The Eurasian minnow:

Post-glacial dispersal history

and

recent invasion patterns in Norway

by

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19 December 2012

Johannes Holmen

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List of papers

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Paper II: Holmen, J.; Vøllestad, L. A.; Jakobsen, K. S.; Primmer, C. R. 2009. Cross-species amplification of 36 cyprinid microsatellite loci in *Phoxinus phoxinus* (L.) and *Scardinius erythrophthalmus* (L.). *BMC Research Notes* **2**, 248.

Paper III: Holmen, J.; Jakobsen, K. S.; Primmer, C. R.; Vøllestad, L. A. Phylogeography and post-glacial dispersal of the Eurasian minnow with an emphasis on Norway. Manuscript.

Paper IV: Holmen, J.; Jakobsen, K. S.; Primmer, C. R.; Vøllestad, L. A. A native species turns invasive – the Eurasian minnow. Manuscript.

Paper V: Holmen, J.; Jakobsen, K. S.; Primmer, C. R.; Vøllestad, L. A. Multiple invasions of Eurasian minnow *Phoxinus phoxinus* in two Norwegian lake systems: implications for gene diversity and genetic structure. Manuscript.

Introduction

Following upon the release of Charles Darwin's successful book *On the Origin of Species* (Darwin 1859), Alfred Russel Wallace wrote *The Geographical Distribution of Animals* (Wallace 1876). Both Darwin and Wallace had, through their extensive travelling and explorations, understood that the geographical distribution of species was determined by a number of factors. Not only were there climatic zones and particular ecosystems in which certain species would thrive, constraints on dispersal would also effectively limit species distribution. Much of this thinking was coupled with their even more famous work concerning evolution and, in particular, natural selection. Wallace was among the first to put forward the hypothesis that species formation would occur more frequently in the presence of biogeographic barriers. Species distribution, the geographical confinements of species, has been a topic of great interest in biology ever since.

The emergence of plate tectonics theories and cladistics has been important for the development of biogeographical theory through the 20th century (Flessa 1980; Hallam 1981; Humphries 2005). The hypothesis that new lineages have appeared through history as a result of large-scale geological events separating environments with oceans, rivers or mountain ranges, is now widely accepted. It is popularly referred to as the vicariance theory, contrasting, but not excluding, the geodispersal theory, which relies on dispersal and gene flow (see e.g. Lieberman 2005 for further discussion). Traditional cladistics was based on geology and palaeontology as well as modern species morphology, often dealing with high level taxonomy. While most major findings using these methods persist as valid, modern methods involving molecular genetics have vastly increased the range of knowledge into biogeography. Exploiting the usually much higher resolution and precision of molecular genetics as opposed to morphology, studies of taxon distribution and phylogeny have been extended to deal with lower taxonomic levels as well (Hillis 1987; Avise 1989).

The inclusion of phylogeny and population genetics in biogeography brought about the term phylogeography. While the term first appeared in the 1970's when the first molecular methods became available, it did not really draw much attention until the development of polymerase chain reaction (PCR) in 1984. Further method streamlining through the following decade (Bartlett & Stirling 2003) made molecular genetics laboratory work considerably more efficient. Investigations of individual and population relatedness have since then been

explored at an increasing pace, following the development of more sophisticated molecular markers.

With the ever growing sophistication of molecular genetics methods, they are increasingly used in an expanding number of biological fields. As a result, the volume of population genetics literature has exploded during the last couple of decades. Population genetics has been employed to better understand historical events such as ancient dispersals responsible for today's biogeographical patterns, as exemplified in **Paper III**, recent translocations of individuals between populations (gene flow, discussed in **Paper IV**), as well as other evolutionary mechanisms, such as selection and genetic drift, the latter briefly dealt with in **Paper V**.

Conservation biology

The diverse field of conservation biology can roughly be divided into two fields: The scientific field seeking to describe and understand causes of changes in the native species composition within a more or less defined geographical area, and the management field which aims at altering or conserving the species composition within such an area (Macdonald & Service 2007). For the scientific field, historical changes evident through findings of biological remnants, study of written material or population genetics alterations due to selection processes, migrations or simply population size variations, are often described. In addition, conservation biologists also hypothesize about possible future changes in species compositions. Both descriptions of past and predictions of future events provide wildlife and nature management with tools for their work. A well-accepted view is that nature management should seek to conserve nature with its species composition as close as possible to what it was like before human interference (Hunter & Gibbs 2007). However, a certain degree of subjectivity is often unavoidable within this field, and it is repeatedly seen that species of high economical or emotional value are more likely to receive attention than less conspicuous species. This sometimes leads to management actions that may be beneficial to the species of human interest, while it can be detrimental to other species (Kjærstad & Arnekleiv 2011).

A rather new benefit of population genetics is its application in conservation biology (Macdonald & Service 2007). Species of particular conservation concern have typically low numbers of individuals within populations (Ellstrand & Elam 1993; Stephens & Sutherland

1999) and may often experience inbreeding depression (Crnokrak & Roff 1999). On the other hand, populations may have adapted to a particular local environment, making external gene flow less advantageous at the local scale (Champagnon *et al.* 2012; Mosca *et al.* 2012). These subjects can easily be monitored through genetic screening on a population level, substantiating the basis for sound management decisions.

Species invasions

The definition of invasive species used by the European Commission (EC) is "Invasive Alien Species are animals and plants that are introduced accidently or deliberately into a natural environment where they are not normally found" (http://ec.europa.eu/). Furthermore, the EC emphasizes invasive species' negative impacts on the economies of member countries. Usually, invasive species are discriminated from species exhibiting range-shifts in that the latter encompass natural dispersal, in many cases facilitated by climate change (Phillips et al. 2010; Sorte et al. 2010).

Biogeographical barriers are important limiters for species distribution, especially when habitats separating populations are hostile. Dispersal of species across such biogeographical barriers may lead to large changes in the receiving ecosystem. This is because the native species have not evolved in the presence of the invading species, and thus may not be able to cope with the new levels of predation, competition or infection. Ever-increasing dispersal of species due to increased cargo and passenger transport around the globe leads to biotic homogenization in many areas of the world, many of the new species being reported to impose negative and sometimes detrimental effects on native species (Lodge 1993; Callaway & Aschehoug 2000; Rahel 2000). Some authors refer to fossil data and claim that temporal changes in species distribution is nothing new, but as Mooney & Cleland (2001) point out, the rate at which these changes have occurred in recent times has grown tremendously.

The most commonly listed criteria for a species to be termed invasive are a) the species is non-indigenous in the focal area, and b) the species have or will potentially induce negative effects on the indigenous species and/or impose negative effects on human health and welfare. Terms of invasive ecology are discussed further in Colautti & MacIsaac (2004). Newly introduced species impose ecological effects on native species, ranging from positive to negative, the latter of which would put the tag invasive on the species (see e.g. Westman

2002; Wonham *et al.* 2005; Nakata *et al.* 2006; Borroto-Paez 2009; Ostermann-Kelm *et al.* 2009; Branch *et al.* 2010; Minden *et al.* 2010).

Freshwater biological communities are in many cases especially susceptible to negative effects from the introduction of invasive species. Due to water bodies' inherent physical characteristics, they are, to limnic organisms, either totally separated by uninhabitable areas or interconnected by relatively narrow corridors that may impede migration or in fact render it impossible. Members of separated ecosystems often experience far less inter-specific competition and predation, and thus utilize a broader range of niches. In the case of a biological invasion, native species are more susceptible to experiencing negative impacts than the same species in a more complex ecosystem (Lodge 1993). Species-poor ecosystems usually occur in areas where limnic ecosystems have only existed for a phylogeographically limited period, such as areas exposed after the last glacial period. However, topological features in many cases make human-mediated transportation of the invading species a requisite for an invasion. Time span since an area was transformed into a suitable habitat is then of less importance.

Knowledge of the historical biogeography of a species often provides important information for management authorities. If the species in question is a protected species or an invasive species, such information is often essential for making informed management decisions. In the case of an invasive species, such information is often crucial to prevent further dispersal. Estoup & Guillemaud (2010) argue that detailed knowledge of invasion routes is crucial to establish knowledge of the evolutionary and environmental factors required for the invasive species, thus providing management strategies for prevention or control of further invasions.

The minnow – a "native invader"

In Norway, invasions of the small cyprinid fish the Eurasian minnow *Phoxinus phoxinus* (hereafter minnow) have proven most negative to native fish populations where a single species constituted the complete native fish fauna. Often, the native species is brown trout *Salmo trutta*, and the invaded water bodies consisted greatly of minnow-suitable habitat (Museth *et al.* 2007). Minnows rarely impose severe negative effects upon other fish species in multi-species communities (Rask *et al.* 2000; Taugbøl *et al.* 2002). This feature is common for most ecosystems (Lodge 1993; Mooney & Drake 1989).

The minnow is a member of the most species-rich vertebrate family, Cyprinidae. Its total native distribution stretches from eastern Siberia to Spain and the British Isles, being common throughout large parts of the European and northern Asian continents (Borgstrøm *et al.* 1996; Museth *et al.* 2007). The minnow is a native species to Norway. However, its original distribution was rather limited, confined to low altitudes in the eastern parts of the country, mainly in three different areas at the extremes of a continuous Fennoscandian distribution (Huifeldt-Kaas 1918; Museth *et al.* 2007; see **Paper IV**, Figure 1). While the exact boundaries of the native minnow distribution may be disputed in some areas, it shares its distribution with a number of other freshwater fish species. These species seem to have had a common post-glacial immigration wave through the Ancylus Sea and Swedish river systems (Figure 1).

Due to significant topographic challenges, many freshwater systems in Norway could not be reached by any fish species. However, humans quickly started carrying live fish between water bodies to establish new harvestable populations. Salmonid fishes, in particular the brown trout, were abundant as well as being a nutritious and tasty food source, leading these species to achieve a widespread distribution across the Scandinavian Peninsula. Such movement has a more than 1000-year history (Huitfeldt-Kaas 1918).

The widespread dispersal of the minnow is much more recent, and in many regions of Norway, the minnow is today considered an invasive species. Judging by its success in the newly invaded areas, the minnow's native distribution is believed to be limited by the land elevation after the last glacial period rather than by its habitat requirements (Huifeldt-Kaas 1918). Through the last half of the 20th century, stories of collapsed high mountain trout populations surfaced more frequently, triggering suspicions of the increasing distribution of the minnow into these regions to be an important factor. The minnow has expanded its Norwegian distribution at a rapid pace since the early 20th century and is now present in all 19 counties of Norway (Hesthagen & Sandlund 1997; Museth *et al.* 2007).

The minnow rarely forms dense populations within its native distribution areas where it lives in sympatry with a number of species, some of which often are efficient predators or competitors (Saltveit & Brabrand 1991). In these locations, the minnow never reach densities at which it imposes severe competition upon other fish species. In contrast, many of the introduced populations experience only limited predation since the brown trout, and sometimes arctic char *Salvelinus alpinus*, are the only species present. In addition, many

mountain lakes of southern Norway are shallow, and they are typically oligotrophic, leaving little organic sediments to cover coarse rocky substrate. This makes a very suitable habitat for the minnow. Indeed, the minnow form very dense populations in many of these rather newly invaded lakes, making competition with existent indigenous species more likely (Museth *et al.* 2007).

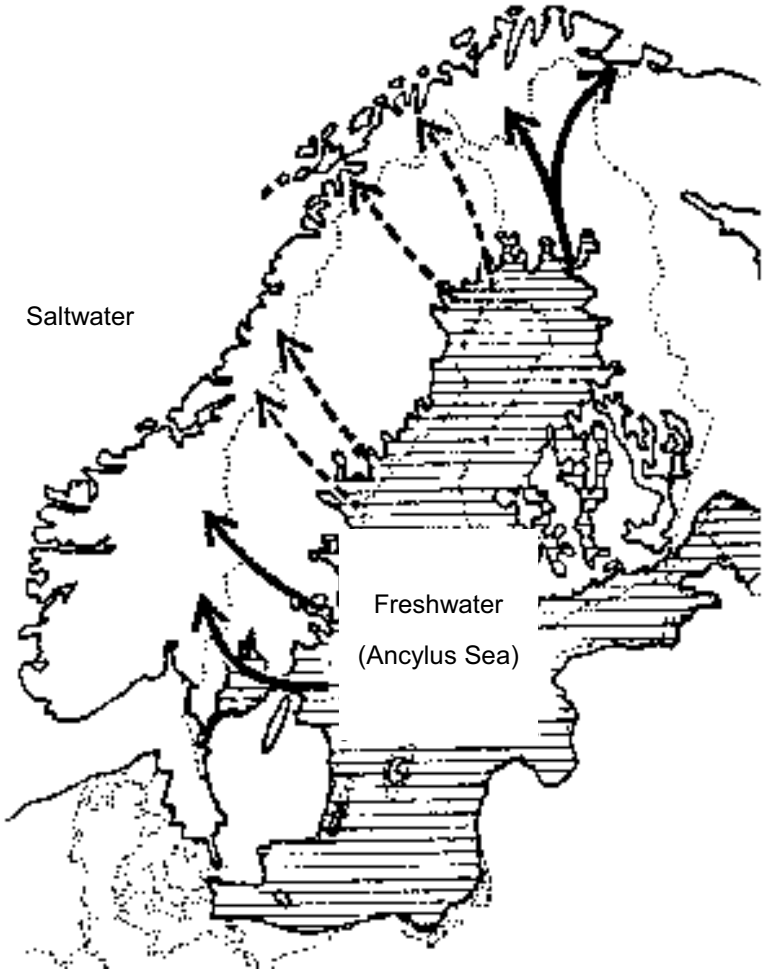

Figure 1. Extent of the Ancylus Sea approximately 8-9000 BP and major immigration routes of freshwater organisms on the Scandinavian Peninsula. Figure modified after Økland & Økland (1999).

In 1997, Norwegian fisheries management authorities outlined a plan for the prevention of continued distribution of minnows (Skurdal *et al.* 1997), emphasizing the need for knowledge

about dispersal history. It is widely recognized that most of the present day dispersal of the Norwegian minnow is exacerbated by man; however, rarely intentionally. Such man-induced dispersal is most probable to occur at small geographic scales – with movement from water body to water body at local or regional scales. However, based on available information, it has not been possible to infer the mode of dispersal of minnow into the new areas. Due to incidents of European anglers being caught in the act of fishing with live minnow bait in Norway (Erik Garnås, freshwater fish administrator at the County Governor of Buskerud, Norway, pers. comm.), there is a possibility that the source populations may come from mainland Europe. In order to investigate this, an understanding of the current phylogenetic relationship among European minnow populations and their post-glacial large-scale movements is needed. Hopefully, such information would reduce the chances of drawing false conclusions about the source populations for the various newly established populations.

A number of different hypotheses regarding means of dispersal have been put forward, most of them involving human activities. Live bait angling is efficient and has been popular among trout anglers in Norway (Borgstrøm 1973; Saltveit & Brabrand 1991; Taugbøl *et al.* 2002; Hesthagen & Sandlund 2006). During the 20th century, travelling habits developed, making it easier for people to bring live bait fish from one watercourse to another. Release of live fish that were not used for angling could establish new populations. Hydropower development has been tremendous during the same period, often involving tunnel drilling between water bodies as well as stocking of trout as mitigation measures. Hitchhiking with stocked brown trout and migrations through water transfer tunnels have likely happened in quite a few locations, both potential sources for the establishment of new populations (Hesthagen & Sandlund 1997). A few cases of intentional establishment of minnow populations also exist, but they are rare (Hesthagen & Sandlund 1997). Some also claim that dispersal by animals, e.g. waterfowl may play an additional role (NJFF 2012). Even though general means of dispersal for the minnow in Norway during the last century are well known, most often, there was no way to decipher how and from where the minnow was introduced to a specific location.

Effects of minnow introductions

Some studies show minnows to have a significant negative effect on other fish species, in particular brown trout (Brittain *et al.* 1988; Museth *et al.* 2007). Literature about the minnow as a competitor is scarce and, as far as can be retrieved, no studies on the subject have been conducted outside Fennoscandia. Particularly in locations of high fish species richness,

minnows seem to have little if any negative effect on trout growth and survival. While some argue that the minnow is a serious competitor for brown trout (Borgstrøm *et al.* 1996; Hesthagen & Sandlund 1997; Hesthagen & Sandlund 2004; Museth *et al.* 2007), others dispute this (Taugbøl *et al.* 2002).

The minnow is a small fish, rarely exceeding 10 cm in body length. It prefers cold, well-oxygenated water and feeds mainly on benthic invertebrates. Spawning usually takes place in May or June, but may be delayed to July in parts of Norway. Competition for food is considered the main cause of trout recruitment decline in lakes where the minnow has been introduced, as shown in (Borgstrøm *et al.* 1996), especially in ecosystems originally consisting of one or only a few fish species (Museth *et al.* 2007). Considerable diet overlap between minnow and brown trout is found in a Norwegian mountain lake (Museth *et al.* 2010), but the authors discuss whether this results from forced diet overlap or abundance of resources, and are therefore cautious towards drawing conclusions about the degree of competition. Other findings from this lake, such as reduced recruitment and individual growth of brown trout (Borgstrøm *et al.* 2010), as well as density reductions of important benthic invertebrates, lead the authors to interpret the diet overlap as forced. Absence of alternative prey species and habitats means that the brown trout likely experience negative effects from competition with the minnow in this particular location.

In most cases involving fresh water systems, it will be difficult or impossible to eradicate invasive species after they have become established. Exceptions do exist, and in particular, the piscicide rotenone was used to eradicate invasive minnow populations inhabiting several small lakes on the Hardangervidda mountain plateau in South Norway around the turn of the millennium (Norwegian Ministry of the Environment 2007). Special focus was drawn towards these locations as the minnow potentially could disperse from them across the continental divide and invade large areas of western Norway. The minnow was absent for about ten years and the treatment was considered a success, but the minnow has once again invaded this area (NJFF 2011). Eradications have typically larger success rates within small biotic confinements, and therefore, management measures concerning invasive species will most often involve actions to prevent further dispersal or to control population densities.

Multiple introduction events

High genetic diversity is expected to be an advantage for a pioneering species related to its larger potential for adaptation to new environments (Frankham 2005). However, a newly established population of a pioneering (or invading) species is often based on few individuals, highly restraining the population's genetic diversity (Nei *et al.* 1975). In the case of multiple immigration events, especially if those events are sourced from geographically distant locations, the resulting genetic diversity is usually significantly increased (Dlugosch & Parker 2008; Facon et al. 2008; Zalewski *et al.* 2010). The greater the genetic distance between the two or more source populations, the greater the resulting genetic diversity will become for the establishing populations. However, if local adaptations of the different source populations imply e.g. different timing of reproduction, the new species will either establish as two independent populations filling different niches, or one of them will go extinct. Such prezygotic reproductive barriers may lead to formation of new species and usually demand separation for a very long time (see e.g. Lowry *et al.* 2008). In most cases, prezygotic barriers will not exist, and multiple introductions will add to the new population's genetic diversity.

Outline of the thesis

Application of cross-species microsatellite amplification

Even with the vast and rapid evolution of molecular genetics, molecular marker development is still considered rather costly and labour consuming, involving cloning and sequencing. In many cases, the development stage surpasses the actual study with respect to financial and occupational effort. Therefore, starting in the 1990's, several studies have shown that flanking sequences of microsatellite markers often are conserved such that existing primers produce amplified microsatellites in related species (Primmer *et al.* 1996; Tong *et al.* 2002; **Papers I** and **II**). Given a reasonably high success rate, cross-species amplification procedures may come across as a cost- and labour-effective way of establishing a set of neutral molecular markers.

Phylogeography and post-glacial dispersal

Quaternary geologists have taught us that large areas of the northern hemisphere were covered with ice during a period of about 110 000 years ago until about 10 000 years ago (Donner 2005). This period, termed the Weichselian epoch in Northern Europe, left all of Scandinavia

and northern areas of the European continent uninhabitable for most living organisms. After glacier retraction during the following couple of thousands of years, species moved northwards to establish new populations in the ever-increasing open areas (see e.g. Reinhammar & Hedren 1998; Painter *et al.* 2007). Succession rates as well as availability of nutrition were important determinants of the speed in which species would disperse and establish themselves, but for many species, constraints on suitable habitats in geographically intermediate habitats were equally important.

Freshwater organisms have in general restricted ability to expand their area of distribution. Bodies of freshwater are typically separated by terrestrial areas were most freshwater organisms do not survive. Migrations along water courses, i.e. along rivers from one lake to the next, are theoretically conductable, but waterfalls often render at least upstream migrations challenging or impossible. During the melt-off period after the last glacial period, lake and river systems in northern continental Europe as well as in Scandinavia were rapidly changing. Through damming of large water bodies by glaciers actions and the increasing land elevation, water runoff directions were altered repeatedly in most drainages. Thus, during this period, freshwater organisms had a greater possibility for dispersal through freshwater systems than what is the case today.

The Scandinavian Peninsula was, except perhaps for a few Nunataks, entirely covered by an ice sheet during the Weichselian glaciation (Boulton *et al.* 2001; Paus *et al.* 2006). All freshwater species now present in this area resided in various refugia during this period – and recent studies have identified a number of potential refugia for freshwater fishes (Nesbø *et al.* 1999; Koskinen *et al.* 2000; Østbye *et al.* 2005). **Paper III** discusses some hypotheses of Weichselian freshwater refugia locations based on phylogeography studies. While different studies (and species) produce slightly different results, general conclusions agree on some common areas: One refugium was present in today's Russia, near the Ural mountain range, and another on the plains of central Europe. For some species, more western refugia were probably also present. All likely refugia that were sources of present freshwater populations in Northern Europe were situated not far from the edge of the ice sheet at its maximum coverage during the Weichselian glaciation.

The patterns and speed of species re-establishment after glacier retraction have been discussed in a number of studies throughout the history of written biology. Attention has been drawn towards both pioneer primary production species inhabiting relatively recent local glacier

retractions (Viereck 1966; Matthews & Whittaker 1987; Whittaker 1993; Stöcklin & Bäumler 1996), as well as on a larger regional or even continental scale. In particular, some studies highlight the northward expansion of species distribution areas in North America and Eurasia after the last glacial period (Blackburn 1952; Einarsson 1963; Davis 1969; Likens & Davis 1975). One common method for the latter studies has been to date findings of biological remnants, e.g. pollen or bones, using the ^{14}C method. Using such data, maps of historical species distribution can be drawn (Wright *et al.* 1963; Brubaker *et al.* 2005). However, in the last twenty years or so, modern molecular genetics techniques have made possible the investigation of phylogeography based on relationship among present populations. Initially, allozymes were the preferred molecular marker (Riffel *et al.* 1995; Roderick 1996; Toumi & Lumaret 1998), but methods utilizing e.g. AFLP, RFLP, microsatellites, SNP or sequencing of mitochondrial or chloroplast DNA have gained popularity (Verspoor *et al.* 1999; Eggert *et al.* 2002; Brumfield *et al.* 2003; Cavers *et al.* 2003; Timmermans *et al.* 2005; Krystufek *et al.* 2009).

Allele frequency data, such as provided by single nuclear polymorphism (SNP) or microsatellites, are excellent genetic markers when comparing relatively closely related populations due to their high mutation rate (Nei & Kumar 2000). However, microsatellites may impose problems when comparing populations that have been isolated from each other for an extended number of generations. Microsatellite mutations are rarely unidirectional; thus a number of mutations within an allele may lead to the exact same allele length as the original one, a phenomenon termed homoplasy (see e.g. Jarne & Lagoda 1996; Estoup *et al.* 2002). The probability increases with the number of mutations that has happened, and thus, also with time since populations split, the effect levelling out at an asymptote (Garza & Freimer 1996). Running a clustering test on a set of such distantly related populations may thus result in erroneous results (Goldstein *et al.* 1995; Coates *et al.* 2009). For that reason, genetic markers exhibiting lower mutation rates are generally preferred if the most recent common ancestors of the investigated populations are believed to be ancient. In such situations, mtDNA sequences have proven valuable, especially the more variable regions, e.g. the D-loop, cytB, cytC, and others. Still, care has to be taken when using these markers for phylogeny, as some studies find less reliable results when they are employed for more distantly related species (Larizza *et al.* 2002; Yang & Speller 2006).

Source population assignments

The Scandinavian distribution of the minnow is typical for the earliest post-glacial immigrants of obligate freshwater fish species. Westwards dispersal has effectively been limited by the Scandinavian mountain range, running along the length of the Scandinavian Peninsula. While the minnow's distribution in Scandinavia has probably been relatively stable over several thousand years, the 20th century has seen a rapid expansion, and particularly many populations have become established in the mountains of southern Norway. Local topography renders self-dispersal impossible, and human-induced dispersal is important in most cases.

I wanted to assign newly established populations to specific source populations. Sampling of potential source populations was carried out in most major watercourses of Norway holding native populations, as well as in many continental European populations. **Paper IV** presents my findings, which in general shows strong correlations between genetic and geographical distances. A few exceptions were detected; most notable was a native minnow area between the lakes Mjøsa and Randsfjorden in south-east Norway that has likely been the source of many new minnow populations.

Population genetics of newly established populations

Six of the introduced minnow populations had a D-loop haplotype that proved to be very rare among Norwegian native populations, suggesting a possible foreign origin. In two of these populations, the common Scandinavian haplotype occurred in parallel, leading me to believe they were founded by multiple introductions. I investigated the minnows' genetic clustering within these two lakes and compared their genetic diversity with that of introduced minnow populations based on single introduction events (**Paper V**).

Main objectives

The main goals of the study can be summarized in three sections:

1. Establish a better understanding of phylogeography and post-glacial dispersal routes of the minnow, including identification of potential refuges. Emphasis was to be put on the history of minnow populations of the Scandinavian Peninsula.
2. Identify source populations or regions of a number of non-native Norwegian minnow populations.

3. Investigate genetic differentiation or mixing in non-native populations founded by multiple immigration events.

Selected as the appropriate tool for the investigations, modern molecular genetics methods were believed to produce data through a large resolution range, and thus be able to bring me closer to my goals. At the time I began my work on the minnow, very few molecular markers had been developed for this species, and the ones that were available had quite limited resolution. The preferred method for my work involved microsatellite genotyping, thus a set of suitably variable microsatellites were developed (**Papers I** and **II**).

In order to identify the potential sources for the newly established minnow populations it was necessary to have good background knowledge about native minnow phylogeography, especially at the more local Norwegian scale. This was done by sampling and genotyping minnows from Norway and other areas of Europe (**Paper III**). Based on this, the different hypotheses for recent minnow dispersal in Norway were investigated, by sampling and genotyping minnow from a large number of newly established populations (**Paper IV**). Local scale population structure of introduced minnows was studied in two lakes supporting populations likely based on multiple introductions from different source populations (**Paper V**). In these populations, I also looked into genetic diversity compared with that of populations likely based on a single introduction event.

Summary of methods

Population sampling

To increase likelihood of locus polymorphism detection, samples for the cross-species studies were preferably chosen from sites situated far apart (**Papers I** and **II**), such as Northern and Southern Europe. However, for some of the species studied in **Paper I**, such samples were not obtainable.

For the post-glacial dispersal study in **Paper III**, tissue samples were obtained from large parts of the minnow's total distribution area throughout Europe. Among the sample sites, central Europe, the Black Sea area, the Iberian Peninsula and the Moscow area were not covered by the ice sheet during the Weichselian period, and were thus potential refugia. Samples sites in Fennoscandia and the southern shores of the Baltic Sea, on the other hand, were all inhabited by freshwater fish after the Weichselian period.

To investigate more recent dispersal of the minnow in Norway, additional samples were obtained from water bodies in which minnow introduction had taken place during the 20th century, i.e. non-native sites (**Paper IV**). Most of these sites were mountainous lakes and streams in southern Norway.

At an early stage in the study, two very different mtDNA D-loop haplotypes were sequenced among Norwegian minnows. While only one common haplotype was detected among native samples, both were found among non-native sites. However, only two sites, both being lakes located to the mountainous region of southern Norway, were found to have both sequences. These two lakes were sampled in detail at a later stage: Several sampling sites were identified in each lake, and for one of them, two upstream lakes known to contain minnows were also sampled (**Paper V**).

All populations were sampled from 1997 to 2003 using simple fish traps or backpack electric fishing equipment. Foreign samples were provided by fellow researchers doing field work in respective areas, while Norwegian samples were partly provided by other field workers, partly collected by myself and the co-authors of the present papers.

Laboratory methods

At an early stage, an approximate 250 bp sequence of the mitochondrial control region (D-loop) was revealed to be conserved among all sampled Norwegian native minnow populations (constituting clade A in **Paper III**). This haplotype also prevailed among introduced populations, but a very different haplotype was sequenced from some introduced populations, suggesting them to originate from foreign source populations. Other haplotypes, up to twelve bp different from the common Norwegian one, were sampled from populations in France and Austria, but none of them resembled the rare haplotype. A second haplotype was later found in one of my sampled populations, but nevertheless, higher resolution was required to reveal Norwegian phylogeography.

The Cyprinidae family is the most species-rich vertebrate family, many of which are well-studied. Different microsatellite loci reported in a number of species were tested for cross-amplification in the minnow, as described in **Papers I** and **II**. Typically, a touchdown PCR procedure was employed in the testing phase, in which the annealing temperature began at the recommended temperature for the original species, and then reduced by 0.5 °C per cycle for twenty cycles. Fifteen additional cycles at this lower temperature were added, making a total of 35 cycles. The advantage of this method is two-sided: First, detection rate increases when lowering annealing temperature, making it possible to identify microsatellite loci that will anneal only at lower temperatures than in the source species. Repeated low annealing temperature may however lead to false amplification or too much noise to identify actual microsatellite amplification. Second, the nature of the touchdown procedure means that loci annealing at lower temperatures only have the potential of amplifying for a smaller number of cycles than those annealing at higher temperatures. Thus, among amplifications at lower annealing temperatures, only those that produce very clear bands will be detectable, decreasing both the chance of false detections as well as the risk of losing true amplifications to the noise floor of random segments.

In this study, further PCR optimization was required for most loci in order to produce reliable results and peaks of reasonably similar heights. In total, twelve loci out of 160 tested cross-amplified and were polymorphic in the minnow. Polymorphism differed greatly among loci, and when amplified using minnow samples throughout Europe, they ranged from three to 60 alleles.

Genetic analyses

Scoring of genotypes was performed with the software provided for the two sequencers involved, i.e. Genotyper 2.5 on results from the ABI377 (Applied Biosystems) and Genetic Profiler 1.5 on results from the MegaBACE 1000 (GE Healthcare). Mitochondrial DNA sequences were run on an ABI 3730 sequencer and visualised with Sequence Scanner 1.0 (Applied Biosystems).

Alignment and phylogeny

Due to the low resolution of the D-loop mtDNA within Norwegian populations, sequence data analyses were employed for the large scale phylogeography study only. Sequence alignment was a straightforward task, as sequences differed by no more than 24 mutational steps, i.e. less than 10 %. However, due to the absence of many intermediate sequences, phylogeny analyses were more demanding. Substitution model was selected on the basis of MrModel tests run on the bioportal web page (www.bioportal.no), phylogeny analyses were run on software packages available online, employing neighbour-joining, maximum likelihood and parsimony methods (www.phylogeny.fr). In addition, minimum spanning networks were created using TCS 1.21 (Clement *et al.* 2000).

Genotype data were treated in two different ways for phylogeny purposes. First, traditional F_{ST}-based methods comparing predefined geographical units were employed. Here I used FSTAT 2.9.3.2 (Goudet 1995) and GENEPOP 4.0 (Raymond & Rousset 1995a; Rousset 2008) softwares. Second, I used a Bayesian method using Monte Carlo Markov Chain (MCMC) convergence. For this method, the individual is the starting point from which genetic clusters are constructed, possibly across sampling locations. Here, the softwares STRUCTURE 2.2 (Pritchard *et al.* 2000) and BAPS 4.0 (Corander *et al.* 2004) were both employed.

Assignment tests

Assignment test were performed using WHICHRUN (Banks & Eichert 2000). Only populations from which I had a minimum of 17 individual tissue samples were included as potential sources. Average assignment likelihoods were calculated on a population level. Due to obvious bottleneck events and the loss of alleles during the establishment phase, assignment likelihoods were in general low. For the introduced populations, I therefore chose

to compare relative assignment likelihoods among all potential source populations. Furthermore, for each introduced population, likelihood values were compared with self-assignment likelihoods, potentially revealing the significance of the relative assignments.

Within population genetics

Population-based genetic structure on lake/reservoir as well as watercourse level was studied by calculating pairwise F_{ST} values and genetic diversity ($1\text{-}Q_{inter}$) with GENEPOP on the web and its derivatives (Raymond & Rousset 1995a; Raymond & Rousset 1995b; Rousset 2008). The software STRUCTURE (Pritchard *et al.* 2000) was employed to establish individual-based genetic clustering.

Main results

Cross-species amplification success

Out of the 156 tested microsatellite loci in **Papers I** and **II**, 64 amplified in the minnow, 35 of them being polymorphic. Out of these, only twelve loci had sufficient enough quality and strength and unambiguous scoring to be used for population genetics analyses. Three of the tested zebrafish loci had an overall observed heterozygosity (H_O) in the range of 0.20-0.40, while the fourth zebrafish locus' H_O was only 0.01. Among 17 tested central stoneroller *Campostoma anomalum* loci, five ended up being used after selection and optimization, their observed heterozygosities ranging from 0.47 to 0.88, most of them in the upper region. Two markers developed for the common carp *Cyprinus carpio* were also quite diverse with heterozygosities above 0.50, while the sole marker originally developed for goldfish *C. auratus* had very little diversity: $H_O = 0.05$.

The developed set of twelve microsatellite markers proved to be an ample tool for fine-scale population genetics analyses (**Paper IV**), and it was used with success even for studies of within-population genetic diversity in populations founded through multiple immigration events. On a larger scale, clustering analyses failed to resolve European phylogeny when all 38 native populations were included. Here, Fennoscandian samples split to three clearly distinct clusters, while the rest of Europe clustered together. As the D-loop haplotypes revealed (see below), European samples from around the continent were distinctly different and far removed genetically, suggesting that microsatellites are not the correct tool for large scale phylogeny studies on the minnow. Interestingly though, when running clustering analyses on all but the Fennoscandian samples, clusters are more less the same as what is suggested by the D-loop analyses. One noticeable exception is the Spanish sample (no. 38 in **Paper III**), which has the identical D-loop sequence as those of the Rhine drainage and the upper reaches of the Danube drainage, but forms a distinct cluster using microsatellite data.

Clades and clusters on a European level

Mitochondrial DNA sequencing revealed deep splitting of European minnow populations. Among the sampled populations, four distinct clades stand out. In central Europe, one western (termed C in **Paper III**) and one eastern (B) clade is separated with a minimum of 10 mutations. Within these clades, haplotypes differ with up to 8 mutations. Fennoscandian

populations, along with one population sampled near Moscow, all have the same common haplotype and constitute a separate clade (A). This clade is a minimum of seven mutation steps removed from either of the central European clades. A fourth clade (D) consists of two haplotypes sampled at the same location northeast of the Black Sea. This clade is a minimum of six mutation steps different from clade C, and at least 10 and 13 mutation steps different from clades A and B, respectively. The position of haplotype 6b, sampled in River Lysakerelva in Oslo, southeast Norway, is somewhat unclear, and it may belong to either of clades C or D. I have in **Paper III** chosen to include haplotype 6b in clade C, due to the greater probability of closer relatedness with western European populations than with populations from Caucasus.

Norwegian minnow phylogeography

Within Norwegian native minnow populations, clustering analyses reveal four distinct groups. One is restricted to watersheds draining to the Oslofjord west of Oslo; the second consists of populations of the Trysil and Glomma watersheds, close to the Swedish border in south-eastern Norway, the third is a single sampling site in mid-Norway; the fourth consists of native samples from far north in Norway. Samples 7 through 10 in **Paper IV**, collected north and east of Oslo, did not easily cluster.

Source population assignments

Few individuals from the sampled non-native populations assigned unambiguously to specific source populations (Figures 4 and 5, **Paper IV**). However, quite a few non-native populations tended to assign to one or more of the closest situated native populations. Besides that, many South-Norwegian non-native populations displayed relatively high assignments to native populations 5, 6 and 8-10. Also, a couple of Mid- to North-Norway non-native populations (34 and 35) assigned strongly to source populations 13-16 from the Trysil drainage, which is situated a long ways south.

Genetic diversity and structure of multiply introduced populations

Both D-loop haplotypes A and B were found at all but one of a total of nine sample sites in the two watercourses studied in **Paper V**. In the Lægreid/Ørteren system, introduced minnows seemed to have gone through complete genetic mixing. Here, no splitting of clusters was detected, and pairwise F_{ST} values between sampling sites were below 0.007. In the

Vinstra system, the three reservoirs mainly clustered to three separate groups. However, as shown in Figure 1 and 4 in **Paper V**, the sampling site v3 clustered more closely with the upstream reservoir v2 than to other sampling sites in its own reservoir. None of the nine sampling sites showed resemblance with the potential source populations in River Lysakerelva and River Sørkedalselva.

Genetic diversities as calculated $1\text{-}Q_{inter}$ values were on average significantly larger in native minnow populations than in non-native populations. However, this was not the case for those non-native populations in which two different D-loop haplotypes were detected, suggesting multiple introduction events. Indeed, non-native populations with two haplotypes had on average significantly higher genetic diversity than non-native populations with only one haplotype.

Discussion

Main findings

Glacial refugia of the minnow are believed to have been in a range of freshwater bodies south and east of the Weichselian glacial extent. My findings suggest refugia of Baltic populations were in the Danube watershed region, while the Scandinavian populations were mainly established by immigrants from the east. Post-glacial dispersal routes from these refugia, following glacier retraction, were probably partially parallel with those of other cold-water tolerant freshwater species, such as the bullhead *Cottus gobio* (Hänfling *et al.* 2002), the chub *Squalius cephalus* (Seifertová *et al.* 2012), the grayling *Thymallus thymallus* (Koskinen *et al.* 2000) and the perch *Perca fluviatilis* (Nesbø *et al.* 1999).

As expected, my results did suggest that quite a few non-native Norwegian populations were established by nearby native populations. However, native populations sampled from one region in south-eastern Norway, between the large lakes Mjøsa and Randsfjorden (native populations 8, 9 and 10, Figure 2 in **Paper IV**), seemed to have a disproportionately large probability of being sources for many non-native populations. Dispersal events over larger distances have likely been facilitated by human activities, like live bait angling or stocking of fish.

There are no signs of prezygotic reproductive barriers between distantly related clades of minnow inhabiting common water systems. Such coexistences are probably the result of multiple invasions, and while there are tendencies of clear genetic structuring in one out of five investigated lakes/reservoirs, this is maintained by continued gene flow from an upstream lake. Through what may be genetic mixing of different clades, the resulting populations have greater genetic diversity than populations based on single event invasions.

Cross-species PCR amplification

Cross-species amplification tests have proved to be a valuable tool for obtaining useable microsatellite markers in a number of taxa. The concept involves running PCR on DNA from a different species than the microsatellite primers were designed for, and the amplification success has been reported to range widely, from above 60 % within genera to below 10 % within classes (see Barbará *et al.* 2007 for a review). In many cases however, cross-amplified

loci turned out to be monomorphic among the focal populations and are thus worthless as tools for phylogenetic studies. The success rate of a cross-species amplification study relies heavily on the relatedness between the species for which primers were developed and the focal species. It is expected that amplification success rates will be higher when species belong to a common low taxonomic level. Despite this, some taxa seem to preserve flanking regions better than others. A general review of cross-species amplification studies from several taxa is given in Barbará *et al.* (2007).

Cross-species amplification successes observed in **Papers I** and **II** were comparable to similar studies conducted on a number of taxa. However, in a specific study on cyprinids, Dubut *et al.* (2010) reported mean cross-species success rates for 41 loci as high as 95.1 % on a wide range of cyprinids.

In **Paper II**, I briefly discuss the relevance of cross-species amplification studies for phylogenetic analyses. However, while I find tendencies for more closely related species to cross-amplify, results are not conclusive. My somewhat conflicting results in this matter emphasize the importance of employing a sufficiently large number of markers if cross-species amplification is to be used for phylogeny studies.

Post-glacial history of the European minnow

Large areas of relatively lowland topography south and east of the glacial maximum were likely refugia for the minnow during the glaciations. As suggested by a modern topographic map of Europe (Figure 2), high mountain ranges such as the Pyrenees, the Alps and the Carpathians, restricted northwards dispersal from the Iberian Peninsula, Italy and south-eastern Europe. Today's Baltic Sea drainage basin covers an area southwards to the Carpathians and coincides largely with Poland's borders with Czech Republic, Slovakia, Ukraine and Belarus, as well as Finland's and the Baltic countries' borders with Russia and Belarus. While some watershed boundaries coincide with high mountain ranges, others are elevation maximas in otherwise relatively flat landscapes, and post-glacial migrations may very well have taken place across such boundaries.

Based on the theory of molecular clocks (Zuckerkandl & Pauling 1962), minnow populations in central Europe diverged before the Weichselian glaciation, but probably during the epoch of Pleistocene glaciations. Due to clear divergence among geographically closely situated

populations, there are reasons to believe that divergence could be large also in other glacial refugia for the minnow. A much less hilly topography in Ukraine and Russia than in central Europe may however have allowed higher gene flow across larger areas, restricting genetic divergence. The main Fennoscandian minnow clade (clade A in **Paper III**) originated with high certainty from areas east of the glacial maximum, but due to only one sampled population in this potential source region, the exact refugium cannot be pinpointed. It remains uncertain if clade A distribution covers larger areas in Russia (and surrounding areas).

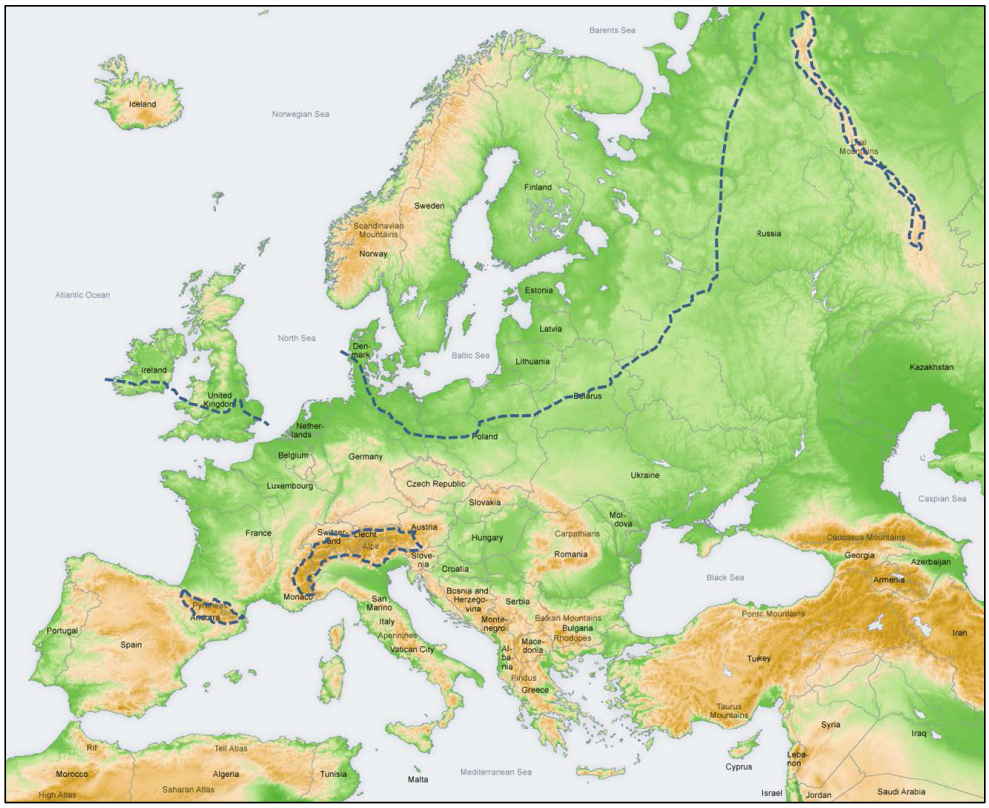

Figure 2. Topographic map of Europe. The map is collected from Wikimedia Commons, created using the Generic Mapping Tools (http://gmt.soest.hawaii.edu/). Dashed lines denote approximate Weichselian glacial maximum, a modification of the original map.

The rare Fennoscandian haplotype (6b in **Paper III)**, is suspected to originate from land areas surrounding the English Channel, either in England or France. This assumption is based on the quite distant relatedness of at least six mutation steps from clade C, which inhabits the Rhine and upper Danube tributaries, and the lack of samples from Great Britain and western France. Furthermore, Nesbø *et al.* (1999) argue that perch, a species with a distribution very

similar to the minnow, may have immigrated from the south to the Scandinavian Peninsula through freshwater drainages across a temporary post-glacial land bridge, an area now covered by the North Sea. Confusing enough, haplotype 6b is also six mutation steps removed from clade D, sampled in the north Caucasus region, within the Black Sea drainage. Post-glacial dispersal to Fennoscandia from areas closer to the Black Sea was not highly unlikely due to the mostly flat topography of the continent between the Black Sea and the Baltic Sea, but as far as today's literature on freshwater fish post-glacial dispersal is concerned, no other species have undergone dispersal along this route. Therefore, I believe that the glacial refugium of populations with the 6b haplotype was in an area not covered by my sampling scheme. In any case, more sampling of native populations, both in Norway and continental Europe is needed to pinpoint the origin of this unique haplotype.

Post-glacial history of the Norwegian minnow

Post-glacial Fennoscandian immigration of freshwater species started soon after the first melt water rivers appeared along the coast, providing habitat for species tolerant of saltwater and low temperatures. In particular, species such as salmon *Salmo salar*, brown trout, arctic charr *Salvelinus alpinus* and three-spined stickleback *Gasterosteus aculeatus* were among the pioneering species. While the minnow certainly is cold water tolerant, it does not survive particularly high salinities (Kottelat & Freyhof 2007), meaning that the species immigrated to the Scandinavian Peninsula from the east through the Ancylus Sea. The minnow was, along with grayling, pike *Esox lucius*, perch *Perca fluviatilis*, whitefish *Coregonus lavaretus*, nine-spined stickleback *Pungitius pungitius* and burbot *Lota lota* among the first fish species to immigrate fresh waters in this region (Huitfeldt-Kaas 1918). The Scandinavian mountain range runs along almost the entire length of the Scandinavian Peninsula, making most rivers too steep for upstream fish migrations. While the highest and steepest mountains are situated in Norway, the mountain range includes the western parts of Sweden's northernmost two thirds extent, stopping fish migrations before crossing the border. However, south-eastern and north-eastern parts of Norway have always been relatively smooth lowlands, and the first freshwater species that immigrated to the Scandinavian Peninsula through the Ancylus Sea were thus able to reach that far. Continued land elevation following glacier retraction prevented species of later immigration waves from reaching as far west or north.

Within Norway, a rare haplotype (6b in **Paper III**) have a distinctly different origin than the common clade A. While a specific Weichselian refugium for the 6b haplotype is not clear, it

is not unlikely that the only native 6b population sampled was founded by immigrants from the south, i.e. north-western continental Europe. The 6b haplotype is too different from any of my sampled potential source populations to pin-point a refugium, and the microsatellite data are generally too fine-scaled to resolve phylogeography on a European scale. Immigration from the continental North Sea coast or the English Channel region however, cannot be ruled out.

The minnow populations in Norway are structured into four main clusters, relating to post-glacial immigration (**Paper III)**. All Norwegian populations belonging to clade A have probably an eastern origin and immigrated through the Ancylus Sea and Swedish rivers after glacier retraction. Due to today's genetic clustering into regions, each encompassing non-connected watercourses, there is reason to believe that the minnow did not undergo complete mixis in the Ancylus Sea. Rather, the minnow dispersed in temporally and/or geographically non-overlapping waves through the Ancylus Sea from a number of glacial refugia, or the huge distances within the Ancylus Sea slowed down or prevented genetic mixing. Both scenarios would lead to regional clustering in Norway.

None of the four clusters seemed to share close ancestry with foreign samples, not even with the Moscow sample belonging to clade A. The three eastern clusters have probably immigrated from east, through the Ancylus Sea. These clusters are geographically distributed so that they correlate well with post-glacial topography and drainages. E.g. native samples 11-16 cluster together, even if they consist of samples from two separate large drainages, Trysil and Glomma. Shortly after deglaciation, both rivers drained to a bay of the Ancylus Sea, which today is Lake Vänern in Sweden, explaining this genetic cluster. River Glomma took later a different course to run into the ocean in south-eastern Norway. The sampled mid-Norwegian population constituted a separate genetic cluster, as did native samples from Finnmark in northern Norway.

Fluctuating climate during the epoch of the Ancylus Sea, as well as differing glacial refugia and continued land elevation, limited the dispersal possibilities of later immigrants. It is generally believed that the native distribution of the eastern freshwater fish immigrants on the Scandinavian Peninsula correlates with timing of immigration. Earlier immigrants got further west and north and inhabited several areas along Norway's north-south axis, while later immigrants reached no further than south-eastern Norway (Figure 1). Without speculating too much, the theory of parallel immigration of many freshwater fish species should not be ruled

out. The Ancylus Sea was a huge lake, and differing temperatures within its water body, especially between north and south, may have facilitated the immigration of different species of fish to the different parts of the peninsula.

Identifying source populations and current dispersal routes

The last century or so, Norway has experienced a tremendous dispersal of the minnows to non-native waters. Most new populations have established in mountain lakes and slow-running streams, far from the minnow's native distribution areas. Given the distances and the elevation of the new habitats, human-induced dispersal is a probable explanation. There are several hypotheses of dispersal means, but live bait angling and fish stocking remain the most viable ones. To better understand dispersal mechanisms, I investigated relatedness among minnows from native and non-native populations, both on an individual and on a population level. In addition, specific assignment analyses were performed to pinpoint source populations or regions.

Assignment values suggest that genetic and geographic distances between non-native and native minnow populations are correlated, but nonetheless, an overwhelmingly large number of the sampled non-native populations assigned to a group of potential source populations sampled in an area between lakes Mjøsa and Randsfjorden (Figure 4, **Paper IV**). However, this figure presents relative assignment values among all sampled potential source populations, and the absolute values are in many cases very low. When compared with self-assignment values, only a few non-native populations remain high and thus show clear signs of relatedness (Figure 5, **Paper IV**). Nonetheless, many non-native populations seem to originate from this particular region. In addition, introduced populations 34 and 35 assigned clearly to native populations of the Trysil drainage (14, 15 and 16), situated several hundred kilometres south, making another exception from the usual geographic and genetic correlation.

Genetic diversity – a competitive advantage?

I hypothesized that no genetic structuring would be evident in the two investigated watercourses in **Paper V**, even if two very different D-loop haplotypes were detected. In one of the investigated reservoirs, however, there were tendencies of clear genetic structuring, probably maintained by continued immigration from an upstream lake. Here, pairwise F_{ST}

values between the upstream lake and respective sampling sites in the downstream lake were strongly correlated with distance (Figure 3, **Paper V**). I conclude therefore that the observed structuring is not a consequence of reproductive barriers, but of the continued gene flow acting strongly on one part of the population. With time, the downstream population will likely homogenize as its genetic composition evolves and gets more similar to that of the upstream population.

There is considerable theoretical as well as empirical evidence of the benefits of phenotypic plasticity or genetic diversity of a population when facing an unstable or unpredictable environment (see e.g. Gibbs & Dyck 2009, Jacobs & Latimer 2012, Tan & Gore 2012 for discussions). After immigrating to a new environment, the founding population usually go through a considerable genetic bottleneck, removing much of the genetic diversity of the source population (Allendorf & Lundquist 2003). It used to be general consensus that this loss of genetic diversity may prevent or at least slow down the adaptation of an invasive (or pioneering) species (Nei *et al.* 1975). However, more recent studies have taken into account the effects of multiple (or repeated) invasions of a species into a new habitat. Through multiple invasions from a common source, a larger selection of the available alleles will persist in the new population, hence reducing bottleneck effects. More importantly, when an introduced population originate from different sources, the resulting mixed population may display an even larger genetic diversity than either of its source populations (See e.g. Kelly *et al.* 2006 for a discussion), possibly leading to heterosis, the competitive advantage of hybrids (Birchler *et al.* 2010). On the other hand, some authors argue that such genetic mixing may lead to outbreeding depression (Tymchuk 2007; Jourdan-Pineau 2012).

While I have no actual fitness measurements to correlate with genetic divergence estimates, I found that those non-native populations that had both haplotypes A and B (**Paper V**) displayed significantly higher genetic diversity than those that had only one haplotype. In fact, genetic diversities of the former were on par with those of native populations. Bottleneck tests and estimates of effective population sizes did not provide much basis to estimate the number of settlers that first arrived. However, many of the non-native populations are found in quite remote mountainous areas of Norway, implying that whatever the means of dispersal, the initial number of individuals were small. Therefore, the genetic diversities observed in the multiply introduced populations is rather interesting, and I conclude in **Paper V** that while it is important to prevent further dispersal of an invasive species, management authorities

should also implement measures to avoid continued gene flow into populations of invasive species. By denying continued gene flow into the system, the bottlenecked population of the invasive species may impose a lesser threat to the existing biota.

Epilogue

Hundreds of runs through the STRUCTURE software (Pritchard *et al.* 2000) reveal the importance of including a sufficient number of iterations. In a recent paper, Gilbert *et al.* (2012) present suggestions for guidelines using the software as well as for the interpretation method in Evanno *et al.* (2005). It is stressed that the number of burnin iterations and MCMC repetitions are high enough, and that repeated runs for a dataset will increase the likelihood of obtaining the correct number of clusters (*K*). Depending on the complexity of genetic structuring of the included individuals, a premature abortion of a STRUCTURE run may give a completely different result than a longer run due to the likelihood of a specific value of *K* fluctuating erratically during a run's first phase (Figure 3). In any case, such situations often reflect complex genetic patterns adding to the picture, rather than opposing results. Typically, obtaining a lower likelihood value of a particular *K* when more iterations are run, favouring a higher value of *K*, is likely due to a further subdivision of existing clusters. While conclusions of differing phylogenetic division depths can be deciphered from this, it can never serve as a substitute for adequate sampling and genetic marker choice.

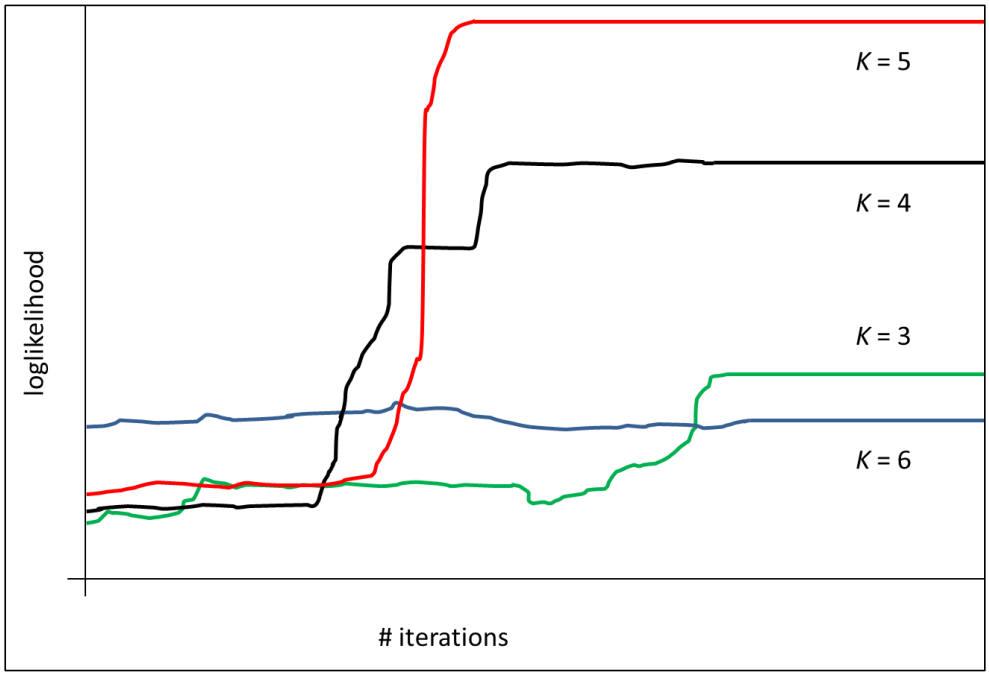

Figure 3. Hypothetical development of loglikelihoods for four different *a priori* values of *K* through the iterations of a STRUCTURE run.

In the case of the Norwegian minnow, phylogeographic relationships between native and introduced populations seem to be obscured by a combined action of multiple introductions and bottleneck events. Most introduced populations reveal probable regions for their origins, but precise dispersal patterns remain unclear. More thorough population sampling and development of additional markers can perhaps produce more tangible conclusions, but for most introduced populations, pinpointing of specific source populations will remain challenging.

Invasion biology is a rapidly growing field, being fuelled by ever increasing international travelling and goods trade. The importance of genetic diversity and evolution for a species' survival success in its native or new habitat has earned increased attention, and many modern ecological studies involve such considerations. In this thesis, such effects are only superficially treated, but it is of my belief that species- and population-specific knowledge of these subjects will play an important role in future management of invasive species.

References

Allendorf FW, Lundquist LL (2003) Introduction: Population biology, evolution, and control of invasive species. *Conservation Biology* **17**, 24-30.

Avise JC (1989) A role for molecular genetics in the recognition and conservation of endangered species. *Trends in Ecology and Evolution* **4**, 279-281.

Banks M, Eichert W (2000) Computer note. WHICHRUN (version 3.2): a computer program for population assignment of individuals based on multilocus genotype data. *Journal of Heredity* **91**, 87-89.

Barbará T, Palma-Silva C, Paggi GM, Bered F, Fay MF, Lexer C (2007) Cross-species transfer of nuclear microsatellite markers: potential and limitations. *Molecular Ecology* **16**, 3759-3767.

Bartlett JMS, Stirling D (2003) *PCR protocols* Humana Press, Totowa, NJ.

Beaumont MA (1999) Detecting population expansion and decline using microsatellites. *Genetics* **153**, 2013-2029.

Birchler JA, Hong Y, Chudalayandi S, Vaiman D, Veitiac RA (2010) Heterosis. *The Plant Cell* **22**, 2105-2112.

Blackburn KB (1952) The dating of a deposit containing an elk skeleton found at Neasham near Darlington, County Durham. *New Phytologist* **51**, 364-377.

Borgstrøm R, Brittain JE, Hasle K, Skjolaas S, Dokk JG (1996) Reduced recruitment in brown trout *Salmo trutta*, the role of interactions with the minnow *Phoxinus phoxinus*. *Nordic Journal of Freshwater Research* **72**, 30-38.

Borgstrøm R, Museth J, Brittain JE (2010) The brown trout (*Salmo trutta*) in the lake, Øvre Heimdalsvatn: long-term changes in population dynamics due to exploitation and the invasive species, European minnow (*Phoxinus phoxinus*). *Hydrobiologia* **642**, 81-91.

Borroto-Paez R (2009) Invasive mammals in Cuba: an overview. *Biological Invasions* **11**, 2279-2290.

Boulton GS, Dongelmans P, Punkari M, Broadgate M (2001) Palaeoglaciology of an ice sheet through a glacial cycle: the European ice sheet through the Weichselian. *Quaternary Science Reviews* **20**, 591-625.

Branch GM, Odendaal F, Robinson TB (2010) Competition and facilitation between the alien mussel *Mytilus galloprovincialis* and indigenous species: Moderation by wave action. *Journal of Experimental Marine Biology and Ecology* **383**, 65-78.

Brittain JE, Brabrand Å, Saltveit SJ, Bremnes T, Røsten E (1988) The biology and population dynamics of *Gammarus lacustris* in relation to the introduction of minnows, *Phoxinus phoxinus*, into Øvre Heimdalsvatn, a Norwegian subalpine lake. Report in Norwegian. Laboratory for Freshwater Ecology and Inland Fisheries, Oslo, 30 p.

Brubaker LB, Anderson PM, Edwards ME, Lozhkin AV (2005) Beringia as a glacial refugium for boreal trees and shrubs: new perspectives from mapped pollen data. *Journal of Biogeography* **32**, 833-848.

Brumfield RT, Beerli P, Nickerson DA, Edwards SV (2003) The utility of single nucleotide polymorphisms in inferences of population history. *Trends in Ecology & Evolution* **18**, 249-256.

Callaway RM, Aschehoug ET (2000) Invasive plants versus their new and old neighbors: a mechanism for exotic invasion. *Science* **290**, 521-523.

Cavers S, Navarro C, Lowe AJ (2003) Chloroplast DNA phylogeography reveals colonization history of a Neotropical tree, *Cedrela odorata* L., in Mesoamerica. *Molecular Ecology* **12**, 1451-1460.

Champagnon J, Elmberg J, Guillemain M, Gauthier-Clerc M, Lebreton J-D (2012) Conspecifics can be aliens too: A review of effects of restocking practices in vertebrates. *Journal for Nature Conservation* **20**, 231-241.

Clement M, Posada D, Crandall KA (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology* **9**, 1657-1659.

Coates BS, Sumerford DV, Miller NJ*, et al.* (2009) Comparative performance of single nucleotide polymorphism and microsatellite markers for population genetic analysis. *Journal of Heredity* **100**, 556-564.

Colautti RI, MacIsaac HJ (2004) A neutral terminology to define 'invasive' species. *Diversity and Distributions* **10**, 135-141.

Corander J, Waldmann P, Marttinen P, Sillanpaa MJ (2004) BAPS 2: Enhanced possibilities for the analysis of genetic population structure. *Bioinformatics* **20**, 2363-2369.

Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics* **144**, 2001-2014.

Crnokrak P, Roff DA (1999) Inbreeding depression in the wild. *Heredity* **83**, 260-270.

Darwin C (1859) *On the origin of species by means of natural selection,: or the preservation of favoured races in the struggle for life.* J. Murray, London.

Davis MB (1969) Climatic changes in southern Connecticut recorded by pollen deposition at Rogers Lake. *Ecology* **50**, 409-422.

Dlugosch KM, Parker IM (2008) Founding events in species invasions: genetic variation, adaptive evolution, and the role of multiple introductions. *Molecular Ecology* **17**, 431-449.

Donner J (2005) *The Quaternary history of Scandinavia.* Cambridge University Press, Cambridge ; New York.

Dubut V, Sinama M, Martin J-F, Meglécz E, Fernandez J, Chappaz R, Gilles A, Costedoat C (2010) Cross-species amplification of 41 microsatellites in European cyprinids: A tool for evolutionary, population genetics and hybridization studies. *BMC Research Notes* **3**:135.

Eggert LS, Rasner CA, Woodruff DS (2002) The evolution and phylogeography of the African elephant inferred from mitochondrial DNA sequence and nuclear microsatellite markers. *Proceedings of the Royal Society of London B. Biological Sciences* **269**, 1993-2006.

Einarsson T (1963) Pollen-analytical studies on vegetation and climate history of Iceland in late and post-glacial times. In: *North Atlantic Biota and their History* (eds. Löve A, Löve D), pp. 255-365. Pergamon.

Ellstrand NC, Elam DR (1993) Population genetic consequences of small population size: implications for plant conservation. *Annual Review of Ecology and Systematics* **24**, 217-242.

Estoup A, Guillemaud T (2010) Reconstructing routes of invasion using genetic data: why, how and so what? *Molecular Ecology* **19**, 4113-4130.

Estoup A, Jarne P, Cornuet J-M (2002) Homoplasy and mutation model at microsatellite loci and their consequences for population genetics analysis. *Molecular Ecology* **11**, 1591-1604.

Evanno G, Regnaut S, Goudet J (2005) Detecting the number of clusters of individuals using the software structure: a simulation study. *Molecular Ecology* **14,** 2611-2620.

Facon B, Pointier J-P, Jarne P, Sarda V, David P (2008) High genetic variance in life-history strategies within invasive populations by way of multiple introductions. *Current Biology* **18**, 363-367.

Flessa KW (1980) Biological effects of plate tectonics and continental drift. *BioScience* **30**, 518-523.

Frankham R (2005) Genetics and extinction. *Biological Conservation* **126**, 131-140.

Garza JC, Freimer NB (1996) Homoplasy for size at microsatellite loci in humans and chimpanzees. *Genome Research* **6**, 211-217.

Garza JC, Williamson EG (2001) Detection of reduction in population size using data from microsatellite loci. *Molecular Ecology* **10**, 305-318.

Gibbs M; Dyck H (2009) Reproductive plasticity, oviposition site selection, and maternal effects in fragmented landscapes. *Behavioral Ecology and Sociobiology* **2009**, 1-11.

Gilbert KJ, Andrew RL, Bock DG, Franklin MT, Kane NC, Moore JS, Moyers BT, Renaut S, Rennison DJ, Veen T, Vines TH (2012) Recommendations for utilizing and reporting

population genetic analyses: the reproducibility of genetic clustering using the program STRUCTURE. *Molecular Ecology* **21**, 4925-4930.

Goldstein D, Linares A, Cavalli-Sforza L, Feldman M (1995) Genetic absolute dating based on microsatellites and the origin of modern humans. *PNAS* **92**, 6723-6727.

Goudet J (1995) FSTAT version 1.2: a computer program to calculate F-statistics. *Journal of Heredity* **86,** 485-486.

Hallam A (1981) Plate tectonics, biogeography and evolution. *Nature* **293**, 31-32.

Hänfling B, Hellemans B, Volckaert FAM, Carvalho GR (2002) Late glacial history of the cold-adapted freshwater fish *Cottus gobio*, revealed by microsatellites. *Molecular Ecology* **11**, 1717-1729.

Hesthagen T, Sandlund OT (1997) Endringer i utbredelse av ørekyte i Norge: årsaker og effekter. In: *NINA-NIKU Fagrapport* (ed. Heggberget TG), p. 16. NINA-NIKU, Trondheim. Report in Norwegian.

Hesthagen T, Sandlund OT (2004) Fish distribution in a mountain area in south-eastern Norway: human introductions overrule natural immigration. *Hydrobiologia* **521**, 49-59.

Hesthagen T, Sandlund OT (2006) NOBANIS - Invasive Alien Species Fact Sheet: *Phoxinus phoxinus*. From: Online database of the European network on invasive alien species – NOBANIS www.nobanis.org.

Hillis DM (1987) Molecular versus morphological approaches to systematics. *Annual Review of Ecology and Systematics* **18**, 23-42.

Huifeldt-Kaas H (1918) Ferskvandsfiskenes utbredelse og indvandring i Norge, med et tillæg om krebsen Centraltrykkeriet, Kristiania. In Norwegian.

Humphries CJ (2005) *Cladistics biogeography*. John Wiley & Sons, Ltd.

Hunter ML, Gibbs JP (2007) *Fundamentals of conservation biology* Blackwell, Malden, Mass.

Jacobs BS, Latimer AM (2012) Analyzing reaction norm variation in the field vs. greenhouse: comparing studies of plasticity and its adaptive value in two species of *Erodium*. *Perspectives in Plant Ecology Evolution and Systematics* **14**, 325-334.

Jarne P, Lagoda PJL (1996) Microsatellites, from molecules to populations and back. *Trends in Ecology & Evolution* **11**, 424-429.

Jourdan-Pineau H, Folly J, Crochet P-A, David P (2012) Testing the influence of family structure and outbreeding depression on heterozygosity-fitness correlations in small populations. *Evolution* **66**, 3624-3631.

Kelly DW, Muirhead JR, Heath DD, Macisaac HJ (2006) Contrasting patterns in genetic diversity following multiple invasions of fresh and brackish waters. *Molecular Ecology* **15**, 3641-3653.

Kjærstad G, Arnekleiv JV (2011) Effects of rotenone treatment on lotic invertebrates. *International Review of Hydrobiology* **96**, 58-71.

Koskinen MT, Ranta E, Piironen J, Veselov A, Titov S, Haugen TO, Nilsson J, Carlstein M, Primmer CR (2000) Genetic lineages and postglacial colonization of grayling (*Thymallus thymallus*, Salmonidae) in Europe, as revealed by mitochondrial DNA analyses. *Molecular Ecology* **9**, 1609-1624.

Kottelat M, Freyhof J (2007) Handbook of European freshwater fishes. Publications Kottelat, Cornol, Switzerland. 646 p.

Krystufek B, Bryja J, Buzan EV (2009) Mitochondrial phylogeography of the European ground squirrel, Spermophilus citellus, yields evidence on refugia for steppic taxa in the southern Balkans. *Heredity* **103**, 129-135.

Larizza A, Pesole G, Reyes A, Sbisà E, Saccone C (2002) Lineage specificity of the evolutionary dynamics of the mtDNA D-Loop region in rodents. *Journal of Molecular Evolution* **54**, 145-155.

Latter BDH (1972) Selection in finite populations with multiple alleles. III. Genetic divergence with centripetal selection and mutation. *Genetics* **70**, 475-490.

Lieberman BS (2005) Geobiology and paleobiogeography: tracking the coevolution of the Earth and its biota. *Palaeogeography, Palaeoclimatology, Palaeoecology* **219**, 23–33.

Likens GE, Davis MB (1975) Post-glacial history of Mirror Lake and its watershed in New Hampshire, U.S.A.: an initial report. *Verhandlungen Internationale Vereinigung Limnologie* **19**, 982-993.

Lodge DM (1993) Biological invasions: lessons for ecology. *Trends in Ecology & Evolution* **8**, 133-137.

Lowry DB, Modliszewski JL, Wright KM, Wu CA, Willis JH (2008) The strength and genetic basis of reproductive isolating barriers in flowering plants. *Philosophical Transactions of the Royal Society B* **363**, 3009-3021.

Macdonald DW, Service K (2007) Key topics in conservation biology. Blackwell Publishing, Malden, Massachusetts.

Matthews JA, Whittaker RJ (1987) Vegetation succession on the Storbreen Glacier foreland, Jotunheimen, Norway: a review. *Arctic and Alpine Research* **19**, 385-395.

Minden V, Jacobi JD, Porembski S, Boehmer HJ (2010) Effects of invasive alien kahili ginger (*Hedychium gardnerianum*) on native plant species regeneration in a Hawaiian rainforest. *Applied Vegetation Science* **13**, 5-14.

Mooney HA, Cleland EE (2001) The evolutionary impact of invasive species. *Proceedings of the National Academy of Sciences of the United States of America* **98**, 5446-5451.

Mooney HA, Drake JA (1989) Biological invasions: a SCOPE program overview. In: *Biological invasions: a global perspective* (ed. Drake JA), pp. 491-506. John Wiley & Sons, New York.

Mosca E, Eckert AJ, Di Pierro EA, Rocchini D, La Porta N, Belletti P Neale DB (2012) The geographical and environmental determinants of genetic diversity for four alpine conifers of the European Alps. *Molecular Ecology* **21**, 5530-5545.

Museth J, Borgstrøm R, Brittain JE (2010) Diet overlap between introduced European minnow (*Phoxinus phoxinus*) and young brown trout (*Salmo trutta*) in the lake, Øvre

Heimdalsvatn: a result of abundant resources or forced niche overlap? *Hydrobiologia* **642**, 93-100.

Museth J, Hesthagen T, Sandlund OT, Thorstad EB, Ugedal O (2007) The history of the minnow *Phoxinus phoxinus* (L.) in Norway: from harmless species to pest. *Journal of Fish Biology* **71**, 184-195.

Nakata K, Nakaoka T, Goshima S (2006) Negative effects of the invasive brown trout *Salmo trutta* on freshwater crustacean species: predation on the endangered Japanese endemic crayfish species *Cambaroides japonicus* by the trout. *Nippon Suisan Gakkaishi* **72**, 447-449.

Nei M (1972) Genetic distance between populations. *The American Naturalist* **106**, 283.

Nei M, Kumar S (2000) Molecular evolution and phylogenetics. Oxford University Press. New York.

Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution* **29**, 1-10.

Nei M, Tajima F, Tateno Y (1983) Accuracy of estimated phylogenetic trees from molecular data. *Journal of Molecular Evolution* **19**, 153-170.

Nesbø CL, Fossheim T, Vøllestad LA, Jakobsen KS (1999) Genetic divergence and phylogeographic relationships among European perch (*Perca fluviatilis*) populations reflect glacial refugia and postglacial colonization. *Molecular Ecology* **8**, 1387-1404.

NJFF (2011) News story on Norwegian Association of Hunters and Anglers' home page: http://www.njff.no/portal/page/portal/njff/nyhet?element_id=133885981&displaypage=TRUE

NJFF (2012) News story on Norwegian Association of Hunters and Anglers' home page: http://www.njff.no/portal/page/portal/njff/artikkel?element_id=175782018&displaypage=TRUE

Norwegian Ministry of the Environment (2007) Strategy on invasive alien species. Report, 57 pp.

Økland J, Økland KA (1999) *Vann og vassdrag 4 – Dyr og planter: Innvandring og geografisk fordeling*. Book in Norwegian. Vett & Viten. 200 p.

Østbye K, Bernatchez L, Naesje TF, Himberg KJM, Hindar K (2005) Evolutionary history of the European whitefish *Coregonus lavaretus* (L.) species complex as inferred from mtDNA phylogeography and gill-raker numbers. *Molecular Ecology* **14**, 4371-4387.

Ostermann-Kelm S, Atwill E, Rubin E, Hendrickson L, Boyce W (2009) Impacts of feral horses on a desert environment. *BMC Ecology* **9**, 22.

Painter JN, Siitonen J, Hanski I (2007) Phylogeographical patterns and genetic diversity in three species of Eurasian boreal forest beetles. *Biological Journal of the Linnean Society* **91**, 267-279.

Paus A, Velle G, Larsen J, Nesje A, Lie Ø (2006) Lateglacial nunataks in central Scandinavia: biostratigraphical evidence for ice thickness from Lake Flåfattjønn, Tynset, Norway. *Quaternary Science Reviews* **25**, 1228-1246.

Phillips BL, Brown GP, Shine R (2010) Life-history evolution in range-shifting populations. *Ecology* **91**, 1617-1627.

Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: A computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity* **90**, 502-503.

Primmer CR, Møller AP, Ellegren H (1996) A wide-range survey of cross-species microsatellite amplification in birds. *Molecular Ecology* **5**, 365-378.

Pritchard JK, Stephens M, Donnelly P (2000) Inference of population structure using multilocus genotype data. *Genetics* **155**, 945-959.

Rahel FJ (2000) Homogenization of fish faunas across the United States. *Science* **288**, 854-856.

Rask M, Appelberg M, Hesthagen T, Tammi J, Beier U, Lappalainen A (2000). Fish status survey of Nordic lakes: species composition, distribution, effects of environmental changes. TemaNord, Report 2000:508.

Raymond M, Rousset F (1995a) GENEPOP (Version 1.2): Population genetics software for exact tests and ecumenicism. *Journal of Heredity* **86**, 248-249.

Raymond M, Rousset F (1995b) An exact test for population differentiation. *Evolution* **49**, 1280-1283.

Reinhammar L-G, Hedren M (1998) Allozyme differentiation between lowland and alpine populations of *Pseudorchis albida* s.lat. (Orchidaceae) in Sweden. *Nordic Journal of Botany* **18**, 7-14.

Riffel M, Storch V, Schreiber A (1995) Allozyme variability of brown trout (*Salmo trutta* L.) populations across the Rhenanian-Danubian watershed in southwest Germany. *Heredity* **74**, 241-249.

Roderick GK (1996) Geographic structure insect populations: Gene flow, phylogeography, and their uses. *Annual Review of Entomology* **41**, 325-352.

Rousset F (2008) GENEPOP'007: a complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* **8**, 103-106.

Saltveit SJ, Brabrand Å (1991) Ørekyt. En litteraturoversikt om økologi og utbredelse i Norge. Report in Norwegian LFI 130:1-21.

Shriver M, Jin L, Boerwinkle E, Deka R, Ferrell RE, Chakraborty R (1995) A novel measure of genetic distance for highly polymorphic tandem repeat loci. *Molecular Biology and Evolution* **12**, 914-920.

Skurdal J, Hartvigsen R, Hesthagen T, Vøllestad A, Aas Ø (1997) Et krafttak mot ørekyte. Report in Norwegian. Østlandsforskning, Lillehammer. 35 p.

Sorte CJB, Williams SL, Carlton JT (2010) Marine range shifts and species introductions: comparative spread rates and community impacts. *Global Ecology and Biogeography* **19**, 303-316.

Stephens PA, Sutherland WJ (1999) Consequences of the Allee effect for behaviour, ecology and conservation. *Trends in Ecology and Evolution* **14**, 401-405.

Stöcklin J, Bäumler E (1996) Seed rain, seedling establishment and clonal growth strategies on a glacier foreland. *Journal of Vegetation Science* **7**, 45-56.

Takezaki N POPTREE version for DOS.
http://www.bio.psu.edu/People/Faculty/Nei/Lab/Programs.html

Tan L, Gore J (2012) Slowly switching between environments facilitates reverse evolution in small populations. *Evolution* **66**, 3144-3154.

Taugbøl T, Hesthagen T, Museth J, Dervo B, Andersen O (2002) Effekter av ørekytintroduksjoner og utfiskingstiltak - en vurdering av kunnskapsgrunnlaget, pp. 1-31. Report in Norwegian. NINA - NIKU, Lillehammer.

Timmermans MJTN, Ellers J, Mariën AGH, Verhoef SC, Ferwerda EB, VanStraalen NM (2005) Genetic structure in *Orchesella cincta* (Collembola): strong subdivision of European populations inferred from mtDNA and AFLP markers. *Molecular Ecology* **14**, 2017-2024.

Tong J, Wang Z, Yu X, Wu Q, Chu KH (2002) Cross-species amplification in silver carp and bighead carp with microsatellite primers of common carp. *Molecular Ecology Notes* **2**, 245-247.

Toumi L, Lumaret R (1998) Allozyme variation in cork oak (*Quercus suber* L.): the role of phylogeography and genetic introgression by other Mediterranean oak species and human activities. *Theoretical and Applied Genetics* **97**, 647-656.

Tymchuk WE, Sundström LF, Devlin RH (2007) Growth and survival trade-offs and outbreeding depression in rainbow trout (*Oncorhynchus mykiss*). *Evolution* **61**, 1225-1237.

Verspoor E, McCarthy EM, Knox D, Bourke EA, Cross TF (1999) The phylogeography of European Atlantic salmon (*Salmo salar* L.) based on RFLP analysis of the ND1/16sRNA region of the mtDNA. *Biological Journal of the Linnean Society* **68**, 129-146.

Viereck LA (1966) Plant succession and soil development on gravel outwash of the Muldrow Glacier, Alaska. *Ecological Monographs* **36**, 182-199.

Wallace AR (1876) *The geographical distribution of animals*. Macmillan, London.

Westman K (2002) Alien crayfish in Europe: negative and positive impacts and interactions with native crayfish. In: *Invasive aquatic species of Europe: distribution, impacts and management* (eds. Leppäkoski E, Gollasch S, Olenin S). Kluwer Academic, Dordrecht.

Whittaker RJ (1993) Plant population patterns in a glacier foreland succession: pioneer herbs and later-colonizing shrubs. *Ecography* **16**, 117-136.

Williamson-Natesan E (2005) Comparison of methods for detecting bottlenecks from microsatellite loci. *Conservation Genetics* **6**, 551-562.

Wonham MJ, O'Connor M, Harley CDG (2005) Positive effects of a dominant invader on introduced and native mudflat species. *Marine Ecology - Progress Series* **289**, 109-116.

Wright HE, Winter TC, Patten HL (1963) Two pollen diagrams from southeastern Minnesota: Problems in the regional late-glacial and postglacial vegetational history. *Geological Society of America Bulletin* **74**, 1371-1396.

Yang DY, Speller CF (2006) Co-amplification of cytochrome b and D-loop mtDNA fragments for the identification of degraded DNA samples. *Molecular Ecology Notes* **6**, 605-608.

Zalewski A, Michalska-Parda A, Bartoszewicz M, Kozakiewicz M, Brzeziński M (2010) Multiple introductions determine the genetic structure of an invasive species population: American mink *Neovison vison* in Poland. *Biological Conservation* **143**, 1355-1363.

Zuckerkandl E, Pauling LB (1962) Molecular disease, evolution, and genetic heterogeneity. In: *Horizons in Biochemistry* (eds. Kasha M, Pullman B). Academic Press, New York. pp. 189-225.

I

II

BMC Research Notes

Short Report

Cross-species amplification of 36 cyprinid microsatellite loci in *Phoxinus phoxinus* (L.) and *Scardinius erythrophthalmus* (L.)

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Abstract

Background: To conduct phylogeographic or population genetic studies, an adequate number of DNA markers for the focal species are required. Due to severe unavailability of genotype markers of any kind for the species Eurasian minnow (*Phoxinus phoxinus* L.) and rudd (*Scardinius erythrophthalmus* L.), we set out to attempt cross-amplification of a set of microsatellite loci from related species.

Findings: We tested 36 cyprinid microsatellite loci for cross-species amplification in minnow and rudd. Fifteen species-locus combinations produced amplifications in minnow, seven being polymorphic, while 18 combinations amplified in rudd, nine of these being polymorphic.

Conclusions: The positive cross-species amplifications present potential contributions to the establishment of genetic marker sets for population genetics studies of the two focal species.

Findings

Microsatellites are widely used for population genetics purposes, especially when the scope of the study involves comparing closely related individuals. This is mainly due to their high mutation rates and to the potential of acquiring large amounts of data through relatively labour-thrifty multi-marker panel runs on capillary electrophoresis sequencers. However, utilization of microsatellites demands knowledge about their flanking sequences generated through library construction and/or PCR cloning approaches [1] to construct adequately sized annealing primer pairs. The flanking regions of microsatellites usually mutate at a much slower rate than the microsatellites themselves and will in many cases be identical across a species' range of distribution. They may even be conserved well enough through evolution to serve as primer templates for closely related species (see e.g. [2-4]).

The diverse family Cyprinidae, the most species-rich family of all vertebrates, has been paid only limited attention in population genetics studies. In the few studies available, the primary focus has been on a few species that have shared the status of being either commercially important or popular game fish; exemplified by studies on common carp (*Cyprinus carpio* L.) [5-7], goldfish (*Carassius auratus* L.) [8], European chub (*Leuciscus cephalus* L.) [9] and the genetic model species zebrafish (*Danio rerio* Hamilton) [10]. Therefore, for the great majority of cyprinids genetic

markers are unavailable. In an earlier study, Holmen *et al*. [4] established a platform for optimization of microsatellite markers for six different cyprinids based on cross-species amplification of markers that initially were developed for *D. rerio* and central stoneroller (*Campostoma anomalum* Rafinesque). In this subsequent study, the platform was extended for two of the six species; Eurasian minnow (*Phoxinus phoxinus* L.) and rudd (*Scardinius erythrophthalmus* L.). We tested 36 microsatellite loci developed for the five cyprinid species fathead minnow (*Pimephales promelas* Rafinesque) [11], silver barb (*Barbonymus gonionotus* Bleeker) [12,13], common carp (*Cyprinus carpio carpio* L.) [14], *Anaecypris hispanica* (Steindachner) [15], and goldfish (*Carassius auratus auratus* L.) [16] for amplification of minnow and rudd DNA. Primer design incorporated the original primers with the addition of GTTT to the 5' end of one primer of each pair facilitating reasonably consistent adenylation of the 3' end of the forward primer [17] (see Additional file 1). We tested two samples of each focal species, yielding a total of 144 PCR reactions. To ascertain the polymorphism of a potential microsatellite locus, it is advisable to include more than only two samples of the focal species; however, we selected samples from populations situated far apart to increase the probability of polymorphism detection; minnow samples were from Norway and Spain, rudd from Norway and Italy.

We extracted genomic DNA from ethanol preserved fin tissue using a salt extraction protocol outlined by Aljanabi & Martinez [18]. Further, we performed PCR reactions on MJ Research PTC-100/PTC-200, Techne or Biometra thermal cyclers. In the total volume of 10 μl, PCR reactions contained 20-100 ng genomic DNA, 20 μM dNTP, plus 1 μM fluorescently labelled dUTPs (R110, R6G or TAMRA FdUTP; Applied Biosystems), 0.5 μM of each primer, 1× BioTaq buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl, 0.1% Tween-20; all buffer concentrations), 1.5 mM $MgCl_2$, and 0.1 units of BioTaq DNA polymerase (Bioline). PCR protocols were constructed with annealing temperatures and durations of incubations from published recommendations for the source species in mind. However, all PCR reactions were transformed to 'touchdown' procedures; starting with a relatively high annealing temperature, gradually decreasing it for each cycle and eventually keeping a fixed annealing temperature for a number of cycles towards the end. Details of the PCR protocols for all markers are given in Additional file 1.

PCR products were pooled with a loading buffer and size standard mix (MegaBACE 10× Running buffer and MegaBACE ET400-R Size Standard, GE Healthcare, formerly Amersham Biosciences) and electrophoresed using a MegaBACE 1000 sequencer (GE Healthcare). Genotypes were scored using Genetic Profiler 1.5 (GE Healthcare). Scoring results were classified according to their amplification quality level, as outlined in Primmer & Merilä [19]: 1: 1 or 2 alleles observed in a single individual, with little stuttering observed; 2: 1 or 2 alleles, moderate stutter; 3: 1 or 2 alleles, considerable stutter; 4: multiple bands and/or smear; 5: no amplification. Due to the possible confusion between true microsatellite alleles and other amplifications, bands having no trace of a weaker band one repeat below were included in category 4, even when only one or two bands were observed. Note that no positive controls were used in these runs.

Thirty-three out of the total of 72 heterologous locus-species combinations resulted in products of amplification quality 3 or better (Table 1). Of these successful combinations, 15 (seven polymorphic) were recorded for minnow and 18 (nine polymorphic) for rudd. Interestingly, all eight successful amplifications with *C. carpio* loci were polymorphic for both target species, while only 24% of the remaining amplifying loci were polymorphic. Average amplification successes in Holmen *et al*. [4] were 40% in rudd and 49% in minnow, while the corresponding figures in the current study were 50% and 42%, respectively. Some of the amplified loci were later optimized for population genetics studies in minnow (Holmen *et al*., in prep.), the selection being based on the number of alleles revealed in this study, amplification quality, and, in order to fit into an already half completed panel, the size range in which alleles appeared. Thus, only *MFW1*, *MFW17*, and *GF11* have been further optimized and amplified in 1660 minnows from 72 sampling sites across Europe (Table 2). These three loci produced reasonably strong, unambiguous peaks after some optimization, and were included in the population studies. However, *GF11* proved to exhibit very little variation; in fact it was monomorphic in 55 sampling sites, and thus the amount of genetic information from this locus was very limited. Within-population deviations from Hardy-Weinberg equilibrium were tested for using Genepop [20,21]. For these tests, only those 44 sampling sites that consisted of at least 17 individuals were included. *MFW1* was polymorphic for all of these sampling sites, while *MFW17* was polymorphic in all but one. For these two loci, none and one, respectively, of the samples deviated from Hardy-Weinberg equilibrium at the Bonferroni-adjusted 0.05 significance level. For *GF11*, only eleven samples were polymorphic, and out of those one was in Hardy-Weinberg disequilibrium. To specifically test for the presence of null alleles, ML-NullFreq [22] was employed. Using the Bonferroni-adjusted 0.05 significance level, one and four out of the 44 samples indicated the presence of null alleles in *MFW1* and *MFW17*, respectively. For *GF11*, six out of the eleven polymorphic sampling sites indicated the presence of null alleles, further emphasizing the limited value of this locus in population genetics studies. Unfortunately, further information of the tested loci is presently unavailable for *S. erythrophthalmus*.

Table 1: Details of cross-species amplification of 36 cyprinid microsatellites in *P. phoxinus* and *S. erythrophthalmus*

Source species	Locus	Repeat motif	Focal species					
			P. phoxinus			*S. erythrophthalmus*		
			A	Size (bp)	Q	A	Size (bp)	Q
P. promelas	Ppr101	AC	0	-	5	1	395	1
	Ppr102	AC	0	-	5	0	-	5
	Ppr103	AC	0	-	5	0	-	5
	Ppr104	AC	1	122	2	0	-	5
	Ppr105	AC	0	-	4	1	222	1
	Ppr106	AC	0	-	4	0	-	5
	Ppr107	ACAG	1	200	2	1	266	1
B. gonionotus	Bgon8	AC	0	-	5	2	138-178	2
	Bgon13	GT	0	-	4	0	-	5
	Bgon17	AC	1	149	3	0	-	5
	Bgon22	TCC	0	-	5	4	103-151	3
	Bgon69	TG	0	-	5	1	260	2
	Bgon75	AC	1	78	3	0	-	5
	Bgon79	CA	0	-	4	3	154-208	2
C. carpio	MFW1	CA	4	166-226	2	3	172-178	2
	MFW5	CA	0	-	5	2	103-107	2
	MFW17	CA	2	191-195	1	2	183-187	2
	MFW18	CA	0	-	5	0	-	5
	MFW19	CA	2	222-226	2	4	191-203	2
	MFW24	CA	2	137-161	2	0	-	5
	MFW28	CA	0	-	5	0	-	5
A. hispanica	II04	GT	0	-	5	0	-	5
	IV04	CA*	0	-	5	1	168	3
	IV34	CA*	1	108	1	1	109	1
	IV46	TG*	0	-	5	0	-	5
	X44	CA*	1	148	2	1	148	2
	XII02	CA*	1	88	2	0	-	5
	XIII40	GT*	0	-	4	0	-	4
	XIV13	GT/GA*	0	-	5	0	-	4
	XIV31	CA*	2	93-149	2	2	93-147	2
	XV28	CA*	0	-	5	2	160-172	3
C. auratus	GF1	TG	2	90-226	1	0	-	4
	GF11	TG*	3	149-161	2	1	161	3
	GF17	TG	1	114	1	0	-	5
	GF20	TG	0	-	5	0	-	5
	GF29	TG*	0	-	5	1	109	1

Number of alleles recorded (A), size (bp), and quality of the amplified product (Q: 1, little stutter; 2, moderate stutter; 3, considerable stutter; 4, multiple bands or smear; 5, no amplification) are given. Asterisks denote non-continuous repeat motif sequences.

The likelihood that primer pairs developed for one species should amplify in a second species is higher the more closely related the two species are. On that general basis, one can assume the relative success rate among a number of cross-species amplification attempts. Cyprinidae taxonomy is rather complex. Although the family has traditionally been organized into several subfamilies, each comprising one or more lineages which in turn include a number of genera, and most lineages and genera are generally accepted as being monophyletic, there is controversy regarding the monophyly of some subfamilies [23]. Thus, we had few obvious expectations regarding amplification successes in the present cross-species study. *S. erythrophthalmus*, *P. phoxinus*, *P. promelas*, and *A. hispanica* all belong to the subfamily Leuciscinae, but Hänfling & Brandl [24] considered the genus *Phoxinus* to be a sister taxon to a Leuciscinae-Alburninae lineage. *C. carpio*, *C. auratus*, and *B. gonionotus* all belong to the Cyprininae

Table 2: Population genetics parameters for the loci *MFW1*, *MFW17* and *GF11* in *P. phoxinus*, based on amplifications in 1660 individuals from 72 sampling sites across Europe

Locus	Number of alleles	N_E	H_E	H_O	F_{IS}
MFW1	31	6.52	0.85	0.66	-0.02
MFW17	38	4.15	0.76	0.53	0.02
GF11	3	1.08	0.07	0.05	0.26

subfamily. *P. promelas* and *A. hispanica* loci were thus expected to amplify with a reasonably high rate in *S. erythrophthalmus* and slightly lower in *P. phoxinus*, and indeed, the success rates, defined as the proportions that produced peaks with amplification quality 3 or better, were 43% versus 29% and 50% versus 40% in favour of *S. erythrophthalmus* for the two source species, respectively. Loci from the Cyprininae subfamily were expected to produce a lower success rate in both target species. This was not the case, however, as amplification success ranged from 29% to 57%. Notably, the number of loci examined is too low for any differences observed to be statistically significant. The results should therefore not be regarded as a contribution to the lineage discussion within Cyprinidae.

The present and our previous study [4] points out the usefulness of cross-species amplification of microsatellites in Cyprinidae to establish markers for population genetics studies. More specifically, the findings in these two papers have provided the authors with a useful set of markers for phylogeography and population genetics studies of the minnow and will hopefully contribute to fellow researchers' related work as well.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
JH carried out the molecular genetics laboratory work and drafted the manuscript. LAV conceived the study. KSJ helped design laboratory procedures. CRP had the major input to the design of the study. All authors read, contributed to and approved the final manuscript.

Additional material

Additional file 1
Primer sequences and PCR protocol details of all loci investigated
Click here for file
[http://www.biomedcentral.com/content/supplementary/1756-0500-2-248-S1.DOC]

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References
1. Refseth UH, Fangan BM, Jakobsen KS: **Hybridization capture of microsatellites directly from genomic DNA.** *Electrophoresis* 1997, **18(9):**1519-1523.
2. Primmer CR, Møller AP, Ellegren H: **A wide-range survey of cross-species microsatellite amplification in birds.** *Mol Ecol* 1996, **5(3):**365-378.
3. Tong J, Wang Z, Yu X, Wu Q, Chu KH: **Cross-species amplification in silver carp and bighead carp with microsatellite primers of common carp.** *Mol Ecol Notes* 2002, **2(3):**245-247.
4. Holmen J, Vøllestad LA, Jakobsen KS, Primmer CR: **Cross-species amplification of zebrafish and central stoneroller microsatellite loci in six other cyprinids.** *J Fish Biol* 2005, **66(3):**851-859.
5. Desvignes JF, Laroche J, Durand JD, Bouvet Y: **Genetic variability in reared stocks of common carp (*Cyprinus carpio* L.) based on allozymes and microsatellites.** *Aquaculture* 2001, **194(3-4):**291-301.
6. Fuchs H, Schlee P, Blusch J, Werner T, Stein H, Rottmann O: **Phylogenetic studies in cyprinid species from central Europe by cytochrome b sequences of mitochondrial DNA.** *J Appl Ichthyol* 2000, **16(2):**79-82.
7. Zhou JF, Wu QJ, Ye YZ, Tong JG: **Genetic divergence between *Cyprinus carpio carpio* and *Cyprinus carpio haematopterus* as assessed by mitochondrial DNA analysis, with emphasis on origin of European domestic carp.** *Genetica* 2003, **119(1):**93-97.
8. Zhou L, Wang Y, Gui JF: **Molecular analysis of silver crucian carp (*Carassius auratus gibelio* Bloch) clones by SCAR markers.** *Aquaculture* 2001, **201(3-4):**219-228.
9. Larno V, Launey S, Devaux A, Laroche J: **Isolation and characterization of microsatellite loci from chub *Leuciscus cephalus* (Pisces: Cyprinidae).** *Mol Ecol Notes* 2005, **5(4):**752-754.
10. Shimoda N, Knapik EW, Ziniti J, Sim C, Yamada E, Kaplan S, Jackson D, de Sauvage F, Jacob H, Fishman MC: **Zebrafish genetic map with 2000 microsatellite markers.** *Genomics* 1999, **58(3):**219-232.
11. Ardren WR, Miller LM, Kime JA, Kvitrud MA: **Microsatellite loci for fathead minnow (*Pimephales promelas*).** *Mol Ecol Notes* 2002, **2(3):**226-227.
12. Kamonrat W, McConnell SKJ, Cook DI: **Polymorphic microsatellite loci from the southeast Asian cyprinid, *Barbodes gonionotus* (Bleeker).** *Mol Ecol Notes* 2002, **2(2):**89-90.
13. McConnell SKJ, Skibinski DOF, Mair GC: **Microsatellite markers from a commercially important South-east Asian cyprinid, the silver barb (*Barbodes gonionotus* Bleeker).** *Mol Ecol Notes* 2001, **1(3):**135-136.
14. Crooijmans RPMA, Poel JJVd, Groenen MAM, Bierbooms VAF, Komen J: **Microsatellite markers in common carp (*Cyprinus carpio* L.).** *Animal Genetics* 1997, **28(2):**129-134.
15. Salgueiro P, Carvalho G, Collares-Pereira MJ, Coelho MM: **Microsatellite analysis of genetic population structure of the endangered cyprinid *Anaecypris hispanica* in Portugal: implications for conservation.** *Biol Conserv* 2003, **109(1):**47-56.
16. Zheng W, Stacey NE, Coffin J, Strobeck C: **Isolation and characterization of microsatellite loci in the goldfish *Carassius auratus*.** *Mol Ecol* 1995, **4(6):**791-792.
17. Brownstein MJ, Carpten JD, Smith JR: **Modulation of non-templated nucleotide addition by tag DNA polymerase: Primer modifications that facilitate genotyping.** *Biotechniques* 1996, **20(6):**1004-1010.
18. Aljanabi SM, Martinez I: **Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques.** *Nucleic Acids Res* 1997, **25(22):**4692-4693.
19. Primmer CR, Merilä J: **A low rate of cross-species microsatellite amplification success in Ranid frogs.** *Conserv Genet* 2002, **3(4):**445-449.
20. Raymond M, Rousset F: **Genepop (Version-1.2) - Population-Genetics Software for Exact Tests and Ecumenicism.** *J Hered* 1995, **86(3):**248-249.

21. Rousset F: **Genepop'007: a complete re-implementation of the Genepop software for Windows and Linux.** *Mol Ecol Resour* 2008, **8(1):**103-106.
22. Kalinowski S, Taper M: **Maximum likelihood estimation of the frequency of null alleles at microsatellite loci.** *Conserv Genet* 2006, **7(6):**991-995.
23. Howes GJ: **Systematics and biogeography: an overview.** In *Cyprinid fishes - Systematics, biology and exploitation* Edited by: Winfield IJ, Nelson JS. London: Chapman & Hall; 1991:1-33.
24. Hänfling B, Brandl R: **Phylogenetics of European cyprinids: insights from allozymes.** *J Fish Biol* 2000, **57(2):**265-276.

III

IV