



Evolutionary distinctness of grayling (*Thymallus thymallus*) inhabiting the Adriatic river system, as based on mtDNA variation

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The northern part of the peri-Adriatic river system is inhabited by a native grayling population. In comparison with the Danubian population most of the Adriatic population is regarded as morphologically distinct and therefore recognized as racially differentiated. DNA sequence analysis of the 394-bp fragment of the mtDNA control region and 363-bp fragment of the cytochrome *b* gene region from grayling specimens of the Adriatic, Danubian and Atlantic river systems revealed nine composite mtDNA haplotypes. The genetic divergence of the Adriatic and the Danubian-Atlantic haplotypes ranged from 2.52 to 3.10% and from 3.38 to 3.92% in the cytochrome *b* gene and control region, respectively. On the basis of sequence data an attempt was made to elucidate the phylogeography of the Adriatic population. The molecular data indicate an ancient monophyletic origin and also confirm its distinct position within the *Thymallus thymallus* species, in spite of the fact that stocking with the Danubian type occurred in the Adriatic river system. Therefore the Adriatic population deserves special attention. As a first step in its preservation, its formal distinction by using the name Adriatic grayling is proposed.

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ADDITIONAL KEY WORDS: mitochondrial DNA – control region – cytochrome *b* gene, phylogeography, conservation biology.

INTRODUCTION

The peri-Adriatic river system harbours a variety of endemic freshwater fishes (Giuffra, Bernatchez & Guyomard, 1994; Durand, Persat & Bouvet, 1999; Bianco, 1986). On the basis of fossil records, palaeogeography and palaeoecological data it was proposed that some of these represent relict populations probably derived from primary Paratethian fishes (Bianco, 1990). The peculiarity of the peri-Adriatic ichthyofauna dictates its thorough survey, not only for conservation purposes, but also to reconstruct and improve our understanding of the different patterns of early freshwater fish colonization and dispersal throughout this region.

A geographically limited district of the peri-Adriatic area, confined to the Po river basin in Italy and the Soča river with its tributaries in Slovenia, is inhabited by grayling (Teleostei; Salmoniformes; Salmonidae: *Thymallus thymallus*). In comparison with the Danubian populations of grayling this population is regarded

as morphologically distinct (Janković, 1962) and as such recognized by many authors as a racially differentiated endemic population.

In addition to the peri-Adriatic district, grayling dominate the Danubian and Atlantic river systems, inhabiting most waters of central and northern Europe (Northcote, 1995). Its natural range is restricted to clean and swift-running watercourses with undamaged habitats. During the last few decades populations throughout Europe have been seriously affected by environmental degradation, over-fishing and predation by piscivorous birds (Uiblein *et al.*, 2000; Bertok & Budihna, 1999). The intra-specific diversity and long-term survival of grayling rely on maintaining local populations. Due to a constant decline of the population, stocking is and will probably remain the chief conservation action. Domestic strains of grayling with different degrees of genetic relatedness to the local populations have been purchased and used for stocking. For instance, strains originating from the Sava river (Danubian drainage) have been introduced into the Soča river (Adriatic drainage) and also dispatched to several fish farms in Italy, Austria and

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Germany (Bertok & Budihna, 1999). However, stocking may result in a number of problems, such as outbreeding depression and swamping of local gene pools, resulting in the replacement of wild and possibly locally adapted populations by non-adapted hatchery stocks (Hansen & Loeschcke, 1994). The best donor stocks for supplementary stocking are those most closely related to the threatened populations. Therefore, the ability to assess the relatedness based not only on morphological characters but also at the DNA level between grayling populations is of great importance.

Population studies based on molecular genetic data of *T. thymallus* appear to be relatively rare. Uiblein *et al.* (2000) surveyed the genetic structure in populations from southern Austria, based on the mtDNA control region, in order to devise appropriate management units. RFLP analysis of mtDNA ND genes, the cytochrome *b* gene and the control region was utilized to infer post-glacial dispersal routes into and within northern Europe (Koskinen *et al.*, 2000). For the purposes of conservation and management programmes Gross *et al.* (2001) investigated the genetic variation between three drainages in Bavaria analysing the ND 1/3/4 gene cluster of mtDNA and nuclear DNA markers. All the studies confirmed the genetic distinctness of geographically remote populations.

A pronounced phenotypic variation, particularly in southern Europe, was revealed by biometric analysis (Janković, 1960; Surre, Persat & Gaillard, 1986), colour patterns (Persat, 1982) and biochemical analysis (Persat, 1996). In spite of the fact that there has been no complete and methodological study, morphological differences observed between grayling from the Adriatic and those from the Danubian basin should be mentioned (Janković, 1960; Sabbadini, 2000). A morphological comparison of these two populations from the region of former Yugoslavia indicated statistically significant meristic differences (Janković, 1960). Sabbadini (2000) also confirmed that all the available data from the population study show that there are real racial differences between populations from the Black Sea and the Adriatic river system expressed in characters such as the number of radii in the pectoral fins, the number of pyloric caeca and many other traits. Apart from significant differences in a number of meristic characters, some peculiar external features concerning mainly the colour of the body, fins and tail are characteristics of the Adriatic population (for references see Sabbadini, 2000).

Several genetic studies in the past showed that the Mediterranean populations of many fish species differ considerably from those in the Danubian and Atlantic river systems [*Salmo trutta* (Giuffra *et al.*, 1994; Bernatchez, 2001), *Leuciscus cephalus* (Durand *et al.*, 1999; 2000;), *Luciobarbus sp.* (Machordom & Doadrio,

2001)]. However, no molecular analysis has been performed on grayling populations originating from the Adriatic area and southern Europe, which is considered an important refugium for several vertebrates during the Pleistocene glaciation. Therefore, in this study we propose addressing the following questions:

- (1) Could the endemicity of the Adriatic population, established on morphology, also be confirmed at the DNA level?
- (2) Does the distinctness of the Adriatic population justify its special protection in regard to the present practice of stocking?

Additionally, we performed the phylogenetic analysis to understand the early history of grayling, with emphasis on colonization patterns that caused the present distribution in southern Europe, particularly in the Adriatic river system. Genetic variation was estimated on the basis of mtDNA sequence data. In order to get an insight into genetic variability of the coding as well as the non-coding regions, a portion of the cytochrome *b* gene and the control region of mtDNA was analysed.

MATERIAL AND METHODS

SAMPLE COLLECTION AND DNA EXTRACTION

Grayling were sampled in the Adriatic and Danube basins. Additional samples were provided from the Atlantic and Mediterranean drainage (Table 1, Fig. 1). Blood samples were taken from the lateral vein of anaesthetized animals. Genomic DNA was extracted from erythrocytes following the protocol of Medrano, Aasen & Sharrow (1990).

DNA AMPLIFICATION, SEQUENCING AND SEQUENCE ANALYSIS

PCR amplification of an approximately 2400-bp segment, comprised of the cytochrome *b* gene and the control region, was performed using primers HN20 (Bernatchez & Danzmann, 1993) and C-Glu (Cronin, Spearman & Wilmont, 1993). PCR amplifications were performed in a programmable thermocycler PTC-100 (MJ Research, Inc.). A total volume of 30 μ l contained 1 μ M of each primer, 0.2 mM of dNTP, 1.5 mM of MgCl₂, 1 \times PCR buffer, 1 U of Taq polymerase (PE Applied biosystems) and 100 ng of genomic DNA. The following thermal profile was used: denaturation (95°C, 3 min) followed by 30 cycles of strand denaturation (94°C, 1 min), primer annealing (53°C, 30 s) and DNA extension (72°C, 1.5 min). Amplified DNA fragments were run on a 1.5% agarose gel and were purified from the gel using QIAEX II Gel Extraction Kit (QIAGEN).

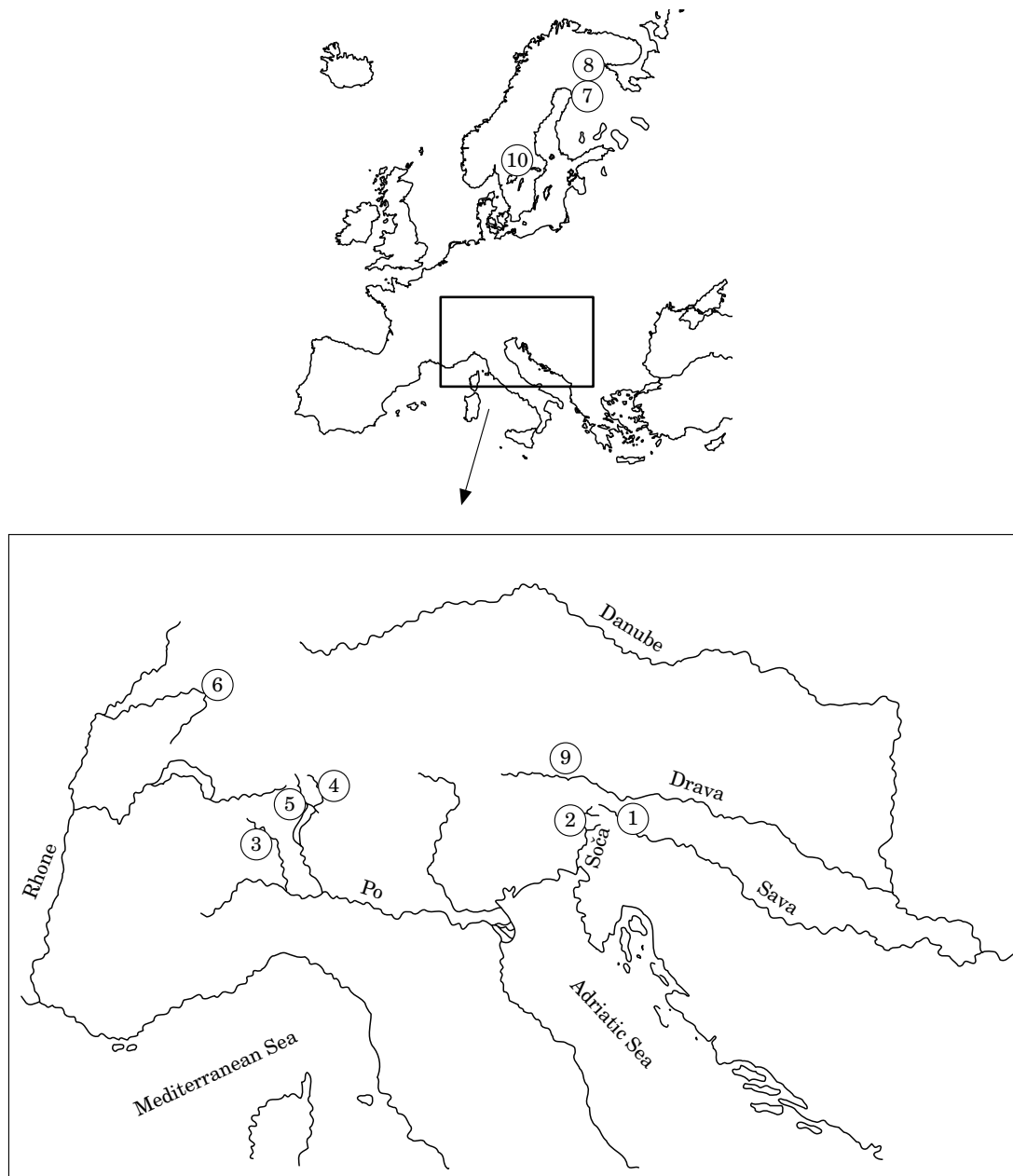


Figure 1. Grayling sampling locations: 1 – Sava; 2 – Soča; 3 – Sessia; 4 – Ticino; 5 – Maggia; 6 – Doubs; 7 – Iijoki and 8 – Tenojoki river. Locations where sampling was performed by Uiblein *et al.* (2000) are also marked: 9 – affluents of the Drava river, 10 – grayling hatchery in Sweden.

Sequencing reactions were prepared using BigDye Terminator Ready Reaction Mix (PE Applied Biosystems) according to the manufacturers recommendations. In order to allow the comparison of mutation rate within the coding and the non-coding mtDNA regions, a 363-bp segment at the 5'-end of the cytochrome *b* gene and a 394-bp segment of the control region were sequenced for all 65 individuals. The sequencing primer at the 5'-end of cytochrome *b* gene

was C-Glu (Cronin *et al.*, 1993). Sequencing of the 5'-end of the control region was carried out using an internal primer RIBa: 5'-CAC CCT TAA CTC CCA-AAG CTA AG-3. Termination PCR reactions were performed on a programmable thermocycler PTC-100 (MJ Research, Inc.) under the following conditions: 10 s denaturation at 96°C, 5 s annealing at 50°C and 4 min extension at 60°C, repeated for 30 cycles. The amplified, fluorescently labelled and terminated DNA

was precipitated with sodium acetate and ethanol and analysed on the ABI PRISM 310 automated sequencer.

The sequencing data of the 394-bp and 363-bp fragments of the control region and the cytochrome *b* gene respectively were combined and subjected to a distance analysis using the PHYLIP computer package (Version 3.5c, Felsenstein, 1993). Sequence divergences were calculated using the DNADIST programme, applying the Kimura 2-parameter model (Kimura, 1980) and with the transition/transversion ratio of 2. The resulting distance matrices of pair-wise distance comparisons of mtDNA haplotypes were used to build least-square estimates of the phylogenetic network with unconstrained branch lengths (FITCH programme). A phylogenetic tree was also generated from the aligned sequences using the quartet-puzzling, maximum likelihood procedure in the PUZZLE programme, version 4.0.2 (Strimmer & von Haeseler, 1996). It was performed under the HKY model of sequence evolution (Hasegawa, Kishino & Yano, 1985). Support values for each internal branch were obtained with the construction of 10 000 intermediate trees. An arctic grayling (*T. arcticus*) sequence was used as an outgroup. For the graphical representations of tree topologies the Treeview programme (Page, 1996) was applied.

RESULTS

SEQUENCE ANALYSIS

The sequence of a 394-bp segment at the 5'-end of the control region and 363-bp segment at the 5'-end of the cytochrome *b* gene were determined for 65 individuals from eight populations (Table 1, Fig. 1). The mutation rate of two mtDNA segments did not differ appreciably (Table 4). A total of 20 substitutions defining seven haplotypes was detected in the control region segment and 15 variable nucleotide positions determining six haplotypes in the cytochrome *b* gene region (Fig. 2).

The haplotype nomenclature used in this study follows completely that proposed by Uiblein *et al.* (2000), giving the names according to the specimens' geographic origin (Da for Danubian, At for Atlantic and Ad for the Adriatic river system). In their study, six haplotypes of the control region were detected (Da1-5, At1). Haplotypes observed in our investigation were designated Da6-9, At2 and Ad1; haplotype Da4 was the only one that was the same as defined previously by Uiblein *et al.* (2000). Two haplotypes designations in this study do not refer to sample origin (Da8 and Da9, found only in the Soča and Doubs rivers respectively). As revealed by the phylogenetic analysis, these were closely related to the Danubian haplotypes (Figs 2 and 3) and were therefore designated according

to the tree topology rather than to the geographical origin of samples. A combined analysis of the control and cytochrome *b* gene regions revealed nine composite haplotypes (Da4, Da6₁, Da6₂, Da7₁, Da7₂, Da8, Da9, At2, Ad1; the subscripts designate differences in the cytochrome *b* gene region within the same type of control region sequence; Tables 1 and 3).

The sequence variation in both the control and the cytochrome *b* gene regions was biased toward transitions with the ratios 13:7 and 12:3, respectively (Table 2). Of 15 substitutions detected in the cytochrome *b* gene region, 13 occurred at the third base and two at the first base of a codon, but none affected the amino acid sequence. In addition, no amino acid change was detected in the analysed part of the cytochrome *b* gene between *T. thymallus* and *T. arcticus*.

Pair-wise sequence divergence estimations among mtDNA haplotypes varied from 0.25 to 3.92% in the control region and from 0.28 to 3.10% in the segment of the cytochrome *b* gene (Table 4). The highest sequence divergences between Ad1 haplotype, which was characteristic for grayling in two rivers of the Adriatic drainage (Soča, Sessia), and other haplotypes observed, exceed the inter-specific divergences in some salmonid species reported by Berg & Ferris (1984).

MOLECULAR TREES

Both, phenetic (data not shown) and cladistic (Fig. 2) analyses of composite haplotypes of the 5'-ends of the control and cytochrome *b* gene regions were performed. In both cases the main topology of the dendrograms remained the same, showing one major Danubian-Atlantic cluster and two distinct lineages leading to haplotypes Da4 and Ad1 (Fig. 2). The cluster mainly consisted of haplotypes characteristic for the Danubian drainage and also of those found in the Mediterranean (Da9) and Atlantic drainage (At2). As regards haplotypes referring only to the mtDNA control region, a separation of the Atlantic haplotype from the Danubian branching was observed (see Table 4 for distances). The vast majority of haplotypes was geographically confined (Table 1). Haplotype At2 was found exclusively in grayling from the Atlantic river basin and represented the only haplotype found in this group of samples. Haplotypes Ad1 and Da4 were confined to those from the Adriatic river system, and haplotypes Da6₁, Da6₂, Da7₁ and Da7₂ to those from the Danubian river system. However, some of the Danubian haplotypes were also found in the Adriatic, mainly in the areas where the introduction of grayling from the Sava river system was practised (Bertok & Budihna, 1999). All the specimens from the Doubs river were fixed for the haplotype Da9, which was also found in one specimen from the Ticino river.

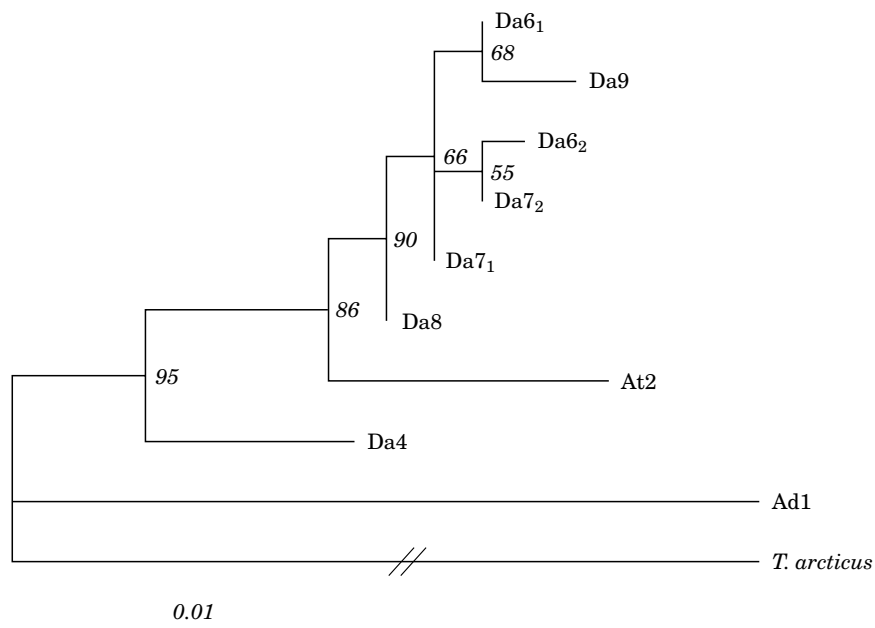


Figure 2. Maximum likelihood tree relating nine *Thymallus thymallus* composite mtDNA haplotypes. Confidence statements (in percent) estimated from 10 000 puzzling steps are shown between the nodes. Composite haplotype of *T. arcticus* (Acc. No. AF319545 and AF319544 for the control region and the cytochrome *b* gene region respectively) represents the outgroup of the tree. The analysis performed by algorithms in NEIGHBOR and FITCH programs of PHYLIP computer package resulted in the identical topology of the tree.

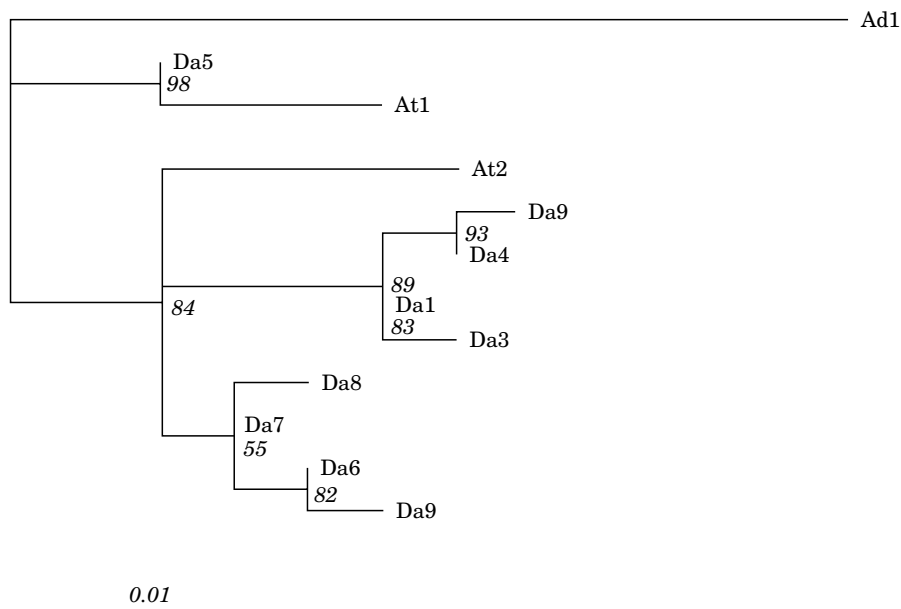


Figure 3. Maximum likelihood tree relating 11 *Thymallus thymallus* mtDNA control region haplotypes (haplotypes Da1-Da5 and At1 as reported in Uiblein *et al.*, 2000). Confidence statements (in percent) estimated from 10 000 puzzling steps are shown between the nodes. Haplotype AD1, which turned out to be very distant from other *T. thymallus* mtDNA haplotypes in previous analysis, was chosen as the outgroup of the tree.

Table 3. Matrix of pairwise distances under the Kimura 2-parameter method for the *Thymallus thymallus* control region and the cytochrome *b* gene region composite haplotypes (below main diagonal) and the observed number of nucleotide substitutions between composite haplotypes (above main diagonal)

	Da6 ₁	Da6 ₂	Da7 ₁	Da7 ₂	Da8	Da4	Da9	Ad1	At2	<i>T. arcticus</i>
Da6 ₁		1	1	2	2	9	2	23	7	35
Da6 ₂	0.13		2	1	3	10	3	24	8	36
Da7 ₁	0.13	0.26		1	1	10	3	24	6	36
Da7 ₂	0.26	0.13	0.13		2	11	4	25	7	37
Da8	0.26	0.40	0.13	0.26		9	4	23	7	35
Da4	1.20	1.33	1.33	1.47	1.20		11	22	14	37
Da9	0.26	0.40	0.40	0.53	0.53	1.47		25	9	37
Ad1	3.11	3.25	3.25	3.39	3.11	2.97	3.39		26	43
At2	0.93	1.06	0.80	0.93	0.93	1.87	1.20	3.52		35
<i>T. arcticus</i>	4.54	4.68	4.68	4.82	4.54	4.81	4.83	5.83	4.53	

Table 4. Matrix of pairwise distances under the Kimura 2-parameter method for the cytochrome *b* gene haplotypes (below main diagonal) and the control region haplotypes (above main diagonal) of grayling. *Equality of the composite haplotypes in one of the separate analysed regions (cytochrome *b* gene region below main diagonal and control region above)

	Da6 ₁	Da6 ₂	Da7 ₁	Da7 ₂	Da8	Da4	Da9	Ad1	At2	<i>T. arcticus</i>
Da6 ₁		*	0.25	0.25	0.51	1.02	0.25	3.39	1.54	6.15
Da6 ₂	0.28		0.25	0.25	0.51	1.02	0.25	3.39	1.54	6.15
Da7 ₁	*	0.28		*	0.25	1.28	0.51	3.65	1.28	6.43
Da7 ₂	0.28	*	0.28		0.25	1.28	0.51	3.65	1.28	6.43
Da8	*	0.28	*	0.28		1.02	0.77	3.39	1.54	6.15
Da4	1.39	1.67	1.39	1.67	1.39		1.28	3.39	2.06	6.42
Da9	0.28	0.55	0.28	0.55	0.28	1.67		3.65	1.80	6.44
Ad1	2.81	3.10	2.81	3.10	2.81	2.52	3.10		3.92	7.56
At2	0.28	0.55	0.28	0.55	0.28	1.67	0.55	3.10		5.86
<i>T. arcticus</i>	2.82	3.10	2.82	3.10	2.82	3.10	3.10	3.98	3.10	

DISCUSSION

OBSERVED HAPLOTYPES AND RELATIONSHIP OF THEIR DISTRIBUTION TO PALEOGEOGRAPHICAL EVENTS

In the relatively small number of populations analysed, nine haplotypes were found, revealing a complex genetic structure. As it was not our objective to provide a comprehensive phylogeography of grayling in Europe, these results allow us to propose some general conclusions about phylogenetic relationships. In spite of the fact that the sampled material in this study was partially affected by stocking activities and that the number of samples obtained at some locations was low, our results show that the distances among grayling mtDNA haplotypes are higher than in brown trout, in some cases even exceeding the values among mtDNA haplotypes of some salmonids on the inter-specific

level. Therefore, placing grayling populations with such an extensive mtDNA differentiation within a single species, without even a subspecies division, may be questionable.

A genetic relationship between haplotypes observed in this study was estimated with both distance-based (data not shown) and maximum likelihood (Fig. 2) phylogenetic inference methods. Both approaches, analysing either composite control and cytochrome *b* gene regions or only the control region, revealed the same tree topology. In order to evaluate the phylogenetic relationship between all the haplotypes observed so far, our results were compared with data already published [Uiblein *et al.*, 2000; haplotypes Da1–Da5 (southern Austria) and At1 (Sweden) in Figure 3; variable nucleotide positions of these haplotypes are given in Table 5]. Since the results of Uiblein *et al.* (2000) refer only to the control region, cytochrome *b* was omitted from detailed phylogenetic analysis. With regard to these

Table 5. Variable nucleotide positions of the control region haplotypes described by Uiblein *et al.* (2000). Numbers above the sequence refer to variable nucleotide positions according to the numbering in the Da6 sequence (Acc. No. AF27069). For other haplotypes variable nucleotides are shown, identity is indicated by dashes

Sequence haplotype	57	76	112	116	218	235	267	293	312	316	353	385
Da6	C	T	G	T	C	T	T	C	C	C	A	A
Da1	—	—	—	—	—	C	—	—	G	—	—	G
Da2	—	C	—	—	—	C	—	T	G	—	—	G
Da3	T	—	—	—	—	C	—	—	G	—	—	G
Da4	—	—	—	—	—	C	—	T	G	—	—	G
Da5	—	—	A	A	T	T	—	T	G	T	—	A
At1	—	—	A	A	T	T	C	T	A	T	G	A

data three main phylogenetic groups were observed: two Danubian-Atlantic clusters and a phylogenetic lineage leading to the Adriatic haplotype (Fig. 3).

The Adriatic population

The morphological characterization of the Adriatic population has already been established (Janković, 1960; Sabbadini, 2000) and represents a good argument that this population may be considered as a divergent lineage. However, phenotypic appearance is certainly not an absolute indicator of population differentiation, since its interpretation is subjective and may change due to environment, season, etc. Additionally, crossbreeding with introduced non-native grayling has blurred a clear picture of the primary appearance of grayling from the Adriatic district. Nevertheless, the peculiarity of the Adriatic populations is evident also at the genetic level. This was confirmed by our preliminary results from a microsatellite analysis (Sušnik, Snoj & Dovč, 1999) and further supported by the mitochondrial DNA data shown in the present study, confirming our hypothesis that this population is racially distinct.

The most common haplotype seems to be Ad1, found exclusively in the population confined to the Adriatic area. Several haplotypes characteristic of the Sava river population and haplotype Da4, otherwise found in Austrian river populations (see below) were also observed there. Since stocking from Austria and Slovenia has been carried out in the Adriatic area (Sabbadini, 2000) and Ad1 was not found outside it, it is reasonable to assume that this haplotype represents an autochthonous genetic variant which is confined to the North Adriatic basin. In addition, the results obtained by phylogenetic analysis also support this view and further define Ad1 as an ancient monophyletic clade with the earliest branching within haplotypes observed (Figs 2 and 3).

According to mitochondrial DNA variation, a genetic distance between Ad1 and Danubian and Atlantic

haplotypes of the control region ranged from 3.39 to 3.92%. Such extensive differentiation is probably due to early separation of the Adriatic and Danubian-Atlantic lineages from their common ancestor. The uniqueness and peculiarity of several freshwater fishes in river systems of the Italian peninsula and those draining into the Adriatic from within the Balkan peninsula have already been reported [*Salmo trutta marmoratus* (Giuffra *et al.*, 1994), *Leuciscus cephalus* (Durand *et al.*, 1999), *Rutilus rubilio* (Bianco, 1986), *Barbus plebejus*, *B. caninus*, *B. tyberinus* (Tsi-genopoulos & Berrebi, 2000)] suggesting their long separation and independent evolution.

Several hypotheses have been proposed in order to explain the very high level of endemic freshwater fauna existing in Mediterranean drainage. Migration of fishes, aided by the river connection between central and southern Europe in the Miocene or earlier was first proposed (Banareescu, 1973). However, there is no geological evidence that this river connection existed. Bianco (1990) proposed that Mediterranean endemic fishes could be regarded as having evolved from pure Paratethyan ancestors which colonized the Mediterranean area via the Adriatic-Pannonian sea-connection. The theory suggests that the Mediterranean was flooded by freshwater discharged from the Paratethys during the Messinian, about 5 Mya (the 'Lago Mare' event) resulting in a brackish or freshwater environment. This would have allowed freshwater fish dispersion from the Paratethys into the Mediterranean. Later, the return of marine waters caused a general extinction of all freshwater fishes and the species inhabiting the peri-Mediterranean ancient rivers were the only ones to survive. There is some palaeontological evidence supporting this hypothesis. In several peri-Mediterranean basins, fossils of Danubian primary fishes are not older than the Messinian. This would suggest that grayling could have participated in this early colonization of the Mediterranean drainages as well. Assuming that this palaeogeographic event occurred and in view of the

Table 6. Minimal and maximal values of genetic distances between groups of *Thymallus thymallus* mtDNA haplotypes. Comparison of *T. thymallus* with *T. arcticus* mtDNA haplotypes was also performed. The numbers in parentheses refer to the average genetic distances between two groups of mtDNA haplotypes

	Cytochrome <i>b</i>	Control region	Cytochrome <i>b</i> /control region
Ad1/other haplotypes	2.52–3.10 (2.93)	3.38–3.92 (3.57)	2.97–3.52 (3.25)
<i>T. thymallus</i> / <i>T. arcticus</i>	3.10–4.26 (3.48)	5.86–7.56 (6.43)	4.66–5.96 (4.94)
Ad1/ <i>T. arcticus</i>	4.26	7.56	5.96
Between <i>T. thymallus</i> except Ad1	0.28–1.67 (0.89)	0.25–2.06 (1.02)	0.13–1.87 (0.71)

mtDNA divergence between Ad1 and the other haplotypes, a molecular clock for grayling mtDNA can therefore be roughly calibrated as 0.68 to 0.78% nucleotide substitution per Myr. These data match the results obtained by Martin & Palumbi (1993), who estimated a substitution rate of salmon mtDNA of 0.5–0.9% per Myr.

However, phylogeographic studies on other freshwater fish species (Tsigenopoulos & Berrebi, 2000; Durand *et al.*, 2000) suggest that the grayling may have reached the north Italian/Adriatic area as a result of river capture, which operated during the Pleistocene and may have occurred between low valleys joining the opposite sides of mountain ranges in the Alps. Our results are not congruent with this latter view and, instead, tend to support the scenario proposed by Bianco (1990). It seems probable that the Adriatic population may be regarded as a relict Paratethyan element isolated since the late Miocene.

It may seem contradictory that an old lineage such as the Adriatic population exhibits no intra-population genetic variation within the most variable mtDNA region. The first possible explanation is that due to a low number of samples and populations analysed, some of the existing polymorphism remained undiscovered. However, it should also be noted that the habitats have been relatively small and, due to their distribution over the Alpine region, the grayling must have been considerably affected by several Pleistocene glaciations, particularly during the last Würm (20 000–18 000 years ago). Influenced by stochastic events, the Adriatic population had probably gone through many bottlenecks and possibly, due to non-random genetic drift, fixation of a single haplotype occurred.

The Danubian-Atlantic clusters

Two Danubian-Atlantic clusters of mtDNA haplotypes were found when we combined our results with those of Uiblein *et al.* (2000). The first cluster could be further divided into three separate branchings (Fig. 3). The first branch represents haplotypes characteristic of the upper drainage of the Sava river (Da6, Da7 and Da8).

Da9, found in the Doubs river (Mediterranean drainage), was placed into this group as well. Taking into account the fact that the Doubs river was stocked with non-native grayling, the presence of Da9 might be a consequence of stocking rather than synapomorphism shared by the Danubian and Mediterranean populations. The second branch consists of Da1 to Da4, which are confined to the Inn drainage, and the third is represented by At2. The presence of Da4 in the Ticino and Maggia rivers, (both belonging to the Adriatic river system) may result from stocking with grayling originating from Austrian hatcheries.

The second cluster contained Da5 and At1. Da5 was restricted to the population from the Drava drainage; according to Uiblein *et al.* (2000) it represents a highly diverged evolutionary branch, unique to the Austrian region Carinthia.

Two Atlantic haplotypes (At2 and At1) were also placed within both Danubian-Atlantic clusters. Both were associated with the Atlantic drainage (At2 found in Finland and At1 in Sweden). According to the results obtained by phylogenetic analysis, they exhibited paraphyletic rather than monophyletic origin. Two or more lineages packed parapatrically in areas colonized after the last glaciation have already been described for many terrestrial and aquatic organisms (Taberlet & Bouvet, 1994; Wallis & Arntzen, 1989; Nesbø *et al.*, 1999; García-Marín, Utter & Pla, 1999). Based on PCR-RFLP and sequencing analysis of mtDNA fragments (ND1, ND3/4, ND5/6, cytochrome *b*/control region), the same scenario has also been proposed for grayling (Koskinen *et al.*, 2000). It was suggested that the Danubian area did not contribute markedly to the recolonization of grayling into northern glaciated regions (Koskinen *et al.*, 2000; Gross *et al.*, 2001). Nevertheless, the great similarity between Atlantic (At2) and Danubian haplotypes (Da6–9) in the cytochrome *b* gene region (minimum 0.28% nucleotide divergence; Table 4) implied a closer phylogenetic relationship between them than proposed.

A much higher intraspecific diversity in the southern areas than in the recently colonized northern regions has been documented for many freshwater fishes; it

was also observed in this study. Taking into account the level of genetic divergence between Danubian haplotypes (1–1.6%) and in accordance with palaeogeological events, the separation of the Danubian populations could have appeared in the late Pliocene or early stage of Pleistocene, as a consequence of new, isolated habitats, formed within each river system.

CONCLUSION

Among the several lineages of grayling found so far, the Adriatic one seems to be the most divergent. The present results, based on genetic distances, indicate its ancient monophyletic origin. Moreover, its branching appears to be earlier than that of any other phylogenetic groupings. The nucleotide divergence between Ad1 and the other haplotypes, based on the mtDNA control region, ranged from 2.97 to 3.52% (Table 6). This value exceeded the greatest divergence found in arctic grayling, when Russian versus Northern American haplotypes of mtDNA control region were compared (0.5 to 2.4%; Redenbach & Taylor, 1999). In addition, the divergence between grayling from the Adriatic and Danubian drainage in terms of the cytochrome *b* gene was about the same as the divergence between grayling from the Danubian drainage and arctic grayling (Table 6). The present molecular data showed that, according to evolutionary (Wiley, 1978) or phylogenetic species concepts (Cracraft, 1987), the Adriatic population could potentially be regarded as a distinct species or at least as a population on its way to speciation. In our opinion such a population should be given an opportunity to preserve its primary identity and continue its evolution in the sense of adaptation to a specific environment. However, this identity is seriously affected by massive and continuous introduction of foreign strains of grayling. In order to preserve the autochthonous populations, stocking of non-native grayling has to be terminated immediately. Animals meant for stocking or reproduction should be in the first place chosen according to the characteristic phenotype. The final selection should be performed by genotyping based on diagnostic genetic markers.

In order to promote the identification of the Adriatic population it would be appropriate to distinguish it also by its name. Without accurate naming it is impossible to identify various stocks, evaluate their real conservation status and list them as endangered (Kottelat, 1997). With regard to phenotypic and genetic distinctness of the Adriatic population, we suggest a simplified working nomenclature based on geographical distribution (as long as its taxonomic status is not established). As proposed by Sabbadini (2000),

we would also suggest the use of the working name 'Adriatic grayling'.

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