0.5
Specific growth rate

Figure 4.3 *Relationship of mean $\delta^{13}C$ (filled circle; solid line) and $\delta^{15}N$ (clear circle; dashed line) per replicate versus (A) chub specific growth rate (SGR) and (B) the predicted number of completed stable isotope half-lives (Busst & Britton 2018). Solid lines represent their significant relationships according to linear regression. (C): Relationship of chub SGR versus the predicted number of completed stable isotope half-lives per replicate, where the solid line represents the significant relationship according to polynomial regression.*

Table 4.3. *Mean differences for each prey item between the fish species per treatment for the uncorrected (δSI_{actual}) and corrected ($\delta SI_{G0.95}$) stable isotope data (confidence limits are not shown for brevity); Δ are in ‰. Busst & Britton (2016) predicted for chub, $\Delta^{13}C$ on plant-based diets of 4.24 ± 0.13 ‰ and invertebrate diets of 2.74 ± 0.13 ‰, and $\Delta^{15}N$ on plant-based diets of 6.79 ± 0.10 ‰ and invertebrate diets of 4.59 ± 0.23 ‰; and for barbel $\Delta^{13}C$ on plant-based diets of 5.31 ± 0.09 ‰ and invertebrate diets of 3.97 ± 0.14 ‰, and $\Delta^{15}N$ on plant-based diets of 6.43 ± 0.13 ‰ and invertebrate diets of 5.00 ± 0.21 ‰ (see Fig. 4).*

			Putative prey resource					
			Macroinvertebrate		**Macrophyte**		**Terrestrial insects**	
Species	**SI data**	**Treatment**	**$\Delta^{13}C$**	**$\Delta^{15}N$**	**$\Delta^{13}C$**	**$\Delta^{15}N$**	**$\Delta^{13}C$**	**$\Delta^{15}N$**
Chub	δSI_{actual}	C5	2.2	4.8	5.1	4.8	0.9	5.6
		C10	3.9	4.9	6.8	4.9	2.6	5.7
		T1	5.2	5.1	8.1	5.1	3.9	5.8
		T2	6.6	5.3	9.5	5.3	5.3	6.1
		T3	6.1	5.2	9.1	5.2	4.8	6.0
Chub	$\delta SI_{G0.95}$	C5	3.0	4.8	5.9	4.8	1.7	5.6
		C10	2.7	4.6	5.6	4.6	1.4	5.4
		T1	2.0	4.5	5.0	4.5	0.7	5.3
		T2	1.5	4.3	4.4	4.4	0.2	5.1

Table 4.3 (Continued)

			Putative prey resource					
			Macroinvertebrate		Macrophyte		Terrestrial insects	
Species	**SI data**	**Treatment**	**$\Delta^{13}C$**	**$\Delta^{15}N$**	**$\Delta^{13}C$**	**$\Delta^{15}N$**	**$\Delta^{13}C$**	**$\Delta^{15}N$**
		T3	1.5	4.3	4.4	4.4	0.2	5.1
Barbel	δSI_{actual}	T1	7.3	5.8	10.2	5.8	6.0	6.6
		T2	8.7	6.1	11.7	6.1	7.4	6.8
		T3	8.7	6.1	11.7	6.1	7.5	6.9
Barbel	$\delta SI_{G0.95}$	T1	5.0	5.6	7.9	5.6	3.7	6.3
		T2	6.1	5.8	9.0	5.8	4.8	6.6
		T3	4.9	5.7	7.8	5.7	3.6	6.5

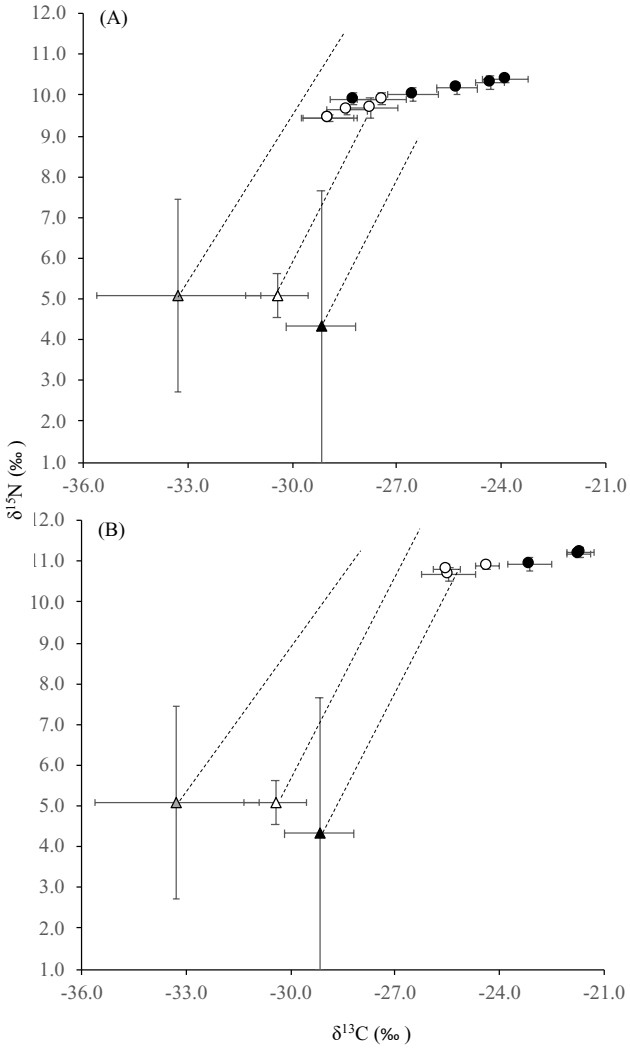

Figure 4.4 *Mean unconverted (δSI_{actual}; filled circle) and converted ($\delta SI_{G0.95}$; clear circles) (± 95 % confidence limits) stable isotope (SI) data for (A) chub and (B) barbel, where clear triangle: mean aquatic macro-invertebrate SI data (n = 15), grey triangle: mean macrophyte*

SI data (n = 3), and black triangle: mean terrestrial invertebrate SI data (n =3). Dashed lines represent the mean fractionation factors of each species with their prey types from Busst & Britton (2016).

The standard ellipse areas (as SEA_B) of both SI_{actual} and $SI_{G0.95}$ data revealed that differences in the isotopic niches of C5 versus all other treatments were not significantly different, with overlap evident in the 95 % intervals around their means (Table 4.4). In all treatments, there were considerable inter-specific differences in the positions of these niches in isotopic space, with no overlap between chub and barbel in T1, T2 and T3 for both SI_{actual} and $SI_{G0.95}$ data (Fig. 4.5A, B). In addition, there were shifts in chub isotopic niche position between C5 and T1 to T3; C5 overlapped with C10 by 99 %, by 19 % with T1, but not overlap at all with T4 and T5 (Fig. 4.5B). For C10, their 95 % intervals around mean SEA_B of $SI_{G0.95}$ was significantly larger than T1 and T2, but not C5 and T3, and had some overlap with all of them (17 to 40 %; Fig. 4.5B).

Table 4.4 *Mean unconverted (δSI_{actual}) and converted ($\delta SI_{G0.95}$) stable isotope data (± 95 % confidence limits) per species treatment, and their mean standard ellipse areas as SEAc and SEA_B (95% credible intervals)*

			δSI_{actual}				**$\delta SI_{G0.95}$**			
Species	**Treatment**	**n**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**SEA_c**	**SEA_B**	**$\delta^{13}C_{G0.95}$**	**$\delta^{15}N_{G0.95}$**	**SEA_c**	**SEA_B**
Chub	C5	10	-28.2 ± 0.4	9.9 ± 0.1	0.96	0.75 (0.42-1.62)	-27.4 ± 0.3	9.9 ± 0.1	0.87	0.73 (0.38-1.48)
	C10	15	-26.5 ± 0.4	10.0 ± 0.1	1.35	1.23 (0.70-2.11)	-27.8 ± 0.4	9.7 ± 0.1	2.17	1.96 (1.11-3.33)
	T1	8	-25.3 ± 0.3	10.2 ± 0.1	0.64	0.50 (0.26-1.11)	-28.4 ± 0.4	9.6 ± 0.1	0.55	0.45 (0.19-0.96)
	T2	13	-23.9 ± 0.3	10.4 ± 0.1	0.59	0.53 (0.28-0.97)	-29.0 ± 0.3	9.4 ± 0.1	0.55	0.51 (0.28-0.92)
	T3	13	-24.3 ± 0.2	10.3 ± 0.0	0.74	0.64 (0.37-1.14)	-28.9 ± 0.2	9.4 ± 0.1	0.79	0.68 (0.38-1.25)
Barbel	T1	12	-23.1 ± 0.3	10.9 ± 0.0	0.87	0.75 (0.40-1.46)	-25.5 ± 0.4	10.7 ± 0.1	1.08	0.96 (0.51-1.88)

Table 4.4 (Continued)

			δSI_{actual}				$\delta SI_{G0.95}$			
Species	**Treatment**	**n**	**$\delta^{13}C$ (‰)**	**$\delta^{15}N$ (‰)**	**SEA_c**	**SEA_B**	**$\delta^{13}C_{G0.95}$**	**$\delta^{15}N_{G0.95}$**	**SEA_c**	**SEA_B**
Barbel	T2	15	-21.7 ± 0.2	11.2 ± 0.1	0.31	0.28 (0.16-0.48)	-24.4 ± 0.2	10.9 ± 0.1	0.34	0.29 (0.18-0.52)
	T3	15	-21.7 ± 0.2	11.2 ± 0.1	0.57	0.51 (0.28-0.87)	-25.5 ± 0.4	10.8 ± 0.4	0.60	0.53 (0.31-0.93)

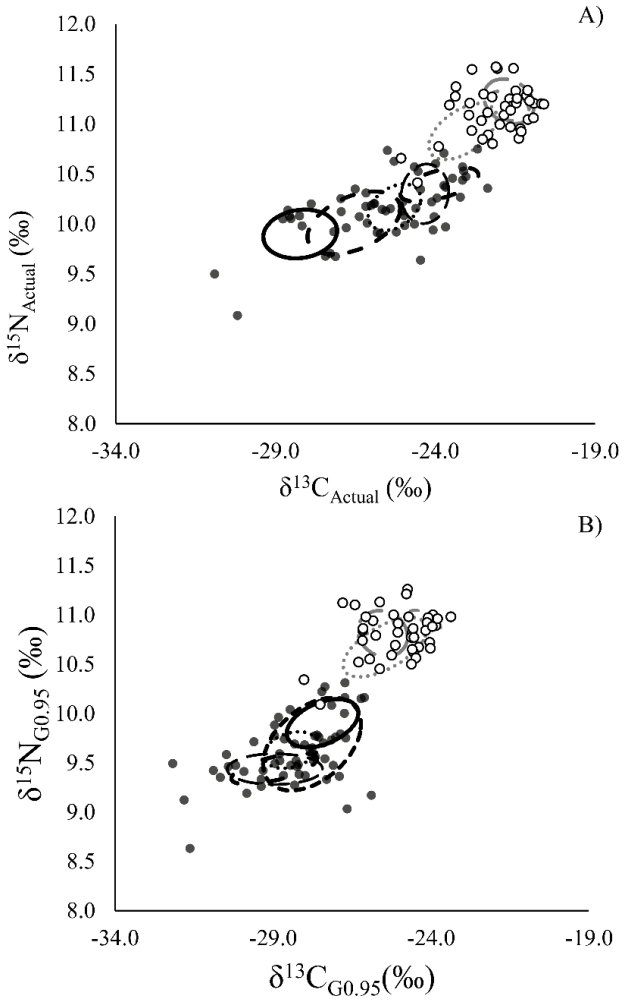

Figure 4.5 *Stable isotope plots for (A) unconverted (δSI_{actual}) and (B) converted ($\delta SI_{G0.95}$) showing the standard ellipse areas (SEA_c) for chub (filled circles; black ellipses) and barbel (clear circles; grey*

ellipses) per treatment, where solid line: C5; solid = C5; dashed: C10; dotted: T1; dot-dashed: T2; and long-dashed: T3.

4.4 Discussion

The presence of coexisting barbel in the sympatric treatments had marked impacts on the growth, isotopic turnover rates and trophic niche sizes of the chub when compared to the allopatric controls. Specific growth rates were significantly reduced in all treatments compared to the C5 control, with these lower growth rates being significantly related to decreased isotopic turnover in the treatments, resulting in the diet of the sympatric chub not being at isotopic equilibrium with their diet in the mesocosms. When the fish stable isotope values were corrected to represent 95 % isotopic turnover since the start of the experiment (i.e. at diet equilibrium), the chub in the sympatric treatments had smaller isotopic niches than C5, with this also evident in their trophic niches (from stomach contents data). Conversely, the isotopic and trophic niches of chub in the allopatric control of C10, where there was twice the number of fish per replicate versus C5, were both larger, despite the reduced growth rates of the fish. These results suggest a fundamental difference in how the ecological consequences of intra- versus inter-specific interactions can manifest (Buoro et al., 2016; Britton et al., 2018).

The results of this experiment that revealed substantial differences in chub growth rates between C5 and the other treatments were considered to primarily be an impact of the increased competition for prey that resulted from the higher fish densities, but with the effect of this being the same for intra- and inter-specific competitive interactions. These results are consistent with Britton et al. (2018), where similar effects were seen in the growth rates of native tench *Tinca tinca* in allopatry versus sympatry with carp *Cyprinus carpio* and goldfish *Carassius auratus*. For *B. barbus*, previous tank-based experiments revealed their growth rates were strongly impacted by density, but with the density-dependent impacts being independent of species (Pegg & Britton, 2011). Across these studies, there is consistency in reduced fish growth rates as the extent of their competitive interactions increase, i.e. the growth is density-dependent (Ward et al., 2006). However, in contrast to here, the differences in density dependent growth did not differ between intra- and inter-specific competitive interactions suggesting some context dependency and/ or species-specific responses in these outcomes.

In contrast to the specific growth rates, there were some marked patterns in the trophic responses of the fish in the treatments. Compared with the allopatric chub treatment C5, the stomach contents data revealed significantly smaller dietary niches in the

species when in sympatry with the alien barbel. Whilst this also had some support from the stable isotope data, there were some overlaps in the extent of the 95 % confidence intervals of SEA_B. These reduced niche sizes suggested chub shifted to be a more specialised diet when sympatric with barbel, a result consistent with the niche variation hypothesis that predicts populations become less generalized in their diet under conditions of increased inter-specific competition (Van Valen, 1965; Thomson, 2004; Olsson et al., 2009). Similar outcomes were evident in native fish communities invaded by *P. parva*, where strong patterns of niche divergence and constriction were detected across a range of spatial scales (Jackson & Britton, 2014; Tran et al., 2015), which were at least partially explained by some of the low threshold, non-linear impacts of *P. parva* on their prey communities (Jackson et al., 2014). However, this niche constriction was only detected in the presence of inter-specific competition; comparison of the trophic niche results of the chub allopatric controls of C5 versus C10 revealed increased niche sizes as intra-specific competition increased. This is also consistent with trophic niche theory that suggests that as resource competition increases, species will exploit a wider dietary base to maintain their energetic requirements (Svanbäck & Bolnick, 2007). Thus, a major finding of this experiment was this fundamental difference between the impact of increased

competition between allopatric (niche expansion) and sympatric (niche constriction) contexts.

These differences in the trophic and isotopic niche sizes of chub between their allopatric and sympatric treatments were despite the isotopic niches of the two species being strongly partitioned (irrespective of whether the uncorrected or corrected SI data were used). These results suggest that the changes detected in chub niche sizes were less likely to relate to their ability to continue to consume their core dietary items, but more likely to be due to the reduced availability of less important items that contributed to their diet on a more occasional basis. However, the experimental design precluded this from being tested. Irrespective, this trophic and isotopic niche partitioning is also evident in other studies that have analysed these species in both experimental and wild settings (e.g. Bašić & Britton, 2016; Gutmann Roberts et al., 2017). For example, in the River Teme, Western England, where alien barbel have been sympatric with chub since the 1970s, the trophic and isotopic niches of the two species tend to be partitioned, with the niche divergence being apparent in their juvenile life-stages (Gutmann Roberts & Britton, 2018a) and then remaining throughout life (Gutmann Roberts & Britton, 2018b). Despite this partitioning, the species do overlap in some aspects of their resource use, such as when they are juveniles when they both consume chironomid larvae

(Gutmann Roberts & Britton, 2018a), supporting the suggestion that the species were competing directly for at least some of the prey resources available in the pond mesocosms here.

The utilisation of two complementary methods of trophic analyses in the study was helpful given that there was an inherent issue with the use of stable isotope data in some of the treatments that related to the extent of their isotopic turnover during the experiment. The turnover rate of stable isotopes within animal tissues varies between tissue types, with faster turnover rates evident in blood and blood plasma compared with white muscle (Vander Zanden et al., 2015; Mohan et al., 2016). In fish, the isotopic turnover rates of $\delta^{13}C$ and $\delta^{15}N$ tend to be slowest in scales, with the rates increasing for fin tissue and then dorsal muscle, but with these rates often varying considerably by species and context (Busst & Britton, 2018). Epidermal mucus tends to show the fastest turnover rates (Church et al., 2009; Winter et al., 2019). Dorsal muscle was used here as the tissue of choice for the SIA, with muscle tending to be the usual tissue used in fish-based studies (Grey, 2006). Also, it was justified by the *a priori* prediction that the fish (due to their size and the duration of the experiment) would complete approximately 4 stable isotope half-lives during the experiment, i.e. approximately 94 % isotopic turnover, where 95 % is considered to be at equilibrium with the new diet (Thomas &

Crowther, 2015), with the predicted chub isotopic turnover rates being 94.8 to 95.9 in C5. In T1 to T3, however, these reduced to 89.7 to 93.0 %, resulting in substantially enrichmed ^{13}C and ^{15}N versus the putative prey resources, presumably due to the remaining influence of their previous diet. Although these data were then corrected, the calculations were based on the *B. barbus* $\delta^{15}N$ turnover rate of Busst & Britton (2018), and thus assumed that: (i) chub has a similar stable isotope turnover rate to barbel; and (ii) the turnover rate of $\delta^{13}C$ in both species is similar to $\delta^{15}N$. Whilst these assumptions were made due to the absence of any other data available on the stable isotope turnover rates for these species, it is acknowledged that this is potentially an issue within these analyses. Nevertheless, the difference in the isotopic niche results were relatively similar for the corrected and uncorrected data, and were consistent with the trophic niche results from the stomach contents data, and so this issue was not considered to be a confound in the experiment.

In summary, this experiment revealed that the impacts of the increasing abundances of coexisting species include increased inter-specific competition that results in dietary specialisation and suppressed somatic growth rates in native species. This result has applicability to manipulations of fish assemblages for angling, whether the species released to enhance fishery

performance is of native or non-native origin, and in situations where there are temporal increases in fish abundance through increased annual recruitment success (Nunn et al. 2007). Although depressed growth rates also result from increased intra-specific competition, increased intra-specific competition resulted in trophic niche expansion and so a shift to a more generalized diet, whereas increased inter-specific competition resulted in niche constriction, so a shift to a more specialised diet. These results thus indicate some important ecological differences in how competitive interactions can manifest within and between species in freshwater fish communities.

Data sharing statement: The data that support the findings of this study will be provided in Bournemouth University's data repository on acceptance.

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CHAPTER V

Influences of angler subsidies on the trophic ecology of European barbel *Barbus barbus*

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Abstract

European barbel *Barbus barbus* is a recreationally important riverine fish that is widely introduced outside of its natural range. Contemporary angling practices for *B. barbus* involve the use of baits based on marine fishmeal (MF). MF is isotopically distinct from freshwater prey via highly enriched $\delta^{13}C$ and thus its dietary influence on *B. barbus* can be tested via differences in fractionation factors ($\Delta^{13}C$). Correspondingly, stable isotope data from 11 riverine *B. barbus* populations tested how their trophic ecology varied across populations according to MF from angling. $\Delta^{13}C$ of fish with macroinvertebrate prey resources varied within and between populations (range 0.90 to 10.13 ‰) and indicated that, within populations, up to 71 % of *B. barbus* had relatively high dietary contributions of MF. These contributions were significantly and positively related to fish length, with MF influences increasingly apparent as fish length increased. Population isotopic niche sizes increased as the dietary influence of MF in that population increased. These results indicated that whilst MF from angling can act as a strong trophic subsidy, its influence varies spatially and with fish length, with its use as a food resource by *B. barbus* generally involving dietary specializations of larger-bodied individuals.

5.1 Introduction

The European barbel *Barbus barbus* (L.) is a fluvial cyprinid fish typically encountered in the middle reaches of European rivers (Huet 1949). Their populations have high recreational value with catch-and-release anglers (Penczak & Sierakowska 2003; Taylor et al. 2004; Britton & Pegg 2011), with this a driver of introductions into waters outside of their native range (Wheeler & Jordan 1990; Taylor et al. 2004; Antognazza et al. 2016). Areas invaded by *B. barbus* include rivers in Western Britain and Italy (Wheeler & Jordan 1990; Antognazza et al. 2016; Zaccara et al. 2014).

The natural diet of *B. barbus* tends to comprise of benthic macroinvertebrates (Gutmann Roberts & Britton, 2018). Despite this, contemporary angling practises for *B. barbus* utilise pelletized marine fishmeal ('pellet'; Bašić et al. 2015; Gutmann Robert et al. 2017). These pellets are commonly used in aquaculture, where their feeding in high quantities promotes fast growth rates via their high protein content (Naylor et al. 2000). In angling for *B. barbus*, pellets of up to 21 mm in diameter are used as both an attractant and hook-bait, and so have the potential to supplement fish diet (Grey et al. 2004; Bašić et al. 2015; Gutmann Roberts et al. 2017). The large size of some of these pellets results in their size-selective

exploitation of *B. barbus*, with fish below 300 mm rarely captured (Amat Trigo et al. 2017).

Novel ecological opportunities can enable individual specialisation in resource use to develop within populations (Britton & Andreou 2016), with examples including when terrestrial insects become available for predation by stream fishes (Syrjänen et al. 2011). Individual trophic specialisation results in the population trophic niche becoming diversified, shifting to consist of sub-groups of specialised individuals (Araújo et al. 2011). In four riverine populations in England, the diets of some large bodied *B. barbus* have been shown to comprise of high proportions of pelletized fishmeal, i.e. they are dietary specialists on this allochthonous resource (Bašić et al. 2015). There was, however, high variability in the contribution by fishmeal to the diets of individuals (Gutmann Roberts et al. 2017). As pellets are selective in the sizes of *B. barbus* capture (Amat Trigo et al. 2017), it is also likely that there will be a strong ontogenetic pattern in the extent of their contribution to diet (Gutmann Roberts & Britton 2018), although this has not been tested. Levels of angling exploitation are also not evenly distributed across river fisheries, with disproportionately high levels of angling exploitation focused on relatively small areas where angling quality is perceived to be highest (Parnell et al. 2010; Post & Parkinson 2012). Correspondingly, the extent to

which angler baits form an allochthonous trophic subsidy for *B. barbus* might also vary spatially.

Stable isotope analysis (SIA) enables the energy sources of riverine consumers to be differentiated between resources derived from freshwater (depleted ^{12}C) and marine (enriched ^{13}C) environments (Jardine et al. 2005; Gutmann Roberts et al. 2017). There tends to be considerable differences in the $\delta^{13}C$ of marine fishmeal pellets and freshwater prey resources (e.g. between 7 and 10 ‰; Gutmann Roberts et al. (2017)). Correspondingly, if a freshwater fish has consumed large quantities of marine fishmeal, their stable isotope (SI) fractionation factors (Δ) with putative macro-invertebrate prey resources should be highly enriched in ^{13}C. Busst & Britton (2016) revealed that when scale tissue was used for SIA in *B. barbus*, maximum $\Delta^{13}C$ with a single formulated food resource was 5.3 ‰. Thus, if the $\Delta^{13}C$ of an individual fish with their putative macroinvertebrate prey exceeds this Δ, it would be assumed that an alternative, highly ^{13}C-enriched source has been a strong contributor to its diet, such as marine fishmeal. Whilst mixing models can predict diet composition from SI data of consumers and their putative prey resources (e.g. Jackson et al. 2012), these models require SI data from a range of putative prey. However, for many sampled fish populations, these data are often limited or absent, limiting the application of these

models.

The aim of this study was to thus utilise a SI data-set ($\delta^{13}C$, $\delta^{15}N$) based on 11 riverine *B. barbus* populations to quantify how their trophic ecology varies spatially, and how it varies with fish size (as fish fork length) and in relation to the use of marine fishmeal in angling. Across the populations, the extent of SI data on putative food resources varied considerably, preventing use of mixing models to predict diet composition. Instead, variability in $\Delta^{13}C$ was used to infer the extent to which *B. barbus* diet was being influenced by freshwater macroinvertebrates versus marine fishmeal (*cf.* Methods, Results). Objectives were to: (1) assess the utility of fractionation factors to discriminate between macroinvertebrate and marine fishmeal in diets of *B. barbus*; (2) test relationships in fractionation factors of *B. barbus* with macroinvertebrates and marine fishmeal within and between populations, and according to fish length; and (3) determine trophic (isotopic) niche sizes of populations and test the drivers influencing inter-population differences.

5.2 Methods

5.2.1 Sample collection and SI analysis

The study was based on the stable isotope data ($\delta^{13}C$, $\delta^{15}N$) of *B. barbus* sampled from 11 rivers in England completed

between 2013 and 2017 (Fig. 5.1; Table 5.1). Angling for *B. barbus* in these rivers was all catch and release. The dataset included unpublished data as well as some that have been used previously (Table 5.1) and comprised populations from both the *B. barbus* indigenous and non-indigenous range of England (Table 5.1; Antognazza et al., 2016). The sampled *B. barbus* were collected by electric fishing and/ or catch-and-release angling. During sampling, captured *B. barbus* were measured (fork length, nearest mm), and between 3 and 5 scales removed and transferred to a paper envelope. For 9 of the 11 populations, samples of macroinvertebrates were collected concomitantly by kick-sampling (disturbance of the substrate by kicking, with displaced benthic macroinvertebrates captured downstream in a net) (Table 5.1).

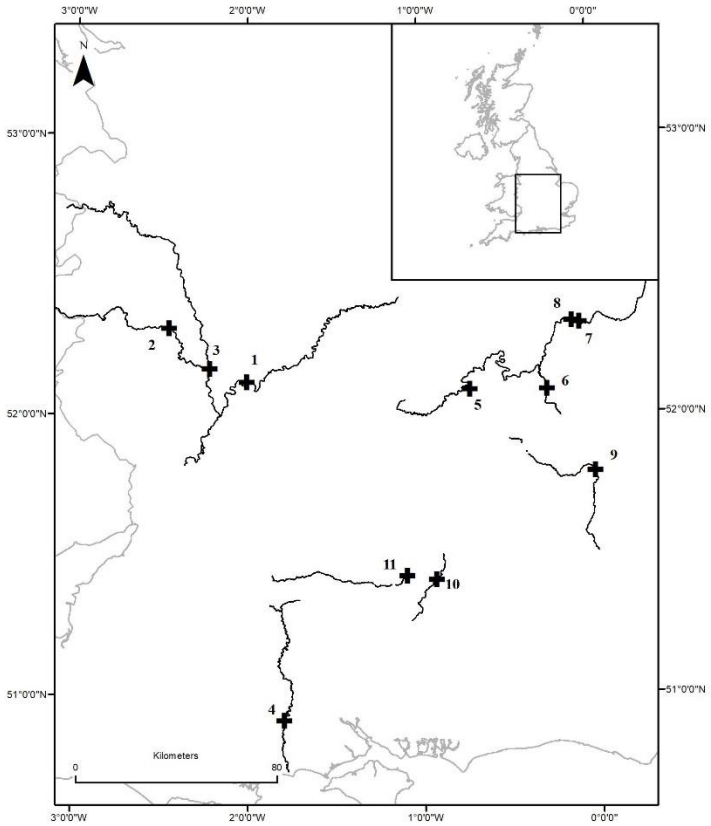

Figure 5.1 *Approximate locations in Britain (inset) of the 11 B. barbus populations used in the study, where: 1: Warwickshire Avon, 2: River Teme, 3: River Severn, 4: Hampshire Avon, 5: River Great Ouse, 6: River Ivel, 7: Chub Stream, 8: Trout Stream, 9: River Lee, 10: River Loddon and 11: River Kennet.*

Table 5.1 *Overview of the 11* Barbus barbus *populations used in the study. (In 'River', W. Avon = Warwickshire Avon, H. Avon = Hampshire Avon; 'Basin', S = River Severn, GO = Great Ouse, HA = Hampshire Avon, TH = Thames; 'Range', NI = non-indigenous, I = non-indigenous; Method, A = angling, EF = electric fishing. Note L = fork length, mm; $\delta^{13}C$ and $\delta^{15}N$ are all in ‰, 'MI' = macroinvertebrate; and 'Source' indicates whether the SI data have been used previously; U = unpublished, 1 Gutmann Roberts et al., (2017); 2 Gutmann Roberts & Britton (2018); 3 Bašić & Britton (2016); 4 Bašić et al., (2015).*

River	Basin	Range	n	Method	Mean L	L range	Mean $\delta^{13}C$	$\delta^{13}C$ range	Mean $\delta^{15}N$	$\delta^{15}N$ range	MI sample	Source
W. Avon	S	NI	18	A	637 ± 62	282 - 850	-26.1 ± 1.1	-28.4 - -21.2	16.2 ± 0.9	11.9 - 18.7	Y	U
Teme	S	NI	122	A/ EF	400 ± 79	105 - 690	-25.4 ± 0.9	-28.6 - -20.1	12.3 ± 0.2	10.7 - 13.5	Y	1
Severn	S	NI	69	A	591 ± 27	272 - 800	-23.4 ± 0.5	-27.04 - -19.4	12.6 ± 0.2	10.5 - 14.9	Y	1,2
H. Avon	HA	NI	25	A	660 ± 30	550 - 800	-26.9 ± 0.5	-29.6 - -24.7	11.4 ± 0.5	10.0 - 13.7	Y	4
Great Ouse	GO	I	7	EF	399 ± 107	188 - 643	-27.4 ± 0.5	-28.3 - -26.2	20.5 ± 0.2	20.1 - 20.8	Y	3
Ivel	GO	I	11	EF	513 ± 118	250 - 785	-26.2 ± 0.9	-28.3 - -24.1	21.4 ± 0.8	19.5 - 23.8	N	3
Chub Stream	GO	I	8	EF	204 ± 20	166 - 258	-27.2 ± 0.6	-28.1 - -26.0	16.5 ± 0.8	15.4 - 18.9	Y	3
Trout Stream	GO	I	6	EF	159 ± 17	142 - 197	-22.8 ± 0.7	-24.1 - -22.03	13.4 ± 0.8	12.2 - 14.9	Y	3

Table 5.1 (Continued)

River	Basin	Range	n	Method	Mean L	L range	Mean $\delta^{13}C$	$\delta^{13}C$ range	Mean $\delta^{15}N$	$\delta^{15}N$ range	MI sample	Source
Lee	TH	I	20	EF	319 ± 44	202 - 435	-25.6 ± 0.7	-27.9 - -23.8	17.8 ± 0.8	14.3 - 20.6	N	U
Loddon	TH	I	7	A	403 ± 182	80 - 655	-23.6 ± 1.7	-27.3 - -20.2	13.1 ± 1.8	10.3 - 17.0	Y	U
Kennet	TH	I	9	A	631 ± 37	550 - 710	-25.0 ± 1.5	-28.3 - -22.7	11.3 ± 0.6	10.2 - 12.9	Y	4

The *B. barbus* SI data were derived from their scale samples, where scales have a longer isotopic turnover rate than their muscle and fin tissue (Busst and Britton 2018). Thus, scale SI data provides information on the long-term diet of the fish (e.g. 6 months, although this will vary with fish size and the different contributions of growth and metabolism to isotopic turnover; Busst & Britton 2018). In the SIA, scale decalcification was not performed prior to their analysis. Whilst comparisons of acidified versus non-acidified scales have revealed significant differences in their isotopic data, the actual changes tend to be minor with, for example, Ventura & Jeppesen (2010) showing that the process produced mean changes in $\delta^{13}C$ (± SD) of 0.2 ± 0.1 and in $\delta^{15}N$ of −0.2 ± 0.2, with conclusions that these changes were not biologically relevant. Moreover, these minor changes in SI values by scale acidification compare to the mean differences here between macro-invertebrate and fishmeal pellets (the primary food resources of the *B. barbus* used here) of 8.2 ± 0.8 ‰ for $\delta^{13}C$ and 5.9 ± 2.2 ‰ for $\delta^{15}N$ (Table 5.2). It is, therefore, considered unlikely that the analytical process of the scales had a material influence on the ability of the study to discriminate between fish mainly feeding on macroinvertebrates versus fishmeal pellets.

Preparation for SI involved the cleaning of scales in distilled water and then, using dissecting scissors, removing the very

outer portion of the scale (Bašić et al. 2015). This was to ensure the scale material being analysed was from the most recent growth of each fish (Hutchinson & Trueman 2006). For the macro-invertebrate samples, sorting was to species, with a minimum of three replicate samples analysed per species, and where a sample comprised of between one and three individuals (dependent on body size) (Bašić et al. 2015). Samples from a range of pelletized marine fishmeal ('pellet' hereafter) were also analysed, where a minimum of three samples per product was analysed. All samples were dried to constant mass at 60 °C and then analysed at the Cornell Isotope Laboratory, New York, U.S.A. SI analytical details were as per Busst and Britton (2018), with lipid correction not necessary as C:N ratios indicated very low lipid content (Post et al. 2007).

Prior to some of the data analyses and testing, the *B. barbus* SI data had to be corrected. This was because of differences between the populations in the values of $\delta^{13}C$ and $\delta^{15}N$ of the macroinvertebrates that meant their data could not be compared without correction (Olsson et al. 2009; Jackson & Britton 2014). For each population, this process involved conversion of $\delta^{15}N$ to trophic position (TP) and $\delta^{13}C$ to corrected carbon (Ccorr) (Olsson et al. 2009; Jackson & Britton 2014). Before these calculations could be completed, a common group of macroinvertebrates was identified across all of the samples that

were also highly probable to be an important prey item for *B. barbus*. As per Gutmann Roberts and Britton (2018), the chosen macro-invertebrate was the amphipod *Gammarus pulex*. This macroinvertebrate is ubiquitous in British rivers and tends to form an important dietary component for cyprinid fishes (Macneil et al. 1999), including *B. barbus* (Bašić et al., 2015; Gutmann Roberts & Britton, 2018).

Conversion of $\delta^{15}N$ to TP was through $TPi = [(\delta^{15}N_i - \delta^{15}N_{base})/3.4]+2$, where TP_i was the trophic position of the individual fish, $\delta^{15}N_i$ was the isotopic ratio of that fish, $\delta^{15}N_{base}$ was the isotopic ratio of the primary consumers (macro-invertebrates), 3.4 was the fractionation between trophic levels and 2 was the trophic position of the baseline organism (Post 2002). The $\delta^{13}C$ data were converted to $\delta^{13}Ccorr$ by $\delta^{13}C_i - \delta^{13}C_{meaninv}/CR_{inv}$, where $\delta^{13}C_{corr}$ was the corrected carbon isotope ratio of the individual fish, $\delta^{13}C_i$ was the uncorrected isotope ratio of that fish, $\delta^{13}C_{meaninv}$ was the mean invertebrate isotope ratio (the 'baseline' invertebrates) and CR_{inv} is the invertebrate carbon range ($\delta^{13}Cmax - \delta^{13}Cmin$; Olsson et al., 2009).

5.2.2 Data analysis and statistical testing

Across the 11 populations, the *B. barbus* samples were collected by electric fishing and/ or angling, comprised of fish between

80 and 850 mm, and were collected in different years. Thus, to understand how river, sampling method, fish length and year of sampling affected the SI data, linear mixed models (LMM) were used. Due to the non-comparable nature of the raw SI data between rivers (due to variable macroinvertebrate SI data; Table 5.2), the corrected data (Ccorr and TP) had to be used in these models. Correspondingly, they could only be completed using data from the 9 *B. barbus* populations where macroinvertebrate data were available (Table 5.2). In LMMs, Ccorr or TP was the dependent variable, the independent variable was either sampling method, river or fish length (depending on the test), covariates were sampling, river, year or fish length (depending on the independent variable), and river was used as the random variable (except when the model was testing differences between rivers). Model outputs were the significance of the overall test, the significance of covariates, and the mean values of Ccorr and TP (adjusted for the effects of the covariates) with their pairwise comparisons (with Bonferroni adjustment for multiple comparisons). Where a covariate had consistent non-significant values in all models, it was removed from all final LMMs. The final LMMs were also checked to ensure they met the test assumptions (e.g. the errors have constant variance, are independent, and are normally distributed). Where uncorrected data were used in univariate

tests at the population level (e.g. differences in the range of *B. barbus* isotope data between sampling methods) then, after checking for normality, either ANOVA (normal distribution) or Mann Whitney U tests (non-normal distribution) were used, with checking that model assumptions were also met.

Table 5.2 *Mean stable isotope data of macro-invertebrates per river (‰) used to calculate* B. barbus *fractionation factors sampled from 9 rivers. Note that the mean $\delta^{13}C$ of fishmeal pellets used in the study was -22.12 ± 0.53 ‰ (range -23.19 to -20.17 ‰) and $\delta^{15}N$ was 7.31 ± 1.02 ‰ (range 4.10 to 9.40 ‰).*

River	Basin	Mean $\delta^{13}C$	Mean $\delta^{15}N$
W. Avon	S	-30.3 ± 1.4	14.8 ± 0.4
Teme	S	-29.5 ± 0.8	10.3 ± 0.5
Severn	S	-29.0 ± 0.4	12.3 ± 2.5
H. Avon	HA	-32.9 ± 1.5	9.5 ± 0.8
Great Ouse	GO	-29.4 ± 0.9	14.1 ± 0.7
Chub Stream	GO	-30.0 ± 1.3	17.1 ± 1.1
Trout Stream	GO	-31.1 ± 0.9	16.2 ± 0.6
Loddon	TH	-31.0 ± 0.5	16.5 ± 0.1
Kennet	TH	-29.9 ± 0.2	7.6 ± 0.2

The uncorrected SI data for each fish per population were used to calculate their fractionation factor (Δ) with their macro-invertebrate data ($\Delta^{13}C$_macroinvertebrate; $\Delta^{15}N$_macroinvertebrate) by subtracting their $\delta^{13}C$ and $\delta^{15}N$ values from the mean macroinvertebrate values. The utility of $\Delta^{13}C$_macroinvertebrate and $\Delta^{15}N$_macroinvertebrate to discriminate between fish feeding primarily on

macroinvertebrates and marine fishmeal was tested using data from Gutmann Roberts et al. (2017). In that study, stable isotope Bayesian mixing models had predicted the proportion of marine fishmeal in the diet of *B. barbus* sampled from the lower River Teme/ Severn. Here, linear regression tested the relationship between the Δ^{13}C_macroinvertebrate and Δ^{15}N_macroinvertebrate of these fish with their predicted proportion of marine fishmeal in diet. Note that due to the results, all subsequent analyses focused only on use of Δ^{13}C and δ^{13}C (*cf.* Results). The regression coefficients (*a*, *b*) were then used in the equation FM = (Δ^{13}C_macroinvertebrate × *b*) + *a*, where FM = the proportion of marine fishmeal in diet, to predict the proportion of fishmeal in the diet at Δ^{13}C_macroinvertebrate = 5.3 ‰ (Busst & Britton 2016; Gutmann Roberts et al. 2017). The Δ^{13}C of 5.3 ‰ is from Busst & Britton (2016), who determined the fractionation factors of *B. barbus* in relation to a range of formulated feeds and revealed that the maximum Δ^{13}C of *B. barbus* with a known food resource was 5.3 ± 0.09 ‰. Thus, where Δ^{13}C_macroinvertebrate exceeded 5.3 ‰, it was assumed that the main dietary item of that fish could not be macroinvertebrates. The relationship of Δ^{13}C_macroinvertebrate with fish length was then tested across the dataset, enabling the proportion of fish per population whose Δ^{13}C_macroinvertebrate exceeded 5.3 ‰ to be

determined. Values of $\Delta^{13}C$_pellet were then calculated for each fish using a mean $\delta^{13}C$ value of fishmeal pellets, and with these values then tested for their relationship with $\Delta^{13}C$_macroinvertebrate.

The isotopic niches of the *B. barbus* populations were then estimated using the corrected SI data (Ccorr and TP). These niches were based on 'standard ellipse areas' (SEA), calculated using the package 'Stable Isotope Bayesian Ellipses in R' (R v 3.4.2; SIBER v 2.1.3; Jackson et al., 2011; Jackson et al., 2012; R Core team, 2014). The SEA metric of each population represents the core 40 % of their isotopic data and so is a bivariate measure of the distribution of individuals in isotopic space that represents a population's typical resource use (Jackson et al., 2011; Jackson et al., 2012). Two measures of SEA were calculated. The first was SEA_C, whose calculation accounts for small samples sizes that were generally encountered in the datasets (Jackson et al. 2012). The second was SEA_B, the Bayesian standard ellipse area, as it enables the 95% credible intervals to be determined around the estimate gained from the posterior distributions. Correspondingly, estimates of SEA_B were produced by applying the corrected SI data in a Bayesian framework (*cf.* Parnell et al. 2013). The calculations used vague Inverse-Wishart priors on the covariance matrix and vague normal priors on the means

(Parnell et al. 2013). The posteriors were estimated with the software 'Just Another Gibbs Sampler' (JAGS v4.3.0., Plummer, 2003), with this run for two chains with 20000 iterations, removing 10000 for burn-in and thinning by a factor of 10. Convergence of the chains was checked with the coda package (Plummer et al., 2006) and the Brooks–Gelman–Rubin diagnostic (Gelman and Rubin, 1992; Brooks and Gelman, 1998). Significant differences in the size of Bayesian isotopic niches between populations were inferred when ≥ 95% of posterior draws for one niche were smaller than the other.

The influence of variability in Ccorr (as the range (maximum – minimum values) and coefficient of variation of Ccorr per population) on isotopic niche size was then tested using linear regression. Note that throughout the paper, whenever errors around the mean are presented, the values are 95 % confidence limits unless stated otherwise.

5.3 Results

5.3.1 *Influence of fish length, sampling method, year and river on stable isotope data*

In the LMMs, the covariate of sampling year always had non-significant effects (P = 0.83 to 0.97), so was omitted from all final models. The final LMMs testing the effect of sampling method on the corrected stable isotope data were significant

(Ccorr: $P < 0.01$; TP: $P < 0.01$), with the effect of fish length as a covariate not significant ($P = 0.38$ and $P = 0.28$ respectively). Angled fish had significantly higher values of Ccorr and TP than those sampled by electric fishing (Ccorr: 1.98 ± 0.70 versus 0.59 ± 0.97, $P < 0.01$; TP: 2.75 ± 0.14 versus 2.29 ± 0.22, $P < 0.01$). The LMMs testing differences in the corrected stable isotope data between rivers were also significant (Ccorr: $P < 0.01$; TP: $P < 0.01$). In the models, the effect of fish length as a covariate was significant for Ccorr ($P < 0.01$) but not TP ($P = 0.41$); sampling method was not a significant covariate in either model (Ccorr: $P = 0.45$; TP: $P = 0.45$). Across the rivers, the River Kennet had the highest mean value of Ccorr (adjusted for the effects of covariates) that was significantly higher than all other rivers (Table 3). For TP, fish in the Great Ouse had the highest mean values (4.0 ± 0.3) (Table 5.3). The LMM testing the effect of fish length on Ccorr was not significant ($P = 0.89$), with the effect of sampling method also not significant ($P = 0.22$). However, the LMM testing the effect of length on TP was significant ($P < 0.02$), where the effect of sampling method was also significant ($P = 0.02$).

Table 5.3 *Mean values (adjusted for the effects of covariates in LMMs) of corrected carbon (Ccorr) and trophic position (TP) for* Barbus barbus *sampled from 9 rivers.*

River	Mean Ccorr	TP
W. Avon	1.3 ± 0.7	2.4 ± 0.2
Teme	3.4 ± 0.5	2.6 ± 0.3
Severn	2.3 ± 0.4	2.6 ± 0.1
H. Avon	0.5 ± 0.7	2.6 ± 0.2
Great Ouse	6.7 ± 1.1	4. ± 0.3
Chub Stream	2.4 ± 0.9	1.2 ± 0.3
Trout Stream	3.0 ± 1.0	3.6 ± 0.3
Loddon	4.9 ± 1.2	1.1 ± 0.3
Kennet	9.4 ± 1.0	3.1 ± 0.3

The uncorrected stable isotope data over all 11 rivers revealed that as the length range increased in the sampled *B. barbus*, their $\delta^{13}C$ range also generally increased ($R^2 = 0.56$; $F_{1,9} = 11.57$, $P < 0.01$), but this was not apparent in $\delta^{15}N$ ($R^2 = 0.03$; $F_{1,9} = 0.30$, $P = 0.60$) (Fig. 5.2). Where the samples contained fish captured by angling, the range of both stable isotopes was not significantly different to samples that only comprised of fish sampled by electric fishing (Mann Whitney U test: $\delta^{13}C$ $Z = -1.83$, $P = 0.08$; $\delta^{15}N$: $Z = -0.74$, $P = 0.47$; Fig. 5.2).

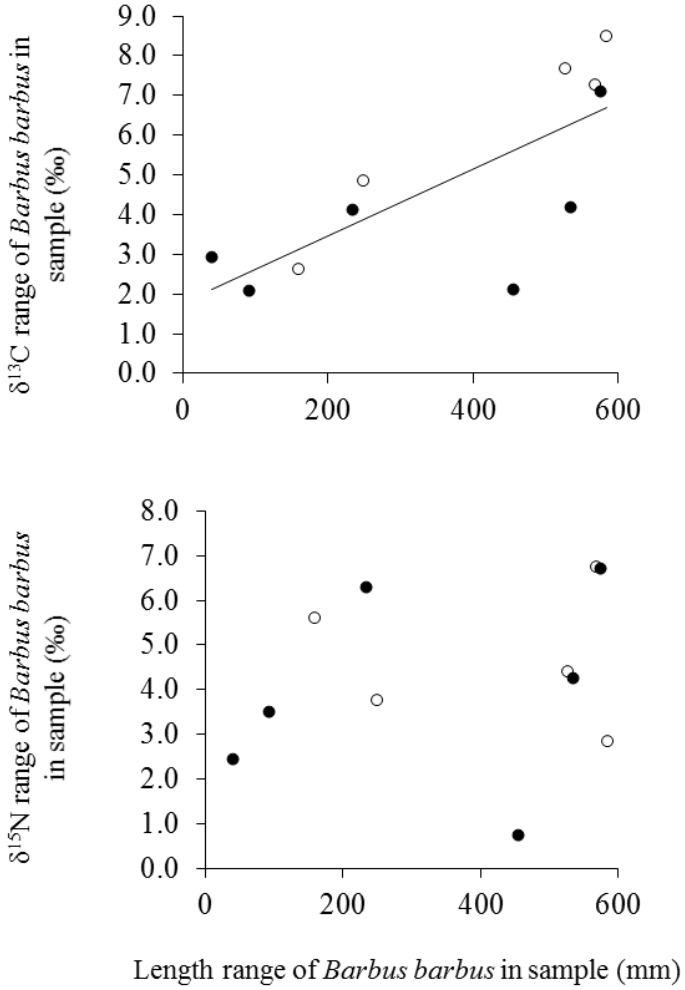

Figure 5.2 *Relationships between length range of* Barbus barbus *per population and the range of their $\delta^{13}C$ and $\delta^{15}N$ data. All ranges represent the difference between the maximum and minimum values in samples. Black circles indicate the sample was only collected by electric fishing, open circles indicate the sample included fish captured by angling.*

5.3.2 Predicting contributions of marine fishmeal to **Barbus barbus** *diet*

The relationship of the predicted proportion of marine fishmeal in the diet of 17 *B. barbus* from the lower River Teme and Severn (Gutmann Roberts et al., 2017) and the Δ^{13}C_macroinvertebrate of these fish was significant (R^2 = 0.78, $F_{1,15}$ = 54.44, P < 0.01; Fig. 5.3). Use of the regression coefficients (*a* = -0.24, *b* = 0.10) in the regression equation revealed that the Δ^{13}C_macroinvertebrate value of 5.31 ‰ was equivalent to a diet comprising 32 % fishmeal; at Δ^{13}C_macroinvertebrate = 10.00 ‰, this proportion of dietary fishmeal increased to 80 % (Fig. 5.3). The relationship of the predicted proportion of marine fishmeal in diet and Δ^{15}N_macroinvertebrate was also significant (R^2 = 0.76, $F_{1,15}$ = 22.45, P < 0.01; Fig. 5.3). However, due to the low δ^{15}N values of marine fishmeal (mean 4.33 ± 0.26 ‰) versus the macroinvertebrates (12.30 ± 2.51 ‰), then this was a negative relationship. Following Fig. 3, Δ^{13}C_macroinvertebrate was thus considered a significant predictor of the proportion of marine fishmeal in *B. barbus* diet. As the ^{13}C stable isotope is also generally used to discriminate between consumer energy sources (especially marine versus freshwater) then the remaining analyses focused on only Δ^{13}C.

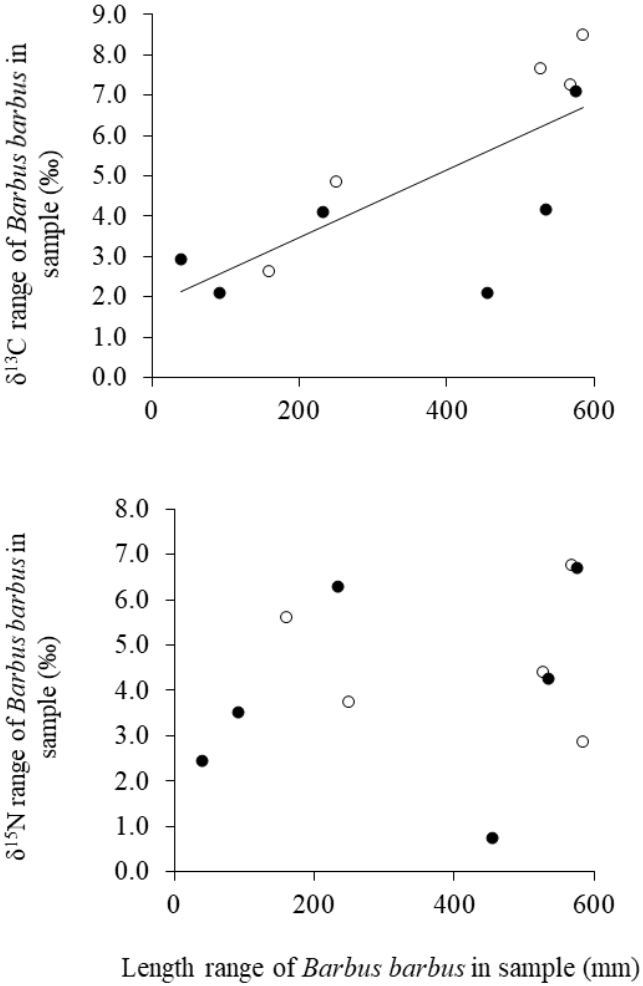

Figure 5.3 *$\Delta^{13}C$_macroinvertebrate (open circle) and $\Delta^{15}N$_macroinvertebrate (filled circle) versus predicted proportion of marine fishmeal in the diet of 17 B. barbus from the lower River Teme/ Severn, where the solid line represents the significant relationship between the variables according to linear regression.*

5.3.3 Stable isotope fractionation of Barbus barbus *from food resources*

The LMM testing the effect of sampling method on $\Delta^{13}C$_macroinvertebrate was not significant (P = 0.89), with the effect of length as a covariate not being significant (P = 0.18). The LMM testing the effect of fish length on $\Delta^{13}C$_macroinvertebrate was significant (P < 0.01), where the effect of sampling method as a covariate was not significant (P = 0.39). This significant influence of fish length on $\Delta^{13}C$_macroinvertebrate was then explored further by a LMM testing the differences in $\Delta^{13}C$_macroinvertebrate between fish of < 300 mm and > 300 mm. The model was significant (P < 0.01), with the effect of sampling method as a covariate also being significant (P = 0.04). The mean $\Delta^{13}C$_macroinvertebrate (adjusted for the effects of covariates) of fish < 300 mm was 2.8 ± 0.8 ‰ versus 5.4 ± 0.3 ‰ for fish > 300 mm.

In the 9 populations with macro-invertebrate data available (Table 5.2), only 53 % of all fish had $\Delta^{13}C$_macroinvertebrate within 5.3 ‰, the maximum predicted Δ for *B. barbus* (Fig. 5.4; Busst and Britton 2016). All *B. barbus* with $\Delta^{13}C$_macroinvertebrate exceeding 5.3 ‰ were at least 394 mm in length (Fig. 5.4). This pattern in $\Delta^{13}C$_macroinvertebrate was significantly related to fish length ($R^2 = 0.31$, $F_{1, 259} = 118.82$, P < 0.01); all of the fish with $\Delta^{13}C$_macroinvertebrate exceeding

5.3 ‰ were at least 394 mm fork length (Fig. 5.5). The proportions of fish with Δ^{13}C_macroinvertebrate exceeding 5.3 ‰ also varied between the rivers, ranging from 0 to 71 % (0 to 83 % for fish > 300 mm) (Table 5.4). For each individual *B. barbus* with a high Δ^{13}C_macroinvertebrate value, their Δ^{13}C_pellet range ranged from -2.89 to 5.3 ‰ (versus 5.4 to 10.1 ‰ for Δ^{13}C_macroinvertebrate).

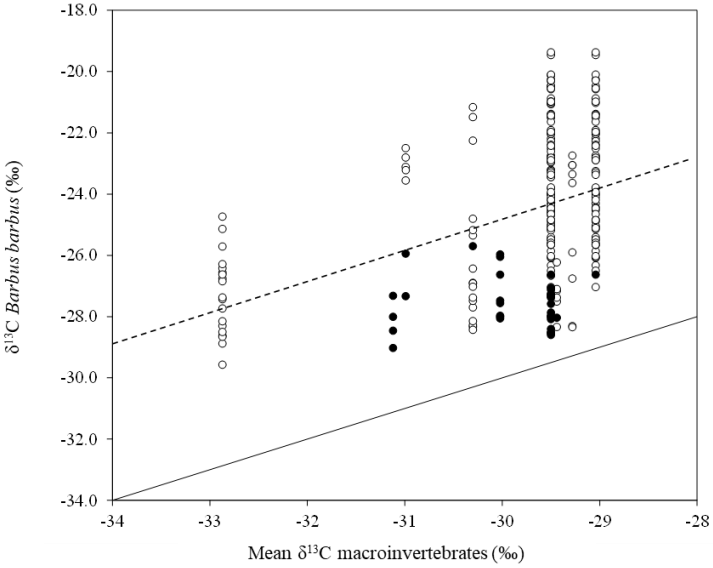

Figure 5.4 *Mean δ13C of macroinvertebrates versus δ13C of individual* Barbus barbus*, where filled circle=fish of<300mm and opem circle=fish ≥300 mm. Solid line represents the 1:1 line and the dashed line represents the maximum Δ13C_macroinvertebrate according to Busst and Britton (2016) (5.31‰).*

Table 5.4 *Proportion of* Barbus barbus *with $\delta^{13}C$ fractionation factors with macro-invertebrates within the range of the species (Busst & Britton 2016) (NP) and those exceeding the maximum fractionation factor with macroinvertebrates (P) for all fish and then only those exceeding 300 mm in length.*

		All fish		Fish > 300 mm	
River	**Basin**	**% NP**	**% P**	**% NP**	**% P**
W. Avon	S	77.8	22.2	76.5	23.5
Teme	S	49.2	50.8	39.2	60.8
Severn	S	49.3	50.7	48.5	51.5
H. Avon	HA	42.1	57.9	42.1	57.9
Great Ouse	GO	100.0	0.0	100.0	0.0
Chub Stream	GO	100.0	0.0	-	-
Trout Stream	GO	100.0	0.0	-	-
Loddon	TH	28.6	71.4	16.7	83.3
Kennet	TH	44.4	55.6	44.4	55.6

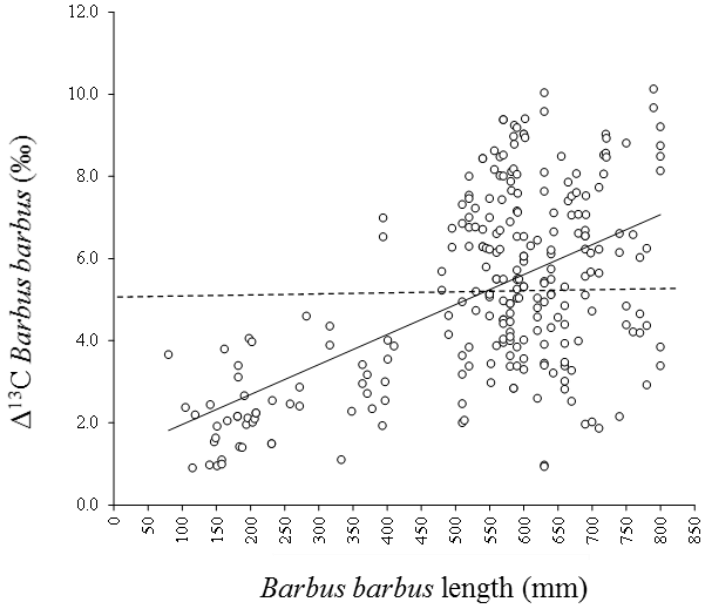

Figure 5.5 *Lengths of individual* Barbus barbus *versus $\Delta^{13}C$_macroinvertebrate. The solid line represents the significant relationship between the variables according to linear regression and the horizontal dashed line represents the maximum $\Delta^{13}C$_macroinvertebrate according to Busst and Britton (2016) (5.31 ‰).*

5.3.4 Isotopic niche size

The corrected SI data enabled the isotopic niches to be determined for the 9 populations. This revealed variability in the isotopic niche size across the populations (Table 5.5). The largest niche was for the River Loddon population (Table 5.5). The Loddon data were omitted from further analyses (it was considered an outlier due to its small sample size in

combination with fish present < 100 mm, a contrast to the other populations). Testing using linear regression then revealed that as the range in Ccorr and the coefficient of variation of Ccorr increased, so too did the size of the isotopic niche (Ccorr range: $R^2 = 0.52$; $F_{1,6} = 6.62$, $P = 0.04$; CV: $R^2 = 0.79$; $F_{1,6} = 23.12$, $P < 0.01$; Fig. 5.6).

Table 5.5 *Isotopic niche sizes (as standard ellipse areas, SEA) of 9 populations of* Barbus barbus*. Details on basin and range as per Table 1.*

River	Basin	Range	Length range (mm)	SEA_c	SEA_B (95% CI)
W. Avon	S	NI	282 - 850	0.75	0.95 (0.52-1.43)
Teme	S	NI	105 - 690	0.94	0.95 (0.65-1.26)
Severn	S	NI	272 - 800	0.53	0.54 (0.42-0.67)
H. Avon	HA	NI	550 - 800	0.35	0.35 (0.19-0.52)
Great Ouse	GO	I	188 - 643	0.52	0.52 (0.17-0.96)
Chub Stream	GO	I	166 - 258	0.15	0.17 (0.07-0.30)
Trout Stream	GO	I	142 - 197	0.49	0.73 (0.32-1.24)
Loddon	TH	I	80 - 655	2.62	2.75 (0.94-5.16)
Kennet	TH	I	550 - 710	0.77	1.41 (0.59-2.40)

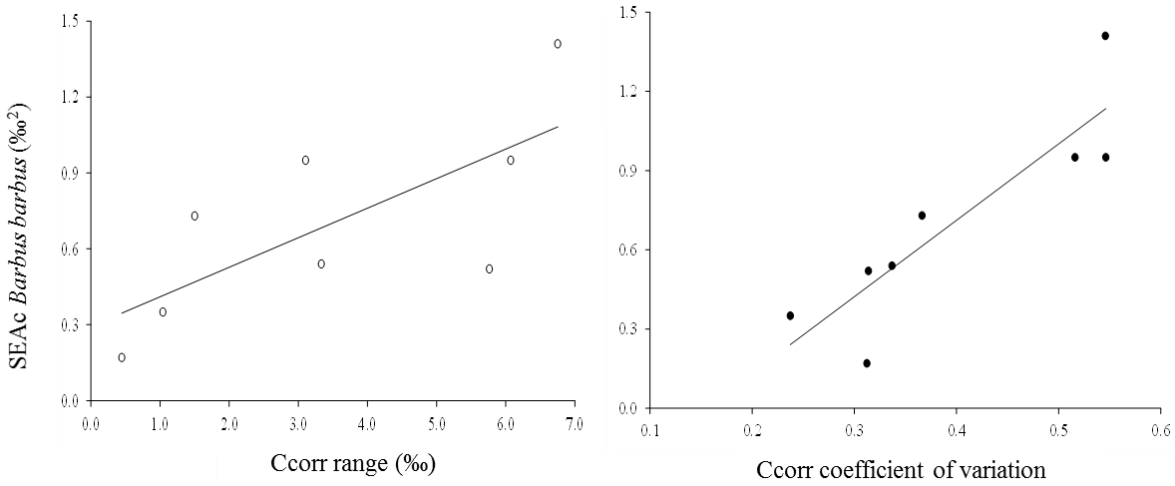

Figure 5.6 *Range of the corrected carbon stable isotope (Ccorr; open circle) and coefficient of variation of Ccorr versus the isotopic niche size (as SEAc). The solid line represents the significant relationship between the variables according to linear regression.*

5.4 Discussion

In these *B. barbus* populations, fish that were larger had a greater probability of being enriched in ^{13}C and whose isotopic difference ($\Delta^{13}C$) with macroinvertebrate $\delta^{13}C$ was elevated. There was, however, high variability within and between rivers over the extent to which the diet of larger fish was based on marine fishmeal, indicating that even where this trophic subsidy was available, only some fish specialised their diet on this subsidy (Gutmann Roberts et al. 2017). Fish captured by angling also had significantly higher $\Delta^{13}C$_macroinvertebrate values than those electric fished. Between rivers, there were considerable differences in the proportions of fish with elevated $\Delta^{13}C$_macroinvertebrate values, indicating higher consumption of fishmeal pellets. Whilst this was at least partially related to the sampling method and the lengths of captured from that river, it would also depend on the extent of angling practised on each river, as this determines the amount of pelletized marine fishmeal being released by anglers and so the extent to which it would be available for consumption by *B. barbus* (Gutmann Roberts et al., 2017).

The assessments of the influence of marine fishmeal on *B. barbus* diet were completed using calculations of $\Delta^{13}C$. This was used in preference to stable isotope mixing models to

predict data composition (Jackson et al. 2012; Phillips et al. 2014), due to differences in the extent of putative prey SI data available across the sampled populations. The use of $\Delta^{13}C$ here was possible due the marine fishmeal baits being substantially enriched in ^{13}C versus freshwater macroinvertebrates (differences approximately 7 to 10 ‰). Thus, despite $\Delta^{13}C$ of macroinvertebrates and pelletized fishmeal being relatively similar (Busst & Britton 2016), it was initially assumed that fish that fed mainly on macroinvertebrates would have considerably more negative $\delta^{13}C$ values and substantially lower $\Delta^{13}C$_macroinvertebrate than fish that fed mainly on pelletized fishmeal. This was then tested using data from the River Teme and Severn (Gutmann Roberts et al. 2017), with the results revealing that individual fish with a $\Delta^{13}C$_macroinvertebrate of 5.3 ‰ (the maximum $\Delta^{13}C$ recorded in *B. barbus* with a known food resource; Busst & Britton 2016) had a diet predicted to comprise of 32 % pelletized fishmeal that increased to 80 % when $\Delta^{13}C$_macroinvertebrate was 10.0 ‰. Bašić et al. (2015) did, however, reveal that the diet of adult *B. barbus* can also comprise small fishes and invasive crayfish, yet SI data on these resources were absent for the majority of the populations used here. Although this could have been a concern, in Bašić et al. (2015) the SI data of these prey resources were heavily associated with the freshwater macroinvertebrate energy

pathway and were thus ^{13}C-depleted and highly distinct from the marine fishmeal resources. Correspondingly, the use here of $\delta^{13}C$ and $\Delta^{13}C$ to discriminate between influences of freshwater prey versus marine on *B. barbus* diet was still considered highly appropriate, despite the potential for some freshwater prey resources to be missing.

The application of $\Delta^{13}C$ to the 9 *B. barbus* with macroinvertebrate data available revealed that for fish below 394 mm, $\Delta^{13}C$_macroinvertebrate was always below 5.3 ‰ (the highest $\Delta^{13}C$ of Busst & Britton (2016)). Only at larger body sizes did their values of $\Delta^{13}C$_macroinvertebrate become more ^{13}C-enriched, with a maximum $\Delta^{13}C$_macroinvertebrate of 10.1 ‰. This $\Delta^{13}C$_macroinvertebrate and ^{13}C enrichment in the larger fish was thus assumed to be through these fish consuming relatively high quantities of angling-derived marine fishmeal. This assumption was supported by other studies on some of these *B. barbus* populations that had revealed no other putative food resources such enriched in ^{13}C (*cf.* Bašić et al., 2015; Gutmann Roberts et al., 2017; Gutmann Roberts & Britton, 2018). It was also supported by a number of studies demonstrating that the strong influence of marine fishmeal in the diet and trophic ecology of freshwater fauna can be traced through foodwebs using $\delta^{13}C$ (Grey et al. 2004; Marcarelli et al. 2011; Jackson et al. 2013; Roussel et al. 2018).

Across the 9 populations with macroinvertebrate data available, there was high variability in Δ^{13}C_macroinvertebrate values. There were four populations where Δ^{13}C_macroinvertebrate values suggested the *B. barbus* prey resources were all primarily of freshwater origin. The samples from the Warwickshire Avon and River Great Ouse both included fish over 394 mm, but only 23 % of fish in the Avon and 0 % from the Great Ouse had Δ^{13}C_macroinvertebrate values exceeding 5.3 ‰. The Chub and Trout Stream also had no fish with Δ^{13}C_macroinvertebrate values exceeding 5.3 ‰, but this was most likely related to their samples only comprising fish < 300 mm. In the five other rivers, between 51 and 71 % of all fish had Δ^{13}C_macroinvertebrate values exceeding 5.3 ‰. These results thus suggest that the dietary utilisation by *B. barbus* of this angling trophic subsidy varied spatially. This was likely to relate to differences in the intensity of *B. barbus* angling effort that affected the quantity of marine fishmeal being released into these rivers. Evidence suggests that recreational anglers allocate fishing effort based on perceived fishing quality and travel time (Post & Parkinson 2012). Whilst the Warwickshire Avon and Great Ouse are both close to urban centres, the Avon has been renown for the quality of its angling for smaller cyprinid species (Hickley 1986), with angling effort for *B. barbus* being relatively low (personal observations, the authors). Whilst the

River Great Ouse has been renown for producing specimen-sized *B. barbus* (e.g. The Times, 2004), genetic analyses have revealed these fish were all stocked (Antognazza et al., 2016). Moreover, these large fish are no longer present due to natural mortality and have not been replaced by either natural recruitment or other stocked fish (Bašić & Britton 2016). This recruitment failure is likely to be due to poor spawning habitat (Bašić et al. 2017; 2018). Consequently, in the last decade, angling effort for *B. barbus*, including the use of marine fishmeal, has declined sharply in the river due to the perception by anglers of decreased angling quality (Post & Parkinson, 2012).

As well as being variable between populations, values of Δ^{13}C_macroinvertebrate varied considerably within populations, including in fishes above 394 mm, where values varied between 0.9 and 10.1 ‰. This variability was also apparent in other *B. barbus* studies where mixing models have predicted diet composition from SI data (Bašić et al., 2015; Gutmann Roberts et al., 2017). Thus, where marine fishmeal was present as an angler trophic subsidy, some individual trophic specialisation on this subsidy was apparent (Britton & Andreou, 2016). The consumption of this marine fishmeal by some individuals then increased the sizes of their population niches. This finding aligns to Araújo et al. (2011) who outlined

that individual specialisation results in population trophic niches becoming more diversified, shifting to comprise of sub-sets of trophically specialised individuals (Araújo et al., 2011). What was not apparent is why individual fish vary their use of this subsidy and this requires further investigation.

Contemporary angling practises for other cyprinid fishes (such as carp *Cyprinus carpio*) now also include the use of energy rich, formulated feeds (Mehner et al. 2018). Substantial quantities of these feeds are now released into many European freshwaters. For example, individual freshwater anglers in Germany have been estimated as using 7.3 kg bait year^{-1} (Arlinghaus 2004). For anglers specifically targeting large *C. carpio* in Germany, the average amount of bait released was 215 kg per angler per year (Niesar et al. 2004). Per hour of fishing, freshwaters anglers introduce approximately 150 g of bait (Niesar et al., 2004; Arlinghaus, 2004). Consequently, the release of energy-rich angler baits into freshwaters provides a strong trophic subsidy that can supplement fish diet (Specziár et al. 1997; Arlinghaus & Niesar 2005; Bašić et al. 2015). Whether this is considered beneficial for the fish and fishery might then depend on the fishery management objectives. If the management objective is to provide faster growing fishes to enhance catch-and-release angling via increasing the opportunity for anglers to capture larger individuals then this

trophic subsidy can be viewed positively, with encouragement for anglers to introduce more of this bait. This is because these subsidies can directly increase fish production (Schreckenbach & Brämick 2003; Niesar et al. 2004), potentially also altering population demographics via increasing the body mass of individual fishes (Arlinghaus & Niesar, 2005). Indeed, in *B. barbus*, individuals increased in condition and had higher food conversion ratios when fed a formulated feed rather than Chironomid larvae (Kamiński et al. 2010). However, if the management objectives are to provide more natural angling experiences, such as for anglers whose main motivations for angling are non-catch related (Arlinghaus 2006), then the use of these baits as a trophic subsidy might be viewed as being less beneficial as it results in fish diet becoming associated with artificial enhancement.

In summary, the application of on $\Delta^{13}C$ to a number of *B. barbus* populations enabled the influence of marine trophic subsidies on their isotopic ecology to be assessed. The results suggested that where present as a trophic subsidy, marine fishmeal had some substantial influences on *B. barbus* diet and, correspondingly, their isotopic niche size. However, this influence varied spatially and with body size, indicating its exploitation as a dietary resource by *B. barbus* was not universal

and involved large bodied individuals specializing on this subsidy.

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CHAPTER VI

Genetic and phenotypic displacement of an endemic *Barbus* complex by invasive European barbel *Barbus barbus* in central Italy

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Abstract

Invasions of alien fishes can result in considerable consequences for native biodiversity, including local extinctions of native species through genetic introgression. In Italy, the alien European barbel *Barbus barbus* was first detected in 1994. It has since undergone range expansion, raising conservation concerns on their impacts on endemic *Barbus* species, including *Barbus plebejus* and *Barbus tyberinus*. Here, the genetic and phenotypic consequences of *B. barbus i*nvasion in the Tyrrhenian and Adriatic basins of central Italy were assessed by comparing 'invaded' with 'uninvaded' river sections that remain free of *B. barbus* due to barriers preventing their upstream dispersal. In both basins, uninvaded sites were confirmed as *B. barbus* free, but the endemic populations had low genetic variability. In the invaded sections, haplotype and nucleotide diversity was relatively high, with introgression skewed towards *B. barbus* genes, with the barbel populations comprising of only 4 % and 23 % of *pure B. tyberinus* and *B. plebejus* respectively. Relatively high morphological differentiation was apparent between pure *B. tyberinus* and hybrid forms, whilst differences were less apparent between pure *B. plebejus* and their hybrid forms. Thus, the endemic *Barbus* species only persist in areas that remain free of invasive *B. barbus*, with this only due to river structures

that impede their upstream movements. As these structures also limit the effective population size of the endemic species, conservation plans must reconcile *B. barbus* dispersal prevention with the need to increase the population connectivity of the endemics.

6.1 Introduction

The invasion of freshwater ecosystems by alien fishes can result in considerable consequences for native biodiversity, including local extinctions of endemic and native species (Gozlan et al. 2010; Jackson et al. 2017; Mollot et al. 2017). These consequences can result from the trophic interactions of the invader with native species that lead to increased predation and competition pressure (David et al. 2017; Jackson et al. 2017), the foraging behaviours of the invader that modify the habitat characteristics through ecological engineering (Mollot et al. 2017), and the transmission of novel pathogens (Sheath et al. 2015). In addition, genetic introgression between the invader and native species can result in the loss of genetic integrity of populations of ecologically important native species (Hanfling et al. 2005; Hayden et al. 2010; Meraner et al. 2013; Geiger et al. 2016). Consequently, invasive alien fish represent a considerable global challenge, requiring effective management

and regulation (Pimentel et al. 2000; Dlugosch and Parker 2008; Estoup and Guillemaud 2010).

The management and regulation of invasive species can be strongly informed by their invasion genetics (Hänfling 2007). Information on the introduction history of the invader, its biogeographic source, population connectivity, and mixing of the species in both the native and invasive range can inform knowledge on its genetic diversity in the invasive range, how this diversity varies spatially, and help identify the introduction pathways (e.g. Lawson Handley et al. 2011; Bock et al. 2015; Hardouin et al. 2018). A further genetic consideration is where the invasion process is being facilitated by hybridization, where the invader undergoes introgression with populations of taxonomically similar native species. This can result in the rapid evolution of invasiveness, with a consequent loss of native genetic diversity and locally adapted genotypes (Rhymer and Simberloff 1996; Brennan et al. 2014; Bock et al. 2015; Morais and Reichard 2018). This is particularly common in fish, especially in species of the Cyprinidae family (Scribner et al. 2001), where the widespread incidence of interspecific hybridization among closely related species has been widely observed (Scribner et al. 2001). This potentially leads to new invasive hybrid lineages that may out-compete native parental genotypes through the production of more vigorous hybrids

(Hanfling 2007). It can also result in higher adaptive capacity to altered environmental conditions that are driven by anthropogenic exploitation of the freshwater resources (e.g. habitat fragmentation due to dam and weir construction, increased environmental pollution) (e.g. Oziolor et al. 2019).

These issues of anthropogenic hybridisation and introgression are increasingly apparent in Italian river basins where, during the last century, environmental degradation has increased dramatically at a time when there has also been multiple and recurrent introductions of freshwater fishes, especially of cyprinids (Gherardi et al. 2008; Castaldelli et al. 2013; Bianco, 2014; Carosi et al. 2017a; Lanzoni et al. 2018). Introductions of cyprinid fishes have resulted in ecological impacts including trophic niche overlap, habitat shifts, and extirpations of native populations (Vilizzi 2012). There have also been frequent events of genetic introgression between native and exotic species (Kottelat and Freyhof 2007). This is especially the case between co-generic *Barbus* species, with the recent introduction of the exotic European barbel *Barbus barbus* (Linnaeus, 1758) resulting in introgression with endemic *Barbus* species (Meraner et al. 2013; Zaccara et al. 2014). The European barbel, a fluvio-lacustrine cyprinid naturally distributed in central Europe (e.g. Danube basin), has habitat preferences of medium-large flowing rivers that are characterized by laminar flows and

relatively warm temperatures (Kottelat and Freyhof 2007). These habitat preferences are shared with endemic Italian barbels (common barbel *Barbus plebejus* Bonaparte, 1839 and Tiber barbel *Barbus tyberinus* Bonaparte, 1839). The natural distributions of these Italian endemic barbel vary; *B. plebejus* inhabits the Adriatic basins of Padano-Venetian district (PV), while *B. tyberinus* is present in Tyrrhenian basins within the Tuscany-Latium district (TL) (*sensu* Bianco 1995). *B. barbus* was first reported in Italian waters in 1994 in the Po River, with the species surmounting the Alps through 'mixed cyprinid stocking' events (Meraner et al. 2013). Its subsequent range expansion and invasion of several Italian river basins has been assisted by unregulated releases by recreational anglers (Zerunian 2002). In the Po River, impacts of hybridization between *B. barbus* and endemic *Barbus* species has been well documented (Meraner et al. 20013; Zaccara et al. 2014; Piccoli et al. 2017). Since 1998, *B. barbus* has been present in the Tyrrhenian and Adriatic basins of central Italian peninsula (Mearelli et al. 2000), where its hybridization with native *B. plebejus* and *B. tyberinus* is considered likely (Buonerba et al. 2015; Carosi et al. 2017b).

The aim of this study is, therefore, to use the river basins of central Italy that are populated by *B. plebejus* and *B. tyberinus* to assess their genetic and phenotypic responses to the invasion

of *B. barbus*. Through molecular and morphological assessment of barbels in these basins, important knowledge on the impact of invasive *B. barbus* will be generated that can then be used by policy-makers and practitioners to limit its further diffusion, including of its hybrid forms.

6.2 Materials and Methods

6.2.1 *Sampling locations and methods*

Putative purebred populations of *B. tyberinus* and *B. plebejus*, and populations in basins where *B. barbus* is present, were sampled in the Tyrrhenian (Tiber River) and Adriatic (Metauro River) basins respectively (Fig. 6.1, Table S6.1). In these rivers, both uninvaded and invaded areas have recently been recorded (Zaccara et al. 2019b). In both basins, one invaded and one uninvaded site was selected. In the Tiber basin, the invaded *B. tyberinus* site was in the Paglia River (here after referred as TL*i*), where *B. barbus* has been recorded since 1998 (Carosi et al. 2017b). The non-invaded site in the Tiber River was in the Montacchione Stream (here after referred as TL*p*), a tributary of the Paglia River that is isolated from the main channel by the presence of two weirs with a head of approximately 2 m that prevents the upstream movement of *B. barbus* (Carosi et al. 2017b; Zaccara et al. 2019b). In the Metauro River basin, invaded *B. plebejus* were collected from the Candigliano River,

where *B. barbus* has been present since 2005 (Lorenzoni et al. 2006). The non-invaded site was the upper section of the Metauro River basin (i.e. Bosso Stream, here named PV*p*), that was isolated from *B. barbus* invasion by three weirs with heads of between 0.4 and 1 m (Zaccara et al. 2019b). In general, these tributaries are characterised by highly variable flow regimes, especially in summer where flows can be very low due to a combination of drought and abstraction (for irrigation and hydropower production).

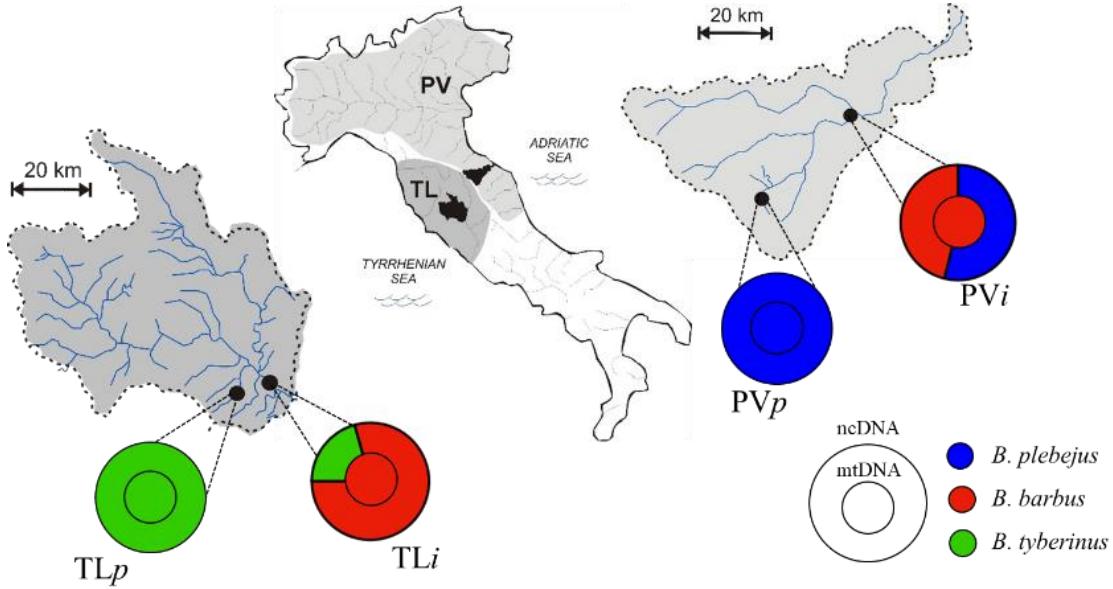


Figure 6.1 *Sampling sites of* B. tyberinus *(uninvaded TLp and invaded TLi) and* B. plebejus *(uninvaded PVp and invaded PVi) populations, collected in Tyrrhenian (TL, Tuscany-Latium) and Adriatic (PV, Padano-Venetian) basins respectively (see Table S1). Pie charts indicate the species frequency according to genetic attribution (mtDNA inner circle and ncDNA outer circle).*

The barbel populations were sampled at each site using electric fishing during July 2019. Following their capture, fish were held in aerated tanks of water. Then, under general anaesthesia (MS-222), fish were photographed (left side; Nikon D300 camera (24–85 mm lens) positioned by a tripod on a table with a millimetric scale), measured (total length, nearest mm), weighed, and a biopsy of the caudal fin taken from a sub-sample of each population (approximately 20 specimens per site). The fin clips were preserved in 90% ethanol and stored at 4° C prior to DNA extraction. Following their recovery to normal behaviour, the fish were released to their approximate location of capture.

6.2.2 *Morphological analyses*

A total of 167 fish were used for morphological analyses. From their images, eight morphometric and four meristic traits were analysed (*sensu* Zaccara et al. 2019a; Supplementary material: Fig. S6.1A), with their phenotypic characters (spot/dot/pigmentation presence on the body, and all fins and fin colour) also recorded. Twenty-eight landmarks (LMs) were used for geometric morphometric analyses of body shape within the R Geomorph function "digitize2d" (Adams et al. 2018; Fig. S6.11B). In the images, the positioning of caudal fin was important in ensuring their associated LMs could be used in

these analyses (17-28; see supplementary material Fig. S6.1B). Generalized Procrustes analysis, as implemented in MorphoJ software (Klingenberg 2011), removed any non-shape variation that had resulted from variation in fish position, orientation, and size. In the same software, shape variations between the four populations were analysed by canonical variate analyses (CVA), with Mahalanobis distances calculated using permutation tests (10,000 replicates). Morphometric traits were standardized to the overall mean standard length to reduce the effects of size and allometry (Beacham 1985). Pairwise comparison on morphological traits between the four populations was performed using analysis of variance (ANOVA) and Tukey post hoc tests, as implemented in PAST software (Hammer et al. 2001).

6.2.3 *Molecular analysis and DNA polymorphism*

Total genomic DNA was extracted from 102 individuals using a proteinase K digestion, salting-out method (Aljanabi and Martinez 1997). Mitochondrial control region (D-loop) sequences were amplified by polymerase chain reaction (PCR) using D-loopsxF and D-loopdxR (Antognazza et al. 2016) primer pairs, with an 869bp length fragment analysed. As *Barbus* species are tetraploid, we sequenced the nuclear DNA (nDNA) growth hormone paralog-2 (GH-2) using specific

primers developed for other European species of *Barbus* and *Luciobarbus* (F- GTACTATAGTAAGCAGAAATGG and R-AGTGGSAGGGAGTCGTTC; Gante et al. 2011). The GH-2 *locus* was selected as it is polymorphic and suitable for phylogenetic and population genetic analyses (Moyer et al. 2009; Gante et al. 2011; Buonerba et al. 2015).

Both PCR reactions were performed using Multiplex PCR kits (Qiagen) in 10 µl reaction volumes that contained approximately 10 ng of template DNA and 0.25 µM of each primer pair. Thermal cycling was performed as follows: denaturation of 15 minutes at 95 °C, followed by 30 cycles (D-loop) and 35 cycles (GH-2) of 30 s at 94 °C, 90 s at 55 °C and the extension step at 72 °C for 90 s, with the final elongation at 72 °C for 10 min. PCR products were purified using ExoSAP-IT™ (USB) and directly sequenced by MACROGEN Inc (http://www.macro gen.org) using a 3730XL DNA Sequencer. The nucleotide sequences of mitochondrial D-loop haplotypes and nuclear GH-2 alleles were deposited in the GenBank database (Accession numbers: MT385872-MT385896 for the D-loop and MT385897-MT385938 for the GH-2).

Alignment of all sequences was carried out automatically by Clustal W (Thompson et al. 1994), as implemented in Bioedit software (Hall 1999), and further checked manually to eliminate remaining ambiguities. For the nuclear *locus*, the individual fish

that were exclusively characterised by single nuclear polymorphisms (SNPs) (i.e. homozygotes for one barbel species) were solved by phasing the sequences using DNAsp (Librado and Rozas 2009), while specimens with alleles of different lengths due to insertions or deletions (indels) (i.e. interspecific heterozygotes) were manually phased by analysing the forward and reverse sequences, as detailed in Flot et al. (2006). Genetic variability was estimated for each species by calculating the number of haplotypes (h), the number of polymorphic sites (S), the haplotype diversity (H), and the mean number of nucleotide differences (π) for both D-loop mtDNA and the GH-2 nDNA locus, using DNAsp software (Librado and Rozas 2009).

6.2.4 *Phylogenetic analyses*

Maximum likelihood (ML) and Bayesian inference (BI) methods were used for all phylogenetic analyses inferred on both the D-loop and GH-2 datasets. The best-fit nucleotide substitution model was selected by the corrected Akaike Information Criterion (AIC) in jModeltest 2.1.7 (Darriba et al. 2012). For the D-loop dataset, the model used was HKY+I+G, while HKY+I was employed for the GH-2 dataset. ML analyses were performed using GARLI software (Zwickl 2006; Bazinet et al. 2014) with 1000 bootstrap replicates (i.e. btp). The BI was

applied using MrBayes v.3.2.6 (Ronquist et al. 2012), with four independent runs (10^6 generations with a sampling frequency of one tree for every 100 generations), each with four chains (three hot and one cold). All runs reached convergence (average standard deviation of split frequencies below 0.01). The posterior distribution of trees was summarized in a 50% majority rule consensus tree (burn-in of 25%), with statistical support expressed as posterior probability (i.e. pp).

To definitively establish the phylogenetic taxonomic attribution of the barbel samples (i.e. differentiating the native and non-native individuals) (Tsigenopoulos et al. 2002), diagnostic sequences of native *B. plebejus* and *B. tyberinus* (Buonerba et al. 2015; Zaccara et al. 2019b), and of the alien *B. barbus* (detected from pure allopatric populations from English basins (Antognazza et al. 2016) and Italian basins (Zaccara et al. 2019b)) were retrieved from GenBank. These data were included in the analyses of both the mitochondrial and nuclear datasets (see supplementary material Table S6.2 and Table S6.3 for D-loop and GH-2 sequences used respectively). This step also enabled possible introgression between the endemic and invasive species to be traced. Two rheophilic *Barbus* species were selected as outgroups: *Barbus meridionalis* Risso, 1827 (AJ388417) for D-loop and *Barbus caninus* Bonaparte, 1839 (KF963432) for GH-2. A minimum spanning network was also

created from both D-loop and GH-2 multiple alignment using a statistical parsimony criterion, as implemented in PopART v 1.7 software (Leigh and Bryant 2015).

6.2.5 *Population genetic structure*

For each sampling site, allelic polymorphisms, expressed as nucleotide diversity index (π), were calculated for each species using DNAsp software. To compare the connectivity between populations within the Tyrrhenian and Adriatic basins (*B. tyberinus* and *B. plebejus* respectively), and between invaded Tyrrhenian and Adriatic sampling sites (*B. barbus*), the genetic differentiation was tested using the fixation index Φ_{ST} (Weir & Cockerham 1984). Its significance ($P < 0.05$) was assessed by permuting haplotypes between populations 3,024 times, as implemented in Arlequin v 3.5 (Excoffier and Lischer 2010).

6.3 Results

6.3.1 *Morphological analyses*

The canonical variate analyses (CVA) plot revealed the four populations clearly separated along the CV1 axis, with TL*i* individuals distinct from individuals in the other three groups (Fig. 6.2). This axis explained shape variations associated with the head, caudal fin and body depth. In TL*i*, the specimens (identified genetically as hybrids *B. tyberinus* × *B. barbus*) had

deeper bodies and longer snouts with a different mouth orientation (i.e. ventral) and longer tail lobes. Specimens from the pure *B. plebejus* and *B. tyberinus* populations (PV*p* and TL*p*, respectively) were separated along the CV2 axis, where shape variations were in head, caudal fin and body depth: TL*p* fish displayed more fusiform and slender bodies, smaller heads and caudal lobes both smaller and more rounded compared to PV*p* fish. Even here, the main source of variation referred to the fish head and caudal fin that was both shorter and more rounded in TL*p* than in PV*p* individuals. The group of fishes from PV*i* partially overlapped with the PV*p* group. The maximum Mahalanobis distance (9.4) was between the TL*i* and the other three populations, while the minimum value (6.6) was recorded between PV*p* and PV*i* populations.

As morphometric traits, pre-orbital distance (POD) was significantly longer in PV*i* and TL*i* specimens than in fish from the other two sites (Tukey, $P < 0.05$; Table 6.1). The length of ventral fin (LVF) and the height of the first dorsal fin ossified ray (HDOR1) differed significantly between all the four populations (Tukey, $P < 0.05$), with increasing values from TL*p*, PV*p*, and PV*i*, up to TL*i* fish. The length of the pectoral fin (LPF) was significantly different in the TL*p* fish to the other sites (Tukey, $P < 0.05$), except those from TL*i*. The number of scales on the lateral line (NSLL) and above the lateral line was

significantly lower in TL*p* and TL*i* specimens (Tukey, $P < 0.05$), while NSLL was significantly higher in the PV*p* specimens (Tukey, $P = 0.02$) (Table 6.1).

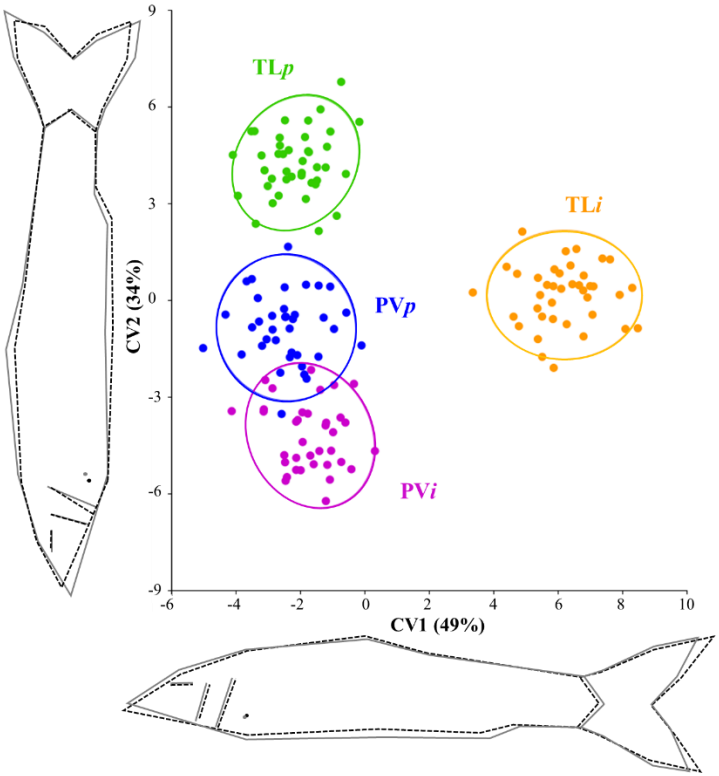


Figure 6.2 *Canonical variate analysis (CVA) output of the body shape comparison between B. tyberinus (uninvaded TLp and invaded TLi) and B. plebejus (uninvaded PVp and invaded PVi) populations. Wireframe graphs indicate the shape changes along each axis (from grey to dashed black).*

Table 6.1 *List of the measured morphometric and meristic traits, and the mean (±standard deviation) values per site for the pure* B. plebejus *(PVp), pure* B. tyberinus *(TLp) and their hybrids (*B. barbus × B. tyberinus *in TLi and* B. barbus × B. plebejus *in PVi). Sample size is reported.*

		PV*p*	**PV*i***	**TL*i***	**TL*p***
		***N* = 41**	***N* = 40**	***N* = 42**	***N* = 44**
Morphometric traits (cm)					
Total length	TL	17.3±4.0	14.9±5.9	15.9±3.6	16.7±5.2
Eye diameter	ED	0.7±0.1	0.6±0.2	0.6±0.1	0.6±0.1
Pre-orbital distance	POD	1.3±0.3	1.3±0.5	1.4±0.3	1.3±0.4
Mouth-operculum distance	MOD	3.5±0.8	3.1±1.2	3.2±0.8	3.3±1.0
Length of pectoral fin	LPF	2.7±0.7	2.2±0.9	2.5±0.6	2.7±0.8
Length of ventral fin	LVF	2.1±0.5	1.9±0.7	2.1±0.5	1.9±0.6
Length of anal fin	LAF	2.3±0.7	2.1±0.8	2.2±0.6	2.5±1.0
Height of the first dorsal fin ossified ray	HDOR1	2.4±0.6	2.2±0.9	2.5±0.6	2.2±0.7
Height of the third dorsal fin ossified ray	HDOR3	1.9±0.4	1.5±0.6	1.7±0.4	1.7±0.5

Table 6.1 (Continued)

		PV*p*	**PV*i***	**TL*i***	**TL*p***
		***N* = 41**	***N* = 40**	***N* = 42**	***N* = 44**
Meristic traits					
Number of dorsal fin branched rays	NDBR	8±0	8±0	8±0	8±0
Number of scales on the lateral line	NSLL	64±3	60±4	56±2	56±3
Number of scales above the lateral line	NSALL	13±1	13±1	12±1	11±1
Number of scales under the lateral line	NSULL	9±1	9±1	8±1	8±1

All of the fish from PV*i* and TL*i* had scales with pigmentation on the edge and most also had dots (Table 6.2). In contrast, some fish from TL*p* had spots on the body and with the ventral and anal fins being different colours (Table 6.2); along with almost half of the TL*i* specimens, they also had a grey dorsal fin. Moreover, the caudal fin was mostly grey/orange in these TL*p* individuals, while it was orange in individuals from PV*p* (Table 6.2).

Table 6.2 *List of phenotypic characters concerning spot/dot/pigmentation presence and fin colour for the barbel populations of the four sites sampled, expressed as percentages (%)*

Phenotypic traits		**PV*p***	**PV*i***	**TL*i***	**TL*p***
Dots on body	no	100	100	100	100
	yes	0	0	0	0
Spots on body	no	98	92	90	66
	yes	2	8	10	34
Scale edge pigmentation	no	100	0	0	100
	yes	0	100	100	0
Dots on scales	no	73	0	17	98
	yes	27	100	83	2
Dots on dorsal fin	no	17	35	45	89
	yes	83	65	55	11
Dots on anal fin	no	100	100	95	100
	yes	0	0	5	0
Dots on caudal fin	no	51	40	64	70

Table 6.2 (Continued)

Phenotypic traits		**PV*p***	**PV*i***	**TL*i***	**TL*p***
Dots on caudal fin	yes	49	60	36	30
	orange	100	100	100	27
Ventral fin colour	grey	0	0	0	52
	orange/grey	0	0	0	21
	orange	100	100	100	27
Anal fin colour	grey	0	0	0	41
	orange/grey	0	0	0	32
	orange	0	5	5	0
Dorsal fin colour	grey	0	5	43	86
	orange/grey	100	90	52	14
	orange	80	70	57	11
Caudal fin colour	grey	0	0	0	5
	orange/grey	20	30	43	84

6.3.2 *Phylogenetic attribution*

The complete D-loop alignment, obtained from 102 barbels, consisted of a total length of 869 bp that identified 25 haplotypes. The multiple alignment of 188 GH-2 sequences, obtained from 94 barbel (GH-2 sequencing failed for 8 fish), identified 42 haplotypes. Sequence analyses of the GH-2 nuclear *locus* yielded a 1030 bp-long alignment, where several indels of different length (1 bp up to 95 bp) were assumed to maximize base identity in flanking conserved sequence blocks

(see Table 6.3). The maximum likelihood and Bayesian phylogenetic analyses performed on the D-loop and GH-2 datasets (including 'reference sequences' from GenBank of the native and non-native species; Tables S6.1, S6.2), provided congruent tree topology. This revealed three evolutionary lineages that were attributed to *B. plebejus*, *B. tyberinus* and *B. barbus* (Fig. 6.3 a, b) and allowed the assignment of our novel sequences to native and non-native barbels. Specifically, the *B. plebejus*, *B. tyberinus* and *B. barbus* clades were largely supported by both the mtDNA and nDNA data (pp>0.9) (Fig. 6.3 a, b). Among the 25 mitochondrial D-loop haplotypes, seven and three haplotypes clustered as *B. plebejus* and *B. tyberinus* respectively, and 15 as *B. barbus*; among the 42 GH-2 haplotypes, 17 were *B. plebejus*, eight were *B. tyberinus* and 17 were *B. barbus*.

Table 6.3 *Sequence polymorphism at mitochondrial and nuclear loci per species. N: number of sequences, h: number of haplotypes excluding gaps, H: haplotype diversity, π: nucleotide diversity (expressed in %), S: number of polymorphic sites, SD: standard deviation.*

Locus	**Species**	**Length**	**Indel (size in bp)**	***N***	**h**	**H ± SD**	**π (%)± SD**	**S**
GH-2	all	1030	6 (6, 13, 95, 22, 1, 1)	188	42	0.87 ± 0.02	1.20 ± 0.30	50
	B. tyberinus	1023	2 (6, 1)	40	8	0.57 ± 0.09	0.08 ± 0.02	6
	B. plebejus	899	5 (13, 95, 22, 1, 1)	78	17	0.52 ± 0.07	0.14 ± 0.03	21
	B. barbus	1029	1 (1)	70	17	0.77 ± 0.01	0.50 ± 0.04	22
D-loop	all	869		102	25	0.91 ± 0.01	2.30 ± 0.06	64
	B. tyberinus	869		22	3	0.12 ± 0.11	0.05 ± 0.03	4
	B. plebejus	869		25	7	0.84 ± 0.03	0.18 ± 0.02	7
	B. barbus	869		55	15	0.86 ± 0.03	0.31 ± 0.04	20

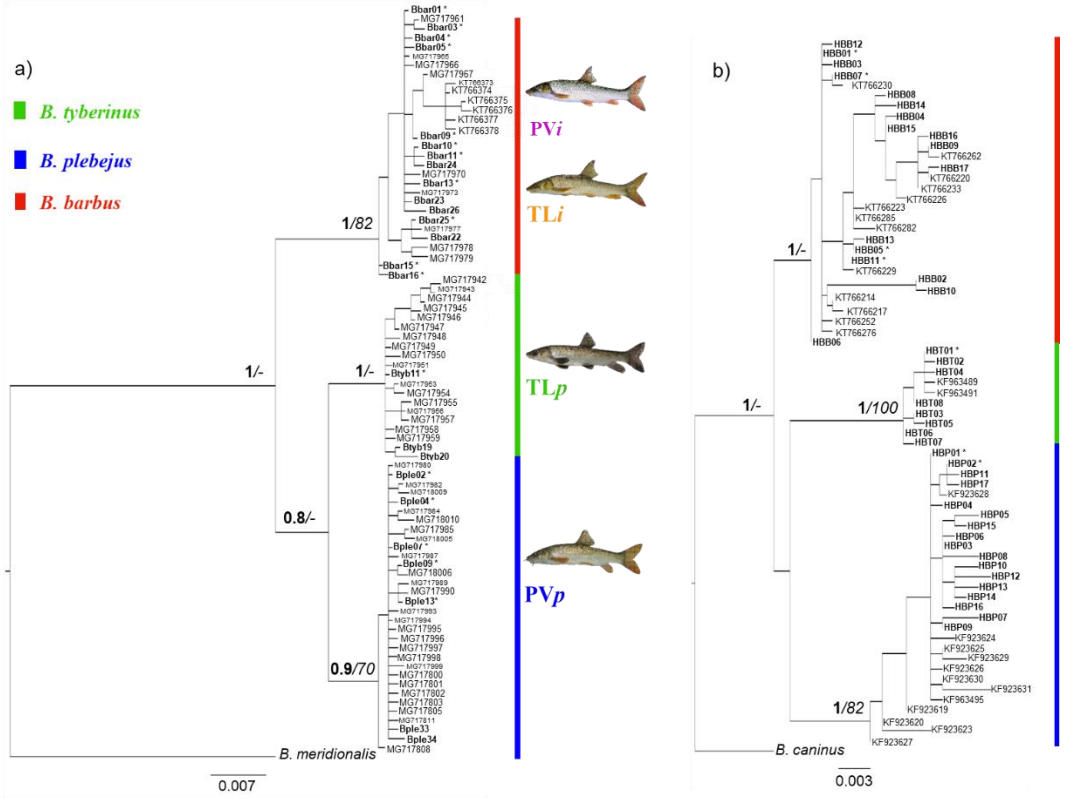
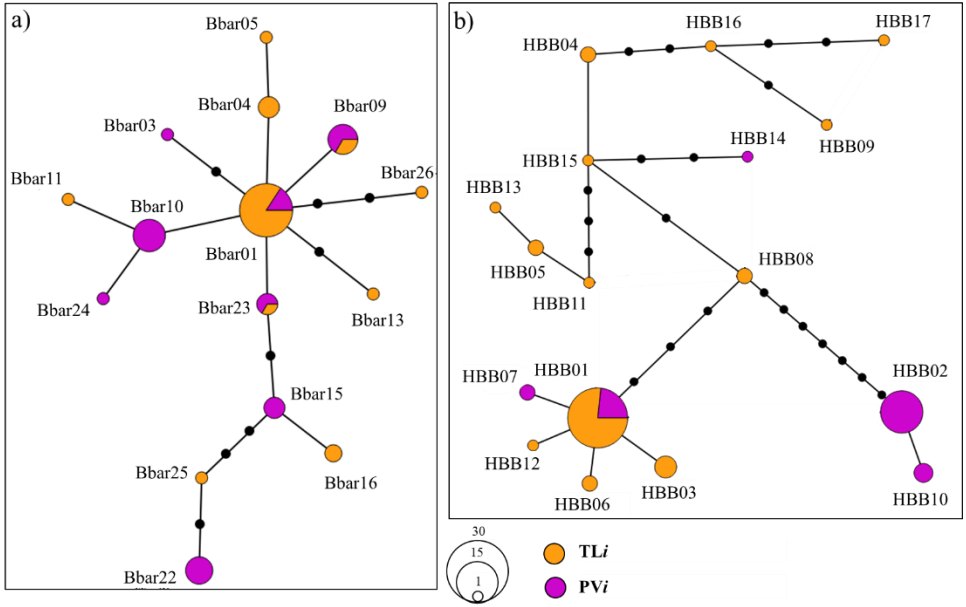

a)
B. tyberinus
B. plebejus
B. barbus
1/82
1/-
1/-
0.8/-
0.9/70
B. meridionalis
0.007
PVi
TLi
TLp
PVp
b)
1/-
1/100
1/82
B. caninus
0.003

Figure 6.3 *a) Bayesian tree for D-loop mtDNA, and (b) Maximum likelihood tree for GH-2 nDNA haplotypes. Statistical support for the major clades is expressed as posterior probability (pp) and bootstrap (btp) values, indicated in bold and italic respectively. Colored bars indicate current species assignation. The haplotypes scored in this study are in bold, whereas the haplotypes retrieved from GenBank are indicated by their accession number (Supplementary material Table S2, S3); * indicates haplotypes previously recorded). Morphology of each lineage is reported (i.e.* B. plebejus *in PVp;* B. tyberinus *in TLp);* B. barbus *is represented by two hybrid forms with* B. tyberinus *and* B. plebejus *(i.e. in TLi and in PVi, respectively).*

6.3.3 *Genetic variability and Minimum spanning networks*

The mitochondrial and allelic diversity varied considerably among the species; *B. barbus* had the highest levels of nuclear and mitochondrial polymorphisms (H = 0.77 and π = 0.50%; H = 0.86 and π = 0.31 respectively), whereas the lowest levels were recorded in *B. tyberinus* (H = 0.57 and π = 0.08%; H = 0.12 and π = 0.05 respectively) (Table 6.3). In the network analyses of *B. barbus* D-loop and GH-2 haplotypes (n = 15 and 17 respectively), the most frequent haplotypes (Bbar01 and HBB01, respectively) were shared in both the Adriatic (PV*i*) and Tyrrhenian (TL*i*) invaded sampling sites (Fig. 6.4). This pattern was also reflected in two more D-loop haplotypes (Bbar09 and Bbar23) (Fig. 6.4). There were four and five private haplotypes detected at PV*i* in the GH-2 and D-loop dataset respectively (Fig. 6.4a), whilst 12 and 7 private haplotypes were detected in these at TL*i*, all separated by up to 15 mutational steps (Fig. 6.4b).

Figure 6.4 *Minimum spanning networks of* B. barbus *mitochondrial (D-loop (A)) and nuclear (GH-2 (B)) recorded in Adriatic (PVi) and Tyrrhenian (TLi) invaded population. Circles represent haplotypes and size is proportional to the frequency of each haplotype. Black dots represent missing haplotypes.*



6.3.4 *Status of* B. barbus *invasion within Tyrrhenian and Adriatic basins*

The nuclear and mitochondrial genetic composition of each population are shown in Figure 1, with the haplotype distribution and frequencies provided in Supplementary material (Table S6.4 and Table S6.5 for D-loop and GH-2 respectively). Mitochondrial and nuclear sequences obtained from PV*p* and TL*p* populations confirmed the absence of *B. barbus* haplotypes and the exclusive presence of *B. plebejus* and *B. tyberinus* haplotypes respectively (Fig. 6.1, Table S6.4, Table S6.5). In contrast, in the PV*i* and TL*i* populations, all of the D-loop sequences (i.e. 26 and 29 respectively) belonged to the *B. barbus* clade, while the allelic frequency of GH-2 *B. barbus* sequences ranged between 46 and 79 % respectively (Fig. 6.1, Table S6.6). The nuclear sequences thus revealed different admixture between native and alien species, from hybrids (34 % *B. barbus* × *B. tyberinus* in TL*i*; 62 % *B. barbus* × *B. plebejus* in PV*i*) to pure strains for *B. barbus* haplotypes (62 % and 15 % in TL*i* and PV*i*, respectively). Only 4 % and 23 % showed both GH-2 alleles for *B. tyberinus* and *B. plebejus* respectively (see Table S6.6).

Values of molecular indices (haplotype and nucleotide diversity) were the lowest in both native *B. plebejus* and *B. tyberinus* pure populations (i.e. PV*p* and TL*p* respectively), and

were the highest in mixed populations (PV*i* and TL*i*) for both native and exotic alleles (Table 6.4). Genetic differentiation between pure populations of the native species and introgressed populations were all significant: i) in *B. plebejus* between PV*p* and PV*i* ($\Phi_{ST} = 0.22$; $P < 0.001$); and ii) in *B. tyberinus* between TL*p* and TL*i* ($\Phi_{ST} = 0.24$; $P < 0.001$). Major values of genetic differentiation were also recorded between *B. barbus* in PV*i* and TL*i* ($\phi_{ST}=0.51$; $P < 0.001$).

Table 6.4 *Molecular indices calculated for the nuclear GH-2 alleles for pure* B. plebejus *(PVp),* B. tyberinus *(TLp) and their hybrids (*B. barbus × B. tyberinus *in TLi and* B. barbus × B. plebejus *in PVi): haplotype diversity (H), nucleotide diversity (π, expressed in %), with relative standard deviations. N= number of total alleles for sampling sites; in brackets the number of alleles per species.*

Species	Indices	PV*p* ***N* = 50**	PV*i* ***N* = 52**	TL*i* ***N* = 58**	TL*p* ***N* = 28**
B. plebejus	π (%)	0.02 ± 0.01 (50)	0.30 ± 0.05 (28)		
	H	0.19 ± 0.01 (50)	0.88 ± 0.01 (28)		
B. tyberinus	π (%)			0.16 ± 0.02 (12)	0.03 ± 0.01 (28)
	H			0.90 ± 0.01 (12)	0.27 ± 0.01 (28)
B. barbus	π (%)		0.43 ± 0.06 (24)	0.30 ± 0.06 (46)	
	H		0.66 ± 0.01 (24)	0.69 ± 0.01 (46)	

6.4 Discussion

Our morphological and genetic results confirmed hybridization between the endemic and alien *Barbus* species in the main watercourses of both the Tyrrhenian and Adriatic basins of central Italy. However, in areas of these watercourses that were considered inaccessible to *B. barbus* due to structures in the river preventing their upstream movement, the results revealed the persistence of 'pure' *B. tyberinus* and *B. plebejus* populations, so confirming the uninvaded status of these areas.

A complex of cryptic species, the *Barbus* species complex in Italy has high morphological similarity that prevents their straightforward taxonomic differentiation in the field (Geiger et al. 2016; Zaccara et al. 2019a). This similarity is likely to have resulted from an evolutionary lack of divergence that was driven by the ecological uniformity of Italian rivers (Livi et al. 2013; Buonerba et al. 2015; Geiger et al. 2016; Zaccara et al. 2019b). Introductions of the ecologically analogous and alien *B. barbus*, which has high potential for genetic introgression with congeners, have enhancedthese taxonomic identification issues, especially because their hybrids' morphological traits are rarely described (see Geiger et al. 2016). While any descriptions of hybrid versus pure species morphologies should be treated cautiously, as they were based on just one

mitochondrial marker and one nuclear genetic locus, there was strong separation between the native fluvio-lacustrine barbel phenotypes that enabled an initial and tentative morphological description of the hybrids to be made. These revealed that the *Barbus* species inhabiting the Tyrrhenian slope (i.e. *B. tyberinus* in TL*p*) were characterized by more fusiform and slender bodies with a smaller head, different mouth orientation (sub-ventral) and shorter and more rounded tail lobes. These morphological variations also distinguished the hybrid phenotypes from the endemic morphotypes (i.e. *B. tyberinus, B. plebejus*), with differences more marked for hybrids in the Tiber River system than those inhabiting the Adriatic slope. Fish in TL*i* showed the greatest morphological differentiation from that of the reference native species (i.e. *B. tyberinus* in TL*p*), while barbels from PV*i* showed little differentiation from the corresponding endemic morphotype (i.e. *B. plebejus* in PV*p*). For the other morphological traits, the pre-orbital distance and the length of the first ossified dorsal ray and ventral fins were lower in *B. tyberinus* and *B. plebejus*, with the highest values measured in the hybrid morphotypes. Correspondingly, across this morphological gradient, the hybrids tended to have more extreme benthic specialized forms (e.g. having longer snouts and ventral mouths, deeper bodies and longer dorsal, ventral and caudal fins). Similarly, a cline was observed in the number

of scales along the lateral line, a commonly used meristic trait for discriminating between *Barbus* species (Bianco 2003a,b; Lorenzoni et al. 2006; Kottelat and Freyhof 2007). The lowest scale number was in the Tiber pure population (i.e. 53-59) and the highest in the *B. plebejus* populations (i.e. 61-67), with hybrids showing intermediate values that match those for invasive *B. barbus* (from literature 53-62; Kottelat and Freyhof 2007). Finally, hybrids were characterized by the pigmentation of the scale edge, a trait typical of the alien *Barbus*, but that was absent in the Italian endemics.

The genetic pattern of both pure populations, characterised by low variability and dominated by just one haplotype, suggest recent periods of low effective population size, promoting local genetic drift (Grant and Bowen 1998). This is supported by general natural population reductions that have resulted from angler exploitation and, especially, from hydrological fluctuations in summer when scarce rainfall and excessive water abstraction cause widespread river droughts. Furthermore, the fish populations in the upstream areas have become increasingly isolated due to the construction of numerous barriers (mainly weirs) that impede their movements. This has limited their spawning migrations and restricted gene flow between downstream and upstream areas, reducing the dispersion of private haplotypes of native species that have

remained confined to downstream populations, and generally reducing the genetic variability of upstream populations. Nevertheless, these barriers have also appeared beneficial by preventing the further upstream dispersal of *B. barbus*.

Conversely, the genetic signal of invasive *B. barbus* (high H and low π), which was similar in both Adriatic and Tyrrhenian populations, was consistent with a recent invasion history (started in the 1990s) that started with several haplotypes. The invasion of both basins probably occurred as a result of the general practice of 'multiple introductions' of fish for angling (i.e. multiple founder events) (Meraner et al. 2013). Although these anthropogenic actions initially favored the fast spread of *B. barbus*, its more recent range expansions have been through natural diffusion in the downstream areas of these rivers.

Despite evidence for introgression does not necessarily mean that there has been displacement of one species by another one (or even that it shows the ability to do so), we did detect that *B. barbus* has invaded and largely displaced native congeners through introgression, and producing small - but distinct - morphological changes in the invaded populations (as described above). In contrast to the Adriatic basin (i.e. Metauro River, PV*i*), *B. barbus* alleles in the Tyrrhenian basin (i.e. Paglia river, TL*i*) strongly outnumbered the native alleles that were detected exclusively in a low number of fishes. This nearly complete

genotype and phenotypic displacement of the endemic Tiber barbel by *B. barbus* may be due to several factors. The first is the hydrographic structure. The Tiber River basin, for which Paglia (TL*i*) is one of the main tributaries, has a dendritic-shaped network extended on a large surface area (17375 km^2). This configuration may have favored the natural diffusion of *B. barbus* by allowing the fish to spread more easily to large parts of the basin using the hydrographic connections. In contrast, the Metauro River basin (PV*i*) has a relatively limited hydrographic network (1325 km^2) and, as with all Adriatic basins of central Italy, it flows independently to the sea, limiting the ability of invasive *B. barbus* to disperse naturally between Adriatic rivers. A second factor may relate to resident time of the alien *B. barbus* in the two basins. The higher number of introgressed fish in PV*i* population is indicative of the more recent hybridization - after 2005 - where first generation (F1) hybrids were dominant (Meraner et al. 2013), which tend to decrease in later hybrid generations (Baack & Rieseberg 2007). Indeed, we detected the highest proportion of pure *B. barbus* in the Paglia River, where the first record of *B. barbus* dated back to 1998. The final factor may relate to degraded water quality and habitat alteration that impacted the sustainability of the natural *B. tyberinus* populations in TL, providing the ecological niche space for the invasive *B. barbus* to utilize. It should be noted

that it is likely that it was the interaction of these factors that resulted in these outcomes, rather than one factor acting in isolation.

In both the Tyrrhenian and Adriatic basins, introgression was skewed toward *B. barbus* mtDNA. This situation has been described as a 'mother species' effect (*sensu* Wirtz 1999), which can be explained by the unequal size between the invader and the native species, where the larger females (i.e. *B. barbus*) are favoured in spawning rather than smaller ones (*B. plebejus* and *B. tyberinus*). Indeed, in other hybrids of the *Barbus* genus, the prevalence of mtDNA was observed for the larger females (*B. barbus* × *B. meridionalis* (Chenuil et al. 2004); *B. barbus* × *B. carpathicus* (Lajbner et al. 2009). This might be a consequence of a sexual selection mechanism that allows only the larger females to be fecundated or also by a higher relative fecundity of the larger species, given *B. barbus* females may produce more eggs than the native species (Banarescu et al. 2003; Bianco 2003a, b; Meraner et al. 2013).

The pattern of hybridization that resulted from *B. barbus* invasion can lead to adaptation through the establishment of novel genotypes and morphologies, in which the hybrids (especially in Tyrrhenian basin) are showing phenotypic traits outside of the trait range of the endemic parental species, which can be a consequence of an adaptative allele introgression

(Whitney et al. 2006), or a transgressive segregation that has resulted in new traits (Rieseberg et al. 1999). The observed morphological changes may be a response to different river characteristics (i.e. level of degradation, flow regime) (e.g. Corse et al. 2009; Samways et al. 2010; Corse et al. 2015) and might be indicative of different trophic resource and habitat uses (Costedoat et al. 2007; Cunha et al. 2009). This potentially results in introgressed *Barbus* populations having a greater adaptive capacity and higher resilience to the anthropogenically altered rivers than the pure endemic fish, especially as the non-native genes are derived from an ecologically analogous congener. This could help ensure the *Barbus* genus can continue to persist in these modified rivers in future. Indeed, many recent studies allude to the adaptive role of hybridisation (Costedoat et al. 2007; Pfennig et al. 2007; Reyer 2008; Hayden et al. 2010) that can drive biodiversity responses to environmental variation (Scribner et al. 2001). Therefore, it is also possible that the introgression is leading to a species erosion process where the phenotype and genotype of the alien are prevalent when compared to the native ones due to the higher fitness of the invader driving a species substitution process (Ward et al. 2012).

In conclusion, our results emphasize the importance of combining morphological (both with traditional traits and using

geometric morphometrics) and genetic (analyzing both mitochondrial and nuclear DNA) approaches in the analysis of cryptic species complexes of cyprinid taxa such as *Barbus* spp., especially when a co-generic invader is present. It was likely that the morphologies recorded in the two populations invaded by alien *B. barbus* (PV*i* and TL*i*) may reflect initial and final displacement stages of the endemic morphotypes and genotypes in the Adriatic and Tyrrhenian basins respectively. This suggests that reliance on using fish body shape to identify the initial invasion stages of *B. barbus* is insufficient, as phenotypic differences might not be evident until the later stages of the invasion. This has important implications for the effective management for this cryptic invasive species, as it suggests it requires the use of molecular tools for its detection in the early invasion stages. Future studies should always analyse the invasion mechanisms, as these shed light on the ecological and trophic factors, which facilitate widespread hybridisation. Then, the improvement of detailed morphological and genetic studies should help in identifying the parental hybrid taxa and allow the mapping of the distribution of gene flow between the endemic species and invader. This knowledge could then provide the basis of an adaptive management tool to limit *B. barbus* invasion and contribute to the long-term conservation of endemic barbels.

6.5 Supplementary material

6.5.1 *Supplementary figures*

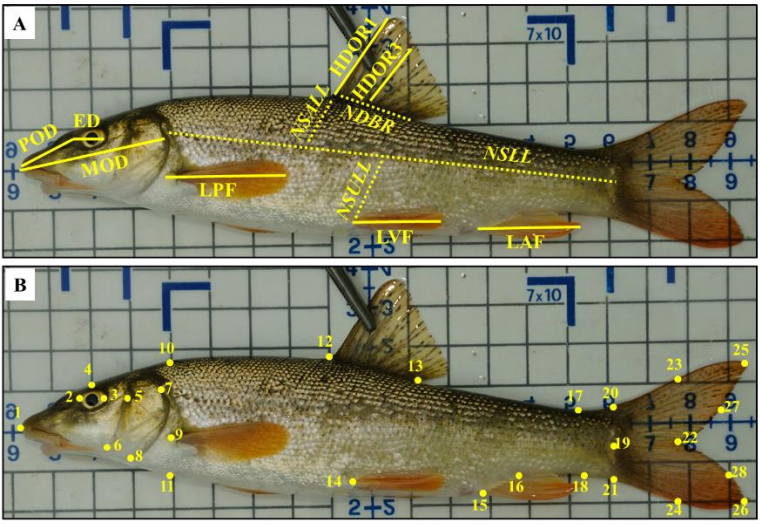


Figure S6.1 (*A) Eight morphometric (ED, eye diameter; HDOR1, height of the first dorsal fin ossified ray; HDOR3, height of the third dorsal fin ossified ray; LAF, length of anal fin; LPF, length of pectoral fin; LVF, length of ventral fin; MOD, mouth-operculum distance; POD, pre-orbital distance) and four meristic traits (NDBR, the number of dorsal fin branched rays; NSLL, the number of scales on the lateral line, and on rows above – NSALL - and under – NSULL - the lateral line) considered for morphological analyses. (B) Position of the 28 landmarks used for body shape analysis: (1) anterior tip of snout, (2, 3) anterior and posterior end of the eye, (4) orthogonal projection on the dorsal profile of the eye centre, (5) lateral projection of the eye centre on the insertion of the operculum, (6) intersection of the operculum at the lateral profile, (7, 8) ventral and dorsal end of gills, (9) anterior insertion of pectoral fin, (10, 11) orthogonal projections on the dorsal and ventral profile of the anterior insertion*

of pectoral fin, (12,13) anterior and posterior insertion of dorsal fin, (14) insertion of pelvic fin, (15, 16) posterior and anterior insertion of anal fin, (17, 18) anterior attachment of dorsal and ventral membrane of caudal fin, (19) base of middle caudal rays, (20, 21) orthogonal projections on the dorsal and ventral profile of the base of middle caudal rays, (22) fork, (23, 24) orthogonal projections on the dorsal and ventral profile of fork, (25, 26) end of the upper and lower lobe of caudal fin, (27, 28) lateral projections of anterior attachment of dorsal and ventral membrane of caudal fin.

6.5.2 *Supplementary tables*

Tables S6.1 *Sampling sites of* B. tyberinus *(uninvaded TLp and invaded TLi) and* B. plebejus *(uninvaded PVp and invaded PVi) populations, collected in Tyrrhenian (TL) and Adriatic (PV) basins respectively (see Fig. 1). For each site, water course and geographic coordinates are reported. Sample size for morphological and genetic (nDNA and mtDNA) analyses are also indicated.*

Basin		Water course	Pop ID	Geographic coordinates	Morphology	mtDNA	nDNA
Adriatic	Metauro	Bosso	PV*p*	43°31'3.14"N 12°33'17.89"E	41	25	25
	Metauro	Candigliano	PV*i*	43°38'8.59"N 12°42'41.32"E	40	26	26
Tyrrhenian	Tevere	Paglia	TL*i*	42°43'38.88"N 12° 7'43.00"E	42	29	29
	Tevere	Montacchione	TL*p*	42°42'44.39"N 12° 5'37.88"E	44	22	14
Total					167	102	94

Table S6.2 *D-loop mtDNA diagnostic sequences of the native B. tyberinus and B. plebejus and the exotic B. barbus retrieved from GenBank and used in the phylogenetic analyses (see Fig. 3). For each sequence, species, GenBank accession number, refence, geographic source. * haplotype overlap with sequences produced in this study.*

Species	GenBank code	Reference	Geographic Source	*Haplotype
B. tyberinus	MG717942	Zaccara et al., 2019b	Italy	
	MG717943	Zaccara et al., 2019b	Italy	
	MG717944	Zaccara et al., 2019b	Italy	
	MG717945	Zaccara et al., 2019b	Italy	
	MG717946	Zaccara et al., 2019b	Italy	
	MG717947	Zaccara et al., 2019b	Italy	
	MG717948	Zaccara et al., 2019b	Italy	
	MG717949	Zaccara et al., 2019b	Italy	
	MG717950	Zaccara et al., 2019b	Italy	
	MG717951	Zaccara et al., 2019b	Italy	
	MG717952	Zaccara et al., 2019b	Italy	Btyb11*
	MG717953	Zaccara et al., 2019b	Italy	
	MG717954	Zaccara et al., 2019b	Italy	
	MG717955	Zaccara et al., 2019b	Italy	
	MG717956	Zaccara et al., 2019b	Italy	
	MG717957	Zaccara et al., 2019b	Italy	
	MG717958	Zaccara et al., 2019b	Italy	
	MG717959	Zaccara et al., 2019b	Italy	

Table S6.2 (Continued)

Species	GenBank code	Reference	Geographic Source	*Haplotype
B. barbus	MG717960	Zaccara et al., 2019b	Italy	Bbar01*
	MG717961	Zaccara et al., 2019b	Italy	
	MG717962	Zaccara et al., 2019b	Italy	Bbar03*
	MG717963	Zaccara et al., 2019b	Italy	Bbar04*
	MG717964	Zaccara et al., 2019b	Italy	Bbar05*
	MG717965	Zaccara et al., 2019b	Italy	
	MG717966	Zaccara et al., 2019b	Italy	
	MG717967	Zaccara et al., 2019b	Italy	
	MG717968	Zaccara et al., 2019b	Italy	Bbar09*
	MG717969	Zaccara et al., 2019b	Italy	Bbar10*
	MG717970	Zaccara et al., 2019b	Italy	Bbar11*
	MG717971	Zaccara et al., 2019b	Italy	
	MG717972	Zaccara et al., 2019b	Italy	Bbar13*
	MG717973	Zaccara et al., 2019b	Italy	
	MG717974	Zaccara et al., 2019b	Italy	Bbar15*
	MG717975	Zaccara et al., 2019b	Italy	Bbar16*
	MG717976	Zaccara et al., 2019b	Italy	Bbar25*
	MG717977	Zaccara et al., 2019b	Italy	
	MG717978	Zaccara et al., 2019b	Italy	
	MG717979	Zaccara et al., 2019b	Italy	

Table S. 6.2 (Continued)

Species	GenBank code	Reference	Geographic Source	*Haplotype
B. barbus	KT766373	Antogranzza et al., 2016	England	
	KT766374	Antogranzza et al., 2016	England	
	KT766375	Antogranzza et al., 2016	England	
	KT766376	Antogranzza et al., 2016	England	
	KT766377	Antogranzza et al., 2016	England	
	KT766378	Antogranzza et al., 2016	England	
B. plebejus	MG717980	Zaccara et al., 2019b	Italy	
	MG717981	Zaccara et al., 2019b	Italy	Bple02*
	MG717982	Zaccara et al., 2019b	Italy	
	MG717983	Zaccara et al., 2019b	Italy	Bple04*
	MG717984	Zaccara et al., 2019b	Italy	
	MG717985	Zaccara et al., 2019b	Italy	
	MG717986	Zaccara et al., 2019b	Italy	Bple07*
	MG717987	Zaccara et al., 2019b	Italy	
	MG717988	Zaccara et al., 2019b	Italy	Bple09*
	MG717989	Zaccara et al., 2019b	Italy	
	MG717990	Zaccara et al., 2019b	Italy	
	MG717991	Zaccara et al., 2019b	Italy	Bple13*
	MG717992	Zaccara et al., 2019b	Italy	Bple13*
	MG717993	Zaccara et al., 2019b	Italy	

Table S. 6.2 (Continued)

Species	GenBank code	Reference	Geographic Source	*Haplotype
B. plebejus	MG717994	Zaccara et al., 2019b	Italy	
	MG717995	Zaccara et al., 2019b	Italy	
	MG717996	Zaccara et al., 2019b	Italy	
	MG717997	Zaccara et al., 2019b	Italy	
	MG717998	Zaccara et al., 2019b	Italy	
	MG717999	Zaccara et al., 2019b	Italy	
	MG718000	Zaccara et al., 2019b	Italy	
	MG718001	Zaccara et al., 2019b	Italy	
	MG718002	Zaccara et al., 2019b	Italy	
	MG718003	Zaccara et al., 2019b	Italy	
	MG718004	Zaccara et al., 2019b	Italy	Bple04*
	MG718005	Zaccara et al., 2019b	Italy	
	MG718006	Zaccara et al., 2019b	Italy	
	MG718007	Zaccara et al., 2019b	Italy	
	MG718008	Zaccara et al., 2019b	Italy	
	MG718009	Zaccara et al., 2019b	Italy	
	MG718010	Zaccara et al., 2019b	Italy	
	MG718011	Zaccara et al., 2019b	Italy	

Table S6.3 *GH-2 nDNA diagnostic sequences of the native B. tyberinus and B. plebejus and the exotic B. barbus retrieved from GenBank and used in the phylogenetic analyses (see Fig. 3). For each sequence, species, GenBank accession number, refence, geographic source. * haplotype overlap with sequences produced in this study.*

Species	GenBank code	Reference	Source	Haplotype label
B. tyberinus	KF963487	Buonerba et al., 2015	Italy	HBT02*
	KF963488	Buonerba et al., 2015	Italy	HBT01*
	KF963489	Buonerba et al., 2015	Italy	
	KF963490	Buonerba et al., 2015	Italy	HBT01*
	KF963491	Buonerba et al., 2015	Italy	
	KF963492	Buonerba et al., 2015	Italy	HBT01*
B. plebejus	KF923618	Buonerba et al., 2015	Italy	
	KF923619	Buonerba et al., 2015	Italy	
	KF923620	Buonerba et al., 2015	Italy	
	KF923621	Buonerba et al., 2015	Italy	HBP01*
	KF923622	Buonerba et al., 2015	Italy	HBP02*
	KF923623	Buonerba et al., 2015	Italy	
	KF923624	Buonerba et al., 2015	Italy	
	KF923625	Buonerba et al., 2015	Italy	
	KF923626	Buonerba et al., 2015	Italy	
	KF923627	Buonerba et al., 2015	Italy	
	KF923628	Buonerba et al., 2015	Italy	
	KF923629	Buonerba et al., 2015	Italy	

Table S6.3 (Continued)

Species	GenBank code	Reference	Source	Haplotype label
B. plebejus	KF923630	Buonerba et al., 2015	Italy	
	KF923631	Buonerba et al., 2015	Italy	
	KF963493	Buonerba et al., 2015	Italy	HBP02*
	KF963494	Buonerba et al., 2015	Italy	HBP01*
	KF963495	Buonerba et al., 2015	Italy	
	KF963496	Buonerba et al., 2015	Italy	HBP01*
	KF963497	Buonerba et al., 2015	Italy	HBP01*
B. barbus	KT766209	Antognazza et al., 2016	England	HBB07*
	KT766214	Antognazza et al., 2016	England	
	KT766217	Antognazza et al., 2016	England	
	KT766220	Antognazza et al., 2016	England	
	KT766223	Antognazza et al., 2016	England	
	KT766226	Antognazza et al., 2016	England	
	KT766229	Antognazza et al., 2016	England	
	KT766230	Antognazza et al., 2016	England	
	KT766233	Antognazza et al., 2016	England	
	KT766252	Antognazza et al., 2016	England	
	KT766262	Antognazza et al., 2016	England	
	KT766276	Antognazza et al., 2016	England	
	KT766282	Antognazza et al., 2016	England	

Table S6.3 (Continued)

Species	GenBank code	Reference	Source	Haplotype label
	KT766285	Antognazza et al., 2016	England	
	KT766287	Antognazza et al., 2016	England	HBB01*
	KT766288	Antognazza et al., 2016	England	HBB05*
	KT766289	Antognazza et al., 2016	England	HBB11*
	KT766290	Antognazza et al., 2016	England	HBB09*

Table S6.4 *D-loop mtDNA haplotype distribution in invaded and uninvaded populations.*

Species	Haplotype	GenBank code	PV*p*	PV*i*	TL*i*	TL*p*
B. tyberinus	Btyb11	MT385879				20
	Btyb19	MT385880				1
	Btyb20	MT385881				1
B. barbus	Bbar01	MT385882		3	16	
	Bbar03	MT385883		1		
	Bbar04	MT385884			3	
	Bbar05	MT385885			1	
	Bbar09	MT385886		4	2	
	Bbar10	MT385887		7		
	Bbar11	MT385888			1	
	Bbar13	MT385889			1	
	Bbar15	MT385890		3		
	Bbar16	MT385891			2	
	Bbar22	MT385892		5		
	Bbar23	MT385893		2	1	
	Bbar24	MT385894		1		
	Bbar25	MT385895			1	
	Bbar26	MT385896			1	
B. plebejus	Bpleb02	MT385876	6			
	Bpleb04	MT385872	5			
	Bpleb07	MT385874	5			
	Bpleb09	MT385875	5			
	Bple13	MT385878	2			
	Bple33	MT385873	1			
	Bple34	MT385877	1			
TOTAL			25	26	29	22

Table S6.5 *GH-2 ncDNA haplotype distribution in invaded and uninvaded populations.*

Species	Haplotype	GenBank code	PV*p*	PV*i*	TL*i*	TL*p*
B. plebejus	HBP01	MT385914	45	9		
	HBP02	MT385915	3	2		
	HBP03	MT385916		4		
	HBP04	MT385917	2			
	HBP05	MT385918		1		
	HBP06	MT385919		1		
	HBP07	MT385920		1		
	HBP08	MT385921		1		
	HBP09	MT385922		1		
	HBP10	MT385923		1		
	HBP11	MT385924		1		
	HBP12	MT385925		1		
	HBP13	MT385926		1		
	HBP14	MT385927		1		
	HBP15	MT385928		1		
	HBP16	MT385929		1		
	HBP17	MT385930		1		
B. tyberinus	HBT01	MT385931			5	22
	HBT02	MT385932				4
	HBT03	MT385933				2
	HBT04	MT385934			2	
	HBT05	MT385935			2	
	HBT06	MT385936			1	
	HBT07	MT385937			1	
	HBT08	MT385938			1	
B. barbus	HBB01	MT385897		5	27	
	HBB02	MT385898		13		
	HBB03	MT385899			4	
	HBB04	MT385900			2	
	HBB05	MT385901			2	

Table S6.5 (Continued)

Species	Haplotype	GenBank code	PV*p*	PV*i*	TL*i*	TL*p*
B. barbus	HBB06	MT385902			2	
	HBB07	MT385903		2		
	HBB08	MT385904			2	
	HBB09	MT385905			1	
	HBB10	MT385906		3		
	HBB11	MT385907			1	
	HBB12	MT385908			1	
	HBB13	MT385909			1	
	HBB14	MT385910		1		
	HBB15	MT385911			1	
	HBB16	MT385912			1	
	HBB17	MT385913			1	
TOTAL			50	52	58	28

Table S6.6 *Introgression pattern of invaded populations (TLi and PVi) detailing the mitochondrial (D-loop) and nuclear (GH-2 allelles A and B) combinations of each sample. Haplotypes, taxonomic attribution and GenBank accession number are provided.*

Population	Sample ID	Dloop Haplotype	Dloop taxa	GB code	GH2 Haplotype_A	GH2_A taxa	GB code	GH2_Haplotype B	GH2_B taxa	GB code	nDNA Genotype
PVi	Mt1	Bbar09	*B. barbus*	MT385886	HBP01	*B. plebejus*	MT385915	HBP01	*B. plebejus*	MT385915	Bp/Bp
PVi	Mt3	Bbar22	*B. barbus*	MT385892	HBP03	*B. plebejus*	MT385916	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt4	Bbar01	*B. barbus*	MT385882	HBP15	*B. plebejus*	MT385918	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt5	Bbar10	*B. barbus*	MT385887	HBP03	*B. plebejus*	MT385916	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt6	Bbar09	*B. barbus*	MT385886	HBB07	*B. barbus*	MT385915	HBB07	*B. barbus*	MT385915	Bb/Bb
PVi	Mt8	Bbar10	*B. barbus*	MT385887	HBP13	*B. plebejus*	MT385926	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt9	Bbar10	*B. barbus*	MT385887	HBP17	*B. plebejus*	MT385930	HBB10	*B. barbus*	MT385915	Bp/Bb

Table S6.6 (Continued)

Population	Sample ID	Dloop Haplotype	Dloop taxa	GB code	GH2 Haplotype_A	GH2_A taxa	GB code	GH2_Haplotype B	GH2_B taxa	GB code	nDNA Genotype
PVi	Mt10	Bbar01	*B. barbus*	MT385882	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
PVi	Mt11	Bbar03	*B. barbus*	MT385883	HBP05	*B. plebejus*	MT385918	HBB10	*B. barbus*	MT385915	Bp/Bb
PVi	Mt12	Bbar23	*B. barbus*	MT385893	HBP01	*B. plebejus*	MT385915	HBP01	*B. plebejus*	MT385915	Bp/Bp
PVi	Mt16	Bbar23	*B. barbus*	MT385893	HBP06	*B. plebejus*	MT385919	HBB01	*B. barbus*	MT385897	Bp/Bb
PVi	Mt21	Bbar09	*B. barbus*	MT385886	HBB02	*B. barbus*	MT385914	HBB14	*B. barbus*	MT385915	Bb/Bb
PVi	Mt24	Bbar09	*B. barbus*	MT385886	HBP03	*B. plebejus*	MT385916	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt25	Bbar15	*B. barbus*	MT385890	HBP02	*B. plebejus*	MT385915	HBB01	*B. barbus*	MT385897	Bp/Bb
PVi	Mt26	Bbar22	*B. barbus*	MT385892	HBP01	*B. plebejus*	MT385915	HBP01	*B. plebejus*	MT385915	Bp/Bp
PVi	Mt29	Bbar10	*B. barbus*	MT385887	HBP03	*B. plebejus*	MT385916	HBP07	*B. plebejus*	MT385920	Bp/Bp

Table S6.6 (Continued)

Population	Sample ID	Dloop Haplotype	Dloop taxa	GB code	GH2 Haplotype_A	GH2_A taxa	GB code	GH2_Haplotype B	GH2_B taxa	GB code	nDNA Genotype
PVi	Mt30	Bbar15	*B. barbus*	MT385890	HBP09	*B. plebejus*	MT385922	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt31	Bbar22	*B. barbus*	MT385892	HBP02	*B. plebejus*	MT385915	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt32	Bbar10	*B. barbus*	MT385887	HBP10	*B. plebejus*	MT385923	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt33	Bbar24	*B. barbus*	MT385894	HBP11	*B. plebejus*	MT385924	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt34	Bbar01	*B. barbus*	MT385882	HBP12	*B. plebejus*	MT385925	HBB02	*B. barbus*	MT385914	Bp/Bb
PVi	Mt36	Bbar22	*B. barbus*	MT385892	HBP01	*B. plebejus*	MT385915	HBP01	*B. plebejus*	MT385915	Bp/Bp
PVi	Mt37	Bbar15	*B. barbus*	MT385890	HBB01	*B. barbus*	MT385897	HBB10	*B. barbus*	MT385915	Bb/Bb
PVi	Mt38	Bbar22	*B. barbus*	MT385892	HBP08	*B. plebejus*	MT385921	HBP01	*B. plebejus*	MT385915	Bp/Bp
PVi	Mt39	Bbar10	*B. barbus*	MT385887	HBP14	*B. plebejus*	MT385927	HBB02	*B. barbus*	MT385914	Bp/Bb

Table S6.6 (Continued)

Population	Sample ID	Dloop Haplotype	Dloop taxa	GB code	GH2 Haplotype_A	GH2_A taxa	GB code	GH2_Haplotype B	GH2_B taxa	GB code	nDNA Genotype
PVi	Mt40	Bbar10	*B. barbus*	MT385887	HBP16	*B. plebejus*	MT385929	HBB02	*B. barbus*	MT385914	Bp/Bb
TLi	PA01	Bbar11	B. *barbus*	MT385888	HBT01	*B. tyberinus*	MT385931	HBB11	*B. barbus*	MT385907	Bt/Bb
TLi	PA04	Bbar01	B. *barbus*	MT385882	HBB04	*B. barbus*	MT385900	HBB05	*B. barbus*	MT385901	Bb/Bb
TLi	PA05	Bbar16	B. *barbus*	MT385891	HBT01	*B. tyberinus*	MT385931	HBB06	*B. barbus*	MT385902	Bt/Bb
TLi	PA07	Bbar13	B. *barbus*	MT385889	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA08	Bbar05	B. *barbus*	MT385885	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA09	Bbar23	B. *barbus*	MT385893	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA10	Bbar01	B. *barbus*	MT385882	HBT01	*B. tyberinus*	MT385931	HBB03	*B. barbus*	MT385899	Bt/Bb
TLi	PA11	Bbar01	B. *barbus*	MT385882	HBT05	*B. tyberinus*	MT385935	HBB01	*B. barbus*	MT385897	Bt/Bb

Table S6.6 (Continued)

Population	Sample ID	Dloop Haplotype	Dloop taxa	GB code	GH2 Haplotype_A	GH2_A taxa	GB code	GH2_Haplotype B	GH2_B taxa	GB code	nDNA Genotype
TLi	PA12	Bbar01	B. *barbus*	MT385882	HBB08	*B. barbus*	MT385904	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA13	Bbar25	B. *barbus*	MT385895	HBB01	*B. barbus*	MT385897	HBB09	*B. barbus*	MT385905	Bb/Bb
TLi	PA15	Bbar04	B. *barbus*	MT385884	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA16	Bbar01	B. *barbus*	MT385882	HBT07	*B. tyberinus*	MT385937	HBB08	*B. barbus*	MT385904	Bt/Bb
TLi	PA17	Bbar01	B. *barbus*	MT385882	HBB03	*B. barbus*	MT385899	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA18	Bbar01	B. *barbus*	MT385882	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA19	Bbar09	B. *barbus*	MT385886	HBT04	*B. tyberinus*	MT385934	HBB01	*B. barbus*	MT385897	Bt/Bb
TLi	PA20	Bbar04	B. *barbus*	MT385884	HBB17	*B. barbus*	MT385934	HBB05	*B. barbus*	MT385901	Bb/Bb
TLi	PA21	Bbar01	B. *barbus*	MT385882	HBT04	*B. tyberinus*	MT385934	HBB01	*B. barbus*	MT385897	Bt/Bb

Table S6.6 (Continued)

Population	Sample ID	Dloop Haplotype	Dloop taxa	GB code	GH2 Haplotype_A	GH2_A taxa	GB code	GH2_Haplotype B	GH2_B taxa	GB code	nDNA Genotype
TLi	PA22	Bbar01	B. *barbus*	MT385882	HBB04	*B. barbus*	MT385900	HBB03	*B. barbus*	MT385899	Bb/Bb
TLi	PA23	Bbar01	B. *barbus*	MT385882	HBB15	*B. barbus*	MT385911	HBB03	*B. barbus*	MT385899	Bb/Bb
TLi	PA24	Bbar01	B. *barbus*	MT385882	HBT08	*B. tyberinus*	MT385938	HBB01	*B. barbus*	MT385897	Bt/Bb
TLi	PA25	Bbar26	B. *barbus*	MT385896	HBT01	*B. tyberinus*	MT385931	HBT06	*B. tyberinus*	MT385936	Bt/Bt
TLi	PA27	Bbar01	B. *barbus*	MT385882	HBB16	*B. barbus*	MT385912	HBB06	*B. barbus*	MT385902	Bb/Bb
TLi	PA28	Bbar04	B. *barbus*	MT385884	HBT01	*B. tyberinus*	MT385931	HBB01	*B. barbus*	MT385897	Bt/Bb
TLi	PA33	Bbar16	B. *barbus*	MT385891	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA34	Bbar01	B. *barbus*	MT385882	HBB12	*B. barbus*	MT385908	HBB13	*B. barbus*	MT385909	Bb/Bb
TLi	PA38	Bbar09	B. *barbus*	MT385886	HBT05	*B. tyberinus*	MT385935	HBB01	*B. barbus*	MT385897	Bt/Bb

Table S6.6 (Continued)

Population	Sample ID	Dloop Haplotype	Dloop taxa	GB code	GH2 Haplotype_A	GH2_A taxa	GB code	GH2_Haplotype B	GH2_B taxa	GB code	nDNA Genotype
TLi	PA39	Bbar01	B. *barbus*	MT385882	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA40	Bbar01	B. *barbus*	MT385882	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb
TLi	PA42	Bbar01	B. *barbus*	MT385882	HBB01	*B. barbus*	MT385897	HBB01	*B. barbus*	MT385897	Bb/Bb

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CHAPTER VII

Biological and trophic consequences of genetic introgression between endemic and invasive *Barbus* fishes

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Abstract

Genetic introgression with native species is recognized as a detrimental impact resulting from biological invasions involving taxonomically similar invaders. Whilst the underlying genetic mechanisms are increasingly understood, the ecological consequences of introgression are relatively less studied, despite their utility for increasing knowledge on how invasion impacts can manifest. Here, the ecological consequences of genetic introgression from an invasive congener were tested using the endemic barbel populations of central Italy, where the invader was the European barbel *Barbus barbus*. Four populations of native *Barbus* species (*B. plebejus* and *B. tyberinus*) were studied: two purebred and two completely introgressed with alien *B. barbus*. Across the four populations, differences in their biological traits (growth, body condition and population demographic structure) and trophic ecology (gut content analysis and stable isotope analysis) were tested. While all populations had similar body condition and were dominated by fish up to 2 years of age, the introgressed fish had substantially greater lengths at the same age, with maximum lengths 410-460 mm in hybrids versus 340-360 mm in native purebred barbel. The population characterized by the highest number of introgressed *B. barbus* alleles (81%) had the largest trophic niche and a substantially lower trophic position

than the other populations through their exploitation of a wider range of resources (e.g. small fishes and plants). These results attest that the genetic introgression of an invasive congener with native species can results in substantial ecological consequences, including potential cascading effects.

7.1 Introduction

Interspecific hybridization is a widespread process in animal communities that has been suggested to negatively affect species through depressing the fitness of hybrids (i.e. outbreeding depression) (Rhymer and Simberloff 1996). However, growing evidence now suggests that hybridization has driven speciation in a wide range of taxa (Seehausen 2004; Baack and Rieseberg 2007; Svardal et al. 2019) and, consequently, its role in evolution has been reconsidered.

While being an important evolutionary force, introgressive hybridization can create considerable conservation issues (Allendorf et al. 2001; Brennan et al. 2014), especially when anthropogenic activities, such as habitat modification (e.g. Chafin et al. 2019) and species introductions (e.g. Ward et al. 2012), result in the mixing of previously isolated species. This is particularly true when one of the formerly isolated species is an endemic with a narrow distribution range and/or the two species are taxonomically similar (Huxel 1999; Hänfling et al.

2005). Hybridization can even trigger the invasion process (Hovick and Whitney 2014; Roy et al. 2015), with hybrids potentially outperforming parental taxa through the novel combination of parental traits (Seehausen 2004) and/or expressing new traits through transgressive hybridization (Rieseberg et al. 1999).

Invasion driven hybridization, resulting from the introduction of alien species into communities where taxonomically similar native species are present, is increasingly recognized as a threat to the genetic integrity of many native species (Huxel 1999; Gaskin and Kazmer 2009; Kovach et al. 2015). Current knowledge on the genetic introgression of invasive and native species has tended to focus on the underlying genetic mechanisms, with less consideration given to how the introgression alters the functional traits and ecological interactions of the hybrids in relation to their parental species (Matsuzaki et al. 2010; Toscano et al. 2010; Hayden et al. 2011).

A model to study the ecological consequences of invasive hybridizing species is represented by the European barbel *Barbus barbus* (Linneus 1758), a cyprinid riverine species native to central Europe, that has been introduced into other European areas, including Italy (Bianco and Ketmaier 2001) and Western Britain (Wheeler and Jordan 1990) via anglers or

angling orientated stocking events. While this invader has no congeners present in Britain, limiting the genetic introgression concerns (Britton and Pegg 2011), four native *Barbus* species are present in Italy. Two of these, *B. caninus* Bonaparte 1839 and *B. balcanicus* Kotlik, Tsigenopoulus, Rab and Berrebi 2002, inhabit the upper reaches of rivers. In contrast, *B. tyberinus* Bonaparte 1839 and *B. plebejus* Bonaparte 1839 populate the middle/lower reaches of Italian rivers, in habitats that are also preferred by B. barbus (Carosi et al. 2017). All these native Italian barbels are generalist benthivores and so their diet tends to be dominated by benthic macroinvertebrates (e.g. dipteran larvae; Tancioni et al. 2001; Piria et al. 2005; Corse et al. 2010), with proportions of other food items varying according to availability (Piria et al. 2005).

Hybridization between *Barbus* species has been widely documented as both natural events (e.g. Tsigenopoulos et al. 2002; Buonerba et al. 2015), and following invasions (Meraner et al. 2013; Geiger et al. 2016). When hybridization occurs in natural contact zones, it is usually limited to that area but, in the case of the genetic admixture between invasive *B. barbus* and native Italian barbels, it has been found to be more widespread, with a tendency to form a complete 'hybrid swarm' (e.g. Meraner et al. 2013; Zaccara et al. 2014). Moreover, these Barbus hybrids are fertile and a range of hybrid forms may be

present across multiple generations, including backcrossed individuals (Meraner et al. 2013). There is thus the possibility that hybrid barbel have a fitness at least equal (or higher) to the parental species (Pfennig et al. 2007). Given that this introgression can result in morphological differences between the purebred and hybrid forms (Zaccara et al. 2020), questions over how morphological shifts alter the interactions of hybrids with other species and their environment, including their utilization of trophic resources, have arisen.

Therefore, the aim of this study was to test the biological and trophic consequences of genetic introgression across populations of endemic *Barbus* species invaded by *B. barbus*, with comparisons to uninvaded populations. The objectives were to test differences between purebred and introgressed *Barbus* populations in relation to their: (1) somatic growth rates, body condition and population demographic structure (i.e. biological traits); and (2) diet composition and trophic ecology (e.g. trophic niche size and trophic position), enabling the assessment of their functional roles (Davis et al. 2012; Carvalho et al. 2019; Pacioglu et al. 2019). We posit that: (1) introgressed fish will have biological traits at least equal to those of the parental species; and (2) introgressed fish will have larger trophic niche sizes that differ in their trophic positions compared with native parental species, with this potentially

related to alterations in their functional morphology (Zaccara et al. 2020).

7.2 Material and methods

7.2.1 *Sampling strategy and sites description*

Sampling was performed at four representative sites located in central Italy (Fig. 7.1). Two of these were selected where impassable weirs have prevented *B. barbus* invasions and thus purebred populations of the endemic *B. tyberinus* and *B. plebejus* were present (Zaccara et al. 2020). They were located in the species respective distribution range: the Tuscany-Latium (TL) and the Padano-Venetian (PV) ichthyo-geographic districts for *B. tyberinus* and *B. plebejus* populations respectively (Bianco, 1995). The other two sites were located within the same river catchments (see below) but where each of the two native species has introgressed with *B. barbus* (Chapter VI; Zaccara et al. 2020) following its invasion of the middle and lower reaches since at least 1998 and 2005 (i.e. their first detections in these basins; Lorenzoni et al. 2010; Lorenzoni and Esposito 2011) in TL and PV districts respectively. Therefore, for each ichthyo-geographic district, there was one purebred ("*p*") population (generally located in the upstream section), and one invaded and introgressed ("*i*") population (in the lowland section). Pure vs. hybrid status of populations have

already been tested using mitochondrial (D-loop) and nuclear (growth hormone 2; GH-2) DNA markers (see Zaccara et al. 2020). Thus, PV*p* and TL*p* were known to be populated by purebred *B. plebejus* and *B. tyberinus* respectively. Mitochondrial DNA analyses had revealed that barbel in PV*i* and TL*i* were all of hybrid origin (*B. plebejus* × *B. barbus* and *B. tyberinus* × *B. barbus*, respectively), while at the analysed nuclear marker, a different proportion of *B. barbus* alleles was detected between the two invaded populations, resulting in a higher number of *B. barbus* alleles (81%) in TL*i* than PV*i* (68%) (Zaccara et al. 2020).

Sites in TL were situated within the Paglia River basin and were the Paglia River (TL*i*) and the Montacchione Stream (TL*p*) (Fig. 7.1), where the latter is a tributary isolated from the main river by the presence of two weirs of over 2 m high. This basin is characterized by impermeable soils, with watercourses flowing in upland areas (Lorenzoni et al. 2010). Sites in PV were located within the Metauro River basin, being the Candigliano River (PV*i*) and the Bosso Stream (PV*p*) (Fig. 7.1); these sites were separated by three weirs, ranging in height from 0.4 to 1 m. This basin has a mountainous upper section that cuts across an area of steeply folded bedrock (Lorenzoni and Esposito 2011).

All of these watercourses were characterised by marked flow rate oscillations throughout the year and a high susceptibility to drought periods in summer, which are aggravated by water abstraction for irrigation and drinking water supply. The Montacchione sub-basin has a volcanic origin, while the other three are siliceous. Downstream sites (i.e. TL*i* and PV*i*) were characterised by a wider riverbed than the upstream sites (approximately 15 m *vs.* 5 m), which results in major vegetation cover of the latter that provides shading even during summer droughts (Fig. 7.1).

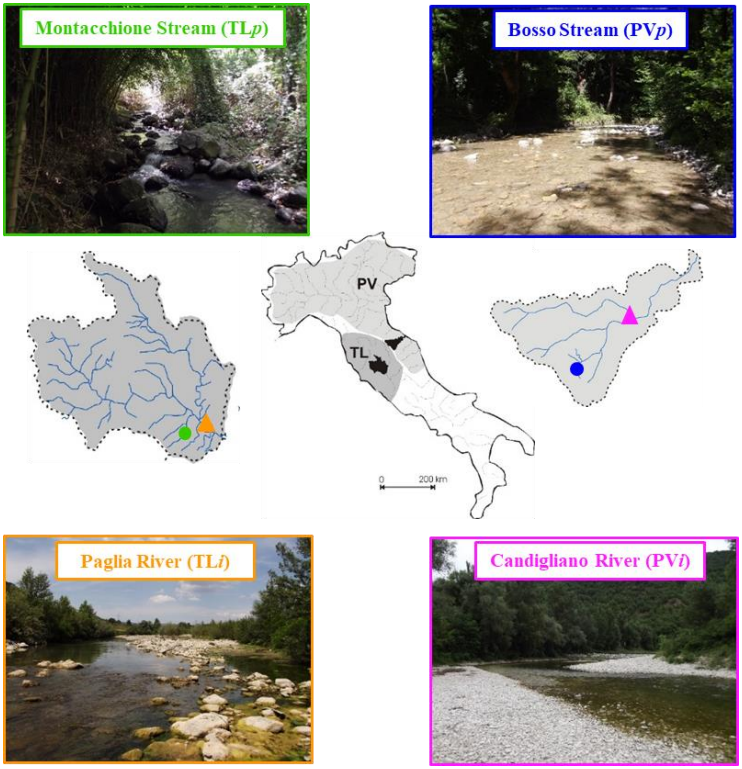

Figure 7.1 *Location and pictures of the four sampled rivers located within the Tuscany-Latium (TL) and the Padano-Venetian districts (PV) where 'i' and 'p' indicate sites where barbel hybrid ('i'; indicate by colored triangles on map) and purebred populations ('p'; indicated by colored circles on map) were found.*

7.2.2 *Fish and macroinvertebrate communities sampling and characterisation*

Quantitative sampling of the fish communities was completed in July 2019 by electrofishing using DC electric current (2500 W). To estimate fish density, a two-pass electrofishing approach was implemented (Moran, 1951; Zippin, 1956) involving the survey of a longitudinal transect of length 60 to 112 m (according to river size) in a downstream-upstream direction, applying the same sampling effort twice. No stop nets were in place, but river morphology was used (significant reduction of riverbed width (i.e. mesohabitat change) or weirs) to determine the end of the transect. Following their capture, fishes were anaesthetised, identified to species where possible (including non-*Barbus* species), measured for total length (to 1 mm) and weight (to 0.1 g).

A quantitative multi-habitat approach (Buffagni et al. 2005) was then used to sample benthic macroinvertebrate (BMI) communities using a Surber sampler (0.1 m^2 area, 500 µm mesh). Once collected, BMI samples were preserved in formalin (4%) and, then, in the laboratory, were sorted into families whose density (individual/m^2) was determined by counting individuals. For each sampling site, the fish and BMI assemblages were characterised through the calculation of three common metrics: total density (i.e. number of individuals per

m^2), richness (fish: number of species; BMI: number of families) and diversity (Shannon-Wiener index - H; Shannon 1948). The Bray-Curtis index (Bray and Curtis 1957) was used to quantify the compositional dissimilarity between BMI samples, where values ranged from 0 (completely similar) and 1 (completely dissimilar). These analyses were completed within the Past software (Hammer, Harper, and Ryan 2001).

7.2.3 *Barbel biological traits*

From all barbel, after measuring, three to five scales were removed from the left side for age determination. This was performed under a stereomicroscope coupled with a camera, with images stored within an archive built with the image analysis system IAS 2000 (QEA's IASLab® software). Ageing of scales from the images was carried out by two operators independently, discarding unreadable or dubious scales. Length at age relationships of each population were then fitted to the von Bertalanffy growth model (von Bertalanffy 1938) according to:

$$TL = L_\infty (1\text{-}\exp (-K (t\text{-}t_0)))$$

Where TL is the total length (mm) of each fish in cm at time t, L_∞ is the theoretical maximum length, K is the rate of approach to the maximum length, and t_0 is the theoretical age at which TL= 0. To assess possible differences in theoretical growth

parameters between populations, different non-linear models were fitted using von Bertalanffy equation in the fisheries assessment R package 'FSA' (Ogle et al. 2020; R core team, 2019) following a hierarchical approach (Ogle 2013). This consisted in starting with a general model in which L_∞, K and t_0 were calculated for each population independently and subsequently simplifying the model by keeping constant initially one and then two parameters at a time, finishing with a model where all the three parameters were in common. The best-fit model was then selected according to the Akaike's information criterion (AIC; Burnham and Anderson 1998). Differences in length-at-age between populations were tested through AMOVA in R ('*dplyr*' package, Wickham et al. 2020), with age and length kept as dependent variables and sampling site retained as independent variable.

Length-weight relations (LWRs) in each population was also estimated using the following linear regression model:

$$\log_{10} W = a + b \log_{10} TL$$

Where W is the weight in grams of the fish, TL is the total length in mm, *a* is the intercept of the regression curve and *b* is the regression coefficient (slope). ANCOVA was also used to test for differences in LWR across the populations, with differences from isometric growth (i.e. $b = 3$) tested for each

population using t-tests. LWR models obtained for each population were then used to back calculate fish weight, and residuals (differences between observed and predicted weight) were tested for significant differences (one way ANOVA) in fish body condition between the populations (Jakob et al. 1996).

7.2.4 *Barbel diet determination through Gut Content Analysis (GCA)*

A subsample of barbel (approximately 20 fish per site of age >1+ and up to 4+, and lengths between 69 and 279 mm) were selected for gut content analysis (GCA). These fish were euthanized (anaesthetic overdose, MS-222), placed on ice and brought to the laboratory. After their defrosting, the fish were dissected and their guts preserved in ethanol (70 %) prior to analysis. As barbel do not have a differentiated stomach, then the entire digestive tract ('gut') was examined, involving weighing and then emptying the tract of its contents into a Petri dish, with prey items viewed under a dissecting microscope (x 5 to x 50 magnification). Prey were initially identified to the lowest possible taxonomic level before being grouped into 15 categories according to their taxonomic affinities. Food items with a low frequency, low specific abundances (< 5 %) and/or occurred in only one population, were grouped into broader categories (i.e. terrestrial organisms, other aquatic BMI, fish bones). As the actions of the pharyngeal teeth of barbel makes

the separation of their ingested prey difficult, this prevents the effective use of gravimetric or numeric methods and, consequently, the relative-fullness method was selected (Hyslop 1980). This method is recommended as one of the election methods in relative diet composition studies, as it can produce robust data despite its subjective nature (Amundsen and Sánchez-Hernández 2019). Accordingly, gut fullness was estimated on a scale from empty (0 %) to full (100 %), with the volumetric percentage of each food item then estimated by eye and summed up to reach the total fullness. The feeding activity of the fish in each population was then tested comparing the vacuity index (I%; calculated as the proportion of fish with empty stomachs in each sample) and the mean volume of gut contents.

The fish feeding strategy was then assessed following the method proposed by Costello (1990), in its modified version (Amundsen et al. 1996), where the frequency of occurrence and prey-specific abundances of each food category are calculated and used to plot graphs. Visual inspection of the plots indicates prey importance, feeding strategy and, ultimately, how each individual contributes to the trophic niche of the population by specialising on specific dietary items (i.e. within phenotypic contribution) or not (i.e. between phenotypic contribution). Correspondingly, the frequency of occurrence of each food

category for each population was calculated as the percentage of fish with prey *i* in their stomachs against the total number of fish with contents in their guts (% $F_i = N_i / N$ x 100). Prey-specific abundances were calculated as the volume occupied by prey item *i* (S_i) in all the guts against the total gut volume comprising prey *i* (% $P_i = \sum S_i / \sum S_{ti}$; Amundsen et al. 1996).

The GCA data were then analysed for diet composition and niche width area per population. Data were arcsine square root transformed and non-metric multidimensional scaling (nMDS) was performed with 40% standard ellipses representing the core population trophic niche (Gutmann Roberts and Britton 2018), as implemented in the R package '*vegan*' (Oksanen et al. 2019). A Bray-Curtis distance matrix was built before PERMANOVA ('*adonis*' function) was used to test for differences in barbel's diet between the four populations. SIMPER analysis was then applied to detect the contribution of each food item to the dissimilarities. The Shannon-Wiener diversity index (H) was also calculated within the same package (i.e. *vegan*), and ANOVA and Tukey pairwise test available in R were used to test for differences in H between the four populations.

7.2.5 *Stable Isotope Analysis (SIA) of barbel populations and putative food resources*

For stable isotope analyses, the fish used differed to those used in the GCA but were collected simultaneously. This was partly due to logistical reasons relating to both sample collection, it was mainly due to logistical problems with the stable isotope analyses relating to the Covid-19 lockdown that prevented the fish from the GCA being analysed in a timely manner for the purposes of this study. Consequently, the barbel analysed for their stable isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) comprised of 10 fish per site, where all fish were between age 1+ and 4+ years old and lengths between 98 and 244 mm. As these fish were returned alive to the river then scales were used as the tissue for analyses, as they represent non-lethal alternatives to muscles (Busst et al. 2015) and are indicative of diet composition over considerable timeframes (> 3 months) (Busst and Britton 2018). Three to five scales were removed from the left side (above the lateral line and below the dorsal fin) and then placed in paper envelopes until processing.

To provide SIA data for the baselines and putative prey, BMI, biofilm (periphyton), benthic algae and fine and coarse particular organic matter (FPOM and CPOM respectively) were sampled on the same date of the fish. A dedicated BMI sample was collected at each site with the same method used to

characterize the BMI communities, but this was put on ice and frozen upon arrival in the laboratory. After defrosting, five families that were present at all sampling sites were selected (Supplementary material Table S7.1) and three replicates of each family (comprising of 1 up to 10 individuals, according to the size) were processed. Biofilm was brushed from the upper side of six stones randomly picked up at each sampling site and then collected in 500 ml water. Samples were frozen until processing in the laboratory, where each sample was divided in three replicates and filtered on glass-fibre filters (0.7 μm pore size). Two litres of turbid water were collected moving fine substrate with hands for FPOM collection, and then three replicates were filtered on glass-fibre filters. CPOM (mainly decaying leaves) and benthic algae (except for TL*p*) were randomly collected by hand at each sampling site.

Preparation for SIA of fish scales, BMI and benthic algae involved rinsing with distilled water before being oven dried at 60°C for 48 h, with this drying also performed for the biofilm, FPOM and CPOM samples. For the scales, a preliminary step was added that involved the excision of the outer portion of each scale for analysis, as this reflects the collagen produced in the last growth season and not in previous life stages (Hutchinson and Trueman 2006). The stable isotope ratios of carbon (^{13}C:^{12}C; reported as $\delta^{13}C$) and nitrogen (^{15}N:^{14}N, reported as

$\delta^{15}N$) of the fish, putative prey and baseline samples were then analysed at the Cornell Isotope Laboratory, New York (USA). Across the four sites, 142 samples were analysed: 40 fish, 57 BMI (five families) and 45 primary producers (Supplementary material Table S7.1). Samples were ground to powder, weighed (to nearest 1000 μg) and put in tin capsules, before being analysed on a Thermo delta V isotope mass spectrometer (IRMS) coupled with a NC2500 elemental analyser. Data accuracy and precision were tested every 10 samples reporting an overall standard deviation for internal animal standard (deer) of 0.08 for $\delta^{15}N$ and 0.03 for $\delta^{13}C$. The C:N ratios of all animal samples were below 3.5 and so did not require lipid correction (Skinner et al. 2016).

The SIA data were initially tested for any effects of length on $\delta^{13}C$ and $\delta^{15}N$ (as a proxy of ontogenetic effects on diet) in each population through linear regressions as implemented in R. A Bayesian approach available in the R package 'tRophicPosition' (Quezada-Romegialli et al. 2018) was then implemented to calculate trophic position at population level and to test for differences between purebred and hybrid populations' TPs. As barbel are mainly invertivores, BMI were used as the baseline data. However, as the analysed BMI were not always distinguishable from each other based on their stable isotope ratios (i.e. their standard deviation overlapped; Table

S7.1), then these resources were pooled, resulting in one baseline model being implemented. The trophic discrimination factor used for $\delta^{15}N$ (i.e. $\Delta^{15}N$) was 4.2 ‰ ± 0.2 ‰, with this specific to scales for *B. barbus*, derived experimentally from individuals that had fed on an invertebrate-based diet (Busst and Britton 2016). The probability that the posterior distribution relative to each population's TP was higher or smaller than others ($\alpha = 0.05$) was used to test for differences.

To enable individual comparisons between the different rivers, barbel $\delta^{15}N$ ratio was converted to TP according to Olsson et al. (2009):

$$TP = 2 + \delta^{15}N_{barbel} - \delta^{15}N_{meanBMI} / 4.2$$

Where TP and $\delta^{15}N_{barbel}$ are the trophic position and the nitrogen ratio of each fish and $\delta^{15}N_{meanBMI}$ is the mean nitrogen ratio of the benthic macroinvertebrates and 2 is the trophic position of this latter (i.e. primary consumers). Although it is recommended to estimate consumer TPs through the use of baseline taxa that are long-lived (e.g. bivalves and snails) (Post 2002), there were insufficient densities of these taxa in the samples to enable this. Similarly, for *Barbus* $\delta^{13}C$, conversions to corrected carbon (C_{corr}) utilised the $\delta^{13}C$ data of the BMI using the following equation (adapted from Olsson et al. 2009):

$$C_{corr} = [(\delta^{13}C_{barbel} - \Delta^{13}C) - \delta^{13}C_{meanBMI}] / CR_{BMI}$$

Wherein $\delta^{13}C_{barbel}$ is the carbon value of each fish, $\Delta^{13}C$ is carbon tissue-specific trophic discrimination factor for *B. barbus* fed an invertebrate diet (Busst and Britton 2016), $\delta^{13}C_{meanBMI}$ is the mean carbon ratio of all the benthic macroinvertebrates sampled for SIA and CR_{BMI} is the carbon range (δ^{13}Cmax - δ^{13}Cmin) of the same macroinvertebrates (Olsson et al. 2009). ANOVA implemented in R was used to test for differences in carbon source between populations.

The isotopic niches of each population were then built using two approaches in the SIBER R package (Jackson et al. 2011), the maximum likelihood Standard Ellipse Area (SEA) and the Bayesian estimate of the standard ellipse area (SEA_B). SEA_B tested for significant differences in niche width between populations and it was obtained through Markov Chain Monte Carlo simulations (10^4 iterations per group), with differences calculated as the probability that the posterior distribution relative to each population niche was larger or smaller than others ($\alpha = 0.05$). Maximum likelihood estimate of SEAs were used to plot the niches in the isotopic space, where they represent the population 'core' niche (40 %), and enabled identification of the extent of isotopic niche overlap between the different barbel populations (Jackson et al. 2012).

7.3 Results

7.3.1 *Characterization of fish and benthic macroinvertebrate communities*

The fish communities of the four sites differed considerably in terms of composition, density and richness (Supplementary material Table S7.2). At PV*p*, the dominant species were *Telestes muticellus* (Bonaparte 1837) and *Cottus gobio* L., at PV*i*, *Gobio gobio* (L.) was most abundant, at TL*p* it was *Barbus tyberinus*, and at TL*i* it was *Padogobius nigricans* (Canestrini 1867). These taxa are all native, except for *G. gobio* at PV*i*. At PV*i*, three of eight fish species present were alien and at TL*i*, five of eight were alien (Table S7.2). All fish species at PV*p* and TL*p* were native, except for two salmonid species (Atlantic lineage of *Salmo trutta* L. and *Oncorhyinchus mykiss* Walbaum 1792, respectively). At TL*i* there was the highest density of fishes followed by PV*p*, with both sites having a relatively lower diversity (H = 0.5; Table S7.2) than the other two sites (H > 0.9; Table S7.2).

Similarly, the composition of the BMI communities varied between the four sampling sites (Supplementary material Table S7.3), with values of the Bray-Curtis index ranging from 0.54 (PV*p* *vs.* TL*i*) to 0.96 (TL*p* *vs.* PV*i*). The TL*p* community differed the most from the other communities (Bray-Curtis

index > 0.87) and was dominated by the gastropods Lymnaeidae and Planorobidae (Table S7.3). At PV*i* there was the highest BMI density while the lowest was in TL*p* (Table S7.3). At PV*p* BMI community was relatively more diverse and richer than at the other sites (S < 19; H < 1.6; Table S7.3).

7.3.2 Barbel age structure and condition

Across the four populations, seven age classes (0+ to 6+) were present at fish lengths of 38 to 286 mm. Fish of 5+ and 6+ years were only present in the purebred barbel populations (TL*p* and PV*p*), with fish in the introgressed populations only to a maximum age of 3+ (TL*i*) and 4+ (PV*i*) years (Fig. 7.2a). The most frequent age classes present were 1+ in hybrid populations and 2+ in purebred populations. As the length data were not homogeneously distributed in terms of number of individuals per age class, theoretical growth model calculations were performed on the mean total lengths, where data on the age 5+ and 6+ fish not included as they were not present in all the populations. The model in which L_∞ and t_0 varied across the populations while K remained constant (K = 0.24 ± 0.03 standard error) was selected as the best-fitting model, indicating that the introgressed barbel (both at TL*i* and PV*i*) had significantly larger maximum theoretical lengths than purebreds (Table 7.1). MANOVA showed significantly

different length-at-age between sites for age classes from 1+ to 4+ (Pillai's trace = 0.5; $F_{2, 245} = 31.67$, $p < 0.001$), with hybrids having greater mean lengths at ages equal and/or greater than 2 (Fig. 7.2b).

Length-weight relations (LWRs) varied significantly across the populations (ANCOVA: $F_{3, 293} = 1430$, $p < 0.001$) and within each population, LWR models were highly significant ($R^2 \geq 0.96$, $p < 0.001$; Table 7.1). Allometric negative growth (i.e. $b < 3$; t-test $p < 0.05$) was detected in all populations except for TL*p* ($b = 3.0$). Conversely, body condition indices were all around zero (Table 7.1) and did not vary between barbel populations (ANOVA $F_{3, 294} = 1.35$; $p > 0.05$).

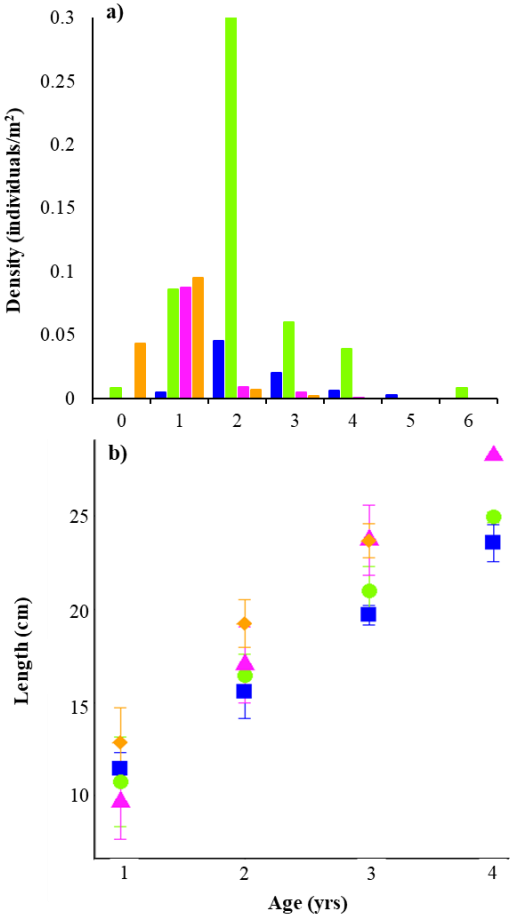

Figure 7.2 (*a) Age class structure of barbel at each site where bars indicate density (individuals/m²) for each age class (0+ to 6+) of fish sampled at PVp, TLp, PVi and TLi respectively. (b) Mean total lengths (and standard deviations) of barbel of ages 1 to 4 sampled at PVi (pink triangles), TLi (orange diamonds), PVp (blue squares) and TLp (green circles) sites.*

Table 7.1 *Mean ± standard error of length-weight relation (LWR) parameters with relative body index (BI) and maximum theoretical lengths (L_∞) and theoretical age at which the total length of the fish is equal to 0 (t_0) calculated by the best-fitting von Bertalanffy (1938) model for introgressed (PVi and TLi) and purebred populations (PVp and TLp). The rate of approach to L_∞ remained constant (K = 0.24 ± 0.03) between the populations and it is not reported in the table. N= number of barbel analysed per population; a = intercept of the LWR regression curve, b = regression coefficient (slope), R^2= determination coefficient of the LWR regression curve.*

		LWR parameters					
Population	**N**	***a***	***b***	**R^2**	**BI**	**L_∞**	**t_0**
PV*p*	41	0.015 ± 0.19	2.83 ± 0.07	0.98	0.01 ± 0.01	34.4 ± 1.8	-0.60 ± 0.10
PV*i*	72	0.016 ± 0.17	2.78 ± 0.07	0.96	0.01 ± 0.03	45.7 ± 3.1	0.03 ± 0.07
TL*p*	44	0.011 ± 0.10	2.99 ± 0.04	0.99	0.01 ± 0.01	35.9 ± 1.7	-0.50 ± 0.10
TL*i*	141	0.014 ± 0.07	2.80 ± 0.03	0.99	0.01 ± 0.02	41.1 ± 2.8	-0.55 ± 0.1

7.3.3 Barbel diet composition and dietary niche

The fish analysed for GCA were not significantly different in length across the rivers (ANOVA $F_{3,77} = 0.84$; $p > 0.05$). The vacuity index (I %) ranged between 0 % (TL*p*) to 21 % (TL*i*) (Table 7.2), with mean gut fullness being the highest and the lowest in the same rivers respectively (ANOVA: $F_{3, 69} = 14.86$; $p < 0.001$). The most frequent food items in barbel diets across all sites were aquatic insect larvae, particularly Chironomidae (Supplementary material Fig. S7.1). Feeding strategy plots (Fig. S7.1) indicated generalized feeding behaviour in all populations, with all barbel frequently consuming certain prey items (e.g. Chironomidae and Simulidae), but with some differences in the contributions of others (e.g. Mollusca, terrestrial organisms and plants). However, an exception was in TL*i* and PV*i*, where there was some dietary specialization through some individuals feeding on fish (Fig. S7.1). This resulted in considerable differences in diet composition among sites (Fig. 7.3), with significant differences in the population trophic niches (PERMANOVA test: $F_{3, 69} = 14.75$, $R^2 = 0.40$; $p < 0.001$). The widest trophic niche was in TL*i* and then PV*p* (as shown by 40% ellipses in the nMDS analysis, Table 7.2; Figure 7.3a). All pairwise comparisons revealed significant differences in niche composition between the populations ($p_{adj} < 0.01$), with the highest overall average dissimilarity in the diet of TL*i*

barbels (≥ 75.6 %; Table 7.3). The diet of hybrids in TL*i* lacked the items that were frequent and abundant in the diets of the other populations (e.g. Mollusca, Hydropsichidae and other Trichoptera), while consuming food categories (e.g. fish bones and plants) that were absent or infrequent in the other populations (Table 7.3; Fig. S7.1). Barbel at PV*p* and PV*i* had the lowest average dissimilarity, with some overlap in their dietary niches evident in the nMDS analysis (Fig. 7.3a). Although there were significant differences in H between the diets of the barbel populations (ANOVA: $F_{3, 69} = 11.76$; $p < 0.001$), pairwise comparisons indicated these were only significant between TL*i* and the other sites (Tukey test, $p_{adj} < 0.001$) (Table 7.2). Although there were significant differences in *H* between the diets of the barbel populations (ANOVA: $F_{3, 84}=14.4$; $p<0.001$), pairwise comparisons indicated these were only significant for between TL*i* and the other sites (Tukey test, $p_{adj}<0.001$) (Table 7.2). Feeding strategy plots (Supplementary material Fig. S7.1) indicated generalized feeding behaviour in all populations, with all barbel frequently consuming certain prey items (e.g Chironomidae, other Diptera, Baetidae), but with some differences in the contributions of others (e.g. Mollusca, terrestrial organisms). However, an exception was in TL*i*, where there was some dietary specialization through their feeding on fish (in agreement with the SIMPER analysis).

Table 7.2 *Mean vacuity index (I %) and mean percent gut fullness, Shannon-Wiener diversity index of diet (H) and dietary niche width estimated as 40% nMDS ellipse area for purebred (TLp and PVp) and hybrid (TLi and PVi) barbel populations. Number of fish analysed for GCA per population (N), mean total length (TL) and relative range (mm) are also given.*

Population	N	Mean TL (range)	I	Mean Gut Fullness (%)	H	Niche nNMDS
TL*p*	22	180 (91-285)	0	85 ± 14	1.66	0.13
TL*i*	19	159 (69-241)	21	32 ± 27	0.86	0.62
PV*i*	20	160 (72-279)	10	60 ± 28	1.38	0.20
PV*p*	20	155 (110-252)	10	62 ± 25	1.39	0.43

Table 7.3 *SIMPER analysis results of gut contents between each couple of sampling sites (overall average dissimilarity is reported within brackets) for 12 macroinvertebrate cathegories (when family is indicated, family name is preceded by order) and three broader categories composing the diet of introgressed (PVi and TLi) and purebred (PVp and TLp) barbel. C % = percentage contribution of each food item to the overall average dissimilarity.*

	PV*p vs.* PV*i*	**TL*p vs.* TL*i***	**PV*p vs.* TL*p***	**PV*i vs.* TL*i***	**PV*p vs.* TL*i***	**PV*i vs.* TL*p***
	(59.2)	**(81.0)**	**(61.9)**	**(75.6)**	**(78.4)**	**(65.9)**
Item			**C%**			
Plecoptera Leuctridae	2.5	2.3	3.3	0.4	2.5	2.2
Ephemeroptera Baetidae	16.5	4.8	5.9	18.6	6.4	11.4
Other Ephemeroptera	6.2	3.5	5.0	4.9	6.2	3.8
Trichoptera Hydropsychidae	7.2	0.3	1.3	7.4	1.2	5.5
Other Trichoptera	4.2	7.1	7.9	5.7	5.7	7.0
Diptera Chironomidae	13.7	10.5	9.4	10.4	18.1	6.3
Diptera Limoniidae	12.1	2.4	8.8	0	11.7	2.5
Diptera Simuliidae	13.7	4.2	7.5	18.9	11.6	11.6

Table 7.3 (Continued)

	PV*p* *vs.* PV*i*	**TL*p* *vs.* TL*i***	**PV*p* *vs.* TL*p***	**PV*i* *vs.* TL*i***	**PV*p* *vs.* TL*i***	**PV*i* *vs.* TL*p***
	(59.2)	**(81.0)**	**(61.9)**	**(75.6)**	**(78.4)**	**(65.9)**
Item	**C%**					
Other Diptera	0.3	6.6	6.8	0	0.3	6.6
Coleoptera Elmidae	4.4	4.2	4.7	1.0	4.4	4.2
Crustacea Gammaridae	0	8.7	9.2	0	0	8.8
Mollusca	8.0	18.1	15.6	5.2	7.0	15.6
Other macroinvertebrates	3.1	6.1	3.1	8.5	7.8	2.1
Fish bones	1.4	6.3	0	9.8	8.4	1.0
Terrestrial organisms	1.6	9.1	9.5	1.4	0.8	8.9
Plants	5.0	5.6	2.0	7.7	7.8	2.7

7.3.4 Stable isotopes, barbel trophic position and isotopic niches

Across the four sites, $\delta^{13}C$ BMI varied, with the carbon range being between 1.6 ‰ (TL*i*) and 6.1 ‰ (PV*p*) (Fig. 7.4; Table S7.1). FPOM was particularly ^{13}C-enriched in all rivers except for TL*p* (Table S7.1). Values of $\delta^{15}N$ were more similar for both BMI and primary producers between TL*p* and PV*i* (Fig. 7.4, Table S7.1), while there was an enrichment of ^{15}N at TL*i*. In the barbel populations, there was no evidence of significant ontogenetic shifts in $\delta^{15}N$ and $\delta^{13}C$ (Supplementary material Table S7.4), except for in PV*p* where $\delta^{13}C$ decreased as fish length increased. There was no significant difference in the length of the fish analysed between rivers ($F_{3, 36}$=0.15, $p > 0.05$).

A significantly lower trophic position (as indicated by posterior probability distributions) was detected for barbel in TL*i* compared to TP in the other populations (TP 2.4 *vs*. > 2.8; Table 7.4). No significant differences were found in C_{corr} between rivers ($F_{3, 36} = 0.84$, $p > 0{,}05$) and length was subsequently removed due to its non-significant effect ($p > 0.05$). The isotopic niche size of the barbel was significantly larger in TL*i* and smaller in TL*p* (as indicated by posterior distributions of the core isotopic niche as SEA_B), with the niches being similarly sized in PV*p* and PV*i* (Table 7.4). In general, the positions of

these niches in the isotopic space did not overlap except for PV*p* and PV*i* that shared the 6% of their core niches (Figure 7.3b).

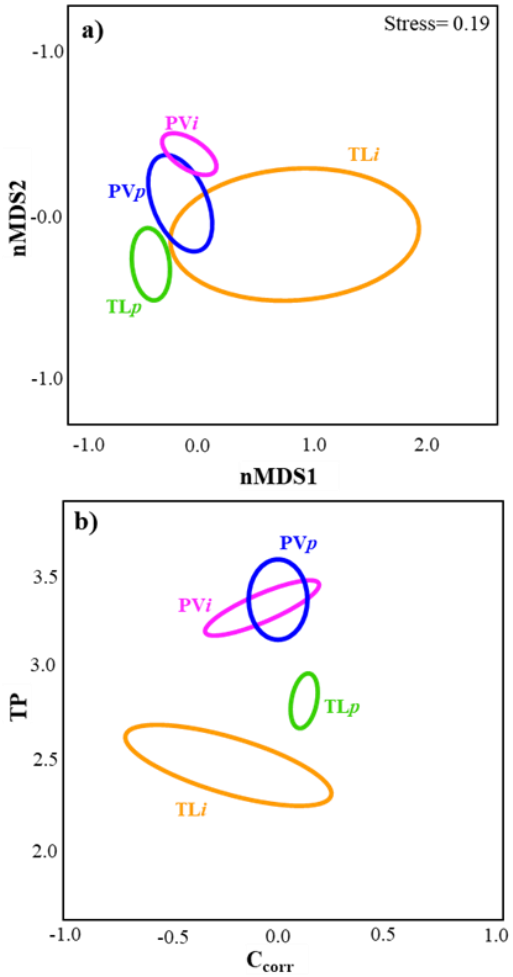

Figure 7.3 *a) Non-metric multidimentional scaling (nMDS) graph showing the dietary niches built as standard ellipses enclosing 40% of the gut content data within each population. b) Isotopic niches of each barbel population built on the corrected stable isotope data as maximum likelihood standard ellipse area (SEA) enclosing 40% of the data for introgressed (PVi = pink and TLi = orange) and purebred (PVp=blue and TLp= green) barbel populations.*

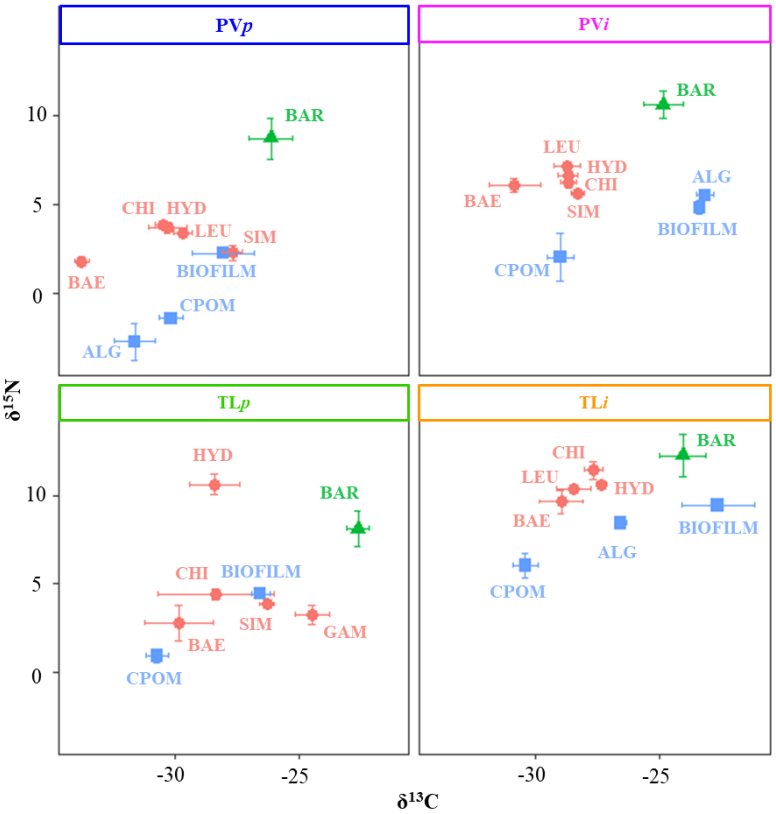

Figure 7.4 *Stable Isotope means with standard deviations (bars) of barbel (green triangles), macroinvertebrates (pink circles) and primary producers (blue squares) collected at four sites. BAR= barbel; macroinvertebrates: BAE= Baetidae, CHI= Chironomidae, HYD= Hydropsichidae, LEU= Leuctride, SIM= Simuliidae; primary producers: CPOM= coarse particulate organic matter, ALG=benthic algae.*

Table 7.4 *Mean raw stable isotope ratio ± standard deviation, Bayesian estimate of trophic position (TP) with relative 95% credible intervals and corrected carbon values (Ccorr) ± standard deviation of each barbel population together with estimate of the isotopic niche breath calculated as Bayesian standard ellipse area SEA_B (95% credible interval). Number of samples analysed (N) and mean total length (TL) in mm and relative range (in brackets) are provided.*

River	N	Mean TL (range)	$\delta^{15}N_{muscle}$	TP	$\delta^{13}C$	C_{corr}	SEA_B
PV*p*	10	164 (103-242)	8.9 ±1.1	3.4 (2.9-4.2)	-26.1 ± 0.9	0.0 ± 0.1	0.12 (0.06-0.23)
PV*i*	10	161 (98-239)	10.8 ± 0.7	3.0 (2.7-3.7)	-24.8 ± 0.8	-0.1 ± 0.3	0.11 (0.06-0.23)
TL*p*	10	172 (106-244)	8.3 ±1.0	2.8 (2.2 -3.4)	-22.6 ± 0.4	0.1 ± 0.1	0.04 (0.02-0.08)
TL*i*	10	168 (100-241)	12.4 ± 1.2	2.4 (2.1-2.8)	-24.0 ± 0.9	-0.2 ± 0.6	0.50 (0.25-0.98)

7.4 Discussion

The testing of the ecological consequences of introgressive hybridisation of the endemic *B. tyberinus* and *B. plebejus* following *B. barbus* invasion revealed substantial changes in both the biological traits and trophic characteristics of hybrids that potentially indicate ecological impacts on a wider ecosystem scale. The growth characteristics of the hybrids (*B. barbus* × *B. tyberinus* and *B. barbus* × *B. plebejus*), including their maximum theoretical lengths and lengths at age, were higher in the invaded populations than in the purebred populations, as per the prediction. These results are also similar to those from previous studies on *B. barbus* hybrid populations of central (Carosi et al. 2017) and northern Italy (Meraner et al. 2013), and suggest an element of hybrid vigour. Indeed, similar patterns of hybrid vigour have been recorded in other interbreeding fish species, such as *Cyprinodon pecosensis* and its congener *C. variegatus* (Rosenfield et al. 2004), the Japanese strain of *Cyprinus carpio* and its domestic exotic lineage (Matsuzaki et al. 2010), and *Abramis brama* and *Rutilus rutilus* (Toscano et al. 2010; Hayden et al. 2011). Moreover, hybrid vigour has been documented in a range of other animal and plant species (Pfennig et al. 2007; Hovick and Whitney 2014). The increased size of barbel hybrids may enhance recruitment through a higher number of eggs being spawned (Philippart and

Berrebi 1990; Meraner et al. 2013; Gutmann Roberts et al. 2020) compared to the smaller native purebreds. This, along with the related vigour of the introgressed progenies, potentially helps to explain the rapid expansion of hybridization in invaded population by *B. barbus*. Alternatively, the larger size of alien barbel and its hybrids may play an active role in sexual selection, with larger females being more attractive to barbel males than the smaller native females (Meraner et al. 2013).

The second prediction concerned the differences in trophic ecology between hybrids and purebred barbel populations and was tested using a combination of gut contents (GCA) and stable isotope (SIA) analyses. These techniques are considered to be largely complementary (e.g. Nolan and Britton 2018) and are often used together in fish trophic studies (e.g. Locke et al. 2013; Hamidan et al. 2016), although they do reflect two different aspects of animals feeding ecology that can result in discordant outcomes (e.g. Pacioglu et al. 2019). Where GCA represents a dietary snapshot of an individual in real time, representing the prey consumed in the preceding hours, SI data integrate spatial and temporal dietary components over a period of days to months, depending on the actual tissue analysed (Vander Zanden et al. 2015). Here, scale material was used that, in *B. barbus*, has a relatively slow isotopic turnover rate compared to other tissues (Busst and Britton 2018), thus the

temporal aspect of the diet being indicated was likely to be several months. Despite these core methodological differences, the two methods were consistent in demonstrating some considerable differences in the diet composition and trophic niche of the TL*i* hybrid population compared to their reference parental population (TL*p*), and, conversely, only minor differences between the PV*i* hybrid population and its reference purebred population (PV*p*). The introgressed barbel of TL*i* differed to the other three populations studied in their relatively high proportions of small fishes and plants in their diet, which resulted in a relatively lower trophic position. The diets of the other populations were all dominated by benthic macroinvertebrate prey. These differences were then reflected in their trophic niche size, with the hybrids in TL*i* having the broadest isotopic and trophic niches.

The relatively high proportion of prey fishes in the diet of the introgressed barbel of TL*i* aligns to some *B. barbus* populations having diets in which prey fishes are present, albeit usually in low frequencies (Piria et al. 2015; Gutmann Roberts et al. 2017). Recreational anglers also frequently capture larger individuals on baits comprising of high proportions of marine fishmeal, suggesting that fish prey are attractive to adult *B. barbus* (De Santis et al. 2019). The barbel of TL*i* were the only population here where small benthic fishes were detected at

relatively high frequency in diet by GCA, despite considerable overlap in the body sizes present, and the presence of small benthic fishes in all sites. The diet of the TL*i* hybrid barbel had the lowest diversity (in terms of the Shannon-Wiener index) of all populations, but as these fish had both plant and fish material present then they actually had the widest trophic niche. Moreover, TL*i* had the highest fish density and a lower macroinvertebrate density than PV*i* site, so these data suggest that the hybrids of TL*i* preyed upon smaller fish through the combination of their high availability and relatively lower availability of macroinvertebrate resources, a pattern that was not evident elsewhere (Supplementary material Table S7.2 and S7.3). We can thus speculate that the hybrids in TL*i* were primarily consuming common food resources in the site rather than preferentially selecting small fishes as dietary items. However, it highlights both their diet plasticity and a shift in their functional feeding guild (Noble et al. 2007) from primarily being insectivorous (Oberdoff et al. 1993; Corse et al. 2005; Piria et al. 2015) in other sites to being omnivorous in TL*i*. This functional shift is potentially important in the context of assessments of their ecological impact (Cucherousset and Olden 2011).

In terms of their age structure and growth, the hybrid populations were relatively similar, despite their trophic

differences. These results are consistent with morphological analyses that were conducted on the same populations (Zaccara et al. 2020), where the TL*i* barbel showed a marked morphological differentiation from the purebred *B. tyberinus* in their body shape, whereas the hybrids of PV*i* resulted relatively similar to the morphology of the PV*p* barbel phenotype. Indeed, the functional morphology of fish is an important driver of their diet (Klingenberg et al. 2003) through its strong influence on their ability to capture and handle different prey, and so can strongly influence the foraging habits utilised and their efficiency in prey capture (Webb 1984). Variation in the trophic ecology of different hybrid classes (i.e. differences in the extent of introgression) has been detected in hybrids between native Japanese *Cyprinus carpio* lineages and non-native strains (Matsuzaki et al. 2010). Invasion history (e.g. time since the first introduction), propagule pressure, habitat structure and disturbance are all factors that may contribute to the different genotypic composition and ecology of hybrid populations (Hayden et al. 2011; Corse et al. 2015). Thus, future studies may involve a higher number of populations representative of different habitat conditions and populations with different genotypic structure to verify to which extent the pattern observed in this study are driven by changes in the genotype

and phenotype, versus those driven by differences in their environment, including in prey availability (Corse et al. 2015).

In summary, the results here provide evidence that the genetic introgression that follows the invasion of *B. barbus* with native congeners can result in substantial ecological shifts between the purebred and hybrid fish. In one population, the morphological change in the hybrids resulted in their exploitation of different prey resources, although the extent to which this was also driven by differences in prey availability was unable to be tested. In this population, the hybrids also grew to considerably larger sizes and had larger lengths at age. These results highlight for the first time that *B. barbus* invasion not only results in the introgression with congeners with consequent genetic pollution, but these introgressed fish can then interact quite differently within the receiving communities than their parental non-hybridised fish, indicating that invasive hybridisation is, potentially, a major driver of ecological change.

7.5 Supplementary material

7.5.1 *Supplementary figures*

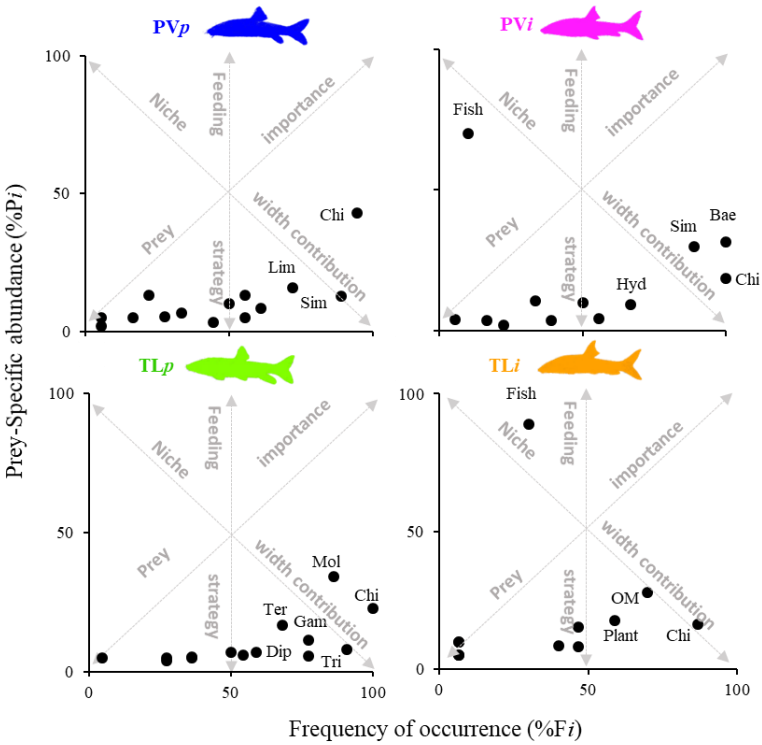

Figure S7.1 *Feeding strategy plot (Costello et al., 1990; Amundsen et al., 1996) of each barbel population based on gut contents. Points indicate food items and name of the most frequent (%Fi ≥ 59 %) or abundant items (%Pi ≥ 70%) are specified where: Fish = fish bones; Bae = Baetidae; Chi = Chironomid larvae; Dip = other Diptera; Gam = Gammaridae; Hyd = Hydropsichidae; Lim = Limonidae; Mol = Mollusca; OA= other aquatic organisms; Plant = aquatic vegetation; Ter = terrestrial organisms; Tri= other Trichoptera. Prey importance (rare to dominant) increases along the diagonal from the bottom left to the upper right while feeding strategy changes along the vertical*

from the bottom up (generalist to specialist) and individual contribution to the trophic niche (i.e. between or within phenotypic contribution to the niche width) increases along the diagonal from the bottom right (high within phenotype contribution) to the upper left (high between phenotype contribution). See Amundsen et al., 1996 for further details on graph interpretation

7.5.2 *Supplementary tables*

Table S7.1 *Mean ± standard deviation of $\delta^{15}N$ and $\delta^{13}C$ ratio of primary consumers (benthic macroinvertebrates, BMI) and primary producers (benthic algae, biofilm, coarse particulate organic matter (CPOM) and fine particulate organic matter (FPOM)) collected at the four sampling sites. Each category was represented by three replicates (N = 3).*

Site	Group	Definition	$\delta^{13}C$	$\delta^{15}N$
PV*p*	BMI	Baetidae	-33.7 ± 0.3	1.8 ± 0.2
		Chironomidae	-30.5 ± 0.3	3.9 ± 0.3
		Hydropsichidae	-30.3 ± 0.8	3.7 ± 0.3
		Leuctridae	-29.7 ± 0.4	3.4 ± 0.2
		Simulidae	-27.7 ± 0.4	2.3 ± 0.4
	Primary producers	Benthic algae	-31.6 ± 0.8	-2.7 ± 1.0
		CPOM	-30.1 ± 0.5	-1.4 ± 0.3
		Biofilm	-28.1 ± 1.2	2.3 ± 0.1
		FPOM	-9.9 ± 0.4	1.9 ± 0.4
PV*i*	BMI	Baetidae	-30.8 ± 1.0	6.1 ± 0.4
		Chironomdae	-28.6 ± 0.3	6.2 ± 0.3
		Hydropsichidae	-28.7 ± 0.4	6.6 ± 0.2
		Leuctridae	-28.7 ± 0.5	7.2 ± 0.2
		Simulidae	-28.3 ± 0.3	5.6 ± 0.2
	Primary producers	Benthic algae	-23.1 ± 0.4	5.5 ± 0.3
		CPOM	-29.0 ± 0.5	2.0 ± 1.4

Table S7.1 (Continued)

Site	Group	Definition	$\delta^{13}C$	$\delta^{15}N$
PV*i*		Biofilm	-23.4 ± 0.2	4.8 ± 0.4
		FPOM	-8.4 ± 0.2	1.5 ± 1.5
TL*p*	BMI	Baetidae	-29.8 ± 1.4	2.8 ± 1.0
		Chironomidae	-28.3 ± 2.3	4.4 ± 0.3
		Gammaridae	-24.4 ± 0.7	3.3 ± 0.5
		Hydropsichidae	-28.4 ± 1.0	10.6 ± 0.6
		Simulidae	-26.3 ± 0.3	3.8 ± 0.1
	Primary producers	CPOM	-30.7 ± 0.4	1.0 ± 0.4
		Biofilm	-26.5 ± 0.4	4.4 ± 0.2
		FPOM	-23.1 ± 0.1	1.9 ± 0.3
TL*i*	BMI	Baetidae	-29.0 ± 0.9	9.6 ± 0.7
		Chironomidae	-27.7 ± 0.4	11.4 ± 0.5
		Hydropsichidae	-27.3 ± 0.2	10.6 ± 0.2
		Leuctridae	-28.4 ± 0.7	10.4 ± 0.2
	Primary producers	Benthic algae	-26.6 ± 0.2	8.4 ± 0.1
		CPOM	-30.4 ± 0.5	6.0 ± 0.7
		PP	-22.6 ± 1.5	9.4 ± 0.1
		FPOM	-10.9 ± 0.9	6.0 ± 0.8

Table S7.2 *Fish species assemblage at each sampling site with relative density (individuals/m2) and total density (N), species richness (S) and Shannon-Wiener diversity index (H). Superscript letters indicate exotic species (e) or translocated ones (t) (i.e. those native to PV and introduced in TL).*

		PV*p*	**PV*i***	**TL*p***	**TL*i***
	N	3.40	1.97	0.78	8.27
	S	6	8	5	8
	H	0.5	1.4	0.9	0.5
	Density ind/m^2				
Family	**Species**				
Cobitidae	*Cobitis bilineata*		0.24		
Cottidae	*Cottus gobio*	1.84			
Cyprinidae	*B. plebejus*	0.08			
	B. barbus × *B. plebejus*^{e}		0.10		
	B. barbus × *B. tyberinus*^{e}				0.15
	B. tyberinus			0.51	
Gobiidae	*Padogobius bonelli*		0.38		
	Padogobius nigricans				7.00
Gobionidae	*Gobio gobio*^{e}		1.08		
	Pseudorasbora parva^e				0.02
Leuciscidae	*Alburnus alborella*^{t}		0.04		0.01
	Leuciscus lucumonis			0.02	
	Protochondrostoma genei^t		0.014		1.00
	Sarmarutilus rubilio	0.02	0.01	0.04	0.01
	Squalius squalus	0.06	0.11	0.01	0.06
	Telestes muticellus	1.40			
Salmonidae	*Oncorhynchus mykiss*^{e}			0.21	

Table S7.2 (Continued)

	Density ind/m²		
Family	**Species**		
	Salmo trutta[e]	0.01	
Siluridae	*Silurus glanis*[e]		0.02

Table S7.3 *Macroinvertebrate assemblage (family level) found at each sampling site with relative density (individuals/m^2), and total density (N), family richness (S) and Shannon-Wiener diversity index (H).*

		PV*p*	**PV*i***	**TL*i***	**TL*p***
	N	1094	5662	1212	848
	S	24	19	15	17
	H	2.0	1.5	1.6	1.5
	Density (ind/m2)				
Class/Order	**Family**				
Amphipoda	Gammaridae		1	6	83
Bivalvia	Spheriidae				16
Coleoptera	Others	9	8	62	5
Diptera	Chironomidae	285	845	530	85
	Simuliidae	24	2267		17
	Others	24	2	11	6
Ephemeroptera	Baetidae	88	1415	74	10
	Caenidae		68	123	
	Ephemerellidae	86			
	Ephemeridae	2			4
	Heptageniidae	19	24	7	2
	Leptophlebiidae	92	9	29	
Gastropoda	Lymnaeidae	6	10		481
	Planorbidae				128
Odonata	Gomphidae	1	6		
Oligochaeta	Lumbricidae	8	5		2
Plecoptera	Leuctridae	354	168	64	1

Table S7.2 (Continued)

	Density (ind/m2)				
Class/Order	**Family**				
Trichoptera	Hydropsychidae	64	805	300	
	Philopotamidae	27	4		
	Rhyacophilidae		25	6	6
	Others	5			2

Table S4 *Results of the regression models testing the relationships between fish length (mm) and stable isotope ratios ($\delta^{15}N$ and $\delta^{13}C$) tested as a proxy of ontogenetic effects on diets for purebred (PVp and TLp) and hybrid (PVi and TLi) barbel populations.*

Dependent variable	Population	$F_{1, 8}$	R^2	p
$\delta^{15}N$	PV*p*	0.01	0.01	> 0.05
	PV*i*	0.05	0.01	> 0.05
	TL*p*	1.64	0.17	> 0.05
	TL*i*	0.31	0.04	> 0.05
$\delta^{13}C$	PV*p*	30.13	0.79	< 0.001
	PV*i*	0.01	0.01	> 0.05
	TL*p*	2.62	0.24	> 0.05
	TL*i*	0.06	0.01	> 0.05

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CHAPTER VIII

Genetic and morphological analyses revealed a complex biogeographic pattern in the endemic barbel populations of the southern Italian peninsula

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Abstract

The Italian peninsula is a biodiversity hotspot, with its freshwater fish fauna characterized by high levels of local endemism. Two endemic fluvio-lacustrine fishes of the genus *Barbus* (barbel, family Cyprinidae) have allopatric distributions in the Tyrrhenian and Adriatic basins of Italy. *Barbus plebejus* inhabits the mid- to northern Adriatic basins, while *B. tyberinus* is widespread in all central-northern basins draining into the Tyrrhenian Sea. For basins in Southern Italy draining into the southern parts of these seas, there remains a knowledge gap on their barbel populations due to no previous genetic and morphological studies, despite their apparent biogeographic isolation. Correspondingly, this study quantified the presence and distribution of barbels in the Adriatic and Tyrrhenian basins of Southern Italy through genetic and morphological analyses of 197 fish sampled across eight populations. Testing of how local isolation has influenced the evolution and persistence of these populations was completed by examining sequence variation at two mitochondrial loci (cytochrome b and D-loop) and performing geometric morphometric analyses of body shape, plus measuring 11 morphometric and meristic characters. Phylogenetic and morphological analyses revealed the presence of two genetically distinct lineages that differed significantly from adjacent *B. tyberinus* and *B. plebejus*

populations. These two new taxa, here described as SI1 and SI2 *Barbus* lineages, are highly structured and reflect a complex mosaic biogeographic pattern that is strongly associated with the underlying hydrographical scenarios of the basins. The geographic isolation of these basins thus has high evolutionary importance that has to be considered for maintaining endemism.

8.1 Introduction

The species richness of southern European freshwaters, including the peri-Mediterranean area, is higher than in central and northern Europe, resulting in these freshwaters having high conservation value (De Figueroa, Fenoglio, & Sanchez-Castillo, 2013). Biogeographically, the region is highly structured with, for example, the freshwater fish diversity between Southern Europe and Northern Africa comprising 23 different ecoregions (Abell et al., 2008; Geiger et al., 2014). Within this, more than 50 native freshwater fish are currently listed as present in the Italian peninsula (Bianco, 2014). The presence of a large number of rare taxa within this relatively small area was strongly influenced by geological and hydrological events during the glacial cycles of the Pleistocene (Bianco, 1995b, 1998; Hrbek & Meyer, 2003). These events resulted in the formation of three distinctive ichthyo-geographic

districts that are characterized by distinct evolutionary histories in species of the Cyprinidae family (Bianco, 1990, 1995a).

To date, fish biogeographic studies in the Italian peninsula have generally focused on the northern and central regions (e.g., Buonerba et al., 2015; Carosi, Ghetti, Forconi, & Lorenzoni, 2015; Livi et al., 2013; Marchetto, Zaccara, Muenzel, & Salzburger, 2010; Meraner et al., 2013; Stefani, Galli, Zaccara, & Crosa, 2004; Zaccara et al., 2019; Zaccara, Stefani, & Delmastro, 2007). These studies have centered on the Padano-Venetian (PV) district of the Italian northeast region, including basins flowing into the upper and middle Adriatic Sea (north of the Vomano River in Abruzzo Region and the Krka River in Croatia), and on the Tuscano-Latium (TL) district of central western region, including all basins draining into the middle Tyrrhenian Sea (Bianco, 1990, 1995a). Conversely, the Apulo-Campano (AC) district of the southern region of Italy, which includes all basins flowing into southern Adriatic, southern Tyrrhenian, and Ionian seas (Bianco, 1990, 1995a; Figure 1), has received little research attention. For studies that have been completed, evidence suggests the AC district has long been isolated, and so might have been less influenced by lowered sea levels that occurred during Pleistocene period than basins further north (e.g., Bianco, 2014; Ketmaier et al., 2004), such as

the paleo-Po drainage (Bianco, 2014; Buonerba et al., 2015; Livi et al., 2013; Stefani et al., 2004; Zaccara et al., 2019).

Testing the evolutionary effects of the isolation of the southern Italian hydrographic basins, and the potential patterns and processes relating to vicariance events and local dispersal, can be completed using their cyprinid fish communities, as these generally show strong patterns of local endemism (Avise, 2000; Kottelat & Freyhof, 2007; Reyjol et al., 2007; Zardoya & Doadrio, 1999). Cyprinid fishes are widespread throughout all peri-Mediterranean districts, but have limited capability of moving between hydrographic basins due to impassable watershed boundaries, coupled with low saline tolerances that result in coastal areas being effective barriers to their mixing. Among cyprinid fishes, barbels (species of the genus *Barbus*) have been used widely to study regional biogeography patterns and dynamic changes in continental and inland waters due to their marked diversity, wide distribution, and varied ecology (Buonerba et al., 2015; Gante, 2011). The genus *Barbus* includes species adapted to a variety of freshwater habitats, ranging from small mountain streams to large and slow-flowing rivers and lakes (Kottelat & Freyhof, 2007).

In the Italian peninsula, three barbel species are considered endemic (Kottelat & Freyhof, 2007): common barbel *Barbus plebejus* Bonaparte, 1839; Tiber barbel *Barbus tyberinus*

Bonaparte, 1839; and *Barbus caninus* Bonaparte, 1839. The habitat preferences of common and Tiber barbels are for larger, slower flowing rivers that are characterized by laminar flows and relatively warm temperatures (Kottelat & Freyhof, 2007). *Barbus plebejus* and *B. tyberinus* have an allopatric distribution in the Adriatic and Tyrrhenian basins, respectively (Buonerba et al., 2015; Zaccara et al., 2019). *Barbus plebejus* is widespread in the Adriatic basins (PV district), with an approximate southern limit of its range localized between the Tronto and Vomano rivers (Bianco, 1994, 2003a; Kottelat & Freyhof, 2007). Conversely, *B. tyberinus* is distributed in the main Tyrrhenian basins (Bianco, 2003b). *Barbus caninus* Bonaparte, 1839 is a small-sized rheophilic barbel (total length up to c. 25 cm) that inhabits mountain brooks in the PV district (Kottelat & Freyhof, 2007; Tsigenopoulos & Berrebi, 2000). In recent studies, *B. plebejus* and *B. tyberinus* have been confirmed as distinct species based on genetic (Buonerba et al., 2015) and morphological differences (Lorenzoni et al., 2006; Zaccara et al., 2019), despite their similar fluvio-lacustrine ecology. To fill this considerable knowledge gap on the endemism of barbels in the AC district, the aim here was to test how local hydrographic history has influenced the evolution and persistence of the fluvio-lacustrine barbels in the southern Italian peninsula. Mitochondrial sequence data and morphological analyses were

applied to examine the extent of diversification of the barbels in the AC district compared with barbel populations in northern and central Italy. The results were then used to construct further hypotheses based on biogeographic scenarios that might have influenced patterns of endemism in the southern Adriatic and Tyrrhenian Sea hydrographical networks.

8.2 Materials and Methods

8.2.1 *Sampling*

A total of 197 specimens of *Barbus* spp. were sampled in AC district between 2017 and 2018 with local authority permission. Fish were sampled from three sites in the Tyrrhenian basins and from five sites in the Adriatic basins. The Tyrrhenian sites were the basins Liri- Garigliano (T1) and Volturno (T2), both close to TL district boundary, and Sele (T3) basin, located in the southern part. The Adriatic sites were in the Aterno-Pescara (A1) basin that represents the first Adriatic drainage in AC district, and the Sangro (A2), Biferno (A3), Fortore (A4) up to Ofanto (A5) basins (see Table 8.1; Figure 8.1).

Sampling of the fish was completed using electric fishing. Captured specimens were removed from the water and then held in aerated tanks of water. Under general anesthesia (MS-222), the fish were attributed to a species according to their phenotypic characteristics (e.g., colouration pattern, spot form

and size, fin color; Kottelat & Freyhof, 2007; Lorenzoni et al., 2006), enabling recognition of the *B. tyberinus* phenotype as per Bianco (1995b). Each fish was then measured (fork length, nearest mm), and a biopsy of the anal fin was taken, preserved in 90% ethanol, and stored at 4°C for subsequent DNA extraction. For morphological analyses, fish were also photographed (left side) using a Nikon D300 camera (24–85 mm lens) positioned by means of a tripod on a table with a millimetric scale. The fish were then placed into another aerated water tank and, following their recovery to normal behaviour, were released back into the river.

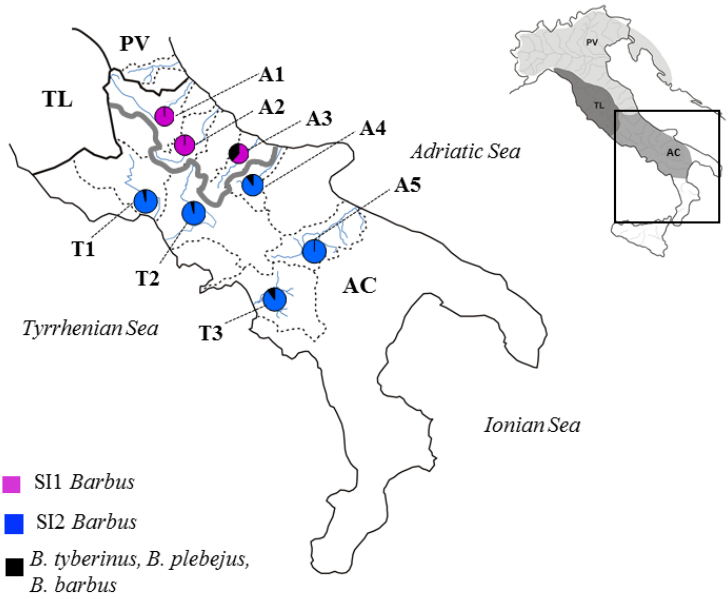

Figure 8.1 *Map of sampling sites in South Italy, detailing SI1 and SI2* Barbus *lineages boundary within the AC district. Biogeographic boundaries between the three Italian ichthyo-geographic districts (PV = Padano-Venetian; TL = Tuscano-Latium; AC = Apulo-Campano*; sensu *Bianco, 1990) are also reported in the insert. The colours of pie charts represent the frequency of phylogenetic lineages: black for B. plebejus, B. tyberinus, and B. barbus, while SI1 and SI2 Barbus lineages in purple and blue, respectively. Detailed frequencies are reported in Table 1. The asterisk indicates the Vomano basin*

Table 8.1 *Sampling site locations (expressed with ID code), watershed, river basin and the number of individuals of each species sampled by site, attributed through D-loop mtDNA phylogenetic tree. The sampling site position, geographic coordinates and barbel composition has been indicated also in Figure 8.1.*

ID code	watershed	Basin	Latitude - Longitude	SI1 *Barbus*	SI2 *Barbus*	*Barbus tyberinus*	*Barbus plebejus*	*Barbus barbus*
A1	Adriatic	Aterno-Pescara	42°10′25.85″N-13°49′51.35″E	24				
A2		Sangro	42°05'29.76"N-14°34'75.82"E	23				
A3		Biferno	41°43'21.41"N-14°43'26.94"E	13			8	
A4		Fortore	41°33'13.20"N-14°52'33.92"E		27	3		
A5		Ofanto	41°07′39.23″N-15°54′62.24″E		20			
T1	Tyrrhenian	Liri-Garigliano	41°52'38.92"N-13°27'11.12"E		25		1	
T2		Volturno	41°38′12.53″N-14°10′20.98″E		23			1
T3		Sele	40°29'27.8"N-15°12'25.2"E		26		3	

8.2.2 *Molecular data*

Total genomic DNA was extracted from all individuals using a proteinase K digestion, followed by sodium chloride extraction and ethanol precipitation (Aljanabi & Martinez, 1997). Two sets of primers were used to amplify mitochondrial control region (D-loop) and cytochrome *b* (cyt *b*) gene (Livi et al., 2013). D-loop sequences were obtained from the 197 individuals and used for all genetic analyses, while cyt *b* sequences were analyzed for a subsample of 26 fish, selected as a representative pool of the fish with specific D-loop haplotypes. The mtDNA D-loop fragment of 871 bp length was amplified using D-loopsxF and D-loopdxR (Antognazza, Andreou, Zaccara, & Britton, 2016; Rossi et al., 2013) primers pair, while cyt *b* gene using L15267 and H16461 (Briolay, Galtier, Brito, & Bouvet, 1998). Both PCR reactions were performed using Multiplex PCR kit (Qiagen) in 10 μl reaction volume containing approximately 10 ng of template DNA and 0.25 μM of each primer pair, using the same thermal cycle protocol (c.f. Zaccara et al., 2019). PCR products were purified using ExoSAP-IT™ (USB) and directly sequenced by MACROGEN Inc (http://www.macro gen.org) using a 3730XL DNA Sequencer. All new haplotypes generated in this study were deposited in the GenBank database (Accession number MK728797–MK728821; MG718025–MG718026).

8.2.3 *Phylogenetic analyses*

Multiple alignments of all sequences were automatically carried out through ClustalW within Bioedit software (Hall, 1999), with polymorphic sites then checked manually. Identical sequences were collapsed into haplotypes in order to facilitate computational processes, as implemented in DnaSP v 5.0 (Librado & Rozas, 2009) software. Computation of mitochondrial phylogeny was performed independently for each gene on nonredundant haplotypes and on combined cyt *b* and D-loop fragments dataset. For all phylogenetic analyses, two different phylogenetic inference methods were used as follows: maximum likelihood and Bayesian analyses. The former was conducted in GARLI v 2.0 (Bazinet, Zwickl, & Cummings, 2014; Zwickl, 2006) software, applying the specific setting for best evolutionary models. This was identified using Akaike's information criterion, as implemented in JModelTest v 2.1.10 (Darriba, Taboada, Doallo, & Posada, 2012): GTR + I (Lanave, Preparata, Sacone, & Serio, 1984; Rodriguez, Oliver, Marin, & Medina, 1990) and HKY85 (Hasegawa, Kishino, & Yano, 1985) for cyt *b* and D-loop, respectively, and HKY85+I+G (Hasegawa et al., 1985) for the combined dataset. The GARLI tree searches were performed under the default settings. Support was assessed with 1,000 bootstrap replicates

in GARLI, under the same settings as the best-tree searches. The resulting bootstrap support values were mapped onto

8.2.4 *Minimum spanning network, genetic diversity, and demography*

A minimum spanning network was created from the multiple D-loop sequences alignment produced in this study using a statistical parsimony criterion as implemented in PopART v 1.7 software (Leigh & Bryant, 2015). The levels of genetic variation within any new endemic lineages were then calculated by estimating nucleotide differences and haplotype diversity using DnaSP v 5.0 software. To visualize their historical demographic trends, mismatch analyses were performed, as implemented in Arlequin v 3.5 (Excoffier & Lischer, 2010) software, testing the sudden demographic expansion model through sum-of-squared deviation values (SSD) in a coalescent algorithm simulation over 1,000 pseudo-replications with statistical significance ($p < .05$). To test the isolation between populations (within and between Tyrrhenian and Adriatic basins), population genetic differentiation was calculated using the fixation index ΦST (Weir & Cockerham, 1984) and its significance assessed ($p < .05$) by permuting haplotypes between populations 3,024 times, as implemented in Arlequin v 3.5.

8.2.5 *Morphological data*

The morphology of the barbel specimens was analysed by measuring seven morphometric and four meristic traits as per Zaccara et al. (2019) (Figure 8.2a). Geometric morphometric analyses of body shape were performed by measurements of 16 landmarks (LMs) from the digital images within the R Geomorph function "digitize2d" (Adams, Collyer, & Kaliontzopoulou, 2018; Figure 2b). Attention was dedicated in positioning of caudal fin in order to include caudal fin LMs in the geometric morphometric analyses (9, 10, and 11; see Figure 8.2b), in agreement with Zaccara et al. (2019), obtaining results that were unchanged when caudal fin LMs were excluded. To strengthen the morphological differences between evolutionary barbel lineages, these data were combined with those from closely related taxa in central Italy (i.e., *B. tyberinus*, *B. plebejus*, and *B. barbus*; Zaccara et al., 2019). Non-shape variation, introduced through variation in position, orientation, and size, was mathematically removed using generalized procrustes analysis, as implemented in MorphoJ software (Klingenberg, 2011). Shape variations were then analyzed by canonical variate analyses (CVA). Mahalanobis distances were calculated using permutation tests (10,000 replicates). Morphometric traits were standardized to the overall mean standard length (Beacham, 1985) to reduce the effects of size

and allometry. Pairwise comparison on morphological traits was then recorded between taxa and between populations by performing the analysis of variance (ANOVA) followed by the Tukey post hoc test. These analyses were carried out using PAST software (Hammer, Harper, & Ryan, 2001).

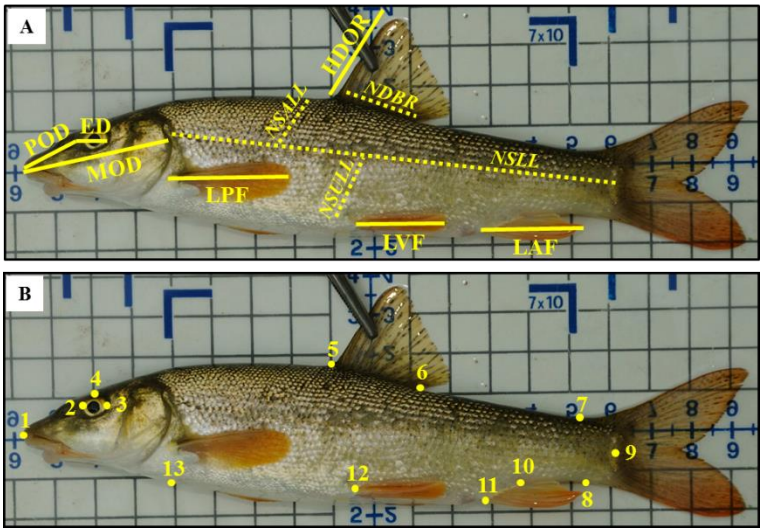

Figure 2. (A) *Seven morphometric (ED, eye diameter; HDOR, height of the third dorsal fin ossified ray; LAF, length of anal fin; LPF, length of pectoral fin; LVF, length of ventral fin; MOD, mouthoperculum distance; POD, preorbital distance) and four meristic traits (NDBR, the number of dorsal fin branched rays; NSLL, the number of scales on the lateral line, and on rows above—NSALL— and under—NSULL—the lateral line) considered for morphological analyses. (B) Position of the 16 landmarks used for body shape analysis: (1) anterior tip of snout, (2, 3) anterior and posterior end of the eye, (4) orthogonal projection on the dorsal profile of the eye center, (5, 6) anterior and posterior insertion of dorsal fin, (7, 8) anterior attachment of dorsal and ventral membrane of caudal fin, (9, 10) end*

of the upper and lower lobe of caudal fin, (11) "furca" of caudal fin, (12) base of middle caudal rays, (13, 14) posterior and anterior insertion of anal fin, (15) insertion of pelvic fin, and (16) orthogonal projection on the ventral profile of the (anterior) insertion of pectoral fin.

8.3 Results

8.3.1 *Multiple alignments and phylogeny*

Across the 197 barbel, 26 haplotypes were identified in the 871 bp length of the multiple D-loop alignment, of which 19 were new and deposited in GenBank (under Accession numbers: MK728797–MK728815) as detailed in Table 8.2. There were 26 variable nucleotide positions detected, of which eight were singletons and 18 were parsimony informative sites. Partial cyt *b* sequences of 714 bp length were obtained from each new D-loop haplotype; in the multiple alignment, 22 variable sites (21 singletons and one parsimony site) were scored and seven new haplotypes detected (GenBank accession numbers: MK728816–MK72821; MG718025– MG718026, see Table 8.2).

Maximum likelihood and Bayesian analysis of the mitochondrial cyt *b* sequences separated out the all fluvio-lacustrine and rheophilic *Barbus* (*B. barbus*, *B. plebejus*, *B. tyberinus*, *B. caninus*, and *B. balcanicus*) species well, but as they did not clearly resolve the evolutionary relationships, they

showed unresolved polytomy (Figure 8.3). Within the fluvio-lacustrine species cluster, D-loop and combined phylogenetic trees (Figure S8.1 and S8.2) were congruent, clustering 16 fish as *B. barbus*, *B. plebejus*, *B. tyberinus*, and, for the first time, two new *Barbus* monophyletic lineages in the AC district. These lineages are named here as "South Italy 1" (SI1) and "South Italy 2" (SI2) *Barbus* lineages. In the D-loop phylogenetic tree, the haplotypes recorded in Vomano River (c.f. Zaccara et al., 2019) were clustered in SI1 *Barbus* lineage.

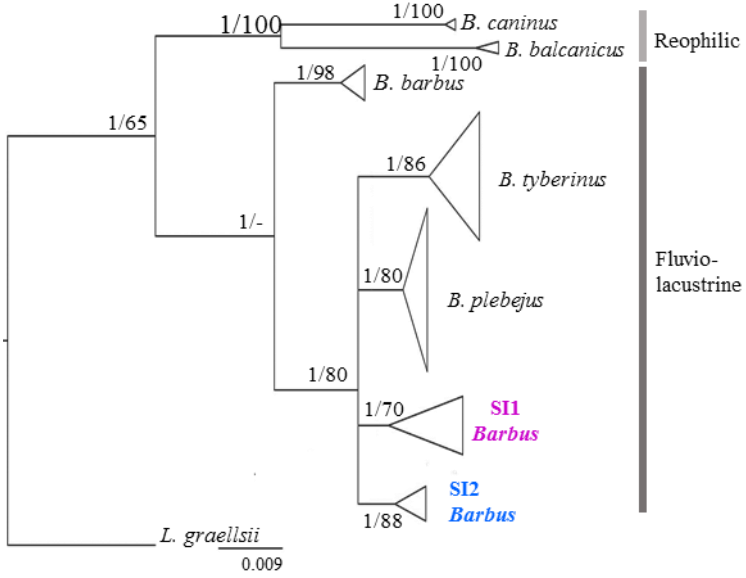

Figure 8.3 *Phylogenetic tree built upon cyt b sequences (714 bp length). Statistic support is given and expressed both as posterior probability and bootstrap values. The tree was rooted on* Luciobarbus graellsii *(GenBank accession number JN049525)*

Table 8.2. *Haplotype distribution and frequencies of D-loop mtDNA fragment (871 bp length) of 181 barbels belonging to SI1 and SI2 Barbus lineages*

		Adriatic basins					Tyrrhenian basins					
***Barbus* lineages**	**D-loop haplotype**	**A1**	**A2**	**A3**	**A4**	**A5**	**T1**	**T2**	**T3**	**Tot**	**D-loop GB acc. n**	**Cyt *b* GB acc. n**
SI1	BSI101	22	12							34	MK728797	MG718025
	BSI102			13						13	MK728798	MK728816
	BSI103		11							11	MK728799	MG718025
	BSI104	1								1	MK728800	MG718026
SI2	BSI201					15		12	22	49	MK728802	MK728817
	BSI202				22					22	MK728808	MK728819
	BSI203						12		1	13	MK728809	MK728821
	BSI204						13			13	MK728810	MK728817
	BSI205							3	1	4	MK728811	MK728817
	BSI206					2		1		3	MK728812	MK728817
	BSI207							2	1	3	MK728813	MK728817
	BSI208							1		1	MK728814	MK728817
	BSI209					3		1	1	5	MK728815	MK728817
	BSI210							1		1	MK728803	MK728817
	BSI211							1		1	MK728804	MK728820
	BSI212							1		1	MK728805	MK728817

Table 8.2 (Continued)

		Adriatic basins					Tyrrhenian basins					
***Barbus* lineages**	**D-loop haplotype**	**A1**	**A2**	**A3**	**A4**	**A5**	**T1**	**T2**	**T3**	**Tot**	**D-loop GB acc. n**	**Cyt *b* GB acc. n**
SI2	BSI213				4					4	MK728806	MK728819
	BSI214				1					1	MK728807	MK728819

The uncorrected p-distance values calculated on the cyt *b* sequences between the SI1 and SI2 *Barbus* lineages and European (*B. barbus*) barbel were 3.9% and 3.6%, respectively. It is noteworthy that SI *Barbus* lineages were more similar to *B. plebejus* (1.5%–1.8%) than to *B. tyberinus* (2.1%–2.4%) and that the inter-lineage uncorrected p-distance between SI1 and SI2 *Barbus* lineages (1.7%) was in a middle position (Table 8.3).

Table 8.3 *Uncorrected p-distances (expressed as percentage) calculated on 714 bp length of cyt b mtDNA for five fluvio-lacustrine Barbus lineages (*B. barbus*,* B. plebejus*,* B. tyberinus*, SI1, and SI2* Barbus; *see Figure 1)*

Lineages	***B. barbus***	***B. plebejus***	***B. tyberinus***	**SI1 *Barbus***	**SI2 *Barbus***
B. barbus	0.23 ± 0.11				
B. plebejus	3.87 ± 0.14	0.29 ± 0.1			
B. tyberinus	4.16 ± 0.23	2.13 ± 0. 20	0.39 ± 0.17		
SI1 *Barbus*	3.86 ± 0.43	1.82 ± 0.43	2.41 ± 0.41	0.87 ± 0.53	
SI2 *Barbus*	3.55 ± 0.19	1.52 ± 0.18	2.10 ± 0.20	1. 69 ± 21 0.36	± 0.15

8.3.2 *Networks, genetic diversity, and demography of South Italy lineages*

In the network analyses of the complete mitochondrial D-loop dataset, the SI1 and SI2 *Barbus* lineages ($N = 181$) were linked

by more than 13 mutational steps and revealed some distinct patterns. The SI1 *Barbus* lineage (N = 60) was composed by five new haplotypes that were connected by up to seven mutational steps, with the most frequent BSI01 positioned in the middle of the radiation (Figure 8.4). The SI2 *Barbus* lineage (N = 121) showed a larger number of haplotypes (i.e., 14), with the two most frequent haplotypes (BSI201 and BSI202) separated by four mutational steps (Figure 8.4). Genetic diversity of the SI1 and SI2 *Barbus* lineages had values of nucleotide diversity (π) of 0.001 and 0.003, and haplotype diversity (H) of 0.61 and 0.78, respectively. The mismatch distribution analyses do not support a sudden expansion model for both lineages (SSD = 0.007, p = .58 in SI1 and SSD = 0.0283, p = .22 in SI2), as they revealed multiwave trends (Figure S8.3).

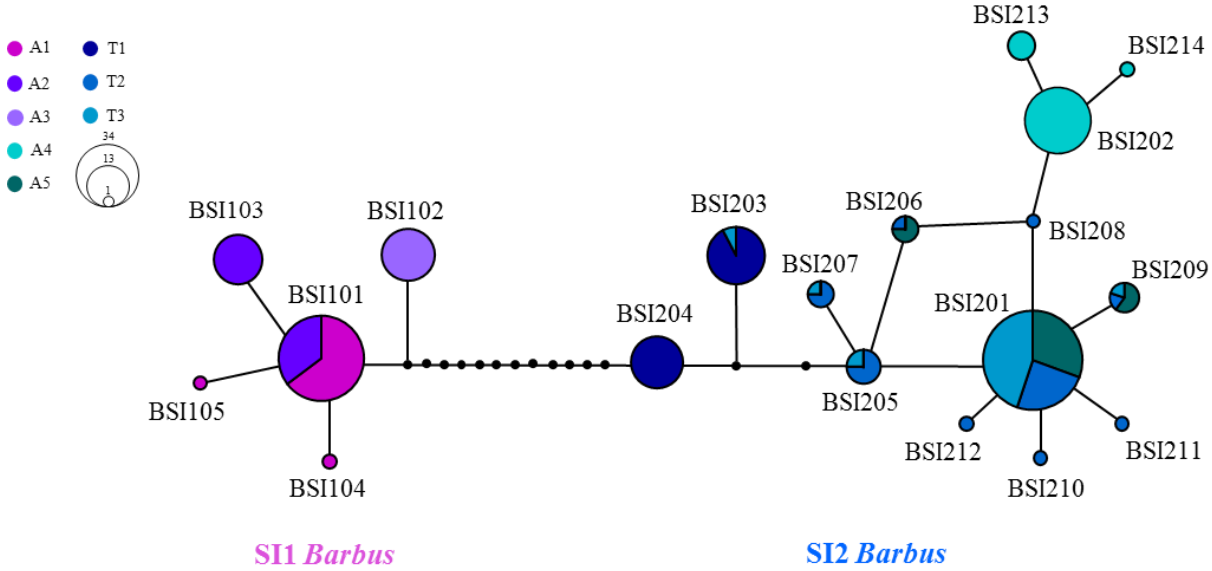

Figure 8.4 *Parsimony network obtained from D-loop sequences (871 bp length) belonging to South Italy Barbus lineages (SI1 and SI2; see Table 2). Circle size is proportional to haplotype frequencies. Colors indicate Adriatic (A1 = Aterno-Pescara; A2 = Sangro; A3 = Biferno; A4 = Fortore; A5 = Ofanto) and Tyrrhenian (T1 = Liri-Garigliano; T2 = Volturno; T3 = Sele) populations.*

8.3.3 *Haplotype distribution and population structure*

In the AC district, the SI1 and SI2 *Barbus* lineages showed an allopatric distribution. The SI1 *Barbus* lineage was recorded in middle Adriatic basins (from A1 up to A3), whereas the SI2 *Barbus* lineage was present both in the three middle Tyrrhenian basins (T1, T2, and T3) and in the two most southern Adriatic basins (A4 and A5; see Figure 8.1 and Table 8.1). Genetic differentiation between the SI1 *Barbus* lineage of the three middle Adriatic populations revealed high genetic structure, with significant ϕST values over 0.39 ($p < .01$; Table S8.1). Genetic differentiation was also recorded between the five populations of the SI2 *Barbus* lineage, with ϕST values ranging between 0.71 and 0.89 ($p < .01$). Among the AC district barbel populations, only the A5, T2, and T3 populations were dominated by the BSI201 haplotype (SI2 *Barbus* lineage; Figure 8.4) and did not show significant differentiation ($p > .05$; Table S8.1).

8.3.4 *Morphological pattern among lineages and among populations*

The geometric morphometric analyses of the CVA plot revealed there was partial visual separation in body shape morphology in the two SI *Barbus* lineages (Figure 8.5). This was supported by Mahalanobis distances that ranged between 3.26 and 4.96 (all *p*

< .05). Variations along the CV1 (54%) were mainly associated with the eye diameter, the depth of the posterior body, and the shape of the caudal fin; those along the CV2 (22%) were mainly associated with the overall fish body shape. The SI1 and SI2 *Barbus* lineages were partially separated from each other along both axes, as also indicated by the Mahalanobis distance value (MD = 3.27). Comparisons with the other two Italian *Barbus* species revealed the SI1 *Barbus* lineage had a higher overlapping position with *B. tyberinus* (MD = 3.26) than with *B. plebejus* (MD = 3.59). The SI2 *Barbus* lineage was more separated from both *B. tyberinus* (MD = 3.58) and *B. plebejus* (MD = 4.01). Both SI *Barbus* lineages showed the highest Mahalanobis distance values against *B. barbus* (MD = 4.09 and 4.96 with SI1 and SI2 *Barbus* lineages, respectively), and, in the case of SI2 *Barbus* lineage, a complete separation with the exotic *B. barbus* was observed in the CVA plot.

The ANOVA results (Table 8.4) and Tukey post hoc test for the pairwise comparison on morphological traits (Table S8.2) revealed statistical distinction ($p < .05$) between the SI1 and SI2 *Barbus* lineages for all the analysed traits, except for the number of dorsal fin branched rays and the number of scales on the lateral line. Both lineages had values of the latter character that were not statistically different from *B. tyberinus* ($p > .05$). Moreover, no significant differences were recorded between

SI1 *Barbus* lineage and *B. tyberinus* for any of the morphometric traits ($p > .05$), except for the height of the third dorsal fin ray ($p < .05$). The SI2 *Barbus* lineage was not statistically different from *B. plebejus* ($p > .05$), both for all the morphometric traits and for the number of dorsal fin branched rays.

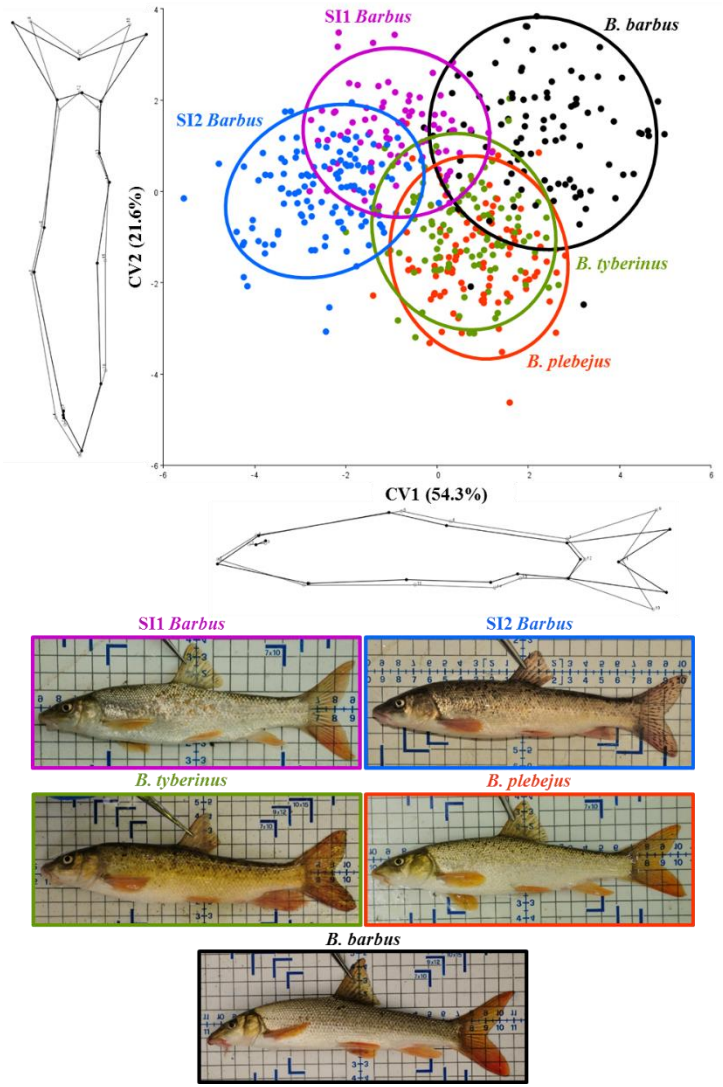

Figure 8.5 *Canonical variate analysis (CVA) output of the body shape comparison between the Barbus lineages detected in this study (SI1 and SI2) and* B. tyberinus*,* B. plebejus*, and* B. barbus *species*

from Zaccara et al. (2019). Wireframe graphs indicate the shape changes along each axis (from gray to dashed black). A sample photograph is shown for each taxon

Although the ANOVA results did not indicate relevant morphological differences among the barbel populations in southern Italy (most $p > .05$), the geometric morphometric analyses of the CVA plot indicated some visual separation (i.e., CV1 = 45% and CV2 = 27%; Figure 8.6). The barbel populations from the Tyrrhenian basins (T1, T2 and T3) were localized in the III quadrant of the CVA plot, while the Adriatic populations were in the I and II quadrants. Differences associated with the eye, and the anal and caudal fins, were detected along the CV2 axes that partially separated populations that were attributed to the SI1 *Barbus* lineage (A1, A2, and A3) from those attributed to the SI2 *Barbus* lineage (A4, A5, T1, T2, and T3). The minimum Mahalanobis distance (MD = 3.95) was recorded between the T2 and T3 populations, belonging to two contiguous Tyrrhenian basins, while the maximum value (MD = 10.50) was found between T1 and A2 populations (Table S8.3), inhabiting two basins located at similar latitude but on the opposite sides of the Italian peninsula (Figure 8.1).

Table 8.4 *List of morphometric and meristic traits, number of individuals (N), mean ± standard deviation and minimum-maximum range for Barbus groups detected in this study and by Zaccara et al. (2019). Data of morphometric traits were transformed according to Beacham (1985) formula. ANOVA results (F) showing differences among the five* Barbus *groups are also reported; all p-values were <0.001. See Table S8.2 for post-hoc comparison results*

		SI1 ***Barbus*** ***N =85***	**SI2** ***Barbus*** ***N = 121***	***B. tyberinus*** ***N = 107***	***B. plebejus*** ***N = 96***	***B. barbus*** ***N = 96***	**ANOVA** **F**
Morphometric traits (cm)							
Eye diameter	ED	0.67±0.11 (0.46-1.03)	0.62±0.10 (0.41-0.91)	0.66±0.12 (0.36-0.95)	0.62±0.13 (0.37-1.02)	0.73±0.14 (0.48-1.14)	**13.9**
Preorbital distance	POD	1.53±0.40 (0.78-2.86)	1.22±0.33 (0.57-2.39)	1.50±0.46 (0.60-2.71)	1.33±0.45 (0.55-2.84)	1.78±0.48 (0.93-3.03)	**25.8**
Mouth-operculum distance	MOD	3.69±0.79 (2.37-6.31)	3.15±0.66 (1.88-5.14)	3.62±0.82 (1.83-5.85)	3.31±0.89 (1.70-6.39)	4.03±0.89 (2.38-6.12)	**19.0**
Length of pectoral fin	LPF	3.07±0.68 (1.68-5.60)	2.58±0.57 (1.35-4.02)	2.87±0.68 (1.07-4.68)	2.59±0.83 (1.12-5.22)	3.29±0.81 (1.86-5.30)	**18.6**

Table S8.4 (Continued)

	SI1 ***Barbus*** ***N =85***	**SI2** ***Barbus*** ***N = 121***	***B. tyberinus*** ***N = 107***	***B. plebejus*** ***N = 96***	***B. barbus*** ***N = 96***	**ANOVA** **F**	
Morphometric traits (cm)							
Length of ventral fin	LVF	2.36±0.56 (1.17-4.17)	1.97±0.43 (1.01-3.04)	2.22±0.56 (1.04-3.81)	2.02±0.62 (0.88-4.08)	2.71±0.69 (1.44-4.49)	**27.5**
Length of anal fin	LAF	2.77±0.73 (1.30-5.18)	2.30±0.67 (1.23-4.21)	2.79±0.93 (1.18-5.20)	2.37±0.89 (1.09-5.93)	2.99±0.72 (1.65-4.92)	**14.4**
Height of the third dorsal fin ossified ray	HDOR	2.03±0.52 (1.13-3.74)	1.62±0.37 (0.89-2.69)	1.83±0.41 (1.01-3.03)	1.66±0.54 (0.67-3.33)	2.10±0.50 (1.15-3.54)	**21.1**
Meristic traits							
Number of dorsal fin branched rays	NDBR	7.9±0.4 (7-9)	8.0±0.3 (7-9)	8.1±0.3 (7-9)	7.8±0.5 (7-9)	8.1±0.3 (7-9)	**7.4**
Number of scales on the lateral line	NSLL	55.8±4.1 (50-70)	55.3±2.8 (49-62)	56.0±3.5 (50-66)	62.6±3.8 (53-71)	56.9±3.5 (49-68)	**70.7**
Number of scales above the lateral line	NSALL	11.1±1.1 (9-14)	11.7±1.1 (9-15)	12.2±1.3 (10-16)	13.4±1.1 (10-16)	12.2±1.0 (10-15)	**55.3**

Table S8.4 (Continued)

	SI1 ***Barbus*** ***N =85***	**SI2** ***Barbus*** ***N = 121***	***B. tyberinus*** ***N = 107***	***B. plebejus*** ***N = 96***	***B. barbus*** ***N = 96***	**ANOVA** **F**	
Meristic traits							
Number of scales under the lateral line	NSULL	7.9±0.8 (6-10)	8.7±0.8 (7-11)	8.5±1.1 (6-13)	9.3±1.0 (7-12)	8.4±0.8 (7-10)	**30.9**

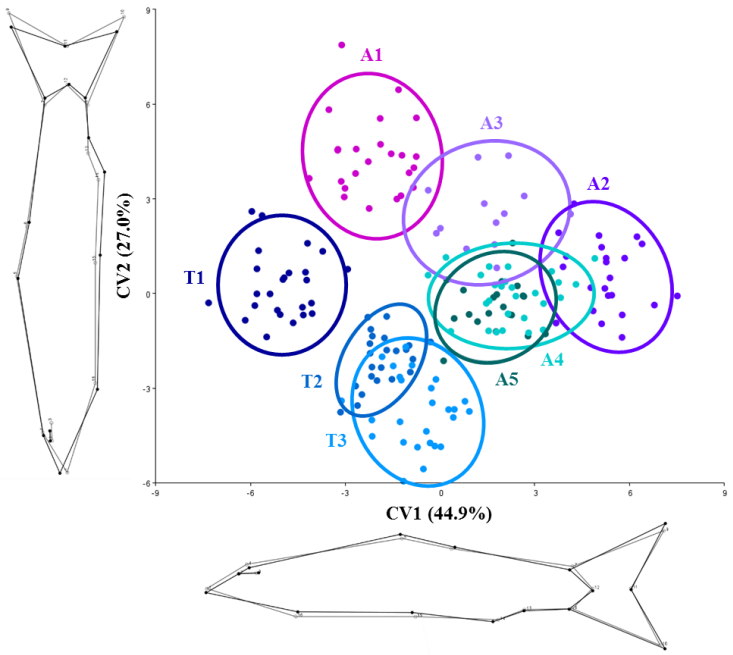

Figure 8.6 *Canonical variate analysis (CVA) output of the body shape comparison between the eight populations of Barbus considered in the present study (see Figure 1). Wireframe graphs indicate the shape changes along each axis (from light to dark grey).*

8.4 Discussion

Through the combined analyses of phylogeny, population genetic structure, distribution and characterization of morphological variability, the results revealed the first evidence for two allopatric *Barbus* evolutionary lineages in the AC district of Southern Italy that were also characterized by distinct morphotypes. These results raise a number of questions relating

to their biogeography and their genetic and morphological differences.

Regarding their biogeography, their genetic and morphological variations may reflect the hydrographic and landscape evolution. The phylogenetic analyses revealed the existence of two new lineages that were only partially identifiable in the field and are considered in the literature as the *B. tyberinus* phenotype (Bianco, 2014). Furthermore, the allopatric distribution of the two new fluvio- lacustrine barbel taxa (SI1 and SI2 *Barbus*) confirms the complex mosaic pattern recorded across the north and central Italian peninsula, where the allopatric origins and dispersion routes of the species have been primarily influenced by distinct geological events (Buonerba et al., 2015; Zaccara et al., 2019). In the north-western Adriatic basins (PV district), the widespread distribution of *B. plebejus* occurred during the glacial cycles that promoted low sea level and low river connections (Buonerba et al., 2015; Meraner et al., 2013). The extended paleo-Po basin reached the meso-Adriatic ditch in the central Adriatic Sea (Bianco, 1990), joining rivers of the two Adriatic slopes (*c.f.* Italian and Balkan peninsula), and resulted in wide genetic admixture of *B. plebejus* (Bianco, 2014; Buonerba et al., 2015; Meraner et al., 2013). In the upper-middle Tyrrhenian basins (TL district), fluvial connection within the rivers systems occurred due to the

considerable extension of the hydrographic network along mountain and high hill environments, with this enabling more effective upstream colonization and widespread distribution of *B. tyberinus* (Carosi, Ghetti, La Porta, & Lorenzoni, 2017; Lorenzoni et al., 2006; Zaccara et al., 2019) up to the Liri-Garigliano basin (T1) where the SI2 *Barbus* lineage was recorded for the first time. The allopatric distribution of these two species confirms there were specific biogeographic boundaries between districts along the Tyrrhenian and Adriatic slopes, constituted by the Rivers Liri and Vomano (see Figure 1), respectively. This biogeographic scenario has been demonstrated for more vicarious species, such as Volturno spined loach (*Cobitis zanandreai* Cavicchioli, 1965) and Italian bleak (*Alburnus albidus* Costa, 1838; Kottelat & Freyhof, 2007). The causes of this biogeographic split may be related to local differences in low sea level drainage patterns, although differences in habitats and in biotic interactions might also have been involved.

The results of the population genetic structure have also demonstrated a nonhomogeneous history in the AC basins, showing the presence of unexpected biogeographic boundary that crossed the Apennine watershed. Across the Italian peninsula, the mosaic biogeographic pattern of the genus *Barbus* was likely to be associated with the differing

hydrographic structure of the basins. For example, the SI1 *Barbus* lineage appeared to originate and only be maintained in basins A1 to A3 (Pescara River up to Biferno River of the middle Adriatic). These basins were not part of the paleo-Po expansion Bianco, 1990), and so they remained isolated from the widespread dispersion of *B. plebejus* that occurred in the upper Adriatic basins (*c.f.* PV district). Within this restricted area, the SI1 *Barbus* lineage had high levels of genetic variability and was thus highly structured. These results suggest that climatic, hydrological, and geological factors probably shaped their local isolation and did not result in dispersion events via temporary connections (Forneris, Merati, Pascale, Perosino, & Tribaudino, 2016). Although the hydrogeographic layout of the AC region is congruent with the current topographic and geological pattern, the main distribution of watercourses has also been influenced by its lithological structure from previous geomorphological stages (Amato, Cinque, & Santangelo, 1995). Current knowledge on the geomorphological evolution of the southern Apennine chain has shown an asymmetric profile of the watershed line, with a retreat of the Tyrrhenian side and progression of the Adriatic side (Brancaccio & Cinque, 1992; Brancaccio et al., 1991). The temporary change in the draining path occurred between Sele (T3) and Ofanto (A5) basins, promoted by temporary river

capture events or transitory mountain lakes, that might help explain the actual distribution of the SI2 *Barbus* lineage in both the southern Tyrrhenian basins (from T1 to T3; i.e., from Liri-Garigliano to Sele basins) and the southern Adriatic basins (A4 and A5; i.e., Fortore and Ofanto basins; Alvarez, 1999), as also reflected by the absence of genetic structure.

Regarding the congruence of the genetic and morphological data, these Italian fluvio-lacustrine barbels, representing a complex of cryptic species, were only partially identifiable by morphology, with their morphological and molecular divergence not always well correlated across the species (Bianco, 1995b; Kottelat & Freyhof, 2007; Livi et al., 2013; Lorenzoni et al., 2006; Zaccara et al., 2019). Despite this lack of congruence between the genetic and morphological approaches, there was nevertheless some significant correlation between evolutionary lineages and body shape. The two SI *Barbus* lineages were significantly differentiated from each other for all morphological traits, except for the number of dorsal fin branched rays and the number of scales on the lateral line, as per Antal et al (2016). Moreover, looking at the dimension of the eye and at the caudal fin lobes, the barbel populations could be morphologically differentiated.

In conclusion, within the hydrogeographic units of the AC district of Southern Italy, there is high genetic structure in the

barbel populations that can be related to the isolation of the basins, resulting in very limited gene flow between them. The limitation in dispersion was due to minimal river capture events in the upstream part of the basins that, due to their typically Mediterranean regime, are characterized by low discharge, and thus, the fish were unable to mix due to insurmountable geographical barriers. Consequently, the AC district can be considered as unique in relation to the biogeography of their endemic barbel populations, with their geographic and hydrological isolation from basins further north being important in this. These results emphasize that, across this district, the evolutionary processes of the endemic barbels have favoured a mosaic pattern, although it is suggested that this requires further work by use of an enlarged dataset, including studies on other freshwater taxa. Although we recorded a limited presence of *B. barbus*, *B. tyberinus*, and *B. plebejus* fish in the AC district, subsequent anthropic manipulation and translocations could still cause genetic admixture (i.e., hybridization) between *Barbus* species in future. If this happens, it is likely to remain undetected along this complex of cryptic species and will potentially lead to the loss of local endemism. Consequently, these results highlight the necessity for any fish and fishery management programmes in this region to recognize the inherently high conservation value of these endemic barbels and

avoid undesirable mixing with other barbels through, for example, fish stocking exercises.

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Data availability statement

All new haplotypes produced in this study have been deposed in GenBank under accession numbers: MK728797–MK72821; MG718025–MG718026.

8.5 Supplementary material

8.5.1 Supplementary tables

Table S8.1 *Pairwise ϕ_{ST} values calculated for South Italy populations collected in Adriatic (A1 up to A5) and in Tyrrhenian (T1 up to T3) basins (see Figure 1A). In bold significant values (p< 0.01).*

	SI1 *Barbus* lineage				**SI2 *Barbus* lineage**			
Pop code	**A1**	**A2**	**A3**	**A4**	**A5**	**T1**	**T2**	**T3**
A1								
A2	**0.39**							
A3	**0.95**	**0.87**						
A4	**0.99**	**0.98**	**0.99**					
A5	**0.98**	**0.97**	**0.98**	**0.80**				
T1	**0.96**	**0.95**	**0.96**	**0.89**	**0.79**			
T2	**0.96**	**0.95**	**0.95**	**0.71**	0.01	**0.72**		
T3	**0.98**	**0.97**	**0.98**	**0.81**	0.00	**0.79**	0.01	

Table S8.2 *Results of the Tukey post-hoc test (Q and p values, the latter within brackets) for the pairwise comparison of the morphological traits (see Figure 2A) between the SI1 and SI2 Barbus lineages versus the other Barbus groups (see Table 4). In bold significant values (p < 0.05).*

	ED	POD	MOD	LPF	LVF	LAF	HDOR	NDBR	NSLL	NSALL	NSULL
SI1 *Barbus vs* SI2 *Barbus*	**4.49 (0.013)**	**7.12 (<0.001)**	**6.75 (<0.001)**	**6.82 (<0.001)**	**6.86 (<0.001)**	**5.84 (<0.001)**	**8.75 (<0.001)**	1.22 (0.912)	1.41 (0.856)	**5.11 (0.003)**	**8.48 (<0.001)**
SI1 *Barbus vs B. tyberinus*	0.77 (0.983)	0.57 (0.994)	0.88 (0.972)	2.82 (0.270)	2.54 (0.376)	0.30 (1.000)	**4.36 (0.018)**	**4.34 (0.019)**	0.66 (0.990)	**10.00 (<0.001)**	**6.91 (<0.001)**
SI1 *Barbus vs B. plebejus*	**4.50 (0.013)**	**4.67 (0.008)**	**4.66 (0.009)**	**6.62 (<0.001)**	**6.00 (<0.001)**	**4.96 (0.004)**	**7.95 (<0.001)**	2.35 (0.459)	**18.85 (<0.001)**	**20.45 (<0.001)**	**15.84 (<0.001)**
SI1 *Barbus vs B. barbus*	**4.42 (0.015)**	**5.86 (<0.001)**	**4.19 (0.026)**	3.15 (0.169)	**6.04 (<0.001)**	2.83 (0.265)	1.39 (0.865)	3.83 (0.052)	2.97 (0.221)	**10.01 (<0.001)**	**5.31 (0.002)**
SI2 *Barbus vs B. tyberinus*	3.72 (0.065)	**6.55 (<0.001)**	**5.87 (<0.001)**	**4.00 (0.037)**	**4.32 (0.019)**	**6.15 (<0.001)**	**4.39 (0.016)**	3.12 (0.177)	2.07 (0.585)	**4.90 (0.005)**	1.58 (0.799)
SI2 *Barbus vs B. plebejus*	0.02 (1.000)	2.45 (0.415)	2.09 (0.576)	0.20 (1.000)	0.86 (0.974)	0.88 (0.972)	0.80 (0.980)	3.56 (0.086)	**20.26 (<0.001)**	**15.34 (<0.001)**	**7.36 (<0.001)**
SI2 *Barbus vs B. barbus*	**8.91 (<0.001)**	**12.98 (<0.001)**	**10.94 (<0.001)**	**9.97 (<0.001)**	**12.90 (<0.001)**	**8.68 (<0.001)**	**10.14 (<0.001)**	2.62 (0.344)	**4.38 (0.017)**	**4.91 (0.005)**	3.17 (0.164)

Table S8.3 *Mahalanobis distances among the eight southern Italian populations, associated to CVA in Figure 8.4.*

	SI1 *Barbus* lineage				**SI2 *Barbus* lineage**			
Pop code	**A1**	**A2**	**A3**	**A4**	**A5**	**T1**	**T2**	**T3**
A1								
A2	8.58							
A3	5.60	5.47						
A4	7.34	5.58	5.29					
A5	7.19	5.59	6.37	5.35				
T1	5.91	10.50	7.72	8.06	7.73			
T2	6.91	8.28	6.69	6.13	6.32	5.75		
T3	8.36	7.52	7.36	6.25	5.85	6.36	3.95	

8.5.2 Supplementary figures

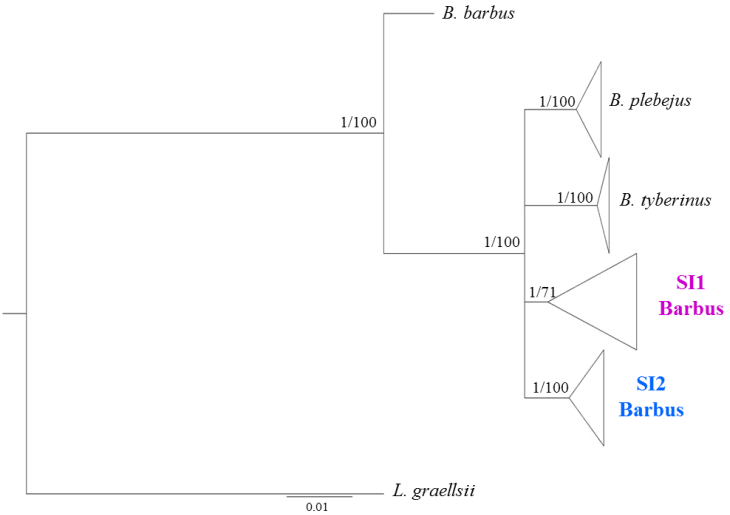

Figure S8.1 *D-loop phylogenetic tree built on 871 bp length haplotypes, produced in this study and retrieved from GenBank (c.f. Zaccara et al., 2019). Statistic support is given and expressed both as posterior probability and bootstrap values. The tree was rooted on Luciobarbus graellsii (GenBank accession number MG827110).*

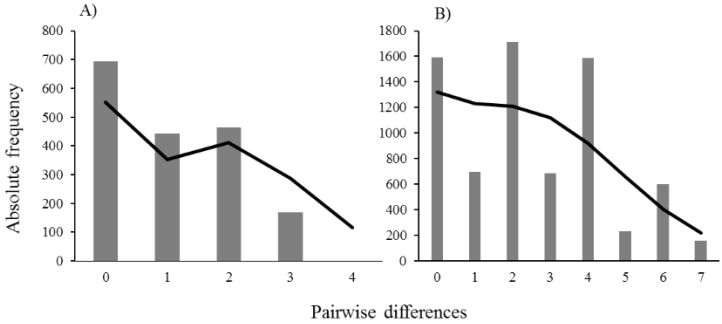

Figure S8.2 *Mismatch distribution trends for SI1 (A) and SI2 (B)* Barbus *lineages. Solid lines represent the estimated trend expected under a model of sudden demographic expansion*

8.6 References

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CHAPTER IX

Cryptic diversity within endemic Italian barbels: revalidation and description of new *Barbus* species (Teleostei: Cyprinidae)

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Abstract

Along the Apulia-Campania ichthyogeographic district of Southern Italy, two fluviolacustrine lineages of the *Barbus* genus have been recently detected through phylogenetic inferences based on mitochondrial DNA. Here we propose the formal description of the new *taxon Barbus samniticus* sp. nov., and the revalidation of *Barbus fucini* Costa, 1953 as a full species, both endemic to southern Italian basins. Molecular analyses provided evidence of four monophyletic clades at a mitochondrial level, while the nuclear dataset highlighted the strict evolutionary relation between *B. plebejus sensu stricto* and the new taxa, converged in *B. plebejus* complex clade. The diagnosis, based on morphological and geometric tools, allowed us to discriminate these cryptic *Barbus* taxa from the already established native Italian fluviolacustrine barbels (i.e. *Barbus tyberinus* and *Barbus plebejus*). At a morphological level, *B. samniticus* sp. nov. and *B. fucini* could be discriminated by the greatest maximum body height and by the longest pre-orbital distance respectively. Both new species have longer ventral and pectoral fins than *B. plebejus* and *B. tyberinus*, a larger caudal fin than *B. tyberinus* and a lower number of scales along the lateral line than *B. plebejus*.

9.1 Introduction

In freshwater ecosystems, environmental pressures (e.g. flow regime variations, substrate and habitat characteristics, physico-chemical conditions) play an active role in modelling freshwater fish morphology (Sagnes & Statzner, 2009; Franssen et al., 2013; Samways et al., 2015) that can result in both phenotypic plasticity within species (Samways et al., 2015) and converge of similar morphotypes between species (Thacker & Gkenas, 2019). As a result, species that display little appreciable morphological differences (i.e. cryptic or pseudo-cryptic) whose identification requires expert taxonomists (Kottelat & Freyhof, 2007) may be misidentified and/or ascribed to already known *taxa* causing an underestimation of biodiversity richness (Geiger et al., 2014). The combined use of molecular and morphometric tools allows cryptic lineages to be detected and these knowledge gaps to be filled (e.g. Costedoat & Gilles, 2009; Antal et al., 2016; Zaccara et al., 2019a). Fish of the genus *Barbus* Daudin, 1805 are a group of medium to large size (mean length of 30 cm) cyprinid fish that thanks to their different phylogeographic structuring (i.e. high level of endemism) and varied ecology have been widely used in biogeographic studies (Marková et al., 2010; Buonerba et al., 2015; Levin et al., 2019). Mitochondrial relationships within *Barbus* genus have been largely resolved in the past 20 years.

Nevertheless, owing to their similar morphologies, description of new species is still ongoing (e.g. Levin et al., 2019; Güçlü et al., 2020).

In Italy among four native species, two are recognised as reophilic (*Barbus caninus* Buonaparte, 1839 and *Barbus balcanicus* Kotlík et al., 2002) and two as fluviolacustrine (common barbel *Barbus plebejus* Buonaparte, 1839 and the Tiber barbel *Barbus tyberinus* Buonaparte, 1839). The fluviolacustrine species are vicariant and mutually populate two ichthyogeographic districts separated by the Apennine mountain chain: *B. plebejus* is distributed in the Padano-Venetian (PV) district that comprises all the basins flowing into the North and middle Adriatic Sea until the Tronto River in Italy and the Krka River in Croatia; *B. tyberinus* inhabits the Tuscany-Latium (TL) district (Zaccara et al., 2019b) that includes Italian rivers flowing into the middle Tyrrhenian Sea, from the Magra River to the Tiber River. In southern Italy, Bianco et al. (1995) suggested the distinction of an additional district (named Apulia-Campania; AC) populated by *B. tyberinus* (Bianco et al., 1995; Lorenzoni, 2006a; Kottelat & Freyhof, 2007), south to the TL up to the Sele River basin along the Tyrrhenian slope and in the southern Adriatic slope from Vomano up to the Ofanto River (Lorenzoni, 2006a; Kottelat & Freyhof, 2007). Basins flowing into the Ionian Sea instead were

not originally populated by barbel (Bianco, 1995; Gallo et al., 2012; Bianco, 2014).

B. plebejus and *B. tyberinus* have similar morphologies (Livi et al., 2013; Geiger et al., 2016; Zaccara et al., 2019b) and they are discriminated by the number (and therefore size) of scales along the lateral line, which are usually less numerous and bigger in *B. tyberinus* (typically 47-63) than in *B. plebejus* (typically 62-78) (Bianco, 1995). Recently, through the application of molecular tools, phylogenetic studies highlighted erroneous field attributions. In Middle Adriatic basins, morphological traits of barbel populations (i.e. barbel in the Esino River) supported *B. tyberinus* phenotype while phylogenetic analysis identified them as members of the *B. plebejus* lineage (Livi et al., 2013; Zaccara et al., 2019b). Moreover, in the AC district, two new mitochondrial lineages were recorded in both Tyrrhenian and Adriatic basins (Zaccara et al. 2019a): i) a first lineage (SI1 *sensu* Zaccara et al., 2019a) is present in rivers draining into the Middle Adriatic Sea, from Vomano River to Biferno River, covering the Northern Apulia-Campania (NAC); ii) a second lineage (SI2 *sensu* Zaccara et al., 2019a) populates the southernmost basins of the Adriatic (i.e. Fortore and Ofanto) and Tyrrhenian (from the Liri-Garigliano to the Sele River) slopes, covering the Southern Apulia-Campania (SAC).

Moreover, species attribution of barbel in Italy is complicated further by the invasion of the exotic fluviolacustrine European barbel *Barbus barbus* (Linnaeus, 1758) that is able to generate hybrids progenies through introgressive hybridisation with the native species (Meraner et al, 2013; Piccoli et al., 2017; Zaccara et al., 2020), resulting in intermediated morphologies to that of the parental species (Geiger et al., 2016; Zaccara et al., 2020).

Consequently, the aim of this study is to solve the taxonomic situation of the two new *Barbus* lineages, using both genetic and morphological approaches. Genetic analysis was performed on mitochondrial (cytb mtDNA) and nuclear DNA (Growth Hormone nDNA) markers with the aim to reconstruct the evolutionary relations between the new lineages and the endemic Italian species. Then, geometric morphometrics and morphological analyses were carried out with the aim to maximize differences among the four taxa, taking care to restrict analysis to specific age class and purebred populations, to reduce allometric bias and to avoid any introgressed form with the alien barbel *B. barbus*, respectively.

For the NAC lineage (SI1 *sensu* Zaccara et al., 2019a), we provide the formal description as a new endemic species named *B. samniticus* sp. nov. as no correspondence with already described taxa was detected. Conversely, our data indicate that

the SAC lineage (SI2 *sensu* Zaccara et al., 2019a) could correspond with *Barbus fucini* Costa 1853 that for several years has been synonymised as *B. tyberinus* (Bianco, 1995) and for which here we propose the revalidation as a full species.

9.2 Material and methods

9.2.1 *Sampling strategy and fish samples*

During autumn 2019, an *ad hoc* sampling campaign was dedicated to collect fish from representative localities of *B. samniticus* sp. nov. (N= 12) and *B. fucini* (N = 9), identified in the Sangro River (NAC basin) and the Liri River (SAC basin), respectively (coloured circles in Figure 1 and sampling sites n. 5 and n. 9 in Table S9.1) using electric fishing. Fish were photographed on the left side using a Nikon D300 camera (24–85 mm lens) positioned by means of a tripod on a table with a millimetric scale. An excision of a fin clip for genetic analysis was also performed. Then, fish were euthanized with an overdose of MS-222, fixed for two days in formaldehyde 10% and preserved in 60% ethanol. Before fixation, a small portion of muscle was retained and preserved in 100% ethanol and is available for future genetic analysis. Acronyms of scientific institutions in which new material is stored follow Kottelat et al. (1993) except for the Natural History Gallery of Casalina (University of Perugia) (GSN).

An additional 35 samples of fin-clips and photos, were also collected from three rivers in NAC, and from four rivers in SAC (N=15 and N=20 for *B. samniticus* sp. nov and *B. fucini*, respectively) (Figure 9.1; Table S9.1).

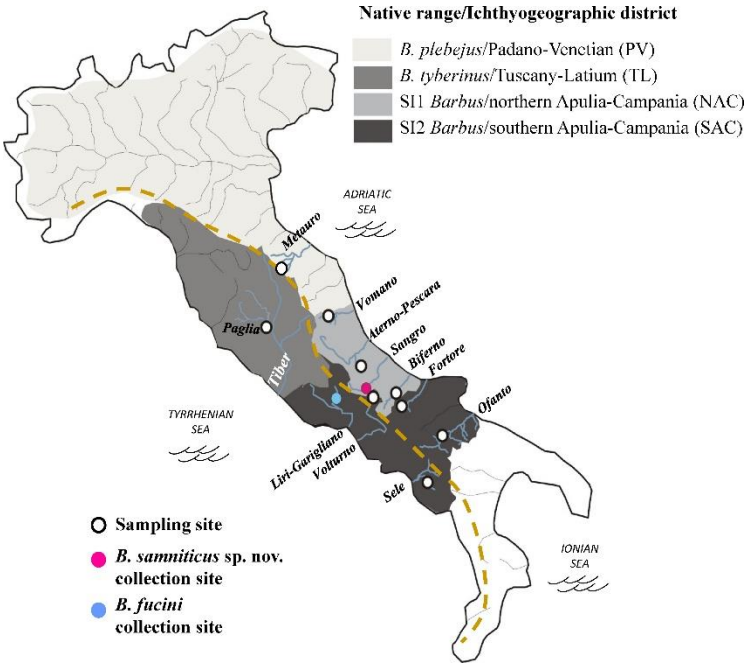

Figure 9.1 *Map of the native ranges (and corresponding ichthyogeographic districts) of the four Italian fluviolacustrine barbel lineages with location of the Appenine mountain chain, which divides central and southern Italian basins in Tyrrhenian and Adriatic drainages, indicated by a brown dashed line. Sampling sites from which comparative material (e.g., scales, fin-clips and/or photos) was sampled are shown as open circles whilst collection sites of* Barbus samniticus *sp. nov. (i.e., SI1* Barbus*) holotype and paratypes and* Barbus fucini *(i.e., SI2* Barbus*) non-type specimens are represented*

by pink circle (Sangro River) and blue circle (Liri River), respectively (see Table S9.1 for further detail).

9.2.2 *Molecular analyses*

Total genomic DNA was extracted following a salting-out method (Aljanabi & Martinez, 1997). The mitochondrial gene *cytb* was amplified through polymerase chain reactions (PCR) using primers L15267 and H16461 (Briolay et al., 1998) and a fragment of 1121 bp was analysed. As barbels are tetraploid fish, we selected and amplified the nuclear Growth Hormone paralog-2 (GH-2) using primers specifically developed for *Barbus* and *Luciobarbus* genera (F-GTACTATAGTAAGCAGAAATGG and R-AGTGGSAGGGAGTCGTTC; Gante et al. 2011). Amplifications were performed for both *loci* using the Q5 High-fidelity Master Mix (New England Biolabs Inc.) in 10 µl reaction volume containing approximately 10 ng of template DNA and 0.25 µM of each primer pair. PCR profile was set with an initial denaturation at 98°C for three minutes followed by 35 cycles of 30s at 98°C, 90s at 55°C and 90s at 72°C, concluding with a final extension step at 72°C for 10 minutes. Amplicons were then purified using ExoSAP-IT™ (USB) and sequenced by MACROGEN Inc (http://www.macro gen.org) using a 3730XL DNA Sequencer.

Sequences were automatically aligned using Clustal W (Thompson et al., 1994) as implemented in Bioedit software (Hall, 1999), and further checked manually to eliminate ambiguities. For the nuclear *locus*, sequences were phased using the PHASE algorithm available in DnaSP 6 (Rozas et al., 2017). All the sequences produced are deposited in GenBank database under accession numbers MT454508-MT454560 and MT454561-MT454618 for *cytb* and GH-2, respectively.

Mitochondrial and nuclear datasets were enriched with 105 (MG495775- MG495922; KC465928-KC465949 in Meraner et al., 2013) and 25 (KF923619-KF923631; KF963487-KF963498 in Zaccara et al., 2014 and Buonerba et al., 2015 respectively) sequences, respectively including available Italian fluviolacustrine species (*B. plebejus* and *B. tyberinus*) (Tables S9.2 and S9.3).

The best fit evolution model for the phylogenetic analysis was estimated for each data set in JModelTest v 2.1.10 (Darriba et al., 2012) and HKY and F81+G models were selected according to the Akaike's information criterion (AIC) for the *cytb* and the GH-2 dataset respectively.

Bayesian inference was then used for phylogenetic reconstruction as implemented in MrBayes 3.1.2 (Ronquist et al., 2012) software. Four independent Markov Montecarlo

coupled chains (MCMC) were run with 10^6 generations, sampling topologies every 100 generations and discarding as burn-in the first 25% generations.

The rheophilic species *Barbus caninus* was used as outgroup available under Genbank accession numbers AF112124 and KF963432 for the mtDNA and the nDNA datasets, respectively.

Uncorrected *p-distances* were calculated in MEGA X (Kumar et al., 2018) for each species and used as proxies of species divergence levels (Doadrio et al., 2002). In both mitochondrial and nuclear datasets, a minimum spanning network (MSN) was built using a statistical parsimony criterion as implemented by the software TCS v 1.18 (Clement et al., 2000) that fixes at 95% the maximum connection steps, corresponding to 13 mutation events.

9.2.3 *Morphological analyses*

Morphological analyses were performed on *B. samniticus* sp. nov and *B. fucini* photos taken from fish of similar age (i.e. only age classes 2+; 3+; 4+) in order to reduce potential allometric bias as much as possible. The morphological data-set was also enriched by 35 and 28 photos of purebred *B. tyberinus* and *B. plebejus* (Figure 9.1 and Table S9.1 respectively; Zaccara et al., 2020).

From each photo, twenty-eight landmarks were captured using the R Geomorph function "digitize2d" (Adams et al., 2018; Figure S9.1a). Generalized Procrustes analysis allowed the removal of non-shape variation, introduced through variation in position, orientation, and size, as implemented in MorphoJ software (Klingenberg, 2011). With the same software, shape variations between species were analysed by canonical variate analyses (CVA), and Mahalanobis distances (MDs) were calculated using permutation tests (10,000 replicates).

A total of 29 traits (14 morphometric and 15 meristic traits) were analysed (Figure S9.1b). Meristic traits included also phenotypic characters concerning spot/dot/pigmentation presence on the body and all the fins, and fin colour. Morphometric traits were standardized to the overall mean standard length (Beacham, 1985) to further reduce the effects of size and allometry. Pairwise comparison on morphological traits between the four species was performed by means of the analysis of variance (ANOVA) followed by Tukey post hoc test, as implemented in PAST software (Hammeret al., 2001). Moreover, a linear discriminant analysis (LDA) was carried out using all the morphological traits (except for those without variation) after standardization to detect differences between species, and MDs were calculated.

9.3 Results

9.3.1 *Molecular variability and genetic relation among taxa*

Fifty-three *cytb* sequences were obtained (N=25 and N=28 for *B. samniticus* sp. nov. and *B. fucini*, respectively) producing a multiple alignment of 1121bp length. We detected 45 variable nucleotide positions, 32 of which were parsimony informative sites and 13 singletons, obtaining 17 haplotypes (10 and 7 for *B. samniticus* sp. nov and *B. fucini*, respectively). With the exclusion of five individuals for which the amplification of the nuclear GH-2 *locus* failed, 96 alleles of 890bp length were obtained, grouping in 10 haplotypes (3 from *B. samniticus* sp. nov. and 7 from *B. fucini*), characterised by 10 polymorphic sites of which 7 were parsimony informative sites and 3 singletons.

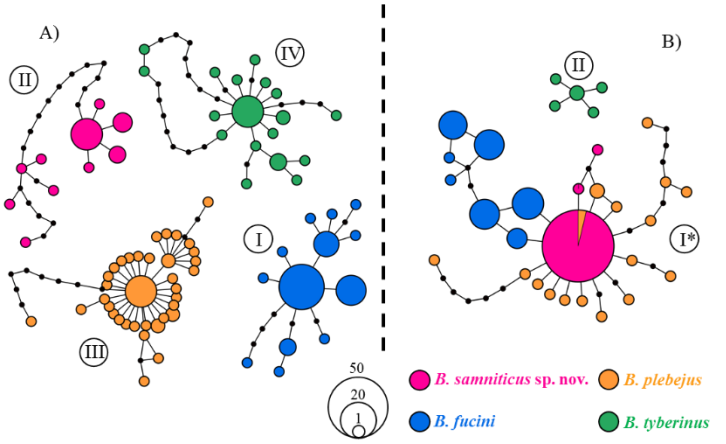

Figure 9.2 *Parsimony minimum spanning networks (MSNs) of the four Italian fluviolacustrine barbel species built on A) 1121 bp mitochondrial cytb sequences and on B) 1021 bp nuclear GH-2 sequences. Colors indicate mtDNA lineages (Figure S9.2a) while roman numbers correspond to the phylogenetic clade identified for each DNA marker respectively (Figure S9.2). Asterisk identifies* Barbus plebejus *complex clade at the nDNA that included* B. samniticus *sp. nov. and* B. fucini.

Phylogenetic trees based on *cytb* and GH-2 markers provided distinct topologies (see Figure S9.2). In the mtDNA tree (Figure S9.2a; Table S9.2) four monophyletic groups (pp=1) were recovered identifying *B. plebejus* (clade III), *B. tyberinus* (clade IV) and the two new evolutionary lineages (clade II and I respectively): *B. samniticus* sp. nov. (SI1 clade *sensu* Zaccara et al., 2019a) and *B. fucini* (SI2 clade *sensu* Zaccara et al., 2019a). The nuclear GH-2 phylogenetic tree (Figure S9.2b) showed the presence of two clusters (pp=1): *B. tyberinus* (clade II), congruent with the mtDNA, and *B. plebejus* complex (clade

I*) where *B. samniticus* sp. nov. and *B. fucini* converged with *B. plebejus sensu stricto* Figure 9.2b).

Uncorrected *p-distances* calculated for the *cytb* dataset evidenced similar levels of differentiation between the four *Barbus* taxa (Table 9.1), with the highest value recorded between *B. samniticus* sp. nov. and *B. tyberinus* (2.2%) and the lowest between each of the two AC lineages and *B. plebejus* (1.7%). At the GH-2, *p-distances* calculated between the two clades *B. tyberinus* and *B. plebejus* complex was 2.5% (±0.2).

Table 9.1 *Uncorrected pairwise nucleotide distances (p-distances) represented as percent mean ± standard deviations of the four Italian fluviolacustrine barbel species at the mitochondrial Cytb marker (cf Figure 9.2; Figure S9.2).*

	***B. samniticus* sp. nov.**	***B. fucini***	***B. plebejus***	***B. tyberinus***
Cytb				
B. samniticus sp. nov.	0.5 ± 0.6			
B. fucini	1.8 ± 0.2	0.2 ± 0.3		
B. plebejus	1.7 ± 0.2	1.7 ± 0.3	0.2 ± 0.2	
B. tyberinus	2.2 ± 0.2	1.8 ± 0.3	1.8 ± 0.3	0.3 ± 0.3

The MSN built on the mtDNA recovered 4 independent networks (Figure 9.2a) corresponding to the 4 phylogenetic lineages detected in the phylogenetic reconstruction (Figure S9.2a), while only two networks resulted from the nDNA that

matched the *B. tyberinus* (clade II) and the *B. plebejus* complex (clade I*) clades of the nuclear phylogenetic tree (Figure S9. 2b). Within this latter group, *B. plebejus sensu stricto* and *B. samniticus* sp. nov. shared a same haplotype whilst *B. fucini* resulted connected to them by 1 up to 7 mutational steps (Figure 9.2b).

9.3.2 *Morphological comparison between Italian barbel species*

All the four species detected by genetic analyses of mitochondrial DNA resulted clearly separated for their body shape in the CVA graph (Figure 9.3), with a higher proximity recorded between *B. samniticus* sp. nov. and *B. fucini* (MD = 6.3) than between the other two already known species (MD = 7.4). *B. samniticus* sp. nov. and *B. fucini* were greatly separated from *B. tyberinus* along both the CVA axis (MD = 9.1 and 9.6 respectively), mainly differentiating for the shape of the caudal fin (see the detail in Figure 9.3). The same occurred also for *B. fucini* from *B. plebejus* (MD = 9.9). *B. samniticus* sp. nov. was instead closer to *B. plebejus* along the CV2 axis (MD = 7.1), indicating a major similarity of caudal fin shape and body depth between these two species.

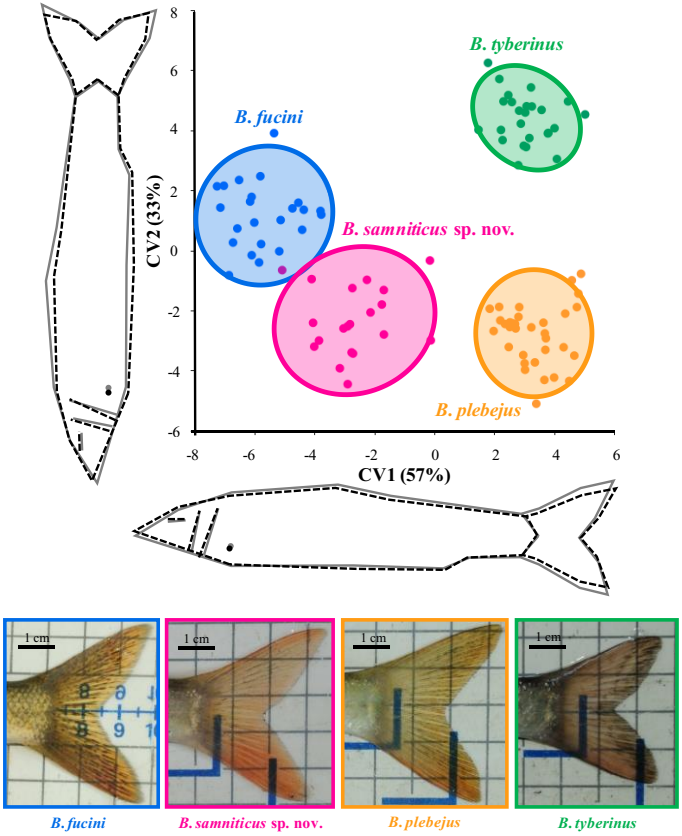

Figure 9.3 *Output of the canonical variate analysis (CVA) on fish body shape carried out between the four Italian fluviolacustrine barbel species. Wireframe graphs indicate the shape changes along each axis (from grey to dashed black). Detail of fin shape in living specimens is reported for each species.*

Also the result of the LDA carried out with morphometric and meristic traits showed a major overlap between *B. samniticus* sp. nov. and *B. fucini* (MD = 3.6) (Figure 9.4). Only the

maximum height and the pre-orbital distance were significantly different between the two species being respectively greater and shorter in *B. samniticus* sp. nov. than in *B. fucini* (ANOVA and Tukey test, $p<0.05$; Table 9.2). Both these species could be discriminated from *B. plebejus* and *B. tyberinus*: the former has longer ventral and pectoral fins and base of the caudal fin than the latter. Between the four different species, *B. samniticus* sp. nov. had the greatest maximum height and *B. fucini* the longest pre-orbital distance (ANOVA and Tukey test, $p<0.05$; Table 2). In contrast to the CVA output, in the LDA both the AC species resulted mainly separated from *B. plebejus* (MDs: *B. samniticus* sp. nov.-*B. plebejus* = 6.5, *B. fucini*-*B. plebejus* = 7.6), displaying a significantly higher number of scales on, above and under the lateral line (see detail in Figure 9.4), a greater minimum height, a larger fork depth, and a lower length of anal fin (ANOVA and Tukey test, $p < 0.05$; Table 9.2).

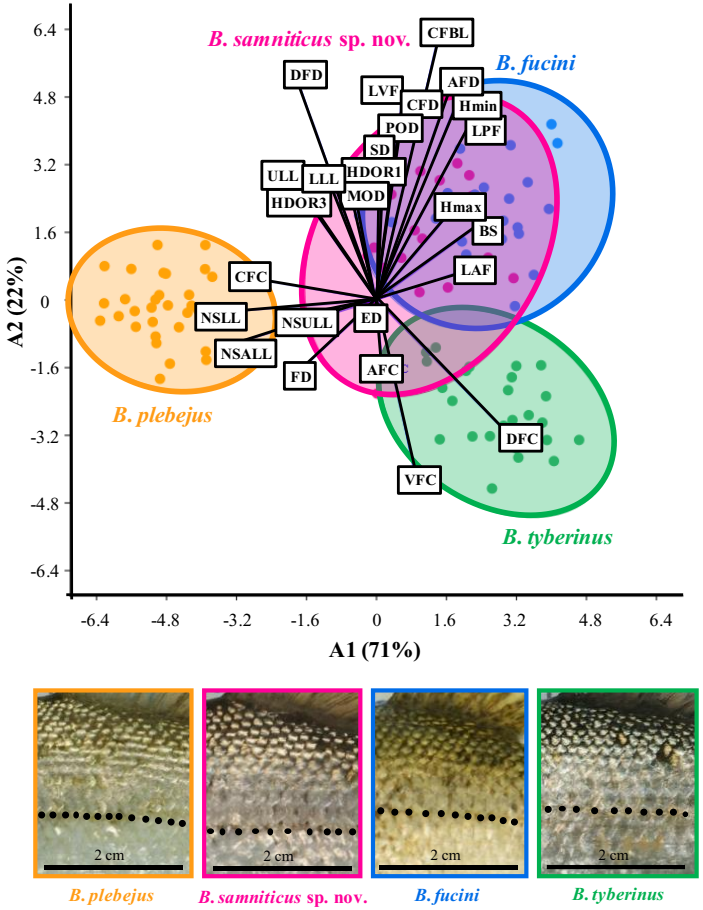

Figure 9.4 *Output of the linear discriminant analysis (LDA) carried out with morphometric and meristic traits (see Tables 9.2 and 9.3) between the four Italian fluviolacustrine barbel species. Detail of scales along lateral line (NSLL) is reported for each species.*

Table 9.2 *List of morphometric and meristic traits (mean ± standard deviation, minimum and maximum within brackets) for the four Italian fluviolacustrine barbel lineages. Significant differences (Tukey test, p<0.05) after data standardization (Beacham, 1985) are shown using different superscript letters.*

		B. samniticus sp. nov. N=20	***B. fucini*** N=25	***B. plebejus*** N=35	***B. tyberinus*** N=28
Morphometric traits (cm)					
Standard length	Lst	16.31 ± 1.59 (13.27-18.40)	15.03 ± 1.68 (11.57-17.93)	15.23 ± 2.99 (11.07-21.99)	16.37 ± 3.19 (12.71-22.60)
Eye diameter	ED	0.68 ± 0.07 (0.55-0.81)	0.68 ± 0.06 (0.57-0.80)	0.68 ± 0.10 (0.48-0.88)	0.70 ± 0.08 (0.56-0.88)
Pre-orbital distance	POD	1.46 ± 0.17 (1.09 + 1.72)	1.41 ± 0.21 (0.91-1.75)	1.36 ± 0.28 (0.86-1.96)	1.41 ± 0.28 (1.09-1.96)
Mouth-operculum distance	MOD	3.68 ± 0.34 (3.02-4.15)	3.50 ± 0.44 (2.45-4.34)	3.50 ± 0.68 (2.37-5.15)	3.63 ± 0.68 (2.90-4.93)
Length of pectoral fin	LPF	3.12 ± 0.28 (2.55-3.56)	2.94 ± 0.36 (2.16-3.39)	2.73 ± 0.60 (1.87-4.06)	2.96 ± 0.50 (2.27-3.96)
Length of ventral fin	LVF	2.39 ± 0.24 (1.98-2.77)	2.22 ± 0.25 (1.70-2.53)	2.12 ± 0.43 (1.50-3.04)	2.17 ± 0.37 (1.60-2.99)

Table 9.2 (Continued)

		B. samniticus sp. nov. N=20	***B. fucini*** N=25	***B. plebejus*** N=35	***B. tyberinus*** N=28
Morphometric traits (cm)					
Length of anal fin	LAF	2.79 ± 0.32 (2.23-3.27)	2.60 ± 0.49 (1.68-3.59)	2.38 ± 0.58 (1.51-4.13)	2.85 ± 0.78 (1.89-4.58)
Height of the dorsal fin ossified ray	HDOR1	2.70 ± 0.22 (2.24-3.21)	2.41 ± 0.31 (1.62-2.86)	2.45 ± 0.51 (1.69-3.62)	2.47 ± 0.41 (2.01-3.38)
Height of the third dorsal fin branched ray	HDOR3	2.00 ± 0.18 (1.61-2.25)	1.80 ± 0.25 (1.33-2.16)	1.89 ± 0.37 (1.29-2.78)	1.86 ± 0.32 (1.48-2.56)
Fork depth	FD	1.63 ±0.20 (1.28-1.98)	1.46 ± 0.18 (1.17-1.88)	1.66 ± 0.35 (1.13-2.72)	1.62 ± 0.24 (1.33-2.13)
Length of the upper lobe of caudal fin	ULL	3.83 ± 0.36 (3.24-4.43)	3.52 ± 0.41 (2.73-4.22)	3.73 ±0.73 (2.69-5.66)	3.50 ±0.58 (2.79-4.72)
Length of the lower lobe of caudal fin	LLL	3.70 ± 0.33 (3.04-4.30)	3.45 ± 0.43 (2.61-4.22)	3.56 ± 0.70 (2.55-5.43)	3.45 ± 0.58 (2.86-4.67)
Length of caudal fin base	CFBL	2.43 ± 0.28 (1.98-2.97)	2.30 ± 0.29 (1.75-2.75)	2.07 ± 0.49 (1.38-3.12)	2.06 ± 0.46 (1.60-3.26)

Table 9.2 (Continued)

		B. samniticus sp. nov. N=20	***B. fucini*** N=25	***B. plebejus*** N=35	***B. tyberinus*** N=28
Morphometric traits (cm)					
Minimum height	Hmin	1.84 ± 0.19 (1.52-2.20)	1.70 ± 0.20 (1.31-2.12)	1.60 ± 0.31 (1.18-2.28)	1.67 ± 0.31 (1.35-2.33)
Maximum height	Hmax	3.65 ± 0.38 (2.77-4.42)	3.22 ± 0.35 (2.37-3.92)	3.13 ± 0.59 (2.32-4.46)	3.43 ± 0.58 (2.67-4.61)
Meristic traits					
Number of dorsal fin branched rays	NDBR	8 ± 0.3 (7-8)	8 -	8 -	8 -
Number of scales on the lateral line	NSLL	57 ± 3.9 (51-67)	56 ± 3.7 (51-65)	64 ± 3.2 (58-71)	56 ± 2.8 (52-63)
Number of scales above the lateral line	NSALL	11 ± 1.0 (10-13)	11 ± 1.3 (9-14)	13 ± 0.9 (11-15)	12 ± 0.8 (10-13)
Number of scales under the lateral line	NSULL	8 ± 0.7 (7-9)	8 ± 0.7 (7-10)	9 ± 0.8 (8-11)	8 ± 0.6 (7-10)

Moreover, *B. tyberinus* specimens were distinguished by the colour of the anal, dorsal, and ventral fins (mainly grey) and for the reduced presence of dots on scales and fins (Table 9.3). Similar to *B. plebejus*, *B. samniticus* sp. nov. and *B. fucini*, were differentiated from *B. tyberinus* by the greater height of the dorsal fin ossified ray and length of both the upper and lower lobes of the caudal fin (MDs: *B. samniticus* sp. nov. *B. tyberinus* = 4.7, *B. fucini*-*B. tyberinus* = 4.9, *B. plebejus*-*B. tyberinus* = 7.8) (ANOVA and Tukey test, $p<0.05$; Table 9.2). *B. samniticus* sp. nov. and *B. plebejus*, had a greater height of the third dorsal fin branched ray than *B. tyberinus*, while *B. fucini* had a longer mouth-operculum distance than *B. tyberinus* (ANOVA and Tukey test, $p<0.05$; Table 9.2). *B. fucini* had also a greater maximum height than *B. plebejus* (ANOVA and Tukey test, $p<0.05$; Table 9.2).

Table 9.3 *List of phenotypic characters (expressed in percentage) concerning spot/dot/pigmentation presence and fin colour for the four Italian barbel species.*

			***B. samniticus* sp. nov.** N=20	***B. fucini*** N=25	***B. plebejus*** N=35	***B. tyberinus*** N=28
Dots on body	BD	yes	0	0	0	0
		no	100	100	100	100
Spots on body	BS	yes	45	56	3	36
		no	55	44	97	64
Scale edge pigmentation	SEP	yes	0	0	0	0
		no	100	100	100	100
Dots on scales	SD	yes	35	52	26	4
		no	65	48	74	96
Dots on dorsal fin	DFD	yes	70	92	89	14
		no	30	8	11	86
Dots on anal fin	AFD	yes	40	60	0	0
		no	60	40	100	100
Dots on caudal fin	CFD	yes	75	96	49	29
		no	25	4	51	71

Table 9.3 (Continued)

			***B. samniticus* sp. nov.** N=20	***B. fucini*** N=25	***B. plebejus*** N=35	***B. tyberinus*** N=28
		orange	80	96	100	14
Ventral fin colour	VFC	grey	0	0	0	68
		orange/grey	20	4	0	18
		orange	85	96	100	14
Anal fin colour	AFC	grey	0	0	0	50
		orange/grey	15	4	0	36
		orange	0	0	0	0
Dorsal fin colour	DFC	grey	45	36	0	86
		orange/grey	55	64	100	14
		orange	50	0	83	0
Caudal fin colour	CFC	grey	0	4	0	4
		orange/grey	50	96	17	96

9.3.4 *Barbus samniticus* sp. nov.

urn:lsid:zoobank.org:act:E83D2FDF-2954-430D-815F-3FF7B19F21E7

Holotype

NMW 100289, 146 mm SL; Italy (Figures 9.5a and 9.5b): Sangro River at Roccascalegna (Abruzzo Region) 42°05'29.76"N, 14°34'75.82"E; Lorenzoni M. and Carosi A. legit with electrofishing; November 2, 2019. GenBank accession numbers MT454527 and MT454584 for mtDNA cytb and nDNA GH-2 markers respectively.

Paratypes

NMW 100290 (three specimens), 130-172 mm SL; MCSNC/P/5002-5005 (four specimens), 133-156 mm SL; ZFMK 122456-122459 (four specimens), 136-178 mm SL; same locality and data as holotype (Figure 9.5c). GenBank accession numbers: MT454521-MT454526 and MT454528-MT454532 for mtDNA cytb marker, and MT454578-MT454583 and MT454585-MT454589 for nDNA GH-2 marker.

GSN 21_01-02 (two specimens), 173-188 mm SL; Italy: Giardino River at Popoli (Abruzzo Region) 42°10'25.85"N,

13°49'51.35"E; Lorenzoni M. and Carosi A. legit with electrofishing; June 24, 2018.

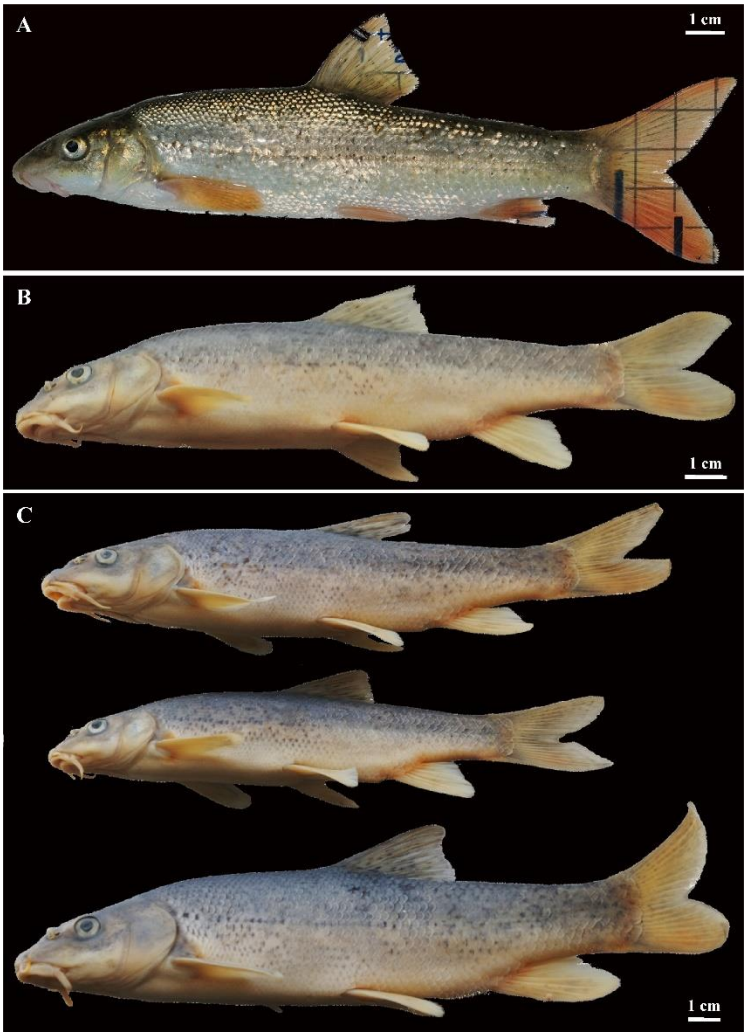

Figure 9.5 *Original pictures of the lateral view of* B. samniticus *sp. nov. A) Live specimen of* B. samniticus *(holotype NMW 100289). B)*

B. samniticus *in 10% formalin (holotype NMW 100289) and C) Paratypes (NMW 100290).*

Molecular diagnosis

At the mitochondrial cytb gene (1121 bp), *B. samniticus* sp. nov. is distinguished from the other three Italian fluviolacustrine *Barbus* species by four diagnostic sites respectively with mean genetic distances comprised between 1.7% and 2.2%. At the nuclear GH-2 locus, *B. samniticus* sp. nov.clusters within the *B. plebejus* species complex (Figure S9.2b).

Morphological diagnosis

Like *B. plebejus*, *B. tyberinus* and *B. fucini* has a weakly ossified last unbranched dorsal-fin ray. The superior margin of the dorsal fin is straight or slightly concave like other Italian fluvio-lacustrine barbels, but some specimens show a margin profile slightly convex. Similar to *B. tyberinus* and *B. fucini* and unlike *B. plebejus*, *B. samniticus* sp. nov. has numerous small irregular shaped black or dark brown dots, smaller than scales that often form large, black or dark-brown spots on the back and flank in juvenile and adults: this pigmentation is more evident in specimens living in clear water, while it tends to disappear in turbid environments.

As detailed in the morphological comparison section, *B. samniticus* sp. nov. can be distinguished from all the other three species by its largest maximum height. *B. samniticus* sp. nov. differs from *B. fucini* by a shorter pre-orbital distance and from *B. tyberinus* and *B. plebejus* for it having longer pectoral, ventral and caudal fins. *B. samniticus* sp. nov. differs from *B. tyberinus* only in that it has longer lobes of the caudal fin, a greater dorsal fin, a major presence of pigmentation dots on scales and fins and a different dominant (orange) colour of the anal, dorsal, and ventral fins. *B. samniticus* sp. nov. differs from *B. plebejus* by having fewer scales on, and fewer rows above and under the lateral line, lower minimum height, smaller fork depth, and greater amount of body spots.

Description

B. samniticus sp. nov. is a medium size species with a moderately deep and slightly compressed body: the maximum and the minimum height of the body are respectively 22% and 11% of the standard length. Dorsal profile arched and ventral profile straight. Convex predorsal profile and straighter postdorsal profile.

The lateral line has 51-67 scales (mode is 56); 10-13 scale rows (mode is 11) between dorsal-fin origin and lateral line, 7-9 scale rows (mode is 7) between anal-fin origin and lateral line. Dorsal

fin with 4 unbranched and 7-8 branched rays (Table 9.2). The anal fin has five branched rays (mode) and 7-8 the pelvic. The formula for the pharyngeal teeth is usually 5.3.2-2.3.5.

The head is conical and is equal to about a quarter of the length of the body (23-25% standard length). The snout is pointed. Mouth inferior with slightly developed lips. Lips with papillae. The lower lip is trilobate with a median lobe not reaching the angle of the mouth; lower lip thicker than upper lip. Maxillary barbels just or not reaching the vertical of posterior margin of pupil. Dorsal-fin origin in front of pelvic-fin origin; the tip of the dorsal fin at the same vertical of the tip of the pelvic fins. Large and fairly forked caudal fin, upper lobe more pointed than the lower one

Coloration

Body coloration is in general greyish-brown on back, the flanks are paler and the abdomen is whitish. Numerous brownish spots, composed of small dots irregularly grouped, are present on the back, flanks and also on the dorsal, anal, and ventral fins.

This livery gives to *B. samniticus* sp. nov. an overall appearance similar to that of *B. tyberinus,* from which it is distinguished for the fins are dominated by orange tones: the dorsal fin is greyish or orange-greyish while other fins are orange-greyish or orange.

Sexual dimorphism

As in other Italian barbels, there is sexual dimorphism and the females have a longer anal fin: only in females, the back of the anal fin reaches the insertion of the caudal fin. During the breeding season, males have small nuptial tubercles all over the body.

Distribution and habitat

Basins of the middle Adriatic slope of the Italian Peninsula between the Vomano and Biferno catchments (Figure 9.1); intermediate and hilly stretches of the Apennine rivers together with other Italian fish species typical of the barbel zone: *Sarmarutilus rubilio* Bonaparte 1837, *Telestes muticellus* Bonaparte 1837 and *Squalius squalus* Bonaparte 1837.

Etymology

From Samnites, ancient Italic people settled in the type locality of the new species.

9.3.5 *Barbus fucini* Costa 1853

Type specimens

Barbus fucini Costa 1853: part 1, sheet 7, figure 1-7 (original description, type locality: Lake Fucino (Abruzzo region, Italy),

holotype: none; lectotype: designated herein, NMW 54799; paralectotype: designated herein, MNHN 192; Synonym *Barbus tyberinus* Bianco, 1995 p. 313; Kottelat, 1997 p. 51; Bianco, 2003b p. 427. The current existence of two probable syntypes has been ascertained in the ichthyological collections of the Natural History museums of Paris and Vienna. The first (MNHN 192, 96 mm SL, Lake Fucino, from Costa collection) was reported by Bianco (1995) with the following short description "The specimen has been dissected, but its right half is still very well preserved. It has 57 scales on LL; 13.5 scales above and 8.5 below LL; 22 circumpeduncular scales; 44 total vertebrae; gill rakers 11 (8+3); D 8. The dorsal fin has 24 very small serrae". The second possible syntype specimen was cited by Kottelat (1997) with the catalogue number NMW 54799 (Figure 9.6) and confirmed by a recent check. In our revalidation of *Barbus fucini* Costa, 1853 we designate NMW 54799 as the lectotype (Figure 9.6) and MNHN 192 as the paralectotype.

Non-type specimens

MCSNC/P/50006-50010 (five specimens), 101-215 mm SL; GSN 21_03-07 (five specimens), 149-179 mm SL; Italy: Liri River at Civita D'Antino (Abruzzo Region) 41°52'38.92"N-13°27'11.12"E; Lorenzoni M. and Carosi A. legit with electrofishing; November 1, 2019 (Figures 9.6c and 9.6d).

GenBank accession numbers MT454552-MT454560 and MT454614-MT454618 for mtDNA cytb and nDNA GH-2 markers respectively.

GSN 21_08-015 (eight specimens), 53-157 mm SL; Italy: Calore River at Castelcivita (Campania Region) 40°29'27.8"N-15°12'25.2"E; Lorenzoni M. and Carosi A. legit with electrofishing; October 12, 2018.

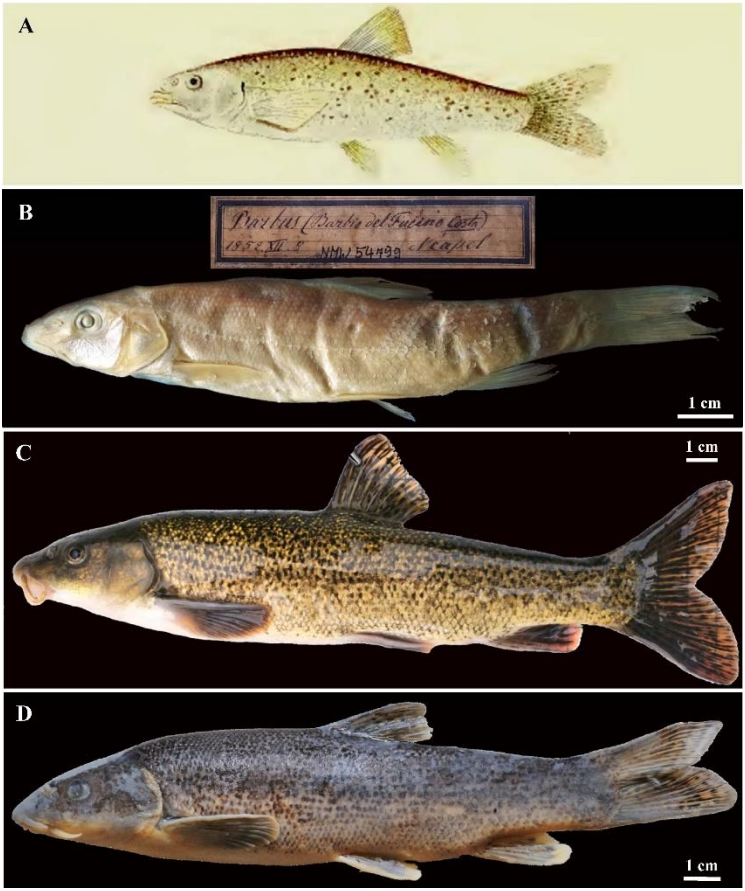

Figure 9.6 *Pictures of the lateral view of* Barbus fucini *Costa 1853. A) Drawing of the main aspect of* Barbus fucini *obtained from the original description by Costa (1853). B) Syntype present at the Natural History Museum of Wien (NMW 54799, 109 mm SL) with its original label. C) Live non-type specimen. D) Non type specimens fixed in 10% formalin.*

Translation of the original diagnosis (Costa, 1853)

"Barbus fucini has a snout sharply pointed, with the snout length (i. e. the distance from the tip of the snout to the upper corner of the operculum) equal to a quarter of the total length. The eye is positioned in a way that the distance from the extremity of the operculum edge to the pupil exceeds the distance from the pupil to the tip of the snout of a half-diameter of the orbit, and the distance from the orbit to the upper lip edge is equal to 2.5 diameters of the orbit itself. The nostrils are close to the eye." […] "The snout is elongated, with the upper jaw protruding out of the lower jaw. Both jaws are covered by fleshy lips. The lower lip is a little rippled. Two pairs of fleshy barbels are present on the snout: two maxillary barbels placed anterior to the nostrils, and two longest mandibular barbels positioned at the corner of the mouth. The mandibular barbels are longer than the longitudinal length of the mouth. The pectoral fins are sharp and composed of 17 rays. The ventral fins are composed of 9 rays, the anal fin of 8 rays. The dorsal fin, with straight edge, consists of 10 rays, the second of which has a posteriorly serrated edge; the serration teeth are visible to the naked eye only when the specimen is dry. The caudal fin is forked and it is composed of 18 rays. The lateral line is straight. The dorsal profile is slightly convex. The body shape is rounded in cross-section and tapered. Body coloration is in general silvery,

yellowish and greyish-brown on back, with brownish spots composed of small dots irregularly grouped, also present on the flanks. These characteristics vary from individual to individual and also according to age. The flanks are paler than the back, the ventral coloration is whitish. The fins have a pale colour with a slight red colouration on the edge. The caudal fin is greyish-brown tending to the violet on the edge. The dorsal fin has a dark colouring that hides small brown spots [...]".

Molecular diagnosis

At the mitochondrial cytb (1121bp) *B. fucini* is distinguished by four diagnostic sites from *B. samnticus* and *B. plebejus* and by five diagnostic sites from *B. tyberinus* and it is distant from the three related *Barbus* species by genetic distances comprised between 1.7 to 2.2%. At the nuclear GH-2 locus (1012 bp), *B. fucini* clusters within the *Barbus plebejus* complex.

Morphological diagnosis

B. fucini is distinguished from all the other three Italian fluviolacustrine species by having a longer pre-orbital distance, in agreement with the original description of the species (Costa, 1853). *B. fucini* differs from *B. samniticus* sp. nov. for a lower maximum height and from *B. tyberinus* and *B. plebejus* by having longer pectoral and ventral fins and base of caudal fin. *B. fucini* differs from *B. tyberinus* only by a longer mouth-

operculum distance, higher length of both the upper and lower lobe of caudal fin, a greater height of the first dorsal fin ossified ray, a major quantity of dots on scales and fins and different dominant colour of anal, dorsal, and ventral fins. *B. fucini* difffers from *B. plebejus* only by having fewer scales on, and rows above and under the lateral line, smaller minimum height and fork depth, greater maximum height, higher number of body spots, and different dominant colour of caudal fin.

Coloration

Body coloration is very similar to *B. tyberinus*, due to the irregular presence of dots on the back and flanks: dots are also found on dorsal, anal, and ventral fins; in this, however, the livery is more different from that of *B. plebejus*.

Sexual dimorphism

The same as the other species of Italian barbel and *B. samniticus* sp. nov. (see above).

Distribution and habitat

B. fucini inhabits basins of the southern Adriatic slope of the Italian Peninsula between the Fortore and Ofanto catchments and basins in the sothern Thyrrenian slope between the Liri-Garigliano basin in the north and Sele basin in the south (Figure 9.1); following Bianco (2003b) it was introduced in the

Mingardo and Bussento Rivers. Along the longitudinal profile *B. fucini* colonizes the intermediate and hilly stretches (barbel zone) in fish assemblage with *Sarmarutilus rubilio*, *Telestes muticellus*, *Squalius squalus, Alburnus albidus* (Costa, 1838) and only in the Volturno River with *Cobitis zanandreai* Cavicchioli, 1965.

9.4 Discussion

Molecular and morphological evidences provided in this study supported the distinction of new *Barbus* taxonomic units within the AC district of Southern Italy from the two already established Italian fluviolacustrine species (*B. tyberinus* and *B. plebejus),* as suggested by Zaccara et al. (2019a). Here we describe the new species *Barbus samniticus* sp. nov. as an endemic to the NAC district, distributed along the middle Adriatic slope of Italy between the Vomano River and the Biferno River (Figure 9.1). Additionally, we suggest the revalidation of *Barbus fucini* Costa, 1853 as endemic species to the SAC district, distributed along the Southern Tyrrhenian slope from Liri-Garigliano basin to Sele-Calore basin and along the Southern Adriatic slope from Fortore to Ofanto basins 8Figure 9.1).

B. fucini is considered in literature as a valid binomial nomenclature (Froese & Pauly, 2018; Fricke et al., 2020;

Roskov et al., 2020), even if it was reputed a junior synonym of *B. tyberinus* (Bianco, 1995, 2003b; Kottelat, 1997). The type locality of *B. fucini* is the Lake Fucino, an extinct lake that was situated in Abruzzo region and reached its maximum extension during the Pliocene when the lake was connected to the Tiber basin through the Salto River. However, water levels of the lake diminished, and it became an endorheic basin without any in-or out- flows until during Roman times (52 AD), an artificial channel was created that connected the lake to the Liri River (Wilkens, 1994). This connection could have allowed fish from the Liri basin to colonise the lake and as such barbel of the Fucino might correspond to the barbel sampled in the SAC area (clade II in the present work; SI2 in Zaccara et al. (2019a)).
The original description of *B. fucini* was made by Costa 1853 on different types without a designation of a holotype, while according to Kottelat (1997) two probable syntypes are present at the Museum National d'Histoire Naturelle of Paris (MNHN) and at the Naturhistorisches Museum of Wien (NMW).

Both morphological and genetic data suggest a closer evolutionary affinity of *B. samniticus* sp. nov. and *B. fucini* with *B. plebejus*. This similarity is highlighted for the first time in this work, as Southern Italian barbel have been always attributed to *B. tyberinus* so far as a result of morphological traits comparisons (i.e. the body colouring and the number of

scale along the lateral line) being used as the main discriminant trait that indeed fall in the range of *B. tyberinus* (Bianco, 1995). In constrast, here we found a closer relation of AC barbels with *B. plebejus* that is especially marked in the nuclear DNA. In fact, although at the mitochondrial *cytb* marker the four fluviolacustrine taxa are recorded as 4 distinct phylogenetic groups (*p-distances* between 1.7% and 2.2%) as also shown by Zaccara et al. (2019a), the nuclear DNA *locus* failed to separate AC species from *B. plebejus sensu stricto* (intragroup *p-distances* between 0.3% ± 0.3). Mito-nuclear discordance may arise from an incomplete lineage sorting that occurs frequently in recently radiated species as new alleles did not have time to fix within the differentiating population and ancestral haplotypes are retained (Galtier & Daubin, 2008). Alternatively, discordance between nuclear and mitochondrial markers may be a result of mitochondrial introgression (i.e. mitochondrial capture; Chan & Levin, 2005) that occurred after an ancient hybridization event, with this widely recognised to be an important factor in the speciation process (Nolte & Tautz, 2010) and common among cyprinids (Scribner et al., 2001; Freyhof et al., 2005).

The four fluviolacustrine barbel species occurring in Italy currently have adjacent geographic distributions. However it is presumable that in the past they have come into wide contact,

especially on the Adriatic side, thanks to the connections offered by the Paleo Po hydrographic network (Maselli et al., 2011), which periodically extended to the south and subsequently retracted, following the marine regression and transgression phenomena that occurred during the various glacial phases (Dias et al., 2014). Although the contact between populations inhabiting the Tyrrhenian and Adriatic slopes divided by the Apennine mountain chain (Figure 9.1) is more difficult, it occurred in the past, thanks to geomorphological evolution of the territory that have allowed events of river capture (Brancaccio et al., 1991; Alvarez, 1999).

9.5 Conclusions

B. fucini and *B. samniticus* sp. nov. are therefore added to the list of endemic species already known for the AC district, such as *Alburnus albidus* and *Cobitis zanandreai* (Bianco, 2014; Zaccara et al., 2019a). Southern Italy is poorly investigated with regard to its fish fauna, and it is most likely that other genus, as well as *Barbus*, can display separate evolutionary lineages than those in the more northern areas. This has been already suggested for the genus *Squalius* (Bianco & Recchia, 1983; Bianco, 2014), *Telestes* (Stefani et al., 2004; Zaccara et. al., 2007; Bianco, 2014) and *Lampetra* (De Cahsan et al., 2020). The present study and previous research (Zaccara et al., 2019a)

also highlight a mosaic pattern in the AC district of Southern Italy in which barbel populations inhabiting the northernmost (NAC) and the southernmost areas (SAC) showed a limited gene flow favoring their isolation and recent speciation. This would suggest the presence of a biogeographic boundary between SAC and NAC that can be related to the non-homogeneous paleogeographic history of these areas and whose presence should be investigated further by analyzing phylogeographic relations of other freshwater *taxa*.

The lack of in-depth knowledge on fish fauna and the high rate of endemism in Southern Italy highlights the need to implement management policies allowing the preservervation of the fish biodiversity of the Apulia-Campania district, which can be also seriously threatened by the introduction of alien species and translocations of fish fauna from other Italian basins (Bianco & Ketmaier, 2001; Lorenzoni et al., 2006b; Bianco, 2014). From the conservation point of view, *B. fucini* and *B. samniticus* sp. nov. should be considered protected species by the Directive 92/43 EC (Habitat Directive), as deriving by taxonomic split from *B. plebejus*, a species included in Annexes II/V.

Acknowledgments

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Fabian Herder and Wesel Serkan (Zoologisches Forschungsmuseum Alexander Koenig - Leibniz Institut für Biodiversität der Tiere, Bonn, Germany) and Sergio Gentili (Centre for the Scientific Museums of the University of Perugia) who kindly provided us with support and information about fish collections stored in their related Institutions. We also would like to thank Franco Recchia of the Abruzzo Region and Pierlisa Di Felice of "Sorgenti del Pescara" Regional Nature Reserve, who provided the fish sampling authorizations (permits number: 01082019DPD023/425 and 31102019/15974).

9.6 Supplementary material

9.6.1 *Supplementary figures*

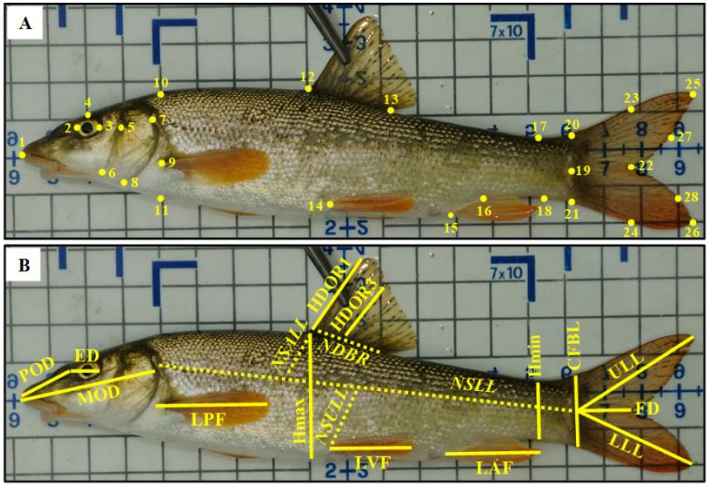

Figure S9.1 *(A) Position of the 28 landmarks used for body shape analysis: (1) anterior tip of snout, (2, 3) anterior and posterior end of the eye, (4) orthogonal projection on the dorsal profile of the eye centre, (5) lateral projection of the eye centre on the insertion of the operculum, (6) intersection of the operculum at the lateral profile, (7, 8) ventral and dorsal end of gills, (9) anterior insertion of pectoral fin, (10, 11) orthogonal projections on the dorsal and ventral profile of the anterior insertion of pectoral fin, (12,13) anterior and posterior insertion of dorsal fin, (14) insertion of pelvic fin, (15, 16) posterior and anterior insertion of anal fin, (17, 18) anterior attachment of dorsal and ventral membrane of caudal fin, (19) base of middle caudal rays, (20, 21) orthogonal projections on the dorsal and ventral profile of the base of middle caudal rays, (22) fork, (23, 24) orthogonal projections on the dorsal and ventral profile of fork, (25, 26) end of the upper and lower lobe of caudal fin, (27, 28) lateral projections of anterior attachment of dorsal and ventral membrane of caudal fin. (B)*

Fourteen morphometric (MOD, mouth-operculum distance; POD, pre-orbital distance; ED, eye diameter; HDOR1, height of the first dorsal fin ossified ray; HDOR3, height of the third dorsal fin ossified ray; Hmin, minimum height; Hmax, maximum height; LAF, length of anal fin; LPF, length of pectoral fin; LVF, length of ventral fin; CFBL, length of caudal fin base; FD, fork depth; LLL, length of the lower lobe of caudal fin; ULL, length of the upper lobe of caudal fin) and four meristic traits (NDBR, the number of dorsal fin branched rays; NSLL, the number of scales on the lateral line, and on rows above – NSALL - and under – NSULL - the lateral line) considered for morphological analyses.

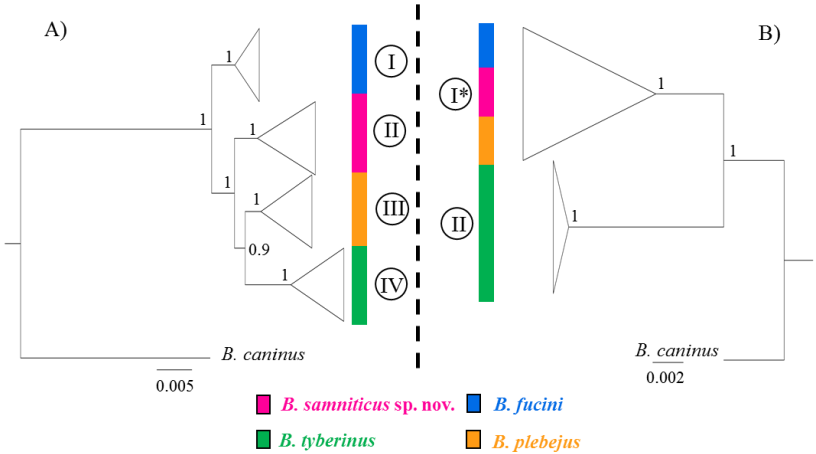

Figure S9.2 *Bayesian phylogenetic trees built on A) cytb mtDNA enlarged dataset (Table S2) and B) GH-2 nDNA dataset (Table S3). Posterior probability values are reported beside each node. Rectangles show species attribution according to the mtDNA while roman numbers indicate the clade identified supported by the phylogenetic reconstruction for each marker. Asterisk indicates Barbus plebejus complex in which B. samniticus sp. nov and B. fucini clustered at the nDNA.*

9.6.2 *Supplementary tables*

Table S9.1 *Sampling sites of the four Italian barbel species in each district (TL=Tuscany-Latium; PV=Padano-Venetian; NAC=Northern Apulia-Campania; SAC=Southern Apulia-Campania) along with geographic coordinates and sampling sizes used for morphological and genetic analyses (both at the mitochondrial cytb and nuclear GH-2 DNA loci).*

						Sampling size		
ID station	**Species**	**District**	**Basin**	**River**	**Geographic coordinates**	**$N_{morphology}$**	**N_{mtDNA}**	**N_{nDNA}**
1[a]	*B. tyberinus*	TL	Paglia	Montacchione	42°42'44.39"N 12°5'37.88"E	28	-	-
2[a]	*B. plebejus*	PV	Metauro	Bosso	43°31'3.14"N 12°33'17.89"E	35	-	-
3	*B. samniticus* sp. nov.	NAC	Vomano	Vomano	42°35'21.56"N 13°40'51.44"E	5	5	5
4			Aterno-Pescara	S.Terenziano	42°10′25.85″N 13°49′51.35″E	5	4	5
5[b]			Sangro	Sangro	42°05'29.76"N 14°34'75.82"E	5	12	12
6			Biferno	Biferno	41°43'21.41"N 14°43'26.94"E	5	4	5

Table S9.1 (Continued)

						Sampling size		
ID station	**Species**	**District**	**Basin**	**River**	**Geographic coordinates**	**$N_{morphology}$**	**N_{mtDNA}**	**N_{nDNA}**
7			Fortore	Tappino	41°33'13.20"N 14°52'33.92"E	5	4	5
8			Ofanto	Ofanto	41°07′39.23″N 15°54′62.24″E	5	5	3
9[c]	*B. fucini*	SAC	Liri-Garigliano	Liri	41°52'38.92"N 13°27'11.12"E	5	9	5
10			Volturno	Vandra	41°38′12.53″N 14°10′20.98″E	5	5	4
11			Sele	Calore	40°29'27.8"N 15°12'25.2"E	5	5	5

[a] Sampling sites of purebred populations of *B. plebejus* and *B. tyberinus* (Zaccara et al. 2020) used as reference for the morphological analyses; [b] sampling site of *B. samniticus* sp. nov. holotype and paratypes; [c] sampling site of *B. fucini* new non-type locality.

Table S9.2 *52 and 105 barbel cytb sequences produced in this study and retrieved from GenBank respectively, used to build the mitochondrial minimum spanning networks. ID station of the sequences produced in this study, GenBank accession number (GB), attributed species, references and haplotype assigned are reported.*

ID Station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Haplotype	Reference
4	PE763	MT454513			1		Bsam1	The Study
6	BF832	MT454517			1		Bsam2	The Study
6	BF837	MT454518			1		Bsam2	The Study
6	BF839	MT454519			1		Bsam2	The Study
6	BF841	MT454520			1		Bsam2	The Study
4	PE764	MT454514			1		Bsam3	The Study
4	PE765	MT454515			1		Bsam3	The Study
4	PE770	MT454516			1		Bsam3	The Study
3	VO657	MT454508			1		Bsam4	The Study
3	VO669	MT454509			1		Bsam5	The Study
3	VO661	MT454510			1		Bsam6	The Study
3	VO663	MT454511			1		Bsam7	The Study
3	VO672	MT454512			1		Bsam8	The Study
5	SA01	MT454521			1		Bsam1	The Study
5	SA02	MT454522			1		Bsam1	The Study
5	SA03	MT454523			1		Bsam1	The Study
5	SA04	MT454524			1		Bsam1	The Study

Table S9.2 (Continued)

ID Station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Haplotype	Reference
5	SA05	MT454525			1		Bsam1	The Study
5	SA06	MT454526			1		Bsam1	The Study
5	SA07	MT454527			1		Bsam1	The Study
5	SA08	MT454528			1		Bsam9	The Study
5	SA09	MT454529			1		Bsam1	The Study
5	SA10	MT454530			1		Bsam1	The Study
5	SA11	MT454531			1		Bsam10	The Study
5	SA12	MT454532			1		Bsam1	The Study
9	LI01	MT454552				1	Bfuc1	The Study
9	LI02	MT454553				1	Bfuc1	The Study
9	LI03	MT454554				1	Bfuc1	The Study
9	LI04	MT454555				1	Bfuc1	The Study
9	LI05	MT454556				1	Bfuc1	The Study
9	LI07	MT454557				1	Bfuc1	The Study
9	LI08	MT454558				1	Bfuc1	The Study
9	LI09	MT454559				1	Bfuc1	The Study
9	LI10	MT454560				1	Bfuc1	The Study
8	OF790	MT454536				1	Bfuc10	The Study
7	FR798	MT454538				1	Bfuc11	The Study

Table S9.2 (Continued)

ID Station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Haplotype	Reference
7	FR803	MT454539				1	Bfuc11	The Study
7	FR816	MT454540				1	Bfuc11	The Study
7	FR824	MT454541				1	Bfuc12	The Study
10	VL868	MT454544				1	Bfuc13	The Study
8	OF775	MT454533				1	Bfuc9	The Study
8	OF782	MT454534				1	Bfuc9	The Study
8	OF783	MT454535				1	Bfuc9	The Study
8	OF793	MT454537				1	Bfuc9	The Study
10	VL864	MT454542				1	Bfuc9	The Study
10	VL867	MT454543				1	Bfuc9	The Study
10	VL872	MT454545				1	Bfuc9	The Study
10	VL876	MT454546				1	Bfuc9	The Study
11	SE915	MT454548				1	Bfuc9	The Study
11	SE917	MT454549				1	Bfuc9	The Study
11	SE921	MT454550				1	Bfuc9	The Study
11	SE927	MT454551				1	Bfuc9	The Study
		MG495895				1	Bfuc2	Rossi et al., 2017
		MG495894				1	Bfuc2	Rossi et al., 2017
		MG495920				1	Bfuc2	Rossi et al., 2017

Table S9.2 (Continued)

ID Station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Haplotype	Reference
		MG495917				1	Bfuc2	Rossi et al., 2017
		MG495897				1	Bfuc3	Rossi et al., 2017
		MG495893				1	Bfuc4	Rossi et al., 2017
		MG495878				1	Bfuc5	Rossi et al., 2017
		MG495875				1	Bfuc6	Rossi et al., 2017
		MG495898				1	Bfuc7	Rossi et al., 2017
		MG495876				1	Bfuc8	Rossi et al., 2017
		MG495913				1	Bfuc9	Rossi et al., 2017
		MG495877				1	Bfuc9	Rossi et al., 2017
		MG495874				1	Bfuc9	Rossi et al., 2017
		MG495922				1	Bfuc9	Rossi et al., 2017
		MG495921				1	Bfuc9	Rossi et al., 2017
		MG495919				1	Bfuc9	Rossi et al., 2017
		MG495918				1	Bfuc9	Rossi et al., 2017
		MG495916				1	Bfuc9	Rossi et al., 2017
		MG495915				1	Bfuc9	Rossi et al., 2017
		MG495914				1	Bfuc9	Rossi et al., 2017
		MG495912				1	Bfuc9	Rossi et al., 2017
		MG495852		1			Btyb1	Rossi et al., 2017
		MG495850		1			Btyb2	Rossi et al., 2017

Table S9.2 (Continued)

ID Station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Haplotype	Reference
		MG495846		1			Btyb3	Rossi et al., 2017
		MG495845		1			Btyb4	Rossi et al., 2017
		MG495841		1			Btyb5	Rossi et al., 2017
		MG495840		1			Btyb6	Rossi et al., 2017
		MG495837		1			Btyb7	Rossi et al., 2017
		MG495835		1			Btyb8	Rossi et al., 2017
		MG495832		1			Btyb6	Rossi et al., 2017
		MG495826		1			Btyb9	Rossi et al., 2017
		MG495824		1			Btyb3	Rossi et al., 2017
		MG495823		1			Btyb6	Rossi et al., 2017
		MG495820		1			Btyb3	Rossi et al., 2017
		MG495819		1			Btyb3	Rossi et al., 2017
		MG495817		1			Btyb3	Rossi et al., 2017
		MG495816		1			Btyb10	Rossi et al., 2017
		MG495815		1			Btyb3	Rossi et al., 2017
		MG495814		1			Btyb3	Rossi et al., 2017
		MG495813		1			Btyb3	Rossi et al., 2017
		MG495812		1			Btyb3	Rossi et al., 2017
		MG495811		1			Btyb3	Rossi et al., 2017

Table S9.2 (Continued)

ID Station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Haplotype	Reference
		MG495808		1			Btyb11	Rossi et al., 2017
		MG495804		1			Btyb3	Rossi et al., 2017
		MG495851		1			Btyb12	Rossi et al., 2017
		MG495849		1			Btyb13	Rossi et al., 2017
		MG495830		1			Btyb12	Rossi et al., 2017
		MG495828		1			Btyb14	Rossi et al., 2017
		MG495796		1			Btyb15	Rossi et al., 2017
		MG495785		1			Btyb16	Rossi et al., 2017
		MG495810		1			Btyb17	Rossi et al., 2017
		MG495809		1			Btyb18	Rossi et al., 2017

Table S9.3 *96 and 25 GH-2 barbel alleles produced in this study and retrieved from GenBank respectively, used to build the nuclear minimum spanning network. ID station of the sequences produced in this study, GenBank accession number (GB), attributed species according to mtDNA, references and haplotype assigned are reported.*

ID station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Reference	Haplotype
3	VO657a	MT454561			2		The study	HBsam1
3	VO661a	MT454562			2		The study	HBsam1
3	VO663a	MT454563			2		The study	HBsam1
3	VO669a	MT454564			2		The study	HBsam1
3	VO672a	MT454565			2		The study	HBsam1
4	PE753a	MT454566			2		The study	HBsam1
4	PE763a	MT454567			2		The study	HBsam1
4	PE764a	MT454568			2		The study	HBsam1
4	PE765a	MT454569			2		The study	HBsam1
4	PE770a	MT454570			2		The study	HBsam1
6	BF832a	MT454571			2		The study	HBsam1
6	BF836a	MT454572			2		The study	HBsam1
6	BF837a	MT454573			1		The study	HBsam1
6	BF837b	MT454574			1		The study	HBsam2
6	BF839a	MT454575			2		The study	HBsam1
6	BF841a	MT454576			1		The study	HBsam1

Table S9.3 (Continued)

ID station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Reference	Haplotype
6	BF841b	MT454577			1		The study	HBsam3
5	SA01a	MT454578			2		The study	HBsam1
5	SA02a	MT454579			2		The study	HBsam1
5	SA03a	MT454580			2		The study	HBsam1
5	SA04a	MT454581			2		The study	HBsam1
5	SA05a	MT454582			2		The study	HBsam1
5	SA06a	MT454583			2		The study	HBsam1
5	SA07a	MT454584			2		The study	HBsam1
5	SA08a	MT454585			2		The study	HBsam1
5	SA09a	MT454586			2		The study	HBsam1
5	SA10a	MT454587			2		The study	HBsam1
5	SA11a	MT454588			2		The study	HBsam1
5	SA12a	MT454589			2		The study	HBsam1
8	OF782a	MT454590				1	The study	HBfuc1
8	OF782b	MT454591				1	The study	HBfuc2
8	OF783a	MT454592				1	The study	HBfuc3
8	OF783b	MT454593				1	The study	HBfuc2
8	OF793a	MT454594				1	The study	HBfuc4
8	OF793b	MT454595				1	The study	HBfuc2

Table S9.3 (Continued)

ID station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Reference	Haplotype
8	FR798a	MT454596				1	The study	HBfuc5
8	FR798b	MT454597				1	The study	HBfuc2
7	FR803a	MT454598				2	The study	HBfuc2
7	FR816a	MT454599				2	The study	HBfuc5
7	FR824a	MT454600				1	The study	HBfuc5
7	FR824b	MT454601				1	The study	HBfuc2
7	FR827a	MT454602				1	The study	HBfuc5
7	FR827b	MT454603				1	The study	HBfuc2
10	VL864a	MT454604				2	The study	HBfuc1
10	VL867a	MT454605				2	The study	HBfuc1
10	VL868a	MT454606				2	The study	HBfuc1
10	VL876a	MT454607				2	The study	HBfuc6
11	SE915a	MT454608				2	The study	HBfuc5
11	SE917a	MT454609				1	The study	HBfuc1
11	SE917b	MT454610				1	The study	HBfuc6
11	SE921a	MT454611				1	The study	HBfuc1
11	SE921b	MT454612				1	The study	HBfuc6
11	SE927a	MT454613				2	The study	HBfuc5
9	LI01a	MT454614				2	The study	HBfuc8

Table S9.3 (Continued)

ID station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Reference	Haplotype
		MT4546						HBfuc
9	LI02a	15				2	The study	8
		MT4546						HBfuc
9	LI03a	16				2	The study	8
		MT4546						HBfuc
9	LI04a	17				2	The study	8
		MT4546						HBfuc
9	LI09a	18				2	The study	8
		KF92361					Zaccara et al.,	
		9	1				2014	HBple2
		KF92362					Zaccara et al.,	
		0	1				2014	HBple3
		KF92362					Zaccara et al.,	HBsam
		1	1				2014	1
		KF92362					Zaccara et al.,	
		2	1				2014	HBlpe1
		KF92362					Zaccara et al.,	
		3	1				2014	HBple4
		KF92362					Zaccara et al.,	
		4	1				2014	HBple5
		KF92362					Zaccara et al.,	
		5	1				2014	HBple6
		KF92362					Zaccara et al.,	
		6	1				2014	HBple7
		KF92362					Zaccara et al.,	
		7	1				2014	HBple8
		KF92362					Zaccara et al.,	
		8	1				2014	HBple9
		KF92362					Zaccara et al.,	HBple1
		9	1				2014	0
		KF92363					Zaccara et al.,	HBple1
		0	1				2014	1
		KF92363					Zaccara et al.,	HBple1
		1	1				2014	2
		KF96349					Buonerba et al.,	
		3	1				2015	HBlpe1
		KF96349					Buonerba et al.,	HBsam
		4	1				2015	1

Table S9.3 (Continued)

ID station	Sample	GB	*B. plebejus*	*B. tyberinus*	*B. samniticus* sp. nov.	*B. fucini*	Reference	Haplotype
		KF963495	1				Buonerba et al., 2015	HBple14
		KF963496	1				Buonerba et al., 2015	HBple15
		KF963497	1				Buonerba et al., 2015	HBple16
		KF963498	1				Buonerba et al., 2015	HBple17
		KF963487		1			Buonerba et al., 2015	HBtyb2
		KF963488		1			Buonerba et al., 2015	HBtyb3
		KF963489		1			Buonerba et al., 2015	HBtyb4
		KF963490		1			Buonerba et al., 2015	HBtyb1
		KF963491		1			Buonerba et al., 2015	HBtyb5
		KF963492		1			Buonerba et al., 2015	HBtyb1

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CHAPTER X

10.1 General discussion and conclusions

Following its intentional introduction in rivers of Italy and western England for angling enhancement, *B. barbus* has successfully established self-sustained populations integrated within the receiving communities. While in western England, no other co-generic *Barbus* species are present, in Italy *B. barbus* have been introduced in rivers populated by endemic *B. tyberinus* and *B. plebejus*, potentially generating different impacts within the two invaded areas. *B. barbus* thus was used in this thesis as a model species to study the contribution of two fundamental mechanisms in driving the impacts of biological invasions: interspecific trophic interactions and introgressive hybridization. This have enabled a contribution enhancing the understanding of the complex mechanisms governing the ecological consequences deriving by biological invasions, which is fundamental for invasive species management while also providing valuable evolutionary information on how species adapt to changing distributions.

Furthermore, crypticism of *Barbus* genus in Italy have been addressed contributing to clarify the conservation status of this valuable fish genus in Italy that is threatened by the invasion of the exotic *B. barbus* as well as by habitat degradation.

10.1.1 *Ecological impacts of* B. barbus *deriving by interspecific interactions*

Data from different experimental approaches demonstrated competition between *B. barbus* and other co-occurring and functionally analogous fishes result in strong niche partitioning. Fish populations tend to specialise on different resources reducing their trophic niche widths in presence of competitors (Chapter III and IV). This mechanism did not vary with fish density. At the same time, intraspecific competition led to an increased generalization of the fish diet (Chapter IV). These outcomes give support to the niche variation hypothesis (Svanbäck & Bolnick, 2007b) and provide experimental evidence that species extinctions through outcompetition following fish introductions is less common (Ricciardi et al., 2013b; David et al., 2017; Jackson et al., 2017c). Contrastingly, a series of trophic outcomes allow species to coexist provided resources are available for the fish to differentiate their diets. These predictions are confirmed experimentally via studies conducted in introduced populations of *B. barbus* in England, where a strong resource partitioning was found between stocked barbel and native fish species in the wild (Gutmann Roberts & Britton, 2018a, 2018b).

Previous study have suggested the influence of energy-rich marine derived trophic subsidies by anglers may have favoured

the co-habitation of barbel within the receiving communities offering alternative resources over which the introduced barbel could have had specialised (Bašić et al., 2015; Gutmann Roberts et al., 2017; Gutman Roberts & Britton, 2018). The survey conducted in this study on the trophic ecology of *B. barbus* in 11 rivers in England inferred with stable isotopes suggests this hypothesis is unlikely. In fact, only some large bodied individuals specialised on this resource and the extent of occurrence of this varied considerably over space according also to angling pressure. Moreover, this was assumed from the distance of the isotopic signal of these barbel from the mean isotopic signature of macroinvertberates. However, the samples of macroinvertebrate analysed for each river was not representative of the entire macroinvertebrate community (i.e. drift and deep were not analysed). Therefore, there is a possibility that large barbel may have fed on different resources that have not been considered in the study (see Chapter VII). In addition, the composition of pelletized meal has changed in the last years, becoming increasingly less constituted of marine derived nutrients (e.g. Hall, 2015; Froehlich et al., 2018), potentially making these resources less trackable with carbon stable isotopes. Therefore, *B. barbus* integration into the receiving communities is more likely the result of the natural

resource partitioning as demonstrated in the experimental approaches.

Despite trophic segregation of niches which reduce interspecific competition between *B. barbus* and ecologically analogous fishes, the resulting trophic rearrangements detected in this study have the potential to alter the trophic structure of the receiving community also through indirect processes (David et al., 2017). The introduction of *B. barbus* in rivers where no co-generic species are present can thus result in cascading effects that need to be further addressed in future studies (Jackson et al., 2017). Benthivorous fishes in fact can alter ecosystem structure through bottom-up or middle-out (i.e. a mix of bottom-up and top-down) effects (Kaemingk et al., 2017), for instance re-suspending nutrients into the water column that facilitates algal blooms and increases water turbidity(bottom-up). This in turn may alter fish community structure favouring benthic feeders over visual feeders like in the case of one of the most introduced species worldwide, the common carp *Cyprinus carpio* (Wahl et al., 2011; Fischer et al., 2013). Although for barbel data on such impacts are limited, a zoogeomorphic hability associated with feeding (Pledger et al., 2014) and breeding activities (i.e. building of spawining nests; Gutmann Robert et al., 2020) has been demonstrated, which can have consequences on macroinvertebrate communities. Therefore,

although *B. barbus* may have a weaker competitive strength than other invasive species (e.g. ide *Leuciscus idus*, Chapter III), its interspecific interactions can result in considerable ecological consequences that should be accounted for when stocking intentionally this species.

10.1.2 *Ecological consequences of* B. barbus *introgressive hybridization*

The genetic and morphological results demonstrated as an introgression of *B. barbus* with the Italian native barbels may result in phenotypic and ecological displacement of native phenotypes (Chapter VI and VII). Regardless the relation with these outcomes to particular environmental conditions for which further study are required, it was highlighted that the process of *B. barbus* introgression is not only eroding the genetic composition of the Italian endemic species but has the potential to generate consistent ecological consequences that involve different traits of the fish.

Two main considerations arise from these results: from one side introgression can act as an important driver in invasion biology while acting on the other side as a strong adaptation force. Both aspects have already been attributed to the hybridization process (e.g. Seehausen, 2004; Hovick & Whitney, 2014) although functional traits consequences are less studied

(Rosenfield et al., 2004; Toscano et al., 2010) and thus this thesis has provided empirical evidence contributing to these important aspects of invasion and evolution.

Regarding the role in driving the invasion impacts, it was found that barbel hybrids can exploit a different trophic niche than the native parental species, foraging at a lower trophic position. This also reflected in a different body shape in hybrids. There is therefore the potential for altered trophic links in the receiving communities with impacts that might spread also on non-barbels members, although this hypothesis needs to be tested. This had been demonstrated for example for hybrids between the California tiger salamander (*Ambystoma californiense*) and the introduced barred tiger salamander (*Ambystoma tigrinum mavortium*) (Ryan et al., 2009).

Concerning the second aspect, the results showing life traits of hybrids are equal to or even enhanced (higher growth rate) compared to native parental species suggest that as the former may be able to persist or perform better in areas where the parental native species are selected against. The advantage of hybrids in degraded environments was experimentally demonstrated in stickleback *Gasterosteus aculeatus* (Best et al., 2017). Due to the degraded state of many Italian rivers, this suggests that hybridization may offer an adaptative potential for

barbel populations, resulting in controversial conservation perspectives (Allendorf et al., 2001)

10.1.3 *Cryptic diversity and conservation implications for Barbus species in Italy*

Two new differentiated fluvio-lacustrine barbel lineages that independently populate basins of southern Italy (Chapter VIII and IX) were recorded.

This, together with the widespread hybridization with *B. barbus*, have several conservation implications for Italian endemic barbel species:

-The actual natural distribution of *B. tyberinus* is more restricted than previously known. Some authors have attributed barbel from southern Italy to this species (Bianco et al., 1995; Lorenzoni et al., 2006; Kottelat & Freyhof, 2007) erroneously expanding its native range (see *B. tyberinus* native range IUCN, 2011). A revaluation of the extinction risk category of this species is thus strongly advised;

-all four Italian *Barbus* species (or taxonomic units) are in danger due to the spread of *B. barbus* and also due to translocation among different Italian rivers (Bianco & Ketmaier, 2001) and as such require adequate protection. This should include halting translocations or at least the use of

molecular tools in order to stock fish only within the distribution area of the local lineage to maintain genetic integrity and local adapted genotypes (Meraner et al., 2013);

-purebred populations of native barbels are likely to have remained only where the expansion of *B. barbus* has been limited by barriers. *B. barbus* presence has been detected also in rivers of southern Italy where the occurrence of future hybridization events with the newly described species cannot be excluded (Chapter VIII). Therefore, conservation plans involving freshwater fishes should reconcile the need to restore fluvial connectivity with the role that isolated headwaters have in offering refuge to native species (Milardi et al., 2018). Moreover, the role in conservation of hybrid populations should be clarified (Allendorf et al., 2001).

10.2 Future conservation actions

The lack of taxonomic, systematic and biogeogrpahic knowledge of freshwater fish species inhabiting different biogeographic district of Italy has led to the deliberate introduction and translocation of fish stocks, altering local community assemblages (Bianco, 2013; Nocita et al., 2017; Lorenzoni et al., 2019). The presence of cryptic taxa and the absence or inacessability of suitable tools for pratictioners to correctly detect species and their natural distribution, have also

substantially contributed. The use of traditional capture-based methods and morphological identification has resulted in inaccurate recognition of species and their distribution despite the importance of this for a correct and sustainable management of fish communities (Radinger et al., 2019). This has been the case for barbels, as it has been demonstrated in this thesis, but it has been potentially the case also for other fishes characterized by high phenotypic plasticity such as minnows (De Santis et al., 2020), Italian riffle daces (Ketmaier et al., 2004) and trouts (Splendiani et al., 2019).

A promising tool that is potentially able to overcome these difficulties is the environmental DNA (eDNA) (e.g. Hänfling et al., 2016; Valentini et al., 2016; Antognazza et al., 2020; Nardi et al., 2020). eDNA surveys are based on the analysis of water samples containing the DNA shed by organisms into the water column through their skin, mucus and other secretions (e.g. feceas, gametes). As such, they do not require the capture of the fish nor are affected by biased field attributions (Radinger et al., 2019). Initially applied to monitor rare taxa or in the early detection of invasive species (e.g. Rees et al., 2014), eDNA has been also proved to be a valid non-invasive and affordable tool for the study of entire freshwater communities in large river systems such as the Volga River (Leucadey et al., 2019) and the Rhône River (Pond et al., 2018).

eDNA apllication would be therefore really helpfull in Italian waters. The metabarconding approach (selection of a standard DNA region; e. g. Pompanon et al., 2011) can be applied and a suitable DNA database for Italian freshwaters fish species inhabiting rivers of different geographic district should be developed. In this way, it would be possible to better define fish community composition, species identity and distribution in cryptic biodiversity-rich rivers of Italy, contributing to:

- define areas (likely headwaters; Milardi et al., 2018), less impacted by IAS and thus requiring conservation;
- define species distribution within biogeographic districts to avoid further translocations;
- identify suitable reproductive stocks (if needed) to be used for restocking plans avoiding the mixing with translocated or alien strains (both purebred and hybrid).

CHAPTER XI

11.1 References (Chapter I, II, X)

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