TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Aquatische Systembiologie

Phenotypic and genetic differentiation of invasive gobies in the upper Danube River

Alexander Fabian Cerwenka

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzende(r):	Univ.-Prof. Dr.agr. Dr. rer.nat.habil. Arnulf Melzer
Prüfer der Dissertation:	1. Univ.-Prof. Dr.rer.nat. Jürgen P. Geist 2. apl. Prof. Dr.rer.nat. Dr.agr.habil. Roland Gerstmeier 3. Univ.-Prof. Dr.rer.nat. Gerhard Haszprunar (Ludwig-Maximilians-Universität München)

Die Dissertation wurde am 26.06.2014 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 23.10.2014 angenommen.

Contents

I List of Figures

II List of Tables

III Preface

This thesis intends to contribute to the basic understanding of evolutionary processes facilitating or hampering the success of invasive species. The thesis comprises eight chapters: Chapter 1 gives insights into threats to species, species-communities and to biodiversity caused by invasive species and emphasizes their potential for the study of evolution and speciation. Chapter 2 provides an overview of the main objectives of this study. Subsequently, patterns and processes of rapid population differentiation are analyzed: Chapter 3 deals with phenotypic changes of two invasive goby species in the upper Danube River and chapter 4 examines the differential development of the genetic constitution of the two species using the same specimens. Chapter 5 highlights the first case of hybridization between two invasive goby species in the River Rhine. Chapter 6 reports the first record of *Babka gymnotrachelus*, a fifth invasive goby species in Germany. Chapters 3 – 6 are based on published peer reviewed research papers. Chapter 7 closes the thesis by presenting a general discussion of the impacts of translocation on the phenotypic and genotypic constitution of non-native organisms. It highlights the rapid differentiation in invasive species and the chances offered by invasions for evolutionary biology research.

IV Summary

Invasive organisms are a major risk to global biodiversity. Nevertheless, they provide highly dynamic evolutionary biology model-systems under natural conditions and in real-time. Several factors affect evolutionary processes simultaneously during invasion and their complex interactions make simplified predictions difficult. Those factors are e.g. phylogenetic constraints (heterogeneous origin of individuals), genetic variation represented in the founder population (the number and genomic variability of founders), and the number of inoculation events. Despite of the multiplicity of factors, coherent evolutionary patterns can be studied, when simultaneous and synoptic invasions of closely related species with different life history traits and invasion characteristics are studied comparatively. Such comparisons are powerful tools to reveal factors that determine the fate and shape of an invasion.

In this study, differentiation of two invasive goby species was analyzed in the upper Danube River, i.e. *Ponticola kessleri* (Günther, 1861) and *Neogobius melanostomus* (Pallas, 1814) (Teleostei, Perciformes, Gobiidae). Potential effects of geographic, ecological and species-specific parameters were investigated using geometric morphometric methods and amplified-length-polymorphisms (AFLPs). To identify native source populations, mitochondrial DNA-haplotype analyses were used in *N. melanostomus*. As the distinction of invasive goby species and their hybrids is difficult using identification keys only, species determination was verified and hybridization was surveyed with AFLPs. The arrival of new invasive species can take place at any time and could ultimately result in additional complexity of species interactions, thus the investigation area was thoroughly screened for the occurrence of additional invasive gobiid species.

The comparative approach revealed that *N. melanostomus* was significantly differentiated on a local, fine-scaled level and showed more pronounced genetic population structure than *P. kessleri*. Unexpectedly, analyses revealed a trisection of the upper Danube River in *N. melanostomus*: The populations of the uppermost and the lowermost margins of the investigation area were more similar to each other than to a large intermediate subpopulation. Genomic differentiation of *N. melanostomus* not only correlated with spatial factors, but also with a nutrition related variable (i.e. white muscle δ^{15}N stable isotope signature), pointing towards local genomic adaptation. On the other hand, habitat quality correlated only with morphometric but not with genomic differentiation in *N. melanostomus*, making phenotypic plasticity probable. Mitochondrial analyses indicated a common origin from the Black Sea region of *N. melanostomus* and thus differentiation is not related to different source-populations. In strong contrast, the comparatively less successful invader, *P. kessleri*, neither exhibited significant phenotypic nor genomic differentiation. Analyses of aberrant gobiid specimens revealed the first record of hybridization between two invasive species on a global scale, i.e. between *N. melanostomus* and *Neogobius fluviatilis* (Pallas, 1814). A thorough morphological investigation of more than 1,000 goby specimens led to the first record of *Babka gymnotrachelus* (Kessler, 1857) in Germany, i.e. identified a novel component to the evolving ecosystem complexity in the upper Danube River and falsified a first record of this species based on misidentified *N. fluviatilis* specimens.

In invasive gobies of the upper Danube River, invasion success is manifested by a combination of life-history characteristics, as e.g. phenotypic plasticity and heritable components, as genomic adaptation to alternative trophic niches and the intimate interaction of both. Speed of differentiation is thereby crucially influenced by intrinsic factors where generalistic, plastic and flexible species characteristics are an important presupposition. Further, extrinsic settings as geographical factors and barriers to gene flow are more critical to differentiation than ecological variables and are crucial for local dissimilar invasion success, as shown in *N. melanostomus*. Lastly, hybridization and the establishment of further non-native species are additional pressures which influence rates of evolution in invasive organisms. Particularly, evidence for rapid population structuring found for the first time in this thesis, is an important contribution in the still young discipline of invasion biology.

V Zusammenfassung

Generell stellen invasive Organismen weltweit eine Bedrohung der natürlich vorkommenden Biodiversität dar. Trotz ihrer negativen Eigenschaften sind sie jedoch auch einzigartige Modellsysteme, um biologische Fragestellungen zu testen und das in Echtzeit und unter natürlichen Bedingungen. Insgesamt sind Vorhersagen zum Erfolg und den Folgen von Invasionen schwer zu treffen, da viele unterschiedliche Faktoren gleichzeitig wirken. Die Herkunft der Organismen, ihre genetische Variabilität, aber auch die Häufigkeit mit der eine Art eingeführt wird, sind nur wenige Beispiele für solche Einflüsse. Betrachtet man jedoch mehrere simultan ablaufende Invasionen im selben Ökosystem, können einzelne Faktoren genauer untersucht werden. Gerade nah verwandte Arten mit unterschiedlichen “life-history“ Merkmalen können wichtige Erkenntnisse für der Invasionsbiologie liefern.

In dieser Arbeit wurden zwei Grundelarten analysiert, die in der oberen Donau invasiv sind: *Ponticola kessleri* (Günther, 1861) und *Neogobius melanostomus* (Pallas, 1816) (Teleostei, Perciformes, Gobiidae). Morphometrische und genetischen Methoden (Amplified-Length-Polymorphisms (AFLPs)) wurden benutzt, um Differenzierungsprozesse invasiver Populationen der beiden Grundelarten zu untersuchen und Einflüsse geographischer, ökologischer und artspezifischer Faktoren zu erforschen. Die Herkunft invasiver Arten kann sich entscheidend auf ihren Invasionserfolg auswirken. Bei *N. melanostomus* wurde sie daher mittels mitochondrieller DNA Analysen eingegrenzt. Grundeln generell und Hybride zwischen Grundelarten sind morphologisch schwer zu unterscheiden. Die Artzugehörigkeit aller in Deutschland invasiven Grundeln wurde daher mittels AFLPs überprüft. Da weitere invasive Organismen jederzeit in ein Ökosystem eingeführt werden können und dieses verändern, wurde das gesamte Untersuchungsgebiet eingehend elektrisch befischt und auf weitere Grundelarten hin kontrolliert.

Es zeigte sich, dass *N. melanostomus* im kleinräumigen Maßstab stärker differenziert war als *P. kessleri*. Auch die Populationsstruktur von *N. melanostomus* war in der oberen Donau stärker ausgeprägt und sowohl morphometrisch, als auch genetisch dreigeteilt: Grundeln an den oberen und unteren Rändern des Untersuchungsgebietes unterschieden sich zueinander weniger stark, als zu Grundeln der dazwischenliegenden Populationen. Die Ernährung hatte ebenfalls einen Einfluss auf die Populationsdifferenzierung von *N. melanostomus*. Die Isotopensignatur für Stickstoff korrelierte mit der Verteilung eines AFLP-Fragments und deutet auf genomische Adaptation hin. Dahingegen wirkte sich das von *N. melanostomus*

bewohnte Habitat nur auf den Körperbau der Grundeln aus, nicht aber auf ihre genetische Variabilität und weist auf phänotypische Plastizität dieser Art hin. Die genetische Vermischung verschleppter Individuen unterschiedlicher Herkunft konnte als Grund für eine Differenzierung von *N. melanostomus* ausgeschlossen werden. Genetisch stammen die invasiven Populationen der oberen Donau von nativen Populationen im Schwarzen Meer ab. Insgesamt war *P. kessleri* die weniger erfolgreiche invasive Grundelart und im Gegensatz zu *N. melanostomus* weder phänotypisch, noch genetisch signifikant differenziert. Die genetische Untersuchungen morphologisch nicht zuordenbarer Grundelindividuen führten weltweit zum erstmaligen Nachweis einer Hybridisierung zweier invasiver Arten: *Neogobius melanostomus* und *Neogobius fluviatilis* (Pallas, 1814). Zuletzt erbrachte die genaue Betrachtung von über 1000 Grundelindividuen den Erstnachweis der Art *Babka gymnotrachelus* (Kessler, 1857) in Deutschland.

Die Gründe für den Erfolg invasiver Grundeln in der oberen Donau sind sehr vielseitig. Verschiedene “life-history“ Merkmale, wie phänotypische Plastizität, aber auch erbliche Gründe, wie die genomische Adaptation an alternative Nahrungsnischen und die Kombination aus beiden Faktoren spielt eine entscheidende Rolle und führte zur Populationsdifferenzierung. Die Geschwindigkeit ist dabei vor allem von intrinsischen Faktoren beeinflusst, wobei generalistische, plastische und flexible Artmerkmale ausschlaggebend sind. Jedoch wirken auch extrinsische Einflüsse. So kann eine Unterbrechung des Genflusses zwischen einzelnen invasiven Populationen die Differenzierungsrate beeinflussen und stark verändern, wie diese Arbeit bei *N. melanostomus* zeigte. Schließlich ändern auch Hybridisierungsereignisse und neue, zusätzliche nicht-native Organismen die Geschwindigkeit der Evolution invasiver Arten. Die vorliegende Arbeit konnte erstmals die schnelle Bildung von Populationsstruktur invasiver Organismen zeigen und liefert damit einen wichtigen Beitrag in der noch jungen Wissenschaft der Invasionsbiologie.

1 Faces of aquatic invasions

Freshwater biodiversity is threatened because of multiple reasons (e.g. Death & Winterbourn, 1995; Ricciardi & Rasmussen, 1999; Gleick, 2003; Dudgeon et al., 2006; Geist, 2011). Most threats are mediated by human activities like pollution and over-exploitation (Sala et al., 2000; Dudgeon et al., 2006; Strayer & Dudgeon, 2010). Frequently, the decline of species in aquatic ecosystems is much greater than in terrestrial ones (e.g. Sala et al., 2000; Vörösmarty et al., 2010). Herein, invasive neozoic species often are one of the major threat to global biodiversity (Dudgeon et al., 2006; Keller et al., 2011). Non-native species can alter entire ecosystems and endanger or eradicate native species and communities (e.g. Sexton et al., 2002; Smith & Bernatchez, 2007) and lead to a homogenization of flora and fauna (Mooney & Cleland, 2001; Petit, 2004). This applies particularly to aquatic ecosystems (Moyle & Light, 1996).

Success of non-native species in novel environments may have several different reasons and numerous hypotheses were designed to explain and predict invasions. Examples are: the enemy release hypotheses, proposing an advantage of invasive species because of an absence of their native enemies (e.g. Colautti et al., 2004), the hypothesis of novel weapons, assuming an advantage of invasive species because of traits that are unknown to native species (e.g. Callaway & Aschehoug, 2000) or the biotic resistance hypothesis (Levine et al., 2004) predicting ecosystems of high biodiversities to be more resistant against invaders.

Besides threats to biodiversity, invasions represent unique natural model-systems for scientific research (Kolar & Lodge, 2002; Strayer et al., 2006). Responses and dynamics of ecosystems, native species and species communities after the introduction of a non-native species have been in the focus of interest (e.g. Therriault et al., 2005; Stepien & Tumeo, 2006; Benkman et al., 2008; Elliott & zu Ermgassen, 2008). Nevertheless, the rapid changes and shifts of alien species characteristics offer a highly informative “natural experiment setup” for evolutionary research. In contrast to most natural range expansions (e.g. after glaciations), evolution can be studied in real-time in invasive species (Kolar & Lodge, 2002; Strayer et al., 2006), representing highly dynamic model-systems.

Non-native species have to cope with several challenges before becoming an invasive species (Sakai et al., 2001; Kolbe et al., 2004; Prentis et al., 2008). In a first step the uptake and the subsequent transportation are an obstacle for most species, followed by the release (the introduction) and establishment into the new ecosystem (Colautti et al., 2004). Only a small part of all introduced non-native species will spread and become invasive (e.g. Williamson & Fitter, 1996; Richardson et al., 2000; Kolar & Lodge, 2002; Jeschke & Strayer, 2005). The ability of becoming a successful invasive species is influenced by several factors, e.g. a broad environmental tolerance, variability of life history and phenotypic traits, and the genetic constitution of the founder individuals (e.g. Tsutsui et al., 2000; Lee, 2002; Sexton et al., 2002; Roman & Darling, 2007; Fig. 1.1). Due to the mostly small population size of initial colonizers and the correlated loss of genetic variation e.g. through genetic drift and inbreeding effects, invasive founder populations are generally thought to be genetically less diverse than their native ancestors. These potential population bottlenecks of introduced species contrast the success of invasive species and has been called the “genetic paradox” (Allendorf & Lundquist, 2003). Shortly after introduction, i.e. during the so called lag-phase, few or no changes in invasive species abundance are observable but nevertheless fast adaptation to new selective forces may act (Bossdorf et al., 2005). In general and particularly on a genetic perspective, the original genetic constitution (Tsutsui & Case, 2001; Lee, 2002; Allendorf & Lundquist, 2003; Roman & Darling, 2007), population genetic effects, as genetic drift or selection (e.g. DeWalt & Hamrick, 2004; Suarez & Tsutsui, 2008) but also gene flow, introgression and hybridization (Ellstrand & Schierenbeck, 2000; Nolte et al., 2005) are thought to be important factors enhancing invasion success. In contrast to this scenario, the genetic diversity of many invasive populations can exceed the genetic diversity of their ancestral native populations (Allendorf & Lundquist, 2003; Dlugosch & Parker, 2008) and demographic bottlenecks can even have a positive effect on founder populations by erasing deleterious alleles (Roman & Darling, 2007).

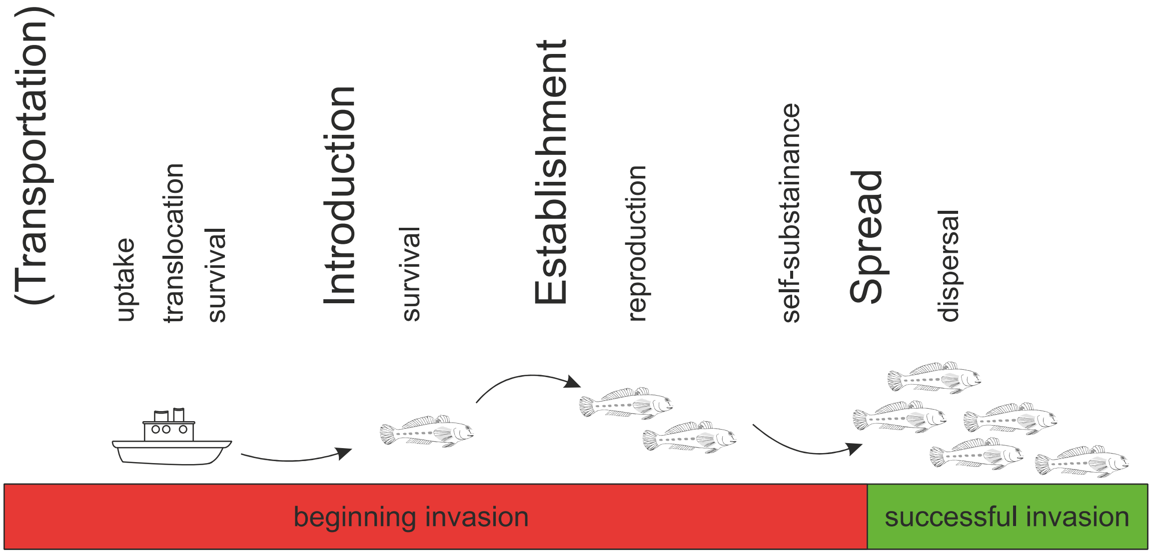

Fig. 1.1: Phases of an invasion and influences non-native species have to cope with.

Speciation is a central topic in evolutionary biology (Hendry et al., 2002). Invasive organism may enable the study of evolutionary processes proceeding speciation during contemporary time scales (Mooney & Cleland, 2001), including rapid changes of their phenotype (particularly life history traits) and their genotype. In non-native species such a rapid differentiation may start directly after being translocated if they were introduced into novel selective regimes. Introgression and hybridization may result of an admixture of differentiated lineages from different origins leading to increased standing genetic variation in the invasive population. In addition, fast adaptive diversification enhanced by phenotypic plasticity can affect rates of contemporary evolutionary change which theoretically may lead to speciation. However, it is difficult to study if the speciation process is already terminated (Grosholz, 2002; Lambrinos, 2004; Bossdorf et al., 2005). Phenotypic and genotypic changes of invasive species therefore may provide ideal research subjects to understand the initial phases of the speciation process.

1.1 Body shape variation of invasive populations and the potential role for phenotypic plasticity

Body shape is often used to investigate phenotypic changes during local adaptation of invasive species (e.g. Huey et al., 2000; Bossdorf et al., 2005; Nolte et al., 2005; Collyer et al., 2007; Fox, Vila-Gispert & Copp, 2007), as it may change shortly after species-introduction. New or altered environmental and ecological forces may fuel this process, but factors contributing to variation have remained understudied (Langerhans et al., 2007; Collin & Fumagalli, 2011). Geometric morphometric methods are especially suitable to investigate phenotypic variation of invaders, and they are sensitive enough to detect even subtle adaptive shape differences (Lawing & Polly, 2010; Klingenberg, 2011; Firmat et al., 2012). Evolution is traditionally defined as a change in allele frequencies and thus the genetic constitution is the presupposition for phenotypic variability and heritable selection, amongst others (Ghalambor et al., 2007). Recently, phenotypic variation and subsequent selection were identified to be important in evolution and adaptation even if they are not induced genetically (e.g. Langerhans & DeWitt, 2004; Walker, 2010). For example, initially non-heritable body shape changes, i.e. phenotypic plasticity, can be caused by environmental parameters. In a second step, the alternative body-shape phenotype (see below) can become subjected to altered selective regimes. These regimes again can trigger heritable shifts of allele frequencies, i.e. the evolution of genetic responses in the population (West-Eberhard, 2005).

Phenotypic plasticity is defined as non-heritable phenotypic variability that is correlated with different environmental conditions (Agrawal, 2001; Fitzpatrick, 2012). It may increase the ability of adapting of invasive populations to new environments (Fig. 1.2), as it provides a mechanism to cope with novel possibly radically different environmental conditions (Via et al., 1995; Yeh & Price, 2004). Phenotypic plasticity may influence the genetic constitution especially of invasive populations because it may accelerate rates of local adaptation or acclimatization (Sexton et al., 2002; West-Eberhard, 2005). However, high levels of phenotypic variability are not necessarily essential for fast differentiation rates, since even under low levels of plasticity fast ecological adaptation may occur (Chevin et al., 2010), but undoubtedly phenotypic variability in new environments will not hamper invasion success (Pigliucci, 2005). To conclude, phenotypic plasticity may theoretically contribute substantially to invasiveness (Williams et al., 1995) but yet very few empirical studies have investigated patterns of phenotypic plasticity and patterns of heritable genetic variance during invasion process (Sexton et al., 2002).

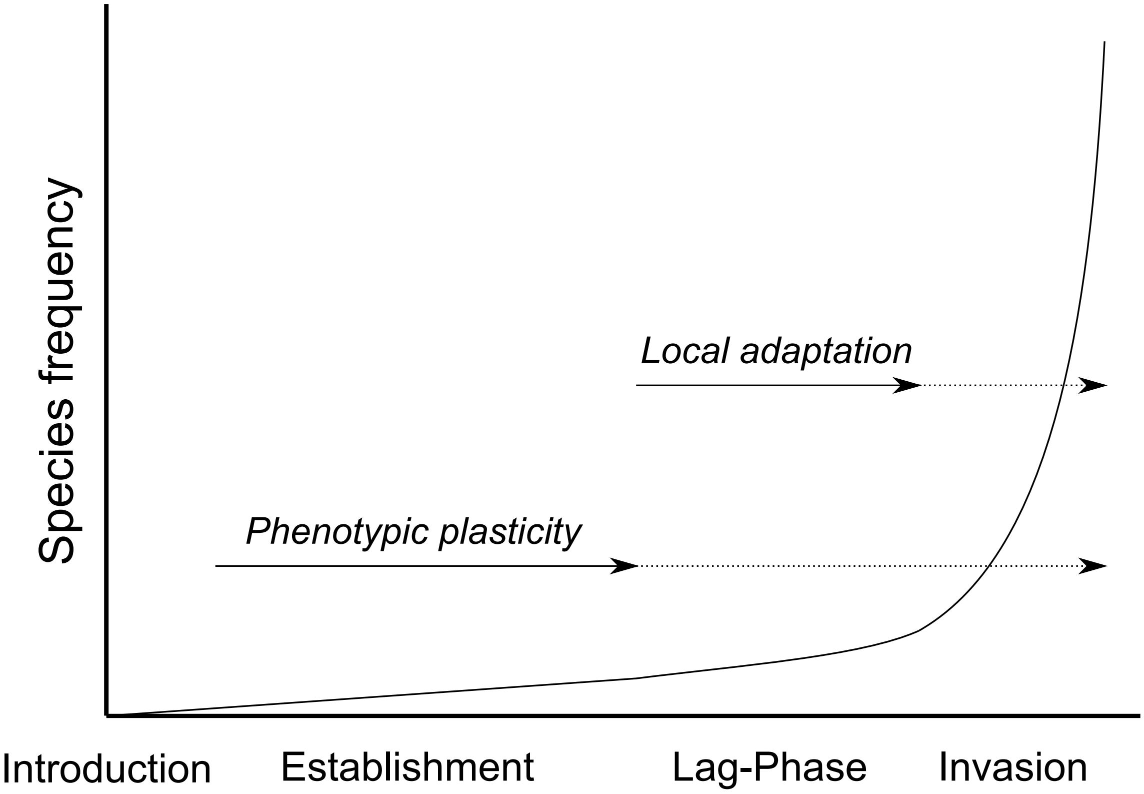

Fig. 1.2: Species frequency during different invasion phases. Phenotypic plasticity primarily is important during the establishment and may facilitate and contribute to local adaptation during the Lag-phase. Figure modified from Sexton et al. (2002).

1.2 Rapid genetic differentiation and diversifying evolution during invasions

Non-native species offer the unique possibility to investigate evolution (differentiation and diversification) under contemporary time-scales (Hendry et al., 2000; Reznick & Ghalambor, 2001). During invasions, the genetic variation often changes (e.g. Kolbe et al., 2004) and evolutionary processes seem to be strong determinants of invasion success (Sakai et al., 2001). In addition, population genetic effects act despite of different propagule pressures, genetic bottleneck-dimensions or levels of admixture of founder populations (Lambrinos, 2004; Björklund & Almqvist, 2010). These common invasion-specific effects influence rates of differentiation (Colautti et al., 2004; Prentis et al., 2008) and discriminate recent invasions and palaeo-evolutionary processes in the past (Gillson et al., 2008).

To become an invasive species, non-native organisms have to spread to adjoining areas after establishment, by definition (Keller et al., 2011). A key factor seems to be the propagule pressure (Parker et al., 2001; Colautti et al., 2004; Lockwood et al., 2005): It is composed by the number of specimens being introduced (propagule size) and the number of introduction events (propagule number). The genetic variability and the genetic diversity of non-native populations thus are related to the propagule pressure and might differ between non-native populations (Lockwood et al., 2005). Another important influence potentially determining the success of non-native species are genetic bottlenecks, i.e. a strong reduction of the population size and of the genetic diversity, in most of the cases (Tsutsui et al., 2000; Allendorf & Lundquist, 2003; Roman & Darling, 2007; Dlugosch & Parker, 2008). The successful colonization despite of a reduced genetic diversity is called the genetic paradox (Roman & Darling, 2007). A reduction of the population size might contribute to deleterious inbreeding effects (inbreeding depression) which might limit survival and population growth (Keller & Waller, 2002). In contrast, the genetic variability of non-native populations could even be increased, e.g. through an admixture of different source populations. Following Verhoeven et al. (2011), this could boost levels of local adaptation and thus change rates of evolution (Colautti et al., 2004; Stepien & Tumeo, 2006; Prentis et al., 2008).

In invasions, (adaptive) evolution starts directly after the introduction of a non-native species (Lee, 2002; Dlugosch & Parker, 2008) and may influence success (Prentis et al., 2008). Effects of rapid evolutionary changes may already accumulate during the lag-phase (Ellstrand & Schierenbeck, 2000). During the subsequent phase of spreading so called allele-surfing effects (Klopfstein et al., 2006) could influence the genetic constitution and increase rates of evolution at leading edge populations (Price & Sol, 2008). Surfing is defined as a rapid increase of alleles with previously low frequencies (Klopfstein et al., 2006; Excoffier & Ray, 2008) and seems to be common and could promote population expansion (Excoffier & Ray, 2008).

Another factor potentially influencing invasiveness is hybridization (Lee, 2002). Hybridization is increasingly being recognized as an important evolutionary force (Barton, 2001), which affects interspecific gene flow and rates of differentiation and diversification (Seehausen, 2004; Hayden et al., 2010; Bezault et al., 2011). Nevertheless, its global importance for adaptation, speciation and evolution is still unclear (Petit et al., 2003; Hayden et al., 2010). Post-hybridization selection may favor newly generated genotypes (Ellstrand & Schierenbeck, 2000; Barton, 2001). Herein, the term “hybridization” is used as defined by Mallet (2005) and is restricted to inbreeding of two genetically distinguishable groups or taxa, and not to a genetic admixture of individuals from different (source) populations. In non-native regions, hybridization may be common and widespread as geographical boundaries may have been omitted (O’Hanlon et al., 1999; Seehausen, 2004). Following Petit (2004) hybridization frequency is underestimated frequently, although inbreeding between non-native and native species is reported in several studies (e.g. Petit et al., 2003; Petit, 2004; Nolte et al., 2005). In contrast, hybridization between several invasive species is not known, yet. Anthropogenic altered rivers may offer ecosystems where hybrids between invaders may become especially successful (Arnold, 1997).

In summary, contemporary evolution of non-native organisms is influenced by several intrinsic factors: propagule pressure (Lambrinos, 2004; Björklund & Almqvist, 2010), inbreeding (Nei et al., 1975; Young & Seykora, 1996; Tsutsui et al., 2000), migration and dispersal abilities (Sakai et al., 2001; Phillips et al., 2006), phenotypic plasticity (Parker et al., 2003) and genetic constitution (Tsutsui et al., 2000; Sakai et al., 2001; Lee, 2002; Roman & Darling, 2007; Prentis et al., 2008). In addition, extrinsic factors may influence gene-flow, e.g. natural or artificial barriers. This may support the generation of partially isolated evolutionary units and non-native organisms offer the possibility to decipher rates of (adaptive) evolution (Petit, 2004; Huey et al., 2000).

1.3 Invasion of the Danube River

For analyzes of the invasion process the selection of the study-system, the study-organism and the time window is essential (Sanders et al., 2003; Strayer et al., 2006). Comparisons of (related) species and populations with potentially different invasion histories offer the possibility to analyze single factors contributing to their success. The linear, one-dimensional topology of riverine ecosystems is ideal to study contemporary evolution in invasive species. It allows the investigation of local differentiation along a simple geographical gradient. The Danube River is the second largest European River and one of the most important European shipping channels. It has been invaded by several non-native species and it has become one of the main spreading corridors of Ponto-Caspian organisms (Corkum et al., 2004; Kovac & Siryova, 2005; Molnár, 2006; Stepien & Tumeo, 2006; van Beek, 2006; Vyskočilová et al., 2007). Ponto-Caspian gobies (Teleostei: Gobiidae) invaded not only the Danube River (Ahnelt et al., 1998; Simonovic, 1999; Stranai & Andreji, 2004; Jurajda et al., 2005; Harka & Bíro, 2007) but also other European freshwater systems (Charlebois et al., 1997; Simonović et al., 2001; Sapota, 2004; van Beek, 2006; Sokołowska & Fey, 2011) and the North American Great Lakes system (Jude et al., 1992; Charlebois et al., 1997; Ricciardi & MacIsaac, 2000; Gutowsky & Fox, 2011; Lynch & Mensinger, 2012). Non-native gobies do not occur continuously throughout the invaded Danubian freshwater system, making a stepping stone spreading mechanism most likely (presumably supported by ballast water of transportation vessels; Wiesner, 2005). At the beginning of this study four invasive benthophiline gobies were reported in Germany, three of which occurred in the upper Danube River. *Proterorhinus semilunaris* (Heckel, 1837) was the first species which was recorded for the first time about 100 years ago (Dönni & Freyhof, 2002). In 1999 *Neogobius melanostomus* (Pallas, 1814) (Fig. 1.3) was found close to Vienna (Corkum et al., 2004) and in 2004 it had established populations in the German section of the upper Danube River (Paintner & Seifert, 2006) and the Rivers Main and Rhine (Kottelat & Freyhof, 2007). *Ponticola kessleri* (Günther, 1861) (Fig. 1.4) was first detected in 1999 in the German section of the Danube River close to the city of Straubing (Seifert & Hartmann, 2000). The species *Neogobius fluviatilis* (Pallas, 1814) is present in the River Rhine, since the year 2008 (Borcherding et al., 2011a). *Babka gymnotrachelus* (Kessler, 1857), a further non-native gobiid species was first recorded in the River Rhine in 2010 (Borcherding et al., 2011b). However, the species identification was not verified using molecular methods.

Fig. 1.3: *Neogobius melanostomus* specimen (tissue collection number: ZSM 40654, BayFi 10910), photographed directly after collection (Photo: A. Cerwenka).

Migration rates and home ranges are thought to be small in adult *N. melanostomus* and indicated between 500 m and four kilometers per year (Ray & Corkum, 2001; Bronnenhuber et al., 2011; Gutowsky et al., 2011; Gutowsky & Fox, 2011; Brownscombe et al., 2012; Kornis et al., 2012). However, Kornis et al. (2012), Bronnenhuber et al. (2011) and Brandner et al. (2013a) describe this species to migrate up to 17 river-kilometers per year in upstream direction. Non-native goby populations are likely characterized by limited gene-flow and it is assumed that populations at single river stretches represent single “evolving units” (Hewitt, 2000) on which different selective forces may act. However, downstream drift, anthropogenic transportation and migration may contribute to an admixture of most probably disconnected populations (Wiesner, 2005; Janáč et al., 2013).

Fig. 1.4: *Ponticola kessleri* specimen (tissue collection number: ZSM 40640, BayFi 10891), photographed directly after collection (Photo: A. Cerwenka).

Knowledge on the origins of non-native populations and their invasion history are important. This applies especially since the genetic diversity of invasive populations can be different and can even exceed the autochthonous one (Dlugosch & Parker, 2008). Genetic variation in non-native populations is influenced by multiple factors, as e.g. the number of inoculation events, number of founding individuals, origin of founder populations, genetic drift and impacts of new selective regimes (Rejmanek & Richardson, 1996; Kolbe et al., 2004; Bossdorf et al., 2005; Dlugosch & Parker, 2008; Prentis et al., 2008). In *N. melanostomus*, an increased genetic diversity is frequently found in non-native populations (Stepien et al., 2005). Brown and Stepien (2008) showed that populations of *N. melanostomus* in the Slovakian section of the Danube River (Serbia) most likely originated from native populations in the region of Odessa, based on analyses of seven microsatellite markers. The origin of invasive populations of *N. melanostomus* in the upper Danube River is still unknown.

The simultaneous study of two non-native (goby) species offers the ideal possibility for an integral study of an invasion process. *N. melanostomus* and *P. kessleri* are closely related, have several common life history traits and a comparable invasion history. Nevertheless both species are characterized by species-specific traits making comparisons promising and particularly suited. In addition, fine-scaled local differentiation may act differentially at geographically proximate areas and rapid changes may account for the success of invasive species (e.g. Lee, 2002; Murphy & Schaffelke, 2003; Fleischer et al., 2008) highlighting the investigation area of the upper Danube River. As very few studies have assessed effects of spatial and environmental factors on population differentiation directly after the very short time slot of first introduction (Sakai et al., 2001; Lee, 2002; Kolbe et al., 2004) this system is optimal for the study on invasional and evolutionary research.

2 Objectives of the study

Specimens of two closely related goby species currently invading the upper Danube River, *N. melanostomus* and *P. kessleri*, were used to investigate basic aspects of evolution and invasion biology. Phenotypic and local genetic differentiation was analyzed along a short river section for both species. It was hypothesized that intraspecific differentiation developed at small geographic scales already shortly after first inoculation and that population differentiation is species-specifically different, i.e., *Ponticola kessleri* being characterized by a comparatively weak population structure than the presumably rapidly adapting *N. melanostomus*, which had invaded the investigated area about five years later. Further, contemporary population structure was tested for correlation with patterns of spatial and local differentiation, i.e. with the locality of sampling location, barriers to gene flow, and with environmental, ecological and individually varying parameters. Further, potential population admixture of primarily diverged lineages from a metapopulation in the Black Sea drainage was assessed. In addition, interspecific hybridization was assumed to have taken place between several invasive goby species and the entire area of the upper Danube River was screened for further non-native gobiid species.

3 Phenotypic differentiation of Ponto-Caspian gobies

A similar version of this chapter was published as: Cerwenka, A. F., P. Alibert, J. Brandner, J. Geist & U. K. Schliewen, 2014. Phenotypic differentiation of Ponto-Caspian gobies during a contemporary invasion of the upper Danube River. *Hydrobiologia* **721**: 269-284.

3.1 Abstract

Evolution is known to act on contemporary timescales and invasive organisms are often used to study rapid evolutionary changes of geno- and phenotypes under natural conditions. The ability and speed of local adaptation is discussed as a key character triggering successful invasions. Variation of body shape among populations of two highly invasive, sympatric Ponto-Caspian goby species (Teleostei: Gobiidae) with a comparable invasion history in the upper Danube River, i.e. *Neogobius melanostomus* and *Ponticola kessleri*, was assessed using geometric morphometric methods. Phenotypic variation established within less than 15 generations was evident in both species. It was mainly correlated with geographical location, but in *N. melanostomus* also with substrate type, an ecological variable reflecting habitat quality. The two species differed in their degree of intraspecific variation which was more pronounced in *N. melanostomus*, the numerically dominant invader in the upper Danube. Body shape variation significantly correlating with geographical rather than ecological variables suggests a heritable component and renders phenotypic plasticity as a lone explanation unlikely. Patterns of body shape similarity among upper Danubian goby populations suggest a stepping-stone rather than a continuous expansion model for both species, where multiple introductions, possibly from various origins, may have shaped differentiation.

3.2 Introduction

Range expanding species are exposed to new environments with novel selective forces and thus provide natural experiments to study evolutionary mechanisms (Kolar & Lodge, 2002;

Strayer et al., 2006). Adaptive responses of expanding species to altered selection regimes in novel environments may be species specific, often remarkably rapid, and can promote speciation (Hewitt, 1996, 2000). Divergent selective pressures acting on the population level, under sympatric or parapatric conditions, can drive adaptation and thus population differentiation, which can serve as basis for ecological speciation (Schluter, 2000; Coyne & Orr, 2004; Gavrilets, 2004; Seehausen, et al., 2008a, b)

After tectonic landscape rearrangements or after glaciations, species ranges may expand rapidly into previously uncolonized areas and change dramatically over short time scales (Bernatchez, 2001; Zamudio & Savage, 2003). In Europe for example, huge drainage areas were re-shaped after the pleistocene glaciations and re-colonized with fish species originating from Ponto-Caspian refugia (Hewitt, 2001, 2004; Griffiths, 2006). These palaeo-range expansions are partially comparable to anthropogenically induced invasions of neobiota, if gradual expansion by (re-)colonizing edge populations has been similar in postglacial and recent invasive processes. Both may offer insights into species evolution. Whereas most palaeo-range expansions terminated in the past, neobiotic invasions are characterized by their contemporary character, i.e. their ongoing evolution in new environments over no more than tens of generations (Lee, 2002; Sax et al., 2007; Vellend et al., 2007; Prentis et al., 2008). Rapid range expansion of non-native species (often at multiple inoculation sites) and their subsequent establishment offer highly informative time-windows for the investigation of phenotypic and genotypic changes (Kolar & Lodge, 2002; Strayer et al., 2006; Keller et al., 2011). Biological invasions therefore provide a semi-experimental setup to study the links between adaptation, divergence, and speciation in contemporary time scales and under natural conditions (Orr & Smith, 1998).

Changes in geno- and phenotype of invasive organisms have been reported in many recent studies (e.g. Huey et al., 2000; Tsutsui et al., 2000; Mooney & Cleland, 2001; Lee, 2002; Bossdorf et al., 2005; Dlugosch & Parker, 2008). Especially changes in body shape may integrate multiple adaptive responses to novel environments. They are partially controlled genetically (Leinonen et al., 2011), but are often also strongly influenced by environmental variables (Langerhans & DeWitt, 2004; Walker, 2010). Phenotypic differentiation between individuals and populations of invading species therefore offers the possibility to decipher the speed of genotypic and phenotypic evolution with regard to the adaptation to novel selective environmental forces and constraints (Moczek & Nijhout, 2003; Lambrinos, 2004; Firmat et al., 2012). In invasive species, adaptive phenotypic plasticity seems to be important, and is thought to be a key factor influencing the success of invasive species in newly occupied environments (Agrawal, 2001). Phenotypic plasticity can slow down directional evolution by retarding selection (West-Eberhard, 2003), or, in contrary, accelerate adaptation. The maintenance of plasticity is supposed to boost the evolution of responses to differing environmental conditions (Pigliucci, 2005). Even moderate phenotypic plasticity can contribute to increased fitness and ecological speciation under various environmental conditions, which are frequently encountered during invasions (Fitzpatrick, 2012). In sum, both phenotypic plasticity and genetically based environmental specialization represent adaptations to environmental heterogeneity. They can potentially cause differential success of invasions, with respective phenotypic responses and adaptations depending on locally different selective pressures (Lind & Johansson, 2007).

Invasive riverine fish populations provide optimal targets to study contemporary adaptive evolution, since the linear, one-dimensional river topology allows the investigation of local differentiation along a simple geographical gradient. Fish body shape is often used to analyze phenotypic adaptation as it rapidly evolves in response to ecological parameters (Langerhans & DeWitt, 2004; Langerhans et al., 2007; Collin & Fumagalli, 2011). As geometric morphometric methods have the potential to identify adaptive shape information (Lawing & Polly, 2010; Klingenberg, 2011), studies of benthic invasive (freshwater) fish species may be particularly suited to reveal finescaled local phenotypic differentiation. Locally divergent selection may act at geographically proximate areas, especially if gene flow between parapatric populations is limited. Thus, extrinsic natural or artificial barriers may lead to partially reproductive isolated evolutionary units (Hewitt, 2000).

This study focuses on two invasive goby species, the Bighead Goby *Ponticola kessleri* (Günther, 1861) and the Round Goby *Neogobius melanostomus* (Pallas, 1814), which appeared in the upper Danube River in 1999 and 2004, respectively (Seifert & Hartmann, 2000; Paintner & Seifert, 2006). The native distribution range of both species is the Ponto-Caspian region, the main source of many aquatic organisms invasive to the Danube River (Hewitt, 2004). These gobies are suspected to have low migration rates, in *N. melanostomus* ranging between 500 m and 4 km per year (Bronnenhuber et al., 2011; Gutowsky et al., 2011; Brownscombe et al., 2012; Kornis et al., 2012), with recent studies suggesting values up to 17 km per year (Brandner et al., 2013a). Introductions occurred most probably by ballast water of transportation vessels (Wiesner, 2005), presumably resulting in comparatively few arriving individuals at a time.

Unfortunately, it is difficult to evaluate, whether invasive populations are the result of rapid population growth after single or after multiple secondarily admixing inoculations (Taraborelli et al., 2010; Brownscombe et al., 2012). Kováč et al. (2009) showed that both species exhibit a precocial life-history in their native brackish water habitat as compared with a less precocial one in invasive populations of the middle Danube River, possibly due to lower ecological specialization. The authors suggest that goby life-history responses in native versus invaded areas originate from plasticity as both species show high variability in all analyzed traits (i.e. with a higher fecundity, higher number of spawning batches and fish maturing at smaller size in non-native populations). *Neogobius melanostomus*, the species with a more precocial life-history, with a more direct ontogeny, lower fecundity, and larger eggs, is suggested to have higher invasive success especially in the first years after invasion. The comparatively less precocial life-history of *P. kessleri* is suspected to pay off over longer time periods because of a better capability to cope with long-term unstable and less predictable environmental changes (Kováč et al., 2009).

This study for the first time tests for simultaneous intraspecific phenotypic differentiation of two sympatric invasive goby species using geometric morphometric methods. Specimens of *P. kessleri* and *N. melanostomus* were sampled along the same comparatively short (~200 km) river section of the upper Danube River. The time lag between collections for this analysis and the first record of invasive gobies is only less than 15 generations. Hence, detectable intraspecific differentiation should be linked either to contemporary evolution after inoculation(s) or to original differentiation of previously allopatric lineages that came into secondary contact in the invaded area. To provide a starting point for discussion on contemporary differentiation of invasive species on a small scale, general hypotheses tested in this study were that (i) phenotypic differentiation correlates with local differentiation, i.e. with sample location within the upper Danube River in both species, *N. melanostomus* and *P. kessleri*; and that (ii) body shape variation correlates with environmental- and fish-specific variables, respectively again in both species. It was further tested whether (iii) potential changes in body shape are similar or dissimilar between species.

3.3 Materials and methods

Field sampling

Fish were collected by electrofishing following a standardized sampling procedure described in detail in (Brandner et al., 2013b). A total of 1,078 specimens (490 *P. kessleri* and 588 *N. melanostomus*) were sampled in 2010 (March 29–October 18) and 2011 (April 11–October 20) at 10 river stretches (*stretch 1 – stretch 10*, recorded from downstream to upstream direction) along the upper Danube River (Fig. 3.1). These specimens were selected out of 6,705 *N. melanostomus* and 182 *P. kessleri*. To reach an approximately balanced sample size per stretch, additional 308 *P. kessleri* were sampled at the respective stretches. Total length (LT) was measured to the nearest 0.1 cm and wet mass was weighted to the nearest 0.2 g. Sex was determined according to the shape of the urogenital papilla following Stránňai (1999) and later verified in the laboratory.

Table 3.1 summarizes the number of goby specimens sampled at each river *stretch* differentiated according to species, *sex*, *bank side* of the river and *habitat quality* (rip-rap, i.e. rock used to artificially armor river shores, or natural gravel). As Kovac and Siryova (2005) showed both species to be different in their development and to further reduce effects of size, only specimens in the range of mature individuals with a LT range from 3.7 to 15.2 cm (mean = 9.7 cm, SD = 1.3 cm) in *N. melanostomus* and from 6.4 to 15.5 cm (mean = 9.8 cm, SD = 1.7 cm) in *P. kessleri* were included. All specimens and tissue vouchers are stored in the ichthyological collection of the Bavarian State Collection Munich (ZSM).

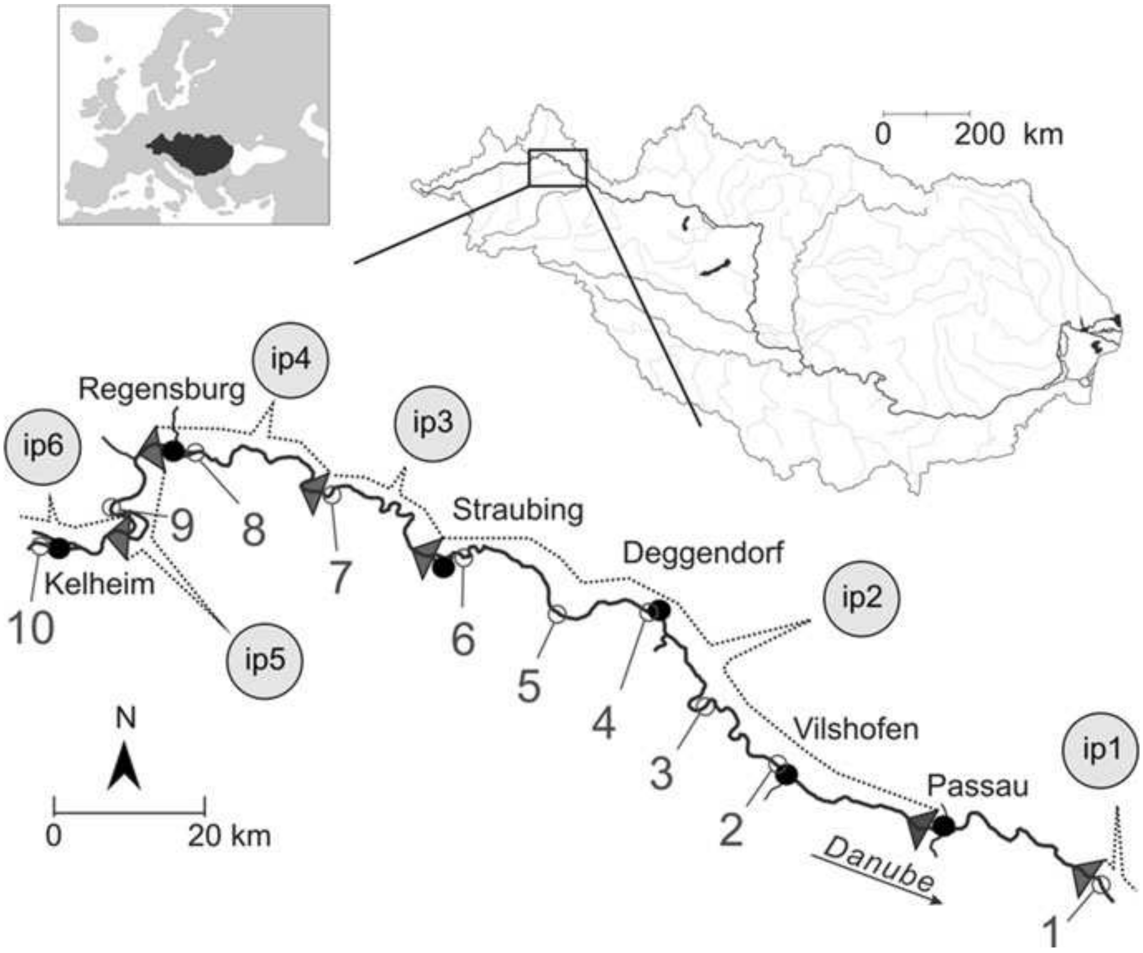

Fig. 3.1: River *stretches 1–10* in the study area of the upper Danube River (upper right part). Bars crossing to the river indicate dams. Populations combined for some analyses to interdam populations (*ip 1–6*) are given in grey shaded circles. Figure modified from Brandner et al. (2013b).

Geometric morphometrics

Fish were photographed (Nikon D60) under standardized conditions, i.e. always applying the same conservation mode, way of positioning and magnification. To avoid errors due to deformation of fish body, the right side of every specimen was placed on fitted styrofoam and fins were fixed with pins. Seventeen landmarks (LMs), modified from L'avrincikova et al. (2005) and Čápová et al. (2008), were positioned on digital photographs using the TPSdig software package (Rohlf & Marcus, 1993; Rohlf, 2006a, b). Positions of the 17 LMs (Fig. 3.2) were based on those used for Cottus sculpins by Nolte and Sheets (2005). Non-shape variation (e.g. differences due to scaling and positioning of fish on pictures) was removed using ‘‘Generalized least squares Procrustes superimposition’’ as implemented in the software morphoJ (Klingenberg, 2011). Procrustes coordinates were then used as shape variables for the computation of principal components (PCs) and all further analyses in morphoJ. PCs are linearly uncorrelated, reducing a multivariate dataset to new synthetic variables (here shape variables) which each account for parts of the original variance in the dataset (for a more detailed description of geometric morphometrics see Zelditch et al. (2004)). Following Adajar et al. (2011) for statistical analyses (except for the calculation of measurement error and linear regression) PCs with an eigenvalue higher than the Joliffe-cut-off were considered relevant and used, whereas components with smaller eigenvalues were excluded. To minimize bias and error, all steps of morphometric analysis were performed by the same operator, including preparation and adjustment of gobies, taking photographs and setting LMs on pictures.

Table 3.1: Number and name of river *stretches*, GPS-coordinates of lower sampling site boundaries and numbers of analyzed goby specimens separated according to *bank side*, *type of substrate* and *sex*.

River Stretch			**Number of Specimens**		
#	**Name**	**GPS-coordinates**	**Bank side (right/left)**	**Substrate (rip-rap/gravel)**	**Sex (female/male)**
1	Engelhartszell	E 13°46'29" N 48°28'32"	32/32 16/17	32/32 5/28	34/30 19/14
2	Vilshofen	E 13°10'44" N 48°38'24"	32/32 22/23	32/32 16/29	31/33 29/16
3	Winzer	E 13°03'08" N 48°43'37"	32/31 25/33	27/36 29/29	31/32 28/30
4	Deggendorf	E 12°59'50" N 48°47'31"	32/28 29/30	24/36 29/30	29/31 33/26
5	Mariaposching	E 12°52'12" N 48°50'28"	31/31 32/26	27/35 28/30	31/31 32/26
6	Straubing	E 12°42'26" N 48°53'34"	28/36 29/29	24/40 27/31	32/32 31/27
7	Geisling	E 12°23'37" N 48°58'51"	36/29 25/38	20/45 34/29	30/35 34/29
8	Regensburg	E 12°10'41" E 12°10'41"	33/32 32/30	31/34 33/29	31/34 36/26
9	Bad Abbach	E 12°00'13" N 48°57'57"	23/22 20/29	10/35 37/12	22/23 24/25
10	Kelheim	E 11°56'27" E 11°56'27"	12/24 5/0	2/34 5/0	21/15 3/2
1-10			291/297 235/255	229/359 243/247	292/296 269/221

Data on *Neogobius melanostomus* (n = 588) are given on the upper part of every cell and data of *Ponticola kessleri* (n = 490) at the lower part, respectively

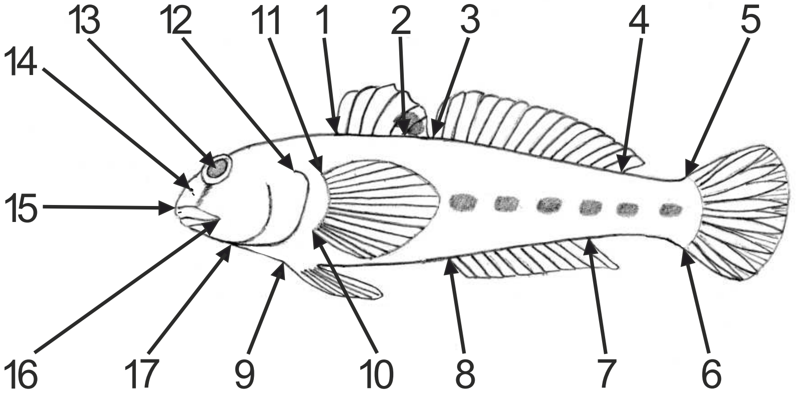

Fig. 3.2: Position and configuration of landmarks (LM; n = 17) set on standardized images of *Neogobius melanostomus* (n = 588) and *Ponticola kessleri* (n = 490). LM 1: anterior insertion of the dorsal fin; LM 2: insertion point of last spine of first dorsal fin; LM 3: injection point of first spin of second dorsal fin; LM 4: injection point of last spin of second dorsal fin; LM 5: dorsal insertion of caudal fin; LM 6: ventral insertion of caudal fin; LM 7: injection point of last spine of anal fin; LM 8: insertion of first spine of anal fin; LM 9: insertion of ventral fin; LM 10: insertion of lower spine of pectoral fin; LM 11: insertion of upper spine of pectoral fin; LM 12: insertion of operculum; LM 13: central point of the eye; LM14: tubular nostril; LM15: junction of premaxillary and ethmoid; LM 16: posteriormost point of the lips; LM 17: injection point of operculum and preoperculum.

Statistical analysis

Following Bailey and Byrnes (1990) and Yezerinac et al. (1992), measurement error negatively influences the efficiency to detect differences in body shape, and is assessed in per cent depicting the proportion of total variance generated by differences in measuring. To calculate the error, random subsamples of each 27 specimens per species were measured and analyzed twice.

Assessment of fish size effects on body shape

As growth is widely isometric in both analyzed gobies [for *N. melanostomus*: see L'avrincikova et al. (2005) and for *P. kessleri*: see Kovac and Siryova (2005)], effects of size on body shape are supposedly small but nevertheless may significantly contribute to shape variation (Parsons et al., 2003). To test for a potential effect of size on shape variation, regression of shape coordinates on centroid size was computed in morphoJ (Drake & Klingenberg, 2008). Following Klingenberg et al. (1998), a permutation test with 10,000 rounds was conducted using morphoJ (Klingenberg, 2011). Further, to analyze if potential body shape differences correlate with analyzed localities, median LT of gobies at single *stretches* was compared using pairwise Kruskal–Wallis tests (Bonferroni corrected).

Assessment of globally significant factors correlating with shape variation

Geographic, specimen-specific and environmental variables potentially associated to shape data were assessed using MANOVA in R (R Development Core Team, 2011). Variables considered were sampling location (*stretches 1–10*, Fig. 3.1), *stretches* summarized according to potential populations separated by dams which may limit species upstream migration (*ip* [interdam population] *1–6*: *ip 1* corresponds to *stretch 1*, *ip 2* summarizes *stretches 2–6*, *ip 3 = stretch 7*, *ip 4 = stretch 8*, *ip 5 = stretch 9*, *ip 6 = stretch 10*, Fig. 3.1), bank side of the river (*bank*: left or right), habitat quality (*substrate*: gravel or rip-rap), sex (*sex*: female or male) *centroid size* as an estimate for body size and fish wet weight (*weight* in g). Variables potentially correlating with body shape were set as dependent and were classified as grouping (i.e. qualitative) or as quantitative variables. *Stretch*, *ip*, *bank*, *substrate* and *sex* were set as qualitative factors for MANOVA and *centroid size* as well as *weight* as quantitative ones respectively. Because multiple factors can act simultaneously on shape and thus make the interpretation of different correlations more difficult (e.g. Andersson et al., 2006), concurrent effects were analyzed through examination of interaction of variables: i.e. effects, which along with the main action of each of two variables arise, if the combination produces additional effects not explainable with the main effects of the two variables alone; here between the *stretch* and *ip* variable on the one hand and the *sex*, *substrate*, *weight* and *centroid size* variables on the other hand. Pillai's trace was used to test for significance of MANOVAs and to compare the magnitude of single factors by comparing amounts of the variance in the dataset related to specific factors.

Model comparisons approach using F-test

Sequential stepwise removal of non-significant variables and non-significant interactions from the fullfactorial model was controlled by ANOVAs testing if there was a significant change between variation explained by MANOVAs before and after exclusion of single variables and interactions.

Assignment and illustration of morphometric variation to geographical clines

Pairwise ANOVAs were used to test for significant morphometric differentiation of goby populations per river *stretch* and per *ip* as mirrored by PC 1–3 values. In *P. kessleri*, analyses were repeated excluding all specimens from *stretch 10* as the sample size of this population was low (n = 5) and because two specimens were extremely meagre females, which had extremely low gonado-somatic index (data not shown), i.e. they most likely were spent and could have influenced overall morphometric variation disproportionally. Discriminant Function Analysis was utilized to examine separation of goby shapes between habitat qualities including a parametric T^2 test using morphoJ. For graphical illustration of body shape in invasive gobies, deformation grids of second PC showing differences between single populations and all remaining specimens were computed.

3.4 Results

Differences in body shape of invasive gobies

In *N. melanostomus*, PC 1 accounted for 40.39% (eigenvalue = 5×10^{-5}) of the variance, PC 2 explained 11.30% (eigenvalue = 1.4×10^{-5}). The value of the Joliffe-cut-off was 2.89×10^{-6} suggesting the inclusion of PCs 1–9 in the downstream analysis. In *P. kessleri*, the PC 1 and PC 2 represented 27.21% (eigenvalue = 3.3×10^{-5}) and 14.22% (eigenvalue = 1.73×10^{-5}) of the variance, respectively. The Joliffe-cut-off was 2.83×10^{-6}, leading to the inclusion of PCs 1–10. Altogether included PCs explained 84.77% of total variance in *N. melanostomus* and 83.04% in *P. kessleri*. The average measurement error of total variation for shape variables concerning all PCs was 5.04% for *N. melanostomus* and 8.09% for *P. kessleri*.

LT of gobies at river *stretches* was not significantly different in both species by pairwise comparisons (Kruskal–Wallis test, Bonferroni corrected, all $p > 0.05$); however, *P. kessleri* were significantly smaller at *stretch 8* compared to specimens at *stretches 3* ($p < 0.001$) and *5* ($p < 0.05$). The relative amount of total variation for which the regression between *centroid size* and all shape variables accounted was 1.05% for *N. melanostomus* and 2.84% for *P. kessleri*. Permutation tests indicated a significant influence of *centroid size* on total variation in both gobies ($p < 0.001$).

MANOVA/ANOVA models

MANOVAs revealed significant correlations of body shape variance in both species with locality data at high resolution (*stretch*) and coarse resolution (*ip*) scales (both: $p < 0.001$). Pillai's trace indicated geographic fine-scaled factors (*stretch*) to be more important than large-scaled (*ip*). Further significant correlations were present, regarding the combination of variables *stretch x substrate* (*N. melanostomus*: $p < 0.001$; *P. kessleri*: $p < 0.05$). Additionally, in *N. melanostomus* only, PCs were first correlated with *sex* ($p < 0.001$) and *substrate* ($p < 0.01$) and second, with the combination of the variables *stretch x sex* ($p < 0.01$), *ip x sex* ($p < 0.05$) and *ip x substrate* ($p < 0.001$). In *P. kessleri*, the combinations of *size x stretch* and *size x ip* showed significant (both: $p < 0.05$) correlations with shape variables. All other variables and combinations of variables did not show significant correlations with variance of body shape PCs (all: $p > 0.05$). Results of MANOVAs and ANOVAs are summarized in Table 3.2.

In a second step, non-significant source of variation indicated by results of MANOVAs was excluded. ANOVAs were used to compare results before and after exclusion. With exception of the variables *size* and *weight* in *N. melanostomus* and the variables size, *bank*, *substrate* and the combinations *ip x sex*, *ip x substrate* in *P. kessleri*, ANOVAs indicated no significant differences (all: $p > 0.05$; see Table 3.2).

Table 3.2: MANOVAs between variables, and between interactions of several variables and body shape represented through the first nine PCs in *Neogobius melanostomus* (n = 588, in the upper part of every cell) respectively the first 10 components in *Ponticola kessleri* (n = 490, in the lower part of every cell).

Variable/interaction between variables	Pillai's trace	Hotelling-Lawley
Centroid size	0.032	0.052
	0.035	0.122
Stretch	0.326	< 0.001
	0.421	< 0.001
Interdam population	0.253	< 0.001
	0.225	< 0.001
Bank	0.007	0.944 ^{A}
	0.041	0.056
Sex	0.058	< 0.001
	0.039	0.075 ^{A}
Substrate	0.041	< 0.010
	0.031	0.204
Weight	0.021	0.270
	0.016	0.724 ^{A}
Stretch:centroid size	0.184	0.086 ^{A}
	0.263	< 0.050
Stretch:sex	0.216	< 0.010
	0.243	0.110 ^{A}
Stretch:substrate	0.261	< 0.001
	0.246	< 0.050
Stretch:weight	0.178	0.131 ^{A}
	0.244	0.100 ^{A}
Interdam population:centroid size	0.102	0.100 ^{A}
	0.149	< 0.050
Interdam population:sex	0.110	< 0.050
	0.138	0.086
Interdam population:substrate	0.142	< 0.001
	0.118	0.060
Interdam population:weight	0.092	0.231 ^{A}
	0.135	0.110 ^{A}
centroid size:sex	0.016	0.484 ^{A}
	0.027	0.321 ^{A}
stretch:centroid size:sex	0.133	0.775 ^{A}
	0.245	0.096 ^{A}

Variables and interactions were excluded stepwise from the model. Influence between the preceding and the consecutive MANOVAs was tested using ANOVAs. Non-significant influences are indicated with an elevated A

Goby body shape differentiation on a spatial scale

To test for shape differentiation associated with individual river *stretches* and *ips,* medians of PC 1, 2 and 3 of populations were compared using ANOVAs. In *P. kessleri* no significant differences were detected for all three PCs. Analyses were repeated excluding all specimens from the population at *stretch 10* (n = 5). The results after exclusion were highly comparable to those using all *P. kessleri* populations. PC 1 accounted for 27.16% (eigenvalue = 3.3×10^{-5}) of the variance, PC 2 explained 14.09% (eigenvalue = 1.7×10^{-5}) and thus no strong overall effects were produced by the few specimens of this population. Differences in mean PC values between the two replicate analyses were most likely caused by two meagre female specimens, which most likely were freshly spent. In *N. melanostomus*, no significant differences were found for pairwise *stretch* comparisons of PC 1 and PC 3 (all: $p > 0.05$), with exception of comparison of PC 1 of *stretch 5* and stretch *10* ($p < 0.01$). Significant differences were found, comparing PC 2 of *N. melanostomus* individuals from *stretch 1* with those from *stretches 2, 4, 7* (all: $p < 0.05$) and *stretch 5* ($p < 0.001$), and comparing *stretch 5* with *stretch 9* ($p < 0.05$). Comparing *N. melanostomus* at *ips*, they differed significantly between *ip 2* and *ips 1* ($p < 0.001$) and *5* ($p < 0.05$), supporting results from pairwise single *stretch* comparisons to a large extent. Deformation grids illustrating major differences of LM configurations were used to visualize variation in body shape according to PC 2. Grids showed differences between single populations and all remaining specimens (Fig. 3.3). *Neogobius melanostomus* (Fig. 3.3a) from *stretch 1* were very short-snouted, specimens at *stretches 5* and *9* had medium snout length and at *stretches 2* and *4* they were long-snouted. *Neogobius melanostomus* from *stretch 1* were long-tailed, populations at stretches *4* and *5* small-tailed and at *stretches 2* and *9* the tail length was intermediate. Individuals at *stretch 9* were characterized by a dorsoventrally compressed head region, by having a pectoral fin insertion almost at the insertion point of ventral fin and by a shallower midbody. Individuals at *ip 1* had a comparatively long snouts and a large head region, whereas *N. melanostomus* at *ip 9* were short-snouted and had a large body height. In *P. kessleri* (Fig. 3.3b), populations at *stretches 5, 8* and *9* were long-tailed and snout length was smaller at individuals from *stretches 1* and *2*.

Goby body shape differentiation on an environmental scale

To test for shape differentiation associated with the environmental variable *substrate*, Discriminant Function Analysis was applied to the total sample of *N. melanostomus* inhabiting rip-rap versus gravel habitats. Shape was significantly different between gobies collected on different substrates (Procrustes distance = 0.00470, $T^2 = 64.85$, $p < 0.01$).

3.5 Discussion

A major question in invasion biology is whether successful invaders are characterized by a higher potential to adapt to local conditions. In this study, body shape divergence along geographical and ecological gradients applying simultaneously to two sympatric invasive goby species, i.e. *N. melanostomus* and *P. kessleri*, showed phenotypic variation potentially reflecting local adaptation established within less than 15 generations since first introduction. Body shape changes may rapidly integrate adaptive responses and can thus lead to divergence of phenotypes among populations (Burns et al., 2009; Fruciano et al., 2011); hence, inter- as well as intraspecific morphometric differentiation may shed light on the species-specific adaptive potential of successful invaders. The present study revealed contemporary phenotypic differentiation being significantly correlated with fine-scaled geographic variation. Amongst others, separation by man-made barriers (dams) correlated with body shape differentiation of both investigated goby species. However, in *N. melanostomus* only, a correlation of body shape divergence with *substrate* reflecting habitat quality related to anthropogenic change was significant. Interspecifically, the two species differed further in their degree of overall morphometric variance, which was more pronounced in *N. melanostomus* than in the slightly longer established *P. kessleri* (Fig. 3; Table 3.2).

Intraspecific differentiation: phenotypic plasticity versus localized genetic differentiation

Ponto-Caspian gobies have invaded a wide range of temperate aquatic systems on earth (Stepien & Tumeo, 2006; Kornis et al., 2012). Both goby species investigated in this study are of Ponto-Caspian origin and have expanded their range into the upper Danube River in about one decade before this study (Corkum et al., 2004; Eros et al., 2005; Polačik et al., 2009; pers. obs.), and are now established in all suitable habitats of the German Danube River accessible to large ships. The contemporary gobiid populations of the upper Danube River consist of multiple phenotypically variable and partially differentiated subpopulations in both invasive gobies. Undoubtedly, the observed phenotypic differentiation has developed within very few generations, similar to a case of invasive *Drosophila* flies (Diptera) (Huey et al., 2000). However, whether phenotypic plasticity (Davidson et al., 2011; Fitzpatrick, 2012), rapid local genetic adaptation (Stelkens et al., 2012), population genetic effects as founder effects and allele surfing during rapid population expansion (Lee, 2002; Travis et al., 2007; Price & Sol, 2008), or divergence due to different ancestry of local populations (Vellend et al., 2007; Prentis et al., 2008) shaped morphometric variation of goby populations, still remains to be evaluated. Population genetic effects and effects of different ancestry could not be assessed without population genetic data. However, subsequent genomic analyses of the same goby specimens studied in here showed increased levels of differentiation in *N. melanostomus* in comparison to *P. kessleri* (Cerwenka et al., 2014a), and a population genetic similarity of *N. melanostomus* analogous to patterns of similarity among populations of the analyzed upper Danube River as revealed by results of this study. Phenotypic differentiation in correlation to the factor substrate found in *N. melanostomus* was not detectable in genetic analyses, hereby rendering phenotypic plasticity more probable, i.e. that individuals with a single genotype express different phenotypes under different environmental conditions (Fitzpatrick, 2012). In addition, in both species no correlation between morphospace and genotype was detectable, making phenotypic plasticity more likely. Kováč et al. (2009) suggest neogobiids to be phenotypically plastic with regard to their life histories; localized morphometric divergence due to phenotypic plasticity might explain the detected phenotypic variation—as opposed to or in combination with rapid local genetic adaptation. Valiente et al. (2010) suggested flexibility in life-history traits to be an important factor for the successful invasion of Andean streams by brown trout. Fox et al. (2007) showed that invasion success of the centrarchid fish *Lepomis gibbosus* (L.) is correlated with higher levels of plasticity in life-history traits, i.e. in maturation and in growth rate of juveniles. Similarly in gobies, *N. melanostomus* was found to reveal high levels of plasticity in these traits (see also: Brandner et al., 2013a) which may contribute to the greater success of this species compared to *P. kessleri*. In the more precocial species, *N. melanostomus* current invasion success is higher when measured in fish density at analyzed shore lines (see also Brandner et al., 2013c). *P. kessleri* exhibits less local phenotypic differentiation than the more precocial species, *N. melanostomus*. In sum, and in *N. melanostomus*, higher levels of phenotypic variability and increased levels of invasion success support conclusions of Fox et al. (2007), at least with regard to the very early phase of the (simultaneous) goby invasion.

As in MANOVAs, an interaction of the geographical variable *stretch* with the ecological variable *substrate* was highly significant in both species, and as the variable *substrate* was significantly correlated with shape variation in only one of the two species, localized phenotypic plasticity as a sole explanation for morphometric population differentiation appears unlikely. Shape differences were detected mainly in the head region (Fig. 3), where they are often ascribed to dietary differences (e.g. Caldecutt & Adams, 1998; O'Reilly & Horn, 2004). Nevertheless, trophic-related shape differences in fishes are commonly correlated to their feeding mode instead of food quality (e.g. Pfaender et al., 2009). As *substrate* and food resource availability correlate in both species and in the investigation area (Brandner et al., 2013b), this interaction could be an indirect hint to conditionally different feeding modes and could be associated to shape responses in different habitats at least in *N. melanostomus*. In addition, water current velocity may trigger phenotypically plastic changes of body shape in different habitats (e.g. Fischer-Rousseau et al., 2010; Haas et al., 2010). In rivers, current velocity is strongly correlated with the factor *substrate*, but the evaluation of this, and possibly other environmental factors, needs further investigation. Another possible explanation is that allopatrically or parapatrically induced population divergence is triggering and genetically accounting for phenotypic divergence of rapidly spreading invasive species. This hypothesis is supported because inter-population (*stretch, ip*) rather than intra-population variables (*substrate*, *bank*) correlate more often in pairwise comparisons with body shape variation, and because geographic variables explain more variance in the data set (measured through Pillai's trace, Table 3.2) in both, *N. melanostomus* and *P. kessleri*. The gobies' limited migration ability (Bronnenhuber et al., 2011; Gutowsky & Fox, 2011; Brownscombe et al., 2012; Kornis et al., 2012; Brandner et al., 2013a) is indeed likely to have constrained genetic admixture between adjacent and even partially isolated populations and therefore could have facilitated localized genetic differentiation, especially if multiple introductions in adjacent areas lead to ad-hoc increased variation (Vellend et al., 2007; Prentis et al., 2008). On the other hand, the significant interaction of the ecological variable *substrate* with geographical variables does not preclude a role of phenotypic plasticity supporting localized adaptive responses.

Morphometric variation along the Danube River

An integrative view on the present results tentatively suggest a morphometric partition of goby populations of the upper Danube River into an uppermost one including a differentiated population at *stretch 9* beyond the influx of the Rhine-Main-Danube canal, a single lowermost one below the influx of the alpine coldwater River Inn, and a large central group upstream from the River Inn influx including a highly differentiated population at *stretch 5* at least in *N. melanostomus*. It is noteworthy that the most downstream population of *N. melanostomus* is significantly differentiated from some central populations, but not from the most distant uppermost ones, hereby contradicting a simple isolation-by-distance or a strict stepping-stone pattern of upstream invasion. The overall pattern rather supports the view that multiple introductions from different sources shaped both the invasion and differentiation process along the upper Danube River. This applies especially since there are no apparent ecological similarities between the most downstream and the uppermost locations potentially explaining similarity through convergent directional selection. Introductions from different populations or subspecies, as indicated for the origin of invasive *N. melanostomus* in the upper Volga but not for the Danube River (Brown & Stepien, 2008), might have contributed to this localized phenotypic and possibly genetic differentiation in the upper Danube River. If substantiated it would support independent results of Firmat et al. (2012) for another freshwater invader, *Oreochromis mossambicus* (Peters, 1852), that phylogeographic constraints dominate morphometric population differentiation of invasive freshwater fishes; however, this hypothesis needs to be tested using different source population samples and genetic data.

Two studies have provided and statistically analyzed data on morphometric variation of *N. melanostomus* within and between native and invasive populations from different native and introduced regions, which are useful for comparison with morphometric variation in the upper Danube River: Simonović et al. (2001) in Serbia and Polačik et al. (2012) in Slovakia. Unfortunately, different morphometric methods were applied in these studies and the one presented here, rendering direct comparisons difficult. Both previous studies found that introduced central Danubian populations differed significantly in several measurements from native lower Danubian ones (Polačik et al., 2012), as well as from Dniestr, Dniepr, Black Sea and Sea of Azov populations (Simonović et al., 2001). Nevertheless, the three Danubian freshwater populations, i.e. the introduced Slovak, the native Bulgarian from the Lower Danube River as well as introduced Serbian population, appeared to be more similar to each other than to brackish water or non-Danubian freshwater populations.

Simonović et al. (2001) showed that *N. melanostomus* from freshwater habitats differ from Black Sea basin populations mainly in postdorsal distance, height of second dorsal fin and ventral disc length. Maximum body height, preanal length and prepectoral length are similar in invasive Danubian but differed significantly between all other analyzed populations. In contrast, Polačik et al. (2012) found native and nonnative goby populations differing significantly in head associated measurements, i.e. upper and lower jaw length, eye diameter, interorbital and predorsal distance. In addition, only females differed between populations in head depth and two non-head measurements (post orbital distance and minimum body depth). Variability in morphometric measurements of *N. melanostomus* found by Polačik et al. (2012) and in this study is partially comparable, because (i) overall body shape variation concerned the same regions (Fig. 3.3a), and because (ii) the head region was variable both in Polačik et al. (2012) and this study. No study tested statistically for morphometric differentiation of *P. kessleri* populations, but Smirnov (1986) pointed out that Black Sea populations of this species are characterized by a comparatively elongated anal fin, lower caudal peduncle and a shorter preanal distance.

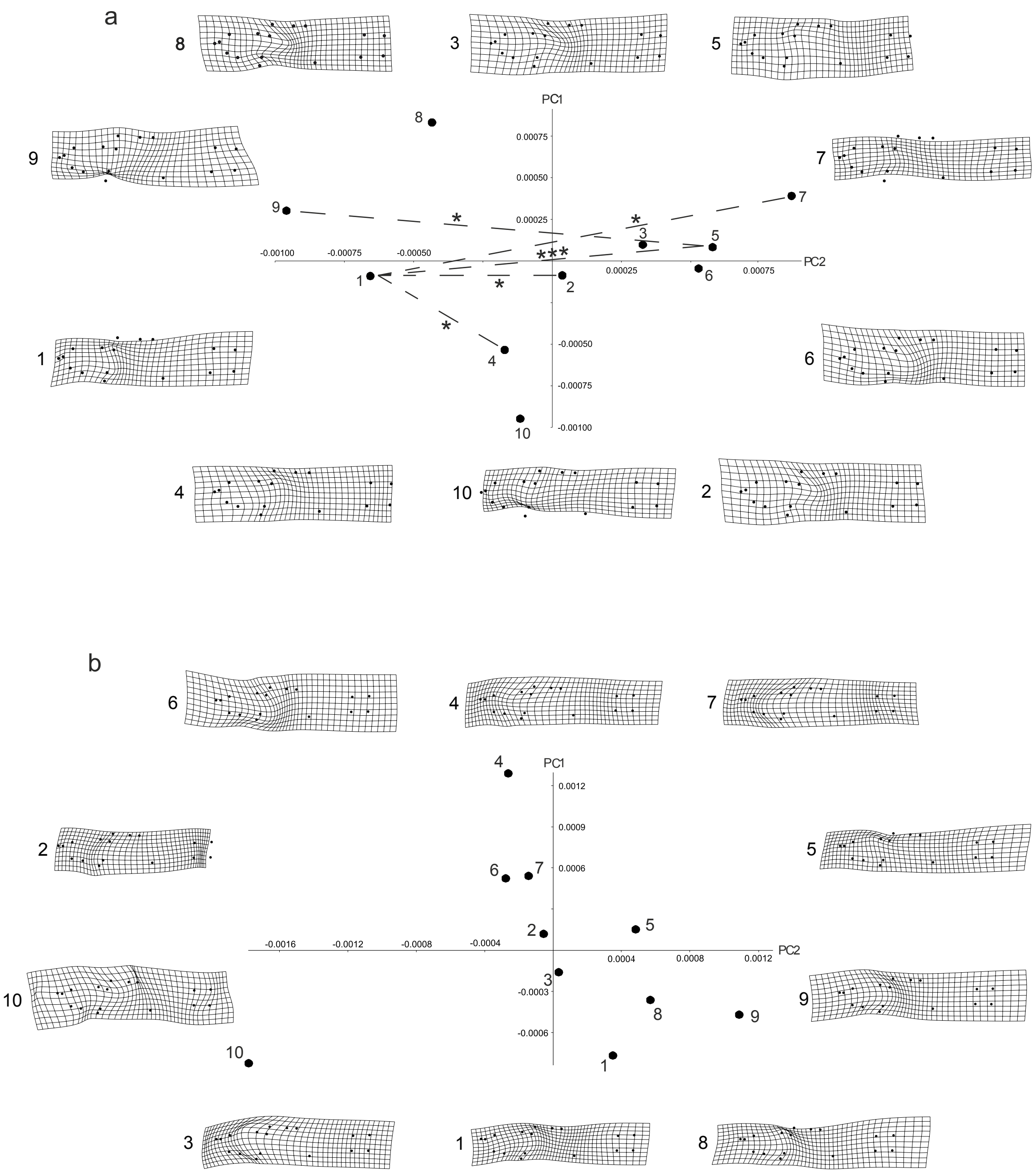

Fig. 3.3: Principal components analyses (PCA) plots and deformation grids of a *Neogobius melanostomus* and b *Ponticola kessleri*. PCA plots show mean Procrustes coordinates of each stretch population (*stretch 1–10*, Arabic numbers) and grids illustrate differences in body shape based on PC 2 of indicated stretches in comparison to all other analyzed gobies. The scaling factor is set to default value of 0.1 using morphoJ. Dashed lines connecting mean Procrustes coordinates indicate significant different body shapes of gobies at river stretches using ANOVAs. Levels of significance are given above the lines (p < 0.05; ** p < 0.01; *** p < 0.001).**

Interspecific differentiation

The almost simultaneous invasion and complete sympatry of the two investigated goby species in the upper Danube River allows a direct comparison of species-specific characters potentially influencing invasion success. Overall morphometric variation as well as qualitative differences in morphometric character variation correlated in both species with locality but only in *N. melanostomus* with habitat quality suggesting that latter species is ecologically more adaptable.

In both species, shape differences were mainly detected in the head region which is most important for food uptake selectivity, as well as proportions of central middle body i.e. the area in between the insertion point of the pelvic fin disc and both dorsal fins, and tail length. Variation was substantially more pronounced in *N. melanostomus* than in *P. kessleri*. Interestingly, both species showed a similar trend of morphometric variation at similar localities: *N. melanostomus* and *P. kessleri* populations from the lowermost sampling stretch of the Danube River expressed a shorter and more compressed body shape (Fig. 3.3). Convergent phenotypic variation could hint to directional selection shaping morphology in both species in a similar way. Specialization on particular food sources could influence body shape (Wimberger, 1992) possibly being one reason of shape variation at single river sections, as e.g. amphipods reach lowest densities at the lowermost sites of the investigation area.

Invasive *P. kessleri* are characterized by a higher degree of trophic specialization as compared with *N. melanostomus* in the upper Danube River (Brandner et al., 2013b). The comparatively small morphometric variation of *P. kessleri* fits with the expectation for a stenotopic specialist of being less variable in ecological relevant characters (Smith & Skulason, 1996). Richards et al. (2006) suggested that phenotypic plasticity could be advantageous in maintaining or even increasing fitness under convenient or unfavorable conditions in invasive plants. Both gobiid species were most probably assigned to the category jack-and-master, increasing their fitness under favorable conditions but maintaining fitness under unfavorable environments. Abundance differed strongly in dependence of environmental conditions, i.e. substrate, along the upper Danube River and phenotypic plasticity probably characterizes both goby species but is most likely more pronounced in *N. melanostomus*. Further invasive goby species present in the upper Danube River, i.e. *Babka gymnotrachelus* (Kessler, 1857) (Haertl et al., 2012) and *Proterorhinus semilunaris* (Heckel, 1837) or present in the River Rhine, i.e. *N. fluviatilis* (Pallas, 1841) are probably best classified as master-of-some-trades, being more dependent on specific conditions (Corkum et al., 2004; Grabowska & Grabowski, 2005; Naseka et al., 2005; Čápová et al., 2008).

Elapsed time is important to be considered in contemporary evolutionary studies of population divergence (Coyne & Orr, 1989; Strayer et al., 2006). Interestingly, *P. kessleri* invaded the upper Danube River area before *N. melanostomus* (Seifert & Hartmann, 2000; Paintner & Seifert, 2006), developed high population densities, which then decreased after the arrival of *N. melanostomus*. Whether the density decline of *P. kessleri* is causally related to a direct competitive advantage of *N. melanostomus*, e.g. due to predation, food competition or due to invasion-related or unrelated ecosystem changes cannot be assessed post hoc. Following Brandner et al. (2013b), *N. melanostomus* may have benefited from former invasions, e.g. the one from *P. kessleri* or *Dikerogammarus villosus* (Sovinsjij, 1894), because non-native species could promote subsequent invasions of other species by reducing spatial heterogeneity (García-Ramos & Rodríguez, 2002; Stockwell et al., 2003) and by positively affecting interspecific interactions between non-native species (Grosholz, 2005). Fast rates of change in body shape in both highly invasive non-native gobies, especially in *N. melanostomus*, arise some support of a positive relationship between invasiveness and speed of adaptation, but this has to be analyzed in time series data including trait-utility analyses of morphological changes. Strong competition between both invaders for food may affect niche width and species abundance which could influence phenotypic variability resulting amongst others from phenotypic plasticity. Both species showed very similar ecological niches, i.e. a high dietary overlap, but *N. melanostomus* having higher plasticity in feeding ecology, a seasonal switch and an ontogenetic diet shift (Brandner et al., 2013b). Comparing both species, *P. kessleri* is further characterized by lower phenotypic variability and lower densities at least in near shore areas of the Danube River (see also Brandner et al., 2013b). Whether this is a result from secondary reduction of phenotypic plasticity due to interspecific competition in invaded areas or due to naturally occurring differences between both species, needs further evaluation. Since interspecific competition between invaders is considered as an important factor in success of non-native species (e.g. Braks et al., 2004), this aspect is especially interesting in the context of the simultaneous invasion of *N. melanostomus* and *P. kessleri*. Invasive species may not only outcompete native species, but also each other. However, competition is notoriously difficult to measure and requires experimental and not only observational approaches. Sexual dimorphism differs in the two species. Significant morphometric differentiation according to sex was detected in

N. melanostomus supporting Sokolowska and Fey (2011) and Kornis et al. (2012), who found males to differ from females in some morphometric characteristics. Similar to Simonovic (1996), who did not detect differences in osteological characters of *P. kessleri* depending on sex, this study detected no sex-related differentiation in this species. Moreover, no other specimen-specific variable (i.e. *size* and *weight*) correlated with morphometric differentiation in both species along the upper Danube River, at least when considered without interacting geographical variables (Table 3.2). In summary, geographic factors seem to be more important than ecological factors in shaping population structure in the invasive gobies of the upper Danube River.

4 Population genetic differentiation of Ponto-Caspian gobies

A similar version of this chapter was published as: Cerwenka, A. F., J. Brandner, J. Geist & U. K. Schliewen, 2014. Strong versus weak population genetic differentiation after a recent invasion of gobiid fishes (*Neogobius melanostomus* and *Ponticola kessleri*) in the upper Danube. *Aquatic Invasions* **9**: 71-86.

4.1 Abstract

Approximately ten to 15 generations after first inoculation, two invasive goby species *Neogobius melanostomus* and *Ponticola kessleri* have dispersed and established rapidly the upper Danube River. Population genomic amplified length polymorphism (AFLP) data show that the genome of the more recent newcomer, i.e. the globally invasive *N. melanostomus*, is significantly differentiated to a comparatively large degree (~ 5%) and exhibits pronounced small-scale population structure along a recently invaded 200 km river section. MtDNA haplotype identity over *N. melanostomus* samples suggests that an admixture of phylogenetically strongly differentiated source populations is unlikely. Fine-scaled local genetic population structure of *N. melanostomus* as deduced from Bayesian assignment tests suggest a trisection of the upper Danube instead of a clinal pattern: one downstream sample is assigned together with distant upstream samples to one population cluster. A second cluster comprises central samples, whereas two samples from the margins of this central region appear to have mixed ancestry. AFLP genome scan results indicate this population structure is strongly correlated with extrinsic (geographic) parameters, i.e. migration barriers of anthropogenic origin. However, divergence of at least one AFLP locus correlates positively with a proxy for trophic differentiation, i.e. variation of white muscle δ15N stable isotope signature. In contrast to *N. melanostomus*, no significant population differentiation was detectable in *P. kessleri* along the analyzed invasion pathway. In genome scans of *P. kessleri*, variation of a single locus is strongly positively correlated with an extrinsic parameter combination but not with any ecological parameter.

4.2 Introduction

Invasive species, by definition, arrive, establish and spread in novel environments within very short time frames (Keller et al., 2011). The study of the evolutionary dynamics of invasions may yield inferences about the causal factors leading to invasion success, a key topic in invasion biology. In addition, invasions may reveal otherwise intractable insights into evolutionary processes, e.g. the first and difficult-to-observe steps towards population differentiation and speciation (Hendry et al., 2000; Reznick & Ghalambor, 2001). Whereas the effects and consequences of invasive species on natural communities have been studied in great detail (e.g. Gozlan et al., 2010; for a summary see Sanders et al., 2003), the evolutionary biology of alien species themselves is increasingly receiving attention (e.g. Sakai et al., 2001; Lambrinos, 2004; Hastings et al., 2005; Dlugosch and Parker, 2007; Hänfling, 2007).

Contemporary evolution and invasion success is hypothesized to be shaped to a large degree by intrinsic characteristics of the source populations (e.g. number and genetic constitution of introduced specimens) (Lambrinos, 2004; Björklund & Almqvist, 2010), but also effects of propagule pressure (Allendorf & Lundquist, 2003; Lockwood et al., 2005; Colautti et al., 2006), inbreeding (Nei et al., 1975; Young & Seykora, 1996), phenotypic plasticity (Parker et al., 2003), life history traits (Tsutsui et al., 2000) and migration and dispersal abilities (Sakai et al., 2001; Phillips et al., 2006). In particular, genetic bottlenecks and founder effects can promote but also restrict the speed of adaptive evolution (Tsutsui et al., 2000; Colautti et al., 2004; Stepien and Tumeo, 2006; Prentis et al., 2008). These effects are not apparent in every invasion (Stepien et al., 2005) and the genetic variability of invasive populations can even exceed the one of the source population (Lockwood et al., 2005). Invasive populations evolve under novel and diverse extrinsic conditions, which may differ not only in their ecology but also in the degree of natural or anthropogenic habitat fragmentation and connectivity (Ricciardi & MacIsaac, 2000; Lambrinos, 2004; Bronnenhuber et al., 2011). The interplay between population-intrinsic and environmental factors may fuel or delay the rate of spatial and/or adaptive diversification by changing locally divergent standing genetic variation (Stepien et al., 2005; Mitchell-Olds & Schmitt, 2006; Novak, 2007; Kolbe et al., 2008). Admixture of different native stocks in a single novel inoculation site or after secondary contact of previously allopatric invasive populations may lead to increased standing genetic variation upon which natural selection might act and may lead to local adaptation (Verhoeven et al., 2011). Thus, range expansion, admixture and/or population genetic diversification are often concurrent (Kolbe et al., 2008; Olivieri, 2009) and common in invasions. Especially in human-mediated introductions (Kolbe et al., 2004; Therriault et al., 2005; Roman & Darling, 2007), and at fronted expanding sites (Price & Sol, 2008) differentiation is widespread and can occur within short time frames.

Over the last two decades, the upper Danube River in Germany (Fig. 4.1) has been invaded by numerous invasive species mostly originating from the Ponto-Caspian region (Gollasch & Nehring, 2006). Among those, invasive goby species (Teleostei: Gobiidae) have reached the region probably in ballast water of freight vessels commuting between the lower Danube (Black Sea) region and the lower Rhine (North Sea) along the Rhine Main Danube canal (RMD-canal) (Wiesner, 2005). Bighead goby, *Ponticola kessleri* (Günther, 1861) was first recorded in the central part of the upper Danube close to the city of Straubing in 1999 (Seifert & Hartmann, 2000). Five years later in 2004, round goby, *Neogobius melanostomus* (Pallas, 1814), was detected simultaneously next to the port town of Passau in the lower reach of the Danube in Germany and again in Straubing (Paintner & Seifert, 2006). Both, *N. melanostomus* and *P. kessleri* have a similar ecology (Eros et al., 2005) and their expansion has been fast and successful in terms of fish densities (Brandner et al., 2013b, c) although they have low natural migration rates and small home ranges (Ray & Corkum, 2001; Brownscombe & Fox, 2012, 2013). *Neogobius melanostomus* is a globally invasive species, which has expanded its range rapidly with and without anthropogenic support (Stepien & Tumeo, 2006; Bronnenhuber et al., 2011; Kornis et al., 2012), whereas *P. kessleri* is restricted to central and eastern Europe (Ahnelt et al., 1998; Borcherding et al., 2011a; Brandner et al., 2013b; Kalchhauser et al., 2013). On a local scale in the upper Danube River, both species have colonized the whole 200 km stretch between Passau and the most recently (2010) invaded uppermost area at the junction of the Danube with the RMD-canal, hereby providing a link between invasive populations of the Rhine and the Danube River (Brandner et al., 2013a; Cerwenka et al., 2014b).

Population genetic analyses of *N. melanostomus* from its native Ponto-Caspian as well as from its invasive Eurasian and North American ranges identified two major native mtDNA-lineages from the Caspian and the Black Sea drainages, each with a high intralineage genetic diversity according to microsatellite results (Stepien et al., 2005). Invasive populations from the middle Danube River in Serbia and Slovakia appeared most closely related to a population sample from Odessa (Black Sea drainage). On average, invasive populations exhibited comparatively low levels of genetic diversity, except for the upper Volga population, which contained haplotypes from both divergent lineages (Brown & Stepien, 2008). Populations from the upper Danube River or the River Rhine have not yet been investigated, and population genetic data for *P. kessleri* are not available yet.

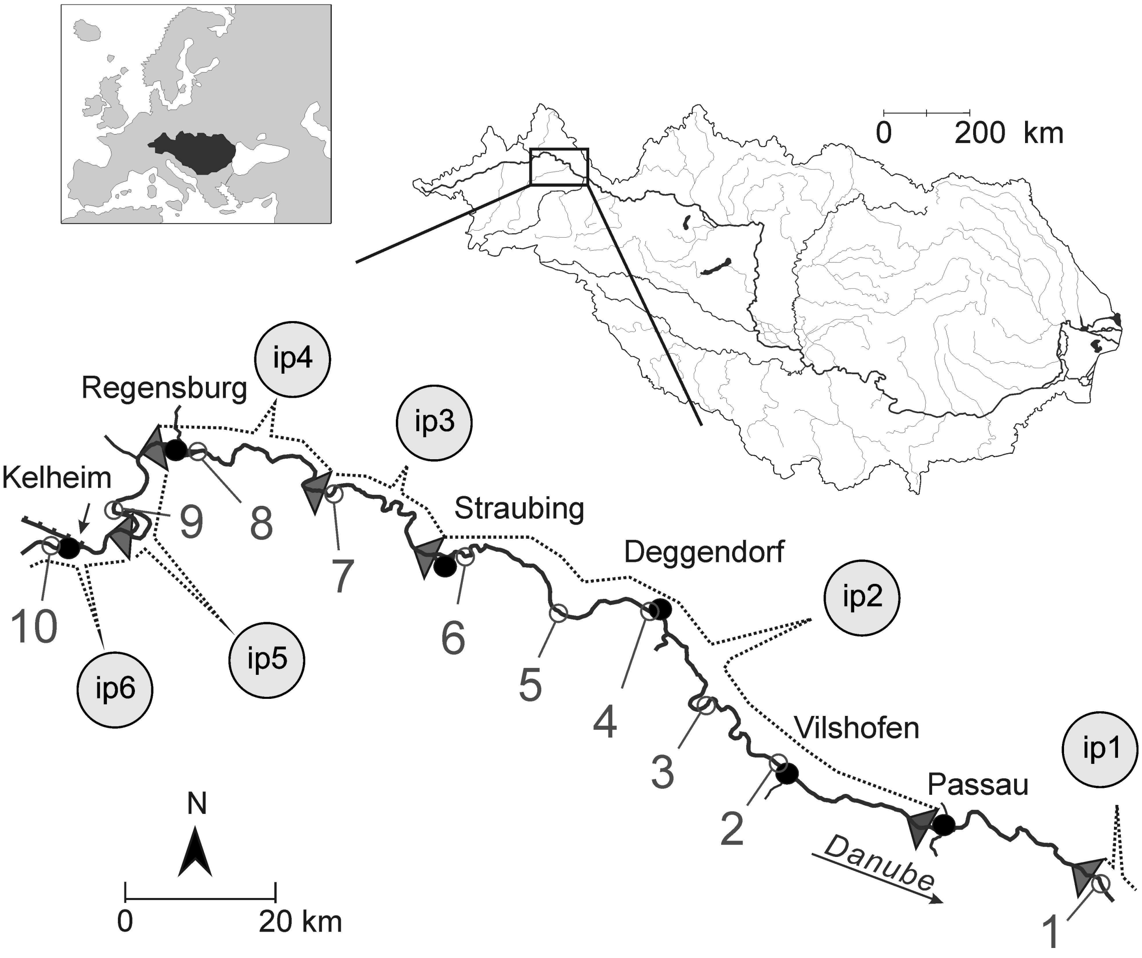

Fig. 4.1: *River stretches 1* to *10* in the study area of the upper Danube River (within the Danube drainage, upper right part). Triangles crossing the river indicate hydroelectrical dams. Populations combined in some analyses to "interdam populations" (*ip 1* to ip *6*) are given in grey shaded circles. The beginning of the RMD-canal at the city of Kelheim is marked by an arrow.

The initial phase of invasions is short and few population genomic studies have assessed the correlation of invasive population differentiation with spatial and environmental factors and genetic admixture (Sakai et al., 2001; Lee, 2002; Kolbe et al., 2004). A comparative approach assessing patterns of population genomic differentiation of two or more invasive species under identical eco-geographical settings could elucidate the relative contribution of intrinsic versus extrinsic ecological and/or geographical factors to invasion dynamics. The present study was designed to compare the dynamics of two simultaneous goby invasions along a small-scale two-dimensional river continuum. The two species are sympatric throughout the investigated river stretch with both recent ("leading edge") and comparatively old ("established") inoculations. The Danube River is intersected by migration-barriers potentially facilitating a rapid built up of locally adapted populations. Using population genomic (Amplified Fragment Length Polymorphism (AFLP)) and to a smaller extent mtDNA data, we investigate baseline population genetics, ancestry and admixture of the upper Danube goby populations and assess the general hypothesis that intraspecific differentiation of two sympatric invasive goby species has developed on a small geographical scale in about ten generations after first introduction. We further hypothesize that the globally less successful species, *P. kessleri* is characterized by a less pronounced local population structure and is therefore less potent for the evolution of local genetic adaptation. In contrast, we expect the highly invasive *N. melanostomus* to exhibit increased local population differentiation correlated with both, barriers to gene flow and ecological parameters.

4.3 Materials and methods

Field sampling, environmental and specimen data

1,053 goby specimens (471 *P. kessleri* and 582 *N. melanostomus*) were sampled from October 2009 to October 2011 at ten river stretches (*stretch 1* (downstream) to *stretch 10* (upstream) along the upper Danube River, Fig. 4.1) and a single site at the River Rhine (near Rees) (Table 4.1). All specimens were collected and processed using an electro-fishing gear following the procedure described in Brandner et al. (2013b, c). Pectoral fin clips were preserved in 96% ethanol p.A. for genetic analyses. All specimens and tissue vouchers were stored in the ichthyology collection of Bavarian State Collection Munich (ZSM). On each sampling site and of each species, two males and two females were collected with a selected total length (L_T) of 8-12 cm. In the field, L_T was measured to the nearest 0.1 cm and sex was determined externally and later verified in the laboratory (for a detailed description, see Brandner et al. 2013b). For each specimen (i) environmental and (ii) specimen-specific parameters were recorded. Environmental parameters were: (i) *stretch 1-10* (i.e. sampled localities which despite of missing information on origin and gene flow are subsequently referred to as “populations”; Fig. 4.1), (ii) *interdam population (ip) 1-6* (i.e. combination of *stretches* separated by artificial dams limiting upstream migration; *ip 1 = stretch 1*, *ip 2 = stretches 2* to *6*, *ip 3 = stretch 7*, *ip 4 = stretch 8*, *ip 5 = stretch 9*, *ip 6 = stretch 10*; Fig. 4.1), (iii) the distance measured in km from the lowermost stretch (*river-km*), (iv) left or right bank of the Danube River (*bank side*), (v) gravel or rip-rap substrate (*habitat type*), and (vi) densities of con- and heterospecific gobies at each sampling site (*competitors*). Specimen parameters were (i) the number of acanthocephalan parasites (*parasite*), (ii) the stable isotopic signatures of muscle tissue ($\delta^{15}N$ and $\delta^{13}C$, determined as in Brandner et al. 2013b) and (iii) body shape measurements (i.e. principal components (*PC*) of geometric morphometric analyses calculated as in Cerwenka et al. 2014b; for *N. melanostomus PC 1-9* and for *P. kessleri PC 1-10*). Table 4.1 summarizes the number of specimens sampled from each stretch differentiated according to specimen and ecological parameters.

Table 4.1: Stretches (i.e. names of localities with the corresponding river system, number of sampling locality and GPS-coordinates of downstream sampling site boundaries) and numbers of analyzed goby specimens separated according to specimen-specific parameters and ecological data. Data on *Neogobius melanostomus* (n = 582) are given at the upper part of every cell and data on *Ponticola kessleri* (n = 471) at the lower part, respectively.

River Stretch			**Number of Specimens**		
Name (River)	**#**	**GPS-coordinates**	**Bank side (right/left)**	**Habitat type (rip-rap/gravel)**	**Sex (female/male/juvenile)**
Engelhartszell	1	E 13°46'29"	30/30	30/30	34/26/0
(Danube)		N 48°28'32"	13/17	5/25	17/13/0
Vilshofen	2	E 13°10'44"	29/36	35/30	32/33/0
(Danube)		N 48°38'24"	19/27	14/32	31/14/1
Winzer	3	E 13°03'08"	32/30	31/31	30/32/0
(Danube)		N 48°43'37"	22/27	26/23	23/26/0
Deggendorf	4	E 12°59'50"	27/31	30/28	29/29/0
(Danube)		N 48°47'31"	25/28	24/29	31/22/0
Mariaposching	5	E 12°52'12"	31/31	32/30	30/32/0
(Danube)		N 48°50'28"	26/23	24/25	27/22/0
Straubing	6	E 12°42'26"	32/25	28/29	29/28/0
(Danube)		N 48°53'34"	26/29	25/30	30/25/0
Geisling	7	E 12°23'37"	29/35	31/33	30/34/0
(Danube)		N 48°58'51"	24/33	30/27	31/26/0
Regensburg	8	E 12°10'41"	33/30	31/32	29/34/0
(Danube)		N 49°00'34"	29/27	27/29	33/23/0
Bad Abbach	9	E 12°00'13"	23/25	38/10	26/21/1
(Danube)		N 48°57'57"	25/38	47/16	30/33/0
Kelheim	1	E 11°53'25"	24/11	33/2	20/15/0
(Danube)	0	N 48°54'27"	5/0	5/0	3/2/0
Rees	1	E 6° 20'12"	8/0	8/0	4/4/0
(Rhine)	1	N51°45'49"	8/0	4/4	3/5/0
all stretches	1-11		298/284	327/255	293/288/1
			222/249	231/240	259/211/1

DNA-extraction, AFLP-genotyping and mtDNA sequencing

DNA from 0.4 – 0.6 cm^2 pectoral fin tissues of samples from 2009 and 2010 was extracted using the Genomic DNA from Tissue Kit (Macherey-Nagel) and of samples collected in 2011 using the DNeasy® Blood and Tissue Kit (QIAGEN). Subsequently, AFLP were detected following Vos et al. (1995) modified by Herder et al. (2008). In order to control for systematic errors due to sample position on 96 well microtiter plates (seven plates for *N. melanostomus* and six plates for *P. kessleri* specimens) in downstream genetic analyses, samples were processed plate by plate. Each plate contained specimen DNA of one goby species from all eleven different localities with the full range of environmental variation (*bank side*, *habitat type*). The season and the year of sampling were present on each plate as far as possible. Samples were AFLP-genotyped with six restrictive amplifications using an ABI 3130 capillary sequencer (PE Applied Biosystem, Foster City, CA, USA) and ROX 500 XL as internal size standard. The primer combinations were *Eco*AGG/*Mse*CTG (Albertson et al., 1999), *Eco*ACA/ *Mse*CAA (Albertson et al., 1999; Barluenga et al., 2006), *Eco*ACA/*Mse*CTG (Barluenga et al., 2006), *Eco*ACT/*Mse*CAA (Geiger et al., 2010), *Eco*AGG/*Mse*CTC (Geiger et al., 2010) and *Eco*ACC/*Mse*CTA (Geiger et al., 2010), fluorescently labeled with HEX and FAM. Bin sets were created with Peak Scanner™ Software Version 1.0 (Applied Biosystems) and peaks were automatically selected and scored using tinyFLP (Arthofer, 2010) with modified adjustments following Geiger et al. (2010). Four *N. melanostomus* and six *P. kessleri* specimens were replicated on each well of the corresponding species after the DNA-extraction.

The complete cytochrome b gene (cytb: 1,138 base pairs (bp)) and partial sequence of the threonine tRNA gene (66 bp) was amplified and partially sequenced for a representative subset of *N. melanostomus* samples (n = 28) from all stretches (four samples from *stretch 6*; three samples from *stretches 10, 8, 7, 5* and *4*; and two samples from *stretches 3-1*). Primers L14724 (Meyer et al., 1990) and H5 (Iwata et al., 2000) were used to PCR amplify a single fragment in 10 µl volume with 5 µl Multiplex Mix (QIAGEN), 1 µl genomic DNA, 0.8 µl of each primer (2.5 nmol), Q-Solution (QIAGEN) and HPLC water. The PCR temperature profile was: 94°C initial denaturation (120 s); 35 cycles with denaturation at 94°C (45 s), annealing at 52°C (30 s) and extension at 72°C (60 s); final extension at 72°C (180 s). PCR products were purified using ExoSAP-IT (USB) and were diluted in 10-20 µl HPLC water. Cycle sequencing was performed using Big Dye 3.1 (Applied Biosystems) with the internal sequencing primer L15066 (Brown & Stepien, 2008), and products were electrophoresed and read using an ABI 3130xl DNA sequencer (Applied Biosystems). Sequences were edited using BioEdit v.7.05.3 (Hall, 1999) after a preliminary alignment using default settings of ClustalW algorithm (Larkin et al., 2007). Resulting cytb haplotypes were compared for sequence identity with 81 cytb haplotypes from the native and introduced range of *N. melanostomus* (Brown & Stepien, 2008). Analyses for cytb haplotypes were not conducted for *P. kessleri*.

Population genomic analyses

Plate-specific effects were reduced by the following pairwise comparisons of peak frequencies after binning. Histograms were computed to visualize differences in frequencies between fragments with the same number of bp using PAST 2.15 (Hammer et al., 2001). All fragments showing higher values of differences than indicated by the first minimum of the according Kernel density were excluded from the following comparisons and the subsequent analyses. Furthermore, following Collin and Fumagalli (2011), all fragments not present on replicated individuals were excluded. This deletion decreases plate-specific effects and increases the likelihood of detecting potentially masked divergence (see Geiger et al., 2010). This procedure results in a comparatively large number of weakly amplified low-frequency AFLP loci being excluded, but is conservative with regard to controlling for type II error based on plate-specific systematic error. Mariette et al. (2002) and Singh et al. (2006) proposed ~200 bands to be sufficient for measuring population genetic variation and differentiation (for a summary see Bonin et al., 2007). Population based pairwise genetic differentiation was measured using analysis of molecular variance (AMOVA) in GenAlEx 6.41 (Peakall & Smouse, 2006) and partitioned in among and within population differentiation. Pairwise ΦPT values (after sequential Bonferroni correction, 9999 permutations) are analogous to F_{ST}-values but applicable to haploid markers, and indicate levels of hierarchical genetic differentiation among populations (Huff et al., 1993). Single pairwise comparisons showing negative values for ΦPT were converted to zero. Differentiation according to *river stretch* and *ip* was assessed in additional AMOVAs.

To test for isolation by distance (IBD) versus a potential influence of anthropogenic barriers to migration (i.e. *dam*), three independent approaches were used. Population differentiation (*stretch* and *ip*) was compared using (i) AMOVAs, (ii) pairwise ΦPT values and (iii) matrix comparisons of pairwise ΦPT values and associated geographic distances between sampling stretches (*river-km*) using Mantel tests in PAST 2.15. The longest continuous free-flowing section of the upper Danube River includes five river *stretches* (i.e. *stretches 2 – 6*) not disrupted by dams. Pearson's correlation coefficients (R) between ΦPT (population differentiation) and *river-km* were calculated within this stretch as well as within our down- and upstream ("sliding window") *stretch sections* of similar length but disrupted by one or more dams (i.e. *stretch 1 – 5, 3 – 7, 4 – 8, 5 – 9, 6 – 10*). Levels of significance for correlation coefficients were computed using 10,000 random permutations. These values should be comparable among all six *stretch-sections* in case of only IBD determining population differentiation, whereas values of *stretch-sections* interrupted by *dams* should be higher if *dams* contributed to population differentiation in addition to geographic distance.

Population genetic structure and AFLP loci linked to genomic regions potentially under divergent selection were further examined using a combination of logistic regression and F_{ST}-outlier based methods. First, F_{ST}-outlier loci were identified using the DFDIST algorithm (Beaumont & Balding, 2004) as implemented in the workbench MCHEZA (Antao & Beaumont, 2011), as well as with BAYESCAN (Foll & Gaggiotti, 2008), both preferentially used for AFLP data (Pérez-Figueroa et al., 2010). In a second step, Bayesian factors were calculated for every marker using BAYESCAN which is based on logistic regressions. Following Mattersdorfer et al. (2012) the threshold to reject the null hypothesis of $\log_{10}$(BF) was set to a value smaller than 0.5, all other adjustments were applied using default settings. Candidate loci identified by DFDIST and BAYESCAN were compared with each other as well as with the selection of candidate loci identified by logistic regressions associated to environmental and specimen-specific parameters using MatSAM Version 2Beta (Joost et al., 2008). Here, potential genetic differentiation was correlated with population-genetic independent environmental or specimen-specific parameters shaping divergent selection; it was tested locus by locus, based on likelihood ratios using "G" and "Wald" tests and the "Cumulated test" (Hosmer & Lemeshow 2000). All p-values were adapted using the conservative Bonferroni correction. The more robust Cumulated test was only performed if both, G and Wald were significant (Joost et al., 2007).

Table 4.2: Number of clusters (K) of *Neogobius melanostomus* (upper part of every cell) and of *Ponticola kessleri* (lower part of every cell) including all loci (all) and excluding the loci potentially under selection (without) detected by DFDIST, BAYESCAN and MatSAM. Clusters are inferred by Ln P(X|Y) with its SD over 9 runs following the method of Pritchard et al. (2000) and ΔK, the second order rate of change of Ln P(X|Y) proposed by Evanno et al. (2005). Numbers in bold indicate most probable numbers of clusters.

K	Ln *P*(X|K) (all)	SD (all)	ΔK (all)	Ln *P*(X|K) (without)	SD (without)	ΔK (without)
1	-16476	<1	NA	-15649	<1	NA
	-29339	<1	NA	-28903	<1	NA
2	-15722	<1	**1067**	-15030	<1	926
	-25671	1	**7081**	-25235	<1	**8227**
3	-15338	1	417	**-14723**	1	952
	-25850	964	<1	**-25106**	112	66
4	**-15337**	1	1	-15173	986	<1
	-25799	709	1	-32313	10731	1
5	-15338	1	<1	-15303	1737	<1
	-26708	3455	1	-32510	10468	1
6	-15339	1	1	-15779	2749	1
	-25560	112	48	-26598	4336	2
7	-15338	1	1	-14724	1	**1290**
	-29757	8370	1	-29787	8191	1
8	-15338	1	1	-15371	1709	1
	-27715	6340	1	-26171	2476	2
9	-15339	1	2	-14724	1	683
	-32244	10410	1	-27371	6736	1
10	-15338	1	1	-14962	715	<1
	-25539	108	63	-32119	11186	1
11	-15338	1	NA	-15175	1353	NA
	-25605	145	NA	-25600	1173	NA

An assumption free, individual based Bayesian algorithm (Falush et al., 2003) implemented in STRUCTURE 2.3.4 (Pritchard et al., 2000) was used to identify genetically distinct population clusters. Individuals were assigned to K populations without prior information on their origin. K = 1 to K = 11 were assessed, each with nine independent replicates with 400,000 MCMC-iterations and a burn-in-value of 200,000. STRUCTURE uses a model-based multivariate analysis and a Bayesian approach under the assumption of Hardy–Weinberg or linkage disequilibrium within each population. However, STRUCTURE appears robust with regard to violations of this assumption (Falush et al., 2003). Calculations were performed using the Bioportal computer service of the University of Oslo (http://www.bioportal.uio.no; Kumar et al., 2009). The most likely number of populations (K) was estimated following Evanno et al. (2005) using STRUCTURE HARVESTER (Earl & vonHoldt, 2012) and the method proposed by Pritchard et al. (2000). To display results graphically CLUMPP version 1.1.2 (Jakobsson & Rosenberg, 2007) and distruct version 1.1 (Rosenberg, 2004) were used. To reveal the potential influence of selection on spatial genetic structuring, all calculations and graphical illustrations were performed once using all loci and once without the candidate loci detected by MatSAM, DFDIST and BAYESCAN.

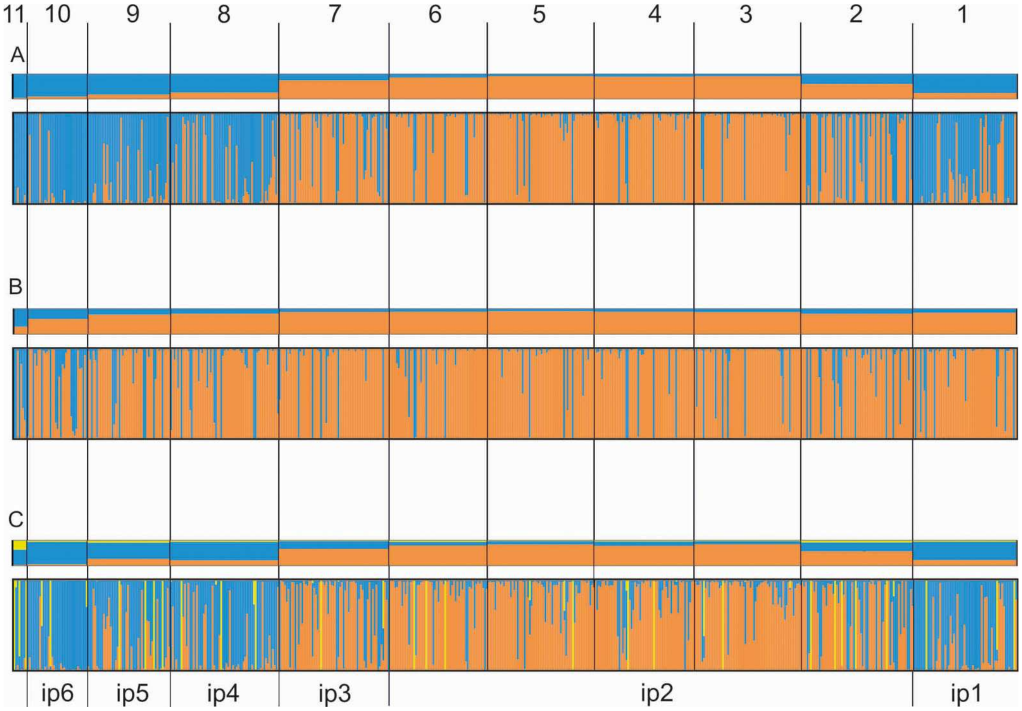

Fig. 4.2: Population structure of *Neogobius melanostomus* identified using STRUCTURE Bayesian assignment analysis, were K is the number of clusters predefined. The uppermost graphs show the population and the lowermost the individual matrix, respectively. A: K = 2 including all loci (n = 189). B: K = 2 and C: K = 7, both without loci potentially under selection (n = 186) identified by MatSAM, BAYESCAN and DFDIST. Numbers of river stretches are indicated at the uppermost and interdam populations (*ip*) at the lowermost part of the figure.

To test for "surfing allele" candidates (sensu Manel et al., 2009) allele frequency distributions were screened under two criteria: (i) they should be outliers detected by DFDIST but not by BAYESCAN and MatSAM (their frequency deviation should not correlate with extrinsic parameters), and (ii) allele frequencies at "leading edge" populations from *stretch 8, 9* or *10* according to Brandner et al. (2013a) should be significantly increased as pairwise compared to "established" populations. Band presence frequencies between ten Danubian "populations" were tested for differences by multiple pairwise non-parametric Kruskal-Wallis tests (Bonferroni corrected).

4.4 Results

Neogobius melanostomus

All 28 partially sequenced cytochrome b haplotypes (862 bp) as well as the three nearly completely sequenced (1,115 bp) haplotypes were identical to each other on the 862 bp stretch as well as to the most common "Black Sea basin" haplotype 1, i.e. there is no indication of mtDNA admixture of phylogeographically different groups in the upper Danube River.

After correction for potential plate-specific effects, the number of detected polymorphic AFLP-bands was 189. Individual band frequency ranged between 7 and 100% (mean = 19%). No fragment occurred with frequencies lower than 5% and 29 fragments were present in more than 95% of all individuals.

STRUCTURE analyses for K = 1 to K = 11 revealed two or seven genetically distinct clusters being most likely. Applying the method of Evanno et al. (2005) it was K = 2 for the complete dataset, whereas after excluding the three candidate loci potentially under divergent selection detected by MatSAM, DFDIST and BAYESCAN, the number increased to K = 7 (Table 4.2, Fig. 4.2). One of the two major clusters combined individuals from disjunct regions, i.e. the uppermost three populations (*stretch 8 – 10*), the River Rhine (*stretch 11*) and the lowermost population (*stretch 1)*; in contrast, most individuals of the central populations (*stretch 2 – 7*) where assigned to the second cluster. Removal of the three candidate loci resulted in a shift of individual assignments to populations of the central stretch. Individuals of "populations" at *stretch 1*, *8* and *9* were almost entirely assigned to the central cluster, and about 20% of the individual genetic constitution of the uppermost Danubian "population" at *stretch 10* was assigned to the cluster from River Rhine (before removal they shared almost 100% of the loci, Fig. 4.2).

The majority (95%) of the genetic variance was explained by within Danubian "population" structure (i.e. by *stretch*), whereas 5% was explained by among *stretch*. Overall ΦPT was 0.05 (Danubian specimens only: $\Phi PT = 0.045$) which is significantly different compared to the variance calculated from randomly generated data (AMOVA all: $p < 0.05$, Danubian "populations" only: $p < 0.001$). Individuals from River Rhine (mean $\Phi PT = 0.176$) showed highest levels of differentiation (mean ΦPT of several pairwise *stretch*-based comparisons ranged between 0.036 at *stretch* 2 and 0.085 at *stretch 9*). Without the "population" from the

River Rhine mean Φ*PT* was smaller and ranged between 0.025 at *stretch 2* and 0.076 at *stretch* 9). All pairwise comparisons were significant (with and without specimens from River Rhine, using Bonferroni corrections and 9,999 permutations: all $p < 0.05$), after exclusion of comparisons showing negative Φ*PT* values and with exception of comparisons between *stretch 6* and *stretch 3* to *5* and of comparison between *stretch 4* and *stretch 6* (all $p > 0.05$). Detailed results are given in Table 4.3.

Estimated differences between allele frequencies among *stretches* was 5% and among *interdam populations* 1%. Pairwise population differentiation of river *stretches* (Φ*PT* values) was not significant for comparisons between *stretches* not disrupted by a dam, i.e. *4* and *3*, *5* and *6*, and between *stretch 5* and *6* (all $p > 0.05$). Overall differentiation was correlated to the geographic distance between *stretches*, when comparing all Danubian "populations" (R = 0.49, $p < 0.05$), and *stretch-sections* interrupted by at least one dam, i.e. *1 – 5* (R = 0.66) and *6 – 10* (R = 0.77, both: $p < 0.05$). Correlations were close to significant for s*tretch-sections 4 – 8* (R = 0.60, $p = 0.05$) and for *5 – 9* (R = 0.66, $p = 0.07$) but not for *stretch-section 3 – 7* ($p > 0.1$) and *stretch-section 2 – 6* ($p > 0.1$) which is not interrupted by a dam.

Candidate loci potentially under selection were identified using three population genetic analysis tools. BAYESCAN identified a total of 15 candidate loci under the criterion of log10(BF) greater than 0.5. Following Jeffreys' interpretation of the Bayes factors, five of those loci were under "strong" selection, one under "very strong" and nine were indicated to be under "decisive selection".

DFDIST detected outliers potentially being under positive selection and outliers possibly having a balancing effect by comparing the calculated F_{ST}-values with simulated ones under neutral conditions. For *N. melanostomus* the overall calculated F_{ST} was 0.045, the simulated one was 0.044. DFDIST suggested 28 loci as candidates under balancing and nine under divergent selection.

MatSAM logistic regressions of extrinsic parameters provided an independent evaluation of BAYESCAN and DFDIST outliers. Seven loci were detected to be potentially under selection. Five loci were associated with parameters related to spatial heterogeneity and barriers to migration: one locus correlated with the parameters *stretch*, *river-km* and *interdam-population*, and four loci to *interdam population*. In addition one outlier was assigned to *isotopic signature of* $\delta^{15}N$ and one to $\delta^{13}C$.

The total number of outliers, thus being candidate loci under divergent selection recognized by all three methods (MatSAM, DFDIST and BAYESCAN) was three. One locus was found to be correlated with all three tested spatial parameters *stretch*, *river-km* and *interdam population*, one single with the spatial parameter *interdam population*, and one single with $\delta^{15}N$ *isotopic signature*.

A single AFLP-locus potentially surfing at the leading edge of *N. melanostomus* of the upper Danube River (i.e. *stretch 10*) was detected being present in 32% of all analyzed individuals at *stretch 10*. This locus was not present in individuals at *stretch 3 – 5*, *7* and *8* and a significantly lower number of individuals had this fragment at *stretch 6* ($p < 0.001$) and *stretch 9* ($p < 0.05$). Higher frequencies were observed at *stretch 2* (12.5%) and at *stretch 1* (15%).

Table 4.3: Pairwise Φ*PT* of goby populations at river stretches according to species. *Neogobius melanostomus* are indicated in the upper right part of the table, whereas *Ponticola kessleri* in the lower left part. Stretches 1 to 10 are part of the Danube River and stretch 11 of the River Rhine. Non-significant differences are indicated by n.s., significant differences according to $p < 0.05$ by *, $p < 0.01$ by ** and $p < 0.001$ by *. Comparisons with negative Φ*PT* were converted to zero.**

stretch	1	2	3	4	5	6	7	8	9	10	11
1		0.022 ***	0.073 ***	0.065 ***	0.07 ***	0.066 ***	0.044 ***	0.011 ***	0.025 ***	0.022 ***	0.147 ***
2	0.016 **		0.018 ***	0.013 ***	0.014 ***	0.013 *	0.013 ***	0.037 ***	0.061 ***	0.036 ***	0.136 ***
3	0.003 n.s.	0.006 n.s.		0.012 *	0 n.s.	0.005 *	0.011 *	0.081 ***	0.121 ***	0.085 ***	0.244 ***
4	0.003 n.s.	0.003 n.s.	0 n.s.		0.004 n.s.	0 n.s.	0.019 ***	0.078 ***	0.117 ***	0.083 ***	0.199 ***
5	0.003 n.s.	0.009 **	0 n.s.	0 n.s.		0 n.s.	0.012 ***	0.08 ***	0.117 ***	0.083 ***	0.223 ***
6	0.006 n.s.	0.006 *	0 n.s.	0 n.s.	0.003 n.s.		0.018 ***	0.077 ***	0.111 ***	0.081 ***	0.195 ***
7	0.003 n.s.	0.009 **	0.006 *	0.007 *	0.011 **	0.001 n.s.		0.045 ***	0.078 ***	0.059 ***	0.197 ***
8	0.01 *	0.016 ***	0.01 **	0.006 *	0.011 **	0.004 n.s.	0.004 n.s.		0.019 ***	0.03 ***	0.143 ***
9	0.02 **	0.021 ***	0.016 **	0.016 ***	0.021 ***	0.009 **	0.007 **	0.005 *		0.038 ***	0.161 ***
10	0 n.s.	0.035 n.s.	0 n.s.	0 n.s.	0 n.s.	0.003 n.s.	0.013 n.s.	0 n.s.	0.012 n.s.		0.119 ***
11	0.021 n.s.	0.072 ***	0.035 n.s.	0.029 n.s.	0.02 n.s.	0.037 n.s.	0.038 n.s.	0.039 *	0.048 *	0.004 n.s.	

Ponticola kessleri

After correction for potential plate effects, the number of detected polymorphic AFLP-fragments was 372 and individual band frequency ranged between 1 and 100% with a mean of 15%. 280 fragments occurred with frequencies lower than 5% and 30 were present in more than 95% of all individuals.

In *P. kessleri* no clusters were recognizable for K = 1-11 in STRUCTURE analyses. As the method of Evanno et al. (2005) cannot be used to estimate the number of clusters for the K extremes (1 and 11) the log probability of Ln P(X|K) was used following Pritchard et al. (2000). However it indicated K = 10 to be most probable (Table 4.2). Excluding the single candidate locus potentially under selection revealed by the three methods used (see below) the highest likelihood for the number of clusters was K = 2, applying the method of Evanno et al. (2005) and K = 3 using the log probability of Ln P(X|K) but K = 2, 6, 8, 9 and 11 showed also comparatively high values of ΔK (Table 4.2). In both cases with and without consideration of the single locus under selection no apparent spatial population structure could be detected (data not shown).

The main part of genetic variance (99%) was explained by "populations" structure (i.e. within *stretch*), whereas 1% was explained by among *stretch*. An exclusion of low sample size populations (n = 8 individuals from River Rhine and n = 5 from *stretch 10*) did not change the molecular variance of the remaining populations. Φ*PT* of all specimens was 0.008 and did not indicate significant population differentiation (AMOVA: $p > 0.05$). However, differentiation was significant when regarding Danubian specimens only (Φ*PT* = 0.007, AMOVA: $p < 0.001$). Mean Φ*PT* values of pairwise comparisons were highest for the River Rhine "population" (mean Φ*PT* = 0.034) and lowest for the one from *stretch 4* (mean Φ*PT* = 0.006). In total, 32 Φ*PT* values of pairwise *stretch* comparisons were not significant (all: Bonferroni corrected, 9,999 permutations, $p > 0.05$). Detailed results are given in Table 4.3.

Differentiation measured in Φ*PT* was 1% among *river stretches* and 2% among *interdam populations*. Φ*PT* values were not significant for comparisons between *stretch 3* and *stretch 4*, *5* and *6*, and for comparison between *stretch 4* and *6*. Φ*PT* values correlated positively to the geographic distance between "populations" (R = 0.36, $p < 0.05$), and to *stretch-section 3 – 7* (R = 0.87), *4 – 8* (R = 0.84) and *5 – 9* (all: $p < 0.05$). Population differentiation within s*tretch-sections 1 – 5* (R = 0.26), *6 – 10* (R = -0.18) and the *stretch-section* not disrupted by a dam (R = 0.05) did not correlate with the distance between *stretches* (both: $p > 0.1$).

BAYESCAN identified four candidate loci potentially under selection using the criterion of log10(BF) greater than 0.5. Following Jeffreys' interpretation of the Bayes factors, three of those loci were under “substantial” and one under “decisive” selection. DFDIST identified 79 loci potentially being under selection, 18 under positive and 61 under balancing selection. Logistic regressions using MatSAM detected a single locus indicating spatially controlled divergence, i.e. for the factors *stretch*, *river-km* and *ip*. All three methods identified this particular locus.

No locus could be identified to potentially surf at the leading edge of *P. kessleri*.

Cytochrome b haplotype analyses of native *P. kessleri* populations are still lacking and thus invasive specimens from the upper Danube River were not analyzed.

4.5 Discussion

Invasive organism structure evolves dynamically and may leave different signatures resulting from intrinsic and extrinsic factors. Population genomic AFLP data of invasive gobies in the upper Danube River show that the more recent newcomer, i.e. the globally invasive *N. melanostomus,* is significantly differentiated to a comparatively large degree (~ 5%) and exhibits pronounced small-scale population structure along a 200 km river section. Local genetic population structure of *N. melanostomus* suggests a trisection: one downstream sample is assigned together with distant upstream samples to a first population cluster, the central samples to a second one, and two samples from the margins of the central region appear to have mixed ancestry. Divergence of at least one locus correlates with a proxy for trophic differentiation, i.e. variation of white muscle $\delta^{15}N$ stable isotope signature in this species. No significant population differentiation of *P. kessleri* is detectable, and in genome scans, variation of only one single locus was strongly correlated with an extrinsic, geographic parameter combination.

The comparison between *P. kessleri* and *N. melanostomus* in the upper Danube River highlights that rapid population differentiation in invasive organisms can be different under identical extrinsic settings. Apparently, the interplay of in- and extrinsic factors e.g. the number of inoculation events, propagule pressure, origin of invaders, and/ or potential genetic admixture acts differentially resulting in interspecifically different evolutionary responses. In addition, intrinsic factors as phenotypic plasticity or different levels of standing genetic variation may act and change the population genetic constitution and therefore the different population genomic basis of non-native species.

Origin of invasive genomic diversity

Invasion success and rapid population differentiation appear to act synergistically, and might enhance the speed of invasion (Grosholz, 2002; Lee, 2002; Björklund & Almqvist, 2010). The genomic constitution of native population(s) potentially contributes to the success of invasive species (Mitchell-Olds et al., 2008; Geneva & Garrigan, 2010). Theoretically, population differentiation may be enhanced, if allopatrically differentiated strains amalgamate into a new (invasive) population, characterized by an instantaneously elevated standing genetic variation (Lucek et al., 2010). Despite being significantly differentiated, mtDNA variation of *N. melanostomus* in the upper Danube is zero, as all analyzed individuals carried the same Black Sea basin haplotype. Therefore, rapid differentiation of this species is most likely not caused by an admixture of phylogenetically strongly distinct source populations from the Caspian and Black Sea basin (i.e. by a Wahlund effect; Björklund & Almqvist, 2010). This has been suggested for an invasive *N. melanostomus* population of the Volga region (Brown & Stepien, 2008). Investigations of native *P. kessleri* populations are still lacking.

However, since this result is based on matrilinearly inherited and comparatively slowly evolving mtDNA only, an admixture of related populations, even of a male Caspian contribution, cannot be excluded completely. Therefore it remains open, whether the observed rapid population differentiation in *N. melanostomus* is the result of multiple introductions of closely related but nevertheless pre-differentiated populations, as it has been shown by several studies in invasion biology summarized by Prentis et al. (2008) and Vellend et al. (2007). Support for this scenario comes from the interspecific comparison, because *N. melanostomus* showed considerably higher overall genetic variability than *P. kessleri*. An alternative explanation, i.e. decreased genetic variability due to genetic bottlenecks in *P. kessleri* potentially restricting differentiation (Stepien & Tumeo, 2006), cannot be ruled out without comparative data for both species from different invasion regions as well as from source populations. Under this scenario, low effective population size and low levels of immigration (Fitzpatrick et al., 2011) would have contributed to lower values of population differentiation in *P. kessleri*. A higher vulnerability to inbreeding (Frankham, 2005) might then explain the observed numerical decrease of *P. kessleri* over the years (own data), independent from effects of interspecific competition.

Factors correlated with invasive population differentiation

Population differentiation may be favored by barriers to gene flow and by IBD (see Meldgaard et al., 2003). In invasive *N. melanostomus* and *P. kessleri*, genetic differentiation was shown across very short geographic distances, and factors correlating with population structure were mostly of geographic nature. Hence, local population structure evolved over very short time spans and few generations, and appears to be supported by extrinsic factors in the upper Danube River. (Anthropogenic) Barriers to gene flow seem to be decisive for locally different success of both gobiid species, even if different source populations would be a major reason for population variation and differentiation. In conjunction with established subpopulations, population genetic effects may promote the rapid evolution of population structure, e.g. “allele surfing”. A rapid increase of previously low allele frequencies in expanding fronted populations (Klopfstein et al., 2006; Excoffier & Ray, 2008) is hypothesized to be typical for invasive populations. Gobies, having an extended spawning period with males guarding nests aggressively and females spawning several times (Charlebois et al., 1997; Corkum et al., 2004; Groen et al., 2012), should be prone to allele surfing through the “Hedgecock effect” (see Hedrick, 2005), where a low number of parental individuals have a high number of offspring. Allele surfing probably is likely to have contributed to local population structure of *N. melanostomus* and was detectable in a strongly differentiated, but yet comparatively young “leading edge” sampling site, i.e. at the uppermost stretch of the Danube River, which was seeded after the year 2009 or even 2010 (Brandner et al., 2013a, b). In contrast, no surfing allele could be detected in *P. kessleri*, underlining the lower genetic and phenotypic (Cerwenka et al., 2014b) variability and the lower population differentiation in this species.

Proxies of trophic and morphometric differentiation as indicators of genomic adaptation

Natural selection may trigger local adaptation even at small geographic scales, but it is often difficult to identify single factors driving local adaptation (Collin & Fumagalli, 2011). In addition, local adaptation can be confounded with genetic signatures of introgression if only F_{ST}-based genome scans are used to identify candidate loci, because allochthonous introgressed alleles in a subsample may mimic selectively favored high allele frequencies (Gagnaire et al., 2011; Mattersdorfer et al., 2012; Gosset & Bierne, 2013). However, in *N. melanostomus* genomic differentiation at a candidate locus has been identified not only on the basis of F_{ST} genome scans but also by logistic regression against a proxy for niche segregation ($\delta^{15}N$ isotopic signature), indicating genomic adaptation to alternative trophic niches. Nitrogen stable isotopes provide a temporally integrated, quantitative perspective on individual diet and are indicative of the relative trophic position of an individual. Although both goby species are generalistic omnivores (Borcherding et al., 2013; Brandner et al., 2013b), the differential variation of *N. melanostomus* in the upper Danube matches with results showing that this species exhibits a greater feeding niche width, and a lower degree of specialization than *P. kessleri*; this possibly reflects a higher degree of individual adaptation to available prey in comparison to *N. melanostomus* (Brandner et al., 2013b).

Nevertheless, the individual trophic niche positions of *N. melanostomus* specimens could be the result of a phenotypically plastic response. *Neogobius melanostomus* is indeed known for its broad diet and high feeding versatility, which could indicate phenotypic plasticity, as deduced from observations in different ecosystems (Gaygusuz et al., 2007). Phenotypic plasticity, where different phenotypes are expressed under different environments (Fitzpatrick et al., 2012),can be important in successfully invaded environments (Agrawal, 2001). In *N. melanostomus* of the upper Danube River phenotypic plasticity is highly probable (Cerwenka et al., 2014b). In addition, population differentiation based on geometric morphometric data revealed a similar geographic trisection into an upper, central and lower part of the River as in the genetic analysis. Habitat parameters and body shape variation were not identified as significant in logistic regression analysis, i.e. no single allele frequency was detected that significantly corresponded to proxies of morphometric PCs or habitat type. This renders phenotypic plasticity more probable, which is considered as an important “jump-starter” directly after inoculation (Collyer et al., 2007). It may drive subsequent directional evolution and it may facilitate rapid adaptive evolution leading to rapid success in invasive species.

Population expansion and spreading

Population structure of non-native species may differ according to expansion mechanisms (e.g. Currat et al., 2008; Bronnenhuber et al., 2011). Results of population structure analyses revealed assignment of *P. kessleri* specimens along the upper Danube River to only a single population which corresponded to comparatively low levels of overall genomic variability. This could either suggest a bottleneck situation at or after inoculation, or an already depauperate native *P. kessleri* stock. The *N. melanostomus* population trisection along the upper Danube River suggests disjunct inoculations from multiple founder populations. The most likely mode of inoculation is by transportation of eggs or larvae in ballast water vessels rather than by active migration since this species has small home ranges and limited migration rates in adults (Bronnenhuber et al., 2011; Gutowsky & Fox, 2011; Brownscombe et al., 2012; Kornis et al., 2012). Population expansion proceeded in upstream direction, with dams acting as barriers to gene flow, in both species. Nevertheless, passive downstream drift of juvenile gobies, may explain the existence of a genetically intermediate population (*stretch 7),* which is located between the central (*stretch* 3 to 6) and the upper part of the upper Danube (*stretch 8* to *10*). Drift has been shown to be significant for invasive gobies (Hensler & Jude, 2007; Hayden & Miner, 2008; Björklund & Almqvist, 2010; Janáč et al., 2013), but its importance is most likely underestimated as compared to active dispersal. Nevertheless, single *N. melanostomus* are known to move long distances at least in upstream direction as described by Bronnenhuber et al. (2011), Kornis et al. (2012) and Brandner et al. (2013a), and hereby could have contributed to an admixture of genetic clusters at least at *stretch 2* and *stretch 7*. Multiple inoculations in combination with subsequent downstream drift and active dispersal may thus have contributed to population admixture and possibly to invasive success.

In conclusion, population differentiation and expansion, as well as factors correlating with it are clearly species-specific in our case. Despite highly similar invasion histories of *N. melanostomus* and *P. kessleri* in the upper Danube River their invasive populations respond differentially to spatial and ecological parameters. The species having a higher variability in life-history traits, phenotype and nutrition (*N. melanostomus*), responds to its novel non-native area by rapid population genomic differentiation on a local level. *Neogobius melanostomus* is by far the most successful of invasive goby species in terms of fastly establishing high density populations on a global scale. The correlation between rapid responses to locally different environments suggests a significant contribution of genomic adaptability to invasion success. Barriers to gene flow conducting to a subdivision of non-native populations increase rather than decrease the potential for local adaptation in “plastic invaders” as *N. melanostomus*. Apparently the less plastic and more inertially invader, i.e. *P. kessleri* is responding less flexible at the genomic level to these extrinsic factors.

5 Interspecific hybridization between invasive goby species

A similar version of this chapter was published as: Lindner, K., A. F. Cerwenka, J. Brandner, S. Gertzen, J. Borcherding, J. Geist and U. K. Schliewen, 2013. First evidence for interspecific hybridization between invasive goby species *Neogobius fluviatilis* and *Neogobius melanostomus* (Teleostei: Gobiidae: Benthophilinae). *Journal of Fish Biology* **82**: 2128–2134.

5.1 Abstract

Two hybrids between the monkey goby *Neogobius fluviatilis* and the round goby *Neogobius melanostomus* from the Rhine River were identified by genotyping and morphological comparison. These are the first records of goby-hybrids outside the parent species' native ranges worldwide.

5.2 Introduction, material and methods, results and discussion

Ponto-Caspian gobies of the subfamily Benthophilinae (Perciformes: Gobiidae) are highly successful invaders of fresh waters both in North America and Europe (Stepien & Tumeo, 2006; Kornis et al., 2012). As Benthophilinae species are not known to undertake long migrations in river systems, their ongoing invasion of lotic ecosystems is most likely triggered by long distance transports of eggs and larvae in ballast-water tanks of cargo ships, traveling between the Black Sea (lower Danube River) and the lower Rhine River through the Rhine–Main–Danube junction (Ahnelt et al., 1998; Wiesner, 2005, Harka & Bíro, 2007). In Germany, they have recently proceeded as far as the upper Danube and the lower Rhine River. Five invasive species are currently known from Germany: bighead goby *Ponticola kessleri* (Günther 1861), round goby *Neogobius melanostomus* (Pallas, 1814), monkey goby *Neogobius fluviatilis* (Pallas, 1814) (Ahnelt et al., 1998; Harka & Bíro, 2007; Borcherding et al., 2011a), tubenose goby *Proterorhinus semilunaris* (Heckel, 1837) (Ahnelt et al., 1998; Harka, 1990) and most recently the racer goby *Babka gymnotrachelus* (Kessler, 1857) (Haertl et al., 2012).

Fish hybrid lineages, *i.e.* of the sculpin genus *Cottus*, have recently invaded the Rhine River system and their hybrid genome may have contributed to their invasion success (Nolte et al., 2005). In contrast, Benthophilinae hybrids have remained unknown from anywhere in their invasion range worldwide, *i.e.* neither from the Rhine nor from elsewhere in Europe or North America. A *N. fluviatilis* × *N. melanostomus* hybrid specimen, however, has been identified based on morphological data (Pinchuk, 1970). It was found in Taman Bay along the Kerch Strait, connecting the Black Sea with the Sea of Azov, lying within both gobies' native range. Individuals from Pinchuk (1970) showed the compact trunk form and the steep snout profile, which is characteristic of *N. melanostomus*, but lacked the species' diagnostic black spot on the first dorsal fin (D1).

This study offers first molecular and tentative morphological evidence for two putative Benthophilinae hybrid specimens (Fig. 5.1) collected from the Rhine River. One specimen was kept and photographed alive in an aquarium and a tissue sample is available [BayFi 11090, *i.e.* collection number of the Zoologische Staatssammlung München (ZSM), Germany, for Bavarian fish tissues], but the specimen was not preserved as a body voucher and therefore no morphometric data are available. The second specimen originally identified as *B. gymnotrachelus* by Borcherding et al. (2011b) was tissue sampled (BayFi 11577) and preserved (ZSM 41266), but no live photograph is available. Despite their shared overall similarity with *N. melanostomus* and *N. fluviatilis*, they did not key out as one of the two species as based on morphometric data given for both subspecies of *N. fluvatilis*, (*i.e.* *N. f. fluviatilis* and *N. f. pallasii*) and for *N. melanostomus* in Pinchuk et al. (2003a, b)*:* The *N. melanstomus*-like D1 spot is present in BayFi 11090, albeit not nearly as dark and rounded as would be expected in a pure bred *N. melanostomus*, but is not present in BayFi 11577. Both specimens have a rather slender tail typical for *N. fluviatilis*, but they differ in their head shape as reflected in the position of the jaw angle, which is below (BayFi 11577) and slightly behind the anterior margin of the eye in specimen (BayFi 11090; judged from the photograph), but certainly not between the anterior margin of the eye and the posterior nostril, as it would be typical for *N. fluviatilis*. Morphometric data for the measurable voucher specimen BayFi 11577 are: nape covered completely with cycloid scales (*N. melanostomus*: nape scales cycloid on anterior and middle nape; *N. fluviatilis*: nape scales ctenoid); pelvic disc as of abdomen length [pelvic origin to anus: 0.85 (*N. melanostomus*: 0.6–0.8; *N. fluviatilis*: > 0.9)]; caudal peduncle depth as of caudal peduncle height: 0.6 (*N. melanostomus*: around 0.66; *N. fluviatilis*: 0.5–0.6); interorbit as of eye diameter 0.94

(*N. melanostomus*: 0.8–1.0; *N. fluviatilis*: > 0.73–0.75); lateral line scale counts not possible due to damage. For a visual comparison of the two putative hybrid specimens with *N. fluviatilis* and *N. melanostomus* see Fig. 5.1.

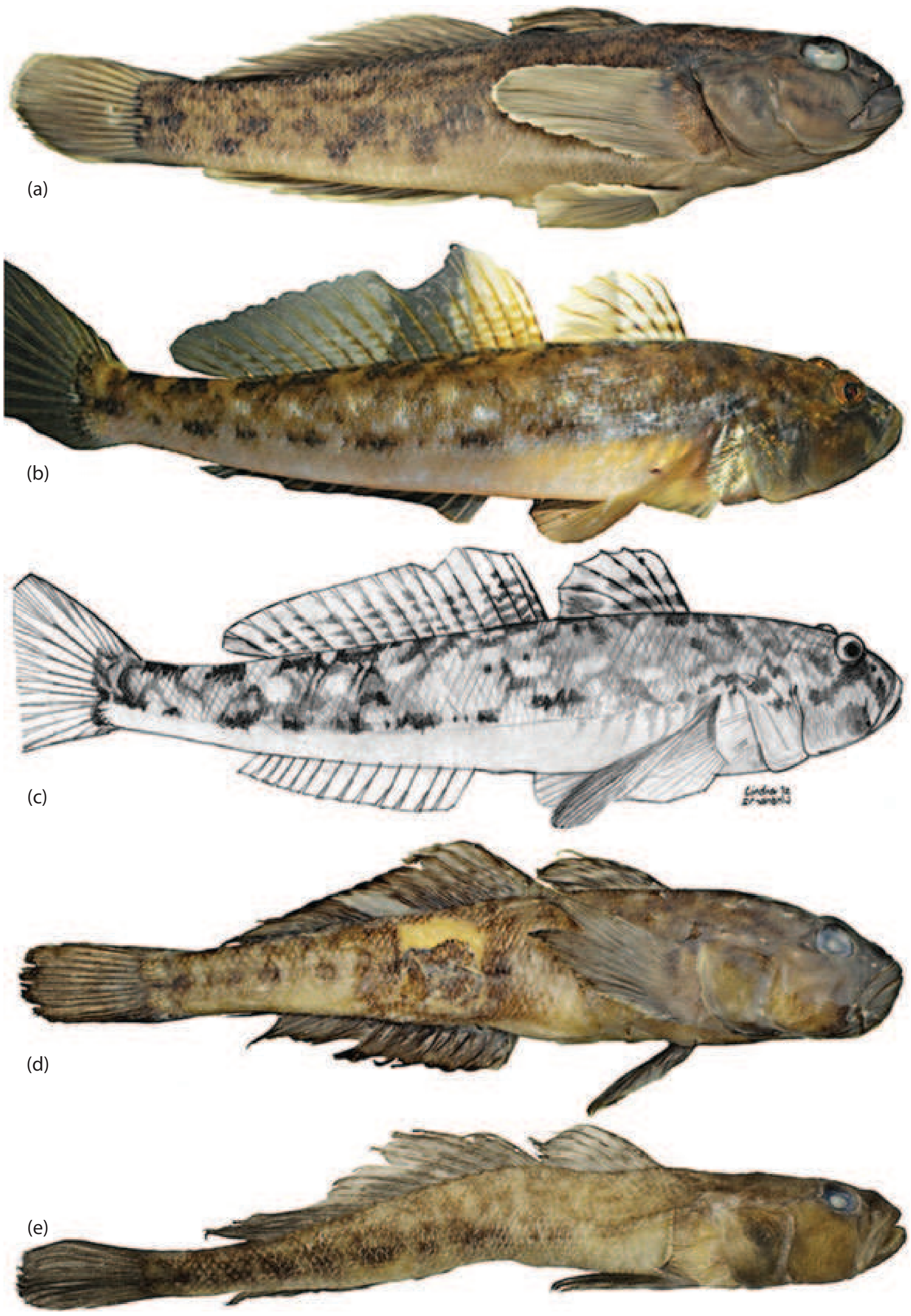

Fig. 5.1: Comparison of hybrid specimens and parental species: (a) *Neogobius melanostomus* ZSM 40237, 87.8mm standard length (LS), (b) hybrid specimen, (BayFi-tissue collection #11090; not preserved, (c) drawing from the above photograph with spot on first dorsal fin D1 pronounced for better visibility, (d) hybrid specimen ZSM 41266 (BayFi-tissue collection #11577), 87.7mm LS and (e) *Neogobius fluviatilis* ZSM 41740 (BayFi-tissue collection #11090), 95.8mm LS.

In order to test for the putative hybrid status of both specimens as suggested by their morphological intermediacy, 45 Benthophilinae specimens of all potential hybrid partners were compared genetically using the amplified fragment length polymorphism (AFLP) method (Vos et al., 1995) as modified and described by Herder et al. (2008). One individual (*N. fluviatilis*, BayFi 11518) was included twice as a quality control. Multiple specimens of the five different Bethophilinae species were included for the direct comparison with the supposed hybrids. An unambiguously identified *B. gymnotrachelus* sample from the Dnieper River (Ukraine) was included as the species status of putative German specimens had not yet been substantiated with genetic methods.

To establish AFLP-genotypes, genomic DNA was digested with the restriction endonucleases MseI and EcoRI followed by ligation of PCR adaptors, specific to the cutting sites of the fragments. First, a selective PCR was performed with one base extension (cytosine and adenine) on each primer (MseI-C and EcoRI-A). Subsequent selective amplifications were performed with two additional bases added to the 3'end of the two primer pairs (MseI/EcoRIDYE): TA/GG and AT/CA in first run and TA/TC and GA/CC in a second run. AFLP fragments were separated on an automated capillary sequencer (ABI 3130) in two separate runs. The resulting electropherograms were generated using the Peak Scanner-software (Applied Biosystems; www.appliedbiosystems.com) and were binned using the program TinyFLP (Arthofer, 2010). The data from the two selective amplifications were combined using TinyCAT (Arthofer, 2010). Fragment presence or absence of AFLP fragments was coded as 0 and 1, respectively, yielding a total of 894 bins. On the basis of the resulting fragment matrix, distances between individuals were calculated with the NeighbourNet algorithm and visualized in a NeighbourNet-phylogenetic-network (Bryant & Moulton, 2004) in the programme SplitsTree4 (Huson & Bryant, 2006). In addition, a principal component analysis (PCA) was performed on the same 0/1 fragment matrix, and PCA plot of PCI and PCII prepared in order to compare an assumption-free clustering pattern with the NeighbourNet network, using the programme PAST (Hammer et al., 2001).

The hypothesis that hybrids exhibit a mosaic of molecular characters (AFLP-bins coded 0 or 1) of both parent species, yielding an intermediate genotype was investigated. Both the NeighbourNet-analysis (Fig. 5.2a) and the PCA (Fig. 5.2b) identified five separate clades or clusters, containing the five Benthophilinae species *P. semilunaris*, *P. kessleri*, *B. gymnotrachelus* and *N. fluviatilis*, as well as *N. melanostomus*. The putative hybrid specimen BayFi 11090 was clearly positioned intermediate between *N. fluviatilis* and *N. melanostomus* in both analyses. The outlier position of specimen BayFi 11577 at a distal position of the *N. fluviatilis* cluster also indicates intermediacy of this specimen, but less clearly and with a more dominant *N. fluviatilis* genome partition. The intermediacy of both specimens is skewed towards *N. fluviatilis*, thus tentatively implying that hybridization occurred within one or more generations in the past, *i.e.* both individuals are likely to be backcrosses with *N. fluviatilis*. A detailed statistical analysis of the genomic partitioning with respect to parental species, however, is not possible without a greater sample size of both hybrids and hybrid partner specimens.

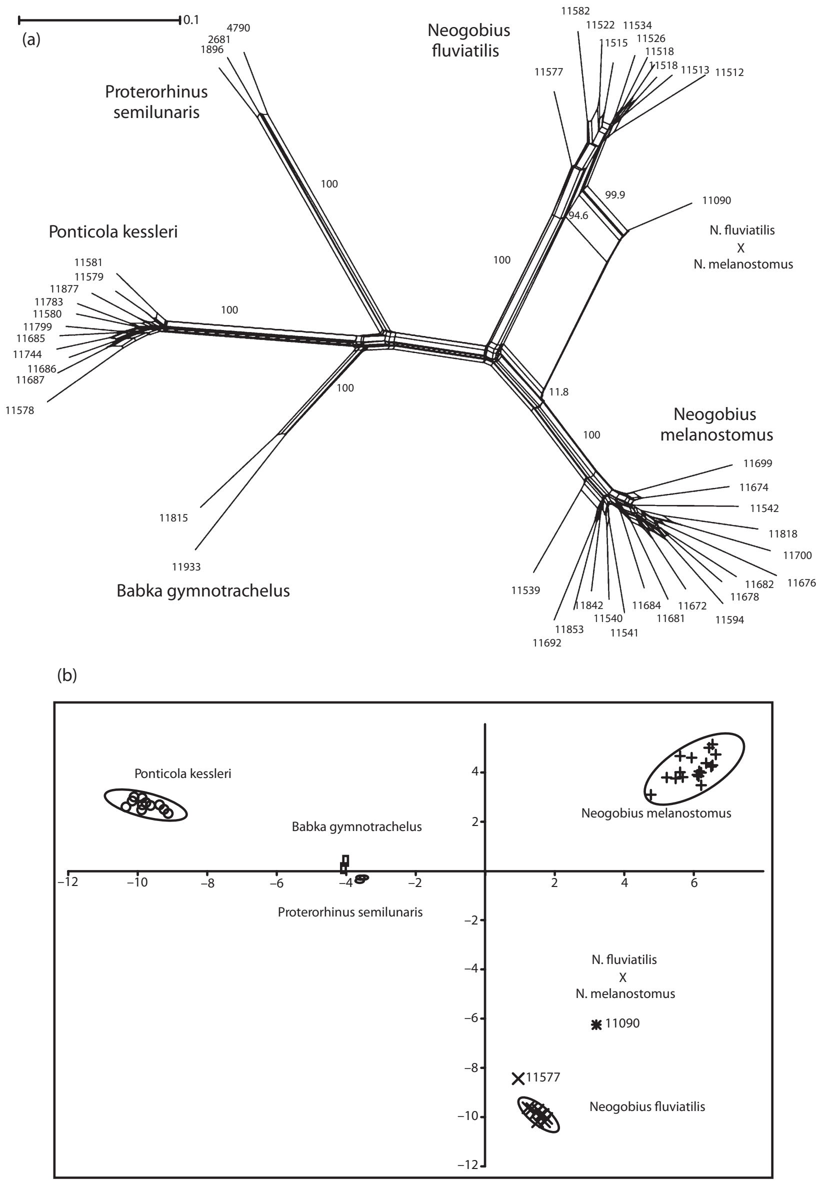

Fig. 5.2: Phylogenetic tree from the NeighbourNet-Analysis: Selected bootstrap values (%) calculated from 1,000 repeats (italics). Individuals are represented by the BayFi tissue collection number of the Zoologische Staatssammlung München; all leaf nodes bear bootstrap values of 100%. Clusters bear species names. (b) Principal component analysis: x-axis: component 1, describing 31.4% of the variance; y-axis: component 2, describing 23.2% of the variance. Clusters with n > 3 bear 95% ellipses. Clusters bear species names. Species are: *Babka gymnotrachelus* (□), *Neogobius fluviatilis* (x), *Neogobius melanostomus* (+), *N. fluviatilis* × *N. melanostomus* (✳), *Ponticola kessleri* (○) and *Proterorhinus semilunaris* (⬭). Selected individuals bear BayFi number.

Invasion biology often faces situations, where an invasive species acts as a hybrid partner of native species, thereby compromising the native congener's genotypes and consequently its autochthonous genetic integrity (Arnold, 1997; Petit et al., 2004). Furthermore, any hybrid genotype expressing a novel phenotype might occupy a novel niche (Fitzpatrick et al., 2011). In this case, the hybrid itself may again become a novel invasive lineage with unique adaptations facilitating the invasion of novel areas (Seehausen, 2004, Nolte et al., 2005). Habitat loss in degraded river ecosystems, which have experienced strong anthropogenic interference and which often have suffered from numerous losses of their autochthonous inhabitants, may facilitate this process, especially in the Rhine River (Nolte et al., 2005). These losses may result in the availability of vacant niches, which can be taken over by preadapted hybrid lineages (Arnold, 1997). Therefore, invasive goby hybrids should be monitored carefully. It is noteworthy, however, that despite intensive fishing efforts in the Rhine River as well as in the Danube River (which yielded > 7,500 goby individuals in the past 3 years), the hybrid specimens we report here are but singletons.

6 First record of *Babka gymnotrachelus* in Germany

Published: Haertl, M., A. F. Cerwenka, J. Brandner, J. Borcherding, J. Geist & U. K. Schliewen, 2012. First record of *Babka gymnotrachelus* (Kessler, 1857) from Germany. *Spixiana* **35**: 155-159.

6.1 Abstract

The Ponto-Caspian racer goby *Babka gymnotrachelus* (Kessler, 1857) is recorded for the first time in Germany from a Danube backwater close to the city of Regensburg, and from the Danube main channel close to the village of Mariaposching. Several specimens were collected and photographed in May and September 2011, and one kept until April 2012 in captivity. Previously reported records of this species from the German River Rhine are male *Neogobius fluviatilis* (Pallas, 1841), or possibly hybrids between different benthophiline goby species.

6.2 Introduction

Ponto-Caspian gobies of the subfamily Benthophiinae Beling & Iljin, 1927 (Teleostei, Gobiidae) are globally invasive and pose serious ecological threats to invaded waters (e.g. Neilson & Stepien, 2009). In Germany, the Rhine system and the Danube were connected by the Main-Danube junction (MD canal) in 1992, and since then have become one of the main dispersal routes for invasive Ponto-Caspian species in Central Europe (Leuven et al., 2009). Until recently four invasive benthophiline freshwater gobies had been reported from the Lower Rhine and/or the Upper Danube in Germany and Austria, i. e. *Proterorhinus semilunaris* (Heckel, 1837), *Ponticola kessleri* (Günther, 1861), *Neogobius melanostomus* (Pallas, 1814) and *Neogobius fluviatilis* (Pallas, 1814) (Copp et al., 2005). A fifth species, the racer goby *Babka gymnotrachelus* (Kessler, 1857), may have been discovered outside of its native range in the middle section of the Danube before 1991 (Hegedis et al., 1991, but see Jurajda et al., 2005), and reached Vienna (Austria) in 1999 (Zweimüller et al., 2000, Ahnelt et al., 2001). Eleven years later it was reported from Germany in the River Rhine (Borcherding et al., 2011b). Racer gobies were now discovered and photographed from a backwater of the

Upper Danube River at Regensburg, and from a groin head habitat in the Danube main channel at Mariaposching, Germany (Fig. 6.1). A closer examination confirmed their preliminary identification and prompted a re-examination of the specimen that was reported as the first record of *B. gymnotrachelus* in Germany (see: Borcherding et al., 2011b).

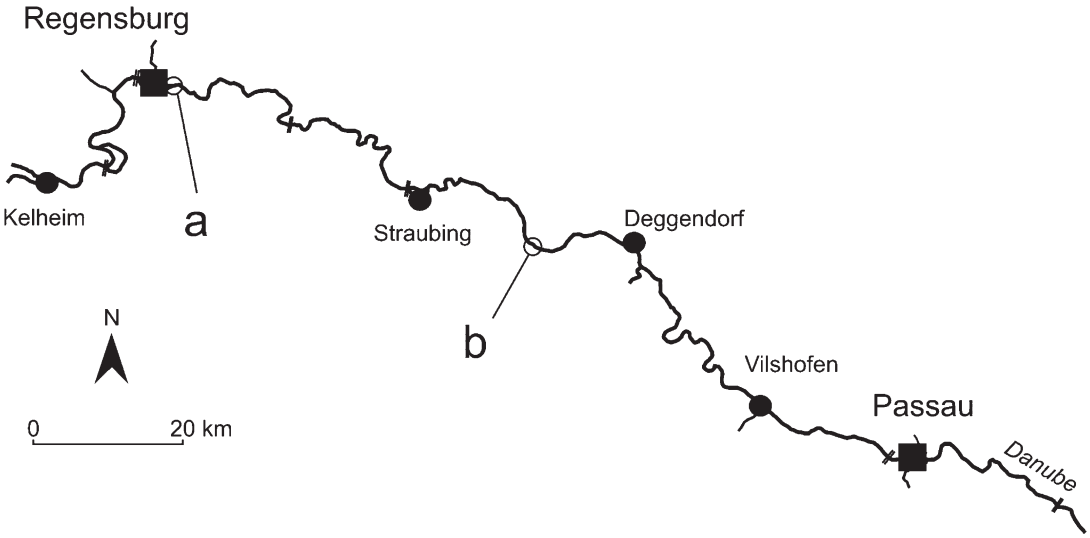

Fig. 6.1: Collection points of *Babka gymnotrachelus* in the upper Danube River (Germany). a, Danube backwater “Almer Grube” opposite the east harbor of Regensburg; b, Danube main channel at Mariaposching.

6.3 Material and Methods

Several specimens identified as *B. gymnotrachelus* were collected from below stones in shallow waters (< 1 m) from a backwater in the River Danube in May 2011 and a single one by electrofishing at a groin head near Mariaposching in September 2011. One male specimen from Regensburg was kept in an aquarium until April 2012, upon it was anaesthetized, preserved and deposited at ZSM. This and the Mariaposching specimen, as well as the one collected and previously identified as *B. gymnotrachelus* in the River Rhine (Borcherding et al., 2011b) were (re-)identified using published keys (Miller & Vasil’eva, 2003, Kottelat & Freyhof, 2007). If not otherwise mentioned, measurements, counts and other characters are taken as described in Schliewen & Kovacic (2008) and compared with literature data (Ahnelt et al., 2001, Pinchuk et al., 2003a,b, Kottelat & Freyhof, 2007) and with comparative material from the native range of *B. gymnotrachelus* and *N. fluviatilis*, as well as with additional *N. fluviatilis* material from the River Rhine.

Babka gymnotrachelus **(Kessler, 1857):**

ZSM 41739 (1 male, 92.2 mm SL), Germany, backwater “Almer Grube” of River Danube close to Regensburg close to an artificial rocky outcrop (49.0127° N, 12.1802° E), M. Haertl, collected May 2011, preserved 19 April 2012. ZSM 41336 (1 female, 78.5 mm SL), Germany, River Danube downriver of Mariaposching at a groin head (48.8255° N, 12.8194° E), J. Brandner, G. Nassel, D. Koeck, 30 September 2011; partly dissected. ZSM 26420 (2 specimens, 53.9-62.0 mm SL), Turkey, Lake Sapanca, M. Winter, 4 June 1984. ZSM 23288 (2 specimens, 52.7-56.8 mm SL), Romania, Lake Crapina, floodplain of the River Danube near Macin, P. Banarescu, 16 November 1964.

Neogobius fluviatilis **(Günther, 1861):**

ZSM 41740 (1 male, 95.8 mm SL), Germany, River Rhine near city of Rees (Rhine-km 843), J. Borcherding, S. Gertzen, S. Staas, 21 September 2010. ZSM 23289 (6 specimens, 83.4-88.0 mm SL), Romania, Lake Crapina, floodplain of River Danube near Macin, P. Banarescu, 24-25 August 1966. ZSM 23863 (12 specimens, 52.6-92.5 mm SL), Romania, Danube estuary at Sulina, P. Banarescu, 15 September 1968. ZSM 41579 (4 specimens, 73.9-80.5 mm SL), Germany, River Rhine near city of Rees (51.7621° N, 6.3408° E), A. Cerwenka, S. Gertzen, J. Brandner et al., 8 August 2011.

Fig. 6.2: *Babka gymnotrachelus* (ZSM 41739), photographed shortly after collection (Photo: M. Haertl).

Fig. 6.3: *Neogobius fluviatilis* (ZSM 41740), photographed shortly after collection (Photo: J. Borcherding).

6.4 Results and conclusions

Measurements and counts of the three benthophiline specimens are reported in Table 6.1. The two Danube specimens ZSM 41739 (Fig. 6.2) and ZSM 41336 key out as *B. gymnotrachelus* in keys provided by Kottelat & Freyhof (2007) and Miller & Vasil'eva (2003), and exhibit almost all applicable diagnostic character states of *B. gymnotrachelus* as reported in the most thorough recent review of the species (Pinchuk et al., 2003a: 266): midline of nape naked in front of preoperculum, otherwise scales cycloid or ctenoid; interorbit one third eye diameter (0.33/0.31); upper lip of rather uniform width and 0.68 and 0.61 times in lateral preorbital area as measured between lip and eye; anterior membrane of pelvic disc without lateral lobes; D1 moderately high, rounded in profile; coloration with oblique dark bands across body; lateral line scales count 59 and 65. The pelvic disc does not reach the anus in specimen ZSM 41739 and is slightly smaller than the diagnostic value given by Pinchuk et al. (2003a), i. e. 0.9 or more than the abdomen length (0.84); in specimen ZSM 41336 it does reach the anus and therefore fits the diagnostic value (0.96); further, the caudal peduncle depth is slightly larger than 0.5-0.6 of its own length (0.76 and 0.71). We conclude, that both specimens are conspecific with *B. gymnotrachelus*, however, with a slightly smaller pelvic disc in one specimen as compared to the character state reported by Pinchuk et al. (2003a).

Using the same keys, the Rhine specimen ZSM 41740 (Fig. 6.3) keys out as *N. fluviatilis*, except that it does not conform to the character state of Kottelat & Freyhof (2007) "first branched ray of second dorsal fin about twice as long as penultimate ray: no, but a bit shorter than first", because the first branched ray is only about 1.5 × as long as the penultimate ray (14.2/11.2 mm). Nevertheless, it exhibits all applicable diagnostic characters of *N. fluviatilis* as reported in the most thorough recent review of the species (Pinchuk et al. 2003b): Nape scaled completely, scales ctenoid; head depth at eyes about equal to width as measured between upper origin of opercles (12.9/13.2 mm); interorbit no more than 0.75 of eye diameter (0.72: 3.8/5.3 mm); angle of jaw below snout between eye and posterior nostril; snout 1.47 times larger than eye (7.8/5.3 mm); upper lip not swollen at angle, 0.4 times in lateral preorbital area as measured between lip and eye (1.8/4.5 mm); pelvic disc 0.94 of abdomen length (18.7/19.8 mm); anterior membrane of pelvic disc with small rounded, lateral lobes, less than 0.2 width of rear edge; D1 high, with acute anterior profile; median fins edged yellowish in breeding males. Lateral line scales count 55. We conclude that specimen ZSM 41740 is a male of *N. fluviatilis* in pre- or postbreeding coloration. We conclude so despite the missing character state in the key of Kottelat & Freyhof (2007), because this character state is not even evident from the photograph of a large *N. fluviatilis* male (page 579). We hypothesize that adult *B. gymnotrachelus* males develop enlarged median fins as a secondary sexual character, as fin shape differences are a kind of sexual dimorphism typical for many adult gobiid species (Horsthemke, 1995).

Table 6.1: Measurements (mm) and meristic counts of *Babka gymnotrachelus* specimens (ZSM 41739, ZSM 41336) and a *Neogobius fluviatilis* specimen previously misidentified as *B. gymnotrachelus* (ZSM 41740).

	Babka gymnotrachelus		***Neogobius fluviatilis***
Distance measurements	**ZSM 41739**	**ZSM 41336**	**ZSM 41266**
Sl, standard length	92.2	78.5	95.8
Tl, total length	114.1	98.4	120.1
Ab, anal fin base	31.2	24.6	31.5
Ad, body depth at anal fin origin	15.4	13.1	14.5
Aw, body width at anal fin origin	10.2	8.8	n/a
Bd, body depth	18.6	14.1	16.0
Cl, caudal fin length	21.9	19.9	24.3
CP, caudal peduncle length	12.4	10.6	12.2
CPd, caudal peduncle depth	9.5	7.6	7.6
D1b, first dorsal fin base	11.5	11.8	14.9
D2b, second dorsal fin base	36.1	31.5	39.1
Hl, head length	26.1	23.2	28.4
HwO, head width between opercles	19.2	13.6	13.2
Hd, head depth	13.4	9.5	12.9
E, eye diameter	5.6	5.2	5.2
SN, snout length	7.5	6.3	8.4
ULl, upper lip length	10.6	8.3	10.6
AULw, anterior upper lip width	2.4	1.9	1.8
LPd, lateral preorbital depth	3.9	3.3	5.0
Chd, cheek depth	8.0	5.2	8.0
PO, postorbital head length	15.3	11.7	16.2
I, interoribital width	2.0	1.6	3.8
IDs, interdorsal space	4.7	3.4	1.0
Pl, pectoral fin length	21.1	16.9	23.9
SN/A, snout to A	51.5	45.1	55.8
SN/AN, snout to anus	46.2	39.0	46.0
SN/D1, snout to D1	28.0	24.8	32.9
SN/D2, snout to D2	46.0	38.6	47.5
SN/V, snout to V	26.9	23.6	31.2
V/AN, pelvic to anus	19.8	17.0	16.5
Vd, body depth at pelvic fin origin	18.6	13.9	14.8
Vl, pelvic fin length	16.6	16.4	18.7
Vw, body width at pelvic fin origin	18.2	13.5	14.0
Counts			
LL, lateral line scale count	59+3	65+4	55+4
D1, spines in the first dorsal fin	VI	VI	VII
D2, spines and rays in the in the second dorsal fin	I/17	I/17	II/17
A, spines and branched rays in the anal fin	I/15	I/14	I/14

6.5 Remarks

In its native range *B. gymnotrachelus* is a typical inhabitant of mud, sand, gravel or muddy-sandy bottoms (Pinchuk et al., 2003b), and it is abundant in backwaters (Kottelat & Freyhof, 2007). Two of the upper Danube records, the one from Austria (Ahnelt et al., 2001) and the one from Regensburg, Germany, are from large backwaters. The specimen from Mariaposching was collected near a groin head, which is adjacent to a soft bottom area with comparatively calm water, and this is the single specimen that has been recorded from main channel habitats despite intensive shallow water electrofishing efforts along the Danube in Germany in 2010 and 2011, which yielded thousands of benthophiline goby specimens (pers. obs. A. Cerwenka and J. Brandner). This preliminary result suggests that monitoring of invasive freshwater fishes should target soft-bottom backwaters and soft bottom main river habitats more intensively, and that the invasive range of *B. gymnotrachelus* may already be larger than previously assumed.

7 General Discussion

7.1 Rapid differentiation, intraspecific hybridization and a newly introduced species

Invasive species offer unique opportunities to the study of evolutionary processes in contemporary time-scales (Hendry et al., 2000; Reznick & Ghalambor, 2001), as novel extrinsic conditions may cause novel evolutionary responses from the invasive species (Ricciardi & MacIsaac, 2000; Lambrinos, 2004). Invasive *N. melanostomus* and *P. kessleri* reacted species-specific to highly similar ecological settings constrained by the environment of the upper Danube River. Notably, local population structure with a potentially adaptive component has been shown to be established in *N. melanostomus* within few generations after first introduction and along a comparatively small geographic gradient along the river continuum. The relevance of this finding for invasion biology is underestimated by a second result, i.e. the combination of detected local population structure with a genomic correlate for local phenotypic adaptation. This finding of rapid *de novo* evolution of a heritable and an apparently adaptive genomic pattern in the more successful of the two studied invaders (*N. melanostomus*) points to a key role of rapid genomic adaptability, which could be the basis for further phenotypic adaptations such as morphological, physiological or behavioral patterns.

Evolutionary responses to changing selective regimes often rely on the level of standing genetic variation in the evolving population, because genomic variation provides the basis for selection (Sakai et al., 2001; Anderson et al., 2011). (Adaptive) Genetic variation in a population can be enhanced instantaneously and dramatically due to introgression and hybridization, and hence hybridization is increasingly recognized as force in divergent species evolution (Mallet, 2008; Kays, et al., 2010; LaRue et al., 2013). However, in the present study a correlation between hybridization, differentiation and population structure of invasive gobies in the upper Danube River was not detected (chapter 4). Instead, hybridization was found involving two invasive goby species (i.e. *N. melanostomus* and *N. fluviatilis*) in the River Rhine, representing the first documentation of hybridization between two invasive vertebrates on a world-wide scale (chapter 5). Hybridization could get of particular relevance in non-native gobies, since an additional Ponto-Caspian species (i.e. *B. gymnotrachelus*) was recorded recently in Germany (chapter 6). The rapid detection of non-native species shortly after their introduction is essential for downstream analyses of invasion processes (Simberloff et al., 2013), as evolution commences immediately and may affect invaders rapidly. Posthoc analyses of the inoculation situation may be difficult and population structuring may develop soon after invasion as shown in this study.

7.2 A two-species comparison: local population structure and rates of genomic evolution

Invasive populations of *N. melanostomus* and *P. kessleri* are morphologically and genetically variable and partially differentiated in the upper Danube River (chapter 3, 4). Differentiation was shown at very proximate areas and among short geographic distances only about one decade after first introduction (chapter 3, 4). Both goby species featured a considerable population structure, but being substantially more prominent in *N. melanostomus* (chapter 4). This applies to the degree of distinctiveness of subpopulations as well as to its spatial structure.

Although having highly comparable invasion histories (Brandner et al., 2013b) the goby species responded differentially to extrinsic settings. Levels of phenotypic and genotypic differentiation were higher in *N. melanostomus*. This is striking since the latter species was introduced about five years later than *P. kessleri* (Paintner & Seifert, 2006).

Extrinsic factors, i.e., barriers to gene flow, may influence the speed of population differentiation and the evolution of population structure to a large extent (Lee, 2002; Stelkens et al., 2012). Nevertheless, the skills of rapidly responding to natural selection in non-native areas are highly depended on the species, i.e. their genetic constitution, and morphological and ecological characteristics, making general predictions difficult (Jeschke et al., 2012; Colautti et al., 2014). However, high rates of flexibility and variability in the genotype and the phenotype seem to correlate to the strength of population structure. Feiner (2012) proposed flexibility and plasticity in the life history strategy of invasive white perch (*Morone americana* Gmelin, 1789) as an important advantage, allowing rapid adaptation to local conditions. This hypothesis is supported by the comparative results in the present study, since population structure was more prominent in the more “plastic invader” *N. melanostomus*. This species is characterized by a higher flexibility in life history traits (Kováč et al., 2009), a more omnivorous and a less specialized feeding strategy (Brandner et al., 2013b) and by a higher variability of its pheno- (chapter 3) and genotype (chapter 4). In addition, densities of *N. melanostomus* were higher (Brandner et al. 2013b, c) and range expansion was faster (Brandner et al. 2013a, b) in the upper Danube region. Both factors indicate a higher invasion success than the syntopic and sympatric *P. kessleri*.

Qualitative differences in the population structure were obvious for both species. A geographic differentiation was not present for *P. kessleri*, whereas *N. melanostomus* populations could be separated according to their geography in three main parts consisting of an uppermost population just upstream the inlet of the Rhine-Main-Danube canal, a large central group upstream from the influx of the River Inn and a lowermost population below this influx. Interestingly the uppermost and the lowermost populations showed higher similarities to each other than the other populations (chapter 3, 4). This spatial population differentiation contradicts a simple isolation-by-distance and a strict stepping-stone pattern of upstream expansion. Instead, anthropogenic barriers seem to support the found population-structure and may limit migration and population admixture. The large ecological differences between the uppermost and the lowermost river-stretches e.g. flow regime, species diversity and habitat composition, contradict the hypothesis of convergent directional selection. Instead, conditions of the invasion history may have shaped the found trisection of the upper Danube River.

The number of inoculation events and the origin of non-native species are known to influence the strength and the quality of differentiation (Bossdorf et al., 2005; Blackburn et al., 2013). In invasive gobies of the upper Danube River, there may have been several inoculations from different native sites. These may have configured their population structure to a large extent, and genetic admixture may have shaped stretches at the border of the population-clusters. This may point towards weak and loose migration abilities of *N. melanostomus*. However, this seems not to be true at the most uppermost stretch, the so called “invasion front” or “leading edge population” were expansion rates were calculated as up to 17 river-km in about one year (Brandner et al. 2013a). Elevated levels of genetic variability (standing or newly generated) most probably have affected the rapid differentiation into significant population structure in *N. melanostomus*. This underlines that increased levels of variability could be advantageous for invasion success (Björklund & Almqvist, 2010).

Genomic correlates indicate rapid directional evolution in non-native gobiid species. Invasion success may be assigned to single genetic loci, which may influence strength and quality of population structure. This could explain the findings of Rollins et al. (2013) that elevated rates of (neutral) genetic variability are not essential for the success of an invasive species. In *N. melanostomus*, a single allele potentially under directional selection of the isotopic signature nitrogen indicates the significance of food resources for the speed of evolution. Thus, and similar to Lee (2002), results underline the importance of only a few genes to invasion success.

Apart from high levels of genetic variability, population genetic effects as “allele surfing” may increase speed of differentiation in rapidly expanding populations. Invasive gobies may be prone to allele surfing, as a low number of individuals may rapidly produce a high number of offspring. Here the chance of reproducing is sweepstakes-like, also known as the “Hedgecock effect” (Hedrick, 2005). Again in *N. melanostomus*, a single locus was detected to “surf” at the expansion edge pointing towards elevated rates of flexibility, variability and differentiation in this species.

7.3 Interaction between phenotypic plasticity and genomic diversity

Invasive gobies of the upper Danube River were variable in their body shape. However, the more precocial species, i.e. *P. kessleri* (Kováč et al., 2009) exhibited the lower phenotypic variation of the two species (chapter 3). Population structure was not detectable in the upper Danube River (chapter 4) and there was no indication for phenotypic plasticity in this species (chapter 3). In contrast, *N. melanostomus* showed higher variability in its body shape and considerable phenotypic differentiation (chapter 3). The spatial population structure revealed by geometric morphometric analyses (chapter 3) was comparable to the one shown by genetic analyses (chapter 4). In addition, phenotypic plasticity is highly probable in this species (chapter 3, 4). It is thought to maintain or increase fitness (Richards et al., 2006) and enhance invasion success (Parker et al., 2003) and may apply to *N. melanostomus*, as fish densities were much higher in comparison to *P. kessleri* (Brandner et al. 2013b, c).

Although genotyping *P. kessleri* populations generally showed more AFLP-fragments, its population genomic diversity and genetic differentiation was lower than in *N. melanostomus*. In addition, spatial population structure was detected in *N. melanostomus* only. Thus, results of genetic and geometric morphometric analyses produced highly consistent patterns.

Considerable genomic differentiation between and within single populations at river stretches was obvious in *N. melanostomus* but not in *P. kessleri*.

Phenotypic plasticity is known to play an essential role in invasion success (Richards et al., 2006; Ribeiro et al., 2007). However, no study has investigated the correlation between phenotypic plasticity and population structuring in invasive organisms, up to now. *N. melanostomus* is characterized by considerable values of population structure and phenotypic plasticity, whereas both were not detected in *P. kessleri*. The species comparison gives a strong hint of the relationship between increased values of phenotypic plasticity and increased speed of population structuring underlining the importance of phenotypic plasticity for invasion success. In concordance, the detected elevated fish densities in newly invaded regions of *N. melanostomus* supports the notion of a higher invasion success of this species.

7.4 Local adaptation facilitated by phenotypic plasticity in combination with genomic adaptation

Phenotypic plasticity and plasticity of life history traits are known to facilitate non-native species to cope with novel situations (e.g. Yeh & Price, 2004) and may support invasiveness, e.g. in gobies: *N. melanostomus* exhibited higher flexibility in its nutrition (Brandner et al., 2013b), trait utilization (Kováč et al., 2009) and body shape characteristics (chapter 3), and showed higher expansion rates in non-native regions (Brandner et al., 2013a). However, causality between correlations and potentially adaptive traits need further experimental research. Nevertheless, the rapid changes in body shape (chapter 3) and genetic constitution (chapter 4) might reflect an elevated potential of *N. melanostomus* to adjust to local conditions and suggest *N. melanostomus* to be the ecologically more adaptable of the two invasive goby species. Overall evidence therefore supports the hypothesis, that fast rates of differentiation and adaptation may be essential for the success of non-native organisms.

In addition to elevated levels of flexibility of several species characteristics, phenotypic plasticity was revealed in *N. melanostomus* of the upper Danube River (chapter 3, 4). Phenotypic plasticity may already have been present in native populations but it could also have been created *de novo* in non-native regions. In both cases and similar to findings of Miner et al. (2005), phenotypic plasticity may be maintained by natural selection. From a genetic perspective, single AFLP-loci potentially under directional selection, indicate rapid genomic adaptation in *N. melanostomus* (chapter 4). However, a more detailed study is needed to analyze this combination of phenotypic plasticity and genomic adaptation which may be essential for (rapid) local adaptation.

7.5 Additional invasive gobies of the upper Danube River

The contemporary upper Danube River fish-biocoenosis is dominated by the two investigated ubiquitous goby species whose impact changed the functionality of the ecosystem properties (Brandner et al. 2013a, b). Interactions between non-native species may shape novel ecosystems and facilitate subsequent invasions, described by the theory of “invasional meltdown” (Simberloff & Von Holle, 1999). In the investigation area, the establishment of a further gobiid invasive species was documented in the present study, i.e. *B. gymnotrachelus*. This increases the number of non-native gobiid species in Germany to five and points towards an invasional meltdown in the upper Danube ecosystem. The detection was only possible through careful morphological comparisons, at which the re-evaluation of the putative *B. gymnotrachelus* specimen of the “first record” by Borcherding et al. (2011b) keyed out as *N. fluviatilis*. This underlines the importance of precise species determination and identification, especially in closely related and difficult to distinguish species groups as Ponto-Caspian gobies and mussels.

The Ponto-Caspian region is a major source of species that became invasive in many parts of the world (e.g. Lowe et al., 2000; Mooney & Cleland, 2001; Gollasch & Nehring, 2006) and provides several typical examples of succession and replacement of invasive species. The zebra mussel *Dreissena polymorpha* (Pallas, 1771) often is followed by the quagga mussel *Dreissena rostriformis bugensis* Andrusov, 1897 (Quinn et al., 2013). Frequently, the so-called “killer shrimp” *Dikerogammarus villosus* (Sovinskij, 1894) seems to offer a nutritional basis for non-native organisms from the Ponto-Caspian region, as e.g. for benthic gobiid fish species. *Neogobius melanostomus,* has followed the *D. villosus* expansion in several non-native regions on both sides of the Atlantic Ocean (Corkum et al., 2004). This may apply especially for the upper Danube River, where an advanced stage of “invasional meltdown” seems to be an appropriate description of the current stage. Nevertheless, additional non-native goby species are highly probable to successfully establish self-sustaining populations and get invasive. *Neogobius fluviatilis,* e.g. is already established in the middle section of the

Danube River (Ahnelt et al., 2001; Prášek & Jurajda, 2005) and in the German section of the River Rhine (Borcherding et al., 2011b). It is highly probable to invade the upper Danube River in the near future and to cause additional threats to the aquatic biodiversity. This underlines the need of a further detailed and intensive monitoring not only of the main river habitats but also of backwaters and habitats not investigated in this study.

7.6 Recommendations for future research

Natural systems are changing due to multiple reasons as climate change, eutrophication and other anthropogenic influences, e.g. non-native species (Davis et al., 2011). Further research is needed on changes and alterations of novel ecosystems, their natural species-communities but also on the identification and detection of non-native and potentially invasive organisms. Controlling, monitoring and management of established invasive and newly arriving non-native species is required to get a more detailed idea of species-characters accountable for invasion success and to limit threats on biodiversity. On the other side, invasions offer unique systems to investigate evolutionary processes under semi-experimental conditions. Especially for the investigation of the first steps towards population differentiation, contemporary evolution and (adaptive) species responses, the detection of non-native species shortly after their introduction is fundamental. Insights into species evolution may become tractable and make invasions useful in the biological field of evolution.

This study describes the early stage of invasion of *N. melanostomus* and *P. kessleri* of the upper Danube River and depicts the actual situation of phenotypic and genotypic differentiation of both species. The results should act as detailed baseline information for investigations of the study area and evolutionary changes in future analyses. This is of especial relevance since ecosystem changes are irreversible (Davis et al., 2011).

Interactions between native and non-native organisms have been in the focus of interest of biologists, e.g. searching for traits determining invasion success (e.g. Grosholz & Ruiz, 1996; Sandra et al., 2014). *Neogobius melanostomus* and *P. kessleri* are highly competitive and show high levels of intra- and interspecific aggression, especially during the period of reproduction and brood care (Dubs & Corkum, 1996; Savino et al., 2007). Their highly comparable diet may even increase interspecific competition (Brandner et al. 2013b). In addition they may compete with native species, similar to interactions observed under experimental conditions between invasive *B. gymnotrachelus* and native *Cottus gobio* L. of the River Vistula (Kakareko et al., 2013). Using (behavioral) experiments investigating and comparing inter- and intraspecific competition of invasive gobies and co-occurring native species with comparable biological requirements could offer details on frequently concurrent species abundances decline of natives.

Invasive organisms perform differently at the expanding edge, the so called invasion front. Brandner et al. (2013a) showed that invasive edge populations of *N. melanostomus* differed from longer established sites in several traits. Plasticity in trait utilization, body shape and genetic constitution seem to be beneficial not only at the beginnings of an invasion but also while spreading and may influence success (Richards et al., 2006). A detailed examination of traits that correlate with success at expanding edge populations in a time-series is still lacking. In addition, results of genetic changes and the detection of a surfing allele in *N. melanostomus* at this site indicate the importance of investigating these populations in more detail to reveal the potential contribution of surfing alleles, spreading rates and changes of life history characters.

Phenotypic plasticity may play an important role not only at leading edge populations but also for the success of non-native species in novel environments (Richards et al., 2006; Lamarque et al., 2013). It may even be essential for rapid adaptive evolution in invasive organisms (Collyer et al., 2007; Forsman, 2014). However, costs may arise for the maintenance of phenotypic plasticity (Dewitt et al., 1998) and may be lost in novel environments (West-Eberhardt, 2003; Richards et al., 2006). More profound investigations of body shape characteristics of *N. melanostomus* and *P. kessleri* are needed to verify this hypothesis. The shape analyses conducted in this study could be complemented by sliding semilandmarks, which are used to quantify curves and surfaces (Gunz & Mitteroecker, 2013).

Phenotypic variability and variability of further intrinsic life history traits are common in invasive organisms (Sakai et al., 2001) and where found to occur in invasive gobies, too (Brandner et al., 2013a, b; chapter 3, 4). Growth rate is an essential life-history trait and may contribute to invasion success of *N. melanostomus* (Gruľa et al., 2012). Scale reads offer a suitable method to categorize individual growth of invasive gobies (Gruľa et al., 2012). Scale circuli counts should be used to determine importance of growth for invasiveness (i.e. plasticity of developmental strategies) and should be applied for *N. melanostomus* and *P. kessleri* populations of the upper Danube River. To complete this study focusing on morphometric and genetic differentiation, scale reads could be used to determine the importance of alternative life history strategies for invasion success: growth rates should be correlated with rates of local differentiation and feeding strategies.

Multiple hypotheses intend to explain and determine invasion success. However, Jeschke et al. (2012) revealed the applicability and empirical support of six current hypotheses of invasion biology to be highly dependent on the species and the environment. Here, a multivariate analysis of all obtained individual goby data is promising to assess fitness proxies, specific parameters and environmental correlates which are accountable for differences in species-specific invasion success. For every analyzed goby specimen individual and specimen-specific data are available from (i) results of body shape and genetic analyses (chapter 3, 4), (ii) fish scale readings, (iii) nutrional analyses (diet and isotopic signature; Brandner et al. 2013a, b). Additional variables are available for (iv) specimen-specific parameters, as e.g. the parasitic load and (v) local conditions (e.g. chemical and physical water conditions, habitat composition, and the fish and the macrozoobenthos biocenosis). An individual based multifactorial approach could be used to analyze common hypotheses in invasion biology and it could be applied to search for proxies for invasion success. Thus, a species-specific comparison should contribute to the basic understanding of single parameters determining invasion success: one of the fundamental questions in invasion biology.

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9 Publication list

Peer reviewed publications in this thesis

Haertl, M., A. F. Cerwenka, J. Brandner, J. Borcherding, J. Geist & U. K. Schliewen, 2012. First record of *Babka gymnotrachelus* (Kessler. 1857) from Germany (Teleostei, Gobiidae, Benthophilinae). *Spixiana* **35**: 155-159.

Lindner, K., A. F. Cerwenka, J. Brandner, S. Gertzen, J. Borcherding, J. Geist & U. K. Schliewen, 2013. First evidence for interspecific hybridization between invasive goby species *Neogobius fluviatilis* and *Neogobius melanostomus* (Teleostei: Gobiidae: Benthophilinae). *Journal of Fish Biology* **82**: 2128-2134.

Cerwenka, A. F., P. Alibert, J. Brandner, J. Geist & U. K. Schliewen, 2014. Phenotypic differentiation of Ponto-Caspian gobies during a contemporary invasion of the upper Danube River. *Hydrobiologia* **721**: 269-284.

Cerwenka, A. F., J. Brandner, J. Geist & U. K. Schliewen, 2014. Strong versus weak population genetic differentiation after a recent invasion of gobiid fishes (*Neogobius melanostomus* and *Ponticola kessleri*) in the upper Danube River. *Aquatic Invasions* **9**: 71-86.

Peer reviewed publications not considered in this thesis

Brandner, J., K. Auerswald, A. F. Cerwenka, U. K. Schliewen & J. Geist, 2013. Comparative feeding ecology of invasive Ponto-Caspian gobies. *Hydrobiologia* **703**: 113-131.

Brandner, J., J. Pander, M. Mueller, A. F. Cerwenka & J. Geist, 2013. Effects of sampling techniques on population assessment of invasive round goby. *Journal of Fish Biology* **82**: 2063-2079.

Brandner J., A. F. Cerwenka, U. K. Schliewen & J. Geist, 2013. Bigger is better: Characteristics of round gobies forming an invasion front in the Danube River. *PLoS ONE* **8**: e73036.

Oral contributions related to this thesis

Brandner, J., A. F. Cerwenka, K. Auerswald, D. Leitzbach, U. K. Schliewen & J. Geist, 2011. Ernährungsökologie invasiver Schwarzmeergrundeln in der oberen Donau: Saisonale Effekte und trophische Einnischung. Jahrestagung 2011 der Deutschen Gesellschaft für Limnologie e.V. (DGL) und der deutschen Sektion der Societas Internationalis Limnologiae (SIL).

Brandner, J., A. F. Cerwenka, K. Auerswald, D. Leitzbach, U. K. Schliewen & J. Geist, 2012. Invasive ponto-caspian gobies in the upper river Danube: Habitat preferences, trophic niches and effects on the food web. Jahrestagung 2012 der Deutschen Gesellschaft für Limnologie e.V. (DGL) und der deutschen Sektion der Societas Internationalis Limnologiae (SIL).

Cerwenka, A. F., J. Brandner, U. K. Schliewen & J. Geist, 2011. Evolutionäre, populationsgenomische und ökologische Mechanismen eines simultan ablaufenden Invasionsprozesses zweier nahe verwandter Fischarten (*Neogobius ssp.*) in der oberen Donau. Jahrestagung 2011 der Deutschen Gesellschaft für Limnologie e.V. (DGL) und der deutschen Sektion der Societas Internationalis Limnologiae (SIL).

Schray, C., A. F. Cerwenka, J. Brandner, U. K. Schliewen & J. Geist, 2012. Abschätzung von Alter und Wachstum anhand von Schuppenmerkmalen bei *Neogobius melanostomus*. Jahrestagung 2012 der Deutschen Gesellschaft für Limnologie e.V. (DGL) und der deutschen Sektion der Societas Internationalis Limnologiae (SIL).

Poster contributions related to this thesis

Brandner, J., D. Leitzbach, A. F. Cerwenka, U. K. Schliewen & J. Geist, 2010. Ernährungsökologie invasiver Grundeln in der oberen Donau. Jahrestagung 2010 der Deutschen Gesellschaft für Limnologie e.V. (DGL) und der deutschen Sektion der Societas Internationalis Limnologiae (SIL).

10 Acknowledgements

During this thesis I was supported by many, many people who helped me with their knowledge and with practical aspects. I want to thank all of them to have made a contribution to the success of this work.

Foremost, I want to grateful thank my supervisor and mentor Prof. Dr. habil. Jürgen Geist. His effort started at the very beginning of this thesis by encouraging and helping me with the funding applications. Afterwards, he guided me during the entire field work but also during the analysis, the publication processes and the writing of my thesis. Thank you very much for all your efforts. I am looking forward for many projects that will follow!

This work was co-supervised by Dr. Ulrich Schliewen from the Bavarian State Collection of Zoology, Department of Ichthyology who gave me great daily assistance concerning scientific questions and who I won as a friend. Thank you for all your sustained assistance!

Further I would like to thank Dr. Jörg Brandner: together, we prepared the funding application and we conducted the entire field sampling. All manuscripts and publications were prepared and worked out in strong collaboration for which I am very grateful. You never rejected a discussion on findings and new directions of our scientific work. Thank you for all the new ideas on our scientific work!

Many people contributed to the success of this thesis and helped with the very intensive field work. I want to thank all students who assisted during the electrofishing and the collection of the material. In this context I thank Jörg Steinhilber for his support in organizing the technical equipment. As successful electrofishing asks for a variety of licenses I would like to thank all the owners of local fishing rights, especially Dr. Gerald Zauner. Thank you for your support concerning the sampling in Austria and thank you for offering my team a place to stay!

My work benefited from the assistance of my appreciated colleagues Dr. Sebastian Beggel, Dr. Matthias Geiger, Dr. Andreas Dunz, Dr. Nicolas Straube, Frederic Schedel and Eva Lodde-Bensch at the Bavarian State Collection and the Aquatic Systems Biology Unit. Thank you for all your helpful comments! I am grateful to Juliane Weiß who made great and important recommendations for analyses of the genetic dataset. The storage of vouchers and specimens at the Bavarian State Collection of Ichthyology was strongly supported by Dirk Neumann. Thank you for your profitable advices and improvements on my field-work! In addition I want to thank Carolin Böker with whom I shared my office almost one year and who got a strong affection to fishes in general and gobies especially. Good look for your own dissertation. Katharina Lindner helped me in the laboratory and decided to do some extra work as she was heavily infected by the molecular research on gobies. Thank you for showing me to promote my activities!

Above all I want to thank my family and Jennifer Zapf for supporting me all along my PhD-study. You helped me during the long time of my thesis by continuously loving me and holding up my crazy affection to fish!