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Genetic characterisation of European grayling populations (*Thymallus thymallus* L.): Implications for conservation and management

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To my family

Contents

List of figures

List of tables

Preface

I structured this thesis on the genetic characterisation of European grayling (*Thymallus thymallus* L.) populations in the following way: A general introduction describing the nature of the problem and the overall aims of the study (chapter 1) is followed by a literature review (chapter 2) intended to provide essential background information concerning the biology, ecology, life history and genetics of the studied species as well as in regard to the recent advances of molecular genetic and statistical analyses in the field of conservation genetics. The chapter ends with a short review of factors contributing to the endangerment of riverine fish populations in general and grayling in particular. Chapters 3, 4 and 5 address three specific case studies in the framework of grayling conservation and population genetics and the species' European phylogeography, each of them representing a full research paper. All papers are published in a slightly modified format, according to the journal requirements. In chapter 6 the specific results of the presented case studies are discussed from a broader population genetic and phylogeographical perspective with special emphasis on the applied molecular markers and in comparison to other fish genetic studies. In addition, the significance of this research for the protection and sustainable management of endangered fish species is highlighted as is the contribution of other research activities. Chapter 6 is completed by an outlook for interesting future fields of research about European grayling concerning approaches both in basic and applied science. The thesis closes with a general summary in English and German (chapters 7 and 8).

1 Introduction

1.1 Statement of problem

According to the IUCN Red List (2006) about 50% of all freshwater fish species in Europe are estimated to be critically endangered, endangered or vulnerable and, about 10% are known to be extinct or are extinct in the wild. As regards Germany, 15 of the estimated 107 presently occurring freshwater fish species are included on the list. However, the IUCN Red List data concerning threatened fish species in Germany is judged to be deficient, in terms of both its quality and topicality (Freyhof & Brunken, 2004). By contrast, in the updated Red List of endangered fish species in Bavaria (Bohl *et al*., 2003), 35 of the 71 species which have been evaluated are considered as threatened. Indeed, over the last two decades there has been a striking decline in the population of riverine fishes in Bavaria and other regions throughout the Alpine area. This is particularly true for the gravel spawning fish of the rivers.

For the European grayling (*Thymallus thymallus*), a strong decline in the population densities has taken place since the mid-1980s (Northcote, 1995; Persat, 1996; Baars, 2000; Gardiner, 2000). This decline was noted at the same time in many rivers throughout the Alpine area, and was reported as well in other regions of the species' European distribution area (e.g. Maitland & Campbell, 1992; Guthruf, 1996; Baars *et al*., 2001). Though there have been times in the past when whole year classes of grayling have been lost (for example due to infectious diseases, extreme spring floods or other impacts such as pollution), the species is known for its potential to recover within a few years to former population densities. However, the steady decline over the last 15 - 20 years appears to be continuing today without evident recovery. The most seriously affected stocks belong to the alpine drainage system of the upper Danube and its tributaries such as the rivers Inn, Isar, Lech or Iller (Steinhörster, 2001).
Specific grayling conservation and support programmes have been initiated by local fishing authorities in several European countries such as Austria, Switzerland, and France due to the increasing endangerment of the species (e.g. Persat, 1996; Uiblein *et al.*, 2001). As early as 1998 the Bavarian Fishing Association (Landesfischereiverband Bayern e.V.) started the Bavarian grayling support program (termed 'Artenhilfsprogramm Äsche'), the first conservation programme for an endangered fish species in Bavaria (Stein & Born, 1999). Several GOs and NGOs including university institutions and conservation and fishery

organisations were commissioned to clarify the causes of the grayling decline in discrete research projects. The final report of this interdisciplinary project was presented in 2003 and can briefly be summarised as follows: Factors such as low water quality, malfunction of natural reproduction due to different types of water regulations in heavily modified rivers or the existing deficiency of suitable spawning grounds, and potential diseases could be excluded as decisive causes of the current decline (e.g. Schubert, 2001; Königsdorfer *et al*., 2001; Sachteleben, 2001; Born, 2001). However, the participating institutions basically agreed that the strong decline of grayling throughout the alpine area could mainly be related to the European-wide growth in the populations of the cormorant (*Phalacrocorax carbo sinenis*) and goosander (*Mergus merganser*). Today, there is general consensus among researchers (both in the field of bird as well as fish conservation) that the high extent of habitat destruction combined with the ongoing predation pressure of the piscivorous birds do not currently allow grayling stocks to recover to a comparable former level. Though completely protected throughout Europe since 1979, a few countries or federal states such as Bavaria allow the shooting of cormorants during the winter, if substantial fishery damage is evident. This is permitted only outside of any type of protected area or bird reserve. However, a needed Europe-wide cormorant action or management plan has, as yet, not been formulated and has failed to date due to the strong conflict between stakeholders of fishery and bird conservation associations (see the 'INTERCAFE' EU-funded project for more information, available at: http://www.intercafeproject.net).

The question still remaining is how to deal with the continuing endangerment of grayling, both now and in the near future? A detrimental combination of several factors such as high predation pressure and negative environmental conditions in addition to inappropriate management by fishermen could easily lead to the irrecoverable loss of the valuable genetic resources of the species, which at present has been hardly investigated and understood (Gross *et al*., 2001). In order to secure the remaining grayling stocks or to restore already extinct populations in suitable habitats, local fishery associations increasingly make demands on appropriate stocking actions. However, according to which principles should sustainable conservation and management of grayling be based? In this regard, we also have to consider the best way to cope with the 'Rio Convention' (Convention on Biological Diversity, 1992), i.e. to conserve biodiversity as well as to develop appropriate measures for sustainable utilisation and management of natural resources. Indeed sustainable utilisation is a principle

that applies to all fishes being affected by angling such as grayling or brown trout. It is therefore urgently necessary to develop and provide ecological and economically practicable guidelines on a scientific basis for sustainable conservation and management of grayling.

Unfortunately, many species such as grayling seldom receive our prime attention until a serious decline of populations is demonstrated. In most cases associated with cost- and time-intensive conservation efforts, a lack of knowledge of ecology and genetics of these species becomes apparent. Therefore, in parallel to ecological studies and within the framework of the above mentioned local grayling support programmes, a genetic conservation approach is needed that identifies and sustains distinct evolutionary lineages in order to protect European grayling intra-specific biodiversity. Such research is important for the conservation of free-living grayling populations, as well as for the development of scientifically based artificial culturing and captive breeding techniques. It is important at the outset to know where and what to protect, to set potential conservation priorities and, especially, to know the biological and economical limitations as well as differing national regulations.

Interestingly, essential elements of the presently accepted (conservation) genetic based concepts applied to endangered species and used for sustainable protection of natural resources have originally been developed for pacific salmon and trout species (Waples, 1991). In the meantime, terms such as evolutionary significant units (ESUs) or management units (MUs) are well-established and have been further developed (Ryder, 1986; Moritz, 1994; Fraser & Bernatchez, 2001). However, the discussion about the scientifically most reasonable and at the time most feasible definition for effective protection of distinct population segments below the species level is not yet been completed (e.g. Crandall *et al.*, 2000; Hedrick, 2001; Ford, 2004). Nonetheless, the concepts of ESUs, MUs or FSUs (functional significant units; van Tienderen *et al.*, 2001) have generally proven their worth and now represent an integral part of molecular ecology and conservation genetics (Frankham *et al.*, 2002). Case studies in population and conservation genetics (e.g. discussed in Kühn, 2004) as well as those in the field of evolutionary biology for salmonid species (reviewed by Ford, 2004) not only provide guidelines for setting of conservation and priority populations but also give valuable information on stock composition, variation of the effective population size, as well as on temporal changes in the genetic composition of populations. Furthermore, genetic tracking of cultured and released individuals permits to verify successful enhancement of local stocks (Tringali, 2006). By addressing these questions, the new research disciplines of conservation genetics and conservation ecology therefore retain the evolutionary potential for

adaptation to future changes in the environment. In addition to the valuable component of conserving biodiversity between and within species, the findings of such research will serve as a basis for sustainable management for both commercially and recreational important species.

The study by Gross *et al*. (2001) provided first insights into the genetic differentiation of European grayling populations across the Danube, Elbe and Main drainages in Bavaria. Following the approach of Gross *et al*. (2001), the present work aims to provide a comprehensive population genetic basis for conservation and management of this species by application of nuclear and mitochondrial markers.

1.2 Aims of the study

The overall aim of this study is the population genetic characterisation of selected grayling populations from the Danube, Rhine/Main und Elbe drainages in Bavaria. By application of highly variable microsatellite markers, the study should clarify the genetic structure between and within grayling populations of different management regimes and assess the genetic impact of grayling stocking measures conducted in the past. In addition, the study aims to identify major evolutionary lineages of European grayling based on the analyses of mitochondrial and nuclear DNA variability in order to investigate the phylogeography and colonisation history of the species in central Europe as well as to allow for a phylogeographical classification of grayling from Bavaria within its European-wide distribution. These baseline genetic data will be used to give recommendations for grayling conservation and to provide guidelines for sustainable management of this endangered fish species.

Specifically the objectives of this study are:

- The application of molecular markers to determine the level of genetic variability, differentiation and gene flow among grayling populations within and between the drainages Danube, Rhine/Main and Elbe.
- The delineation of conservation and management units for the development of appropriate conservation guidelines for *T. thymallus* in Bavaria.

- The extension of European grayling phylogeography by combined application of mitochondrial and nuclear markers in order to:
  i) better understand the contemporary and historical factors that shape the population genetic structure of the species on local and broad scale;
  ii) further clarify the evolutionary and colonisation history of the species in central and northern Europe after glacial perturbations;
  iii) identify potential zones of secondary contact of divergent mtDNA lineages in the contact zones of the major European drainages of Danube, Rhine/Main and Elbe;
  iv) obtain additional data for delineating of appropriate conservation units within and between drainage systems.
- The detection of past stocking activities and discrimination of the impact of human mediated stock transfer from historical gene flow on genetic structure of European grayling.
- The recommendation of conservation strategies for free-living populations and supportive breeding techniques on a genetic basis.

2 Literature review

2.1 Biology and ecology

2.1.1 Systematics and distribution

The genus *Thymallus* belongs to the class Osteichthyes (bony fish), order Salmoniformes, family Salmonidae (salmonids). The Salmonidae family comprises three subfamilies: Salmoninae (char, trout, and salmon), Coregoninae (whitefish and ciscoes), and Thymallinae (grayling) (Kottelat, 1997). The monophyly of this group is not in doubt but determining which fishes are the closest relatives to Salmonidae is a different story altogether (reviewed in Ramsden *et al*., 2003). However, a new topology based on the mitogenomic study by Ishiguro *et al.* (2003) supports the hypothesis that the sister group of Salmonidae is Esociformes (e.g. pike and mudminnows) rather than Osmeroidei (smelts, galaxiids and icefishes). The recent work by Crespi & Fulton (2004) used combined nuclear data to infer the molecular systematics of 30 species of Salmonidae yielding a robust phylogeny with important relationships within the group. Unfortunately not enough nuclear DNA sequence data were available for the genus *Thymallus* in this study. Therefore, there is ongoing uncertainty regarding the molecular phylogenetics of *Thymallus* and its relationship to the other genera of salmonids such as *Salmo*, *Coregonus*, *Hucho*, and *Salvelinus*.

The classification of grayling into species is not yet totally agreed among scientists (reviewed in Gardiner, 2000; Baars *et al*., 2001). Froufe *et al.* (2005) recently presented first insights into the molecular phylogeny of the genus *Thymallus* using sequences from the mtDNA control region and ATPase-6 genes. Based on the combined data, four major (internal) clades were resolved representing two distinct lineages in the Amur basin, one lineage in all remaining Siberian and Mongolian drainages, and one lineage corresponding to European grayling (*Thymallus thymallus*). The study proved the existence of multiple lineages, from both additional internal and terminal clades, within major drainage basins from Asia and underscored the complexity and the historical depth of major phylogeographic events for *Thymallus* within far-eastern Siberia, compared to the probable Pleistocene/Pliocene boundary colonisation of Europe (see Weiss *et al*., 2002; Gum *et al*., 2005). The results by Froufe *et al*. (2005) thus add to the *hitherto* consensus view, that there are two species with extensive ranges, the European grayling *T. thymallus* and the Arctic grayling *T. arcticus* as well as several other species or subspecies in certain areas of central and eastern Asia (see Gardiner, 2000). For example the Mongolian grayling (*T. brevirostris*) is restricted to the

Altai Mountains (Kessler, 1879). Some of the other 'forms' of arctic grayling such as the 'orange spot form' occur to the Southeast of their range in Asia and are classified according as species or subspecies (Froufe *et al*., 2005). Further important forms are described as Amur grayling *T. arcticus grubii* (Dybowski, 1869; Froufe *et al*., 2005 and references therein), Baikal grayling *T. arcticus baycalensis* (Dybowski, 1872; Froufe *et al*., 2003) or Kosogol grayling *T. nigrescens* found in lake Hovsgol (Nikolski, 1957). Based on morphological data, additional potential subspecies were suggested for East Asia and North America (e.g. *T. a. mertensi* in Kamtchatka and *T. a. montanus*, see Knizhin *et al*., 2004; Stamford & Taylor, 2004). Recently two major phylogeographical lineages of arctic grayling occuring in the Lena River (*T. arcticus lenenis* and *T. arcticus pallasii*), and surrounding drainage systems were characterised based on molecular genetic and phenotypic data (Weiss *et al*., 2006). Due to the great variety of grayling species/subspecies found in northern Mongolia and central/eastern Sibiria, Nikolski already assumed in 1975 that the genus *Thymallus* probably had its origin in central Asia around the area of Lake Baikal. From there the different species spread in east- and westward directions across the northern hemisphere and finally reached Europe and, by crossing the Bering Sea, North America (Weiss *et al*., 2006). A comprehensive phylogenetic investigation within the genus *Thymallus* concerning its whole holarctic distribution has yet to be carried out on grayling.

The natural distribution of the European grayling (*T. thymallus*) extends in the west from about the Pyrenees north through France, over to England, throughout Scandinavia and across Northern Russia to the Ural Mountains near the Kara River (Northcote, 1995). In Central Europe grayling occur in rivers throughout the Alpine region from Switzerland to Austria and Northern Italy, as well as in Germany, Czech Republic, Slovakia and Poland. The species is also present in a number of Alpine and Scandinavian cold lakes. To the south, European grayling occur in several regions of the Balkans and from there well north of the Black and Caspian seas on to the southern margins of the Urals. The introductions by fishermen or naturalists, mainly in the 19th century, have extended European grayling's previous distribution into waters of the Tajo River system in Central Spain, in lakes of South-Central Finland and in Southern Scotland (Northcote, 1995).

2.1.2 Habitat

In its entire distribution area, grayling populations can be found in diverse habitats mainly depending on the regional geological parameters of rivers or lakes, the climate zone and other environmental factors (Baars *et al*., 2001). In Central Europe, grayling predominantly occur in rivers but in northern European regions (e.g. in Norway or Finland) and in the Alpine area, the species can be also found in some cold lakes (e.g. Lake Toblach in South Tyrol, Lake Thun in Switzerland). Lake populations usually depend on tributaries for spawning, but inlet spawning was observed as well in a few Alpine lakes and in Norway (Guthruf, 1996; Northcote, 1995). In the Barent Sea, in the White Sea and in the Baltic Sea European grayling can also be rarely found in brackish water along the shores (Salinity up to 3 $^{0}/_{00}$; Abel & Johnson, 1978).

In alpine and subalpine areas, grayling is the dominant species of the so-called grayling zone. It is typically found in the middle and upper river stretches of the hyporhithral. The classification of rivers into zones according to certain key fish species (e.g. trout, grayling, barbel zone etc) is usually applied only in central Europe. Different geological and climate conditions are responsible for other ichtyocenose in northern or southern Europe. Therefore, in these areas the term 'grayling zone' is not common.

Grayling as a reophilic species generally inhabit cool, well-oxygenated, clear rivers and streams preferring flow velocities of 0.7 - 1.1 m/s and a particle size distribution of 1 - 16 mm (Northcote, 1995; Mallet *et al*., 2000; Hochleithner, 2001). According to Dyk (1984) grayling of the Alpine area can be typically found in rivers with headwaters up to 1000 m sea level and maximum water temperatures up to 18 °C (e.g. rivers Isar, Inn, Lech). The species is uncommon in typical trout creeks (> 1500 m sea level). Grayling also inhabits pre-alpine lowland rivers and for a short time is able to tolerate water temperatures up to 23 °C (Dyk, 1984). Well-characterised examples of these lowland type rivers (with formerly noteworthy grayling populations) are the tributaries of the river Isar in the plain area north of Munich. These include such creeks as the Dorfen, Moosach and Sempt. The rivers of the low mountain range in north/northeastern Bavaria and Czech Republic (e.g. in the 'Fichtel- or Erzgebirge'), e.g. the Wiesent of the upper Main system, the Eger of the Elbe and the Fichtelnaab of the Danubian drainage can be classified between the alpine and lowland river type.

Baars (2000) investigated the biology and ecology of grayling populations from the drainage basins of the Danube, Rhine/Main and Elbe in Bavaria. Populations were chosen from a range of habitat types in respect to different biotic and abiotic environmental conditions such as water chemistry, natural food supply or predation pressure. Physical-chemical parameters such as ionic content (water hardness and conductivity as a measure of ionic content), are

mainly determined by the geology of the respective water system. In the primary (granite) rock region of the Bavarian Woods (Bayerischer Wald) water conductivity is for the most part lower than 100 µS/cm (e.g. river Schwarzer Regen), whereas in rivers having their source in the Alps, the high lime content is responsible for water conductivity values between 400 and 700 µS/cm (e.g. river Isar). The lime content considerably affects the pH-value and the acid binding capacity. The pH-value of the south Bavarian rivers generally is in the alkaline range (e.g. Isar pH = 7.5 – 8.6) naturally buffered by the high lime content of the alpine Danubian drainage system. By contrast, rivers largely not influenced by human activities and with very low lime content (e.g. typical pearl mussel rivers, see Geist, 2005) exhibit pH-values from neutral to the slight acid range. Therefore, rivers such as the Schwarzer Regen which flow through a granite rock area (Baars, 2000) show a lower acid binding capacity and average pH-values are about 6 to 6.5. In general, pH-values not lower than 6 and not exceeding 9 are adequate for grayling. This range is normally maintained by all grayling rivers of the German low mountain range in northern Bavaria as well as by the Alpine rivers and those of the Bavarian Woods (Baars, 2000). However, industrial and communal pollution, street runoff or agricultural sediment runoff can lead to significant changes of the ionic content and natural pH-levels of rivers for a certain period of time.

Concerning the structural parameters of typical grayling waters, Dyk (1984) stated that the main habitat of grayling in a river starts where populations of brown trout gradually diminish. The change of shallow and fast flowing river stretches with deep and slow streaming sites (so-called pool and riffle structures) is typical for the course of natural and semi-natural grayling rivers. The flow velocity varies accordingly (Mallet *et al*., 2000). Stony or pebbly ground is dominant in rivers having their sources in the Alps or the lower mountain range of central Europe. In addition, rivers of the lowland type or rivers with new red sandstone in its drainage system can form expanded sandbanks in the grayling zone (Baars, 2000).

Fig. 2.1: Adult male European grayling (*Thymallus thymallus* L.)

2.1.3 Life history traits

Morphology and anatomy

The body of a grayling is spindle-shaped (Figure 2.1). As mentioned above grayling are closely related to whitefish and similar like them have relatively large scales (Crespi & Fulton, 2004). The body colour depends on the habitat, substratum and food source and varies from grey to beige, green and silvery. Depending on the geographical origin and population, adult grayling typically possess more or less little black spots on their body-sides. The key distinguishing feature of the grayling is their large sail-like dorsal fin, which is not found on whitefish or any other salmonid species. The dorsal fin exhibits several ocellar spots and is bigger on the male (reaching to near the adipose fin) than for female grayling. During the spawning time the dorsal fin is intensively coloured, stock specific from red to metallic-violet and green. The literature reveals nothing regarding the grayling's dorsal fin and sexual selection, however given its extraordinary size and colour it can be assumed that it serves as a typical ornament for mate choice.

A detailed overview regarding the major anatomical characteristics of the species concerning body shape, fin rays, colouration, scalation, skeleton, musculature, viscera as well as nervous system and physiology is provided by Baars *et al*. (2001). The following summary includes a few aspects that may be of special interest for the present work as considered from a genetic perspective. Baars (2000) reported that certain Bavarian grayling populations from the Danube, Rhine/Main and Elbe differ significantly in body shape and growth. Both the maximum growth and the ratio of ventral/dorsal to the cranial/caudal length varied substantially between populations originating from these different drainage basins. For

example the ventral/dorsal ratio compared to the total body length of grayling originating from the Elbe drainage was significantly different from that of grayling from the alpine Danubian drainage system (Baars, 2000). Figure 2.2 shows this difference in the ratio of body height, as measured for grayling from rivers Ramsach, Sempt (Danube), Sinn (Main) and river Eger (Elbe). Looking at these anatomical variations one has to take into consideration the basic differences related to sex, i.e. female grayling in general appear more 'compact' in their body shape than males.

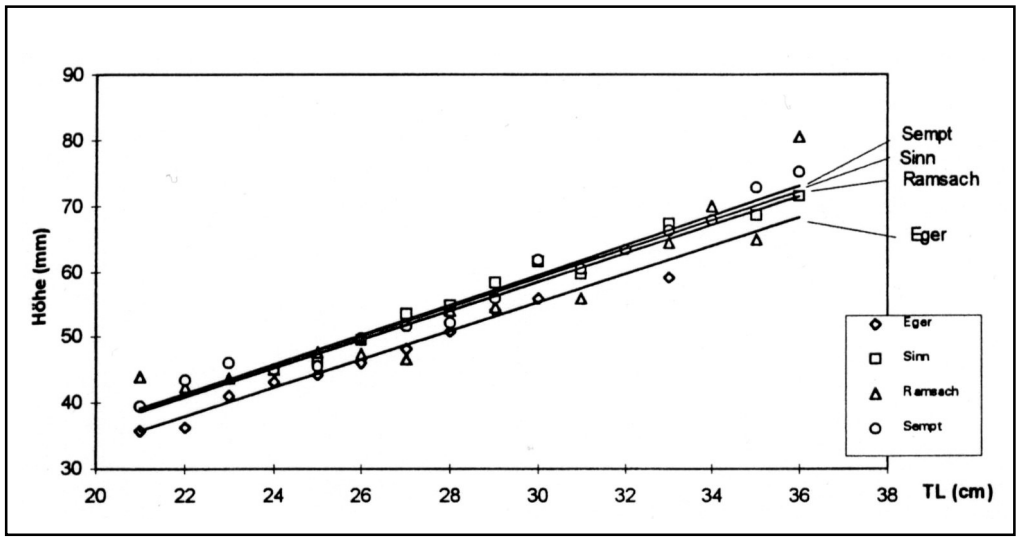

Fig. 2.2: Comparison between relative ratio of body height ('Höhe') for European grayling from rivers Ramsach, Sempt (Danube), Sinn (Main) and Eger (Elbe) according to Baars (2000).

Egg size and fecundity

According to Northcote (1995), in general, the egg size of European grayling seems to be larger than that of Arctic grayling (2.0 to 3.0 mm prior to fertilization and water-hardening, respectively), ranging from 2.5 mm in former Yugoslavia up to 3.5 mm or higher elsewhere (Janković, 1964), and stabilizing at about 4.0 mm after fertilization. These values correspond to those given by Baars *et al*. (2001) with 3.3 to 4.0 mm. The life history data of several salmonids was compared by Hendry & Stearns (2004). In this review the authors specify a range of 2.0 to 4.6 mm for non water-hardened eggs of European grayling. In either case, the diameter of water hardened eggs increases about 1.3 fold compared to the not swelled status, the volume increases about the 2.6 fold.

Fecundity, expressed as number of eggs varies from 10.000 to over 31.000 eggs per kg of body weight (Janković, 1964) depending on the age, body size and nutritional condition of the

female (reviewed in Northcote, 1995; Hendry & Stearns, 2004). According to Herrmann (2001) fecundity of females from three Bavarian rivers was about 7500 – 8700 eggs per kg. Fishery managers today anticipate egg numbers of about 10.000 eggs on average per kg body weight for grayling from central Europe (B. Hornauer, pers. communication).

Thus, there is considerable regional and intra-population variation in egg size and number among female grayling from different stocks of the species' European distribution. Northcote (1995) argues that these differences are due to female body size and age, as well as genetic stock (often expressed regionally among streams) and other controlling factors regulating fecundity (e.g. food supply and food quality, growth rate etc), though not with any great precision. It should be noted that reproductive traits such as egg size and number are strong determinants of fitness and for Atlantic salmon are known to be associated with intense selection (Einum *et al*., 2004). Indeed, for farmed salmon it is known that already after a few generations of captive breeding in a hatchery, selection generally resulted in the production of more but smaller eggs compared to the wild stocks (Heath *et al*., 2003). For brown trout Einum & Fleming (1999) showed that egg size is an important fitness component, with offspring fitness after hatching tending to increase with increasing egg size. However, a female has only a limited amount of energy resources available for egg production and for a given energetic investment (or amount of space), an increase in egg size will inevitably cause a decrease in egg number. Due to this tradeoff, the size of eggs imposes a constraint in egg number and thus presents breeding females with an evolutionary dilemma, arising because maximization of both numbers of eggs and offspring fitness (which is egg size-dependent) is not attainable (for a detailed review of this topic and the evolution of salmon egg size and number see Einum *et al*., 2004).

Similar to egg size and number, Northcote (1995) reported considerable regional variation in age of first maturity for both Arctic and European grayling. In Norwegian populations sexual maturity was reached at an age of 5 - 6 years and both sexes seemed to spawn almost every year thereafter. By contrast all grayling of the Finnish population were sexually mature by 4 years, whereas, in Central Europe and the Alpine area, males and females usually spawn the first time by the age of 3 years (some males and rarely females by 2 years and females by the age of 4 years; Baars *et al*., 2001). Northcote's (1995) hypothesis in respect to a later age of maturity being most probably related to a slower growth rate has, as yet, not been tested empirically. Thus, there is still considerable research needed to clarify the extent to which the reproductive trait 'age at maturity' is determined by environmental and/or genetic factors.

Spawning habitat and spawning behaviour

In contrast to most other salmonid species (salmon, brown trout, charr) grayling are spring spawners. Essentially depending on the habitat (e.g. alpine or lowland river type) and duration and hardness of the winter, the spawning time starts in early March and lasts until end of April. Finally, the exact spawning date of a season is largely determined by the water temperature (Baars *et al*., 2001; Ovidio *et al*., 2004) and may also be influenced by the duration of daylight. According to Maisse *et al*. (1987) the lower boundary of water temperature for ovulation is at about 5 °C. However, Stein (1987) found that grayling inhabiting the Moosach River needed a water temperature of about 8 °C before spawning activities began.

My unpublished observations in a lowland tributary river type of the Isar system showed that predominantly the older and bigger grayling were the first to start spawning activities, at temperatures slightly over 4 °C. For a discrete population or spawning group, spawning usually is completed within a few days, however with sudden cold periods it can be extended up to two or three weeks. In conjunction with the decline of grayling and resulting enhancement programmes, the question arose regarding genetic differences and heritability of the spawning time. Unfortunately scientifically approved studies have not been done in this area. However, casual observations by Baars *et al*. (2001) in a river in northern Germany (which had been stocked with grayling originating from southern Germany) argue, at least to a larger spatial extent, for local adaptation of the spawning time in different habitats. It was observed that several 'mavericks' were consistently ready to spawn about two weeks before the actual spawning time of the majority of the local stock.

Grayling usually perform short upstream migrations to their spawning grounds. Radio telemetric studies carried out in the Ilmenau and Aisne rivers (Lower Saxony and Belgium, respectively) showed, that grayling migrated 5 - 7 km upstream to their spawning areas (Meyer & Pelz, 1998; Ovidio *et al*., 2004). The length of the migrations varies according to Ebel (2000) both between, as well as within, individual rivers and in rare cases, can span up to 50 km (Hanfland, 2002). Apart from the mainstream river, grayling often use smaller tributaries or side-channels for spawning. However, access to these valuable spawning grounds has often become impossible due to numerous migration barriers such as weirs and hydropower stations.

Grayling depend on pebbly substrate at the spawning grounds. Coarse and medium-grained gravel with particle size of 8 – 32 mm is optimal for spawning nests (also called redds for salmonids) and egg development (varying from 0.06 to 64 mm; Guthruf, 1996). In contrast to

other salmonid species, spawning grayling do not produce pronounced pits at their redds. Eggs usually are deposited about 4 - 7 cm deep into the shallow gravel beds where the rivers overflow their banks with average flow velocities of 0.4 - 0.6 m/s (Sempeski & Gaudin, 1995; Meyer & Pelz, 1998; Baars *et al*., 2001). In this process, the female grayling digs its genitalia into the gravel substrate just before the actual egg release.

Fabricius & Gustavson (1955) already described the spawning behaviour of grayling, which is interesting from a genetic perspective. At first, the dominant males appear at the spawning grounds, fight for dominant positions and set up and hold the best spawning territories (mean size 2.6 m^2). Lower rank individuals are thus displaced to the marginal positions of the spawning area (Poncin, 1996). Territory size is inversely dependent on the amount of visual isolation present in selected local areas (stones, boulders, stable large woody debris), low visual isolation resulting in large territory size. Female grayling often remain a few metres downstream in deeper pools and only enter the shallows for short periods to spawn after ovulation. The approach of the female until mating is described in detail in Baars *et al*. (2001). In contrast to trout, female grayling do not deposit all eggs in a single spawning event but repeatedly perform the spawning process (in German called 'Portionslaicher'), which can be distributed over several days (Darchambeau & Poncin, 1997). Fabricius & Gustavson (1955) observed up to 34 distinct spawning acts per female, while males spawned up to 78 times. Thereby females can mate with different males and vice-versa, so that even younger and lower-rank males have a chance to 'come into operation' (Baars *et al*., 2001). In many grayling rivers, the same spawning grounds are used over several years, being often located in small tributaries or side channels of the main river bed (Ovidio *et al*., 2004). In former times (and until about 20 years ago) when the population sizes were at a much higher level in general, people involved in local fisheries regularly reported more than 100 grayling spawners gathering at the well-known spawning grounds (e.g. in the Inn, the Isar or the Lech; Baars *et al*., 2001; F. Fiedler, pers. communication).

Hatching, emergence and fry dispersal

The interstitium within the gravel bed of the spawning redds serves as the first habitat during the egg development stage over several weeks. Flow over these permeable gravel bedforms maintains interstitial oxygenated flow to grayling eggs and the hatched larvae. In grayling-rearing experiments using spawners from different Danubian origins the hatching time was reported to be 20 - 24 days for average water temperatures of 10 °C (B. Hornauer, pers. communication). Maitland & Campbell (1992) for grayling from Britain, and Herrmann

(2001) for the Ammer River grayling give the hatching time as 3 - 4 weeks (180 - 200 degree-days) falling close to the relationship given in Carlstein (1991). Carmie *et al.* (1985) report hatching at 200 and 220 degree-days for 8.5 and 9.0 °C, respectively.

After grayling larvae hatch (size at hatching about 10 - 12 mm, Maitland & Campbell, 1992), they still remain for a time-span of about 120 - 150 degree-days inside the gravel bed, where they exclusively feed on their yolk sac. After this reserve is completely exhausted, larval grayling appear for the first time at the surface of their redds. In this critical stage of development they leave a relatively safe habitat and from then on are exposed to the conditions of the flowing water. In contrast to other salmonids grayling emergence (i.e. the first upwards directed swimming) from their spawning areas is predominantly observed in the morning hours (Baars *et al.*, 2001; Northcote, 1995). In the following hours grayling larvae don't directly migrate after reaching the surface of the gravel. Instead, their relocation to suitable juvenile habitats does not take place until a lag phase in the following night. Under cover of darkness the larvae face a reduced danger of predation during their passage in the open water by passive drift with the water current. Finally, after a development process of 270 - 380 degree-days after fertilisation and with a body length of about 1.7 cm grayling fry for the first time can be observed at their typical habitats, usually in shallow and slower flowing river stretches. For the central European rivers, the soonest that juveniles appear is in April at the riparian areas (if the spawning date fall at the beginning of March).

After swimming up freely and starting to ingest their own, the larvae of grayling are smaller and physiologically less developed as compared to trout or salmon (Baars *et al.*, 2001). Initially the yolk sac is clearly recognisable, the embryonic fin seam has not yet disappeared, and the fin rays are not developed at this stage.

In their natural habitats the larval and juvenile fish have to cope with alternating flow velocities, to hide from potential predators and to quickly learn to make use of the food resources. However, under controlled captive breeding conditions in typical fish hatching facilities, many of these natural selective factors cease to exist: very low and constant streaming, usually no predation (rarely in natural ponds) and as a starter diet, a mixture of frozen plankton and artificial pellets. If a substantial loss of grayling happens after stocking of hatchery-reared fingerlings (at the age 0+ or 1+), it is largely unexplained as to whether this is due to the sudden transposition to the natural conditions (see also below under endangerment).

Age and growth rate

The maximum reachable age of grayling in central Europe is about 10 years according to Baars *et al*. (2001). In Scandinavia, up to 13 year old grayling have been reported (Wooland, 1987). Considering its entire distribution area, Arctic grayling (*T. articus spp*.) don't reach more than about 10 years, similar to European grayling (Northcote, 1995). However, according to De Bruyn & McCart (1974; cited in Northcote, 1995) northern populations of *T. arcticus* in the Yukon Territory, particularly those which are unexploited, are characterised by a much older age composition with a high percentage of fish over 8 years old and some surviving at least up to 22 years. In the latest review by Stearns & Hendry (2004) for European grayling 2 - 28 and for Arctic grayling 2 - 12 years are given. A formal mistake in name probably happened here concerning these two species, the more so as for the native range of *T. thymallus* northeastern Asia is given apart from Europe, which only can come into question for *T. arcticus*. In our central European area of the drainages Danube, Rhine/Main and Elbe, grayling rarely become older than 7 or 8 years (Baars, 2000).

According to Baars (2000), adult European grayling usually reach about 50 cm in total size and then weigh about 1 kg. The presently assumed maximal growth known from catches of a few single individuals is about 70 cm and approximately 2.5 kg. Demoll & Maier in 1941 reported grayling up to 3 kg weight. Fiedler (1991) as well gives account of a maximal weight of 3.1 kg. Interestingly, around the turn of the century, single grayling were caught weighing up to 6 kg (e.g. in the Traun River) and growing up to 1 m in length (Demoll & Maier, 1941). Depending on the time-span of the cold period, during the winter season the growth rates of populations from northern regions generally are lower than those from more southern located drainages (Northcote, 1995). In French rivers, growth of young European grayling increases with temperature up to about 17 °C in summer, and if above this level, growth decreases (Persat & Pattee, 1981).

In Bavaria, Baars (2000) observed considerable lower growth rates for grayling from the more acidic and low-ionic rivers having their sources in primary rock (granitic) regions (e.g. Eger, Regen) compared to grayling populations from the chalk streams of the alpine drainage system (e.g. river Iller). Figure 2.3 shows the different growth rates of grayling populations from six tributaries of the Danubian, Main and Elbe drainage systems. It is largely unknown to what extent these differences depend on genetic or environmental factors. However, there is strong indication, that the produced biomass and consequently the food resources basically are lower in low-ionic granite rivers than in high-lime streams (e.g. Geist, 2005). Common

garden breeding experiments under controlled laboratory conditions could help to clarify this question.

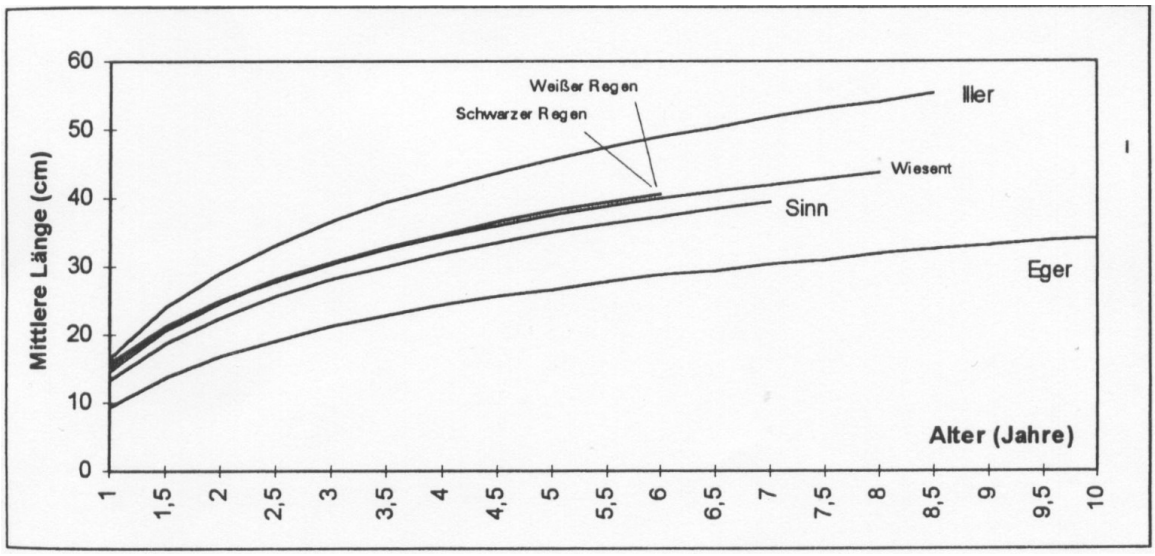

Fig. 2.3: Growth rates of European grayling populations from different drainage systems in Bavaria according to Baars (2000): rivers Iller, Regen (Danube), Wiesent, Sinn (Main) and Eger (Elbe).

Another open question is related to the notably lower maximum weight and growth compared to what was observed 50 years ago. One discussed hypothesis among fishery managers is that this trend may be caused by the (ongoing) strong anthropogenic impact to the body of flowing water such as straightening, impoundment or low amount of water in the original river bed due to canalisations (see below endangerment). Baars *et al*., (2001) argue that the declined living conditions associated with a loss of life expectancy could explain the lower growth of grayling today. However, an argument against this theory holds that due to the strongly decreased populations and population densities over the last 15 - 20 years, the existence of relatively more food resources is available for less fish. This should favour the chance to occasionally catch a big grayling. Instead, a potential selective effect on the growth performance could be expected due to the way of the applied fishery management during the last 50 - 100 years. The practice of using closure times during spawning season and size limits (i.e. the minimum size before a fish is allowed to be caught) for protection of important game fish is reported to be applied since the 16th century (Plomann, 1997). By application of a size limit of 30 - 35 cm as is commonly used for grayling from the alpine river systems anglers inevitably select slower growth rates and early maturation. All fish growing larger than this

size, sooner or later can be caught by fishermen and will be removed from the spawning group (except where 'catch and release' is allowed, but this is forbidden in Bavaria).

Feeding

In the life and year cycle of grayling, the composition of food resources changes according to seasonal circumstances. Basically, both European and Arctic grayling utilise a broad spectrum of macro-invertebrates as food (Northcote, 1995).

After changing from endogenous feed provided by the yolk sac over to an exogenous food supply young-of-the-year grayling at the beginning prefer chironomid larvae. The specialisation to this natural starter diet was observed in different rivers throughout its distribution range (Sumer, 1994; Sempeski *et al.*, 1995; Northcote, 1995; Baars, 2000). Drifting chironomid larvae mostly occur in great quantities in the typical low-current, riparian-close habitats of young grayling for the first 4-6 weeks. At the beginning of active foraging, chironomid larvae and pupae can represent up to 70 - 90% of the daily required food resource (reviewed in Baars *et al*., 2001). A high energy recovery, no hiding response, and an easy to handle, cylindrical body shape as well as their attractive twitching movements may explain the favoured uptake of these as feed. In the diet of older grayling larvae ($\geq$ 20 mm) the proportion of other insects (and their larvae) increases containing for example simulid larvae, dipteran larvae, stonefly and mayfly nymphs or copepods, gammaridae and rotifers, gradually changing to more benthic organisms with age (Baars, 2000; Northcote, 1995; Wooland, 1987).

When foraging, the visual sense of grayling plays a major role. Their eyes are relatively large already during the larval stage which enable them to selectively catch the drifting food if the lightning is sufficient, respectively. Grayling larvae feed during the whole daylight period. Wooland (1987) and Baars *et al*. (2001) described a strong diel periodicity in feeding, with peaks at dusk and dawn, probably governed by the nocturnal peak in invertebrate drift in relation to prey visibility. Feeding is completely stopped during darkness.

Yearlings and older grayling can be characterised as food opportunists. They increasingly ingest benthic organisms such as snails, worms, gammarus spec., caddy-, day-and stonefly larvae (Baars *et al*., 2001; Northcote, 1995). Flying insects are also frequently picked up from the water surface area. The choice of the food is primarily related to the that which is prevailingly on offer and in this way is subject to a dynamics that is adjusted to the life cycle of the feeding organisms. In addition, eggs potentially drifting away from other fish species (e.g. *Salmo trutta* or *Condrostoma nasus*) during the spawning time, can add to the food

spectrum of grayling (Northcote, 1995). Baars (2000) reported that a specialisation of adult fish to certain prey organisms can be observed within a typical grayling pool. Bigger and older European grayling also are known to ingest other small fishes (Baars *et al*., 2001).

Habitat requirements, migration and homing

In order to protect and manage riverine fishes, it is important to know what the characteristics of their selected habitats are in different seasons and ontogenetic stages. European and Arctic grayling are considered as typical gregarious species forming schools and swarms of different age classes (Northcote, 1995). During their under yearling, juvenile, subadult and adult stages grayling undergo a complex cycle of migratory behaviour over their lifespan which involve alternation between at least three major habitats: spawning, wintering and feeding or summer habitats (Northcote, 1995). Migratory and homing behaviour has been studied in much more detail for Arctic grayling (Northcote, 1995). However, recent studies by Mallet *et al*. (2000) and Nykänen *et al*. (2004) showed that general patterns of migratory behaviour as suggested for Arctic grayling do basically also apply to European grayling. Thereby, this trait concerns reproductive, refuge and trophic migrations between wintering, feeding and spawning habitats. The main issues of these migration and habitat requirements for the European grayling will briefly be summarised in the following section.

Under yearlings occupy shallow areas closer than 1.5 m from stream banks and in the upper third of the water column until reaching about 25 - 28 mm, when they move to the deeper benthic habitat characteristic of older grayling (Scott, 1985; Nykänen & Huusko, 2003). Mallet *et al*. (2000) demonstrated that European grayling of three different age-classes (0+, 1+ and adults) had strong preferences for their local habitat in a French river stretch. Large individuals preferred deeper water than small ones. The authors found that water depth segregation between grayling stages in the Ain River is probably due to an intraspecific competition between fish of different sizes and the avoidance of fish predation by young individuals. Similarly, studies on the closely related Arctic grayling showed that water depth segregation was determined by the respective rank of fish in hierarchy, larger dominant individuals being in the deepest habitats (Hughes, 1992; Buzby & Deegan, 2000). Mallet *et al*. (2000) concluded that *T. thymallus* needs various habitat conditions to achieve its entire life cycle.

Nykänen *et al*. (2004) recently investigated changes in movement, range and habitat preferences of adult grayling in a Finish river by radio-tagged monitoring of fish from late summer to early winter. The study revealed that the summer range of grayling in the riffles

(75 ± 146 m) and the autumn range (99 ± 46m) in the pools was relatively small and gross daily movements were equally short in both seasons (about 18 ± 34 m). Grayling shifted by the end of September from the riffle sites to deeper and slower pool sites 0.7 - 1.6 km up- or downstream. In late summer, adult grayling preferred lower water depths (80 - 120 cm) and higher mean velocities (> 40 cm/s) than in autumn (100 - 240 cm and < 30 cm/s, respectively). The results obtained by Nykänen *et al*. (2004) clearly showed that grayling are seasonally local fish with seasonally differing habitat requirements and a strong behaviour of homing for their summer and potential overwintering sites. The fish tendency to gather into deep pools in autumn suggests that lack of suitable winter habitat is a possible bottleneck to many grayling stocks. In areas with few winter pools, grayling stocks can become vulnerable to overfishing and potential predation by piscivorous birds during the cold season (see also below in section endangerment).

In summation, parental spawning, feeding and even wintering habitats may be the same ones used by their offspring so there is the possibility of strong reproductive, trophic and refuge homing in grayling, a life history trait that could be of great significance for their successful management and enhancement.

Population size and density

Until 10 years ago, there seemed to be very few quantitative data on population size or density for European grayling (Northcote, 1995). A Petersen mark-recapture estimate of population size for 2 year and older grayling (> 20 cm) yielded about 110 individuals ha^{-1} in the Swedish river Indalsälven (Henricson, 1984). Concerning grayling biomass, Dujmic (1997) ascertained up to 700 kg/ha in certain stretches of the Mur, one of the most productive Austrian grayling rivers. For the Vöckla, up to 190 kg/ha were reported by Uiblein *et al*. (2001) until a few years ago in certain river stretches. This is in line with Baars (2000) observation in Bavaria that until about 10 - 15 years ago grayling population sizes were highly stock specific with great regional variation. Stock sizes were in a range of 20 to 200 kg/ha. In general the development of population sizes was more stable in the northern Bavarian rivers of the Main-, Danube and Elbe drainages and is until today less critical then throughout the entire Alpine area (Baars, 2000). From the (mostly grey) literature and from catch records of local fishing associations it appears that the average grayling catches, particularly in the alpine Danubian tributaries, were at a relatively high level since the middle of the 1970s lasting until the beginning of the 1980's (e.g. Baars *et al*., 2001; Steinhörster, 2001; Uiblein *et al*., 2001). In the framework of the Bavarian grayling support program (see below for further details of this

project) Steinhörster (2001) analysed the annual catch records (starting in 1975) of fishing associations from 26 alpine Danubian river stretches. The author ascertained a strong population decline that occurred in parallel in the chosen river stretches of the typical grayling zone (see Fig. 2.4). The decline observed in these rivers started approximately at the same time in the middle of the 1980's in a short time-span of a few years. This negative trend is ongoing today and in the meantime also applies to several northern Bavarian rivers that over the last years were still known for their constant and stable grayling population densities (e.g. Fichtelnaab, Sächsische Saale).

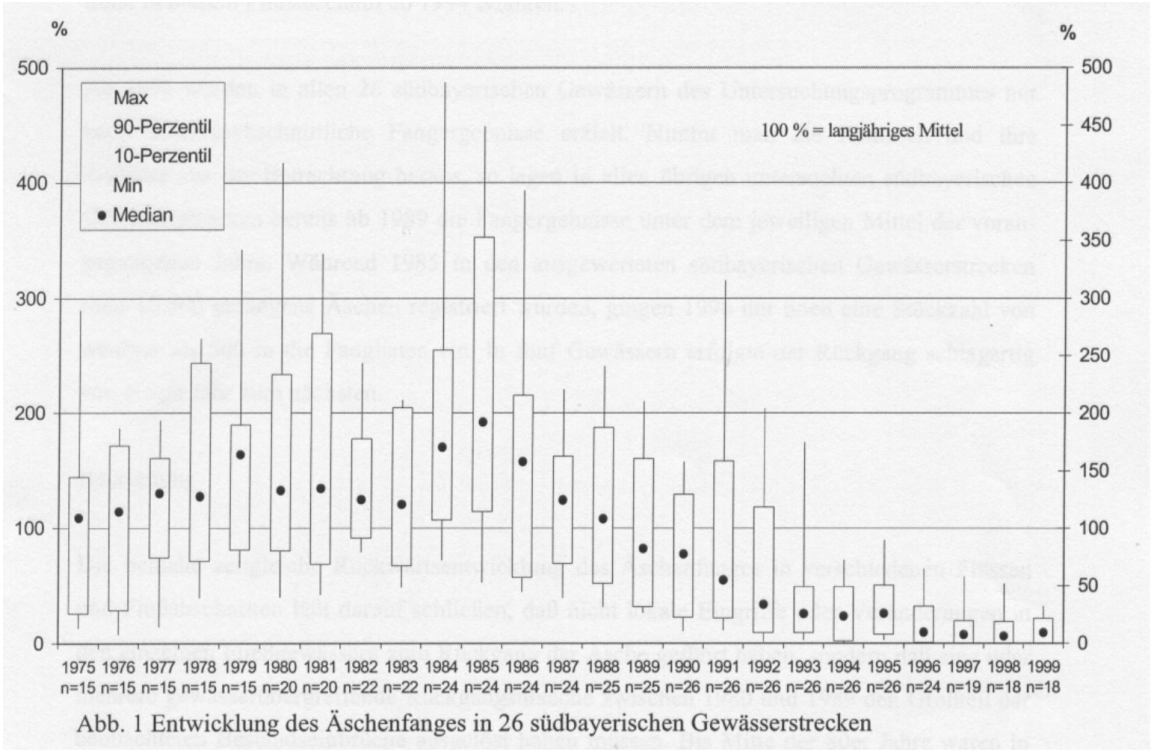

Fig. 2.4: Development of annual European grayling catches from 1975 to 1999 averaged over 26 Bavarian rivers of the Alpine Danubian drainage system according to Steinhörster (2001).

More detailed information on estimates of population sizes and quantitative data for most of the grayling rivers studied in the present work are given in Baars (2000) and Hanfland (2002). It should be noted, that nowadays in certain stretches of formerly densely populated grayling rivers such as Iller, Isar or Ammer, often not more than 10 to 20 spawners can be caught for the purpose of supportive breeding (Herrmann, 2001; O. Born, B. Hornauer pers. communication; B. Gum, pers. observation).

2.2 Genetics

2.2.1 General aspects

Molecular genetic advances

Research opportunities in the well-founded biological discipline of population genetics were significantly improved with the advent of allozyme electrophoresis in the 1960s (Harris, 1966; Lewontin & Hubby, 1966) and have been drastically boosted with the accelerating developments in molecular biology, in particular through invention of the polymerase chain reaction (PCR) (Mullis *et al.*, 1986). Since that time new techniques and genetic markers suitable for population genetic studies have been developed and nowadays are well-established (Sambrook & Russell, 2001). The development and establishment of numerous molecular markers for model species or commercially important species such as zebra fish, Atlantic salmon or rainbow trout (e.g. Shimoda *et al.*, 1999; von Schalburg *et al.*, 2005; Thorgaard *et al.*, 2002) have also greatly facilitated opportunities to investigate other genetically less well characterised organisms. In addition the whole genome sequencing and genome mapping projects for different taxa over the last decade produced and are still producing a huge amount of valuable baseline sequence data (see the Genome Research on All Salmon Project, the GRASP consortium at: http://web.uvic.ca/cbr/grasp/).

The most commonly applied PCR-based techniques for population genetic studies are restriction fragment length polymorphism (RFLP) and nowadays in particular, direct sequencing of the DNA region of interest as well as methods analysing length polymorphism of highly variable nuclear markers (especially genotyping of microsatellite loci). For methodological details see Kühn (2004). If one browses relevant journals like Molecular Ecology or Conservation Genetics other methods denoted by abbreviations such as RAPD, AFLP, SSCP, or DGGE less frequently are applied.

In the present work several previously established microsatellite systems (Gum, 2000) derived from related salmonid species were successfully applied to European grayling. In addition, a panel of markers developed specifically for grayling was used. In summation, a set of 20 polymorphic microsatellite systems listed in Table 3.2 (chapter 3) is now available and can routinely be applied for future grayling monitoring or breeding studies. Today, in the field of freshwater fish genetics, such a broad palette of different kinds of genetic markers is available that it is often possible, in a relatively short time, (i) to transfer suitable markers to genetically less well-characterised species, e.g. to the Danubian salmon (*Hucho hucho*) or to cyprinid

species such as *Chondrostoma nasus* and (ii) to apply these markers in a next step to larger sample sizes and so providing already valuable first baseline genetic data (e.g. Kolahsa, 2006). However, transfer experiments intended to cross-amplify microsatellite markers and subsequent tests for polymorphism are not always successful. For example in the cases of the European pearl mussel (*Margeritifera margaritifera*) and the eastern green lizard (*Lacerta viridilis*) specific microsatellite markers had to be developed because cross-species amplification was impossible or failed (Geist *et al.*, 2003; Laube & Kühn, 2006). For the highly endangered noble crayfish (*Astacus astacus*) species-specific development of markers is necessary as well (Gross *et al.*, in prep.). However, at the same time techniques for direct development of markers turned out to be more effective, thus reducing the expenditure of time. In addition to these advances in marker development, genetic analysis of problematical samples such as various types of archive material (e.g. bones or scale samples) is possible to a large extent now and thus allowing a temporal comparison of the genetic composition of historical and contemporary populations (e.g. Nielsen *et al.*, 1997).

Concerning the intensively studied mtDNA, sequences in some parts of the molecule are highly conserved across species and several sets of 'universal primers' have been developed that allow for the analysis of the same segments in a variety of species (Cronin *et al.*, 1993). In this work the improved mtDNA primer sequences for the chosen ND-1 and ND-5/6 genes and the control region were used. As outlined in Nielsen *et al.* (1998) these sequences were known to work well for several salmonid species such as Atlantic salmon, *Salmo salar* (Nilsson *et al.*, 2001), Arctic charr, *Salvelinus alpinus* (Gross *et al.*, 2004) or Lake whitefish, *Coregonus* sp. (e.g. Hansen *et al.*, 1999).

Computational and statistical advances

Not only have the molecular genetic techniques and possibilities to establish and apply molecular markers been considerably improved over the last decade but at the same time, population genetic theory has continued to evolve and has in many cases led to novel approaches and methods for analysis of empirical data, which are currently revolutionising population genetic data analysis (e.g. Bertorelle & Excoffier, 1998; Pritchard *et al.*, 2000; Beerli & Felsenstein, 1999, 2001; Wilson & Rannala, 2003; Beaumont & Rannala, 2004). Collectively, these methods of modern molecular biology and molecular genetics including high throughput genotyping or sequencing techniques in conjunction with computational advances in the field of statistical population genetics have resulted in unprecedented opportunities for empirical population and conservation genetic studies (reviewed in

Beaumont & Rannala, 2004). Several of these approaches are based on the coalescence theory, where roughly speaking, one 'looks back in time' to estimate the gene genealogy, which contains important information about the history of populations, such as population declines or expansions, e.g. applied in Gum *et al*. (2003) using the 2MOD computer programme (Ciofi *et al.*, 1999). Furthermore, developments in computer technology have made it feasible to conduct highly sophisticated analyses based on Markov Chain Monte Carlo numerical re-sampling techniques for estimating the probability distributions of various population genetic parameters of interest, e.g. migration rates or effective population sizes (e.g. Wilson & Rannala, 2003; Beerli, 2006). In this regard, especially the so called 'assignment methods', which use genetic information (based on multi-locus genotypic data) to ascertain population membership of individuals or groups of individuals, proved to match important biological questions with appropriate techniques (see reviews of assignment methods by Hansen *et al*., 2001b and Manel *et al*., 2005). Doubtless, the most recent progress problems associated with assignment has been made within a Bayesian framework (Beaumont & Rannala, 2004; Beerli, 2006). More precisely, concerning the research on species that are highly disturbed by human activities and environmental impacts, recent computational advances in statistical analyses of genetic data are now enable to effectively address key issues of conservation genetics, such as:

- the assessment of population structure and substructure and inference of the number of distinct gene pools in a data set (Pritchard *et al.*, 2000);
- the identification of the origin of specific individuals or populations (Cornuet *et al.*, 1999; Primmer *et al.*, 2000);
- the estimation of dispersal/migration rates and effective population sizes among populations (Beerli & Felsenstein, 2001; Wilson & Rannala, 2003);
- parentage analysis (e.g. Nielsen *et al.*, 2001);
- the measuring of hybridisation (Anderson & Thompson, 2002);
- the estimation of the proportions of individuals from different source populations contribution to a genetic mixture or admixture (e.g. Beaumont *et al.*, 2001);
- genetic tracking of cultured or released animals (e.g. Tringali, 2006).

All of the above listed aspects are central questions in basic and applied science, but particularly in applied fishery management. A detailed knowledge about the overall composition of a mixture (of individuals from different populations) or an admixture (which results from interbreeding among populations) is required for conservation decision-making because actions of fish stocking are carried out worldwide to a large extent with mostly

unpredictable genetic effects. In the case studies which are presented in this thesis, several of the issues presented above are applied. These approaches include assignment of individuals to populations, testing population bottlenecks (Gum *et al*., 2003), demonstration of the presence of population substructure, and studying hybrid zones (Gum *et al*., 2005, 2006), as well as identification of recent immigrants and admixed individuals (Gum *et al*., 2006). In addition, other statistical developments in population genetic analysis provide valuable tools specifically for prioritisation of populations for conservation. For example, the approach suggested by Petit *et al*. (1998) supplements the genetic characterisation of populations by calculating the heterozygosity contribution (*CT*) of each population to total diversity. This method was successfully applied in Geist & Kuehn (2005) for selection of priority populations of the highly endangered European pearl mussel.

In conclusion, with the molecular genetic and statistical tools available today, research possibilities in the field of conservation genetics are greatly extended as compared with many of the former tree-based studies. However, in general, traditional measures of genetic similarity, such as F_{ST} and genetic distances, are still essential statistical components providing valuable insights into the genetic relationship and overall level of differentiation between populations and should be viewed as a necessary complement to the recent established methods. In this context, for genetic characterisation of other (endangered) species, a integrated approach of the applied genetic (e.g. by using different types of molecular markers as done in this study) and statistical methods is probably best suited to efficiently examine the level of genetic diversity at the individual, population and subspecies level and to estimate the extent of gene flow occurring between the samples.

2.2.2 Grayling genetics

The following short literature review of ‘grayling genetics’ is restricted to studies that used methods and markers corresponding to those applied in the present work, i.e. to genetic studies based on mitochondrial DNA (mtDNA) and microsatellite data.

In recent years the genus *Thymallus* has drawn considerable attention concerning its population genetics, its phylogeography both worldwide (e.g. Koskinen *et al*., 2000; Froufe *et al*., 2003; Stamford & Taylor, 2004; Gum *et al*., 2005; Weiss *et al*., 2006) and on local scale (Koskinen *et al*., 2001; Sušnik *et al*., 2001; Gum *et al*., 2003) as well as from an evolutionary perspective (Koskinen *et al*., 2002c). For the European grayling in particular, a rapid growth in the number of studies could be observed within a relatively short time span both in respect

to the development of molecular genetic markers as well as to large- and small-scale population genetic and phylogeographical studies (e.g. Koskinen *et al*., 2000; Gross *et al*., 2001, Weiss *et al*., 2002, Gum *et al*., 2003, 2005). At this point I will only briefly address and summarise the outcome of the most relevant molecular genetic work which has recently been carried out on European grayling. This is being done mainly in terms of their chronological appearance since the content of most of these studies are discussed in detail in the papers included in this thesis (Gum *et al*., 2003, 2005, 2006).

The first microsatellite markers were developed specifically for grayling by Sušnik *et al*. (1999a,b). At that time and in the following years further markers were established both directly (Sušnik *et al*., 2000) or indirectly through cross-species amplification of polymorphic loci derived from related salmonid species and subsequent tests for polymorphism in grayling (Koskinen & Primmer, 1999; Gum, 2000). After establishment of these markers, the first population genetic studies on a local scale were carried out by Koskinen *et al*. (2001) in Finland and Gross *et al*. (2001) in Bavaria. In this way genetic baseline data were obtained and first insights were gained regarding the level of genetic differentiation between grayling populations from the Danube, Rhine/Main and Elbe (Gross *et al*., 2001). However, these first studies were mostly limited by the sampling of only a few populations (Sušnik *et al*., 1999a; Koskinen *et al*., 2001) or by the application of only a few microsatellite markers (Gross *et al*., 2001). In the following studies by Koskinen *et al*. (2002a) and the present work (Gum *et al*., 2003) both an increase in the number of populations and up to 20 microsatellite loci were used to infer the phylogeography and population genetic structure of the species on its northern and central European distribution.

In parallel to the development of nuclear markers, mitochondrial markers were established and applied for grayling (Koskinen *et al*., 2000; Gum, 2000; Gross *et al*., 2001). Transfer and improvement of conserved mitochondrial primer sequences that were designed to amplify several salmonid NADH (ND) gene regions and the mtDNA control region (e.g. Nielsen *et al*., 1998) provided the necessary tools to effectively investigate the phylogeography of European grayling. Koskinen *et al.*'s (2000) study identified three major evolutionary lineages of grayling with special focus on the species' northern European distribution. Based on mtDNA sequence data Sušnik *et al*. (2001) characterised the evolutionary distinctiveness of grayling from Slovenia proposing a subspecies status for *Thymallus* inhabiting the Adriatic river system. Based on the complete sequencing of the mitochondrial DNA control region, Weiss *et al*. (2002) revealed complex patterns of postglacial re-colonisation for grayling using samples mainly from southern Europe. Combination of the mtDNA data produced in this

work with those previously acquired by Koskinen *et al*. (2000) allowed for a more comprehensive assessment of the species biogeographical structure, distributional history and evolutionary dynamics in central and northern Europe (Gum *et al*., 2005).

Genetic studies based on microsatellite DNA analysis, regarding grayling stocking impact assessment, commenced in 2002. Using contemporary and historical scale samples Koskinen *et al*. (2002b) assessed the spatiotemporal population structure and stocking effects of introduced-to-wild populations among endangered Lake Saimaa grayling in Finland. Despite their finding of clear genetic imprints of stocking, the contemporary populations exhibited evolutionary relationships congruent with the sampling locations, and more than 70% of contemporary individuals were identified to be of pure indigenous origin. In contrast to this result, Sušnik *et al*. (2004) showed that ongoing stocking of grayling originating from the Danube led a loss of at least 50% of the native Adriatic type of grayling in Slovenia. Based on combined data of nuclear microsatellite and mtDNA analysis Gum *et al*. (2006) discriminated the impact of recent human mediated stock transfer from historical gene flow on genetic structure of European grayling (this study, see chapter 5).

2.3 Endangerment of grayling

In the latest revision of the 'Red List of threatened species in Bavaria' grayling is not longer classified as endangered as it was until 1996 but as *critically endangered* (Bohl, 1996; Bohl *et al*., 2003). This category of endangerment is defined as follows: "If the factors responsible for the endangerment and the causation of the decline will further act upon and effective conservation and protection measures are not taken, the species likely is to be threatened with extinction in the near future". Among experts in the field of fishery science and management, grayling currently is considered as endangered at the population level, i.e. some local stocks seriously are threatened with extinction due to few offspring reaching the spawning age.

At the same time it has to be noted that the strong decline of this fish species today is not exclusively a grayling specific phenomenon since it also applies to many other (if not all) typical stream-dwelling fishes such as bullhead (*Cottus gobio*), brown trout (*Salmo trutta*), Danubian salmon (*Hucho hucho*) or sneep (*Chondrostoma nasus*) as well as to several smaller fish species like for example loach (*Nemacheilus barbatulus*) or minnow (*Phoxinus phoxinus*).

2.3.1 Anthropogenic and environmental impacts on stream-dwelling fish populations

The co-action of different factors can lead to a strong decline or even extinction of riverine fish populations (see Fig. 2.5). The most severe of these issues are directly linked to anthropogenically caused impacts such as over-fishing or detrimental alterations to the natural dynamics of river systems.

Comparable to the serious endangerment of many pacific salmonid species in North America (see Lichatowich, 1999; Hendry & Stearns, 2004), the decline of the riverine fish species native to our area is a matter of a long-term negative development. The process certainly goes back to the 19th century and essentially has to do with the complete deformation and modification of aquatic ecosystems caused by humans (Lelek & Buhse, 1992). The increasing effects of anthropogenic river and land utilisation was associated with a significant change of different biotic and abiotic conditions in river habitats. In particular, the past and present intensive efforts and measures to construct dams, weirs and channels, mainly for the purpose of providing energy through hydropower, directly or indirectly had detrimental effects on local fish populations. The long-distance migratory species, salmon and sturgeon, were the first who disappeared; first, because they were often affected by overfishing, and second, because they were critically dependent on the natural dispersal pathways of large connected river systems allowing them to reach the spawning grounds. With the exception of only a few upper river stretches in the Alps (e.g. the Isar upstream of the 'Sylvensteinspeicher' or the upper Lech valley), there is virtually no small or larger river left in Bavaria that is not affected by factors such as construction of dams for water retention or electrical generation, impoundment, straightening, canalisation or diversion (Tombeck, 2006).

For many species, breeding habitats were lost due to the absence of dispersal possibilities to small tributaries caused by restricted access. In addition, significant riparian zones were destroyed due to the loss of the natural dynamics associated with an intact fluvial topography in conjunction with the disappearance of alluvial forests. Current surveying and mapping studies carried out throughout Bavaria suggest about 9000 migrational barriers (Tombeck, 2006), that in upstream direction are impassable for most fish species. Due to the increasing extent of siltational processes caused by impoundment, many spawning grounds of the gravel spawning species and breeding habitats were destroyed (Pulg, 2006).

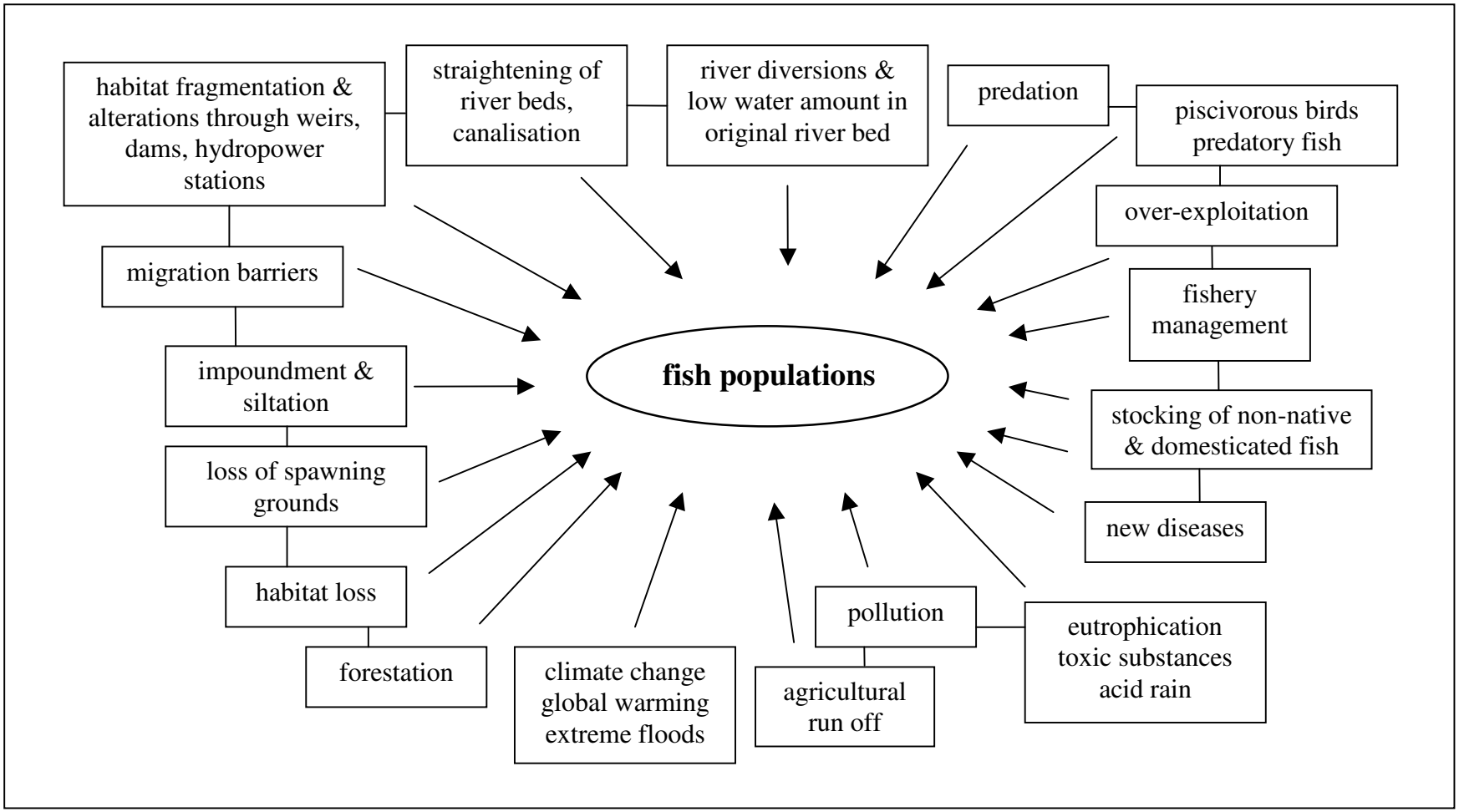

Fig. 2.5: Co-action of different factors leading to the decline and endangerment of riverine fish populations.

Further risk-aggravating factors are caused by sudden water-flow regulation of hydropower stations (so called 'hydropeaking'), and the diversion of major amounts of water to artificial channels. In most cases only a limited amount of water remains in the original river bed (e.g. in the Isar or Alz Rivers).

During the last decade, a combination of more and more severe flood waters and extreme dry periods have occurred due to the climate change. This has probably had a detrimental effect on larval and juvenile fish. Because of global warming in general and the increase of water temperatures, negative effects on cold-water adapted fish species are assumed; however, more research is needed to assess this hypothesis. In addition, it is already known from exposure experiments, that the more intensive UV radiation (associated with the ozone hole) has the potential to seriously harm different freshwater fish like grayling and sneep leading to high losses among juveniles stages of these species (Gross *et al*., 2003).

The direct damage and threat to fish stocks due to sewage or chemical pollution, as was often found in the 1970's and 1980's continuously decreased over the last two decades. Therefore, water quality in general is not considered as a major cause of the overall decline of remnant fish populations. Instead, over-fishing, depending on the species and location, may still play an important role, for the economically most interesting species such as sturgeon, salmon and trout. The effect of size selective angling on the development of populations has possibly been underestimated until recently and is an issue of current research (Arlinghaus & Cooke, 2005; Birkeland & Dayton, 2005).

Fishery mismanagement also can contribute to the decline of natural fish populations or directly cause the loss of local stocks. For example the stocking of non-native fish species can lead to introduction of diseases and parasites (e.g. Bakke *et al*., 1990), displacement of local aquatic fauna through inter-specific competition (e.g. Nisikawa *et al*., 2001) or even extinction of species through direct predation considering the fatal consequences following the introduction of the Nile perch (*Lates niloticus*) into Lake Viktoria (e.g. Kitchell *et al*., 1997). In addition, stocking of domesticated strains as typically used for salmon or rainbow trout has been shown to have the potential to significantly change the genetic integrity of the local gene pool. Depending on the level of introgression and admixture with local wild fish, stocking of domesticated and non-native fish from different ESUs can (ultimately) also lead to the complete loss of locally adapted gene pools (Hindar *et al*., 1991; this study chapter 5). Conventional fishery management, as regards the enhancement motivated stocking of fishes, has only recently been challenged as a result of the involvement of genetics in fish ecology and fishery science.

As explained in more detail in the following chapter, the effect of piscivorous birds (i.e. cormorant and goosander) on the decline of local fish populations is still disputed by people involved in fisheries and bird conservationists. Since the beginning of the 1980s an enormous increase of the European cormorant breeding and wintering populations can be observed (van Eerden & Keller, 2005). However, the actual degree of damage caused by the increased cormorant predation pressure is hard to determine and there are relatively few scientifically approved studies available for the studied area (e.g. Suter, 1995; Keller, 1998; Suter, 1998; Govedic, 2002).

Given the above illustrated factors, it becomes clear that both past and present processes are responsible for the decline of stream-dwelling fish populations. The dysfunction of the ecosystem river is multi-causal. Therefore, in the long-term, only a broad interdisciplinary conservation and management programme taking into account the river systems as a whole can help to secure the endangered remnant self-reproducing populations. In the meantime, several stakeholders from NGOs and GOs have already started to ameliorate the effects of past mistakes. For example, the former problem of insufficient water quality is reduced in many rivers. Recent renaturation projects effectively resulted in a substantial improvement of habitat quality in several river systems (e.g. through deconstruction of weirs and dams and broadening of river beds; Hendry & Cragg-Hine, 1996; Schiemer *et al*., 1999). As exemplified by the successful return of Atlantic salmon to the Rhine River (Klinger *et al*., 2003), riverine fishes have the potential to return and to reproduce, as long as the basic ecological conditions are fulfilled.

2.3.2 Bavarian grayling support programme

The essential findings of the Bavarian grayling support programme (‘Artenhilfsprogramm Äsche’) that was initiated to determine the reasons for the strong decline of grayling have been open to the public since 2003. The final reports of each case study can be obtained upon request from the participating NGOs, the ‘Bund Naturschutz in Bayern e.V.’, the ‘Landesbund für Vogelschutz in Bayern e.V.’, and the ‘Landesfischereiverband Bayern e.V.’ The general summary of the programme’s overall outcome is available on the web: [http://www.lfvbayern.de/] following the link ‘Fischereifachliches’. In the following only the main results of the different projects and investigations are briefly summarised.

Findings concerning the water quality and the influence of structural parameters of stream habitats were largely indisputable among experts. A general amelioration of the water quality was observed particularly in respect to the entry of phosphates or nitrates over the last two decades resulting in a much lower level of eutrophication or processes causing oxygen deficiency as compared to the 1970's and 1980's. Average values of water temperatures and dissolved oxygen were all found to be in an optimum or well-tolerable range for grayling, without restriction. In the programme, several rivers of different status as regards the quality of their structural habitat were chosen. These grayling rivers were affected by anthropogenically caused habitat alterations such as construction of dams, weirs, straightening of the original river bed or real canalisation actions to various degrees. For example the upper stretch of the Ammer represented a largely naturally flowing river without much anthropogenic modifications, whereas some lower stretches of the Iller or smaller streams such as the Dorfen were, in general, heavily affected by hydropower stations, weirs and the straightening of the flow direction. In addition, several semi-natural river stretches e.g. the Schwarzer Regen, were included. However, when finally comparing all the investigated streams, human caused alterations to the water body had to be excluded as a direct (short-term) explanation for the strong decline (Königsdorfer *et al*., 2001; Sachteleben, 2001). Interestingly, in all of these rivers, a natural reproduction of grayling was documented; even in those showing strong impoundment and potential loss of suitable spawning grounds through siltational processes (Schubert, 2001). Further projects addressed the potential impact of fish-toxic substances, changes of the water-chemistry after influx of wastewater and the infection pressure of unknown diseases. Though intensively studied and experimentally tested, no biological or water-chemical substance of content was identified that could have significantly reduced grayling survival in general (Born, 2001). Pathological studies did not indicate any toxic or harmful components to inner organs. There was also no indication of damage or disease among the investigated fish as was tested by virological, bacterial and histological methods. Another approach dealt with the potential detrimental effect of the increased UV radiation on grayling larvae, fry and juveniles (Gross *et al*., 2003). This was especially relevant in spring, which is within the relevant time frame during which grayling reproduction occurres and the intensity of the UV radiation was shown to increase in the last years. First exposure experiments, performed under controlled laboratory conditions and using relatively high UV dosage, resulted in high mortality rates as well as in a significant growth depression of larval grayling. However, further study is needed to determine whether

the effect of the accelerated UV radiation shows similar effects on grayling in their natural habitats.

In summary, it can be concluded that among the factors summarised above, no single factor alone could explain the strong decline of grayling observed to have occurred in parallel in many alpine rivers. However, there was broad agreement that a combination of several of those factors locally have the potential to negatively affect the natural reproduction of grayling and to limit the species' natural recruitment. Nevertheless, a single and decisive reason for the area-wide breakdown since the mid of the 1980's could not be determined from the afore listed research activities. One may ask now: if none of the above mentioned factors, what other possible factors have there been which account for the strong decline of so many stream-dwelling fish species including grayling?

Another important and highly controversial discussed issue within the scope of the Bavarian grayling support programme concentrated on the predation pressure caused by piscivorous birds. More specifically the European wide increasing cormorant (*Phalacrocorax carbo carbo* and *Phalacrocorax carbo sinensis*) and Alpine-wide increase of goosander (*Mergasus merganser*) populations were assumed to have damaging effects on certain susceptible fish species. Indeed, concerning goosander, two studies carried out over consecutive years at the Ammer and Mangfall Rivers (by scaring away the birds through shots fired in the air) showed that the influence of predation by goosander on the development of stock size of juvenile grayling over the winter season can be substantial (Born & Hanfland, 2001; Klein, 2001). However, until the report of the grayling support programme was finalised in 2003, no consensus between experts from fisheries and bird protection agencies or GOs has been reached in respect to the interpretation of the data and concerning the general dimension of the predation pressure caused by goosander and cormorant on grayling. From the Bavarian fishing association perspective, there is no doubt that cormorants and goosanders significantly contributed to the breakdown of many grayling stocks throughout its northern Alpine distribution range. In addition, fishermen argue that the present predation pressure during the winter season does not allow for sustainable recovery of the depleted stock sizes to a comparable former level. Due to this dispute, the representatives of different GOs and NGOs could not agree on the originally planned catalogue of measures intended to secure the remaining grayling populations and to move conservation of grayling forward.

Today, most people involved in the debate acknowledge the fact that the increase of the European cormorant population over the last three decades (since its European-wide protection in 1979) has brought conflicts with human interests, especially in fish-farming areas, coastal inland water and river systems (van Eerden & Keller, 2005). Nonetheless, the debate regarding the potential threat to grayling caused by an increased cormorant wintering population is and will be ongoing in Bavaria as well as in Austria, Switzerland and France. For goosander a similar debate is to be expected in the next years. An objective, scientifically based conclusion of the conflict is urgently needed but seems hard to achieve, not to mention a long-term management plan on a European scale. The situation may be best described by the contribution of Behrens *et al*. (in press) at the 7th international conference on cormorants in Switzerland 2005, entitled: 'Managing the Cormorant - a case study of failure of a European action plan to minimise the conflicts between great Cormorant and fisheries'.

3 Microsatellite variation in Bavarian populations of European grayling (*Thymallus thymallus*): Implications for conservation

published: Gum, B., Gross, R., Rottmann, O., Schröder, W. & Kühn, R. (2003). Microsatellite variation in Bavarian populations of European grayling (*Thymallus thymallus*): Implications for conservation. *Conservation Genetics*, 4, 659-672.

3.1 Abstract

European grayling populations in Bavaria have shown steady declines during the last 10-20 years. In order to provide guidelines for conservation strategies and future management programs, we investigated the genetic structure of 15 grayling populations originating from three major Central European drainages (the Danube, the Elbe and the Rhine/Main) using 20 microsatellite loci. Genetic divergence between the three drainage systems was substantial as illustrated by highly significant heterogeneity of genotype frequencies, high number of drainage-specific private alleles, high between-drainage F_{ST} values, high assignment success of individuals to their drainage of origin and the high bootstrap support for the genetic distance based drainage-specific population clusters. In agreement with earlier studies, microsatellites revealed relatively low levels of intrapopulational genetic diversity in comparison to the overall level of variation across populations. Maximum likelihood methods using the coalescent approach revealed that the proportion of common ancestors was generally high in native populations and that the estimates of N_e were correlated with the genetic diversity parameters in all drainages. The number of effective immigrants per generation (N_em) was less than one for all pairwise comparisons of populations within the drainages, indicating efficient interpopulational reproductive isolation. Based on these findings we recommend a drainage and sub-drainage specific conservation of grayling populations in order to preserve their overall genetic diversity and integrity. For large-scale stocking actions to supplement the declining or to restore the extinct populations, creation of separate broodstocks for major conservation units (ESUs and MUs) is warranted.

3.2 Introduction

The European grayling (*Thymallus thymallus* L.) is distributed over a large part of the European continent. This salmonid species is considered to be culturally important and is highly appreciated by anglers. However, during the last several decades factors such as water-flow regulation, pollution, habitat destruction, predation and over-fishing have contributed to local population-size declines throughout the European distribution range (Persat 1996; Koskinen *et al*., 2001; Sušnik *et al*., 2001; Uiblein *et al*., 2001). To compensate for losses, hatchery fish have been stocked throughout the Danube drainage system, as well as in France, Italy and Slovenia (Baars *et al*., 2001; Sušnik *et al*., 2001). The fishery managers are faced with the problem of preserving the remaining wild populations and there is need to delineate conservation and management units such as Evolutionary Significant Units (ESUs) and Management Units (MUs) as defined by Waples (1991) and Moritz (1994). Distinct MUs can be defined as populations connected by little or no contemporary gene flow, but not separated historically for very long periods of time (e.g. Ryder *et al*., 1986; Waples 1991).

Recent advances in the development of molecular markers have facilitated gaining a better knowledge of the genetic structure of grayling populations and their phylogenetic lineages in Europe. PCR-RFLP analysis and the sequencing of mitochondrial DNA genes have revealed major phylogenetic lineages for European grayling, allowing inference of postglacial expansion routes from hypothetical refugia throughout central and northern Europe (Koskinen *et al*., 2000; Weiss *et al*., 2002). Microsatellite studies of population genetic structure and diversity revealed phylogeographic patterns consistent with the mitochondrial DNA data. In addition these showed low levels of intra-population genetic diversity and substantial divergence between the populations, even between hydrologically connected sampling locations (Koskinen *et al*., 2001, 2002a).

In Bavaria, the grayling is a characteristic species in the upper and middle river stretches of the major Central European drainages: the Danube, the Elbe and the Rhine/Main. However, following dramatic population declines since the mid-1980s (Baars *et al*., 2001), a specific conservation and management program was established (Stein & Born, 1999). Gross *et al.*'s study (2001) on the variability of mitochondrial and nuclear genes and of four microsatellite loci in four wild populations from the Danube, Elbe and Main drainages provided the first insights into the genetic background of grayling in Bavaria. Even though the number of studied populations and nuclear DNA markers were low, both mitochondrial and nuclear data sets identified strong genetic differentiation between the three drainages. Unfortunately, the

numbers of sampling points from the Bavarian area were either low or absent in the studies conducted by Koskinen *et al.* (2000, 2002a) and Weiss *et al.* (2002). Considering that grayling populations have been noted to be highly genetically structured over small geographical scales (Koskinen *et al*., 2001, 2002a) it is obvious that the development of appropriate conservation and management strategies for grayling in Bavaria will require a higher localized sampling density. Here we report variability at 20 microsatellite loci in 15 grayling populations throughout Bavaria presenting a more detailed profile of the levels of genetic variability, differentiation and gene flow among populations within and between the drainages. These data are important not only for delineating the units of conservation and management (ESUs and MUs) but also for better understanding of the contemporary and historical factors that shape the population structure of this species.

Table 3.1: Samples used for the genetic analysis of European grayling (*Thymallus thymallus*) populations from Bavaria.

DRAINAGE Population	Sample size	Status of population
DANUBE		
Ammer	34	wild, supportive breeding
Dorfen	37	wild, supportive breeding*
Inn	11	wild, not stocked
Inn/Gars	39	wild, supportive breeding
Lech	20	wild, supportive breeding*
Ramsach	39	wild, not stocked
S. Regen	32	wild, supportive breeding
Sempt	39	wild, supportive breeding*
Ti. Ache	39	wild, supportive breeding*
RHINE/MAIN		
Erf	15	wild, not stocked
Fr. Saale	15	wild, not stocked
Leinleiter	32	reestablished 30 years ago, supp. breeding
Sinn	39	wild, not stocked
Bodensee	39	hatchery strain
ELBE		
Eger	39	wild, not stocked
Total	469	

* spawners mostly from the same river stretch and to a lesser extent from different tributaries to the Danube

3.3 Materials and methods

3.3.1 Sample collection

A total of 430 individuals of grayling, originating from 14 locations across the drainages of the Danube, the Main (major tributary of the Rhine) and the Elbe in Bavaria, Germany were caught from the wild by electro-fishing in 1999 and 2000 (Fig. 3.1). Additionally, a hatchery strain originating from Lake Constanz (Bodensee) was sampled to represent the upper Rhine drainage. The sampling locations were selected in accordance with the Bavarian state's grayling conservation plan (Stein & Born, 1999).

Six of the sampled populations have never been supplemented by hatchery releases (Stein & Born, 1999) (Table 3.1). Four of the Danubian drainage populations (Dorfen, Sempt, Lech and Ti. Ache) have been enhanced mostly with fish from the same rivers, but to a lesser extent also with fingerlings from other tributaries of the Danube, whereas three populations (Ammer, Inn/Gars and S. Regen) have been augmented using spawners only from the same river stretch (Stein & Born, 1999). The population in the Leinleiter river (Main drainage) was founded approximately 30 years ago by spawners from the adjacent Wiesent river and supplemented by supportive breeding during the last few years. The status of the populations is based on information from the Bavarian Fisheries Association (Landesfischerei Verband Bayern e.V.) and local anglers' associations.

3.3.2 DNA isolation and microsatellite analysis

Genomic DNA was isolated according to the simplified method of Laird et al. (1991)[1].

A total of 20 microsatellite loci were analysed: eight dinucleotide microsatellite loci were developed for Thymallus thymallus (Snoj *et al*., 1999; Sušnik *et al*., 1999a, b) and twelve dinucleotide microsatellite loci were derived from other salmonids and were shown to be polymorphic in grayling (Gross *et al*., 2001; Koskinen & Primmer, 1999, 2001) (Table 3.2). Each polymerase chain reaction (PCR) (15 µl) was composed of 40-60 ng DNA, 1x PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.1 mM dNTPs, 0.2 µM of each primer and 0.3 units of Taq DNA polymerase (MBI-Fermentas).

[1] The basic procedure is explained in 3.6 on page 53.

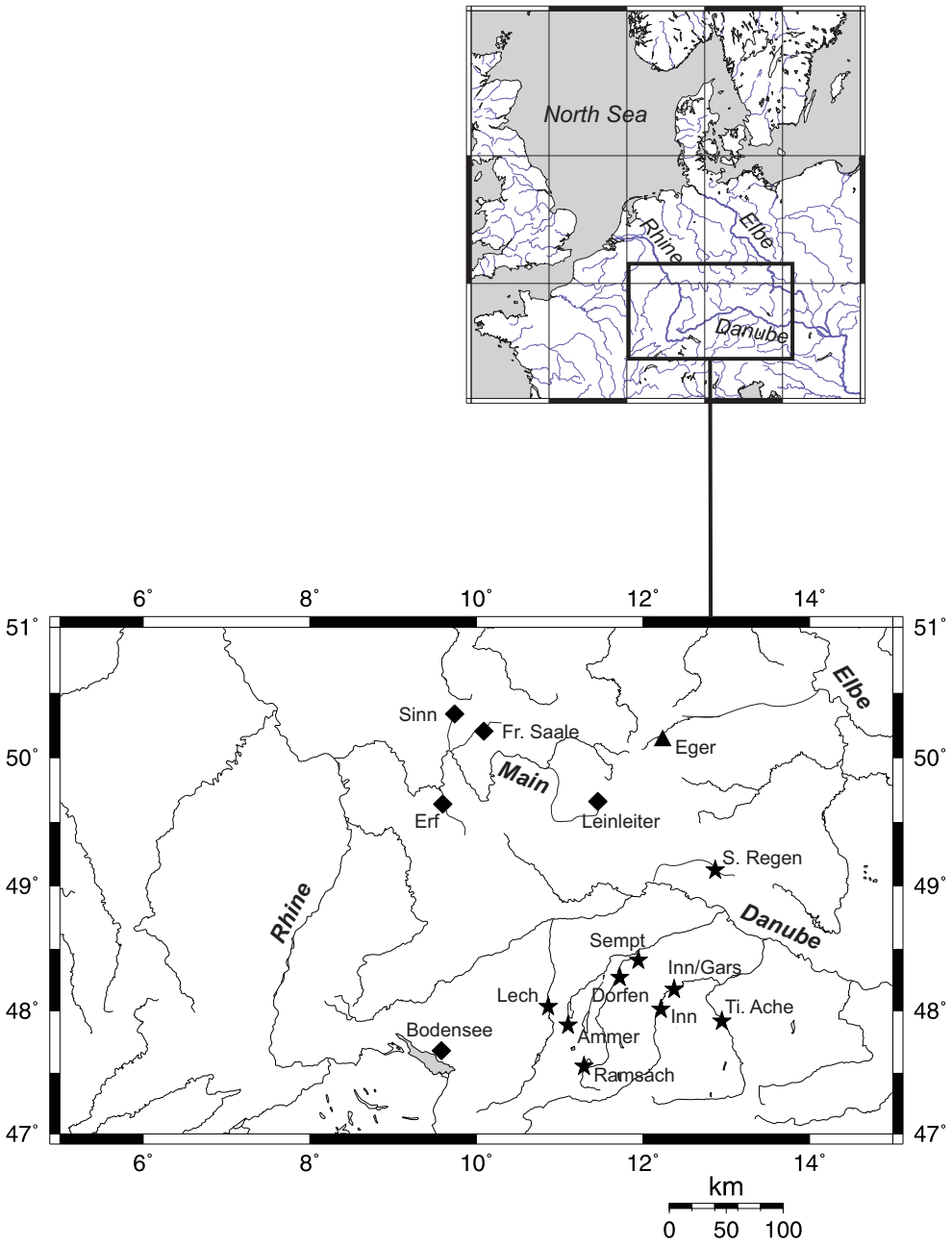

Fig. 3.1: Sampling locations of grayling (*Thymallus thymallus*) in Bavaria (southern Germany). Grayling were collected from drainages Danube (★), Rhine/Main (♦), and Elbe (▲).

We used the following PCR profile: initial denaturation at 94 °C for 3 min, 35 cycles of 30 s at 94 °C, 30 s at annealing temperature (Table 3.2), 30 s at 72 °C and a final extension at 72 °C for 3 min. Thermal cycling was performed in a Biometra Uno cycler.

The forward primers were end-labelled with the fluorescent dye Cy5. The length of the microsatellite alleles was determined by an ALFexpress II DNA analyser (Amersham Pharmacia Biotech) and AlleleLinks version 1.02 software (Amersham Pharmacia Biotech).

Reference samples with known genotypes were included on each gel and internal standards were included in each lane to ensure consistent scoring of genotypes across all gels.

Table 3.2: Characterisation of the studied microsatellite loci in European grayling (*Thymallus thymallus*). Locus and groupings (loci combined and analysed simultaneously), primer sequences, annealing temperature (T_a), range of allele sizes, number of observed alleles (n_A) and average expected heterozygosity (H_e).

Locus	Group	Primer sequences 5'-3'	T_a (°C)	Size range (bp)	n_A	H_e
BFRO004	I	F gctccagtgagggtgaccag R aggccactgattgagcagag	55	157-167	4	0.31
BFRO005	I	F cgcatctgtatgaaaaacct R tggtttggtaggagtttcgt	55	113-141	20	0.62
BFRO007	I	F agacccccaaaaactatgct R taaggtccccaacactacga	55	184-192	3	0.27
BFRO010	I	F ctggagtgcaacgaccacg R catgcaaaaacataatggcgc	55	202-258	20	0.74
SSOSL311	II	F tagataatggaggaactgcattct R catgcttcataagaaaaagattgt	55	108-120	3	0.05
BFRO009	II	F aaattgtccccgttggcaga R acatacaccgcaacacccag	55	241-251	4	0.15
BFRO006	II	F gcctggttttacccttaga R aggcattttacactggcatt	55	136-152	8	0.21
Str85INRA	II	F ggaaggaagggagaaaggt R ggaaaatcaatactaacaa	53	173-181	4	0.41
Ocl8	III	F tagtgttccgtgttcgcctg R caccttccatctctcattccac	54	97-127	14	0.49
One9	III	F ctctctttggctcggggaatgtt R cctacagctatgcattattcaca*	54	182-202	9	0.46
Ogo2	III	F acatcgcacaccataagcat R gtttcttcgactgtttcctctgtgttgag	60	204-258	23	0.71
Str73INRA	III	F cctggagatcctccagcagga R ctattctgcttgtaactagaccta	53	132-162	10	0.55
F43	IV	F agcggcataacgtgctgtgt R gagtcactcaaagtgaggcc	55	118-152	11	0.74
BFRO011	IV	F tgggatgtaacaaccagagc R atgcgtaataatgtcgacag	55	223-257	14	0.50
Sfo8	IV	F caacgagcacagaacagg R cttcccctggagaggaaa	53	194-208	4	0.42
Cocl23	V	F gtttgctgtatgaggatagca R gcattaggtcgttttgtgt	50	237-307	16	0.49
Ssa289	V	F ctttacaaatagacagact R tcatacagtcactatcatc	53	107-121	7	0.41
One2	V	F ggtgccaaggttcagtttatgtt R ccaagcaagcacacctccagg*	60	143-211	25	0.78
One8	VI	F aacattctgggatgacaggggta R ctgttctgctccagtgaagtgga	60	188-282	36	0.78
BFRO017	VI	F gcctagtcttggcaagcatc* R ggactaagcaggccactcaa*	60	129-159	13	0.46

* new primer sequences designed in order to optimise size ranges

3.3.3 Data analysis

FSTAT v. 2.9.3 programme package (Goudet, 2001) was used for calculating allele frequencies and for estimating the expected and observed heterozygosities (H_e, H_o), the number of private alleles (A_{pr}) and the allelic richness A_R (the measure of the number of alleles per locus independent of the sample size). FSTAT was used also for testing the significance of differences in average values of A_R, H_e and H_o among the groups of populations (1000 permutations, one-sided test of the null hypothesis of no difference). GENEPOP v. 3.3 (Raymond and Rousset 1995a) was used to test genotypic distributions for conformance to Hardy-Weinberg (HW) expectations, to test the loci for genotypic disequilibria to calculate F_{ST} values and to estimate the significance of genotypic differentiation between population pairs. All probability tests were based on the Markov chain method (Guo & Thompson, 1992; Raymond & Rousset, 1995b) using 1000 de-memorization steps, 100 batches and 1000 iterations per batch. The sequential Bonferroni adjustments (Rice 1989) were applied to correct for the effect of multiple tests. Analysis of molecular variance (AMOVA) incorporated in ARLEQUIN v. 2.00 (Excoffier et al. 1992) was used to partition genetic variance hierarchically between the Danube and Rhine/Main drainage systems (the Elbe drainage was excluded from the analysis because it was only represented by a single population).

The populations were tested for recent reduction of their effective population size using Wilcoxon sign-rank test as implemented in the BOTTLENECK computer programme (Cornuet & Luikart, 1996), assuming the two-phase model of mutation for microsatellite loci with 5% multi-step changes and variance of 12 as suggested by Piry *et al*. (1999).

We also investigated the relatedness of individuals within populations, effective population size (N_e) and gene flow between populations by using methods that are based on coalescent theory. Relatedness between individuals was estimated based on the *F* value from the 2MOD program (Ciofi *et al*., 1999) that provides information regarding the probability that two genes share a common ancestor within a population. In order to estimate *F* values, a Markov Chain Monte Carlo simulation (100,000 iterations) was computed and the first 10% of the output was discarded in order to avoid bias to the starting conditions. Estimates of N_e and migration rates were calculated by the programme MIGRATE (Beerli & Felsenstein, 2001; Beerli 2002) which provides maximum likelihood estimates of the parameters $\Theta = 4N_e\mu$ (where μ is the rate of substitution per generation at a genetic locus or group of loci) and $4Nm$ (where m is the immigration rate). 20 and 15 individuals were selected randomly from each population of the

Danube and Rhine/Maine drainage systems, respectively and the drainages were analysed separately in order to shorten the computation time. Following the author's suggestions (P. Beerli, personal communication) each locus was analysed separately on parallel computers by using a Brownian motion approximation to the stepwise model and 5 replicates for each locus; the MCMC search employed 10 short chains (sampling 1000 gene trees) and three long chains (10000 gene trees), each time ignoring the first 10^4 steps to ensure parameter stability. Assuming microsatellite mutation rates in the range of 10^{-2}-10^{-4} (Weber and Wong 1993; Schug *et al*., 1997) there is a 10-fold difference of N_e for a given mutation rate. Therefore we followed the recommendation of DeWoody & Avise (2000) to use the relative Θ values rather than absolute estimates of N_e.

The Bayesian approach of population assignment test (Cornuet *et al*., 1999) implemented in the GENECLASS v. 1.0.02 program (Piry & Cornuet, 1999) was applied to estimate both the likelihood of an individual's multilocus genotype occurring in a given population and in the drainage from which it was sampled. Self-classification of individuals was performed using the "leave one out" option. Values of Chi-square were generated to further test the significance of the correct assignments by comparing the observed numbers of correct classifications to the numbers of correct classifications that would be expected by chance. The expectations of numbers correctly classified by chance alone were calculated, assuming equal probability of membership in any single population or drainage system.

Genetic distances between populations were estimated using the Cavalli-Sforza & Edwards (1967) chord distance (D_{CE}) as implemented by the MICROSAT program (Minch *et al*., 1996). This distance measure makes no assumption regarding constant population size or mutation rates among loci and is more likely to generate the correct phylogenetic tree topology than other distance measures (Takezaki & Nei, 1996). The resulting distance matrices (1000 bootstrap replicates) were used to construct an UPGMA dendrogram by using PHYLIP ver. 3.6 program package (Felsenstein 2002). According to Nei (1987) UPGMA is useful for allele frequency data when the evolutionary rate is nearly the same for all populations.

Table 3.3: Microsatellite diversity indices of grayling (*Thymallus thymallus*) populations in Bavaria. Sample size (N), average number of alleles/locus (A), mean allelic richness (A_R) per population, number of private alleles (A_{pr}), expected (H_e) and observed (H_o) heterozygosity, result of Hardy-Weinberg probability test for deviation from expected Hardy-Weinberg proportions (P_{HW}), F value based on the 2MOD programme and estimates of effective population sizes expressed as Theta (Θ) value as implemented in MIGRATE (Beerli, 2002).

Population	N	A	A_R	A_{pr}	H_e	H_o	P_{HW}	F	Θ
DANUBE									
Ammer	34	4.9	3.3	1	0.46	0.41	***	0.210	0.545
Dorfen	37	7.0	4.2	1	0.58	0.53	n.s.	0.047	0.580
Inn	11	4.5	3.8	2	0.47	0.43	n.s.	0.122	-
Inn/Gars	39	5.3	3.3	2	0.44	0.38	***	0.191	0.471
Lech	20	5.1	3.7	0	0.48	0.47	n.s.	0.119	0.512
Ramsach	39	3.9	2.7	1	0.38	0.36	n.s.	0.338	0.359
S. Regen	32	7.2	4.6	6	0.63	0.62	n.s.	0.086	0.628
Sempt	39	5.1	3.7	2	0.57	0.54	n.s.	0.156	0.529
Ti. Ache	39	6.7	4.2	6	0.59	0.60	n.s.	0.075	0.484
Average	*32.2*	*5.5*	*3.7* [a]	*2.3*	*0.51* [a]	*0.48* [a]	-	*0.149*	*0.514*
RHINE/MAIN									
Erf	15	4.2	3.4	0	0.52	0.43	***	0.225	0.602
Fr. Saale	15	3.0	2.7	3	0.47	0.43	n.s.	0.445	0.493
Leinleiter	32	4.1	2.5	3	0.28	0.22	***	0.361	0.330
Sinn	39	4.7	3.1	6	0.44	0.44	n.s.	0.293	0.425
Bodensee	39	3.0	2.3	2	0.32	0.30	**	0.531	0.287
Average	*28*	*3.8*	*2.8* [b]	*2.8*	*0.41* [b]	*0.36* [b]	-	*0.371*	*0.427*
ELBE									
Eger	39	3.2	2.4	9	0.34	0.30	***	0.544	-
Average total	*31.3*	*4.8*	*3.3*	*2.9*	*0.46*	*0.43*	-	*0.250*	-

[a,b] different letters in superscript indicate that a corresponding average parameter value for one drainage is significantly greater ($p < 0.05$) than for another; ** $p < 0.01$; *** $p < 0.001$; n.s., not significant ($p \geq 0.05$)

3.4 Results

3.4.1 Linkage and Hardy-Weinberg equilibrium tests

Linkage disequilibrium was negligible for most samples: one to two pairs of loci out of 190 tests per population (generally not the same loci involved) were in linkage disequilibrium after applying Bonferroni correction for multiple tests. However, the populations Ti. Ache and Dorfen from the Danube drainage and Leinleiter from the Main drainage showed 25, 11 and 8 pairs of loci in linkage disequilibrium, respectively. Six populations displayed significant deviations from the expected HW proportions (deficit of heterozygotes) after applying sequential Bonferroni correction: two populations from the Danube drainage (Ammer and Inn/Gars), three populations from the Main/Rhine drainage (Erf, Fr. Saale and Bodensee) and the Eger population from the Elbe drainage (Table 3.3). Test results for the individual loci are provided as an electronic appendix (supplementary material).

3.4.2 Genetic diversity between and within the drainages

A total of 248 alleles were observed across the 20 microsatellite loci with an average of 12.4 alleles per locus ranging from three alleles at SSOSL311 and BFRO007 to 36 alleles at One8 (Table 3.2). The variation, expressed as the mean allelic richness and expected heterozygosity, was significantly higher ($p < 0.05$) in the Danubian drainage (average A_R = 3.7 and H_e = 0.51) than in the Rhine/Main drainage (average A_R = 2.8 and H_e = 0.41) and the Elbe/Eger population (A_R = 2.4 and H_e = 0.34) (Table 3.3). Within drainages the level of genetic diversity varied relatively strongly depending on the status of the populations (Table 3.3). For example, within the Danube drainage the lowest value of variation was observed in the native Ramsach population (H_e = 0.38), whereas the highest variability was observed in the stocked S. Regen and Dorfen populations (H_e = 0.63 and H_e = 0.59, respectively).

A total of 44 private alleles were detected with 21, 14 and 9 alleles confined to the Danube, Rhine/Main and Elbe drainages, respectively. Within the Danube drainage, the highest number of private alleles was found in the S. Regen and Ti. Ache populations and within the Rhine/Main drainage in the Sinn population (Table 3.3). The Wilcoxon's sign-rank test implemented in the BOTTLENECK program (Cornuet & Luikart, 1996) did not detect significant excess of heterozygosity in the studied populations (except for the Fr. Saale population).

The proportion of common ancestors within a population as inferred from the F values of the 2MOD program was negatively correlated with the genetic diversity parameters A_R and H_e (Spearman rank correlation R = – 0.95 and – 0.82, respectively, $p < 0.001$) and was generally high in native populations such as Ramsach ($F = 0.338$), Fr. Saale ($F = 0.445$), or Eger ($F = 0.544$). The second highest F value was observed for the hatchery strain Bodensee ($F = 0.531$). Within the Danubian group, populations that were supplemented by supportive breeding and, in particular, strains that also were stocked with fish originating from different tributaries showed lower F values (Table 3.3).

The estimates of *Theta* that describe the relative N_e within the drainages (Table 3.3) correlated positively with the genetic diversity parameters A_R and H_e in both drainages (Spearman rank correlation R = 0.69, $p = 0.058$ in the Danube drainage and R = 0.90, $p < 0.05$ in the Rhine/Main drainage).

3.4.3 Genetic differentiation and relationship of populations

The differences in genotype frequencies were highly significant ($p < 0.001$) for most pairwise comparisons of populations except between the Inn and Inn/Gars and between Inn and Lech populations within the Danube drainage. An allele frequency table for all loci and populations is provided as an electronic appendix (supplementary material).

The overall level of genetic differentiation between Bavarian grayling populations was high ($F_{ST} = 0.394$). The Eger population from the Elbe drainage was the most distinct from all other populations (Table 3.4) and its level of differentiation from both the Rhine/Main and the Danube drainage populations was similar (average $F_{ST} = 0.504$ and 0.497, respectively). However, the level of differentiation between the Rhine/Main and the Danube drainages was also high (average $F_{ST} = 0.369$) and the AMOVA analysis of hierarchical gene diversity revealed that 25.7% of the total genetic variation was accounted for by differences between the drainage systems, 10.5% was due to differences between populations within the drainages and 63.9% resided within populations. Within the Rhine/Main drainage, a much greater percentage of variation (26.8%) was attributable to differences between populations than within the Danube drainage (9.0%), indicating that the populations within the Rhine/Maine drainage are more differentiated than the populations within the Danube drainage. However, despite of the low differentiation among the Alpine populations (southern tributaries) of the Danube, they were significantly differentiated (average $F_{ST} = 0.170$, Table 3.4) from the S. Regen population (northern tributary).

The UPGMA phenogram depicting the underlying structure of the D_{CE} chord distance matrix clearly illustrates the high degree of genetic differentiation between the three drainages (Fig. 3.2). The populations clustered with high bootstrap support (98-100%) into three main branches: the Danube, the Rhine/Main and the Elbe (Fig. 3.2). Grayling populations originating from the southern tributaries of the Danube clustered together and formed a closely related Alpine subgroup, whereas the northern tributary (S. Regen) formed a separate, strongly supported branch in the Danubian cluster. Similarly, the northern tributaries of the Main (Sinn and Fr. Saale) formed a separate, well-supported subgroup (bootstrap support 83%) within the Rhine/Main cluster (Fig. 3.2).

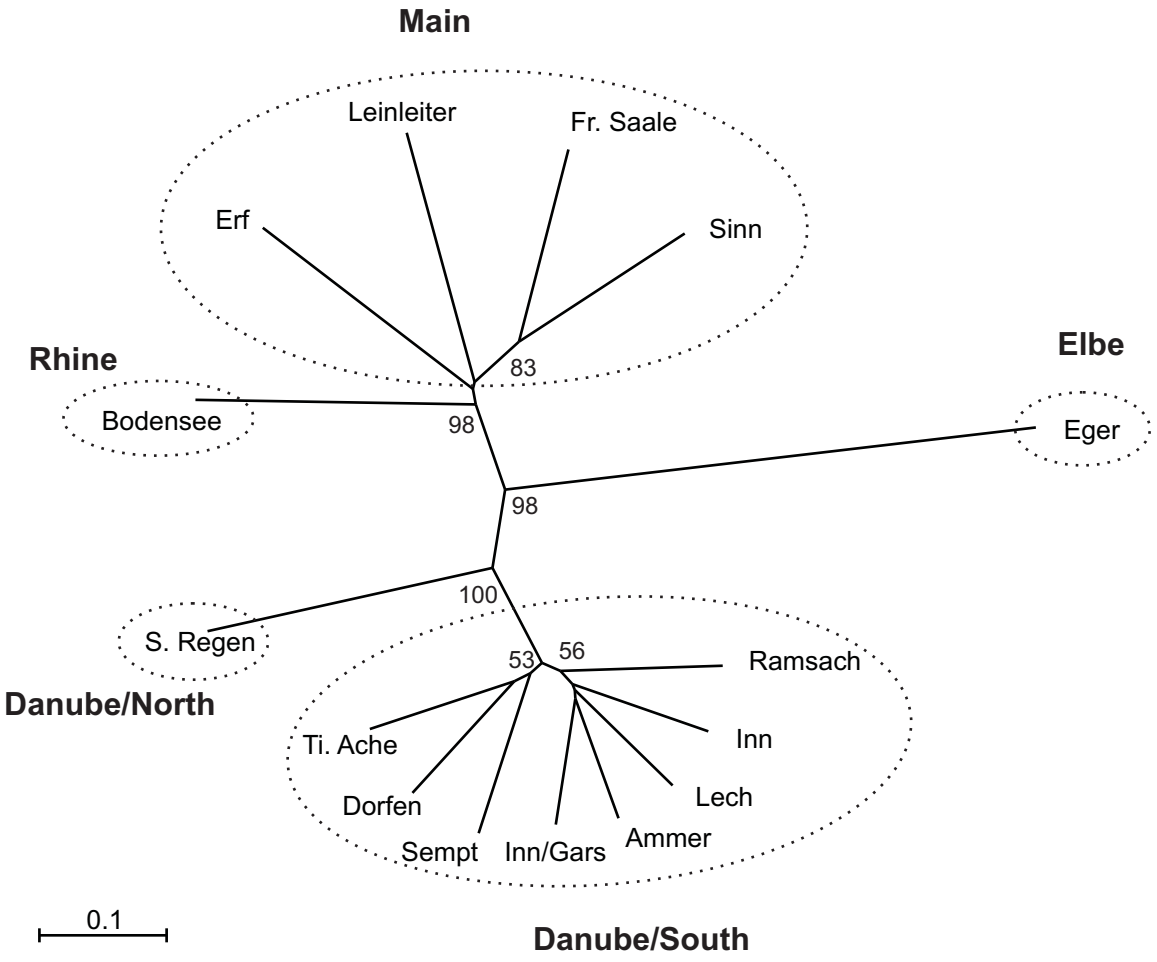

Fig. 3.2: UPGMA dendrogram based on Cavalli-Sforza & Edwards (1967) chord distance for grayling (*Thymallus thymallus*) sampled from the Danube, Rhine/Main and Elbe drainages in Bavaria (river systems indicated by dotted circles). Numbers indicate nodes with bootstrap support above 50% in 1000 replications.

Table 3.4: Pairwise estimates of F_{ST} between populations of European grayling (*Thymallus thymallus*) in Bavaria.

Population	*DANUBE*									*RHINE/MAIN*					*ELBE*
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1 Ammer	-														
2 Dorfen	0.041***	-													
3 Inn	0.011	0.025*	-												
4 Inn/Gars	0.018***	0.060***	0.015	-											
5 Lech	0.023**	0.013	0.010	0.015*	-										
6 Ramsach	0.064***	0.081***	0.069***	0.054***	0.033***	-									
7 S. Regen	0.194***	0.097***	0.168***	0.218***	0.161***	0.252***	-								
8 Sempt	0.099***	0.051***	0.090***	0.126***	0.084***	0.158***	0.142***	-							
9 Ti. Ache	0.038***	0.009*	0.029**	0.049***	0.009	0.079***	0.129***	0.062***	-						
10 Erf	0.287***	0.170***	0.266***	0.303***	0.248***	0.342***	0.173***	0.175***	0.171***	-					
11 Fr. Saale	0.441***	0.287***	0.420***	0.447***	0.409***	0.496***	0.301***	0.303***	0.225***	0.173***	-				
12 Leinleiter	0.515***	0.359***	0.535***	0.519***	0.505***	0.563***	0.373***	0.372***	0.345***	0.307***	0.298***	-			
13 Sinn	0.394***	0.260***	0.382***	0.408***	0.354***	0.439***	0.273***	0.276***	0.232***	0.112***	0.127***	0.243***	-		
14 Bodensee	0.509***	0.356***	0.532***	0.518***	0.490***	0.567***	0.350***	0.378***	0.359***	0.289***	0.339***	0.401***	0.284***	-	
15 Eger	0.557***	0.436***	0.572***	0.552***	0.555***	0.592***	0.451***	0.457***	0.301***	0.453***	0.445***	0.583***	0.462***	0.579***	-

* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

Table 3.5: Results of assignment test for European grayling (*Thymallus thymallus*) populations in Bavaria based on the Bayesian method ('leave one out' option).

Population	*DANUBE*									*RHINE/MAIN*					*ELBE*	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	all
1 Ammer	21	1	1	6	4	-	-	-	2	-	-	-	-	-	-	35
2 Dorfen	2	19	1	-	3	-	1	2	5	-	-	-	-	-	-	33
3 Inn	4	2	-	2	1	-	-	-	3	-	-	-	-	-	-	12
4 Inn/Gars	3	5	6	27	1	-	-	-	5	-	-	-	-	-	-	47
5 Lech	2	4	1	3	4	-	1	-	-	-	-	-	-	-	-	15
6 Ramsach	-	-	2	-	4	38	-	-	4	-	-	-	-	-	-	48
7 S. Regen	-	2	-	-	-	-	30	-	-	-	-	3	-	-	-	35
8 Sempt	-	-	-	-	-	-	-	36	-	-	-	-	-	-	-	36
9 Ti. Ache	2	2	-	1	3	1	-	1	20	-	-	-	-	-	-	30
10 Erf	-	-	-	-	-	-	-	-	-	14	-	-	-	-	-	14
11 Fr. Saale	-	-	-	-	-	-	-	-	-	-	15	-	-	-	-	15
12 Leinleiter	-	1	-	-	-	-	-	-	-	-	-	29	-	-	-	30
13 Sinn	-	-	-	-	-	-	-	-	-	-	-	-	39	-	-	39
14 Bodensee	-	1	-	-	-	-	-	-	-	1	-	-	-	39	-	41
15 Eger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	39	39
Sample size	34	37	11	39	20	39	32	39	39	15	15	32	39	39	39	469
no. correctly classified	21	19	0	27	4	38	30	36	20	14	15	29	39	39	39	370
% correctly classified	61.8	51.4	0	69.2	20	97.4	93.8	92.3	51.3	93.3	100	90.6	100	100	100	78.9
classified by chance	2.3	2.5	0.7	2.6	1.3	2.6	2.1	2.6	2.6	1.0	1.0	2.1	2.6	2.6	2.6	31.3
$p\ \chi^2$	***	***	n.s.	***	n.s.	***	***	***	***	***	***	***	***	***	***	***

*** $p < 0.001$; n.s., not significant

Individual multilocus genotypes were used to assign individuals to their population of origin (Table 3.5). 78.9% of all individuals were assigned correctly to the location from which they were sampled. The proportion of correctly classified individuals was higher in the populations from Rhine/Main drainage (on an average 97%) and in the Eger population from the Elbe drainage (100%) than in the Danubian populations (on an average 67%). Most misclassified fish were found in the less differentiated populations of the southern tributaries of the Danube (Dorfen, Lech, Ti. Ache, Inn) (Table 3.4). No self-classification was observed for the Danubian Inn population. Two individuals from the Danubian Dorfen population were assigned to the Rhine/Main drainage populations (Bodensee and Leinleiter) and three individuals from the Leinleiter population (Main drainage) were assigned to the Danubian S. Regen population (Table 3.5). In a second assignment test (data not shown) we tested the likelihood of each individual's genotype being found in the drainage it was sampled from by pooling populations to groups of drainages. Over all populations, 98.5% of individuals were assigned correctly to their drainage of origin.

Maximum likelihood estimates of the number of effective immigrants per generation (data provided in Table A1; supplementary material) were less than one for all pairwise comparisons of populations within the drainages, suggesting that the equilibrium between the random gene drift and gene flow in studied populations is shifted towards the drift (Wright 1969).

3.5 Discussion

3.5.1 Genetic diversity between and within drainages

Our results indicate that the Bavarian grayling populations have a relatively high level of overall microsatellite diversity with an average of 12.4 alleles per locus and a mean H_e = 0.48 across all populations. However, a substantial proportion (39.4%) of the total genetic variation resided among the populations whereas intra-population diversity was much lower (mean A = 4.8, mean A_R = 3.3 and mean H_e = 0.46). This is consistent with the results of Koskinen's *et al.* (2002a) study on microsatellite variation in grayling populations across Europe (A = 3.5, H_e = 0.41) and can generally be explained by the life history characteristics of this species. Radiotelemetry studies and mark-recapture experiments have demonstrated the strong homing and poor dispersal behaviour of grayling (spawning migrations range from10 to 15 km and out of the spawning season dispersal about 1 km) and the existence of family cohorts (Northcote, 1995; Baars *et al*., 2001). Limited inter-population dispersal of grayling was also

suggested by a microgeographic genetic study of Koskinen *et al.* (2001). Dominant grayling males protect the best spawning grounds and can participate in multiple spawnings with several females (Baars *et al*., 2001). These biological characteristics of grayling lead to a population sub-structure and are likely to have caused the relatively low intra-population genetic variation evidenced in our study.

The levels of genetic diversity in northern Bavarian grayling populations (i.e. within the Rhine/Main drainage and in the Eger population from the Elbe drainage) were significantly lower than those in southern Bavarian populations of the Danube drainage. This is in agreement with the general observation that southern refugial populations display higher levels of variability than the populations in northern recolonised areas which have been affected by bottleneck and founder effects (reviewed in Bernatchez & Wilson, 1998). Koskinen *et al.* (2002a) did not find any significant correlation between the allele numbers and the latitudes of grayling populations in their study; however, their samples included only two populations from the Danube drainage.

As all Danube drainage populations which have been supplemented with hatchery-reared fish exhibit higher values of Θ, A_R and H_e than the native Ramsach population (which has never been stocked), our results are consistent with Wang & Ryman's (2001) conclusions regarding the inference that multiple generations of supportive breeding can increase N_e. The population S. Regen is the Danube population with the highest Θ, A_R and H_e values. The higher level of genetic variability may be explained by the combined influences of supportive breeding and multiple postglacial recolonisations. The Leinleiter population (a tributary of the Main) and the Bodensee hatchery strain displayed the lowest genetic diversity and effective population size. The Leinleiter population was re-established about 30 years ago with a few spawners from the nearby Wiesent river. The number of individuals that founded the Bodensee hatchery broodstock is unknown but it is safe to assume that the low diversity of this strain has also resulted from founder effects as reflected by the second largest coancestry value (F value) among all studied populations.

Substantial linkage disequilibrium was found in three populations (Ti. Ache, Dorfen, Leinleiter). This may be an artefact due to the presence of subgroups within these samples (see Ohta 1982) caused by fish stocking from different tributaries and/or sampling collections of different year classes (Leinleiter). The deviations from the HW equilibrium observed in Ammer, Inn/Gars and Leinleiter might have resulted from a Wahlund effect also caused by genetic heterogeneity between subgroups.

3.5.2 Genetic differentiation between drainages

Genetic divergence between the three drainage systems in Bavaria (29.1% of the total genetic variation) was substantial, and was illustrated by: highly significant heterogeneity of genotype frequencies, a high number of drainage-specific private alleles, high inter-drainage F_{ST} values, a high assignment success of individuals to their drainage of origin and a high bootstrap support for the drainage-specific population clusters based on genetic distance. This high divergence implies a long period of reproductive isolation between grayling populations of the Danube, the Rhine/Main and the Elbe drainages despite their geographical proximity and is generally consistent with the results of mtDNA studies by Gross *et al.* (2001) and Weiss *et al.* (2002). Our microsatellite data, however, indicate that the populations of the Rhine/Main drainage are more closely related to the upper Danube than to the Eger population of the Elbe drainage. This finding contradicts the results of Gross *et al.* (2001), which suggest an early split between the Danubian and the Main-Elbe mitochondrial lineages and a more recent isolation of the Main and the Elbe. While their sampling of the present study area was rather limited (four populations) a closer relationship between Rhine, Rhone and Elbe basin mtDNA haplotypes compared to most of the Danubian haplotypes was also evident from the entire control region sequence data set of Weiss *et al.* (2002). Two Danubian haplotypes formed a clade together with several Swiss Rhenian haplotypes, suggesting some historical gene flow between the upper Rhine and the Danube. This linkage is supported by geological data according to which major connections between upper Danube and upper Rhine have existed until the Riss/Saalian glaciation period (150,000 - 300,000 years BP; Hantke 1993). Similarly, mitochondrial (Englbrecht *et al*., 2000) and microsatellite analysis (Hänfling *et al*., 2002) of bullhead (*Cottus gobio*) also revealed a gene flow between the upper Rhine and the upper Danube but no shared haplotypes between the populations of the Main, the Elbe and the upper Danube. In comparison to bullhead and grayling (which share similar reproduction and limited dispersal behaviour), mitochondrial studies of more mobile species such as chub (*Leuciscus cephalus*) or perch (*Perca fluviatilis*) indicated a more recent postglacial divergence and a lower level of differentiation among the studied drainages (Hänfling & Brandl, 1998; Nesbø *et al*., 1999).

As demonstrated by the S. Regen population, which forms a distinct branch within the Danubian drainage, the genetic differentiation between the northern and the southern tributaries within this drainage is substantial. Within the Rhine/Main group, the Bodensee hatchery strain, originating from a tributary to Lake Constance, was clearly distinct from the

Main River tributaries. Considering F_{ST}, the high number of private alleles distributed among populations across all drainages and the results of the assignment tests, gene flow between populations within drainages appears to be negligible.
However, based on the assignment tests a relatively high proportion of misclassified individuals were observed among Alpine tributaries of the Danube, when each population was tested separately. This could be caused by the colonisation of these river systems following the latest glaciation (10,000 years BP; Hantke, 1993) and/or by stocking rivers such as Lech, Dorfen and Ti. Ache with grayling from several tributaries of the Danube.

3.5.3 Conservation and management implications

One major intention of this study was to identify conservation and management units (MUs) for the development of appropriate conservation guidelines for *T. thymallus* in Bavaria.
Our microsatellite and published mitochondrial DNA data clearly illustrate that grayling populations from three major drainages in Bavaria have been separated for a long time with no gene flow. In addition to the high genetic divergence, the specimens of the Danube, Rhine/Main and Elbe drainages exhibit variations in quantitative characteristics such as body length/height ratio and growth rates (Baars *et al*., 2001). Given that there is also a significant variability in water chemistry (especially lime content) between these drainages (Hantke, 1993), adaptive differences due to different natural selection pressures are to be expected. In conclusion, the Danube, Rhine/Main, and Elbe drainages should be regarded as distinct ESUs, and thus be managed separately.
Additionally, based on the substantial genetic differentiation between the northern and southern tributaries of the Danube drainage as well as due to the habitat variability (i.e. rivers with a high lime content in the south versus primeval rock rivers in the north), these groupings of tributaries should be considered as separate management units. The same applies to the Rhine/Main-ESU, within which the upper Rhine (high in lime content) and the Main (primeval rock rivers) should be regarded as separate MUs.

In order to ensure long-term survival of small and isolated grayling populations, it is important to guarantee a natural level of genetic variation. In this respect, the natural exchange of individuals and gene pools of closely related subpopulations should be supported (e.g. by construction of fish passes). However, if stocking is necessary, translocation of grayling between ESUs should be avoided. Restoration programmes should focus on using

natural stocks of the same MU rather than hatchery-bred stocks, because captive breeding will lead to an adaptation to the ecological conditions of captivity (Vrijenhoek, 1994). A strategy of river-specific supportive breeding will maintain the historically established genetic diversity and integrity of the grayling populations.

3.6 Supplementary material

The following material is available from [http://www.wzw.tum.de/wildbio/gum.htm] under 'Veröffentlichungen'.

Appendix. Genotypic table and allele frequencies of 15 grayling (*Thymallus thymallus*) populations at 20 microsatellite loci. Population names are according to Table 3.1.

Table A1. Maximum likelihood estimates of the number of effective immigrants per generation. Population names are according to Table 3.1.

Simplified DNA isolation procedure according to Laird *et al.* (1991).

The basic method involves three steps:

1. *Lysis*: Addition of 0.5 ml lysis buffer to *c*. 250-300 mg fin-clip sample reduced to small pieces. Digestion is complete within several hours or overnight at 55 °C.
   Lysis buffer: 100 mM Tris-HCL pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg Proteinase K/ml.
   In order to remove tissue residue we performed the recommended centrifugation step (5 min) after complete lysis and transferred the supernatant to a new tube.
2. *Isopropanol precipitation*: One volume of isopropanol is added to the lysate and the samples are mixed or swirled until precipitation is complete (viscosity completely gone).
3. *Recovery of precipitate*: The DNA is recovered by lifting the aggregated precipitate from the solution using a disposable yellow tip. Excess liquid is dabbed off and the DNA is dispersed in an Eppendorf tube containing, depending on the size of the precipitate, 20 to 500 µl of 10 mM Tris-HCl, 0.1 mM EDTA, pH 7,5.

4 Mitochondrial and nuclear DNA phylogeography of European grayling (*Thymallus thymallus*): evidence for secondary contact zones in Central Europe

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4.1 Abstract

Mitochondrial and microsatellite DNA markers were applied to infer the phylogeography, intra-specific diversity and dynamics of the distributional history of European grayling (*Thymallus thymallus*) with the focus on its Central and Northern European distribution range. Phylogenetic and nested clade analyses revealed at least four major mtDNA lineages which evolved in geographic isolation during the Pleistocene. These lineages should be recognised as the basic evolutionary significant units (ESUs) for grayling in Central and Northern Europe. In addition, and in contrast to previous work on grayling, the results of Bayesian analysis of individual admixture coefficients, two-dimensional scaling analysis and spatial analysis of molecular variance provided evidence for a high level of admixture among major lineages in contact zones between drainages (e.g. the low mountain range of Germany), most likely resulting from glacial perturbations and ancient river connections between drainages during the Pleistocene glaciations. Even within river systems a high level of differentiation among populations was revealed as indicated by the microsatellite data. Grayling sampled from 29 sites displayed high levels of differentiation (overall F_{ST} = 0.367), a high number of private alleles and high bootstrap support for the genetic distance-based population clusters across 12 loci. We specifically discuss our results in context of phylogeograpic studies on other European freshwater fish species with habitat preferences similar to those of grayling. Our study shows that both large-scale phylogeographical and detailed genetic analyses on a fine scale are mandatory for developing appropriate conservation guidelines of endangered species.

4.2 Introduction

Climatic fluctuations, geological shifts of rock masses or reversals of river flows drastically affected the natural range and habitat of terrestrial and aquatic animal species during the Quaternary cold periods (Hewitt, 1999, 2000). In Europe, these events left their genomic signatures across both animal and plant taxa, and Northern regions were generally colonised from four major temperate refugia located at the Southern peninsulas of Iberia, Italy and the Balkan-Greece as well as the Ponto-Caspian region (Taberlet *et al.*, 1998; Hewitt, 1999; Kotlík *et al.*, 2004). However, comparisons between case studies also reveal fundamental differences in phylogeographical patterns among regional fauna (Taberlet *et al.*, 1998; Bernatchez & Wilson 1998). In particular the extent and the locations of secondary contact and hybrid-zones of major mtDNA lineages vary greatly among species from formerly glaciated regions (Hewitt, 2001). These hybrid zones are often clustered into suture zones along the Alps, down through Central Europe and across Scandinavia where many expanding genomes met (Hewitt 2004). For any species of interest this requires both large and small scale phylogeograpic data from areas with potentially unique evolutionary histories. Detailed biogeographic information enables inference of the evolutionary relationships between populations and is a pre-requisite for developing appropriate conservation strategies of endangered species (Bernatchez & Wilson, 1998; Avise, 2000).

In the last years, an increasing number of researchers have investigated the phylogeography of European freshwater fishes using mitochondrial DNA (mtDNA) (Durand *et al.*, 1999; Nesbø *et al.*, 1999; Englbrecht *et al.*, 2000; Kotlík & Berrebi, 2001; Bernatchez *et al.*, 2001; Salzburger *et al.*, 2003). In the case of the European grayling (*Thymallus thymallus*), a salmonid species which is distributed over a large part of the European continent, Koskinen *et al.* (2000) identified three major mtDNA lineages for Northern and Central Europe. Weiss *et al.* (2002) revealed complex patterns of colonisation and several potential glacial refugia, mainly from sampling sites in the species' southwestern and southern distribution range in France, the southern Alpine region, in Italy and Slovenia. Unfortunately, the number of sampling sites in the Central European area was either low or absent in these studies leaving a poor understanding of: (i) the phylogeography of *Thymallus thymallus* in Central European drainage systems of the Danube, Rhine/Main and Elbe and (ii) the areas of potential contact zones between different mtDNA lineages, e.g. the northern range of the Alps or along the corridor from Central Sweden and Norway to Southern Germany (Hewitt, 2001; Nesbø *et al.*, 1999; Englbrecht *et al.*, 2000). Microsatellite studies of the genetic structure and diversity of

grayling populations revealed phylogeographical patterns consistent with the mitochondrial DNA. In addition, a low level of intra-population diversity and substantial divergence between populations, even between hydrologically connected sampling locations, was identified (Gross *et al.*, 2001; Koskinen *et al.*, 2002a; Gum *et al.*, 2003).

The European grayling is classified as critically endangered in the entire Alpine area and endangered at the population level throughout its European distribution range due to deterministic factors such as pollution, habitat destruction, river engineering, and local predation by piscivorous birds (updated Red List of Bavaria, see Bohl *et al.*, 2003; Gum *et al.*, 2003 and references therein). Conservation guidelines for recovery projects currently focus on a strategy of drainage-specific management and supportive breeding for stocking or restoration actions in several European countries (Koskinen *et al.*, 2000; Weiss *et al.*, 2002; Gum *et al*., 2003). For the concepts of evolutionarily significant units (ESUs) (Moritz, 1994; Waples, 1995) and functionally significant units (FSUs) (van Tienderen *et al*., 2002) detailed genetic and ecological data (e.g. environmental differences in water chemistry or temperature) are mandatory for establishing appropriate management plans.

This study extends earlier analyses of European grayling phylogeography in order to better understand the evolutionary history of the species. Samples were taken from all over Europe with a focus on potential contact zones of different mitochondrial lineages in Central Germany and adjacent areas where the major European drainages of Danube, Rhine/Main and Elbe are close to each other. Specifically, the goals of the study were i) to further clarify the colonisation history of the species after glacial perturbations, ii) to identify potential zones of secondary contact between divergent mtDNA lineages in Central and Northern Europe and iii) to acquire additional data for delineating of appropriate conservation units within and between drainage systems. We utilized both mtDNA and microsatellite variation to study geographical distribution of distinct phylogenetic lineages and combined our mtDNA data with those of Koskinen *et al.* (2000) in order to obtain a broader coverage of the distribution area. This allowed us to resolve the genetic signatures both of long term isolation in distinct refugia, and postglacial expansion into previously glaciated areas at a fine scale.

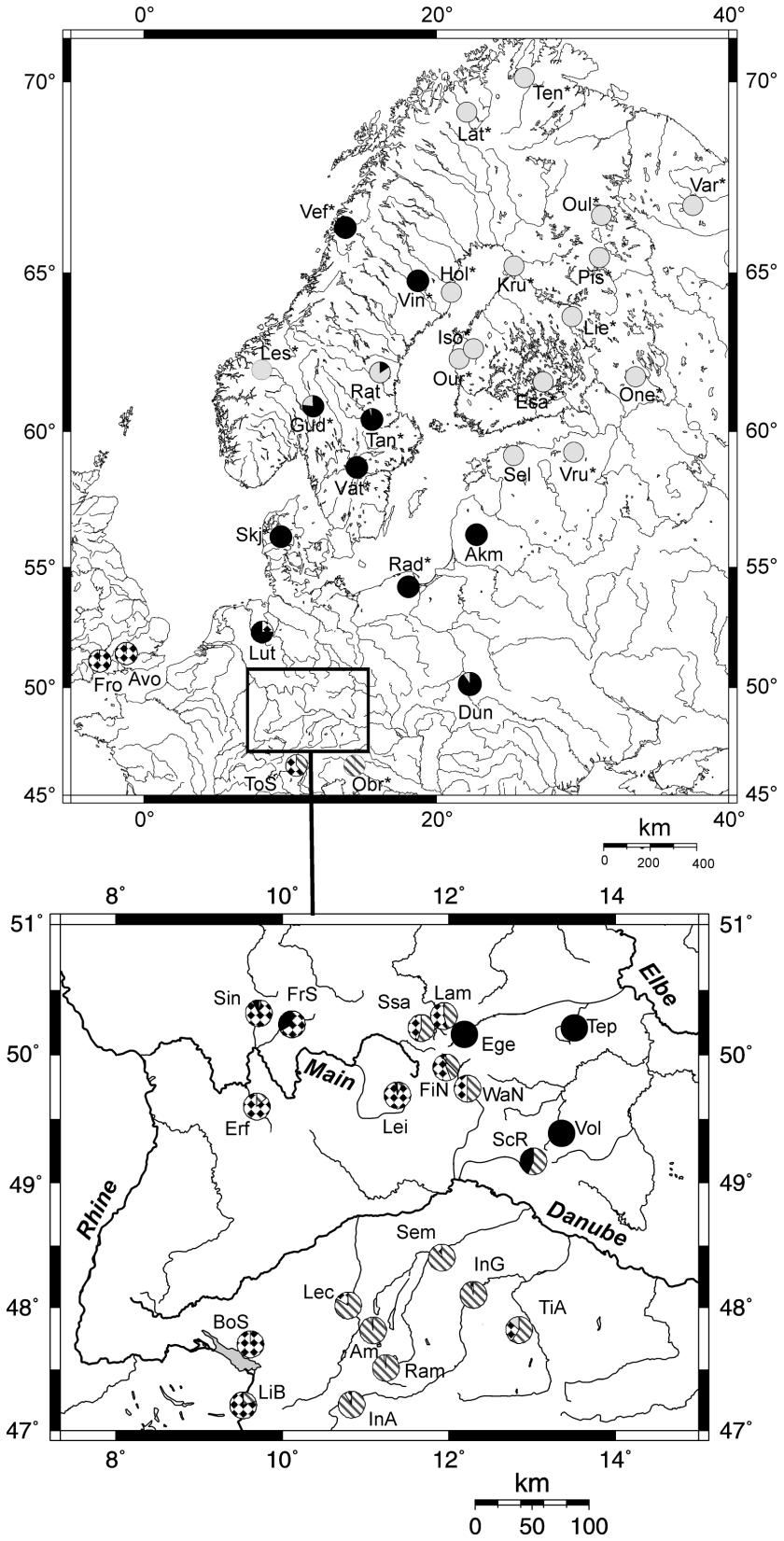

Fig. 4.1: Map of sampling locations with pie diagrams showing the distribution of mtDNA lineage frequencies among studied *Thymallus thymallus* populations. Population codes according to Table 4.1. Codes with * symbol refer to populations analysed by Koskinen *et al.* (2000). For designation of lineages see Fig. 4.2.

Lineage I ○, lineage IIa ⚽, lineage IIb ●, lineage III ◍.

4.3 Materials and methods

4.3.1 Sample collection and DNA isolation

A total of 786 individuals of grayling (*Thymallus thymallus*), originating from 29 locations across Central and Northern Europe were caught from the wild primarily by electro-fishing between 1999 to 2002, or were provided by colleagues as small fin clips preserved in 96% alcohol. (Table 4.1, Fig. 4.1). In addition, one population sample of *Thymallus arcticus*, originating from Alaska, USA, was obtained. The majority of the samples included in this study were wild-caught specimens, the exceptions being the German Bodensee/Lake Constance (BoS) population (hatchery stock produced from locally caught founders) and the Polish Dunajec (Dun) population (hatchery stock produced from spawners from the river Dunajec). Most sampling sites were selected from areas considered to be unaffected by stocking of grayling (Baars, 2000). For populations InA, TiA, Am, Sem, Lec, ScR and Lei, supportive breeding has been practiced by using spawners of local origin (Bavarian Fisheries Association, pers. communication). Total genomic DNA was isolated according to the simplified method of Laird *et al.* (1991).

4.3.2 Mitochondrial DNA analysis

Restriction fragment length polymorphism (RFLP) analysis was performed on two polymerase chain reaction (PCR) amplified segments encompassing the NADH-1 (ND-1) and NADH-5,6 dehydrogenase (ND-5/6) gene regions (primer sequences were obtained from Nielsen *et al.*, 1998). The sizes of the PCR products for these regions are 2013 and 2474 base pairs (bp), respectively. The ND-1 and ND-5/6 mtDNA regions were selected, because they allowed in previous studies to reveal the major evolutionary lineages of grayling in Europe (Koskinen *et al.*, 2000; Gross *et al.*, 2001). PCR reactions (total of 50 µl) were composed as described by Gross *et al.* (2001). The PCR amplification consisted of an initial denaturing at 94 °C for 3 min followed by 35 cycles of denaturing at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min 20 s, followed by a final extension at 72 °C for 5 min. The PCR products were digested with restriction enzymes (MBI Fermentas): *Bsu*RI, *Mva*I, *Hin*fI, *Mbo*I, *Bcn*I, and *Rsa*I for ND-1, and *Bsu*RI, *Sty*I, *Hin*fI, *Alu*I, *Apa*I, *Rsa*I, *Alw*I for ND-5/6, respectively. Fragments were resolved on 2% agarose or 6% polyacrylamide gels. The sizes of the restriction fragments were estimated by comparison to a 100 bp size ladder (MBI Fermentas). PCR-RFLP generated fragment profiles were classified by letters which were then combined to define composite mtDNA haplotype patterns (Table 4.2).

We sequenced the 5´end of the ND-5 gene (529 bp) for each of the 31 composite RFLP haplotypes that we found. Additional individuals were sequenced which showed the same composite RFLP haplotype but originated from different river drainages. The sequence analysis was conducted to examine the phylogenetic relationships between *T. thymallus* haplotypes originating from major Central European river drainages and to enable direct combination with pre-existing ND-5 sequence data for grayling in Northern Europe (Koskinen *et al.*, 2000). ND-5/6 PCR products were purified using a NucleoSpin Extract Kit (Macherey-Nagel) and both strands were cycle-sequenced using Amersham Pharmacia Dye Primer kit with Cy 5.0 labeled 5'-tail. The reverse strand was sequenced by an internal primer designed for this study (5'-TGAGGAGGCGAATATTTGTTG-3'). Sequencing reactions consisted of a 1-min predenaturation at 95 °C and 29 cycles of 95 °C for 30 s, 50 °C for 30 s and 72 °C for 45 s. Sequences were analyzed using an ALF Express II DNA analyser and ALFwin Sequence Analyser version 2.10 (Amersham Pharmacia) software. Sequences included in this paper have been deposited in GenBank under Accession Numbers AY527277- AY527291.

In order to link our mtDNA data set to the Central/Southern European mitochondrial lineages defined based on control region sequence data in Weiss *et al.* (2002), we additionally sequenced the complete mitochondrial control region (1043 bp) and flanking tRNA genes in six individuals representing the four major European mtDNA lineages. For amplification and sequencing of the control region we followed the protocols given by Weiss *et al*. (2002). Sequences have been deposited in GenBank under Accession Numbers AY841355-841360.

4.3.3 Microsatellite DNA analysis

A total of 12 microsatellite loci were analyzed. Seven of the microsatellites (BFRO004, BFRO005, BFRO006, BFRO007, BFRO009, BFRO010, BFRO011) were previously isolated from *T. thymallus* and reported to consist of dinucleotide repeat sequences. The five additional loci F43, Ocl8, Ogo2, One9 and SSOSL311 were derived from other salmonids and are known to cross-amplify polymorphic loci in grayling. Original references for the microsatellites and details of PCR and genotyping procedures are outlined in Gum *et al.* (2003).

Table 4.1: Sampling locations, codes, major river drainage, region, geographical coordinates, sample sizes (*n*), haplotype and nucleotide diversity of European grayling mtDNA. Microsatellite diversity indices of grayling populations. Sample size (*n*), average number of alleles/locus (*A*), mean allelic richness (A_R) per population, number of private alleles (A_{pr}), expected (H_e) and observed (H_o) heterozygosities and the number of putative populations present within each sampling site (*K*).

Population (site)	Code	Drainage/water system	Country of origin	Geographical position	mtDNA *n*	Haplotype diversity	Nucleotide diversity	Microsat. *n*	*A*	A_R	A_{pr}	H_e	H_o	*K*
Inn (Gars)	InG	Inn → Danube	S Germany	48°09' N 12°16' E	50	0.621	0.011	49	5.5	3.3	1	0.42	0.37	1
Inn (Innsbuck)	InA	Inn → Danube	Tyrol, Austria	47°04' N 10°40' E	23	0.244	0.012	23	4.8	3.6	2	0.49	0.55	2
Tittmoninger Ache	TiA	Inn → Danube	S Germany	48°03' N 12°46' E	37	0.855	0.046	39	6.1	4.1	1	0.54	0.54	2
Ammer	Am	Isar → Danube	S Germany	47°52' N 11°09' E	36	0.631	0.009	34	4.2	3.1	1	0.42	0.37	1
Ramsach	Ram	Isar → Danube	S Germany	47°40' N 11°12' E	39	0.498	0.008	39	4.0	2.8	1	0.35	0.34	1
Sempt	Sem	Isar → Danube	S Germany	48°23' N 11°55' E	38	0.657	0.026	39	4.1	3.4	1	0.49	0.48	1
Lech	Lec	Danube	S Germany	47°49' N 10°53' E	18	0.686	0.026	20	4.9	3.5	0	0.43	0.41	1
Schw. Regen	ScR	Danube	S-E Germany	48°58' N 13°07' E	30	0.755	0.078	32	6.8	4.6	4	0.59	0.58	2
Fichtelnaab	FiN	Danube	Central Germany	49°48' N 12°09' E	25	0.621	0.037	25	3.3	2.5	1	0.36	0.33	1
Waldnaab	WaN	Danube	Central Germany	49°47' N 12°01' E	10	0.695	0.046	10	2.4	2.4	0	0.43	0.51	1
Bodensee	BoS	Rhine	S-W Germany	47°34' N 09°36' E	38	0.366	0.007	39	2.4	1.9	0	0.23	0.21	1
Binnenkanal	LiB	Alpine Rhine	Liechtenstein	47°08' N 09°31' E	20	0.656	0.036	19	4.6	3.7	1	0.51	0.55	1
Erf	Erf	Main→ Rhine	Central Germany	49°42' N 09°16' E	15	0.239	0.018	15	4.0	3.5	0	0.51	0.42	1
Fränk. Saale	FrS	Main→ Rhine	Central Germany	50°07' N 09°56' E	15	0.460	0.032	15	2.8	2.6	0	0.46	0.42	1

Tab. 4.1 continued:

Leinleiter	Lei	Main→ Rhine	Central Germany	49°47' N 11°11' E	30	0.188	0.013	31	3.8	2.6	0	0.30	0.24	3
Sinn	Sin	Main→ Rhine	Central Germany	50°09' N 09°38' E	38	0.195	0.012	39	4.2	3.0	1	0.41	0.39	1
Eger	Ege	Elbe	Central Germany	50°07' N 12°00' E	36	0.000	0.000	38	2.7	2.1	3	0.29	0.29	1
Sächs. Saale	SSa	Saale → Elbe	central Germany	50°15' N 11°56' E	20	0.631	0.052	20	3.6	3.0	0	0.49	0.52	1
Lamitz	Lam	Saale → Elbe	Central Germany	50°14' N 12°01' E	13	0.579	0.050	13	3.1	2.9	1	0.45	0.52	1
Tepla	Tep	Elbe	W Czech Republic	50°31' N 13°20' E	19	0.000	0.000	20	2.8	2.4	0	0.32	0.31	1
Volyuka	Vol	Elbe	W Czech Republic	49°16' N 13°37' E	28	0.000	0.000	29	3.4	2.5	0	0.35	0.35	1
Lutter	Lut	Weser	N Germany	52°40' N 10°18' E	8	0.800	0.028	8	2.7	2.7	2	0.42	0.42	1
Toblacher See	ToS	Adige	S Tyrol, Italy	46°42' N 12°13' E	10	0.800	0.052	10	3.9	3.7	1	0.55	0.60	1
Frome	Fro	Atlantic O.	S England	50°42' N 02°21' W	20	0.000	0.000	20	1.8	1.7	0	0.24	0.21	1
Wylye	Avo	Avon	S England	51°04' N 01°51' W	30	0.489	0.007	30	1.6	1.6	0	0.24	0.22	1
Dunajec	Dun	Vistula → Baltic Sea	S Poland	50°01' N 20°59' E	52	0.176	0.014	48	2.9	2.7	2	0.50	0.52	1
Akmena	Akm	Baltic Sea	Lithuania	55°37' N 24°55' E	20	0.000	0.000	20	1.6	1.6	0	0.17	0.18	1
Rätan	Rat	Baltic Sea	Central Sweden	62°27' N 14°32' E	31	0.556	0.034	31	3.1	2.6	5	0.37	0.35	1
Selja	Sel	Baltic Sea	N-E Estonia	59°22' N 26°16' E	31	0.178	0.007	31	1.6	1.3	3	0.10	0.10	1
Featherly Creek*	FeC	Becharof Lake	S Alaska	58°12' N 157°22' W	19	0.000	0.000	-	-	-	-	-	-	-

* *Thy. arcticus*

Table 4.2: Distribution of the mtDNA PCR-RFLP composite haplotypes in 29 *Thymallus thymallus* populations and their correspondence to the ND-5 sequence and haplotype lineage. Population codes are according to Table 4.1.

Haplotype			Composite	Danube											Rhine/Main							Elbe					UK		Baltic Sea				total
ND-1-5/6 RFLP	ND-5 seq	lineage	PCR-RFLP haplotype	Sem	InG	Ram	TiA	Am	Lec	InA	ScR	FiN	WaN	ToS	BoS	LiB	Erf	Sin	FrS	Lei	Lut	Ege	Tep	Vol	Ssa	Lam	Fro	Avo	Dun	Akm	Rat	Sel	
1	Da1	III	dcbaadbdabbha								5																						5
2	Da2	III	caabacddaebca	18	5		4	7	1		6	2		2		1									8	1							55
3	Da2	III	cabbacddaebca		26	24	10	20	7	1		3	5	1		3	2	1							3	5							111
4	Da3	III	cabbacddaebda	13	1	14	2	2						1																			33
5	Da3	III	cbbbacddaebca	3	16		6	6	7	20		6								2													66
6	Da3	III	cabaacddadbca								1																						1
7	Da4	III	cabaacddaebca								5																						5
8	Cw1	IIa	bbbcabicaabba									14	2				13	34															63
9	Cw2	IIa	bbcccbicaabja	2		1	1								29	2																	35
10	Cw2	IIa	bbccabicaabba				2			1						3						1												7
11	Cw2	IIa	bbcccbicaabba							1																								1
12	Cw2	IIa	bbccabibaabba											1																				1
13	Cw3	IIa	bbecbbicaabba																10															10
14	Cw4	IIa	abccbbiiaabba	1	2		2							1																				6
15	Cw5	IIa	abccbbicaabja	1			5								9																			15
16	Cw5	IIa	abccbbicaabba					1	3				2	4		11																		21
17	Cw5	IIa	ebccbbicaabba																									18						18
18	Cw5	IIa	abccbbicaebba										1																					1
19	Cw6	IIa	abccabicaabba																	27	1													28
20	Cw7	IIa	abcdaeicaabba																						9	7								16
21	Cw8	IIa	bbccabiccabba																								20	12						32
22	Ce1	IIb	abbaabcbbdaba								12							3	5	1	1	36	19	28										105
23	Ce1	IIb	bbbaabcbbdaba																		3													3
24	Ce2	IIb	bbbaabcbbhaba																		2													2
25	Ce3	IIb	abbaabcbbdabb																										47	20	5			72
26	Ne2	I	fbbcdcaaaaaab																												19	3		22
27	Ne4	I	gbbcdcbcaabab																													28		28
28	Ne5	I	fbbcdcbaaabab																												7			7
29	Ne6	I	bbbcdcicaabca				5																											5
30	Ne6	I	abbcdcicaabca								1																		5					6

4.3.4 Data analysis

For mtDNA restriction site data, the genetic relationships among haplotypes were analyzed by the distance-based Fitch-Margoliash method (using the average number of nucleotide substitutions per site, d_{ij}, as an estimate of evolutionary distance between the haplotypes) implemented by the program FITCH from the PHYLIP v. 3.57c package (Felsenstein, 1994). Confidence statements on branches were obtained using the SEQBOOT program performed with 1,000 bootstrap replicates. Levels of variation within each population were estimated by calculating the unbiased haplotype diversity (h) and nucleotide diversity (π) (Nei, 1987). Nucleotide-sequence divergence among haplotypes (d_{ij}) and the number of net nucleotide substitutions between all pairs of populations (d_A) were calculated for restriction site data according to Nei (1987) using the software package REAP v. 4.0 (McElroy *et al.*, 1992). The matrix of pairwise d_A values was used to construct a neighbor-joining (N-J) dendrogram showing relationships among populations, using the NEIGHBOR program from the PHYLIP v. 3.57c package (Felsenstein, 1994). Bootstrapping values were computed with the MTDIS program (Danzmann, 1998) using 1,000 randomized data sets produced by SEQBOOT of PYHLIP.

ND-5 sequences were aligned using CLUSTAL_X v. 1.83 (Thompson *et al.*, 1997) and checked visually. To determine the appropriate model of sequence evolution and statistically compare successively nested more parameter-rich models for this data set, the program MODELTEST v. 3.6 (Posada & Crandall, 1998) was used. Analysis were performed with the heuristic search algorithm using the chosen HKY+G model (Hasegawa *et al.*, 1985) to produce a N-J phylogram. To quantify the confidence in the partitioning within the trees we performed a nonparametric bootstrap test using 500 replicates. The phylogenetic analyses were performed using PAUP* v. 4.10b (Swofford, 2003).

To disentangle historical and current impacts on genetic population structure, a nested clade analysis (NCA) was performed on the mtDNA sequence data as proposed by Templeton *et al.* (1995) using GEODIS v. 2.2 (Posada *et al.*, 2000). The cladograms were created by using TCS v. 1.18 software (Clement *et al.*, 2000) with 95% parsimoniously plausible connections between haplotypes (Templeton & Sing, 1993) and nested by hand. A permutational contingency analysis was used to test for significant association between geographical and genetic variation. The null hypothesis is a random geographical distribution of all clades within a nested clade. For interpretation of the GEODIS output, we used the latest Inference Key for the Nested Haplotype Tree Analysis of Geographical Distances (Templeton, 2004).

Dates of evolutionary separation between different mtDNA lineages were estimated using a mtDNA divergence rate of 2% per million years (Myr) based on net genetic distance values (*dc*) to correct within group variation (Avise, 1994). This rate is commonly employed to date divergence times in fish studies (e.g. Bernatchez & Dodson, 1991; Bernatchez *et al.*, 1992).

For nuclear DNA markers, the FSTAT v. 2.9.3 program package (Goudet, 2001) was used to calculate allele frequencies and to estimate the expected and observed heterozygosities (H_e, H_o), the number of private alleles (A_{pr}) and the allelic richness A_R. GENEPOP v. 3.3 (Raymond & Rousset, 1995a) was used to test genotypic distributions for conformance to Hardy-Weinberg (HW) expectations, to test the loci for genotypic disequilibria, to calculate F_{ST} values and to estimate the significance of genotypic differentiation between population pairs. All probability tests were based on the Markov chain method (Guo & Thompson, 1992; Raymond & Rousset, 1995b) using 1,000 de-memorization steps, 100 batches and 1000 iterations per batch. Genetic relationships between populations were studied using the Nei *et al.* (1983) D_A distance that was calculated by the computer program DISPAN (Ota, 1993). This measure was chosen, as it is independent of the mutation models (Nei, 1987) and superior to other distance measures in correct tree topology construction using microsatellites (Takezaki & Nei, 1996). The matrix of D_A-values was used to construct a N-J dendrogram showing relationships among populations, using the PHYLIP v. 3.572c package (Felsenstein, 1994). Bootstrapping (1,000 replications) was performed by the DISPAN program (Ota, 1993). Population Lut was excluded from phylogenetic analysis due to its small sample size of less than 10 individuals.

The presence of intra-population genetic structure was tested using the model-based clustering method of Pritchard *et al.* (2000) implemented in STRUCTURE v. 2.1. This analysis was used to assess if there is indication for the presence of multiple gene pools that can be linked to contemporary phenomena. For each sampling site we evaluated models with *K,* the number of genetically distinct populations, and allowing admixture between populations. For each value of *K*, the MCMC scheme was run with a burn-in period of 50,000 steps and a chain length from 250,000 to 500,000. Multiple runs were performed to assess convergence of $\ln \Pr(X \mid K)$, and the number of populations present was then determined from posterior probabilities of *K* estimated using a uniform prior on $K = \{1,2,3\}$. As recommended by Pritchard et *al.* (2000), a visual inspection of estimated values of *Q* (the membership coefficient for each individual into each population) was performed in all cases, i.e. when estimates of *Q* showed a uniform distribution ($Q \sim 1/K$) it was concluded that a value of $K = 1$ was an appropriate model for the data.

In order to specifically assess the level of admixing between Central European drainages, we additionally calculated individual admixture coefficients for each population from drainages Danube, Rhine/Main and Elbe, assuming a model of $K = 3$. We used a burn-in period of 100,000 steps and then proceeded with 1000,000 MCMC steps. Finally we estimated population level admixture by calculating the mean of the individual admixture coefficients.

To better visualise admixed populations among Central European drainages of the Danube, Rhine/Main and Elbe, we performed a two-dimensional scaling analysis (Kruskal & Wish, 1977) of the matrix of pairwise D_A distances with STATISTICA v. 6.0 (StatSoft Inc.) This analysis permits the genetic relationships among populations to be represented with minimum loss of information and without imposing a bifurcating evolutionary history.

A spatial analysis of molecular variance (SAMOVA, Dupanloup *et al.*, 2002) was used to test for large-scale structuring of the genetic variation without the prior assumption of group composition. The method is based on a simulated annealing procedure that aims to maximize the proportion of total genetic variance due to differences between groups of populations. We initially determined the most likely number of groups (K_{SA}) by repeatedly running the software SAMOVA v. 1.0 (Dupanloup *et al.*, 2002) with two to 10 groups and by choosing those partitions with a maximum F_{CT} value, as suggested by Dupanloup *et al.* (2002). K_{SA} was estimated for i) all populations and ii) Central European drainages with several populations being sampled within each drainage from different areas. We restricted the analysis to mtDNA data only because for microsatellite data the method assumes a strict stepwise mutation model which potentially underlies a strong bias if the number of loci is < 100 (Zhivotovsky *et al.*, 2001). For microsatellite data we performed a hierarchical analysis of molecular variance (AMOVA) (Excoffier *et al.*, 1992) using ARLEQUIN v. 2.0 package (Schneider *et al.*, 2000) by grouping populations according to the results of mtDNA based SAMOVA.

4.4 Results

4.4.1 Mitochondrial DNA diversity and phylogenetic inference

PCR-RFLP analysis of the ND-1 and ND-5/6 mtDNA gene regions of 780 *T. thymallus* individuals originating from 29 distinct European grayling populations revealed a total of 30 composite haplotypes (Table 4.2). One single composite haplotype was revealed for the 19 *T. arcticus* individuals from Alaska. The 13 restriction enzymes resolved a total of 95 restriction sites and 41 variable cut sites in *Thymallus thymallus*. Fragment patterns and approximate sizes of the fragments are available from the corresponding author on request. The highest

number of haplotypes and consequently the highest diversity values were revealed for the Danubian TiA (9), Sem (6) and ScR (6) populations and the Italian ToS (6) population. Populations Ege, Tep and Vol from the Elbe drainage, population Fro from England and population Akm from Lithuania were fixed for distinct haplotypes (Table 4.2). Phylogenetic analysis clustered the mtDNA haplotypes into three distinct groups: lineages I, II and III (Fig. 4.2). Bootstrap support for these three lineages suggested a further division of lineage II into sub-lineages IIa and IIb. The average pairwise RFLP interlineage divergence (d_{ij}) ranged from 4.30% (between lineage I and IIa) to 8.53 % (between lineage IIb and III), whereas average within lineage variation was much lower (ranging from 0.78% to 2.24% for lineages IIb and I, respectively; Table S1 in supplementary material).

Sequencing of 529 bp from the 5'end of the ND-5 gene revealed 19 distinct haplotypes (Table 4.2). Several distinct RFLP composite haplotypes yielded identical sequences for the ND-5 gene (e.g. RFLP haplotypes 15 - 18 all corresponded to the sequence haplotype Cw5; Table 4.2). For phylogenetic analysis we also included six ND-5 sequences previously published by Koskinen *et al.* (2000), and the outgroup sequence of *T. arcticus.* A total of 68 positions were polymorphic of which 63 (11.9%) were found in *T. thymallus* (Table S2, supplementary material). After exclusion of the highly differentiated sequences haplo27a,b found by Koskinen *et al.* (2000) there were 40 (7.6%) variable sites within the *T. thymallus* data set. The transition - transversion ratio was estimated to be 3.5. Figure 3 shows the haplotype network as inferred with the TCS program. The haplotypes in clade 4-1 (Danubian lineage III) were separated by a minimum of 14 steps from clade 4-2 for which parsimony was not supported at the 95% level, demonstrating past fragmentation between the Danubian lineage and clade 4-2 (corresponding to lineages I, II). The network is consistent with the relationships among well supported clades based on the HKY+G distances for ND-5 sequence (phylogram not shown) and PCR-RFLP data (Fig. 4.2). Sequence data also showed a higher interlineage divergence with the average pairwise distances ranging from 0.93% to 3.35%, whereas within lineages, an average divergence of 0.55% was detected in general. The *T. arcticus* haplotype was separated from the *T. thymallus* lineages I, II and III by an overall average pairwise difference of approx. 19% for RFLP and 7.5% for ND-5 sequence data, respectively (Table S1, supplementary material). The very distinct sequence haplotypes 27a,b sampled by Koskinen *et al.* (2000) in Northwestern Russia were more closely related to the *T. arcticus* haplotype and exhibited an average evolutionary divergence from the other *T. thymallus* haplotypes ranging from 6.66% (lineage I) to 7.55% (lineage III).

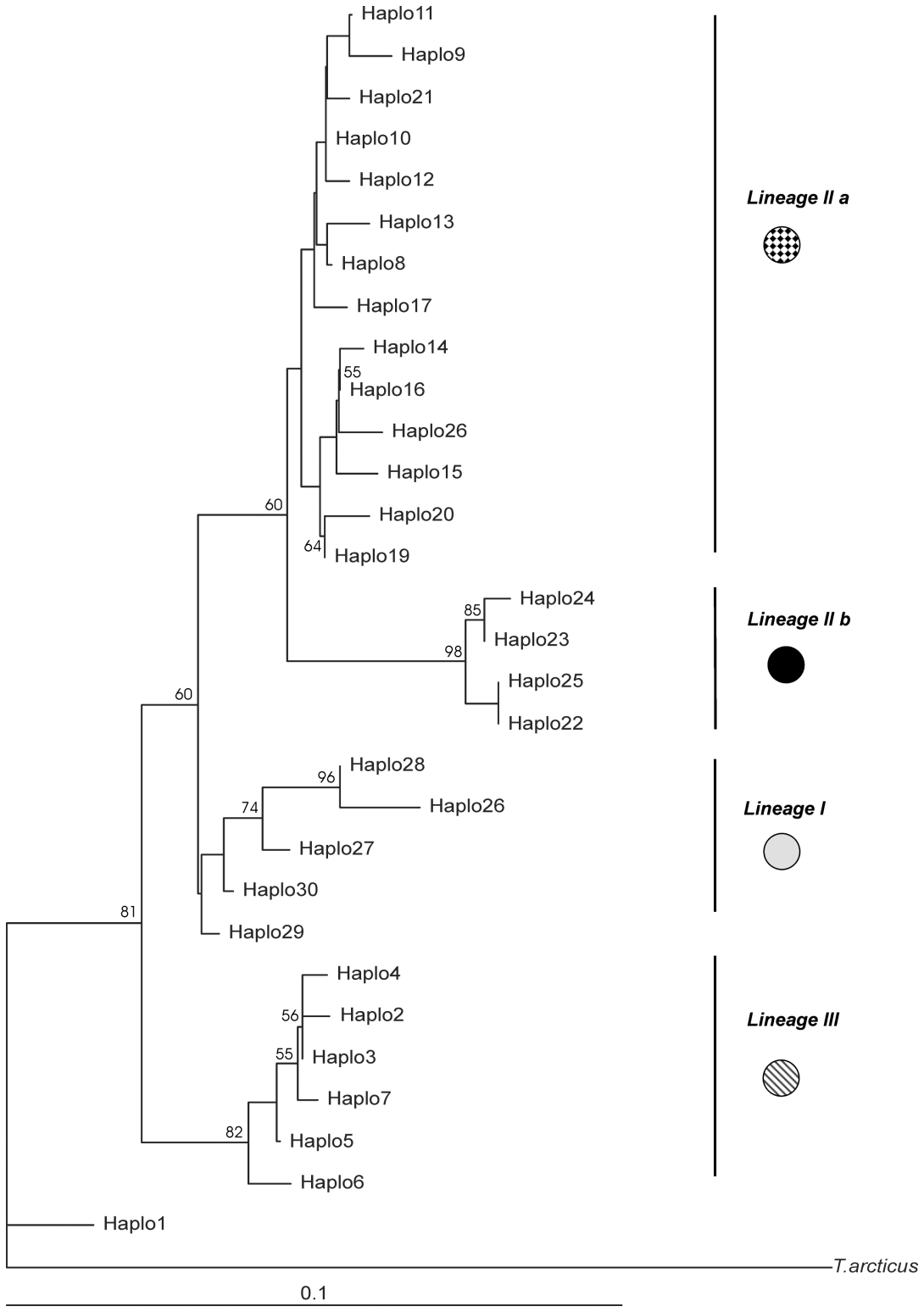

Fig. 4.2: Dendrogram of grayling mtDNA PCR-RFLP composite haplotypes at the ND-1 and ND-5/6 genes estimated by the Fitch-Margoliash method using the average number of nucleotide substitutions per site (d_{ij}) as an estimate of evolutionary distance between the haplotypes. Numbering of haplotypes is according to Table 4.2. Bootstrap values are indicated for statistically supported groupings ($\geq$ 50%). Vertical lines indicate major *Thymallus thymallus* lineages I-III according to the designation by Koskinen *et al*. (2000).

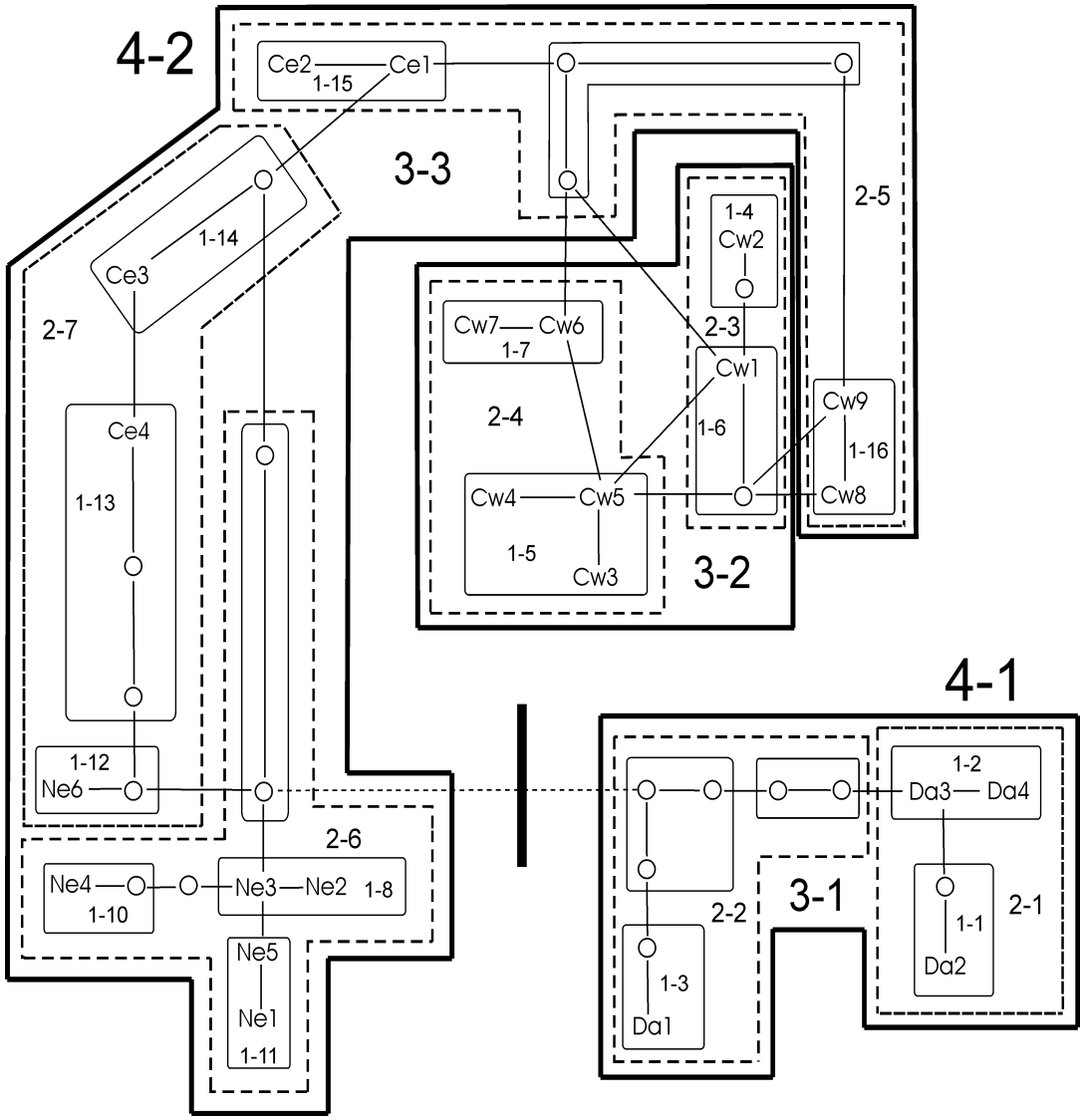

Fig. 4.3: The estimated 95% plausible set of cladograms and associated nested design for the mtDNA ND-5 sequence data found in *Thy. thymallus*. Haplotypes are indicated by letter designation as given in Table 4.2. Each solid line represents a single mutational change that interconnects two haplotype states that has a probability greater than 95%. The thin dashed line indicates a multiple step mutational connection for which the exact interconnections are uncertain and for which parsimony is not supported at the 95% level. Hairline boxes enclose one-step clades, dashed polygons enclose two-step clades, solid lined polygons enclose three-step clades; a thick solid vertical line separates the two four-step clades, 4-1 and 4-2. Technically, these last two clades are greater than four-step clades because of the large mutational distance (a minimum of 14 steps) that separates them, but these clades are the only nondegenerate categories at the level of four-step or above.

Table 4.3: Summary results of the nested clade analysis.

Haplotypes or Clades being tested	χ^2; *P*-value	Inference Chain
Haplotypes in Clade 1-2	87; $P < 0.0001$	1N; 2Y; 3Y; 5N; 6-Two Few Clades (≤ 2) To Determine Concordance = Insufficient Genetic Resolution to Discriminate between Range Expansion/Colonisation and Restricted Dispersal/Gene Flow
Haplotypes in Clade 1-5	106; $P < 0.0001$	1N; 2Y; 3N; 4 = Restricted Gene Flow with Isolation by Distance
Haplotypes in Clade 1-7	44; $P < 0.0001$	1N; 2Y; 3N; 4Y; 9Y; 10 = Geographical Sampling Scheme Inadequate to Discriminate Between Fragmentation and Isolation By Distance
Haplotypes in Clade 1-8	23; $P = 0.0490$	1N; 2Y; 3N; 4 = Restricted Gene Flow with Isolation by Distance
Haplotypes in Clade 1-11	31; $P < 0.0001$	1N; 2Y; 3N; 4Y; 9Y; 10 = Geographical Sampling Scheme Inadequate to Discriminate Between Fragmentation and Isolation By Distance
Haplotypes in Clade 1-15	35; $P = 0.0300$	1N; 2N; 11Y; 12 = Contiguous Range Expansion
Haplotypes in Clade 1-16	9; $P = 0.0050$	1N; 2N; 11Y; 12Y; 13N; 14Y = Sampling Design Inadequate to Discriminate between Contiguous Range Expansion, Long Distance Colonisation, and Past Fragmentation
One step clades in Clade 2-1	57; $P < 0.0001$	1N; 2N; 11Y; 12 = Contiguous Range Expansion
One step clades in Clade 2-3	107; $P < 0.0001$	1N; 2Y; 3Y; 5N; 6-Two Few Clades (≤ 2) To Determine Concordance = Insufficient Genetic Resolution to Discriminate between Range Expansion/Colonisation and Restricted Dispersal/Gene Flow
One step clades in Clade 2-4	115; $P < 0.0001$	1N; 2Y; 3N; 4Y; 9Y; 10 = Geographical Sampling Scheme Inadequate to Discriminate Between Fragmentation and Isolation By Distance
One step clades in Clade 2-5	149; $P < 0.0001$	1Y; 19Y; 20N; Inadequate Geographical sampling
One step clades in Clade 2-6	115; $P < 0.0001$	1N; 2Y; 3N; 4 = Restricted Gene Flow with Isolation by Distance
One step clades in Clade 2-7	166; $P < 0.0001$	1N; 2N; 11Y; 12Y; 13N; 14Y = Sampling Design Inadequate to Discriminate between Contiguous Range Expansion, Long Distance Colonisation, and Past Fragmentation
Two step clades in Clade 3-1	78; $P = 0.0020$	1N; 2Y; 3N; 4Y; 9Y; 10 = Geographical Sampling Scheme Inadequate to Discriminate Between Fragmentation and Isolation By Distance
Two step clades in Clade 3-2	158; $P < 0.0001$	1N; 2Y; 3N; 4 = Restricted Gene Flow with Isolation by Distance
Two step clades in Clade 3-3	645; $P < 0.0001$	1N; 2Y; 3Y; 5Y; 15 = Past fragmentation and/or long Distance Colonisation
Three step clades in Clade 4-2	474; $P < 0.0001$	1N; 2N; 4Y; 9Y; 10 = Geographical Sampling Scheme Inadequate to Discriminate Between Fragmentation and Isolation By Distance

4.4.2 Geographical distribution of mtDNA haplotypes and differentiation among assemblages

Lineage I haplotypes (RFLP 26-30, sequence Ne1-Ne6) were confined to Northern and Northeastern Europe (Fig. 4.1, Table 4.2). Lineage IIa haplotypes (8-21, Cw1-Cw9) prevailed in the Rhine/Main and Alpine Rhine drainage systems, but were also found in England, in the German Weser drainage and in South Tyrol (population ToS). Central European lineage IIb haplotypes (22-25, Ce1-Ce4) were primarily observed in the Elbe and Weser drainages, in the drainage of the Vistula in Poland, in Lithuania, Denmark, Central Sweden and Norway (Fig. 4.1, Table 4.2). Lineage III haplotypes (1-7, Da1-Da4) were predominately found in populations representing the drainage system of the Danube in Germany, Austria and Slovenia.

NCA summary results for the inferred clades are presented in Table 4.3. In the following we only refer to significant associations of geography and haplotypes for important clades at a higher nesting level. For both clade 3-1 (Danubian clade) and 4-2 (haplotypes originating from Central and Northern Europe) no clear conclusion about the evolutionary process with respect to fragmentation or isolation by distance was possible, due to inadequate geographical sampling. For clade 3-2 (Western Europe with haplotypes dominating in the Rhine/Main drainage) restricted gene flow with isolation by distance was inferred. For clade 3-3 including Central-Eastern/North-Western Europe and the haplotypes from England (clades 2-5, 2-6) as well as Northern/North-Eastern Europe (clade 2-6) past fragmentation and/or long distance colonisation was inferred.

Sequencing of the control region in representative samples for linking our data to Weiss *et al.*'s (2002) study revealed that lineage I corresponds to 'Scandinavia' (haplotypes At2, At4; see Weiss *et al.* 2002), lineage IIa can be found in 'Mixed Central Europe' (At18, At20), lineage IIb corresponds to a single haplotype sampled in Denmark (At9) and lineage III corresponds to 'Danube (Northern Alps)' (Da14, Da11).

In addition to the clear geographic distribution of the major grayling lineages in Central and Northern Europe the phylogenetic analysis revealed a substantial level of admixture among highly divergent mtDNA haplotypes within populations (Table 4.2, Fig. 4.4). In particular, populations from areas of potential contact zones of drainages like the low mountain range in Central Germany (along 50° latitude, Fig. 4.1) displayed markedly higher frequencies of haplotypes from different lineages. For instance, populations FiN or WaN from the northern tributaries to the Danube were observed to harbor lineage IIa haplotypes in addition to

Danubian lineage III haplotypes at a frequency of 56% and 50%, respectively (see Table 4.2 for details).

The N-J dendrogram based on RFLP haplotype frequencies of the studied European grayling populations resolved five major population assemblages (Fig. 4.4): (i) a northern/northeastern European group (Sweden, Estonia); (ii) a centraleastern European group consisting of populations from the Elbe and Weser drainage in Germany and Czech Republic, the Vistula drainage in Poland as well as the population from Lithuania; (iii) a centralwestern European group, consisting of populations from the Rhine/Main system and England; (iv) a Danubian group, consisting exclusively of populations from the alpine tributaries to the Danube; and (v) a mixed group, consisting of populations harbouring haplotypes partly from the Danubian lineage III and centralwestern European lineage IIa, which was placed between the Rhine/Main and Danubian population clusters.

4.4.3 Microsatellite DNA variation within and between samples

A total of 152 alleles with an average of 12.7 alleles per locus were observed across the 12 microsatellite loci, ranging from 4 (SSOSL311) to 23 alleles (BFRO010 and Ogo2). Across all populations, the average number of alleles per locus (A) was 3.5, the average mean allelic richness (A_R) 2.8 and the average expected heterozygosity (H_e) 0.39. The estimates of genetic variability are summarized for each population in Table 4.1. Grayling populations from England (Avo), Lithuania (Akm) and Estonia (Sel) displayed remarkably low levels of genetic diversity (A = 1.6, A_R = 1.3 - 1.6 and H_e = 0.1 - 0.24). Variation among the drainages of Danube, Rhine/Main and Elbe was not significantly different (A_R = 3.3, 2.9, 2.6 and H_e = 0.45, 0.37, 0.34, respectively). A total of 31 private alleles were found in 17 of 29 populations. Comparison of drainages revealed 12 private alleles for the Danube (7 and 5 for the southern and northern Danubian tributaries, respectively), 4 for the Elbe and 2 for the Rhine/Main (Table 4.1).

Evidence for intra-population genetic structure (Table 4.1, STRUCTURE analysis) was found in four populations: InA, TiA and ScR consisted of two subpopulations (K = 2) while Lei consisted of three subpopulations (K = 3). However, for the majority of populations a value of K = 1 was chosen as the most appropriate model based on the posterior probabilities of K. Each of the populations with K > 1 also showed significant linkage disequilibrium with 19, 17, 8 and 5 pairs of loci in linkage disequilibrium for InA, TiA, Lei and ScR, respectively (generally not the same loci involved). By contrast, linkage disequilibrium was negligible for the other samples: no disequilibrium was observed between all pairs of loci in 15 of 29

populations analyzed and a maximum of one to three pairs of loci out of 66 tests per population were found to segregate not independently in 10 populations (3.3 significant values can be expected by chance alone at the 5% level).

Significant deviations from the expected HW proportions were observed in seven populations (deficit of heterozygotes) after applying sequential Bonferroni correction: in three populations from the Danube drainage (Am, InA, InG), in three populations from the Rhine/Main drainage (Erf, LiB, Lei), and in the SSa population from the Elbe drainage. Test results for individual loci are provided by the corresponding author upon request.

4.4.4 Microsatellite divergence and admixture analysis among assemblages

Estimates of F_{ST} were highly significant ($p < 0.001$) for all pairwise comparisons of populations with the exception of populations ToS and LiB ($p > 0.05$) and the two Czech populations from the Elbe drainage Tep and Vol ($p < 0.05$). An allele frequency table for all loci and populations is provided as an electronic appendix (Table S3, supplementary material). The overall level of genetic differentiation among European grayling populations was high (mean $F_{ST} = 0.367$). The highest F_{ST}-value (0.810) was observed between the distantly located samples from England and Estonia (Fro and Sel). Within the Danube drainage, differentiation between populations within sub-drainages (e.g. mean $F_{ST} = 0.051$ among alpine populations) was much lower than between northern and alpine tributaries ($F_{ST} = 0.163$). When populations from adjacent rivers of different drainage systems in Central Germany or the Alpine Rhine area were compared, we found both high (e.g. $F_{ST} = 0.256$ between FiN from the Danube and Ssa from the Elbe) and relatively low levels of differentiation (e.g. $F_{ST} = 0.011$ between LiB from the Alpine Rhine and ToS from the Adige drainage).

The N-J phenogram depicting the underlying structure of the D_A distance matrix clearly grouped the studied samples into geographical or drainage-specific assemblages (figure not shown), which were generally congruent with the assemblages for mtDNA RFLP data (see Fig. 4.4). However, the populations from the Elbe drainage (Vol, Tep, Ege) were more clearly separated from the Baltic drainage populations (Dun, Akm, Sel, Rat) than when based on mtDNA RFLP data (92% bootstrap support). Population level admixture analysis indicated that the populations sampled from the contact zones of drainages (such as SSa, Lam, ScR, or LiB) showed a high level of admixture between possible clusters representing the Danubian, Rhine/Main and Elbe gene pools (Table 4.5). The genetic distance based two-dimensional

scaling analysis was in agreement with these results and confirmed also InA and TiA as admixed populations (Fig. 4.5, Table 4.5).

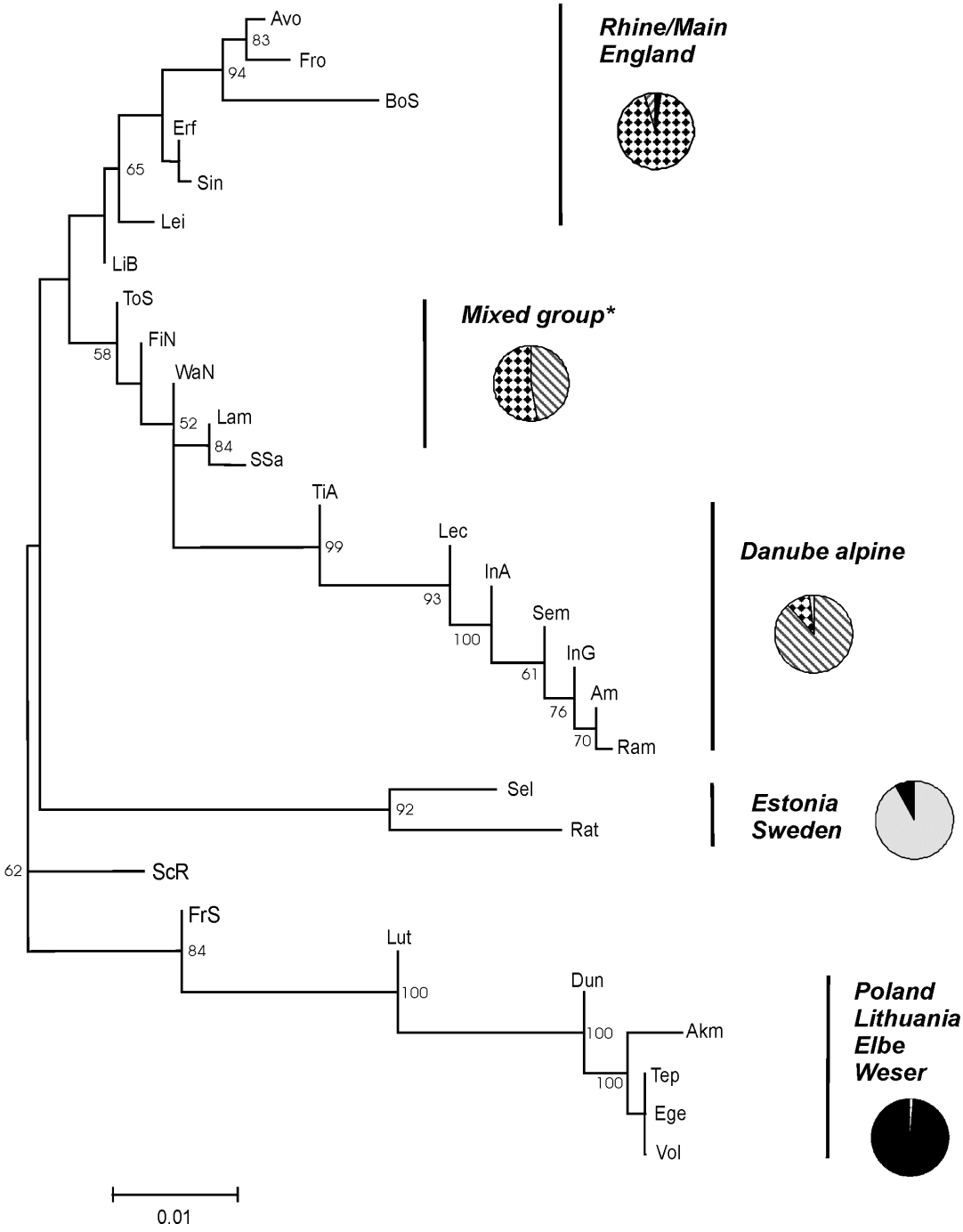

Fig. 4.4: N-J tree of the studied European grayling populations based on the number of net nucleotide substitutions per site (d_A) at the mitochondrial ND-1 and ND-5/6 gene regions. REAP (McElroy *et al.* 1992) was used to calculate the number of nucleotide substitutions per site and nucleotide divergence between all pairs of populations from the restriction site data (Nei & Tajima 1981; Nei & Miller 1990). Population codes are according to Table 4.1. Pie charts represent the relative frequencies of mitochondrial lineages (see Fig. 4.2 for designation of lineages and symbols) for the indicated population clusters.

Table 4.4: Hierarchical analysis of molecular variance based on mtDNA haplotype and microsatellite allele frequencies among European grayling populations grouped according to (i) Central European drainages of the Danube, Rhine/Main and Elbe (K_{SA} = 4) and (ii) all populations (K_{SA} = 5). The number of groups (K_{SA}) was determined by spatial analysis of molecular variance (SAMOVA; Dupanloup *et al.*, 2002) (see text for details).

Type of the DNA marker and population grouping	Among groups			Among populations within groups			Within populations		
	%	*P*	*F* statistic	%	*P*	*F* statistic	%	*P*	*F* statistic
MtDNA									
Central European drainages (K_{SA} = 4)	60.9	0.000	0.609	5.0	0.000	0.127	34.1	0.000	0.659
All populations (K_{SA} = 5)	63.4	0.000	0.634	8.3	0.000	0.227	28.3	0.000	0.717
Microsatellites									
Central European drainages (K_{SA} = 4)	23.5	0.000	0.235	12.6	0.000	0.164	63.9	0.000	0.361
All populations (K_{SA} = 5)	23.2	0.000	0.232	19.9	0.000	0.258	56.9	0.000	0.431

4.4.5 Analysis of molecular variance

The SAMOVA for the mtDNA data revealed maximum F_{CT} values of 0.634 for all investigated populations (K_{SA} = 5) and 0.609 for Central European drainages (K_{SA} = 4) indicating that populations sampled from Northern Europe obviously constitute a discrete group. The five groups revealed by SAMOVA for all sampling locations were composed of i) Danube alpine populations, ii) samples from Elbe, Weser (Lut), Poland and Lithuania, iii) samples from Rhine/Main, England, iv) populations from Estonia, Sweden (Sel, Rat), and v) populations TiA, ScR, FiN, WaN, SSa, Lam, and ToS. Populations from the Central European drainages were classified into 4 groups accordingly: i) Danube alpine, ii) Elbe, iii) Rhine/Main, and iv) TiA, ScR, FiN, WaN, SSa, and Lam. This result indicates that populations such as FiN and ScR (Danube) or SSa and Lam (Elbe) sampled from areas where the headwaters of these drainages come in closest contact and in some cases drained into different watersheds until the late Pleistocene period (e.g. the Upper Main tributaries into the Upper Danube, Hantke 1993), can be more closely related to each other than to other populations from the same drainage.

Although the within-population variation obviously is high for nuclear data as a result of the high variability and high heterozygosity of microsatellites, a doubled amount of the genetic variance was still due to the differences among population groups (~ 23%, Table 4.4) than compared to the differences among populations within groups (12.5%), indicating a clear differentiation between the major Central European drainage systems.

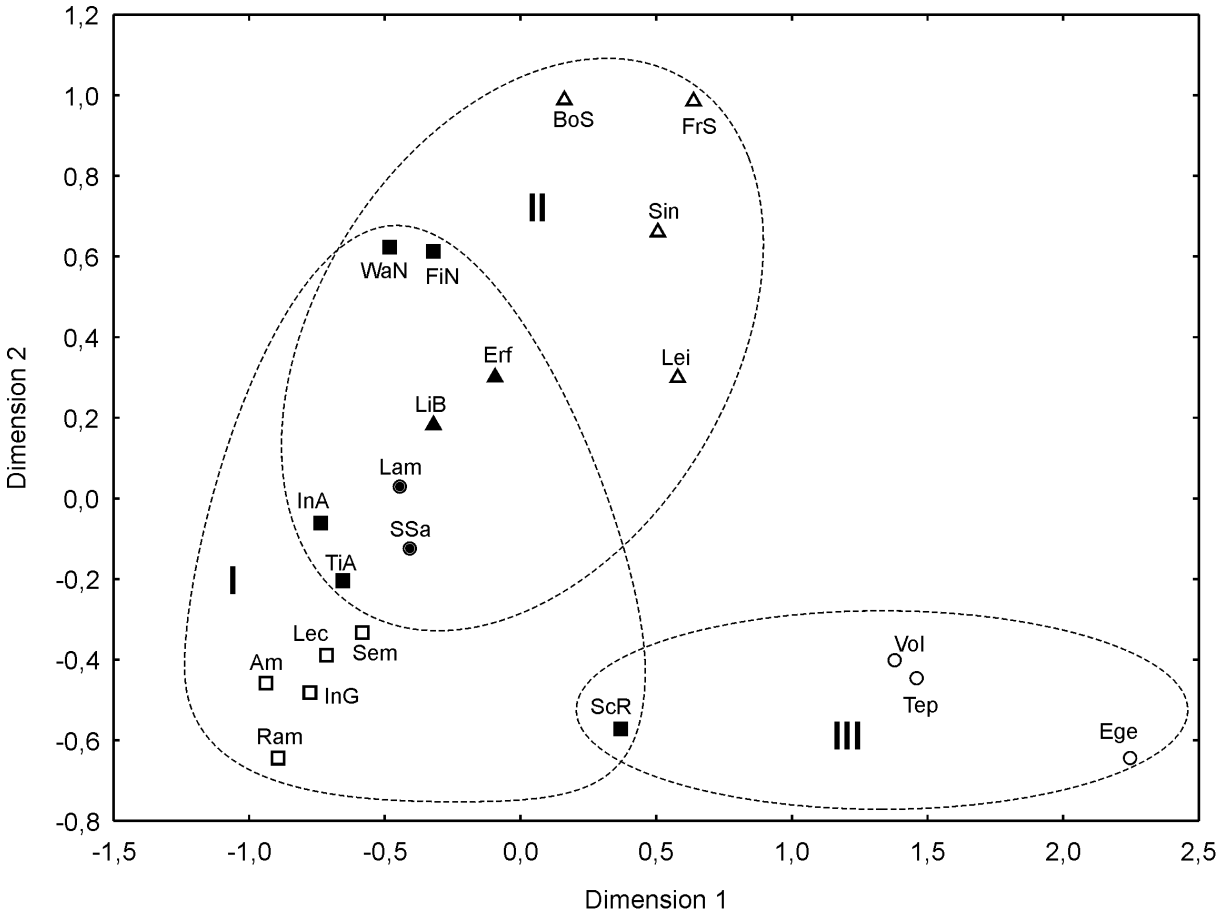

Fig. 4.5: Two-dimensional scaling analysis based on 12 microsatellite loci and Nei's D_A distances (Nei *et al.*, 1983) among pairs of populations. Symbols indicate the geographical origin of samples: Danube (squares), Rhine/Main (triangles), and Elbe (rings). Population codes are according to Table 4.1. Overlapping areas of dashed ellipses indicate admixed populations (filled symbols) with assignment < 85% to one of the clusters (I, II, III) as identified by STRUCTURE analysis (see Table 4.5).

Table 4.5: Estimates of population admixture coefficients (K = 3) for drainages Danube Rhine/Main and Elbe based on the Markov chain Monte Carlo algorithm using STRUCTURE (Pritchard et *al.*, 2000).

Population	*inferred cluster*		
	I	II	III
DANUBE (I)			
Ram	0.979	0.013	0.008
Am	0.935	0.056	0.009
Lec	0.876	0.114	0.010
Sem	0.856	0.121	0.023
InG	0.939	0.050	0.011
TiA	0.743	0.238	0.019
InA	0.728	0.266	0.007
FiN	0.157	0.836	0.007
WaN	0.241	0.752	0.007
ScR	0.406	0.055	0.539
RHINE/MAIN (II)			
BoS	0.008	0.983	0.009
Sin	0.011	0.944	0.045
Lei	0.054	0.858	0.087
Erf	0.281	0.708	0.011
FrS	0.009	0.916	0.075
LiB	0.396	0.592	0.012
ELBE (III)			
Ege	0.010	0.008	0.982
ZV	0.008	0.027	0.965
BT	0.007	0.017	0.976
Ssa	0.579	0.412	0.008
Lam	0.538	0.454	0.008

4.5 Discussion

4.5.1 Diversity and divergence of grayling mtDNA and nuclear DNA

The ranking order of grayling populations based on the estimates of intra-population variation (number of haplotypes/alleles, diversity/heterozygosity), was congruent for the mtDNA and microsatellite data (e.g. correlation between haplotype diversity and H_e was r = 0.6, p < 0.001). Specifically, microsatellites revealed a relatively low level of intra-population variation across all populations (mean A = 3.5, mean H_e = 0.39). This finding is in precise agreement with Koskinen *et al.*'s (2002a) study on microsatellite phylogeography of grayling (mean A = 3.5, *mean* H_e = 0.41). A major reason for the relatively low level of intra-population variation in grayling is probably due to the species' life history characteristics such

as the formation of family cohorts or its spawning behavior (for details see Koskinen *et al.*, 2002 and Gum *et al.* 2003 as well as references therein).

Consistent with earlier studies (Koskinen *et al.*, 2000; Gross *et al.*, 2001), the mtDNA PCR-RFLP and ND-5 sequence haplotypes clustered into three major lineages. Lineage I prevails in Northern and Northeastern Europe (Russia, Finland, Estonia, Northern Scandinavia), while lineage III is confined to the Danube drainage. For lineage II of Koskinen *et al.* (2000), however, a further subdivision into two sublineages IIa (occuring in the Rhine/Main drainage and in England) and IIb (found in Central and Eastern Europe from the Elbe drainage to the Vistula, in Southern Scandinavia and Lithuania) was evident. These observations extend previous phylogeographical knowledge of grayling especially regarding its western and central-eastern European distribution range.

In congruence with mitochondrial data, microsatellites identified strong genetic differentiation of European grayling populations between and within drainages even on relatively small geographical scales, compared to the level across all investigated populations in Central and Northern Europe (e.g. average F_{ST} = 0.367 for all populations and average F_{ST} = 0.268 for Southern Germany). However, the group structure revealed by SAMOVA (K_{SA} = 4) for Danube Rhine/Main and Elbe with one group consisting of populations sampled mainly from the contact zones of these watersheds such as FiN or SSa indicated a higher level of gene flow among Central European drainages than could be expected from former studies (Gum *et al.*, 2003).

Aside from the species' before mentioned life history traits (reviewed by Northcote, 1995), the reasons for these strong intra-specific population differences possibly are associated with the necessity to adapt to different environmental conditions. Grayling from Norway, showed that natural selection caused by temperature regimes in lakes at different altitudes has resulted in local adaptation within 13-18 generations (Haugen & Vøllestad, 2000). Besides selection and mutation, more recent effects of habitat fragmentation (e.g. weirs or hydropower-stations; see Meldgaard *et al.* 2003), increasing environmental stochasticity (e.g. due to springtime floods or increasing water temperatures in hot summers), and/or human introductions (see Sušnik *et al.* 2004) are possible factors leading to genetic stochasticity which subsequently results in population substructure. In this study the presence of multiple gene pools within populations along with substantial linkage disequilibrium was detectable only in populations known to be managed by supportive breeding activities during the last decade (InA, TiA, Lei, ScR). For the majority of populations it is therefore safe to assume that human mediated stock

transfer probably did not play a decisive role as evidenced by the number of populations present within samples, as inferred using STRUCTURE ($K = 1$).

4.5.2 Colonisation history and postglacial dispersal of *T. thymallus*

Based on the applied rate of 2% per million years the Danubian lineage III was separated from lineages I and II about 650,000 (± 150,000) years ago, i.e. after the Günz glaciation (700,000-900,000 years ago). However, as the divergence rate of 2% has to be considered a conservative estimate for the obtained ND-5 sequences (Hansen & Loeschke, 1996a; Churikov *et al.*, 2001), we cannot rule out the possibility that the major split happened before the major Pleistocene glaciation cycles (> 900,000 years). An expansion of *Thymallus* across Southern Europe predating Pleistocene glacial cycles was also suggested by Weiss *et al.* (2002). These observations confirm the theory that Quaternary cold periods are unlikely to be the main reason of the deep divergence among grayling lineages. In addition, fossil evidence corroborates the genetic results of *T. thymallus* inhabiting Europe long before the Pleistocene cold periods (Bănărescu, 1992). Apart from the mentioned deep intra-specific divergence event between the Danubian and the other lineages, the Northern/Northeastern European lineage I and the Central European lineage II diverged approximately 150,000 (±75,000) years ago), clearly predating the late Pleistocene glaciations (Early, Middle and Late Weichselian) which started about 100,000 years ago. Corroborating this finding NCA revealed past fragmentation and/or long distance colonisation for lineages I, II (clade 3-3).

In agreement with Koskinen *et al.* (2000) and Weiss *et al.* (2002) we found that the basin of the Danube served as a major refugium throughout the Pleistocene epoch for grayling populations distributed across the southern areas of Central and Eastern Europe. Additionally the present study showed that the Danubian gene pool did not contribute to the recent colonisation of the Northern or Northeastern European region after the last glacial maximum. The distribution of mtDNA lineage IIb in drainages of Central (e.g. Elbe, Weser) and Eastern Europe (e.g. Vistula) as well as its expansion to Southern Scandinavia (Denmark, Norway and Sweden) clearly points to a Central/Eastern European refugium. It is possible that grayling survived the last Ice Age in some periodically ice-free rivers of the Elbe and/or Vistula drainage or maybe in more Northeastern Balkan drainages. After the retreat of the Scandinavian ice sheet 8,000-10,000 years ago, colonisation of Denmark and Southern Scandinavia became possible and this most likely took place via the Elbe.

Although the geographical position of potential refugia for lineage I (Northern/Northeastern Europe) cannot be determined exactly neither from the results of the present nor previous

studies (Koskinen *et al.*, 2000; Weiss *et al.* 2002), our study suggests that it was probably located north of the Black or Caspian Sea basin. A dense sampling from the drainages of Dnestr, Don or Volga would help to further clarify the colonisation history from Eastern into Central Europe.

The distribution of lineage IIa haplotypes which prevailed in the Rhine/Main drainage points to a previously not described Western European refugium of grayling. As indicated, for example, by the occurrence of unique haplotypes found in some Main or Upper Danube populations of the low mountain range in Central Germany (e.g. FrS, Lei, WaN) and their distinctiveness from lineage IIb haplotypes, grayling most probably persisted throughout the last major Pleistocene glaciation within the Rhine/Main system. A similar scenario was proposed for the cold water tolerant species, *Cottus gobio* (Hänfling *et al.*, 2002). Furthermore, an interglacial or postglacial interbasin expansion via the often cited Rhine/Rhone corridor to the south and vice versa is likely (e.g. in the case of *Cottus gobio*, Riffel & Schreiber 1998; Weiss *et al.* 2002). Our data clearly showed that Southern Britain was colonised via the Rhine as indicated by the close relatedness of the Rhine haplotypes to the haplotypes found in Southwestern England. The growth and melting of glaciers caused changes in the sea level and in general, the sea level was much lower (< 50 m) during the Late Pleistocene/Early Holocene (Milliman & Emery, 1968). Therefore, Britain was connected by a land bridge (depth of the English Channel ~30-40 m) with the continent. Hence, invasion was possible for grayling via the rivers Rhine, Maas, Thames and Scheldt, all of which drained northwards into a common European 'super river' (see Hänfling *et al*., 2002).

Another well-studied region that acted as a source of postglacial colonisation of freshwater fishes into Central Europe is the Adriatic Sea Basin (Bernatchez, 2001; Weiss *et al.*, 2002; Sušnik *et al.*, 2001). However, the present and previous studies (Weiss *et al.*, 2002) could not prove any significant contribution of Slovenian or Italian haplotypes to the colonisation of Central or Northern Europe. Besides the Danubian lineage it rather seems that the above mentioned 'Elbe and Rhine' or 'East Balkan' expansion came to fill most of Central Europe, probably due to an early start after the Ice Age and because the Alps hindered the spread of Italian and Adriatic genomes (see Hewitt, 2001 for a general review).

4.5.3 Zones of hybridization in Central and Northern Europe

A key result of this study is that the majority of sampling locations consisted of haplotypes from more than one of the major mtDNA lineages that came into secondary contact during the interglacial warm periods of the Pleistocene and Holocene. This finding contrasts Koskinen *et*

al.'s (2000) study in which only three sampling locations harbored haplotypes from more than one major lineage. However, their sampling covered mainly Northern Europe.

We found that especially in the headwater region of Central European rivers at relatively low altitudes (i.e. the low mountain range of Central Germany), both results for spatial analysis of molecular variance and estimates of population admixture coefficients indicated ancient rather than modern connections among drainages and reflected glacial perturbations. In these regions periglacial meltwaters produced greatly modified channels between and within drainages (Gibbard, 1988; Hantke, 1993). One major zone of secondary contact among grayling lineages was observed in the German Fichtelgebirge (e.g. populations FiN, Ssa, Lam) where the tributaries of the Danube, the Elbe and the Main come into closest contact (a few km^2) and are known to have changed their flow directions during the Pleistocene Ice Age (e.g. the Upper Main drained into the Upper Danube until 500,000 years ago (Hantke, 1993)). Additional hybrid zones for grayling were detected between the Danube and Elbe watersheds (River ScR); in the Weser drainage where the western and eastern Central European lineages meet (lineage IIa and IIb, respectively); and at the Lake Constance area, where the drainages of the Alpine Rhine and the Upper Danube are closely adjacent (population LiB). The genetic results are supported by geological data, according to which major connections between the Upper Danube and the Upper Rhine have existed until the Riss/Saalian glaciation period (150,000-300,000 years ago; Hantke, 1993). A further important suture zone is located in Southcentral Sweden and Norway (Northern lineage I and Central European lineage IIb). Expansions often met in Southern Scandinavia, where the last of the ice caps melted some 9,000 years ago forming a hybrid zone for a number of species (Taberlet *et al.*, 1998; Hewitt, 2001).

In conclusion, based on our findings in Central Europe and the results of Weiss *et al.*'s (2002) study throughout the species' southern European distribution range, the 'pure lineage situation' for grayling seems the exception rather than the rule.

4.5.4 Comparative phylogeographical inference

In the last few years several freshwater fish species have been studied phylogeographically across Europe and now data for the brown trout (*Salmo trutta*), the perch (*Perca fluviatilis*), the chub (*Leuciscus cephalus*), the bullhead (*Cottus gobio*) the barbel (*Barbus barbus*) or the vairone (*Leuciscus soffia*) are available. Since each taxon has responded independently to Quaternary glaciations (Taberlet *et al.*, 1998) we restrict comparisons to a few species which co-occur with grayling such as bullhead or chub. In comparison to the cold-adapted grayling

and bullhead (Englbrecht *et al.*, 2000), mitochondrial studies of more mobile species such as chub (Durand *et al.*, 1999) or perch (Nesbø *et al.*, 1999) generally indicated a more recent postglacial divergence and a substantially lower level of differentiation among the studied drainages. An origin predating the major Pleistocene glaciations has been suggested for Central European grayling and bullhead populations accordingly (Hänfling *et al.*, 2002; Weiss *et al.*, 2002; this study) and - in contrast to chub and perch - the Main and Elbe groups most probably survived the glaciations in the part of Central Europe which was located between the northern ice sheet and the Alpine glaciers (Bănărescu, 1992). In addition, a postglacial expansion from the Elbe northward to Southern Scandinavia is evident for grayling and bullhead (Hänfling *et al.*, 2002). Interestingly, even though the number of mtDNA lineages in Central Europe varies considerably among grayling, bullhead, brown trout, perch, chub or barbel a common feature of all species is that major lineages came into secondary contact in the same Central European area. Similarly to grayling, the Central European lineages of the barbel met in the Weser (Kotlík *et al.*, 2001), those of the perch or the brown trout in the Upper Danube (Nesbø *et al.*, 1999; Bernatchez *et al.*, 2001) and the only evident contact zone for the chub was in the Elbe (Durand *et al.*, 1999).

To sum up for grayling, although the present and previous studies shed more light on the phylogeography of grayling in Central, Northern and Southern Europe, there are still unresolved questions about the multi-faceted patterns of colonisation of *T. thymallus* in Europe, especially concerning the eastern distribution range and the general complex glacial history of the Ponto-Caspian region (Arkhipov *et al.*, 1995; Kotlík *et al.,* 2004).

4.5.5 Implications for conservation

Corroborating the ND-5 sequence data, mitochondrial PCR-RFLP and microsatellite analyses revealed major Central and Northern European grayling population assemblages and were consistent with earlier studies. Thus, it can be concluded that the major grayling lineages I, IIa, IIb, and III revealed in this study should be regarded as distinct ESUs as defined by Moritz (1994) and Waples (1995). Transfer of stocking material between these highly divergent grayling lineages should be avoided to maintain the historically established genetic diversity. In addition, our data demonstrate that there is a greater degree of phylogenetic diversity within populations, especially in Central European areas of putative refugia and contact zones between watersheds, than was previously appreciated (e.g. Koskinen *et al.*, 2000). Specifically for the area of Central Germany, which was investigated more intensively in the present study, a strict division of populations based on the drainages Danube,

Rhine/Main and Elbe as proposed earlier is not appropriate (Gross *et al.*, 2001; Gum *et al.*, 2003). Here, in particular, we recommend a strategy of river-specific supportive breeding, in order to conserve local forms and to sustain intra-specific diversity. However, the setting of conservation units and conservation guidelines is controversial for regions where populations show a high level of natural admixture among divergent evolutionary lineages and general rules are difficult to propose (reviewed in Allendorf *et al.*, 2001). In any case, the European grayling seems well suited as a model species for future research regarding the biological relevance of genetic differences based on neutral markers and with respect to the increasing interest in adaptive differences at the expression level.

4.6 Supplementary material

The following material is available from [http://www.wzw.tum.de/wildbio/gum.htm] under 'Veröffentlichungen'.

Table S1: (**a**) Pairwise nucleotide substitution matrix (*dij*-values) of 30 PCR-RFLP haplotypes from the mitochondrial ND-1 and ND-5/6 gene regions for the four defined mtDNA lineages and the *Thymallus arcticus* haplotype. **(b**) Pairwise nucleotide substitution matrix for 529 bp of ND-5 sequences from representative individuals of 23 different haplotypes. Indicated are the average (range) of all pairwise combinations in percentages. Intra-group divergence for the four defined lineages are indicated along the diagonal in italics.

Table S2: Variable nucleotide sites across 529 bp of the ND-5 gene 5'end sequence haplotypes. Dots indicate homology with the reference sequence (Ce1). The complete sequences have been entered into GenBank under accession nos AY527277-AY527291.

Table S3: Genotypic table and allele frequencies of 29 European grayling populations at 12 microsatellite loci. Population codes are according to Table 4.1.

5 Discriminating the impact of recent human mediated stock transfer from historical gene flow on genetic structure of European grayling (*Thymallus thymallus* L.)

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5.1 Abstract

Microsatellite markers were first used to partition individuals of European grayling *Thymallus thymallus,* from the Danube, Rhine and Main, and Elbe drainage systems into subpopulations and to estimate individual immigrant ancestries over the last few generations. Subsequently, the studied populations were 'purged' from recent immigrants and the proportions of evolutionary lineages within the 'purged' populations were re-estimated by applying mtDNA markers. The results confirmed a high level of admixture of the divergent mtDNA lineages (i.e. natural secondary contact) in populations sampled at the contact zones of the drainages. In addition, a substantial amount of introgression was observed for several populations that were known to be affected by stocking of European grayling from different origins.

5.2 Introduction

Large-scale phylogeographical studies carried out over the last decade have identified several distinct glacial refugia for the northern hemisphere fish species where populations could diverge genetically in allopatry (Bernatchez & Wilson, 1998; Englbrecht *et al*., 2000; Bernatchez, 2001; Kotlík *et al.,* 2004). Also, several studies have identified specific regions where these genetically distinct lineages naturally came into secondary contact after the last glaciation (Froufe *et al*., 2003; Taylor, 2004; Gum *et al.*, 2005). The dynamics of these naturally occurring genetic interactions have been further complicated by the introgression of exogenous gene pools through human mediated non-native stock transfers or accidental escapes of farmed fishes which may present a considerable threat to the genetic diversity and integrity of native fish stocks (Taylor, 1991; Hindar *et al*., 1991; Hansen *et al*., 1995; Hansen *et al*., 2001a; Fleming *et al*., 2000; Sušnik *et al*., 2004; Vasemägi *et al*., 2005), and also can

result in the transfer of diseases and parasites (Jonsson, 1997). Given the fact that the introduction and stocking of non-native fishes still takes place in many countries, it is necessary to apply and further develop the appropriate tools to (i) distinguish between natural and anthropogenic caused admixture of distinct genetic lineages and (ii) determine the level of introgression of stocked fishes into native gene pools. These results can then be used for phylogeographical inferences and are essential for prioritizing conservation interventions and the setting of conservation units (Allendorf *et al.,* 2001). The exclusion of certain 'stocked' populations from restoration efforts or conservation measures, however, may not always be justified, because the long-term success of many stocking activities cannot be predicted *a priori* (Thorfve, 2002; Ruzzante *et al.,* 2004), and indeed, past stocking practices have not always led to introgression and loss of a formerly distinct population (Mezzera & Largiader, 2001; Hansen, 2002; Ruzzante *et al*., 2004).

Today molecular genetics and the appropriate statistical analyses provide the opportunity to discriminate between long-term gene flow (historical secondary contact) from recent immigration (stocking). As computational constraints have been largely overcome over the past decade (Beaumont & Rannala, 2004), the presently available methods accommodate recent population expansions, nonsymmetrical migration and other complexities that are typical of real biological populations (Beerli & Felsenstein, 2001; Wilson & Rannala, 2003). In particular, several new Bayesian methods have been proposed to allow the combined inference of both the partitioning of individuals into subpopulations (Pritchard *et al*., 2000; STRUCTURE), and the assignment of individual migrant ancestries of individuals into subpopulations (Dawson & Belkhir, 2001; Wilson & Rannala, 2003; BAYESASS). These methods generally require fewer assumptions than estimators of long-term gene flow and can be legitimately applied to non-stationary populations that are far from genetic equilibrium. In addition, recent simulation studies show that the expected accuracy of Bayesian inference is higher when directly compared to competing methods such as maximum likelihood (Beerli, 2006).

For the European grayling (*Thymallus thymallus* L.), stocking and supportive breeding programmes increasingly have been carried out during the last 20 years throughout central Europe with the primary aim of counteracting the strong decline in natural population densities (Baars *et al*., 2001). The species is critically endangered at the population level (Gross *et al.*, 2001; Gum *et al.*, 2003) due to its behaviour and habitat preference, which make it particularly vulnerable to predation by piscivorous birds (Northcote, 1995; Staub *et al.,* 1998). A natural contact zone for three highly divergent evolutionary lineages of European

grayling exists in Bavaria (Gum *et al.,* 2005), where the headwaters of the major central European drainages of Danube, Rhine/Main and Elbe are closely juxtaposed. In these river systems, fishery managers have usually used juvenile fish (0+ to 1+ years; in recent times 1+ to 2+ years) from spawners of the same river stretch to support the depleted stocks (supportive breeding). Fish originating from different river or drainage systems, however, have also been used occasionally for enhancement and this may have affected the genetic structure of native populations. Several populations in this region have been shown to be genetically admixed (Gum *et al.,* 2005), but it remains to be clarified whether this is the result of natural (historical gene flow and secondary contact) or anthropogenic (stock transfer) factors.

In this study the Bayesian methods as proposed by Pritchard *et al*. (2000) and Wilson & Rannala (2003) were applied in order to discriminate between historical secondary contact and recent stocking events. Microsatellite markers were first used to partition individuals into subpopulations and to estimate the posterior probability distributions of individual immigrant ancestries over the last several generations. Then the studied populations were 'purged' from recent immigrants and the proportions of evolutionary lineages within the 'purged' populations were re-estimated by applying mtDNA markers. The presence of multiple mtDNA lineages in these 'purged' populations should more reliably indicate the admixture due to historical secondary contact.

5.3 Sample collection and stocking history

A total of 735 individuals of European grayling (*T. thymallus*) originating from 26 locations across the drainages of the Danube, Rhine/Main and Elbe from Bavaria (Germany), Austria and the Czech Republic were caught from the wild primarily by electro-fishing, or were provided by colleagues as fin clips preserved in 96% alcohol (Table 5.1, Fig. 5.1). In addition to the sampling scheme of Gum *et al.*, (2005) five new populations were included: Iller, Ilm, Isar, Saalach and Weißer Main. Populations were chosen based on different status of management regarding stocking or supportive breeding actions described below. For the majority of locations sampling was carried out during grayling spawning time in March/April in a single year. In order to avoid collection of potential distinct spawning populations the sampled river stretches generally did not exceed 1-2 km (grayling spawners usually congregate close to their spawning grounds). The sex ratio of adult specimen was about 1:1 in all samples, except for population Ilm where only female spawners could be obtained.

Concerning the decline of grayling over the last 20 years the Isar and Iller Rivers have been among the most heavily affected ones (Baars, 2000). Here, a stretch of about 6-8 km had to be sampled to catch a representative number of adult and juvenile grayling including different year classes. Due to the limited number of individuals obtained from the rivers Isar, Leinleiter and Schwarzer Regen in a single year, samples from two consecutive years were collected and combined (Table 5.1). Six of the 23 grayling individuals from river Inn (Innsbruck, population InA) were caught from another tributary of that area (M. Martys, pers. communication). Samples from both the rivers Ammer and Saalach were caught from two closely adjacent river stretches. In order to test for the hypothesis of distinct (spawning) subgroups present within populations Am, Saa, and InA, respectively, individuals originating from these distinct stretches at first were considered as a single population.

Regarding the stocking history of grayling north of the Alps one has to consider that in general there was no need for intensive stocking until the decline started about 20 years ago (Baars, 2000; Hanfland, 2002). In addition, Baars *et al.* (2001) pointed out that the establishment of permanent grayling broodstocks in hatcheries never has been common practice in Bavaria. Instead, grayling spawners are still caught from the wild mostly by electro-fishing. When more intensive stocking started at the end of the 1980ies the southern tributaries of the Danube (rivers Inn, Isar, Lech and Iller and their tributaries) were considerably more affected than the northern Bavarian rivers of the Danube, Main and Elbe drainages. Concerning the Main and Elbe drainages, supportive breeding or stocking actions were reported only in populations Lei and Ssa (see Table 5.1).

In this study, several populations from each drainage were included where no human caused stock transfer has been documented or known (indicated as ‘wild’, Table 5.1). Supportive breeding by stocking of fingerlings of local origin is known for several Danubian populations: Ram, Am, Lec (since 1995), and InG (since the end of the 1980ies). In addition, several populations have also been influenced by stocking of grayling from different origins: Sem (from different tributaries of the Isar system since 40 years), TiA (regular stocking from rivers Alz, Inn, Dorfen since middle of the 1980ies), Ilm (several times from different southern Danubian tributaries but also from the Main drainage since 15-20 years), Isa (regular stocking from both local and different origins from Bavaria until 1999, then stopped), Saa (from 2000 to 2003 stocking of offspring from different southern tributaries of the Danube, since 2003 exclusively supportive breeding), ScR (regular stocking of 1+ from natal broodstock from local hatchery; Baars, 2000).

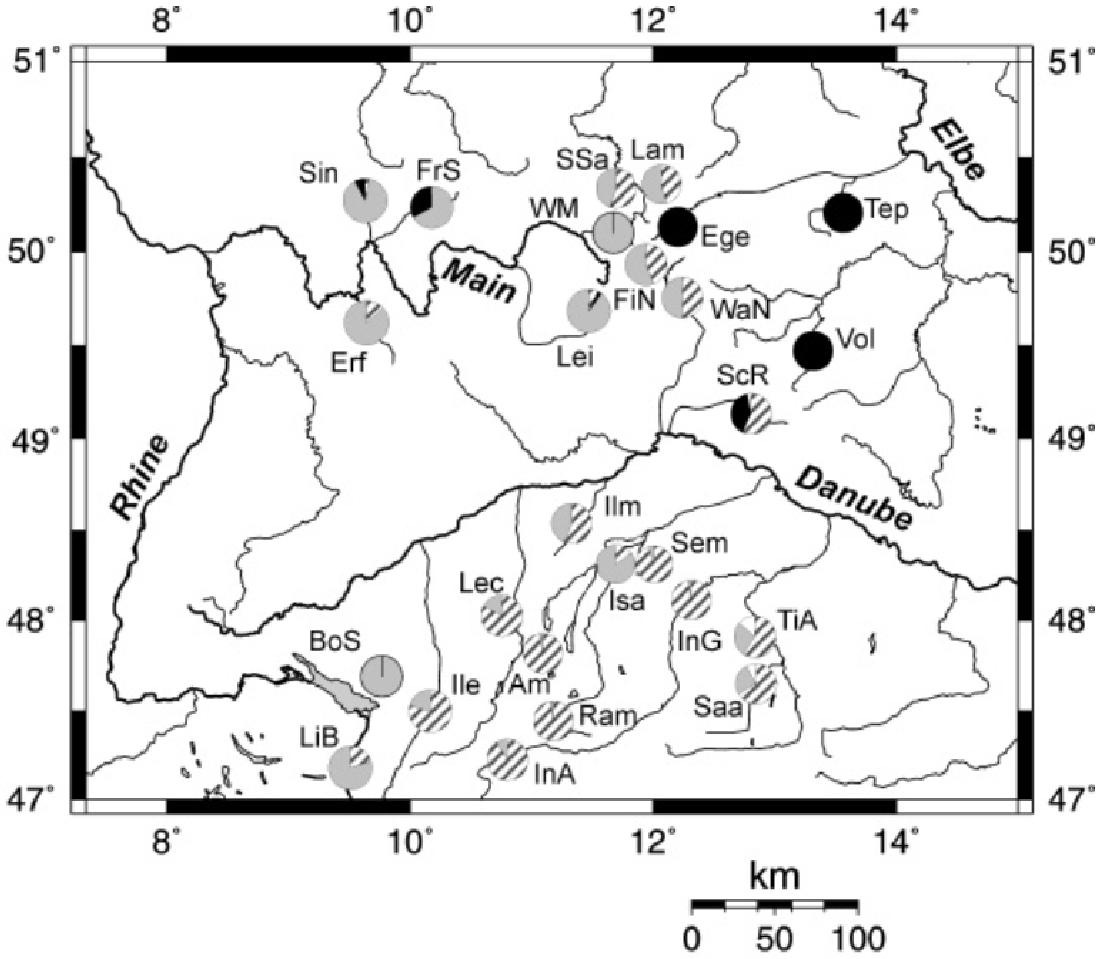


Fig. 5.1: Map of sampling locations with pie diagrams showing the distribution of major mtDNA lineage frequencies among studied European grayling populations. Population codes according to Table 5.1. For designation of lineages see Table 5.2.

Lineage I ◯, lineage IIa◯, lineage IIb ●, lineage III ⊘.

Data concerning the origins of stocked fish and number of stocking events are based on records of the hatchery of the Bavarian Fisheries Association and personal records of local anglers' associations. It should be noted that especially for the bigger rivers Inn, Saalach, Isar, Lech or Iller downstream migration of stocked grayling from upstream stretches is known (pers. observation) and therefore assessment of the stocking history of sampling sites located downstream has to be considered with caution. Hatchery samples BoS, Tep and Vol stem from wild populations (tributaries to the Alpine Rhine and Elbe, respectively) and were transferred to local hatcheries for the purpose of captive breeding (P. Kotlik, E. Bohl, pers. communication).

Table 5.1: Major river drainage, population name, code, sample size, year of sampling, age composition and management status of European grayling (*T. thymallus*) populations. Status 'stocked' means that grayling originating from different rivers were stocked at least once in the last decades, 'wild' means no human mediated stock transfer known (*see text for details of stocking history; [1]supportive breeding; [2] single stocking event in 1990 upstream of sampling site with grayling of Danubian origin; [3] re-established 35 years ago from nearby river Wisent).

Drainage/sub-drainage	Population	Code	Geographical position	N	Year of sampling	Age/year classes	Status
Elbe/Eger	Eger	Ege	50°07' N; 12°00' E	38	1999	adults/mixed	wild
Elbe/Eger	Tepla	Tep	50°31' N; 13°20' E	20	2002	spawners	hatchery
Elbe/Sä.Saale	Sächsische Saale	Ssa	50°15' N; 11°56' E	20	2000	adults/mixed	stocked [2]
Elbe/Sä.Saale	Lamitz	Lam	50°14' N; 12°01' E	13	2002	adults/mixed	wild
Elbe	Volyuka	Vol	49°16' N; 13°37' E	29	2002	spawners	hatchery
Main/Fr.Saale	Fränkische Saale	FrS	50°07' N; 09°56' E	15	2000	spawners	wild
Main/Fr.Saale	Sinn	Sin	50°09' N; 09°38' E	39	1999	adults/mixed	wild
Main/Wisent	Leinleiter	Lei	49°47' N; 11°11' E	30	1999,2000	spawners	stocked [3]
Main	Weißer Main	WM	50°07' N; 11°60' E	22	2004	spawners	wild
Main	Erf	Erf	49°42' N; 09°16' E	15	2000	adults/mixed	wild
Rhine	Bodensee	BoS	47°34' N; 09°36' E	39	2001	all 1+	hatchery
Rhine	Liechtenstein BK	LiB	47°08' N; 09°31' E	20	2001	spawners	wild
Danube/Inn	Inn (Gars)	InG	48°09' N; 12°16' E	50	2000	spawners	wild [1]
Danube/Inn	Inn (Innsbruck)	InA	47°04' N; 10°40' E	23	2002	spawners	wild [1]*
Danube/Inn	Saalach	Saa	47°73' N; 12°88' E	30	2005	spawners	stocked [1]
Danube/Inn	Tittmoninger Ache	TiA	48°03' N; 12°46' E	39	1999	spawners	stocked [1]
Danube/Isar	Ammer	Am	47°52' N; 11°09' E	36	2000	spawners	wild [1]*
Danube/Isar	Isar	Isa	48°40' N; 11°73' E	33	2000, 2001	adults/ 1+	stocked [1]*
Danube/Isar	Sempt	Sem	48°23' N; 11°55' E	39	1999	spawners	stocked [1]
Danube/Isar	Ramsach	Ram	47°40' N; 11°12' E	39	1999	spawners	wild [1]
Danube/Iller	Iller	Ile	47°57' N; 10°22' E	34	2005	adults/mixed	stocked [1]*
Danube/Lech	Lech	Lec	47°49' N; 10°53' E	20	1999	spawners	wild [1]
Danube/Naab	Fichtelnaab	FiN	49°48' N; 12°09' E	25	2002	adults/mixed	wild
Danube/Naab	Waldnaab	WaN	49°47' N; 12°01' E	10	2002	1+ and 2+	wild
Danube/Regen	Schwarzer Regen	ScR	48°58' N; 13°07' E	32	1999, 2000	spawners, 0+	stocked [1]*
Danube	Ilm	Ilm	48°40' N; 11°22' E	25	1999	spawners	stocked [1]*

Table 5.2: Summary information on genetic diversity indices of the studied European grayling populations (see Table 5.1 for population codes).

Pop	MtDNA									Microsatellite loci							
	n	No. Hap.	No. Lin.	Lineage Frequency I	IIa	IIb	III	Hap. div.	Nucleo. div.	*n*	*A*	A_R	A_{pr}	H_E	H_O	P_{HW}	*LD*
Ege	36	1	1	0.00	0.00	1.00	0.00	0.000	0.000	38	2.6	2.4	3	0.29	0.29	*	n.s.
Tep	19	1	1	0.00	0.00	1.00	0.00	0.000	0.000	20	2.8	2.6	0	0.31	0.30	n.s.	n.s.
Ssa	20	3	2	0.00	0.45	0.00	0.55	0.631	0.074	20	3.4	3.0	0	0.49	0.53	n.s.	* (1)
Lam	13	3	2	0.00	0.54	0.00	0.46	0.579	0.072	13	3.1	2.8	1	0.45	0.52	n.s.	n.s.
Vol	28	1	1	0.00	0.00	1.00	0.00	0.000	0.000	29	3.3	2.6	2	0.35	0.35	n.s.	n.s.
FrS	15	2	2	0.00	0.66	0.33	0.00	0.460	0.043	15	2.8	2.7	0	0.45	0.42	n.s.	n.s.
Sin	38	3	3	0.00	0.89	0.08	0.03	0.195	0.016	39	4.2	3.2	3	0.41	0.39	n.s.	* (1)
Lei	30	3	3	0.00	0.90	0.03	0.07	0.188	0.018	30	3.5	2.8	1	0.26	0.22	***	** (2)
WM	22	1	1	0.00	1.00	0.00	0.00	0.000	0.000	22	1.9	1.9	0	0.26	0.25	n.s.	n.s.
Erf	15	2	2	0.00	0.87	0.00	0.13	0.239	0.018	15	4.1	3.7	0	0.51	0.46	*	n.s.
BoS	38	2	1	0.00	1.00	0.00	0.00	0.366	0.009	39	2.4	2.2	0	0.23	0.21	n.s.	n.s.
LiB	20	5	2	0.00	0.80	0.00	0.20	0.656	0.049	19	4.6	4.1	1	0.51	0.55	n.s.	n.s.
InG	50	5	2	0.00	0.04	0.00	0.96	0.621	0.011	49	5.8	3.5	3	0.42	0.40	*	n.s.
InA	23	4	2	0.00	0.09	0.00	0.91	0.244	0.012	23	4.8	3.6	0	0.49	0.55	***	*** (13)
Saa	30	7	4	0.10	0.32	0.02	0.84	0.707	0.057	30	6.3	4.4	2	0.53	0.47	***	*** (22)
TiA	37	9	3	0.14	0.27	0.00	0.59	0.855	0.064	39	6.1	4.3	1	0.54	0.54	*	*** (8)
Am	36	5	2	0.00	0.03	0.00	0.97	0.631	0.012	34	4.2	3.3	0	0.42	0.37	***	*** (2)
Isa	33	4	2	0.00	0.85	0.00	0.15	0.306	0.031	33	3.8	3.1	0	0.42	0.45	n.s.	* (1)
Sem	38	6	2	0.00	0.05	0.00	0.95	0.657	0.026	39	4.1	3.4	0	0.49	0.48	n.s.	n.s.
Ram	39	3	2	0.00	0.03	0.00	0.97	0.498	0.011	39	4.0	3.2	1	0.35	0.34	n.s.	*** (1)
Ile	34	5	2	0.00	0.21	0.00	0.79	0.540	0.038	33	4.4	3.4	1	0.36	0.34	*	*** (9)
Lec	18	4	2	0.00	0.17	0.00	0.83	0.686	0.026	20	4.8	3.7	0	0.43	0.43	n.s.	n.s.

Table 5.2 (continued):

Pop	MtDNA									Microsatellite loci							
	n	No. Hap.	No. Lin.	Lineage Frequency I	IIa	IIb	III	Hap. div.	Nucleo. div.	*n*	*A*	A_R	A_{pr}	H_E	H_O	P_{HW}	*LD*
FiN	25	4	2	0.00	0.56	0.00	0.44	0.621	0.051	25	3.3	2.5	1	0.36	0.33	n.s.	n.s.
WaN	10	4	2	0.00	0.50	0.00	0.50	0.695	0.062	10	2.4	2.7	0	0.43	0.51	n.s.	n.s.
ScR	30	6	3	0.03	0.00	0.40	0.57	0.755	0.119	32	6.9	4.5	2	0.59	0.58	*	*** (2)
Ilm	25	4	2	0.00	0.52	0.00	0.48	0.702	0.064	25	4.3	3.5	0	0.52	0.46	*	*** (2)

MtDNA: sample size (*n*), number of haplotypes (No. Hap.), number of lineages (No. Lin.), frequency of lineages, and haplotype and nucleotide diversity (div.) per population. Microsatellites: sample size (*n*), average number of alleles/locus (*A*), mean allelic richness (A_R) per population, number of private alleles (A_{pr}), expected (H_E) and observed (H_O) heterozygosities, deviations from Hardy-Weinberg equilibrium (P_{HW}), linkage disequilibrium (*LD*) and number of locus pairs involved (in brackets). Significance after application of Bonferroni corrections (* $p < 0.05$; ** $p<0.01$; *** $p < 0.001$; n.s., not significant).

5.4 Methods

5.4.1 DNA isolation and genetic analysis

Genomic DNA was isolated according to the simplified method of Laird *et al.* (1991).
Mitochondrial RFLP analysis was performed on two polymerase chain reaction (PCR) amplified segments encompassing the NADH-1 (ND-1) and NADH-5/6 (ND-5/6) gene regions. These mtDNA regions were selected because they allowed in previous studies to reveal major evolutionary lineages of grayling in Europe (Koskinen *et al.,* 2000; Gum *et al.,* 2005). Details of PCR amplification, RFLP analysis, sequencing mtDNA haplotypes and lineage designation, and phylogenetic analysis are given in Gum *et al.* (2005).
A total of 12 microsatellite loci were analysed which have been used in earlier population and phylogeographic genetic studies on grayling (Gum *et al.,* 2005): BFRO004, BFRO005, BFRO006, BFRO007, BFRO009, BFRO010, BFRO011, F43, Ocl8, Ogo2, One9 and SSOSL311. Original references for the microsatellites and details of PCR and genotyping procedures are outlined in Gum *et al.* (2003).

5.4.2 Data analysis

For nuclear DNA markers, the FSTAT v. 2.9.3 program package (Goudet, 2001) was used to calculate allele frequencies and to estimate the expected and observed heterozygosities (H_E, H_O), the number of private alleles (A_{pr}) and the allelic richness (A_R). GENEPOP v. 3.3 (Raymond & Rousset, 1995a) was used to test genotypic distributions for conformance to Hardy-Weinberg (HW) expectations, and to test the loci for genotypic disequilibria. All probability tests were based on the Markov chain method (Guo & Thompson, 1992; Raymond & Rousset, 1995b) using 1,000 de-memorization steps, 100 batches and 1000 iterations per batch. The data were assessed for the potential genotyping errors, such as null alleles, short allele dominance (large allele dropout), or scoring errors, by using the computer program MICRO-CHECKER 2.2.3 (Van Oosterhout *et al.*, 2006). To better visualise the admixed populations among the drainages of the Danube, Rhine/Main and Elbe, a multidimensional scaling analysis was performed (Kruskal & Wish, 1977) with STATISTICA v. 6.0 (StatSoft, Inc.) based on the matrix of pairwise F_{ST} values which were calculated by FSTAT. This analysis permits the genetic relationships among populations to be represented with minimum loss of information. The optimal number of dimensions (two for our data set) was chosen

based on the plot of stress values against different numbers of dimensions (scree plot available upon request).

The presence of intra-population and inter-population genetic structure was tested using the model-based clustering method of Pritchard *et al.* (2000) implemented in STRUCTURE v. 2.1. This analysis was used (i) to assess if there is indication for the presence of multiple gene pools within populations that can be linked to contemporary phenomena or sampling, and (ii) to infer the uppermost hierarchical level of population genetic structure (or clusters, respectively) represented by the whole data set. For the first approach, the Markov chain Monte Carlo (MCMC) scheme was run with a burn-in period of 50,000 steps and a chain length from 250,000 to 500,000. Multiple runs were performed to assess convergence of ln $\Pr(X \mid K)$, and the number of gene pools present within each sampling site was then determined from posterior probabilities of K estimated using a uniform prior on $K = \{1,2,3,4\}$. As recommended by Pritchard *et al.* (2000), a visual inspection of estimated values of Q (the membership coefficient for each individual into each population) was performed in all cases, i.e., when estimates of Q showed a uniform distribution ($Q \sim 1/K$) it was concluded that a value of $K = 1$ was an appropriate model for the data. For inferring the uppermost hierarchical structure of the sampled distinct gene pools an *ad hoc* statistic ΔK (based on the rate of change in the log probability of data between successive K values) was used according to Evanno *et al.* (2005). Ten independent runs were performed (burn-in 15,000 steps, sampling iterations 150,000, different degrees of admixture allowed, correlated allele frequencies) for each value of K (number of populations) between 1 and 20 [($K \geq 20$ resulted in a strong increase of variance of mean Ln(K)]. The most likely value of K is the one where the change of likelihood from one value to the next drops considerably and consecutive values tend to plateau.

Individual immigrant ancestries were estimated by the Bayesian method of Wilson & Rannala (2003), implemented in BAYESASS v. 1.3., which relies on MCMC techniques. One key advantage compared to earlier methods for detecting recent immigrants is that BAYESASS requires fewer assumptions and allows genotype frequencies to deviate from Hardy-Weinberg equilibrium proportions within populations (indeed, this is often observed in populations affected by human activities). Optimal program settings were determined initially in respect to the total likelihood by repeatedly running the software BAYESASS v. 1.3. using different delta values (0,05; 0,10; 0,15) of migration rate and the level of inbreeding (F), respectively. To

estimate the posterior probability distribution of parameters, the MCMC chain was run for a total of 3,000,000 iterations, discarding the first 1,000,000 iterations as burn in (testing iterations of 30,000,000 did not change the overall results). Samples were collected every 2000 iterations. Posterior probabilities of each individual immigrant ancestry were estimated for each population with the aim to identify non-immigrants, first generation immigrants and offspring of immigrants and non-immigrants. In this study the latter group is termed 'second generation immigrants' (see Fig. 5.4). It should be noted that the method basically allows to detect more distant migrant ancestries dating back several generations ago (see Wilson & Rannala, 2003; B. Rannala, pers. communication). However, the current implementation of BAYESASS does not use information from migrants arriving more than two generations ago to estimate *m* (B. Rannala, pers. communication). In our data set, the natural migration has been extremely limited between most population pairs over the last century (except for Lam/Ssa, FiN/WaN, Saa/TiA) due to numerous impassable weirs and dams within each river system. Therefore, recent immigration rates in our material are expected to primarily reflect stocking effects.

In order to discriminate between the historical and contemporary (i.e., stocking) gene flow, only individuals with a posterior probability > 90% of being non-immigrants were used to re-evaluate the proportion of different mtDNA lineages within populations. This should more reliably reflect the level of admixture of divergent mtDNA lineages due to historical secondary contact. In addition, STRUCTURE was used to specifically assess the level of admixing between drainages Danube, Rhine/Main and Elbe before and after exclusion of the identified immigrants. Individual admixture coefficients for each population were calculated assuming a model of $K = 3$ (representing the uppermost hierarchical structure, i.e. the evolutionary lineages) and using the same running parameters as outlined above for $K = \{1\text{-}20\}$.

Fig. 5.2 (shown on the following page): Two-dimensional scaling analysis based on 12 microsatellite loci and pairwise estimates of F_{ST} among populations. Shown is the result **a) before** ($N = 730$) and **b) after** the exclusion of recent immigrants as identified by BAYESASS ($N = 532$). Symbols indicate the geographical origin of samples: Danube (rings), Rhine/Main (triangles), and Elbe (squares). Filled symbols indicate populations that possess different mtDNA lineages and * next to code indicates where stocking of grayling originating from different drainages was documented at least once.

Fig. 5.2 a)

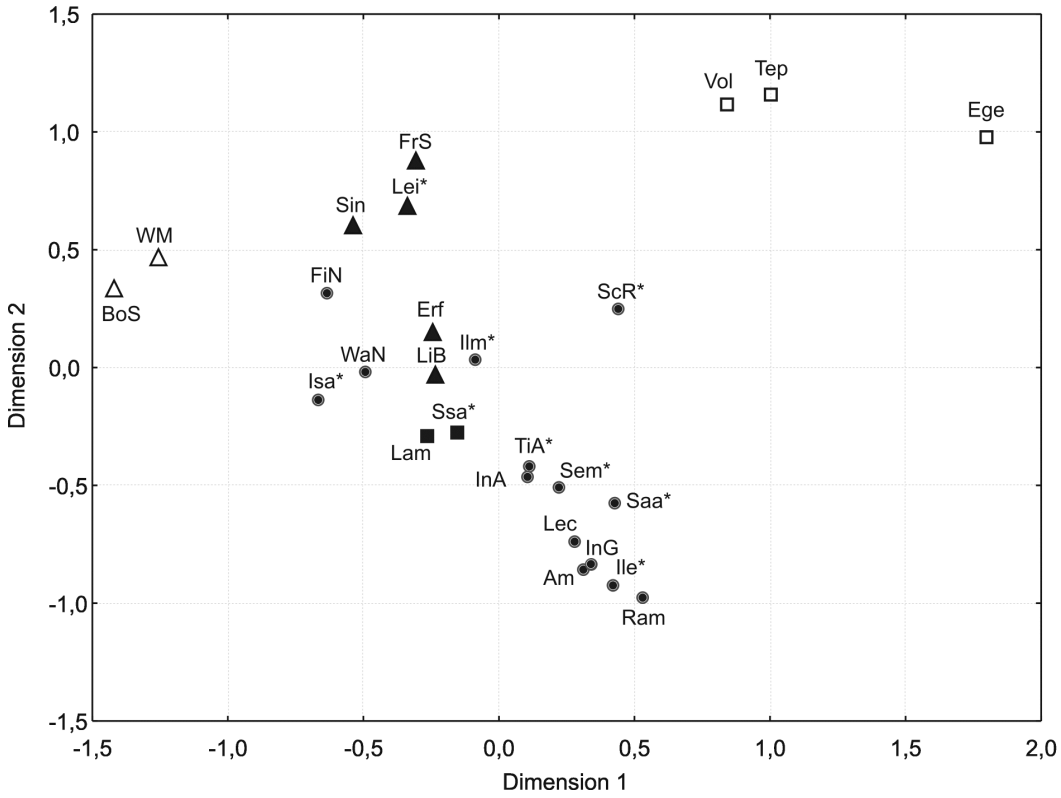
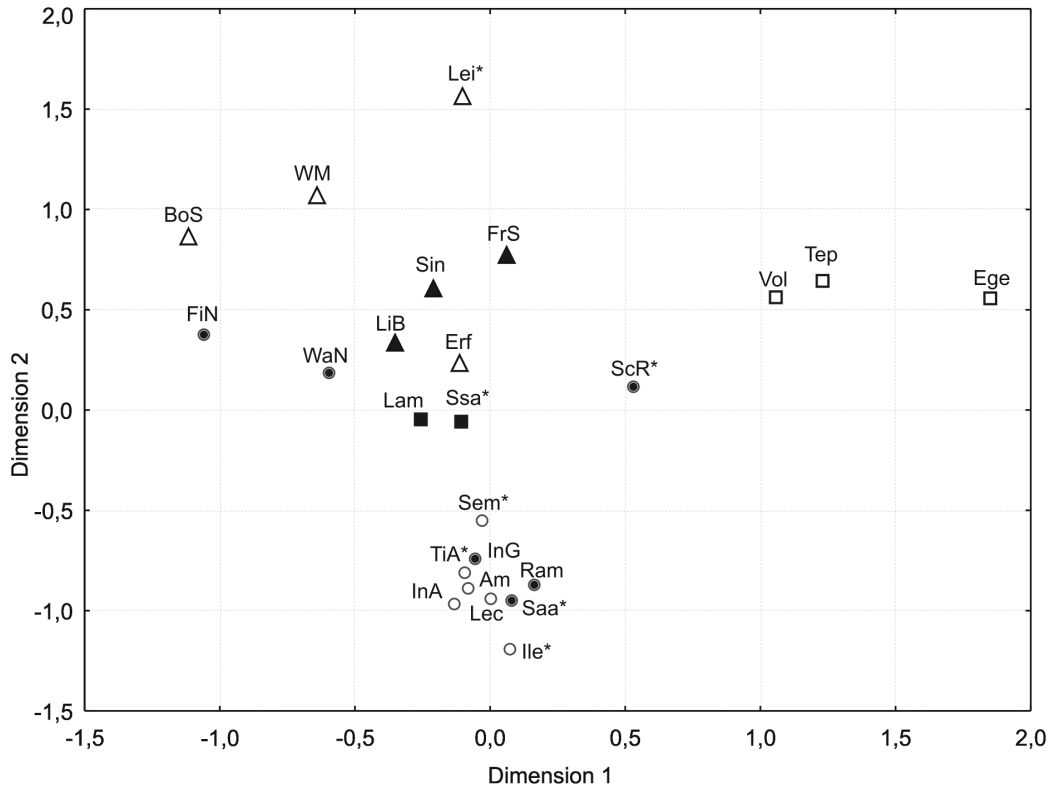


Fig. 5.2 b)



5.5 Results

5.5.1 MtDNA diversity and geographical distribution of mtDNA haplotypes

Previous phylogenetic analyses clustered the mtDNA haplotypes of European grayling into three major lineages I, II and III (Koskinen *et al.*, 2000; Gum *et al.*, 2005). Lineage I was confined to northern Europe, lineage II to central Europe and lineage III was denoted as the Danubian lineage. Gum *et al.* (2005) suggested a further division of central European lineage II into lineage IIa (predominantly found in Rhine/Main drainage in western Europe), and IIb (predominantly found in the Elbe drainage and in eastern, north-eastern Europe). Figure 5.1 shows the geographical distribution of lineages IIa, IIb and III in the studied region and the variable proportions of the lineages within populations (see also Table 5.2). Especially in the contact zones of tributaries to the upper Danube, upper Main and Elbe, populations such as FiN, Lam, WaN and Ssa (area of the Fichtelgebirge) or ScR exhibited about 50% of haplotypes belonging to different lineages (Table 5.2). Mitochondrial RFLP analysis revealed three new composite haplotypes (two belonging to lineage IIa and one to lineage III) for populations Ilm (abccaeicaabba, designation according to Gum *et al.,* 2005), WM (abcdaeidaebba), and Saa (hcbaadbdabbha). Sequencing of the ND-5 region of these haplotypes revealed that these new RFLP haplotypes in Ilm and WM were identical to the sequence haplotypes Cw6 and Cw7, respectively (see Gum *et al.,* 2005, Table 5.2), and the one found in the Saa population resulted in a new ND-5 sequence haplotype designated as DAN05 (GenBank Acc. no. DQ643256). The highest diversity values (Table 5.2) and the highest numbers of haplotypes within the Danubian drainage were found in populations TiA (9), Saa (7), ScR (6) and Sem (6), within Rhine/Main in LiB (5) and within the Elbe drainage in Ssa (3) and Lam (3). Populations Ege, Tep, Vol, and WM were fixed for a single haplotype.

5.5.2 Microsatellite DNA variation within and between samples

The estimates of genetic variability for each population are summarized in Table 5.2. A total of 140 alleles with an average of 11.7 alleles per locus were observed across the 12 microsatellite loci, ranging from 3 (*SSOSL311*) to 23 (Ogo2) (genotypic table and allele frequencies available upon request). Across all populations, the average number of alleles per locus (A) was 4.0, the average mean allelic richness (A_R) was 3.2 and the average expected heterozygosity (H_E) was 0.42. Grayling populations historically more strongly influenced by

human caused stocking activities displayed a significant higher ($p < 0.05$; two-sided test of the null hypothesis of no difference) level of genetic diversity (average $A_R = 3.7$ and $H_E = 0.46$) than populations without documented stock transfers (average $A_R = 2.8$ and $H_E = 0.36$). A total of 21 private alleles were found in 13 of 26 populations. The highest number of unique alleles (3) were found in populations Ege, Sin and InG. Allelic dropouts in the data set would be expected to cause Hardy-Weinberg deviation due to excess of false homozygotes; however, no evidence of short allele dominance or large allele dropout was detected at any of the 12 polymorphic microsatellite loci analysed. Results based on the test by Van Oosterhout *et al*., (2006) indicated potential presence of null alleles only in one population (Ilm) for the highly polymorphic loci BFRO010 and Ogo2 (with the combined probability of all allele size classes at each locus being not significant, $p > 0.05$). As there was no evidence of null alleles present in all other populations investigated it can be assumed that not technical problems but rather the within population genetic structure of samples was responsible for the observed deviations in Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium (LD). Significant deviations from the expected HW proportions were observed in 11 populations (deficit of heterozygotes) and probability tests of LD were significant for 12 of the 26 populations after applying sequential Bonferroni corrections (Table 5.2). After removing first and second generation immigrants (as identified by BAYESASS, see below) for populations Lei and Saa and through separate analysis of sub-groups sampled from different river stretches for Am and InA, respectively, highly significant values for deviations for both HWE and LD disappeared. A Wahlund effect due to presence of different cohorts (through stocking and/or other reasons such as sampling of different year classes) is likely to explain the deviations in the remaining populations such as Ilm, Ile, ScR, TiA or ScR.

5.5.3 Microsatellite divergence and admixture analysis

On the two-dimensional plot of microsatellite-based F_{ST} values, the populations with high frequency of haplotypes from different mtDNA lineages (WaN, FiN, Isa, LiB, Ilm, Lam, Ssa, Erf and ScR) and/or populations where stocking has occurred from different drainages (Lei, Isa, Ilm, ScR, Ssa, TiA, Sem, Saa) were located between the 'pure' Elbe, Danube and Rhine/Main populations, indicating their admixed status (Fig. 5.2a).

The log-likelihood of the genotypic data, L(K) and ΔK, as estimated by the program STRUCTURE, have a clear tendency to plateau for $K > 3$ (Fig. 5.3). Thus, a value of $K = 3$ can be considered as the most appropriate model for the uppermost hierarchical structure of the

data. This also fits well with the three major mtDNA lineages that are found in the studied area of the Danube, Rhine/Main and Elbe drainages (Gum *et al*., 2005).

STRUCTURE analysis, when applied separately for each population, provided evidence for multiple gene pools present within 8 sampling sites: Lei, TiA, Saa, and Ilm consisted of 3 gene pools (K = 3) while Isa, InA, Ile, and ScR consisted of 2 clusters (K = 2). As expected, individuals from populations that historically have been more strongly affected by stocking with fish from different origins (e.g. Isa, Ilm, TiA or Saa) did not form a homogenous gene pool but were unequally distributed over 2 or 3 clusters, respectively. On the other hand, individuals of typical contact zone populations such as FiN or Ssa were also subdivided over 2 clusters but shared about equal proportions of individual admixture coefficients between these clusters (data not shown).

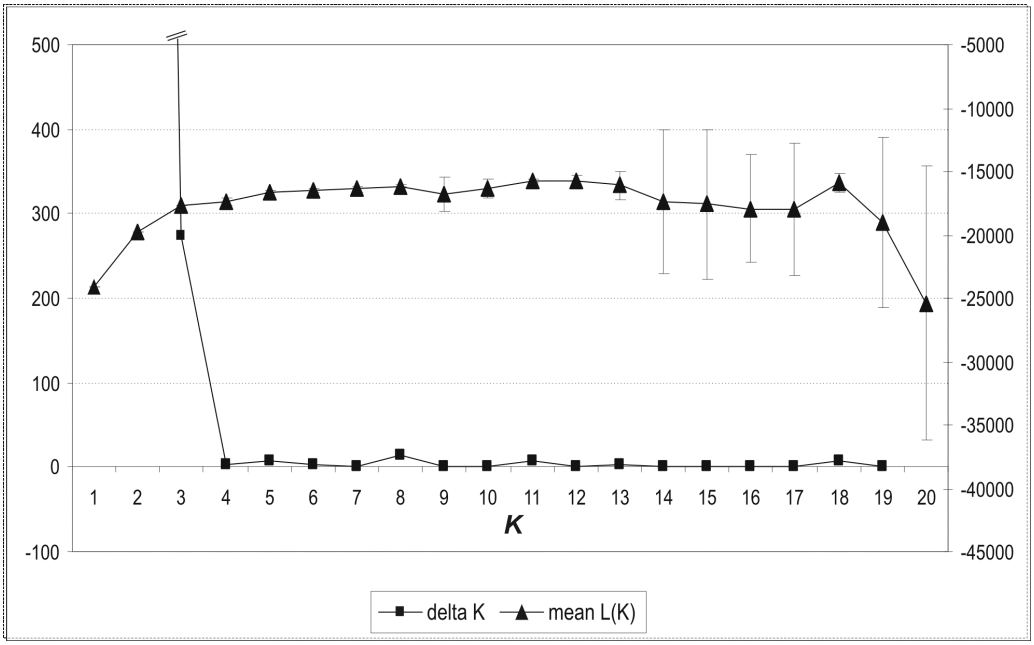


Fig. 5.3: Inference of genetic clusters (K) of the studied grayling populations based on the STRUCTURE algorithm. Mean (± SD) of log likelihood values (10 independent runs), L(K), and the rate of change in the log probability of data between successive values of K, delta K for the whole data set (N = 730). Delta K value for K = 2 (= 3258) not shown on graph due to scale constraint).

5.5.4 Individual immigrant ancestries

Analysis of individual ancestries identified a majority of individuals in most populations as being 'non-immigrants' (Fig. 5.4). However, some populations, especially those defined as 'stocked' in Table 5.1 (e.g. Isa, Ilm, TiA or Saa) showed a substantially higher proportion of individuals with recent immigrant ancestry. In population Ilm, a high number of both introgressed individuals as well as first generation immigrants was identified and only 1 of the 25 individuals remained non-immigrant with > 90% posterior probability (Fig. 5.4). This result certainly is promoted by the fact of forced (i.e., human caused) mating of local and stocked fish that were performed at a local hatchery. A similar scenario has to be proposed for populations TiA, Saa or Sem with the same hatchery involved to carry out the breeding programme. In population Isa, even all sampled grayling were identified as first and second generation immigrants (Fig. 5.4). In populations Lei, Ile, and Saa several individuals (3, 8, and 11, respectively) were identified as first/second generation immigrants and this was in agreement with the STRUCTURE analysis which assigned the same individuals to another cluster with high confidence (data not shown). In addition, based on mtDNA data, all of these individuals belonged to different mtDNA lineages compared to the majority of individuals sampled from these locations.

5.5.5 Discrimination of historical secondary contact from recent human mediated stock transfer

After removing the individuals with recent immigrant ancestries identified under the Bayesian assumption using microsatellite data, the proportions of different mtDNA lineages in the studied populations (except Isa where all individuals were identified as recent immigrants) were re-evaluated (Fig. 5.5a). Substantial changes in the mtDNA lineage composition mainly occurred in Danubian populations which have been more strongly affected by stocking activities (TiA, Ilm, Saa and Ile), but also in some populations where only supportive breeding was known (InA, Lec, Am and the Main population Lei; Fig. 5.5a). With exception of the Erf, the proportion of different mtDNA lineages changed only little in the other populations. Therefore, the high level of admixture of mtDNA lineages which was retained in populations originating from contact zones of the drainage systems (such as FiN or Ssa) can be most probably explained by historical secondary contact. A substantially lower proportion of admixture was retained in populations from other sampling sites designated in Table 5.1 as 'wild' (e.g. Sin, InG, Ram). Though a substantial number of first and second generation

immigrants were removed from populations LiB and ScR (12 and 14, respectively) only small changes in mtDNA lineage proportions were observed. Considering that ScR has been affected by stocking and/or supportive breeding, this suggests that the population obviously possessed divergent mtDNA lineages already prior to the recent human mediated transplantations.

A comparable result was obtained for microsatellite data as indicated by the estimates of population admixture coefficients using STRUCTURE (see Fig. 5.5b). If partitioned into $K = 3$ clusters according to the three drainages systems, again several populations showed clear changes in their cluster composition after removing of individuals with recent immigrant ancestries. Concerning pairwise estimates of F_{ST} based on the purified data set, the southern Danubian populations more clearly formed a separate group than before (Fig 5.2b). In addition, samples Lei and FiN (3 and 4 individuals removed, respectively) obtained a different position compared to figure 5.2a while no considerable change was observed for the remaining populations.

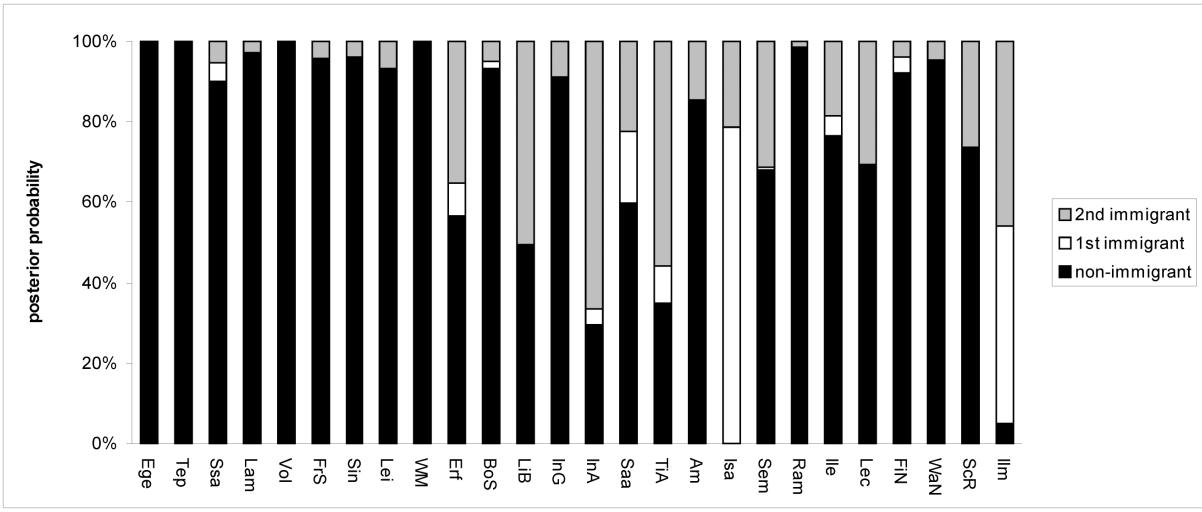


Fig. 5.4: Histogram of posterior probabilities of individual immigrant ancestries summarized for all grayling populations as determined by BAYESASS 1.3 (population grouped by sub-drainage system). Proportion of individuals assigned to its source population are denoted 'non-immigrant' (in black), proportion of an immigrant from a specific population denoted '1st immigrant' (in white) and proportion of an offspring of an immigrant and a non-immigrant denoted '2nd immigrant' (in grey).

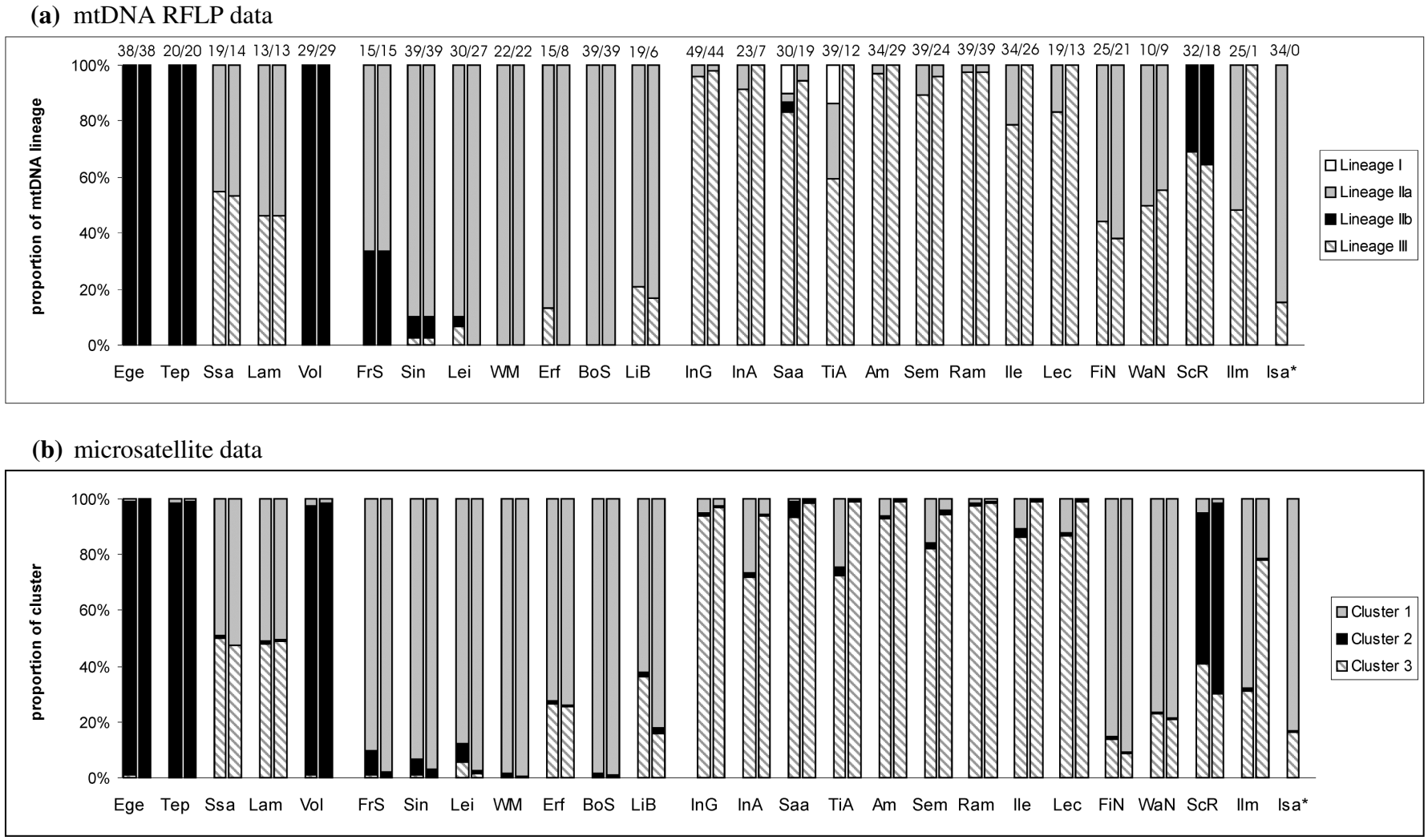


Fig. 5.5: a) Proportions of major mtDNA lineages I, IIa, IIb, and III within European grayling populations from Bavaria, and **b)** estimates of population admixture coefficients using STRUCTURE (K = 3). Shown are the proportions before (left bar) and after (right bar) the exclusion of recent immigrants and/or offspring among local and immigrant fish as identified by BAYESASS (see Fig. 5.2). The sample size before and after exclusion of individuals is given above each bar in Fig. 5.5a. *Only the left bar for population Isa is shown because all individuals were identified as immigrants or introgressed with > 90% probability and consequently excluded.

5.6 Discussion

In this study, the combined analysis of nuclear and mtDNA data allowed to estimate the posterior proportions of divergent mtDNA lineages within grayling populations under different fisheries management regimes, i.e. to assess the level of historical secondary contact by excluding recent immigrants and introgressed individuals from phylogeographical analysis. In agreement with studies on related species such as the brown trout (*Salmo trutta* L.), the present work showed that mtDNA data alone can only indirectly serve as an indicator of introgression (e.g. Weiss *et al.,* 2001; Duftner *et al.,* 2005), and do not allow discrimination between the historical and contemporary gene flow between the populations. Analysis of highly variable nuclear markers in conjunction with new statistical developments, however, has proved to be a valuable tool for identifying recent immigrants (reviewed in Hansen *et al.,* 2001b). Most significantly, it has improved the possibilities for analysing admixtures between stocked and indigenous fishes at the species, population and individual levels (e.g. Primmer *et al*., 2000; Sušnik *et al.*, 2004; Hänfling *et al.*, 2005). Admixture analysis such as the STRUCTURE approach (Pritchard *et al.,* 2000) and the analysis of posterior probabilities of individual immigrant ancestries (BAYESASS, Wilson & Rannala, 2003), however, is dependent on the following conditions (Choisy *et al.,* 2004,): (i) a high degree of differentiation between populations (high F_{ST} values), (ii) a high ratio of native to stocked fish within populations, and (iii) a recent hybridisation event accomplished with a well documented management (stocking) history. Simulation analyses further showed that a larger number of loci (20) reduce bias and the mean square error, and that the posterior probability of each individual immigrant's ancestry can only be estimated with high confidence over the last few generations (Wilson & Rannala, 2003). For the European grayling populations studied, the above proposed necessary premises generally are fulfilled: first, a high level of differentiation is common for grayling in central Europe (average F_{ST} = 0.36; Gum *et al.,* 2005) even between hydrologically connected sampling sites (Koskinen *et al.,* 2002a; Gum *et al.,* 2003); second, natural migration of populations among geographically adjacent tributaries has been virtually impossible during the last century due to human caused habitat fragmentation (in Bavaria alone *c*. 9000 migration barriers such as hydropower stations or dams), and third, enhancement motivated stocking and supportive breeding has been carried out more intensively only for the last two decades.

An important constraint of the applied Bayesian method to discriminate between the historical secondary contact and recent stocking events certainly is the fact that individual immigrant ancestries can only reliably be estimated over the last several generations (Wilson & Rannala, 2003). The present approach, however, is still applicable and valid for the populations where removing of recent immigrants results in the non-admixed sample, as estimated by the proportion of different mtDNA lineages (e.g. populations Ile, Saa, Lei, Lec in our data set; see Fig. 5.5a). Of course, without the availability of historical baseline samples (e.g. scale samples; Nielsen *et al.,* 1997; Koskinen *et al.*, 2002b) or knowledge of the genetic composition of stocked fish (Ruzzante *et al*., 2004; Sušnik *et al*., 2004) safe inferences about the genetic structure of populations can only be extended to few generations ago and they critically depend on the known stocking history. Keeping these limitations in mind the present study on grayling showed that the recent advances in data analysis, such as Bayesian inference (Beaumont & Rannala, 2004) offer great opportunities for a better understanding of the effects of human activities on the genetic structure of wild populations.

5.6.1 Genetic impact of stocking on genetic structure of European grayling

As inferred from the admixture analysis and the posterior probabilities of individual immigrant ancestries, the studied European grayling populations fall into several overlapping categories: 'pure' populations (e.g. Sin, WM, Ege, Ram), several populations with a minor proportion of individuals with recent immigrant ancestries (e.g. Ssa, Lei, FiN), and several populations exhibiting relatively high levels of introgression (e.g. Isa, Ilm, InA, TiA). Even if natural interbreeding would play a minor role (possibly due to different spawning times or selection against hybrid offspring), admixture of exogenous and indigenous gene pools will occur as a result of forced mating through artificial reproduction. In the present study there was a good accordance between the results of genetic analysis and stocking records. For example, it is known that exogenous material of different origin has been introduced into the populations of Ilm, TiA or Saa and a major proportion of spawners from these small river stretches are caught and transferred to a hatchery each year for artificial reproduction. The results of genetic analysis confirmed this by revealing that the vast majority of individuals in these populations were either recent immigrants or introgressed. On the other hand, the study also showed that Bavarian European grayling populations, which have been enhanced by supportive breeding of local stocks (e.g. InG, Ram) and some exogenous material (e.g. ScR, Saa), have resisted the complete erosion of native genes although the documented stocking

procedures could be traced genetically. In Finland, Koskinen *et al.*, (2002b) studied the genetic and ecological impact of stocking on grayling populations, and similar to the report by Hanfland (2002), the stocked individuals exhibited poor survival and reproductive success compared to indigenous grayling. In contrast, Sušnik *et al*. (2004) showed that ongoing stocking of grayling originating from the Danube can lead to a loss of at least 50% of the native Adriatic type of grayling in Slovenia. This observation is in line with the extent of introgression found for some southern Danubian populations of the present study. The unpredictability of the genetic impact of salmonid stocking activities was already pointed out by Hindar *et al*. (1991). The massive introduction of stocking exogenous genetic material into autochthonous gene pools can result in (at one extreme) the displacement of indigenous stocks, negative consequences on the genetic structure of local fish due to interbreeding with cultured fish, increased hybridisation rates with closely related species (Gross *et al.*, 2004), or (at the opposite extreme) no detectable introgression into indigenous gene pools despite substantial introductions (Hindar *et al*., 1991). Moreover, stock transfer often leads to competition and genetic effects on performance traits such as stock size. Indirect effects may implicate earlier spawning leading to little recruitment or cultured fish being maladapted to the environment in which they were released and reduced juvenile survival. Among the direct effects is 'contamination', i.e. introductions of pathogens, for example the threat of Atlantic salmon (*Salmo salar* L.) from Norway through the transfer of Baltic salmon and its associated parasite, *Gyrodactylus salaris* (Bakke *et al.*, 1990).

Important factors that determine the level of impact are the number of stocked fish relative to the native population size (immigration rate) and the selection against the introduced fishes. Fritzner *et al.*'s (2001) study on brown trout concluded that even heavily stocked brown trout populations may be affected substantially less by hatchery trout than anticipated and should not immediately be given low conservation priority. Similar observations by Hansen (2002) shows that even heavily stocked salmonid populations may contain surprisingly low proportions of their gene pool derived from domesticated fishes, as in this case strong selection pressure may be act against the stocked domesticated fish. Angling was also shown to be an important factor influencing the genetic structure of fish populations and their management. Mezzera & Largiader (2001) demonstrated that 'selective angling, in combination with a small legal catch size may have considerably eliminated introduced brown trout and hybrids before spawning at the study sites, and thus may have reduced the introgression of alien genes into the local gene pool.' On the other hand Fleming *et al.* (2000)

investigated the lifetime success and interactions of farmed Atlantic salmon invading a native population and their results indicated that such annual invasions have the potential for impacting population productivity, disrupting local adaptations and reducing the genetic diversity of wild salmon populations.

To sum up, a general rule for success and sustainability of stocking programmes is hard to find, but supportive breeding seems most promising for enhancement and conservation purposes (Wang & Ryman, 2001).

5.6.2 Conservation priorities

In general, the European grayling populations studied in Bavaria serve as a good argument for the necessity of conducting genetic screening of stocked fish populations before making decisions about their conservation priority and status. It cannot be assumed *a priori* that offspring of stocked populations descend solely from hatchery fish and even heavily stocked populations may still contain indigenous genetic material of value for future conservation and restoration efforts. It would, therefore, be poor management to completely exclude some areas or rivers from conservation efforts and management, without determining whether the introgression is natural or anthropogenic and to what extent the native gene pool is still existing (see review by Allendorf *et al.*, 2001).

As outlined above, for economical and ecological reasons, the stocking of cultured (domesticated) fishes is consistently considered detrimental when self-reproducing, indigenous populations are still present (Hindar *et al*., 1991; Allendorf *et al.*, 2001). It must be stressed, however, that there is a large difference between domesticated strains and offspring from spawners caught in the wild and stocked back to their natal rivers (Lynch & O'Hely, 2001). Domesticated strains as typically produced for rainbow trout and Atlantic salmon are hatched over several generations in artificial environments. Specifically, these individuals often exhibit unnatural behaviours, especially concerning their hiding response and antipredator behaviour (Hindar *et al*., 1991; Cross, 1998). No comparable form of domestication has been achieved for grayling in Bavaria yet, despite considerable efforts to create grayling brood stocks in a few hatcheries (M. Hermann, pers. communication). Fortunately, the remaining native gene pools of grayling are currently not threatened by potential extensive releases of offspring from electrofished local or non-local grayling as many local fishing authorities have actually stopped stocking of grayling due to the ongoing cormorant and goosander debate (Cowx, 2003; Behrens *et al.*, in press). Until this problem is

solved, the establishment of captive breeding programmes and more intensive supportive breeding actions will remain at a relatively low level (Bavarian fishing association, pers. communication). This reprieve, however, is being challenged by the advocacy of regional fishing authorities and private angling associations for local breeding programmes and the use of offspring from locally caught spawners for future enhancement. In this respect the data of this work can be used for the identification of non-introgressed individuals or populations that could be used for supportive breeding.

Given the increasing efforts to establish conservation units for European grayling, the genetic discrimination between natural and human caused admixture is of particular interest and critical value in the contact zones of the Danube, Main and Elbe drainages. As has been shown in the FiN and Ssa populations, *c*. 50% proportion of two highly divergent mtDNA lineages within these populations remained constant after the exclusion of recent immigrants. Therefore, it can be concluded that these zones have been long-standing areas of secondary contact among these lineages over an extended period of time due to glacial perturbations. The area of the lower mountain range of the 'Fichtelgebirge', obviously represents a 'hot spot' of aquatic biodiversity in central Europe. Several evolutionary significant units or management units of both vertebrate (Hänfling & Brandl, 1998; Englbrecht *et al*., 2000; Gum *et al.,* 2005) and invertebrate species such as the critically endangered pearl mussel (*Margaritifera margaritifera* L.) intersect here (Geist & Kuehn, 2005). The general outcome of these studies corroborates the need to change the concept of 'single species protection' towards conservation of ecosystems. In addition, further studies within the framework of functional genomics (Ranz & Machado, 2006) are needed in the studied region to clarify the level of local adaptation of specific units to different environmental conditions.

Based on multilocus genotypic data this study showed that it is possible to discriminate long-term historical gene flow (natural secondary contact) from recent immigration and introgression over the last few generations. Through the application of the latest computational achievements (Wilson & Rannala, 2003) Bayesian inference allowed to identify immigrant and introgressed individuals within European grayling populations that were affected by human stocking activities. In combination with small- and large-scale phylogeographic information of the studied area the present work exemplified a way to separate historical and natural from human caused admixture of divergent mtDNA lineages.

This approach could therefore also be applied to other species influenced by human introductions. The question of whether different mtDNA lineages originate from 'man or nature' can adequately be answered, provided that the system under study still consists of a mixture of the founding subgroups where panmixia has not yet been reached.

Thus, the presently available molecular techniques and statistical methods allow for a comprehensive genetic analysis of wild populations, without requiring an exhaustive knowledge of the management history. It is possible to provide *ad hoc* information on important variables such as the number of subgroups present within populations as well as to determine the level of interbreeding and historical gene flow. In this regard the results provide the basis for future management and conservation efforts of European grayling in Bavaria.

6 General Discussion

The unifying concept of the papers included in this thesis is the application of microsatellite and mitochondrial DNA markers for studying the genetic structure of European grayling populations. Until now there are comparatively few studies available that are based on the simultaneous employment of marker sets differing in their evolutionary dynamics in the framework of a conservation genetic approach (e.g. Lu *et al.*, 2001; Barulenga *et al.*, 2006). However, as exemplified in this thesis, the application of two different types of molecular genetic markers are a powerful approach to disentangling both major colonisation events (Gum *et al.*, 2005), and subsequent, more recent mechanisms of population differentiation (Gum *et al.*, 2003, 2006) as well as assessing the genetic effects of recent human manipulations (Gum *et al.*, 2006).

In the following pages, I first discuss briefly some important characteristics of the applied molecular genetic markers and give a short overview about potential analytical and statistical assets and drawbacks. Second, I recapitalise the results that we have obtained within the presented case studies of this work and put the results into a broader population genetic and phylogeographical context. Next, the significance of this research, i.e. in particular the contributions of conservation genetics to the protection and sustainable management of grayling are discussed. Finally, I highlight possible future fields of research for the studied species with special attention to functional genomics and evolutionary biology and sum up the general conclusions reached through the work.

6.1 Application of mitochondrial and microsatellite DNA markers in conservation genetics: general considerations

6.1.1 Mitochondrial DNA

Mitochondrial DNA is the classical genetic marker for phylogeographical studies (e.g. Avise, 1994). The first phylogeographical studies undertaken in the late 1970s and early 1980s used mtDNA as a genetic marker (e.g. Avise *et al.*, 1979) and until today this has remained the preferred molecular marker for testing bio-geographical hypotheses (Avise, 2000). How does this come?

In contrast to nuclear DNA in eukaryote organisms mtDNA is haploid, maternally inherited, (mainly) non-recombining and (mainly) selectively neutral. In addition, the mutation rate in some regions of the mtDNA molecule is higher than in most nuclear DNA regions (Birky *et al*., 1989). However, during recent years some of the features of mtDNA, that were generally taken for granted, have been questioned. This includes features such as selective neutrality (reviewed by Ford, 2002) and its lack of recombination nature (Rokas *et al.*, 2003). Deviations from the latter assumption may lead to severe biases in phylogeny reconstruction (e.g. Schierup & Hein, 2000). The fact that mitochondrial DNA is haploid and maternally inherited has some very important implications for its use as a genetic marker in population studies. The effective population size is only about ¼ that compared to nuclear loci. Therefore, mtDNA is subject to more drift than nuclear loci. Indeed there are some examples showing that the genetic structure is stable on the level of nuclear loci, but temporally unstable on the level of mtDNA (e.g. Hansen & Loeschke, 1996b). The stronger effect of drift also results in a higher level of genetic differentiation when compared to nuclear DNA (Birky *et al.*, 1989). Because mtDNA is inherited maternally, the latter effect can be enforced if gene flow is male-biased. On the contrary, female-biased gene flow will tend to decrease the ratio of difference between differentiation at nuclear loci and mtDNA. Therefore, conclusions solely oriented on mtDNA data have to be considered with caution if gene flow is assumed to be sex-biased (Tonteri *et al*., 2005; Ludt & Kuehn, in prep.).

In either case, due to the relatively high mutation rate and the lack or rarity of recombination, it is reasonably easy to reconstruct the phylogeny of different mtDNA haplotypes. Given a sufficient large random sample, it is supposed in a next step, that the phylogeny of haplotypes also reflects population history. This assumption should be treated carefully as well, because consideration must be given to the fact that mtDNA essentially represents a single locus. Thus, it is not possible to average results from several loci.

Keeping the above mentioned reservations in mind, mtDNA remains the most popular and most useful genetic marker for phylogeography (Avise, 2000). In addition, the recent statistical developments such as nested clade analysis (Templeton *et al.*, 1995) have provided sophisticated tools for discriminating between historical and ongoing gene flow and migration, range expansion and secondary contact (Ludt *et al*., 2004; Gum *et al*., 2005).

6.1.2 Microsatellite DNA

Microsatellite loci are very useful genetic markers for population studies. As mentioned initially microsatellites are available now for many species and an enormous increase of conservation and population genetic studies using these markers have been recorded since the early 1990s (see Kühn, 2004). For population genetic approaches in respect to determining the level of genetic differentiation between populations and recently in particular to assess the genetic variability at the individual level, we ideally need a highly polymorphic, selective neutral (i.e. non-coding) marker, underlying recombination. All these essential characteristics generally are fulfilled by microsatellites. The considerably higher variability of microsatellite compared to (former) allozyme based studies resulted in an increased significance concerning individual based approaches, ranging from analysis of kinship and parentage to assignment of individuals to populations, detection of population bottlenecks and the assessment of differences among populations, either natural or artificial (e.g. reviewed by Luikart & England, 1999; Kühn, 2004; this work). These applications in particular are interesting for species being exposed to strong human caused impacts such as in our case, the high degree of translocations and stocking practices of domesticated and hatchery reared fish species (Gum *et al.*, 2006) or anthropogenic overexploitation acting on natural populations.

In the meantime, numerous studies and books (e.g. Goldstein & Schlötterer, 1999) have been published, dealing only with the specific properties of microsatellite evolution such as mutational processes, repeat motifs and the different mutation models (e.g. Slatkin, 1995; Goldstein & Pollock, 1997; Beaumont, 1999; Estoup & Cornuet, 1999). In the methods section of each of the case studies presented here, we briefly explained reasons for choosing certain statistical measures or algorithms such as the Cavalli-Sforza & Edwards (1967) or Nei *et al.*'s (1983) D_A genetic distance (see chapters 3, 4 respectively). It is therefore not my aim at this point to provide a comprehensive review of the most important aspects concerning microsatellite statistical analyses (see also chapter 2.2 in the literature review). For further background information in respect to microsatellite analyses and various statistical approaches that go beyond the scope of this thesis, one is referred to Kühn (2004). This study recently reviewed all major topics of microsatellite analyses and statistical theories, described in detail their application in conservation genetics, explained the different mutation models, and practically recommended how to use specific genetic software for statistical analyses.

However, despite the usefulness of these markers, they also show limitations, as for example, problems associated with the presence of "null alleles" (e.g. Pemberton *et al.*, 1995). This potential pitfall deserves special attention. Null alleles occur when mutations take place in the primer binding site, i.e. not in the microsatellite repeat unit itself. If one of the two primers is unable to anneal satisfactorily to the target DNA, it can happen that the allele with the mutation is not PCR amplified. The presence of an amplified allele and non-amplifying allele is therefore consequently interpreted as a homozygote, whereas a null allele homozygote would be interpreted as an individual which would not amplify, for example due to low quality DNA. Thus the presence of null alleles at a locus can cause severe problems, in particular in individual based analyses such as relatedness estimation and assignment tests. Therefore, most researchers prefer to discard loci exhibiting null alleles or estimate the null allele frequency and adjust visible allele frequencies in populations (e.g. van Oosterhout *et al.*, 2006). Based on the approach suggested by van Oosterhout *et al.* (2006), there was no indication of potential null alleles present among the chosen microsatellite loci for the grayling populations analysed in this study (Gum *et al.,* 2006).

Microsatellite loci generally are recognised as neutral (non-transcribed markers), so that selection and environmental pressure do not influence their expression directly (reviewed by Kühn, 2004; Ellegren, 2000). Therefore, results based on microsatellite data, although often showing a high level of differentiation (e.g. with $F_{ST} > 0{,}15$ as is often observed between grayling populations), basically do not support conclusions drawn in respect to local adaptation of the studied populations. However, baseline genetic data as acquired by microsatellite analysis or other multiple marker assays can be used as a good starting point for testing selective neutrality and for identifying functional DNA polymorphisms (e.g. Vasemägi & Primmer, 2005). Concerning the latter, Beaumont (2005) showed that analyses based on the distribution of estimates of F_{ST} can provide a useful first step for identifying candidate genes that might be under selection, and explored ways in which this information can be used in ecological and evolutionary studies. In addition, recent studies on human (e.g. Payseur *et al.*, 2002), drosophila (Schlotterer *et al.*, 2003) or Atlantic salmon (Vasemägi *et al.*, 2005b) showed that microsatellites loci indirectly can be successfully used to studying processes of natural selection and adaptation, especially if these markers reveal genetic variation that is linked to the locus of an expressed gene.

6.2 Conservation genetics of European grayling

6.2.1 General aspects

Considering the three case studies in this work, it becomes clear that until the year 2000 virtually nothing was known in regard to population genetics or phylogeography of European grayling. What were the motives for the increasing number of genetic studies related to this species in the last years? Among the general reasons certainly are (i) a broadly increased interest of genetic work in the framework of conservation biology, particularly in conservation genetics (see Kühn, 2004), (ii) the threatened status of grayling in many areas of its European distribution, the species is endangered at the population level throughout the Alpine region, (iii) the rapid advancement of molecular genetic techniques concerning the establishment of genetic markers for non-model organisms, especially microsatellites (e.g. Snoj *et al.*, 1999; Sušnik *et al.*, 1999a) or mtDNA genes (e.g. Nielsen *et al.*, 1998) as well as the opportunity to transfer markers from related species to the species of interest (e.g. Koskinen & Primmer, 1999; Gum, 2000), and iv) the improved possibilities to use a few scales or small fin-clips as source material for fish genetic studies allowing to analyse threatened species without killing them. A further reason for the recent increased interest of doing population genetics for this species is grayling specific: it is the presence of the high level of intra-specific genetic differentiation found among grayling populations. In addition, the species' life history, especially its reproductive and strong homing behaviour, as well as the formation of family cohorts and seasonal aggregations, all contribute to the development of pronounced population structures which can be traced genetically (e.g. Koskinen *et al.*, 2001b, Gum *et al.*, 2003).

6.2.2 Salmonid population genetic structure based on microsatellite DNA analyses

Similar to microsatellites as a population genetic marker, salmonid fishes can be regarded as good model organisms for population genetic research and basic evolutionary questions (e.g. Hendry & Stearns, 2004). In particular, through the establishment of distinct spawning groups, they develop discrete populations, a feature assumed in many models of genetic population structure. Apart from the recently increased genetic work on grayling, to the best studied European salmonid fish species based on microsatellite data include the Atlantic salmon (e.g. King *et al.*, 2001), the brown trout (e.g. Hansen *et al.*, 2002), the Artic charr (e.g. Wilson *et al.*, 2004), and the Lake whitefish (e.g. Østbye *et al.*, 2006).

Concerning the relatively strong intra-specific variability among salmonids, the following key factors may help to explain the comparable high level of genetic differentiation observed in salmonid populations: (i) partially or complete geographical isolation of subgroups present in different drainage systems or lakes (i.e. no gene flow since the geological separation of watersheds), (ii) a strong behaviour to stay at or to return to their natal sites (i.e. limited gene flow even within hydrologically connected river systems), (iii) occupation of various types of habitats such as rivers, streams or lakes at different areas and altitudes potentially underlying strong natural selection (i.e. limited gene flow among differentially adapted populations), and (iv) often, a highly different life history cycle found for populations co-existing in the same environment (e.g. the winter/summer runs for pacific salmon or the migratory and sedentary forms of brown trout). Generally the opposite situation is observed for most marine fishes because of the high level of population connectivity over a much larger area represented by a more or less "single huge habitat" due to the continuous nature of the marine environment. For that reason, marine when compared to freshwater fish species, usually exhibit a lower extent of intra-specific variation or in other words, a much lower level of inter-population differentiation and a high genetic diversity at the individual level (see review by DeWoody & Avise, 2000). This observation has generally been hypothesised to be a result of higher effective population sizes and/or higher inter-population migration rates in marine fish species. Without going into detail, the level of genetic differentiation between grayling populations indeed is of a magnitude higher than for most marine fishes like the Atlantic halibut in the North Atlantic (average F_{ST} = 0.0017, Reid *et al.*, 2005; average F_{ST} for grayling from Europe = 0.367; Gum *et al.*, 2005) and also much higher compared to terrestrial vertebrates, for example the roe deer (average F_{ST} for roe deer in Switzerland = 0.01, Kühn *et al.* (in press).

A main objective of this study was to assess the level of genetic structure and differentiation among European grayling populations originating from different central European drainage systems and to infer its defining mechanisms.

Concerning the relatedness of grayling populations from Bavaria, the first study (Gum *et al.*, 2003) revealed a high level of conformity between the genetic relationships and that which could have been expected from the geographical situation: based on the analysis of 20 microsatellite loci the genetic differentiation of populations coincided well with the investigated tributary systems of drainages Danube, Rhine/Main and Elbe.

In addition, the applied microsatellite systems allowed for an effective assessment of the level of genetic variability at three hierarchical stages:

i) within populations, i.e. at the individual level

ii) between populations within drainages, i.e. at the population or MU level

iii) between drainages or major geographical groups, i.e. at the sub-species or ESU level

More precisely, for European grayling the analysis of molecular variance (AMOVA) revealed that about ¼ of the total genetic variation is explained by differences between major drainage systems, about 11-20 % was due to differences between populations within the drainages and about 57-64 % of the variation resided within populations (Gum *et al.*, 2003, 2005). Although variation among individuals within populations obviously is high for nuclear data as a result of the high variability and high heterozygosity of microsatellites, a substantial amount of the genetic variance is still caused by differences among major geographical groups, indicating a clear differentiation between major central European drainage systems. For comparison in the case of Atlantic salmon about 90 % of the genetic variance is explained by within-population variation of salmon populations from Europe and North America (King *et al.*, 2001). The higher individual genetic variance of salmon compared to grayling most likely is due to a general higher dispersal ability, a different mating behaviour and probably also is related to the much higher habitat connectivity through the marine environment. Salmon spawners are known to frequently stray (Vasemägi *et al.*, 2005a), i.e. they do not always return exactly to their natal sites but also to other adjacent rivers.

Beck (2005) recently investigated microsatellite polymorphism of brown trout (*Salmo trutta*) from central and northern European pearl mussel rivers and generally found a higher level of genetic diversity (average H_e = 0.56) and a lower level of genetic differentiation among populations (overall F_{ST} = 0.179) compared to the studied European grayling populations (average H_e = 0.39, overall F_{ST} = 0.367; Gum *et al.*, 2005). Although a direct comparison of studies based on different species and microsatellite loci has to be interpreted with caution, the overall results still indicate that the population genetic structure of brown trout is less pronounced than for grayling. This finding can be explained by the different life history characteristics of the species (e.g. adult brown trout do not form typical schools as is known for grayling) as well as by spatial and temporal differences in the process of post-glacial re-colonisation (e.g. Bernatchez *et al.*, 2001). For another salmonid species, the European lake

whitefish (*Coregonus lavaretus*), the overall level of differentiation among populations inhabiting the pre-Alpine lakes in Upper Bavaria was found to be relatively low (average F_{ST} = 0.042, Gum, unpublished data) compared to other microsatellite-based whitefish studies (e.g. Lu *et al*., 2001). However, the studied grayling populations from the alpine Danubian tributaries also showed a moderate level of differentiation (F_{ST} = 0.061; Gum *et al*., 2003), suggesting that the studied rivers and lakes north of the Alps were re-colonised by grayling and whitefish similarly after the melting of the glaciers about 10,000 years ago.

What about other, non salmonid fish species in the studied area? Based on microsatellite data, an even higher intra-specific genetic diversity compared to grayling is reported for the bullhead, *Cottus gobio* (overall F_{ST} = 0.49 for central Europe, Hänfling *et al.*, 2002). This bottom-dwelling reophilic species lacks a swim bladder and is known for its highly limited dispersal capacity and strong morphological and genetic differences at very small geographical scales (Nolte *et al*., 2005). For the threespine stickleback (*Gasterosteus aculeatus*) microsatellite genotyping proofed a rapid divergence in postglacial populations depending on the colonised habitat type, drainage and geographical proximity (Reusch *et al.*, 2001; overall F_{ST} = 0.18). In contrast to grayling, stickleback population structure correlated only weakly with drainage system, whereas the primary divergence was among habitat types.

Overall, the observed population genetic results for grayling are in line with the initially described life history characteristics of the species: grayling is known for its characteristic territorial and homing behaviour, the formation of family cohorts, dominant males defending the best spawning grounds (if possible over several years) and the potential to adapt to different environmental conditions within a relatively short time-span (Haugen & Vøllestad, 2000). A high level of conformity concerning the results of grayling population genetics reported in the present and previous studies all confirmed the ‘discrete population character’ of the species between and within river systems (Koskinen *et al.*, 2002a; Gum *et al.*, 2003, 2005). Interestingly grayling develops its pronounced population structures on relatively small geographical scales within hydrologically connected tributary and lake systems (Koskinen *et al.*, 2001b; Gum *et al.*, 2003) as well as within large continuous river systems (Heggenes *et al.,* 2006).

Waples & Gaggiotti (2006) recently empirically evaluated some genetic methods for identifying the number of gene pools and their degree of connectivity in order to deal with the question: ‘What is a population?’ To address this problem, the authors first provided an

overview about different representative definitions of 'population' and related terms by classifying the definitions in a) ecological, b) evolutionary, and c) statistical concepts and d) in other variations such as 'stocks, demes or demographic units'. Comparison of these definitions to what is known regarding grayling genetics and its life history information demonstrates that most of the "commonly used population definitions under both the ecological paradigm (which emphasizes demographic cohesion) and the evolutionary paradigm (which emphasizes reproductive cohesion)" are applicable to the studied grayling populations. Moreover, the simple and often used term "stock", which is defined as "species, group, or population of fish that maintains and sustains itself over time in a definable area", fits well to grayling. In a second step, Waples & Gaggiotti (2006) evaluated the criteria of their population definitions by application of empirical simulations that were based on different numbers of loci (considering low and high variable microsatellites) and individuals. Simulation results were used to assess methods such as assignment tests (e.g. Piry & Cornuet, 1999) and population differentiation or detection of the number of populations without *a priori* information about the sampling collection (Pritchard *et al.*, 2000). Most of the methods tested by Waples & Gaggioti (2006) were applied in the case studies of this work as well. Among them, especially assignment tests and the Bayesian clustering methods (Pritchard *et al.*, 2000; Wilson & Rannala, 2003) were found to be powerful statistical tools to determine grayling's population structure and substructure both within and between major drainages, to assign individuals to their respective population of origin (Gum *et al.*, 2003) as well as to evaluate patterns of contemporary gene flow caused by human introductions (Gum *et al.*, 2006). In light of the above, it is evident that grayling exceptionally well fulfils the necessary "what is a population" criteria from an ecological, evolutionary and statistical perspective.

In conclusion, the results of grayling population genetic studies show that we often have to reconsider classical views concerning strict divisions into species or subspecies categories and in fact may better think in terms of populations and particularly in the case of salmonids, in terms of spawning groups. In addition, depending on the life cycle and habitat requirements of the investigated populations, we should pay attention to the major drainage of origin (i.e. for grayling: ESU level) and connected tributary systems (for grayling: MU level), respectively. Delineation of grayling conservation units will be discussed in more detail under paragraph implications for conservation and management.

6.2.3 Phylogeograpic inference of European grayling with particular reference to salmonids and other European freshwater fish species

Central parts of this work (Gum *et al.*, 2005) as well as other recent studies have concentrated on clarifying the phylogeography of grayling throughout the species' European distribution range by applying mitochondrial DNA markers (Koskinen *et al.*, 2000; Sušnik *et al.*, 2001; Weiss *et al.*, 2002).

In this work, the mitochondrial ND-1 and ND-5/6 fragments were chosen for phylogeographical inference of grayling in Central and Northern Europe because of the following reasons: (i) major evolutionary European lineages could be revealed for salmon, brown trout, and grayling using these mtDNA regions in previous studies (e.g. Nilsson *et al.*, 2001; Gross *et al.*, 2001); (ii) the possibility of directly combining our data with those of previous studies (Koskinen *et al.*, 2000) in order to cover a larger study area and would thus allow a more precise inference about the evolutionary history of grayling from Bavaria; and (iii) the mitochondrial control region (D-Loop) was avoided because this region seems to evolve slower in salmonid as compared to other fish or vertebrate species and generally, it's mutational process is not as yet well clarified (e.g. Donaldson & Wilson, 1999; Churikov *et al.*, 2001). Studying the mtDNA control region, Weiss *et al.*, (2002) in fact found slightly less clear patterns of postglacial colonisation and lineage composition than in the present study. However, additional sequencing of representative samples from different lineages allowed comparison of these two studies and it became clear that both the chosen ND genes and the D-loop are principally suited to reveal the major evolutionary lineages of European grayling. Based on the present study, four major lineages were detected and should be recognised as the basic evolutionary significant units (ESUs) for grayling in central and northern Europe. Direct combination with the data by Weiss *et al.* (2002) and comparison to the Sušnik *et al.*'s study (2001) indicate that from southern to northern Europe at least five major grayling mtDNA lineages have thus far been identified that evolved in geographical isolation during the Pleistocene: 1) an ancient Danubian lineage that can be subdivided into a lineage north and south of the Alps and in a typical Sava lineage occurring in Slovenia, 2) a major western European lineage represented by several sublineages found in the drainages Rhine/Main, Alpine Rhine, Rhone and in the UK, 3) a typical eastern and northeastern European lineage (e.g. dominating in the Elbe, Oder, Vistula drainage), 4) a northern European lineage occurring throughout Scandinavia and western Russia, and 5) a highly divergent Adriatic lineage found only in certain rivers flowing into the Mediterranean Sea.

An essential element of this thesis has focused on the phylogeographical question of whether distinct grayling lineages and contact zones are present in Bavaria and surrounding countries such as Austria and the Czech Republic. The area north of the Alps and the lower mountain ranges in central Germany are of special bio-geographical interest as these regions include important tributaries to the central European drainage systems of the Danube, Rhine/Main and Elbe. The region is known as a secondary contact hotspot where at least three grayling expansion waves have met (see chapter 4 for details). One major expansion started from a south-eastern Danubian refuge (indeed the lower Danube served as a starting point of colonisation into western Europe for many fish species); a second expansion started from an eastern refuge touching Bavaria only at the "Fichtelgebirge" and "Bayerischer Wald", and a third expansion took place from another south-western refuge expanding northward from the Rhone via the Rhine/Main drainage. In addition, regarding grayling, persistence during the last Riss/Salian ice age is assumed for populations from the Main river system (Gum *et al.*, 2005).

Concerning the location of major refugia proposed for grayling in the studied region, both similar and contrasting patterns of (re-)colonisation were observed for other European freshwater fishes. Salmonids certainly are among the fish species that have received most attention for phylogeographical studies in Europe and North America; to mention just a few: Bernatchez *et al.* (1992), Bagley & Gall (1998), Koskinen *et al.* (2000), Bernatchez (2001), Nilsson *et al.* (2001), Østbye *et al.* (2005). In the following, I will briefly compare the results revealed for grayling with other important European salmonid and non-salmonid fishes in respect to major mtDNA lineages, colonisation routes into central Europe and areas of potential contact zones.

With reference to mtDNA based phylogeographical analyses in Europe, one of the best investigated European salmonid fish is the brown trout, *Salmo trutta*. Using fragments of the NADH-5/6 and cytochrome b genes as well as the control region, Bernatchez (2001) investigated brown trout populations throughout its native Eurasian and North African range of distribution. The study revealed five major evolutionary lineages that evolved in geographic isolation during the Pleistocene (considered as the basic ESUs of brown trout). With only a few exceptions, the study did not detect an overlap of divergent lineages within populations; i.e. in contrast to the results of the present study (Gum *et al.*, 2005), generally no secondary contact of major lineages was observed. Bernatchez (2001) concluded the study "provided evidence that for the role of biological factors in addition to that of physical isolation in limiting introgressive hybridization among major trout lineages." However, a serious problem

with this statement is that the study generally did not include a dense sampling at finer geographical scales in potential contact zones of these ESUs as for example from the upper Danube. In fact, we have good reason to assume (similar to grayling) that an admixture of major lineages has happened for brown trout in Bavaria: the designated Atlantic lineage (AT), which is common throughout western, central and northern Europe and the Danubian lineage (DA) naturally co-occur in the region north of the Alps (see also Weiss *et al.*, 2001). However, it remains to be seen if ongoing studies can discriminate natural from human caused admixture of divergent lineages for (in most rivers intensively stocked) brown trout or if we still do have two distinct lineages within certain regions (Geist *et al.*, in prep). In the framework of 'functional genomics', future studies will also have to clarify the relevance of these major trout lineages in regard to conservation and management of the species. In addition, more research is necessary to explore the function of different brown trout lineages as suitable hosts for semi-natural breeding programmes of the critically endangered European pearl mussel. Until now, only the AT lineage was found in pearl mussel rivers (Beck, 2005).

Concerning Atlantic salmon (*Salmo salar*), it has been demonstrated that there is a clear division between the North American and European salmon populations using mtDNA and other classes of molecular markers (e.g. Ståhl, 1987; Verspoor *et al.*, 1999; King *et al.*, 2001). European salmon are further divided into two major groups: the Atlantic and the Baltic salmon (e.g. Verspoor *et al.*, 1999; Nilsson *et al.*, 2001). Despite numerous studies, the post-glacial origin of north European Atlantic salmon is still debated (Tonteri *et al.*, 2005). Considering Baltic Sea salmon, both Atlantic and eastern freshwater refugia have been proposed (Verspoor *et al.*, 1999; Nilsson *et al.*, 2001), as well as combinations of the two (Koljonen *et al.*, 1999). Due to the extinction of the species about a century ago, data for Germany are unfortunately not available. Analysis of the extinct salmon from the Rhine, the drainage from which would have been part of any central European refuge, would be particularly interesting in the latter regard. It could be carried out if archival scale samples or alcohol-preserved material still exist. Regardless of this, the last few years have seen the first returning individuals as a result of the restoration efforts in the Rhine and Elbe systems ("Lachsprogramm 2000"; Klinger *et al.*, 2003), clearly indicating that salmon has the evolutionary potential to re-colonise former suitable habitats.

Another economically important species, the European whitefish *Coregonus laveretus*, was recently investigated in central and northern Europe by Østbye *et al.* (2005) to illuminate its evolutionary history. The study revealed three major evolutionary lineages with a northern

European clade from northwest Russia to Denmark, a Siberian clade from the Arctic Sea to southwest Norway, and a southern European clade from Denmark to the European Alps, reflecting occupation in different glacial refugia. Compared to the major North and South European whitefish clades more genetic groups and clades have been observed for grayling throughout Europe (Weiss *et al.*, 2002; Gum *et al.*, 2005). However, even more intriguing from a scientific perspective than phylogeographic research is the extensive level of phenotypic polymorphism observed in European whitefish. This characteristic of whitefish is triggering evolutionary research at the moment in order to disentangle mechanisms underlying diversification (see Østbye *et al.*, 2006 and references therein). The results point to a process of parallel evolution of ecomorphological traits in whitefish through recurrent postglacial divergence into pelagic and benthic niches in different lakes throughout its European distribution.

For the well-studied bullhead (*Cottus gobio*) Englbrecht *et al.* (2000) suggested a pre-Pleistocene origin of the major central European distribution. Similar to grayling, the first colonisation of the species into western Europe most likely occurred via the ancient lower Danube, with a separate colonisation of the Eastern European territories. In contrast to most other phylogeographically studied European fish species (at least until now), bullhead has largely maintained their population identity despite the strong disturbance caused by the glacial cycles in these areas. On the other hand, a mixing of populations in the lower Rhine River and its tributaries was detected (Nolte *et al.*, 2005). In accordance with the data for grayling from the Main river system and other populations from the lower mountain range in central Germany, mtDNA analyses for bullhead suggested that the latest glaciation cycles did not have a major impact on the general population structure of *C. gobio* in central Europe. Kontula & Vainola (2001), also studying bullhead, Van Houdt *et al.* (2003), and Barulenga *et al.* (2006), studying burbot *Lota lota* (L.), found remarkably similar patterns of major mtDNA lineage distributions with grayling, especially in the more recently re-colonised areas of northern and central Europe.

The study by Durand *et al.*, (1999) on chub (*Leuciscus cephalus*) provided evidence for four major European lineages, which largely evolved in allopatry (two Mediterranean and two north-European sets). In contrast to bullhead and grayling, the authors concluded that the species was eradicated from most of Europe during the maximum ice extend. Different from grayling, a Danubian lineage entered western Europe via the Rhine-Rhone-Loire drainages and during the Holocene also colonised the Elbe northward. A second lineage started from a

Ponto-Caspian refuge to colonise the Baltic area as far as the Oder River. Interestingly, the only evident contact zone of the (western) Danubian and the (eastern) Ponto-Caspian lineage was in the Elbe river without evident mixing. However, due to the limited sampling of populations from this contact zone, future studies at smaller scales are needed to clarify whether natural intermixing among these divergent lineages has occurred.

Four major phylogenetic clades were identified as well for the perch, *Perca fluviatilis* (Nesbø *et al.*, 1999). However, in sharp contrast to chub, several contact zones were recognised between lineages. In the upper Danube, the lineages originating from a western and a southern European refugia meet. In addition and similar to grayling, the southern (Danubian) European refugium probably did not contribute to the re-colonisation of other western and northern European drainages after the last glaciation. Phylogenetic analyses suggested that the southern European mtDNA lineage is the most ancient, and therefore, is probably the founder of all present perch lineages.

For the barbel (*Barbus barbus*), a two-step expansion scenario from the Danubian refuge via the Rhine and Rhone Rivers, during the last interglacial period (Riss/Würm, appr. 13,000-11,500 years ago), similar to chub was suggested (Kotlík & Berrebi, 2001). Secondary contact of the western lineage (expanding from southern France) and the eastern lineage (starting from the Danube) was found in the Rhine and in the Weser rivers.

The above review shows that migration events among regional fish fauna into central Europe were found to be highly species-specific as were the regions of secondary contact among divergent mtDNA lineages. This observation is in general agreement with the review by Bernatchez & Wilson (1998) on comparative phylogeography of Neartic and Paleartic fishes. The different patterns of (re-)colonisation may generally be explained by two major factors: first, the process was critically dependent on the maximum ice coverage and the occupation of similar glacial refugia as well as the geographical situation of ancient drainage systems (Hantke, 1993); second, the revealed differences are to a large extent due to the differences in dispersal ability and environmental tolerance of the studied species, e.g. the known high cold-water tolerance of bullhead and grayling.

However, some common features regarding the overall European fish phylogeography can be found as well. For example, the distributional patterns of haplotype groups of chub, perch, barbel and brown trout indicate that these species were almost completely eradicated from central and western Europe during the major glaciations, followed by re-colonisation of one or

a few refugial lineages. This is in contrast to bullhead and grayling and probably to a lesser extent also for burbot and European whitefish. Another common feature of all of the above mentioned studies, including the European grayling, is the higher genetic variability observed in the south-eastern and south-western distribution (Weiss *et al.*, 2002; this study) and an initial invasion of western Europe starting from the ancient lower Danube. In addition, the recent large-scale phylogeographical studies confirmed the conclusion by Bernatchez & Wilson (1998) that tree topologies were typically deeper for species from non-glaciated regions as compared to northern species, whereas species with partially glaciated ranges were intermediate in their characteristics. The latter observation seems also true for the investigated grayling populations from Bavaria. A relatively high level of relatedness was found among the southern alpine populations (e.g. among populations of the upper Isar, upper Iller, and upper Lech), whose habitats were completely covered by ice until about 10,000 - 12,000 years ago (Hantke, 1993; Gum *et al.*, 2003). By contrast, much deeper tree topologies (i.e. level of differentiation) were observed within the Main system. Therefore, survival of grayling and other cold-water adapted species such as bullhead over the last ice age in the region, that was lying between the alpine and northern European ice sheets, seems likely (Gum *et al.*, 2005; Hänfling *et al.*, 2002).

In 1998, Bernatchez & Wilson concluded that more large scale phylogeographical studies are needed, at both single-species and comparative levels, in order to complete the incomplete phylogeographical picture for both North American and Eurasian fish fauna. In the meantime, for grayling this demand has been well-achieved for its northern (Koskinen *et al.*, 2000), its southern (Weiss *et al.*, 2002; Sušnik *et al.*, 2003) and its mid-European distribution (Gum *et al.*, 2005); however given the limited information from eastern Europe, the picture is still not complete. Future large-scale phylogeographical studies in general are needed to fill the gap concerning data from the huge area ranging from eastern Europe to central and eastern Asia. Once acquired, these data have to be combined with those already existing from European and North American surveys. In this regard grayling and burbot may serve as first examples in a next step towards global phylogeographical surveys (see Froufe *et al.*, 2005; Van Houdt *et al.*, 2005).

6.3 Implications for conservation and management

As outlined above, European grayling has recently been the target of several studies attempting to resolve the distribution and origin of the major phylogeographical lineages. This work also significantly contributed to the growing knowledge of European grayling phylogeography (Gum *et al.*, 2005), but it has been centered on two different issues. First, it has been of interest in elucidating the genetic population structure of a species exhibiting strong homing and discrete populations (Gum *et al.*, 2003). Second, over the last two decades grayling has been increasingly subject to considerable stocking activities, both in Bavaria and elsewhere in Europe. Therefore, discriminating the level of natural secondary contact of divergent evolutionary lineages from immigration resulting from recent human mediated stock transfer and/or potential introgressed fish was approached in a separate attempt (Gum *et al.*, 2006). Regarding the latter issue, it should be emphasised that the actual genetic consequences of stocking grayling from different origins into wild populations is of interest, not only to gain experience in how to solve the problem genetically and statistically, but also to improve management and conservation of the species *per se*; and, it is of general relevance to conservation biology. In the following section, the contribution of conservation genetics to the protection and sustainable management of grayling is summarised and discussed.

6.3.1 Contribution of conservation genetics

Synthesis of the revealed population genetic and phylogeographical results allowed for an effective delineation of ESUs and MUs as defined by Waples (1991) and Moritz (1994). Based on mtDNA and nuclear data of the case studies which are presented (Gum *et al.*, 2003, 2005), three major conservation units can be defined for grayling in the more intensively studied German and central European area: a Danubian ESU (with two MUs in Bavaria: alpine and low-ionic, more acidic tributaries from the north/northeast), a Rhine/Main-ESU (with 2 MUs: one for the Alpine-Rhine and one for the Main) and an Elbe-ESU (all investigated tributaries to the Elbe). In addition, the results provided evidence of a substantial level of historical admixture among major mtDNA lineages in the contact zones between drainages (e.g. in the "Fichtelgebirge" or the Alpine-Rhine catchment at Lake Constance), most likely resulting from ancient river connections between drainages during the Pleistocene glaciations (see chapter 4 for details). Based on these findings, a drainage and sub-drainage specific management of grayling at the population level is recommended. Considering the remaining

self-reproducing populations, any further endangerment (through environmental and/or human caused factors as described initially) of the established grayling conservation units could ultimately result in an irrecoverable loss of valuable components of the evolutionary legacy and overall genetic diversity of grayling. The genetic characterisation of populations is thus a basic requirement for conservation decision making in fish and other endangered organisms (Frankham *et al.*, 2002) and also enables prioritisation of particular grayling stocks for future enhancement programmes or restoration efforts.

Given the long and continuous river and drainage systems, it is important to note that the proposed grayling conservation units (ESUs/MUs) do not end at national boundaries. For example, the grayling MUs proposed for southern and central Germany/Bavaria in most cases directly apply to the same or adjacent rivers of the Danube, Rhine or Elbe drainages flowing through Austria, Czech Republic or Switzerland. Therefore, international cooperation and exchange of knowledge is a must for efficient grayling conservation. In addition, concerning the phylogeographical results revealed for Bavaria, we have to consider the natural contact zones of divergent grayling mtDNA lineages, e.g. in the "Fichtelgebirge" or the area around Lake Constance. These areas represent potential hotspots of grayling biodiversity and deserve closer attention when realising measures of conservation prioritisation and protected areas. In these natural hybrid zones, the development of population management guidelines is more complex than in areas where only a single ESU or MU is found (Allendorf *et al.*, 2001). Here in particular, an exact genetic and ecological knowledge on a fine-scale is crucial for preserving diversity of grayling and biodiversity of the whole aquatic fauna (see Geist, 2005).

What other results of the present work are of concrete use for conservation genetic management of grayling and conservation decision making? The knowledge of historical patterns of gene flow can help to manage dispersal among the anthropogenically fragmented populations. For example the investigated Alpine Danubian grayling populations basically are closely related but natural exchange of migrants between different spawning groups is extremely limited through many dams and impassable weirs. Similar patterns of genetic isolation and connectivity within river systems through recent human caused migration barriers were reported for Danish grayling populations (Meldgaard *et al.*, 2003). Therefore, if fish ladders cannot be constructed in the short-term, the direct transfer of grayling (or their offspring) from downstream to upstream river stretches is recommended and should help to

counterbalance potential negative genetic effects caused by physical isolation. Thus far, no tendency regarding effects of inbreeding or severe bottlenecks has been detected in the central European grayling populations studied (Gum *et al.*, 2003). However, some populations are in danger of moving in this direction as indicated by the existence of extremely isolated spawning groups in upstream located river stretches that are separated by impassable barriers from other spawning sites or downstream stretches (e.g. the Main populations Weißer Main or Leinleiter had the lowest values of heterozygosity). On the other hand, most populations investigated from the Danubian drainage system show a comparatively high level of genetic variability, probably due to (a) historical processes of admixture of divergent evolutionary lineages in regions of secondary contact (Gum *et al.*, 2005) or (b) as a result of the recent human caused stocking actions (Gum *et al.*, 2006).

In contrast to 'supportive breeding', the introduction of non-native or domesticated fish originating from different ESUs or MUs is generally considered as adversely affecting the local populations from a genetic and ecological perspective. Among the detrimental consequences are indirect effects of competition (e.g. for food resources) or direct effects such as introduction of diseases or, if introgression happens, the disruption or loss of locally adapted gene pools (e.g. Hindar *et al.*, 1991; Gross *et al.*, 2004). In this study, the presence of multiple gene pools or population substructure was detected in several rivers and the results pointed to a certain level of interbreeding between stocked and local grayling (Gum *et al.*, 2006). As shown in the case of the Saalach River (alpine Danubian tributary), the results of individual admixture analyses demonstrated the existence of a few introduced grayling originating from the northern European mtDNA lineage. The stocked grayling obviously did not form a homogenous gene pool with the local fish and were identified as first generation immigrants not yet reproducing with the natives (if this is possible at all, e.g. due to different spawning times). Considering the stocking history of other study sites, it becomes clear that repeated and more intensive stocking actions over several years has the potential to cause a substantial level of admixture and, under certain circumstances, can even lead to a substantial loss of the original gene pool (e.g. for the studied Isar population). However, as shown in Gum *et al.* (2006), the complete loss of the native gene pool is supposed to be a worst case scenario. It can normally only happen if the local population suffers from a strong demographic decline and the stocked fish outnumber the locals, keep staying at the stocked river site and successfully reproduce in the local spawning grounds. Regarding natural reproduction, one has to keep in mind that artificial

reproduction, with spawners caught in the wild and mating performed in hatcheries for supportive breeding actions, can also produce admixture if grayling from different origins were stocked before. This is probably the case for seriously depleted stocks of small rivers where a major proportion of spawners are caught and transferred to the hatchery. Nevertheless, it is clear that supportive breeding performed in the strict sense, i.e. taking spawners of exclusively local origin and stocking the offspring back in the same river stretch, is the method of choice for sustainable management and for securing long-term genetic integrity of fish populations (e.g. Ryman *et al.*, 1995).

Similar to grayling, enhancement motivated stocking of non-native brown trout in the upper Danube was judged to be both economically and ecologically inefficient (e.g. Weiss & Schmutz, 1999; Weiss *et al.*, 2001). The authors strongly recommended that its use in a sustainable and conservation-orientated management strategy be reconsidered. This recommendation is in line with Baer (2005), who argues that introduction of domesticated trout into a self-reproducing autochthonous brown trout population is ecologically problematic and is also economically counterproductive in the long term. At the same time, it should be noted that even heavily stocked trout populations may be affected substantially less by hatchery trout than originally anticipated and should not immediately be given low conservation priority (Fritzner *et al.*, 2001). This is confirmed by Mezzera & Largiader (2001), who conclude that selective angling may have considerably eliminated introduced brown trout and hybrids before spawning at the study sites, and thus may have reduced the introgression of alien genes into the local gene pool. As pointed out in this study (Gum *et al.*, 2006), there is a clear difference between stocking of definite domesticated fish, such as the typical world-wide produced strains of rainbow trout, and offspring from spawners caught in the wild. Domesticated fish, compared to their wild counterparts, have been shown in several studies to be subject to the selective conditions of fish hatcheries, often resulting in poor survival rates and lower fitness levels when released (e.g. Metcalfe *et al.*, 2003; Salonen & Peuhkuri, 2004). Concerning the latter observation, Vincent (1960) already reported that domestic fish were spread throughout the water column, whereas the wild stock had a tendency to remain near the bottom of their tank. Furthermore, stocked fish had practically no hiding response, while wild fish would immediately seek concealment. Fraser (1981) found similar evidence in brook trout (*Salvelinus fontinalis*) and speculated that generations of inbreeding in the hatchery have led to a loss of ‘wildness’ and to an inability of the domestic stocks to adapt to ecological conditions

in the wild. Johnsson *et al.* (1996) showed that brown trout raised in a hatchery had a weaker predator response than wild trout during feeding. Thus, anti-predator behaviour may have changed as a consequence of inadvertent selection in the hatchery. Fleming *et al.* (2000) investigated the lifetime success and interactions of farm salmon invading a native population. Their results indicate that annual invasions have the potential for impacting on population productivity, disrupting local adaptations and reducing the genetic diversity of wild salmon populations. In contrast to salmon and rainbow trout, for grayling, no comparable extent of domestication is documented until now in the studied area. However, as shown by Salonen & Peuhkuri (2004) in the case of grayling, second generation hatchery fish already showed a lower level of aggressive behaviour than wild strains. Therefore, the author's conclusion is that 'it would be beneficial to use the progeny of wild fish for re-introductions'.

6.3.2 Contribution of ongoing studies and other fields of research

Aside from the direct implications based on the genetic results of this work, what must additionally be considered to efficiently move forward the conservation and management of grayling? A lot of the traditional practical fishery management of grayling until recently followed the principle of 'trial and error'. In this context and unfortunately for the support of the depleted stocks, even today, the attitude of private fishing associations is sometimes 'good is what is cheap' without examining the sustainability of the applied measures. On the other hand, since profound knowledge in respect to genetics and ecology of the species is of late increasing and at the same time delineation of practically suitable conservation units has become possible, the attitude of fishermen as well as their applied management is gradually changing (E. Roese, B. Hornauer, pers. communication). Most people agree that from an economical and ecological perspective it is more sensible to first think of genetics and ecology before realisation of supplementation measures, restoration of extinct populations or establishment of permanent spawning groups in hatcheries (e.g. Vrijenhoek, 1998; Tringali, 2006).

Ongoing genetic studies on grayling in particular attempt to evaluate the design of the recently initiated captive breeding programmes. By directly comparing natural and artificial reproduction, this research aims to provide the first genetic data for assessment of the human manipulations acting upon the genetic structure of grayling in fish hatcheries. It is assumed (but not scientifically proven) that artificial reproduction by man reduces genetic diversity and

that human caused selection may impact on individual fitness levels and survival chances in the wild when released (Gum *et al.* in prep.). There is still a debate going on concerning the establishment of permanent grayling brood stocks in a fish hatchery (as is common practice for brown trout) or keeping the classical supportive breeding by annually catching the spawners from the wild through electro-fishing. Permanent brood stocks in hatcheries are considered less labour-intensive and more cost-efficient in the long-term. However, if gene pools are not regularly supplemented by grayling taken from the wild, this procedure bears the risk of mal-adaptation to hatchery conditions within a few generations (with all adverse consequences of domestication mentioned above; Salonen & Peuhkuri, 2004). Besides, the longer the fish remain in hatchery facilities, the greater will be the change in the original behavioural characteristics of the fish and the more difficult is a successful and sustainable colonisation of natural habitats after stocking is (Carlstein, 2004).

The initially described typical specialisation of grayling fry to chironomid larvae as a first starter diet may also be considered for development of reasonable captive breeding measures. It is questionable if a complete conversion or habituation of juvenile and adult grayling to artificial fish pellets as commonly fed in trout and salmon aquaculture can be regarded as a suitable measure for production of preferably 'natural' offspring for restoration purposes. Unpublished results, based on first feeding experiments, performed with grayling fry in local hatcheries, indicate that among the different combinations of natural feed and industrially produced fish feed tested (including frozen and living plankton as well as pellets from different manufactures), the best growth and lowest mortality rates were achieved by feeding pure, living plankton (Herrmann, 2001). However, generally higher costs and a higher amount of work are the clear disadvantages of this semi-natural feeding and often simply not enough living plankton is available in early spring. Therefore, a combination of frozen plankton and artificial pellets (smallest particle size) is often applied as a first diet for grayling in practice.

Most river systems in central Europe have been subjected to anthropogenic influence, which in many cases has led to deleterious impacts on important habitat features. These impacts include changes to the stream bed through impoundment and deposition of fine substrate and the restructuring of rivers or modification into canals. In addition, discharge, for example from sewage plants or drainage lines, often substantially reduces water quality and quantity, respectively. In rivers with intensive utilisation of hydropower to gain electricity, often the lion's share of water is redirected to artificial canals (see for example the "Isarkanal") and only

a limited quantity of water remains in the original stream bed (e.g. the Isar in Freising). Therefore, many of the deeper stretches and deeper pools in those rivers disappeared. However, it is exactly these deep areas that are necessary overwintering habitats for grayling and would prove particularly valuable as hiding places in winter, given the increased predation pressure by piscivorous birds during the winter season. Another serious problem directly resulting from hydropower stations relates to an often fluctuating flow regime (the so-called "hydropeaking"), which is known to cause rapidly occuring artificial floods from peaking hydropower operation at times of increased electricity demand. These sudden floods are thought to cause considerable displacement of especially juvenile fish from their shallow, slow-flowing riparian habitats (Schnell, 2006). Thus, degradation and loss of suitable habitats are certainly one of the main factors regulating fish production in fresh water, especially during the juvenile stage, when density-dependent processes are most acute.

Effective management programmes cannot be planned without an understanding of the biological requirements of the species throughout its life cycle. In this context, Riley *et al.* (2006) recently studied the seasonal variation in habitat use by salmon, trout and grayling in a British chalk stream. The work revealed greater differences in the habitats used between the differing age groups of the species than between autumn and winter periods, and the distribution of fish was more strongly dependent on substrate type than on water depth or velocity. For grayling, the 'bigger fish – faster flowing' habitat relationship known from former studies was generally confirmed (e.g. Mallet *et al.*, 2000) and grayling showed a clear preference for the coarsest substrate (gravel) available at all times. Studying migration patterns of grayling by radio-tracking, Ovidio *et al.* (2004) demonstrated that spawning movements and post-spawning homing were similar in three consecutive years. During the pre- and post spawning periods, grayling usually showed small-scale movements in the same pool-riffle sequence and rapidly homed to their established resting-places after reproduction. In addition, individual grayling were found to use the same spawning grounds over several years (Ovidio *et al.*, 2004). This observation clearly stresses the need to detect the different spawning sites in a river and to carefully list the areas that require protection in order to secure natural recruitment of the species.

In summary, the strong reproductive, trophic and refuge homing behaviour of grayling has to be considered carefully for their successful management and enhancement. Improving and maintaining habitat diversity, increasing the amount of overwintering habitat, and assuring that

fish are able to move between the habitats needed in different seasons should all be given attention in grayling conservation.

A comprehensive conservation and management plan for grayling also has to deal with the initially described cormorant and goosander debate. Predation by these piscivorous birds is considered to act inversely on grayling age classes compared to human exploitation through angling; it is predominantly a direct selective intervention on the juvenile and middle age class. Because of the constant predation pressure over the winter season in the last years, many fishing associations throughout the alpine area today choose to stock grayling for enhancement or restoration purposes not earlier than the age of 2+ (i.e. adults > 30 cm in length, which are expected to be too big as suitable prey for goosander). This release of adult fish is carried out, even though a basic principle of applied fishery management in respect to sustainability of stocking actions is that stocking many juvenile fish (e.g. at the fingerling stage) is more efficient than the release of a lower number of adult fish. In any case, the Bavarian fisheries association has for a long time postulated a large scale reduction of the predation pressure caused by these two bird species. Since 1996 the shooting of cormorants has been allowed during the winter season in Bavaria according to the 'Bavarian cormorant regulation' as well as in several other alpine regions, e.g. in Baden-Württemberg or Switzerland. However, until now all recommendations by fisheries advocates concerning a European-wide management have failed because of both national and European bird protection laws and by the resistance of bird conservationists (e.g. Steffens, 2006; see also 'REDCAFE' and 'INTERCAFE', the two EU-funded approaches to European cormorant-fisheries conflicts).

In addition to the environmental factors addressed above, the applied fishery management and the regional angling regulations have the potential of considerably affecting the development of grayling populations. Angling and recreational fisheries in general are currently shown to be important factors influencing the genetic structure of fish populations and their management (Arlinghaus *et al.*, 2002; Cooke & Cowx, 2004). These studies illustrate that not only commercial but also recreational fisheries can contribute substantially to total harvest, particularly among some top predators of the food webs. In addition to the direct effects of overfishing, both Ricker (1981) and Conover & Munch (2002) provide valuable data in respect to the genetic effects of body size dependent fishing rules by studying Pacific salmon and an exploited marine fish (*Menidia menidia*). The selective preference of the young and middle

age classes for reproduction resulted after just 4 generations in a substantial reduction of the overall growth performance and in earlier age of maturity of the *M. menidia* populations (Conover & Munch, 2002). For salmon, selective removal of large fish in commercial fisheries led to dramatic changes in body size: about a 30% decrease in the mean size of pink salmon caught off the coast of British Columbia, Canada, was observed since the 1950s (Ricker, 1981). These results underline the principles of natural selection and show how rapidly evolution is at work subsequent to human impacts (see review by Palumbi, 2001). Additionally, they make clear that management tools that preserve natural genetic variation for a long-term sustainable yield are necessary. Concerning size selective fishing, Birkeland & Dayton (2005), in their review, also pointed to the importance in fishery management of leaving the larger specimens. Summarising recent case studies, the authors demonstrated that older individuals of some fish species produce larvae that have substantially better survival potential than larvae from younger fish. My own unpublished cross-breeding experiments with female grayling of different sizes and age generally confirm this finding. This is important because commercial fisheries and especially recreational fishermen often target the larger fish. Thus, for development of sustainable conservation strategies of endangered game fish species like grayling, potential detrimental consequences of traditional angling practices should not to be underestimated and need to be considered in future decision making. Scientists and managers must develop methods to constrain exploitation, whether commercial or recreational. In several rivers throughout the alpine area, where grayling is endangered at present but in former times represented an interesting and important game fish for fly fishing, angling is in fact nowadays to a large extent restricted or, in some cases, is even completely prohibited. Fishing can be regulated by number and size of the fish that one person is allowed to catch, or by time or by limiting access to certain river stretches. Indeed, the setting of appropriate fishing restrictions when population sizes strongly decline should be a matter of course. In this regard, one important management practice, which is forbidden in Bavaria and other German federal states because of the animal protection law, deserves closer attention: 'catch-and-release'. This practice is common in other European countries and contributes to a sustainable management of fish populations. At the same time, catch-and-release is known to keep people out at the rivers for longer time spans and thus may facilitate – compared to the pure 'put-and-take' practice – to better tracking of short and long-term changes of stock sizes (particularly if a proportion of adult fish is tagged; see also Arlinghaus *et al.*, 2002). Usually the local

fishermen are the first who become aware of problems in the resource they manage, especially if the natural recruitment of important game fish species is affected.

Given the increasing extent of endangerment of grayling and the native freshwater fish fauna in general, what basic conclusion can be drawn by means of this research and considerations presented in this chapter? A key issue, which unfortunately will remain a fundamental problem in the near future and beyond, is degradation and loss of suitable habitats (Lichatowich, 1999). In particular, if the spawning grounds are no longer functioning or simply cannot be reached by the spawning individuals, then even the best intended and sophisticated management plan won't help in the long-term. In this regard, after 15 - 20 years of intensive observation and research, it is doubtless a too short-sighted view, if the only large scale threat to European grayling populations is attributed to the increased populations of cormorants and locally to goosanders. Naturally flowing river systems are the best protection for maintaining the diversity of the native fish species. In addition to this, the design and the revealed genetic results of the present study on grayling including their implications for management and conservation may well serve as an example for studying other endangered fish species in central Europe such as the Danubian salmon (*Hucho hucho*), the sneep (*Chondrostoma nasus*), the sterlet (*Acipenser ruthenus*) or other smaller species like the varione (*Leuciscus souffia*) or the loach (*Nemacheilus barbatulus*).

6.4 Perspectives

From a fishery science and economical perspective, the defined grayling conservation units based on genetic differences found so far at the presumably neutral level (microsatellite data) are particularly meaningful and helpful if they are biologically relevant and correlate with functional variation. Therefore, future case studies need to integrate the environmental variability and extent of genetically fixed adaptation of grayling to their diverse habitats through appropriate experimental design in the framework of functional genomics (Ranz & Machado, 2006). Recent work on Scandinavian populations of European grayling makes clear that the species basically has the potential to quickly adapt to different environmental conditions. First, Haugen & Vøllestad (2000) assessed in their survey the growth and survival effects on maturation patterns in populations of grayling with recent common ancestors. The results suggested that natural selection acting upon grayling populations in three Norwegian lakes exhibiting different annual temperature patterns (lakes are at different altitudes) resulted in significant different maturation patterns within a time-frame of about 18 generations. Later, this finding was generally confirmed by common garden breeding experiments with offspring of wild grayling taken from the same lakes and hatched under controlled laboratory conditions by simulating the different average temperatures regimes (Koskinen *et al.*, 2002c).

Concerning the studied area in Bavaria, studies in progress aim to test the ecological exchangeability of grayling originating from chalk alpine rivers and from populations of the low-ionic, more acidic rivers of the northern Danubian tributaries (e.g. Schwarzer Regen). Although there is good reason to assume that populations from these different geological regions are indeed genetically adapted to their local habitat, this hypothesis still needs to be verified since until now results have been based on selective neutral data and some morphological variation (Baars, 2000). Therefore, future studies need to evaluate fitness related traits as well as determining growth and survival rates of grayling from these different habitats, e.g. by directly cross-breeding individuals from distinct spawning groups or by common garden breeding experiments using eggs or offspring taken from the wild. Another approach that should be addressed in further studies is to test the established grayling ESUs and MUs for significant differences in expression profiles of genes under natural selection. The recent advances concerning the simultaneous analyses of hundreds of expressed genes by application of the DNA chip (so-called microarray) technology offer new and exciting fields of research. First studies show, for example, that the recently established microarray based

methods of expression analyses in Atlantic salmon can successfully be transferred and applied to other non model fish species (e.g. von Schalburg *et al.*, 2005). Salmon species (*Salmo salar* in Europe) are not only of commercial importance but have proved to be valuable model organisms of ecology, evolutionary biology, and genetics (Hendry & Stearns, 2004; Cossins & Crawford, 2005). As also shown in this work (Gum *et al.*, 2003), grayling and other members of the family *Salmonidae* in general are genetically close to each other. Therefore, in our laboratory, a pilot project is attempting to make use of the commercially available Atlantic salmon cDNA slides for analysing experimentally treated grayling samples at the expression level. Regarding the stated hypothesis of grayling populations that are possibly adapted to different environmental conditions, the results may also be used to identify candidate genes under natural selection.

In the context of comparing results based on microsatellite and gene expression data at the population level, Giger *et al.*, (2006) recently demonstrated that global gene expression in brown trout is shaped by life history to a high extent. Microsatellite data proved to accurately resolve the genetic relatedness of populations according to the large-scale geography as well as the fine-scale sampling scheme within river systems. However, by using cDNA microarrays (900 genes analysed), the authors observed significant expression differences between individuals of migratory and sedentary brown trout. The overall result indicated that global gene expression levels within juvenile brown trout from natural populations depend primarily on their future life history – migratory versus residential – rather than their genetic relatedness. A similar approach could be undertaken for studying the immunological reaction at the expression level of different evolutionary lineages of brown trout caused by the glochidia infections of pearl mussels (Geist, 2005).

Moving beyond the scope of this work, further studies on grayling, which are partially already in progress, in the fields of conservation and population genetics as well as evolutionary biology, aim to:

i) clarify the genetic effects of artificial reproduction performed in fish hatcheries by direct comparison of the produced F1 hatchery generation to natural recruitment (i.e. to wild F1 progeny of the same spawning populations), in order to evaluate and optimise commonly applied supportive breeding practices of grayling;

ii) evaluate the temporal stability of the population genetic structure revealed in this survey by re-sampling and re-analysing selected grayling populations of the studied area and in order to permit the estimation of effective population sizes (N_e) of the declining populations by temporal methods (e.g. Williamson & Slatkin, 1999);

iii) test the genetics underlying the co-evolution of mate choice and ornament in the wild, assuming that the remarkable big and colourful dorsal fin of grayling spawners (especially for males) serves as an ornament of sexual selection (see review by Andersson & Simmons, 2006);

iv) learn more about the evolution and tradeoff of egg size and number in respect to the fitness of the offspring given the current considerations to establish permanent grayling broodstocks in hatcheries (e.g. female salmon were shown to produce more but smaller eggs when kept in captivity; Heath *et al.*, 2003).

In addition, species as well as whole ecosystems, which are already stressed due to anthropogenic influence, are especially challenged by the impacts of climate change and other changing environmental conditions, such as increased UV radiation. Because this is an issue of global concern, a central part of future ecological, genetic, and evolutionary studies must be to explore in which way and how quickly single species and ecosystems react to these changing conditions (e.g. Verschuren, 2003). Exploited marine and freshwater fish stocks may be particularly vulnerable to, for example, increased water temperatures or higher UV-A and UV-B radiation, as slight changes in larval and juvenile survival affect recruitment and subsequent stock biomass. Mortality rates are typically high during sensitive periods in early life history stages of many fish species, and should greatly affect the strength of the resulting year class. However, despite some first results of pilot studies, e.g. the mentioned remarkable high mortality observed for juvenile grayling in UV experiments (Gross *et al.*, 2003), there is still not much known about the concrete effects of changing environmental conditions on survival rates of grayling and on fish fauna in general (e.g. Casselman, 2002; Chu *et al.*, 2005). Specifically, in the context of a population genomics approach, it would be interesting to test the extent of selective mortality during early larval stages of grayling and other salmonids. One could compare the relative success of different genotypes by estimating temporal and spatial genetic differentiation in two years with contrasting environmental conditions, in particular temperature.

In addition to the direct effects of global warming (i.e. the higher average air/water temperatures), an increased environmental stochasticity has to be expected as well in the near future. More suddenly alternating weather conditions, in particular longer dry periods or heavy rainfalls causing extreme floods, have already been observed in our temperate area over the last decade (e.g. see the hot summer 2003 or the extreme floods in alpine rivers in August 2005). These abrupt changes of floods and dry seasons in aquatic systems will probably reinforce the increasing endangerment of grayling and other cold-water adapted fish populations given the above described threats due to habitat degradation.

Finally, for development of effective and sustainable conservation strategies, future studies must attempt to provide a broad basis by the integration of genetic, ecological and functional data in order to take the next step towards the long-term objective: evolutionary management (Young, 2004).

7 Summary

The European grayling (*Thymallus thymallus* L.) is a salmonid fish species that typically occurs in the middle and upper stretches of cool, well-oxygenated rivers. The species is endangered at the population level throughout its European distribution range. Especially in alpine rivers grayling populations have shown steady declines during the last two decades, mostly due to habitat destruction and fragmentation and loss of suitable spawning grounds. In addition, increased predation by piscivorous birds, and to some extent, also over-fishing may have contributed to local population breakdowns. Because a single, determinant and plausible factor has not yet been identified, the overall decline of European grayling is assumed to be multi-causal.

In order to provide guidelines for conservation strategies and future management programs, delineation of conservation units is warranted. Therefore, the main objective of this study was to investigate the phylogeography and population genetic structure of grayling, with special focus on the species' central European distribution range, by applying both mitochondrial and nuclear microsatellite DNA markers.

Population genetic results of the first case study, based on the analysis of 20 microsatellite loci, revealed substantial genetic divergence between and within the central European drainage systems of Danube, Rhine/Main, and Elbe which were studied. The genetic differentiation of populations corresponded to the geographical isolation of the drainage and sub-drainage systems. As indicated by the relatively low intra-population genetic diversity, when compared to the overall level of variation across European populations, grayling was found to develop pronounced population structures, a finding which generally fits the behavioural biology of the species.

In a second large-scale phylogeographical approach, sequencing and RFLP analyses of the ND-1/2 and ND-5/6 genes as well as the genotyping of 12 microsatellite markers were used to recognise distinct mtDNA lineages and to examine the phylogeny of grayling throughout central and northern Europe. Synthesis of phylogeographic and population genetic data indicates that at least four major grayling mtDNA lineages that evolved in geographical isolation during the Pleistocene can be identified in central and northern Europe. Three of these divergent lineages naturally co-occur in the more intensively studied area of central Europe north of the Alps, in the drainages of the Danube, Rhine/Main and Elbe. These lineages should be recognised as the basic evolutionary significant units (ESUs) for grayling in

central Europe. In addition, the results provided evidence for a high level of admixture among major lineages in contact zones between drainages (e.g. in southern Scandinavia or in central Germany), most likely resulting from glacial perturbations and ancient river connections between drainages during the Pleistocene.

Since many populations have been reduced throughout Europe there is an increasing need to enhance grayling populations by stocking. Thus, the historically established level of admixture of divergent lineages in certain regions was further complicated by recent human mediated stock transfer. Discriminating the genetic effects resulting from recent human stocking from historical admixture was addressed in a separate case study. Posterior probabilities of each individual immigrant ancestries and Bayesian admixture analyses generally confirmed the level of historical secondary contact of divergent grayling lineages in the contact zones of drainages, but additionally pointed to a substantial amount of introgression for populations that were known to be affected by stocking of grayling from different origins.

Based on the overall findings of this study, a drainage and sub-drainage specific management of European grayling according to the proposed conservation units is recommended, in order to secure the species' genetic diversity and integrity.

This genetic work on grayling demonstrated

i) the benefit of simultaneously using two sets of molecular markers differing in their evolutionary dynamics;
ii) the recent advances in statistical analyses of genetic data in the field of population and conservation genetics, especially concerning individual based approaches; and
iii) that both large-scale phylogeographical studies and detailed genetic analyses on a fine scale are mandatory for developing appropriate conservation guidelines of endangered species.

8 Zusammenfassung

Die Europäische Äsche (*Thymallus thymallus* L.) ist eine Salmonidenart, die typischerweise in den mittleren und oberen Abschnitten sommerkalter und sauerstoffreicher Flüsse vorkommt. Die Art gilt auf Populationsebene im gesamten europäischen Verbreitungsgebiet als gefährdet. Besonders in den Flüssen des Alpenraums zeigen die Äschenpopulationen innerhalb der letzten zwei Jahrzehnte einen kontinuierlichen Bestandsrückgang. Weil ein einzelner plausibler Grund bisher nicht gefunden wurde, ist davon auszugehen, dass der Rückgang der Äschenbestände multi-kausal ist. Für den Zusammenbruch lokaler Bestände werden Faktoren wie Lebensraumzerstörung, Habitatfragmentierung, der gestiegene Prädationsdruck durch fischfressende Vögel und Überfischung verantwortlich gemacht.

Um Richtlinien für Erhaltungsstrategien und zukünftige Management-Programme zu definieren, ist die Beschreibung von erhaltungswürdigen genetischen Einheiten („conservation units“) notwendig. Das Hauptziel dieser Studie war daher die Erfassung der populationsgenetischen Struktur und der Phylogenie der Äsche unter besonderer Berücksichtigung ihres zentraleuropäischen Verbreitungsgebiets. Für die populationsgenetische und phylogeographische Charakterisierung europäischer Äschenpopulationen wurden in den Fallstudien dieser Arbeit sowohl hochvariable Marker des Kerngenoms (Mikrosatelliten) als auch mitochondriale Marker verwendet.

Die populationsgenetischen Ergebnisse der ersten Fallstudie, basierend auf 20 Mikrosatelliten Loci, zeigen eine starke genetische Differenzierung der untersuchten Äschenpopulationen zwischen und innerhalb der untersuchten Flusseinzugsgebiete von Donau, Rhein/Main und Elbe. Die genetischen Unterschiede stimmen mit der geographischen Trennung der Einzugsgebiete überein. Im Vergleich zur Gesamtvariabilität von allen untersuchten Herkünften ist die genetische Variabilität innerhalb der Populationen relativ niedrig. Dies deutet auf ausgeprägte Populationsstrukturen bei der Äsche hin, ein Ergebnis, das generell zur Verhaltensbiologie der Art passt.

In einem zweiten großräumigen Ansatz wurde durch RFLP- und Sequenzier-Analyse der mitochondrialen Gene ND-1/2 und ND-5/6 sowie durch die Genotypisierung von 12 Mikrosatelliten-Loci die Phylogenie und populationsgenetische Struktur der Äsche in Zentral- und Nordeuropa bestimmt. Die Synthese der phylogeographischen und populationsgenetischen Daten weist auf mindestens vier mitochondriale Hauptlinien der Äsche in Zentral- und Nordeuropa hin, die sich in geographischer Isolation während des Pleistozäns entwickelt haben. Drei dieser unterschiedlichen genetischen Linien kommen in dem intensiver

untersuchten zentraleuropäischen Gebiet nördlich der Alpen, entsprechend den Einzugsgebieten von Donau, Rhein/Main und Elbe vor; die vierte Linie beschränkt sich auf Skandinavien und Nordost-Europa. Diese Hauptlinien sollten grundsätzlich als eigene evolutionär bedeutsame Einheiten, so genannte „ESUs“ („evolutionary significant units“), in Zentral- und Nordeuropa angesehen werden. Darüber hinaus wurde bei Populationen aus den Kontaktzonen der Flusseinzugsgebiete, z.B. in Südskandinavien oder im Nordosten und Südwesten Bayerns (Fichtelgebirge, Bodensee), ein hohes Maß an Mischung von unterschiedlichen evolutionären Linien festgestellt. In diesen Gebieten ist der sekundäre Kontakt verschiedener mitochondrialen Linien höchstwahrscheinlich auf historische Prozesse, insbesondere auf die eiszeitlichen Veränderungen und alten Flussverbindungen aus dem Pleistozän, zurückzuführen.
Um die lokal stark zurückgegangenen Äschenbestände zu stützen, wurden in jüngerer Zeit zunehmend Besatzmaßnahmen mit Äschen aus unterschiedlichen Herkünften durchgeführt. Demzufolge wurde der historisch bedingte Grad an Vermischung unterschiedlicher evolutionärer Linien in bestimmten Populationen zusätzlich durch menschliche Eingriffe verstärkt. In der dritten Studie wurde daher auf Basis der Mikrosatelliten und mitochondrialen Daten eine genetische Methode auf Individuenebene etabliert und angewandt, die es ermöglicht, historische Prozesse der Vermischung unterschiedlicher mitochondrialer Linien von jüngerer (durch Fischbesatz bedingte) Immigration in einen Bestand zu unterscheiden. Mit den Mikrosatelliten Daten und einem statistischen Ansatz nach der bayesischen Methode wurde zunächst die Wahrscheinlichkeit der Abstammung eines jeden Individuums berechnet. Nach dem Ausschluss der so identifizierten Immigranten wurden die Anteile an unterschiedlichen mitochondrialen Linien in den untersuchten Populationen erneut geprüft. Die Untersuchung bestätigte den Grad an historischer Vermischung von unterschiedlichen Äschenlinien in den Kontaktzonen der Einzugsgebiete. Populationen, die mit Äschen aus fremden Herkünften besetzt wurden, wiesen zum Teil einen beträchtlichen Anteil an Introgression auf.

Um die genetische Diversität und Integrität der Art zu sichern, ist, basierend auf dem Gesamtergebnis dieser Arbeit, ein Flusseinzugsgebiets- und Zufluss-spezifisches Management der Europäischen Äsche nach den vorgeschlagenen erhaltungswürdigen Einheiten zu empfehlen.

Die vorliegende Arbeit an der Äsche zeigt

i) den generellen Nutzen der gleichzeitigen Anwendung von molekularen Markern, die sich in ihrer evolutionären Dynamik unterscheiden,

ii) die jüngsten Fortschritte der statistischen Analyse von genetischen Daten im Feld der Populations- und Naturschutzgenetik, besonders was Individuen-basierte Ansätze betrifft, und

iii) die Notwendigkeit sowohl großräumiger phylogeographischer Studien als auch genauer genetischer Untersuchungen im kleinräumigen Bereich, um angemessene Richtlinien zum Schutz von bedrohten Arten entwickeln zu können.

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