MOLECULAR ECOLOGY

Molecular Ecology (2010) **19**, 4708–4722 doi: 10.1111/j.1365-294X.2010.04844.x

Range-wide population genetic structure of the European bitterling (*Rhodeus amarus*) based on microsatellite and mitochondrial DNA analysis

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Abstract

An understanding of recent evolutionary processes is essential for the successful conservation and management of contemporary populations, especially where they concern the introduction or invasion of species outside their natural range. However, the potentially negative implications of intraspecific introductions and invasions have attracted less attention, although they also represent a potential threat to biodiversity, and are commonly facilitated through human activities. The European bitterling (*Rhodeus amarus*) is a small cyprinid fish that decreased greatly in its distribution during the 1970s and 1980s and was subsequently included on many European conservation lists. This decline appears to have reversed, and the extent of its distribution now exceeds its former range. We used a combination of 12 microsatellite markers and cytochrome *b* sequences on a large data set (693 individuals) across the current range of the European bitterling to investigate possible scenarios for its colonization of Europe. We show that the inferred history of colonization of Europe was largely congruent between mitochondrial and nuclear markers. The most divergent mtDNA lineages occur in the Aegean region but probably are not reproductively isolated as the Aegean populations also displayed mtDNA haplotypes from other lineages and nuclear data indicated their close relationship to Danubian populations. Much of Europe is currently populated by descendants of two main lineages that came to natural secondary contact in western Europe. An approximate Bayesian computation analysis indicates different dates for admixture events among western and central European populations ranging from the last deglaciation (natural) to the last few centuries (human-assisted translocations).

Keywords: co-evolution, conservation, fish, intraspecific introduction, non-native populations

Received 16 November 2009; revision received 19 August 2010; accepted 25 August 2010

Introduction

The distinction between native and non-native populations is often problematic at the interspecific level (i.e. non-native species introductions). Some species that have been considered invasive and subject to eradication programmes have later been discovered to be native to a region (van Leeuwen *et al.* 2008). Likewise, some species considered to be indigenous, and subjected to legal protection and conservation efforts, have later proven to be recent colonizers via human-assisted dispersal or translocations (Castilla *et al.* 2002). Within species, it is even more challenging to identify native and non-native populations and delineate their natural distributions. Populations from certain regions may be

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competitively superior to those from other parts of the species' range. If individuals from competitively superior populations are translocated outside their range, they can replace locally endemic and previously isolated populations with a consequent loss in genetic diversity (e.g. Kawamura *et al.* 2001). Hitherto intraspecific introductions and invasions have attracted less attention from conservation biologists than interspecific invasions. Despite this, intraspecific invasions represent a potential threat to biodiversity and, like interspecific invasions, are likely to be facilitated through human activities (Mabuchi *et al.* 2008). Indeed, given its cryptic nature, intraspecific introductions may prove to be a more insidious threat (Petit 2004).

The European bitterling (*Rhodeus amarus*) is a small cyprinid fish (subfamily Acheilognathinae) that is common in lotic and lentic habitats throughout Europe (Van Damme *et al.* 2007). It is a thermophilic species with an optimum temperature for its reproduction of 23 °C (Smith *et al.* 2004). *R. amarus* populations decreased considerably during the 1970s and 1980s, especially in western Europe, and were included in most European conservation lists, including the EC Habitat Directive (Lelek 1987; Kirchhofer & Hefti 1996; European Commission 2002). However, the trend recently appears to have reversed; population numbers of *R. amarus* have increased throughout Europe and the extent of its distribution now considerably exceeds its recent formerly described range (Kottelat & Freyhof 2007; Kozhara *et al.* 2007). The recent expansion of *R. amarus* largely appears to be a result of natural dispersal into new habitats (reviewed in Van Damme *et al.* 2007; Kozhara *et al.* 2007), although several cases of human-assisted introduction are well described, both within (Germany) and outside the original range of *R. amarus* (Italy, Denmark, UK, Russia) (Kozhara *et al.* 2007). Notably, *R. amarus* is a parasite of threatened and declining populations of unionid mussels (Karatayev *et al.* 1997; Reichard *et al.* 2006) and, at least in western and central Europe, appears to use evolutionary naive hosts for oviposition (Reichard *et al.* 2007, 2010). Recent research on bitterling has addressed the co-evolutionary relationship between these fish and freshwater mussels (Reichard *et al.* 2006, 2007, 2010). Proper resolution of the phylogeographical and historical status of the European bitterling will be crucial to an understanding of the evolutionary context of their relationship with freshwater mussels and impacts upon them.

Given the strong dependence of *R. amarus* on relatively high water temperatures for reproduction, its range has probably undergone numerous contractions and expansions during Quaternary climate oscillations. While populations in Aegean and Pontic regions have been locally present throughout the Quaternary (Bohlen *et al.* 2006), historical records provide evidence that populations in central and western Europe either established within the last two centuries or fluctuated substantially over relatively short time periods during the last 500 years (Van Damme *et al.* 2007). This historical evidence is supported by relatively low haplotype diversity in bitterling populations in central and western Europe (Bohlen *et al.* 2006). In many European countries, populations of *R. amarus* were only recorded after the end of the Little Ice Age in the mid-19th century, despite detailed accounts of their native fish faunas being available and the unique characteristics of bitterling making records of its distribution especially reliable (Kottelat & Freyhof 2007; Van Damme *et al.* 2007). Hence, it is possible that *R. amarus* may not be native to large parts of Europe, with its presence in western Europe being a consequence of unintentional stocking with the common carp (*Cyprinus carpio*) as a by-product of the formerly widespread trade in live fish for cultivation (Van Damme *et al.* 2007).

Phylogeographical analysis based on a mitochondrial gene (cytochrome *b*) indicates that there are four lineages of bitterling in Europe (Bohlen *et al.* 2006). *Rhodeus amarus* sensu stricto is divided into two lineages: a western lineage (this mtDNA lineage will hereafter be designated as 'WEST') distributed through the River Danube basin and much of western Europe, while an eastern lineage ('EAST') occurs in the Carpathians, the River Vistula and eastern Europe. Two lineages were recently distinguished from *R. amarus* sensu lato as separate species, *Rhodeus meridionalis* Karaman 1924 from the River Vardar in Greece (Bohlen *et al.* 2006) and *Rhodeus colchicus* Bogutskaya & Komlev 2001 from an isolated site in the central Caucasus (Bogutskaya & Komlev 2001). A mixture of WEST and EAST haplotypes has been recorded in some western European populations (Bohlen *et al.* 2006). This finding indicates natural secondary contact of the two lineages after colonization of Europe but may also be a signal of human-assisted introductions. In addition to the trade in juvenile carp for cultivation, the European bitterling has been widely traded as an ornamental fish and for use in pregnancy testing during the 20th century (Wiepkema 1961), making translocations more likely.

Because of their mode of inheritance, mitochondrial markers are more likely to yield erroneous conclusions in phylogeographical analysis because their gene genealogy can differ from the species' history, leading to biased estimates of species phylogeny (e.g. Flanders *et al.* 2009). One means of avoiding this problem is to combine nuclear and mitochondrial markers. However, most nuclear genes have a low level of variability, while hypervariable nuclear markers (such as microsatellites)

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are considered less appropriate for phylogeographical inference because of their tendency towards homoplasious mutations. Nevertheless, high allelic diversity and the use of multiple independent loci can often compensate for the disadvantages of homoplasious evolution (Estoup *et al.* 2002). Several recent studies have successfully applied the combination of microsatellites and mtDNA sequences to the same data set, with the resulting information superior to that obtained through the use of separate sets of markers (e.g. Heckel *et al.* 2005; Flanders *et al.* 2009).

Here we use a large data set, derived from 693 individuals, from across the range of the European bitterling (a total of 26 populations) genotyped at 12 microsatellite markers, with the addition of cytochrome *b* sequences from a subset of individuals, to address four specific questions: (i) Is the history of bitterling colonization of Europe reconstructed using data from nuclear microsatellites congruent with mitochondrial-based analyses? (ii) How are bitterling populations genetically structured across its European range? (iii) Are mitochondrial lineages from the Mediterranean part of the bitterling range reproductively isolated and can they be considered as a separate species? (iv) Are bitterling populations in western Europe descendants of recent or ancient invasions relative to those elsewhere across its range?

Materials and methods

Sampled populations and DNA extraction

Population samples (N = 12–46 fish per locality) were collected at 26 localities in 16 countries (Fig. 1, Table 1) covering almost the entire range of the European bitterling (regardless of species status), including the type locality of *R. meridionalis* in the River Vardar (Bohlen *et al.* 2006). Tissue samples (usually fin clips) from 693 individuals were collected over a 2-year period and were of adults and subadults, which minimized the risk of sampling close relatives because bitterling disperses at the larval stage (Reichard *et al.* 2002). Tissue was stored in 96% ethanol until DNA extraction, performed using the DNeasy Blood & Tissue kit (Qiagen).

Microsatellite genotyping

All samples were genotyped for 12 variable microsatellite loci *Rser01–06*, *Rser08–Rser12* (Dawson *et al.* 2003) and *Rser13* (Reichard *et al.* 2008) in three multiplex PCR sets (Table S1, Supporting information). The reaction mix contained 2 μL of extracted DNA, 0.2 mM dNTPs, 3 mM $MgCl_2$, 1 unit of Taq polymerase (Fermentas), 1× PCR buffer, primers in various concentrations (Table S1, Supporting information) and ddH_2O up to the final volume of 20 μL. The amplification started with initial denaturation at 94 °C (3 min), followed by 30 cycles of denaturation at 94 °C (40 s), annealing at 60 °C (Set 1 and Set 3) or 61 °C (Set 2) (30 s), extension at 72 °C (1 min) and finished with a final extension at 72 °C (7 min). The PCR products (1 μL) were added to a denaturing mixture of size standard (Genescan®, ROX 500; Applied Biosystems) and formamide, and the mix was run on the ABI Prism® 3130 Genetic Analyzer (Applied Biosystems). The length of the DNA fragments was analysed manually by the first author in GeneMapper® v. 3.7 (Applied Biosystems).

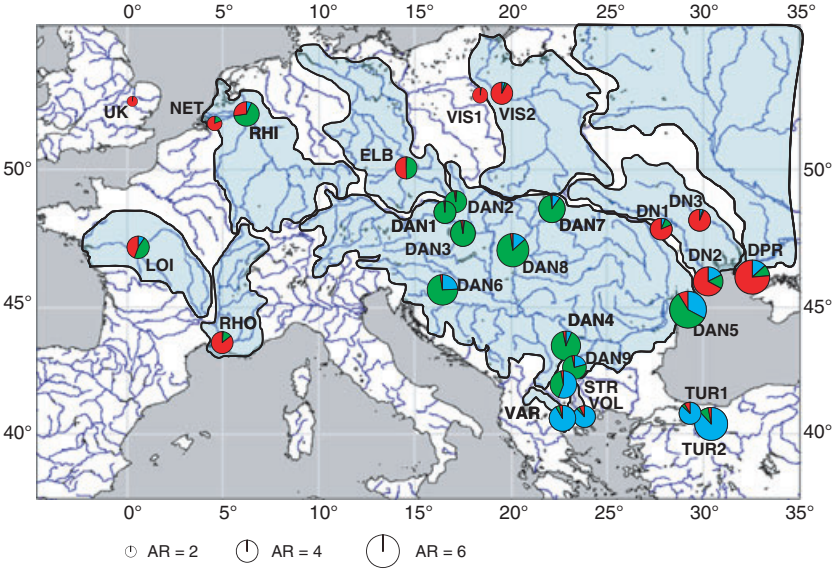

Fig. 1 Map of 26 sampled localities. The colours in pie charts represent the proportional membership of individuals to microsatellite-based clusters inferred from STRUCTURE for K = 3; the size of circles represents the mean allelic richness of nine loci corrected for sample size (see text for more details). Drainages of sampled rivers are shaded.

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Table 1 The sampled localities. Sample size = the number of individuals genotyped at 12 microsatellites; MtDNA = number of partial cytochrome *b* sequences used

Code	Locality	Country	Coordinates	Sample size	MtDNA	Provider	Basin
DAN1	Kyjovka River	Czech Republic	N 48°46′48″ E 17°00′58″	45	—	M. Reichard	Danube
DAN2	Morava River, Čapí, Bažina	Czech Republic	N 48°41′35″ E 16°59′ 56″	34	—	M. Ondračková	Danube
DAN3	Gabčíkovo, Priehradky	Slovakia	N 47°53′00″ E 17°30′00″	15	—	M. Ondračková	Danube
DAN4	Danube, Archar	Bulgaria	N 43°49′14″ E 22°54′ 58″	30	—	M. Ondračková	Danube
DAN5	Danube Delta at Vilkovo	Ukraine	N 45°25′00″ E 29°33′37″	25	8	A. Naseka	Danube
DAN6	Sava-Cazma systém, Glogornica channel at Mostari	Croatia	N 45°46′43″ E 16°29′24″	15	7	J. Bohlen	Danube
DAN7	River Ublianka at village Ubla	Slovakia	N 48°53′56″ E 22°23′26″	21	—	J. Bohlen	Danube (Tisza)
DAN8	Tápio stream, near the village Tápiószele	Hungary	N 47°21′49″ E 19°49′22″	25	7	T. Eros	Danube (Tisza)
DAN9	Palakariya River in village of Shirokiy Dol	Bulgaria	N 42°23′26″ E 23°31′12″	25	—	M. Reichard	Danube
STR	Blavgoevrgrad, Struma river	Bulgaria	N 42°01′33″ E 23°02′52″	33	8	M. Ondračková	Struma
RHI	Grietherother Altrhein NW of city Rees	Germany	N 51°47′17″ E 06°22′17″	27	8	J. Bohlen	Rhine
LOI	Loire River, Mont Jean sur Loire	France	N 47°24′02″ E 00°55′05″	19	8	E. Lasne	Loire
ELB	Labe River near Obříství	Czech Republic	N 50°17′48″ E 14°28′53″	46	7	M. Ondračková	Elbe
DN1	Kubolta River	Moldova	N 48°03′02″ E 27°54′30″	27	—	A. Naseka	Dniester
DN2	Dniester Liman at Krasnaya K.	Ukraine	N 46°19′58″ E 30°06′05″	19	—	A. Naseka	Dniester
DN3	Tashlychka River at Gaivoron	Ukraine	N 48°20′45″ E 29°53′28″	21	6	A. Naseka	Dniester
DPR	Dniprovski Liman near Stara Zburievka village	Ukraine	N 46°29′13″ E 32°26′23″	21	—	J. Bohlen	Dniepr
VIS1	Lake Kociolek	Poland	N 52°37′02″ E 18°28′42″	29	5	M. Przybylski	Vistula
VIS2	Wloclawski reservoir	Poland	N 52°33′00″ E 19°35′00″	24	—	M. Ondračková	Vistula
UK	Wicken Fen	United Kingdom	N 52°19′43″ E 00°15′55″	42	—	M. Reichard	Great Ouse
NET	Netherlands creek Mark, Zevenbergen	Netherlands	N 51°37′34″ E 04°35′08″	16	7	M. Soes	Rhine (delta)
RHO	Durance River	France	N 42°52′00″ E 04°58′00″	12	—	M. Ondračková	Rhone
TUR1	Ömerli Dan Lake	Turkey	N 41°03′11″ E 29°22′05″	31	—	A. S. Tarkan	Black Sea
TUR2	Lake Sapanca	Turkey	N 40°42′46″ E 30°10′30″	24	31	M. Reichard	Sakarya
VAR	Axios (=Vardar) River near Thesalloniki	Greece	N 40°37′06″ E 32°42′36″	29	26	M. Reichard	Vardar
VOL	Lake Volvi	Greece	N 40°40′38″ E 23°27′30″	38	8	M. Koutrakis	Lake Volvi
Total				693	136		

Microsatellite genetic diversity

Genotypic linkage equilibrium between all pairs of loci and confirmation of Hardy–Weinberg equilibrium (HWE) were tested for each locus separately and over all loci for each sampling site with exact tests using the Markov chain methods in GENEPOP 3.4 (Raymond & Rousset 1995). Correction for multiple testing was performed using the false discovery rate (FDR) approach (Benjamini & Hochberg 1995) in QVALUE (Storey 2002).

Genetic variation was estimated over all loci for each sampling site from the unbiased expected (H_e) heterozygosity in GENETIX 4.05 (Belkhir *et al.* 1996–2004). As the observed number of alleles in a sample is highly dependent on sample size, we used the rarefaction procedure implemented in FSTAT 2.9.3.2 (Goudet 2001) to calculate allelic richness (AR). Significant heterozygote deficit and deviation from HWE are often caused by the presence of null alleles (NA). Therefore, we estimated the proportion of NA at each locus and in each population in FREENA (Chapuis & Estoup 2006).

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Population structure based on microsatellite data

Genetic differentiation between sampling sites was quantified by computing pairwise estimators of F_{ST} according to Weir & Cockerham (1984), and their significance was tested by 1000 permutations in GENETIX. The genetic relationships between all genotyped individuals were obtained by factorial correspondence analysis (FCA) using GENETIX. FCA is a form of factor analysis that detects the best linear combination of variables (allele frequencies at different loci) and describes the variation between observations (individuals or populations). The distribution of populations and their genetic structuring was matched with the geographical position of sampling sites and location within drainages.

A Bayesian clustering procedure implemented in STRUCTURE 2.2 (Falush *et al.* 2003) was used to infer the number of distinct genetic populations represented by the samples and the assignments of individuals to these genetic clusters. The Bayesian model assumes K (unknown) populations with different allele frequencies at a set of independent loci. The program was run with 10 independent simulations for each value of K from 1 to 12, each with 10^6 iterations, following a burn-in period of 10^5 iterations. In all simulations, an admixture ancestry model and independent allele frequency model (with $\lambda = 1$) were used. The likelihood of K, i.e. Ln $\Pr(X \mid K)$, was used to infer the most likely number of real populations in the data set using the method of Evanno *et al.* (2005). Alternatively, we forced the assignments of individuals to cluster beyond the number considered to maximize the posterior probability of the data. This approach can be used to reconstruct the hierarchical relationships between populations, as well as to distinguish between historical processes that are likely to shape this structure (Wang *et al.* 2007; Flanders *et al.* 2009). The results of ten replicate runs for each value of K were combined using the Greedy algorithm of CLUMPP 1.1.1 (Jakobsson & Rosenberg 2007), and summary outputs for each value of K were displayed graphically using DISTRUCT v. 1.1 (Rosenberg 2004).

Testing alternative scenarios by ABC modelling

To obtain a more detailed inference on the evolutionary history of central and western European bitterling populations, several potential scenarios were constructed and analysed using the approximate Bayesian computation procedure (ABC; Beaumont *et al.* 2002) in DIYABC 0.8.1 (Cornuet *et al.* 2008). Although there has been debate about the use of ABC in phylogeography (pertaining to the testing of complex phylogeographical models *via* computer simulations, e.g. Templeton 2010 vs. Beaumont *et al.* 2010), we consider the ABC procedure as a useful tool for inferring population history (for theoretical and practical aspects, see recent reviews of Bertorelle *et al.* 2010; and Csilléry *et al.* 2010), and the technique is proving both robust and highly informative (e.g. Verdu *et al.* 2009). For the ABC analysis, six groups of populations were created according to the Bayesian assignment of their genetic structure in STRUCTURE: the northeastern group (NE) consisted of the populations VIS1, VIS2 and DN3 (74 individuals); the Pannonian group (PA) of DAN7 and DAN8 (46 individuals); the Middle-Upper Danube (UD) of DAN1, DAN2 and DAN3 (94 individuals); the River Elbe (EL) formed by a single population (ELB, 46 individuals); and two western European groups, the first (W1) comprised genetically similar NET, UK and RHO populations (70 individuals) and the second (W2) was composed of RHI and LOI (46 individuals). Based on the mtDNA phylogeography from Bohlen *et al.* (2006), the PA and NE groups were considered to have been derived from a hypothetical ancestral population (AN). In total, 32 scenarios in four steps were constructed to test the origin of four population groups (Table 2). The origin of the groups UD, EL, W1 and W2 was investigated by comparison with 6–10 scenarios for each step (Table 2).

To describe the different scenarios, several parameters were used (effective population size, timing of events such as merging, splitting or change in effective population size through time, and rates of admixture in the case of a merging events) whose values were drawn from the minimum–maximum range of priors (Table S3, Supporting information). As a mutation model, we used the generalized stepwise model (GSM; Estoup *et al.* 2002) in which a mutation increases or decreases the number of repeated motifs by one or several units, the latter number being drawn from a geometric distribution. A figure of one bitterling generation per year was used based on Smith *et al.* (2004). The mutation rate (μ) and the parameter for the geometric distribution (P) were drawn from the prior distribution used as default in DIYABC (Cornuet *et al.* 2008; Table S3, Supporting information). Single nucleotide mutations were also considered.

A reference table consisting of 1 million simulated data sets per scenario was created for each step. Subsequently, 1% of the simulated data sets closest to the observed data (using Euclidian distances between each simulated and observed data set) were used to estimate the relative posterior probability (with 95% confidence intervals) of each scenario via a logistic regression estimate (Cornuet *et al.* 2008). Finally, the posterior parameter distributions were estimated from 1% of the closest data sets simulated according to the most likely scenario for each step as chosen previously.

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Table 2 Description of all scenarios used in the approximate Bayesian computation analysis (ABC; Beaumont *et al.* 2002) in DIYABC 0.8.1 (Cornuet *et al.* 2008) to infer colonization history of the central and western European bitterling populations. The best scenario for each of four steps is emboldened. The best scenario of the first step (scenario 1.1) was used to construct all the other scenarios. The relative posterior probabilities and 95% confidence intervals for each scenario were computed via the logistic regression on 1% of the closest data sets to the observed data. UD = DAN1 + DAN2 + DAN3; EL = ELB; W1 = UK + NET + RHO; W2 = RHI + LOI; PA = DAN7 + DAN8; NE = VIS1 + VIS2 + DN3; and AN = a hypothetical ancestral population founding the PA and NE groups (see text for more details)

Step	Scenario	Posterior probability	Credibility interval
Step 1	1.1. only from PA	**0.8429**	**[0.6704, 1.0000]**
Origin	1.2. only from NE	0.0686	[0.0000, 0.1697]
of UD	1.3. only from AN	0.0303	[0.0000, 0.0814]
	1.4. mix NE and PA	0.0165	[0.0013, 0.0316]
	1.5. mix AN and PA	0.0003	[0.0000, 0.0007]
	1.6. mix AN and NE	0.0414	[0.0000, 0.1039]
Step 2	2.1. mix NE and UD	**0.9999**	**[0.9997, 1.0000]**
Origin	2.2. mix UD and PA	0.0000	[0.0000, 0.0000]
of EL	2.3. mix NE and PA	0.0001	[0.0000, 0.0003]
	2.4. only from NE	0.0000	[0.0000, 0.0000]
	2.5. only from UD	0.0000	[0.0000, 0.0000]
	2.6. only from PA	0.0000	[0.0000, 0.0000]
Step 3	3.1. mix NE and UD	**0.6481**	**[0.4646, 0.8317]**
Origin	3.2. mix UD and PA	0.0001	[0.0000, 0.0001]
of W1	3.3. mix NE and PA	0.2961	[0.1237, 0.4685]
	3.4. only from NE	0.0003	[0.0001, 0.0005]
	3.5. only from UD	0.0000	[0.0000, 0.0000]
	3.6. only from PA	0.0000	[0.0000, 0.0000]
	3.7. only from AN	0.0001	[0.0000, 0.0003]
	3.8. mix AN and NE	0.0374	[0.0087, 0.0661]
	3.9. mix AN and UD	0.0153	[0.0014, 0.0292]
	3.10. mix AN and PA	0.0026	[0.0003, 0.0050]
Step 4	4.1. mix NE and UD	0.2449	[0.1044, 0.3854]
Origin	4.2. mix UD and PA	0.0191	[0.0029, 0.0353]
of W2	4.3. mix NE and PA	**0.3912**	**[0.2253, 0.5572]**
	4.4. only from NE	0.0232	[0.0035, 0.0429]
	4.5. only from UD	0.0035	[0.0003, 0.0067]
	4.6. only from PA	0.0102	[0.0019, 0.0184]
	4.7. only from AN	0.0148	[0.0021, 0.0275]
	4.8. mix AN and NE	0.1221	[0.0344, 0.2098]
	4.9. mix AN and UD	0.0605	[0.0156, 0.1053]
	4.10. mix AN and PA	0.1106	[0.0366, 0.1845]

Analysis of mitochondrial DNA

The cytochrome *b* gene was amplified in selected individuals (with the aim of covering the entire study area; see Table 1) using the primers Glu L and Thr H following the protocol of Bohlen *et al.* (2006). The PCR products were sequenced only from one side using the Glu L primer. We combined the sequences obtained in this study with the data set of sequences for *R. amarus*, *R. colchicus* and *R. meridionalis* from Bohlen *et al.* (2006). The haplotypes and their frequencies were identified using DnaSP v. 5 (Librado & Rozas 2009). Median-joining (MJ) networks were constructed in Network 4.510 (Bandelt *et al.* 1999) using an equal transition/transversion ratio.

Results

Microsatellite genetic diversity

Multilocus genotypes from 693 individual samples were obtained with high genotyping success (98.3%). Most of the missing genotypes (80 from 144, 56%) were on the locus *Rser09*, possibly because of the presence of NA. The mean proportion of NA at *Rser09* was almost 9% (Table S1, Supporting information), but it rose to 31% for population DAN9 and 25% for DAN3. The loci *Rser04* and *Rser13* showed high levels of overall polymorphism (93 and 98 alleles, respectively) with pronounced differences between populations (1–31 alleles per population for *Rser04* and 4–35 alleles per population for *Rser13*), which may have significantly biased estimates of mean AR per locus. Consequently, we performed some analyses both with and without *Rser04*, *Rser09* and *Rser13* to achieve unbiased and robust results (hereafter we use the terms '12-loci' and '9-loci' analyses).

Only one pair of loci (*Rser11* and *Rser13*) showed significant linkage disequilibrium in one population (VIS2) after FDR correction. The exact tests in GENEPOP failed to find HWE in 11 of 26 populations in a 12-loci analysis (Table 3). The deficit of heterozygotes was most probably caused by NA at *Rser09*, because only two populations significantly deviated from HWE in the 9-loci analysis (Table 3). In these two populations, the homozygote excess could again be the result of NA at single loci (26% at locus *Rser05* in population DAN5 and 36% at locus *Rser02* in VAR). The values of *He* and AR are shown in Table 3. Mean 9-loci AR per locus estimated for a minimum number of 12 diploid individuals was highly correlated with both the 12-loci *He* (Pearson correlation, $r = 0.797$, $P < 0.001$) and 9-loci *He* ($r = 0.769$, $P < 0.001$). Genetic variation decreased with increasing distance from the Black Sea (Danubian drainage, 9-loci analyses; AR: $R = -0.70$, $P = 0.035$; He: $R = -0.72$, $P = 0.028$) (Fig. 1).

Population structure based on microsatellite data

All but one pairwise F_{ST} were significantly higher than zero ($P < 0.01$), indicating strong population structuring (Table S2, Supporting information). The only

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Table 3 Genetic variation and the results of Hardy–Weinberg equilibrium (HWE) tests in study populations

Locality	He (12)	He (9)	AR (9)	HWE (12)	HWE (9)
DAN1	0.59	0.50	3.81	0.191	0.561
DAN2	0.56	0.46	3.87	0.330	1.000
DAN3	0.65	0.58	4.78	**<0.001**	0.741
DAN4	0.72	0.65	5.33	**<0.001**	0.121
DAN5	0.77	0.71	6.64	**0.001**	**0.013**
DAN6	0.64	0.54	5.36	0.129	0.312
DAN7	0.65	0.56	4.95	0.191	0.905
DAN8	0.71	0.64	5.62	0.118	1.000
DAN9	0.57	0.52	4.60	**<0.001**	0.312
STR	0.67	0.61	5.07	**<0.001**	1.000
RHI	0.74	0.68	4.55	**0.015**	0.432
LOI	0.68	0.64	4.19	**<0.001**	0.966
ELB	0.62	0.56	3.75	0.271	0.966
DN1	0.48	0.41	3.42	0.330	0.741
DN2	0.59	0.49	4.86	0.347	1.000
DN3	0.34	0.29	3.46	0.089	0.312
DPR	0.62	0.52	5.50	0.330	0.980
VIS1	0.30	0.29	2.64	0.426	1.000
VIS2	0.44	0.39	3.85	0.132	0.561
UK	0.26	0.20	1.72	**<0.001**	0.312
NET	0.52	0.47	2.39	0.330	0.966
RHO	0.47	0.50	3.89	**<0.001**	0.844
TUR1	0.54	0.46	3.97	0.390	1.000
TUR2	0.59	0.52	5.97	**0.015**	0.140
VAR	0.62	0.56	4.88	**<0.001**	**<0.001**
VOL	0.57	0.47	4.00	0.063	0.140

He (12), He (9) = mean unbiased expected heterozygosity estimated from 12 or 9 loci, respectively; AR (9) = mean allelic richness per 9 loci corrected for sample size; HWE (12), HWE (9) = the results of exact tests of HWE for data sets of 12 or 9 loci, respectively. The *P*-values of HWE were corrected for multiple testing by false discovery rate approach, and the significant results ($P < 0.05$) are shaded and emboldened.

populations with no evident barrier to gene flow were from the geographically close and connected Rivers Kyjovka (DAN1) and Morava (DAN2) ($F_{ST} = 0.0061$, $P = 0.095$; 9-loci analysis).

The FCA analysis based on allelic frequencies also revealed strong geographical structuring (Fig. 2). Three groups can be defined on the basis of their genetic similarity and geographical position. The first axis separated populations from the Dnieper, Dniester and Vistula basins and populations from western Europe from the Balkan and central European populations. The populations from the area around the Aegean Sea were subsequently separated from those in the Danube and Struma basins along the second factorial axis (Fig. 2).

A similar pattern was also detected in the STRUCTURE analysis. The best models separated the genetic variation into two or three clusters (Fig. S1, Supporting information), with the main division similar to the FCA

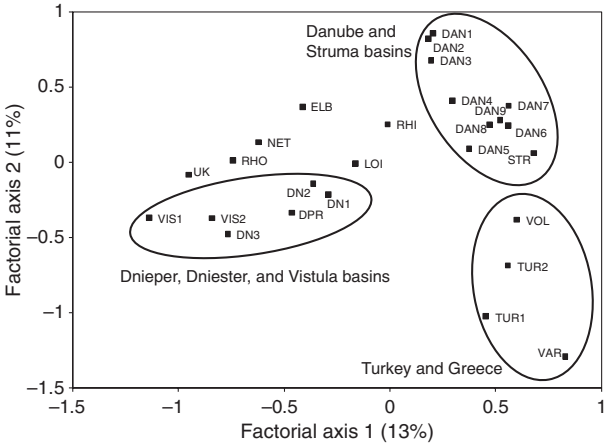

Fig. 2 A two-dimensional plot of the factorial correspondence analysis performed using GENETIX based on 12 microsatellite loci. Three geographical groups are bounded by grey lines.

results. For $K = 2$, the populations from the Dnieper, Dniester and Vistula clustered with three western European populations (Fig. 3). The second cluster was formed by populations from the Balkan Peninsula, Asia Minor and the Danube basin. Populations from the Rivers Elbe (ELB), Rhine (RHI) and Loire (LOI) were markedly admixed from both clusters. For $K = 3$, the 'Danubian' cluster separated from a new cluster that emerged in the southern Balkans and Asia Minor, with strong introgression into populations from the River Struma (STR) and the Danube delta (DAN5). Notably, many western European populations (e.g. LOI, RHI, ELB) were admixed from the 'Danubian' and 'Dnieper-Dniester-Vistula' clusters (Fig. 1). Subsequent increments in the number of clusters up to $K = 10$ recovered structure between central European populations and those from the southeastern part of the Danube basin, revealed similarities between Danubian DAN9 (in the foothills of the Balkan mountains) and the geographically proximate STR population from the River Struma in Bulgaria and separated the population in Lake Volvi in Greece (VOL). Three western European populations (NET, UK and RHO) also clustered together as a separate group, while other western European populations (RHI, LOI) retained strong similarity to Danubian populations. The mixed origin of some western European populations was additionally evident from the distribution of particular alleles at some loci. The best example is the distribution of allele 228 at locus *Rser04* originating from the Dniester basin (Fig. S2A, Supporting information) and allele 126 at locus *Rser13* of Danubian origin (Fig. S2B, Supporting information), both of which were present at high frequencies in populations from the Netherlands and the United Kingdom (NET, UK).

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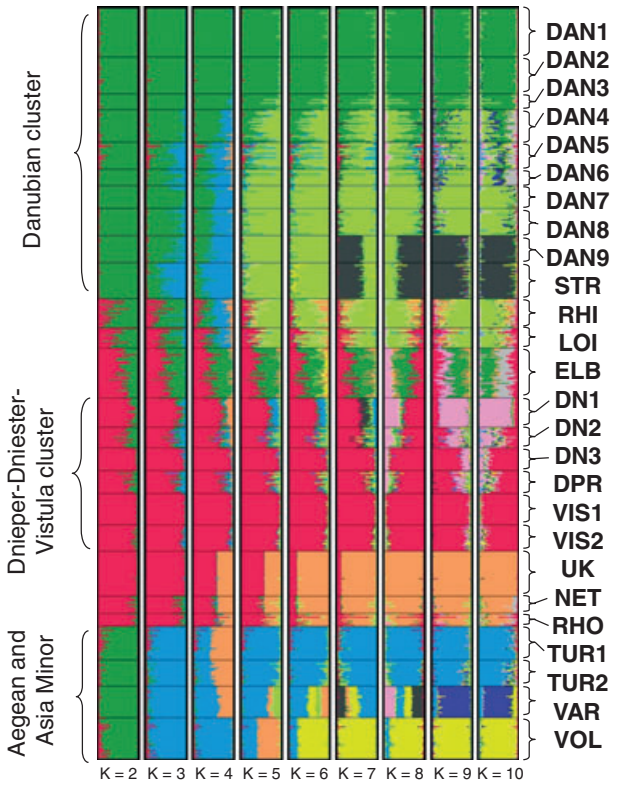

Fig. 3 Population structure estimated in the program STRUCTURE from 693 individuals from 26 localities. Each individual is represented by a vertical line, which is partitioned into K coloured segments, the length of each colour being proportional to the estimated membership coefficient. Black lines separate different populations (labelled on the right side of the figure with abbreviations corresponding to those in Table 1).

Models of the colonization of western Europe

The ABC approach unambiguously revealed the most likely scenarios for the first three steps, although two scenarios received comparable support at the fourth step (Table 2, Figs S3 and S4, Supporting information). The central European populations of bitterling from the Danube basin (group UD, dark green on Fig. 3 for $K = 6$) clearly derived from the Pannonian populations (group PA, light green on Fig. 3), according to scenario 1.1 (posterior probability with a credibility interval of: 0.8429, 0.6704–1.0000). The River Elbe population (group EL) and the western group W1 (UK, NET and RHO) were founded by admixture events between the northeastern populations (group NE) and group UD [scenarios 2.1 (0.9999, 0.9997–1.0000) and 3.1 (0.6481, 0.4646–0.8317), respectively]. The most likely scenario for the fourth step was 4.3 (0.3912, 0.2253–0.5572; Table 2), which indicates that the western group W2 (RHI, LOI) was founded by an admixture of the NE and PA groups. However, scenario 4.1 involving the origin of W2 by an admixture of groups NE and UD was also well supported (0.2449, 0.1044–0.3854; Table 2), with confidence intervals overlapping those of scenario 4.3. The posterior distributions of parameters were estimated on the basis of these scenarios (Tables S4–S7, Supporting information).

The most significant outcomes from the ABC modelling were the following. First, the time of divergence of the PA group from the hypothetical AN (i.e. the common ancestor) was consistently older than the divergence of the northeastern group (NE) (Tpa > Tne). Second, divergence of the NE group (Tne) was comparable to (and perhaps even younger than) the divergence of the middle Danube populations in central Europe (UD) from the PA group (Tud; in three steps from four, Tables S5–S7, Supporting information). The effective population size of the PA group was clearly several times higher than that of the NE group but both groups have experienced a significant expansion in effective population size (NE > NEa and PA > PAa; in three steps from four, Tables S4–S6, Supporting information). Third, the posterior parameter estimation in the second step revealed that the River Elbe (ELB) population was founded by admixture of individuals from NE and UD (Tel) about 1455 years ago (median), with a 95% confidence interval of 212–3729 years ago (Table S5, Supporting information). Fourth, the origin of the ancestral population that gave rise to group W1 (UK, NET and RHO) was estimated to be approximately 7955 (1505–41 896) years ago (Twec), and the effective population size of this group was about 15 335 (4236–60 464) individuals (median, 95% CI; Table S6, Supporting information). Finally, scenario 4.3 suggests that populations RHI and LOI were founded by admixture from NE and Danubian (PA or UD groups) individuals about 18 227 (3558–74 455) years ago (Tres), with an effective population size of the W2 of about 72 140 (37 632–96 913) individuals (Table S7, Supporting information).

Mitochondrial DNA variability

The combined data set was composed of 244 partial (425-bp) cytochrome *b* sequences [136 new sequences from this study + 108 sequences from Bohlen *et al.* (2006)]. Using 69 variable sites, we identified 62 different haplotypes (Table S8, Supporting information; GenBank Accession Numbers HM490028–HM490089). The MJ network showed a significant geographical component to variation (Fig. 4). All but one haplotype from the River Vardar (VAR) formed a separate group, and the same was observed for haplotypes from Lake Volvi in Greece (VOL). Importantly, in both populations, one haplotype was identical to the most widespread

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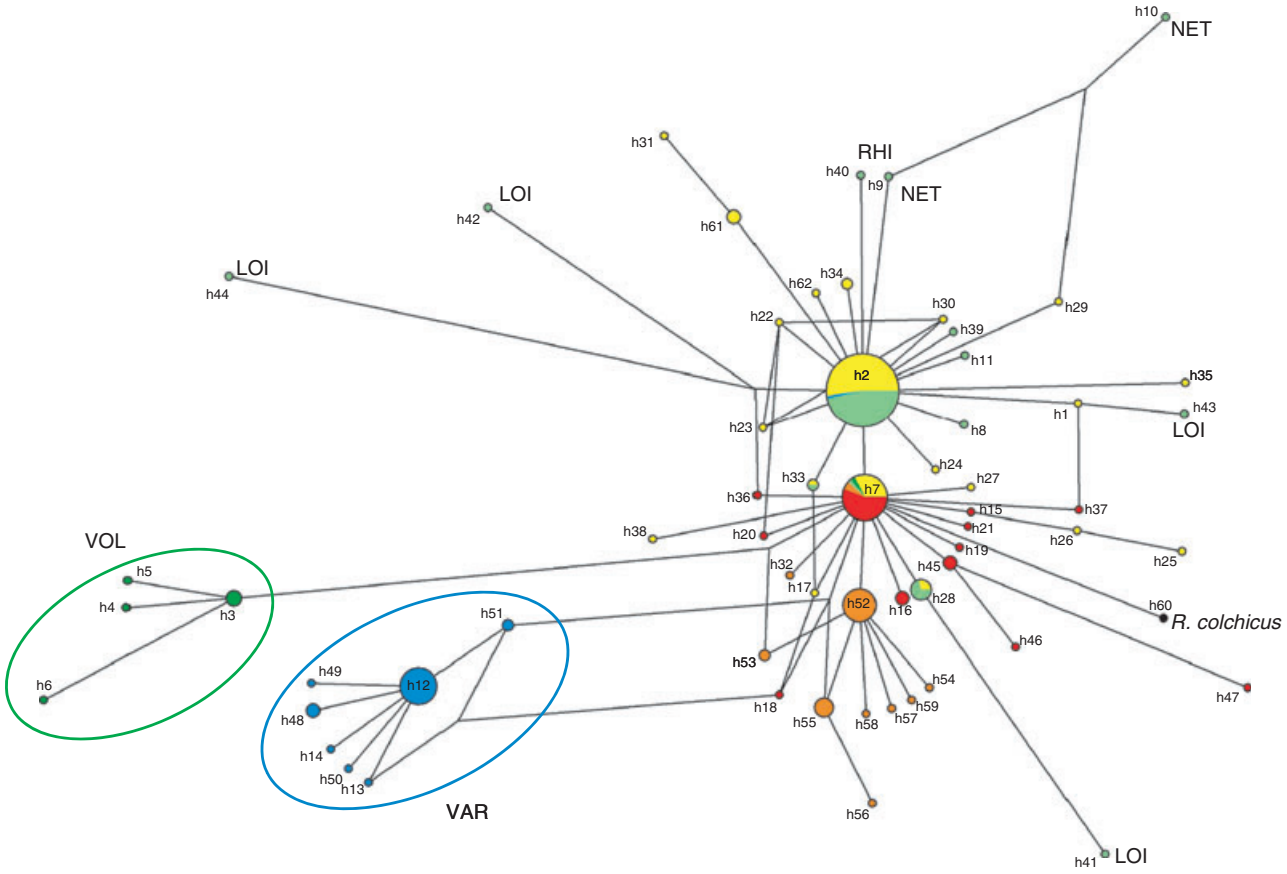

Fig. 4 Median-joining network of 244 sequences of 425 bp of cytochrome *b*. Length of branches is proportional to the number of substitutions along a given branch, and circle size is proportional to haplotype frequency according to information in Bohlen *et al.* (2006) and the present study. Colours indicate geographical origin of samples: yellow—drainages of the Rivers Danube, Struma, Veleka and Drin (corresponding to WEST sensu Bohlen *et al.* 2006); red—drainages of the Rivers Volga, Don, Kuban, Dnieper, Dniester and Vistula (corresponding to EAST sensu Bohlen *et al.* 2006); light green—drainages of the Rivers Elbe, Rhine, Rhône and Loire; dark green—Lake Volvi in Greece; orange—reservoirs in western Turkey (Ömerli and Sapanca); blue—River Vardar; black—*Rhodeus colchicus*, River Notanebi in Georgia. Some of most distant haplotypes found in western Europe (LOI, RHI, NET) and two divergent Greek haplogroups are indicated by locality name.

haplotypes h2 (VAR) and h7 (VOL) (Fig. 4, Table S9, Supporting information). Owing to a lower information content from shorter sequences compared with a previous study, we were not able to divide the remaining sequences unambiguously into WEST and EAST haplotype groups sensu Bohlen *et al.* (2006), although all EAST haplotypes clustered together (Fig. 4). Furthermore, all sequences from Lake Sapanca in Asia Minor formed another separate group. The sequences from sites in western Europe principally corresponded to the most widespread haplotype h2, but some were distant from this central haplotype, especially the haplotypes from the River Loire in France (LOI).

Discussion

We combined mitochondrial sequences and nuclear microsatellites to reconstruct the genetic structure and phylogeographical pattern of the European bitterling, a freshwater fish species used as a model in behavioural and evolutionary ecology, including research on its geographical pattern of host–parasite co-evolution (Smith *et al.* 2004; Reichard *et al.* 2006, 2007, 2010). We demonstrated a high level of differentiation among populations, even within the same drainage (see highly significant F_{ST} values in Table S2, Supporting information), which appears to be a common feature of European freshwater fishes and suggests that genetic drift or local selection plays an important role in the genetic structuring of non-migratory fishes (Triantafyllidis *et al.* 2002; Gum *et al.* 2005). Despite strong population differentiation, forced clustering of individuals demonstrated the hierarchical structure of differentiation. Our analysis broadly confirmed the main outcomes of a previous study based solely on mitochondrial DNA (Bohlen *et al.* 2006) but additionally provided evidence of a high level

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of admixture among major lineages (EAST and WEST) in contact zones between drainages in central and western Europe and estimated dates of admixture events. Our data set also raises a question over the validity of the specific status of *R. meridionalis* from the Aegean region.

Phylogeography of the European bitterling and the status of Mediterranean populations

Two recent studies provided clear evidence that the European bitterling species complex forms a clade separate from the morphologically similar East Asian *Rhodeus sericeus* Pallas (Bohlen *et al.* 2006; Zaki *et al.* 2008) and proposed the existence of at least four glacial refugia of the European bitterling in the areas around the Black Sea (Bohlen *et al.* 2006). While populations from the River Vardar (*R. meridionalis*) and western Caucasus (*R. colchicus*) remained isolated, the populations from the two remaining refugia colonized most of Europe (Bohlen *et al.* 2006). The EAST lineage (sensu Bohlen *et al.* 2006) probably persisted in the Black Sea glacial refugium and colonized northeastern Europe (Fig. 5). The WEST lineage survived in the lower Danube and subsequently colonized the Danubian drainage throughout Europe (Bohlen *et al.* 2006) (Fig. 5). This pattern of colonization is common to other European fishes (e.g. Durand *et al.* 1999; Nesbø *et al.* 1999; Kontula & Väinölä 2001; Perdices & Doadrio 2001) and concordant with our conclusions.

The highly divergent mtDNA lineage from the River Vardar in Greece was proposed as a separate, allopatric species (*R. meridionalis*) by Bohlen *et al.* (2006). We confirmed the presence of a distant haplogroup in the River Vardar and further documented another divergent lineage in Lake Volvi (located 60 km from the Vardar estuary). The Lake Volvi mtDNA lineage is markedly different from the Vardar lineage, as well as from all other European bitterling populations (Fig. 4). The population from Lake Sapanca in Turkey shows a comparable level of divergence, and other unsampled populations from the Aegean region may be similarly distant from each other. Species delineation based only on mtDNA divergence, the approach adopted by Bohlen *et al.* (2006), would result in a species-level designation for all these divergent populations.

However, we believe that there is evidence against assigning high bitterling species endemicity to the western Mediterranean region. First, our mitochondrial analysis revealed that one individual (out of 26 sequenced) in the River Vardar possessed haplotype h2, the most common haplotype in the WEST lineage, and one Lake Volvi individual possessed a haplotype otherwise common in the EAST lineage (h7). Individuals carrying these particular haplotypes were not differentiated from other individuals from their population on nuclear markers (Fig. 3), showing that both populations comprise a mixture of at least two mitochondrial lineages that are not reproductively isolated. This admixture might have resulted from coastal exchanges under low salinity conditions during deglaciations (Grosswald 1980; Ryan *et al.* 1997); notably, the European bitterling is highly tolerant of brackish water conditions (Koutrakis *et al.* 2003). There is also geological evidence of former connections between the Danubian tributaries and the River Vardar (Banarescu 1990) and genetic similarities between Vardar and Danubian fishes (Durand *et al.* 1999; Zardoya *et al.* 1999; Perdices & Doadrio 2001). A second line of evidence against high bitterling species endemicity in the southern part of its range is that nuclear microsatellites revealed the presence of three main population clusters with clear

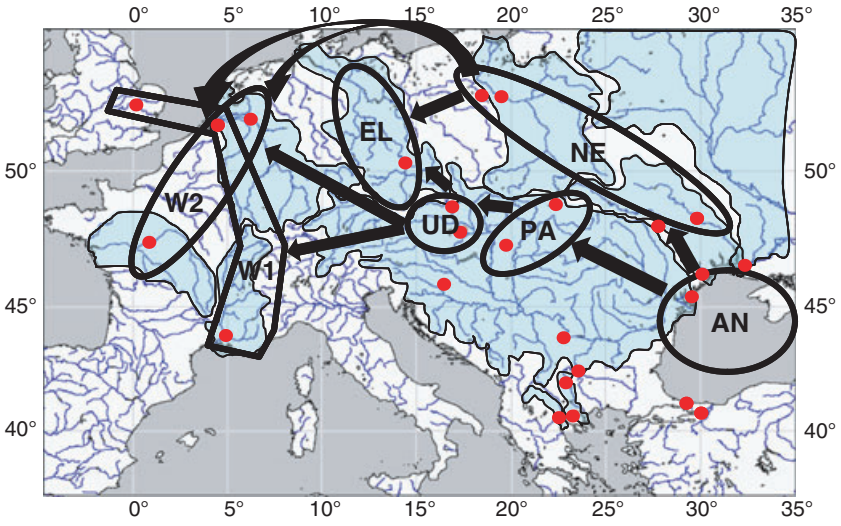

Fig. 5 The most probable colonization scenario for the central and western European bitterling populations. Red dots indicate sampling points, abbreviations AN, PA, UD, NE, EL, W1 and W2 correspond to populations used in ABC analysis. Sampled river drainages are shaded.

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geographical distributions (Fig. 1), northeastern, Danubian (both congruent with EAST and WEST haplotype lineages sensu Bohlen *et al.* 2006) and Aegean. An analysis forced to produce two clusters linked the Aegean group with the Danubian group, rather than separating the Danubian and northeastern groups from the Aegean group (Fig. 3), which would be more consistent with a putative species status of the Aegean populations. Furthermore, evidence of historical admixture is apparent on nuclear genomes in some other populations, especially from the River Struma and the lower Danube (Figs 1 and 3). We hypothesize that coastal and fluvial connections homogenized nuclear genomes in large areas while retaining the divergent mtDNA lineages in restricted populations. Despite admixture with common haplotypes, ancient mtDNA lineages dominate Aegean bitterling populations, perhaps because of the numerical disadvantage of more recent colonizers (Birky *et al.* 1983).

Origin of populations in western Europe and implications for co-evolution and conservation

Two main routes are proposed for the colonization of western Europe by freshwater fishes. The 'western colonization route' suggests dispersal between the Danube and adjacent basins of the Rhine and Rhône during previous interglacial periods (approx. 100 000–130 000 year BP), persistence in a refugium in southern France during the last glaciation and subsequent rapid range expansion from this refugium into the Atlantic and Mediterranean drainages after cessation of the last glaciation (10 000 year BP) (Durand *et al.* 1999; Kotlík & Berrebi 2001; Barluenga *et al.* 2006) (Fig. 5). The 'eastern colonization route' includes the northward spread of lineages from the Ponto-Caspian refugia, followed by dispersal along the coast of the low-salinity Baltic Sea or via connections formed by the circumglacial Mega-Vistula drainage (Grosswald 1980; Starkel 1991) (Fig. 5). Secondary contacts were achieved in various regions of central and western Europe (Durand *et al.* 1999; Kotlík *et al.* 2004, Gum *et al.* 2005; Barluenga *et al.* 2006) (Fig. 5).

Our data on European bitterling are largely consistent with this general scenario but shed more light on the relative timing of admixture events and identified recent contacts between the two lineages, possibly a consequence of human-assisted translocations (Fig. 5). The bitterling is a thermophilic fish that has an optimum temperature for reproduction considerably higher than most other European freshwater fishes (Smith *et al.* 2004). It is generally accepted that such species disappeared from most of western and central Europe during the extremely cold last glaciation (115 000–10 000 year BP; Van Damme *et al.* 2007), but subsequent recolonization remained unexplained. Bohlen *et al.* (2006) suggested natural dispersal, via both main colonization routes, while Van Damme *et al.* (2007) associates the appearance of bitterling in western and central Europe with the onset of carp aquaculture 5–10 centuries ago (Van Damme *et al.* 2007). We showed that both processes probably applied to bitterling populations, with the western groups (W1, W2) colonizing parts of Europe naturally and arising from admixture, while dating of the EL group admixture event is suggestive of human-assisted translocations in recent centuries. Notably, there is evidence that several populations showed rapid expansion of effective population size at different stages during the Holocene (Tables S4–S6, Supporting information).

In the context of the co-evolutionary relationship between European bitterling and their mussel hosts, these data and conclusions have several implications. The first is that populations from the Balkan Peninsula, Asia Minor and Danube basin are significantly older than the Pannonian and middle Danube populations, which in turn are older than the northeastern, western and Elbe groups. A key assumption in recent studies of the co-evolution of European bitterling and freshwater mussel hosts is that the length of association between the two is substantially older in the east of the bitterling's distribution than in central Europe and the west of its distribution (Reichard *et al.* 2006, 2007, 2010). These data support the assumption and reinforce the prediction that mussel populations in the Aegean and Black Sea regions and lower Danube have had longer to evolve responses to exploitation by bitterling, while mussels in western Europe represent relatively recent and naive host populations (Reichard *et al.* 2010). One caveat to this observation is that populations from the middle Danube, designated as group UD in the present study, may be more ancient than formerly suspected (Reichard *et al.* 2007), possibly having persisted since the last European deglaciation. However, in relative terms, these populations are still two orders of magnitude more recent in comparison with populations from the Pontic region.

From a conservation perspective, a second notable outcome of the study is the demonstration that over a relatively large region of western and central Europe, the European bitterling should be considered a native species, in contradiction of Van Damme *et al.* (2007) who argued, based on historical records, that the current distribution of the European bitterling in western and central Europe was largely a consequence of inadvertent introductions by medieval fish traders and did not represent part of its natural range. Still, there is some evidence to support recent, and possibly human-assisted, introductions of European bitterling in Europe from this period, illustrated by populations in the River

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Elbe, in addition to well-documented examples of introductions in the 20th century (Maitland 1972, Kozhara *et al.* 2007).

The significance of intraspecific invasions for conservation

The extinction of indigenous populations by introgression or displacement by non-native populations of the same species has become a serious problem in many species, including some freshwater fishes. Bitterling fishes provide two examples of the dangers of intraspecific invasions. In Japan, an endemic subspecies, the Japanese rose bitterling (*Rhodeus ocellatus kurumeus*), is on the verge of extinction through hybridization and displacement by the Chinese rose bitterling (*R. ocellatus ocellatus*) (Kawamura *et al.* 2001). The greater success of the Chinese rose bitterling in Japan may be linked to its higher tolerance of habitat degradation (Morosawa & Fujioka 2007), and also because female Japanese rose bitterling appear to have a mate preference for male Chinese rose bitterling (Y. Kanoh, unpublished data). A second endangered endemic Japanese bitterling, the Kyushu bitterling (*Rhodeus atremius*), also comprises two subspecies, *R. a. atremius* and *R. a. suigensis*, the latter subspecies in particular has declined dramatically in recent decades. Hybrids of *R. a. suigensis* and *R. a. atremius* are completely fertile (Suzuki 1998), and the collection, transport and reintroduction of both subspecies outside their natural distribution have led to the genetic introgression of the two over parts of their range, severely hampering efforts to conserve them (Miyake *et al.* 2010). In North America, the bull trout (*Salvelinus confluentus*), an endangered salmonid fish, shows a high degree of genetic divergence among populations, which may reflect the evolution of local adaptations (Leary *et al.* 1993). This species has been the target of supplementation programmes, but the use of relatively few parents to generate these hatchery progeny and mixing progeny among populations has reduced genetic variability and led to the loss of local adaptations (Leary *et al.* 1993). Similar cases of intraspecific translocations and their negative consequences are known in other taxa, such as red deer (*Cervus elaphus*) (Frantz *et al.* 2006) and chamois (*Rupicapra rupicapra*) (Crestanello *et al.* 2009).

In the case of the European bitterling, there have been conservation concerns for this species (European Commission 2002), although in recent years the fish appears to have expanded its range, and these concerns may now be unfounded (Kozhara *et al.* 2006, Van Damme *et al.* 2007). However, it is also clear from our results that this species is highly genetically structured across its range. In some regions, we have detected admixture of distinct lineages, possibly facilitated through human introductions. It is also clear that across its range, the bitterling has co-evolved with its mussel hosts to different degrees (Reichard *et al.* 2010). While the immediate future of the European bitterling as a species is secure, indiscriminate transfer of this fish among populations, with the consequent undermining of genetic structuring and co-evolutionary state with its mussel hosts, is to be discouraged.

Conclusions

Our study shows that the history of colonization of Europe by the bitterling was largely congruent between mitochondrial and nuclear markers and that populations are highly genetically structured. The most divergent mtDNA lineages occur in the Aegean region, but probably are not reproductively isolated because the Aegean populations displayed mtDNA haplotypes from other lineages. Most bitterling populations in western Europe are native and bear the genetic signature of both contemporary gene flow and historical separation events. Populations in western and central Europe are relatively recent colonizers compared to populations from Pontic region.

Acknowledgements

We thank numerous colleagues (listed in Table 1) for providing tissue samples. A. Bryjová, B. Bímová and L. Rousková helped with genetic analyses in the laboratory and V. Šlechtová, J. Bohlen and three anonymous referees provided valuable comments. K. Kawamura read and commented authoritatively on the manuscript and we are extremely grateful to him. J.B. and M.R. conceived and designed the study, collected and analysed the data, and drafted the manuscript; C.S. significantly contributed to the framework of the research and manuscript production. A.K. performed ABC analysis. The research was supported by the Grant Agency of Academy of Sciences of the Czech Republic (KJB600930802), the Leverhulme Trust and the Biodiversity Research Centre (LC06073).

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Supporting information

Additional supporting information can be found in the online version of this article.

Fig. S1 (A) Results of Bayesian cluster analysis using the program STRUCTURE showing log probability of the data Ln Pr(X|*K*) as a function of number of putative populations (*K*). (B) The values of Δ(*K*) calculated according to Evanno *et al.* (2005) that were used for selection of the best fitted model.

Fig. S2 (A) Frequency of the allele 228 at locus Rser04 (in blue). (B) Frequency of allele 126 at locus Rser13 (in blue).

Fig. S3 Comparison of the relative posterior probabilities for all scenarios separately for each of four steps.

Fig. S4 Schemes of the best scenarios for each of the four steps.

Table S1 Summary of genotyped microsatellite loci

Table S2 Pairwise F_{ST} estimates between sampled populations

Table S3 The prior distributions of parameters used for description of the 32 scenarios analysed via the approximate Bayesian computation (ABC, Beaumont *et al.* 2002) in DIYABC 0.8.1 (Cornuet *et al.* 2008)

Table S4 Estimations of the posterior distributions of parameters revealed from the approximate Bayesian computation (ABC, Beaumont *et al.* 2002) for the best scenario for the first step (scenario 1.1 in Table 3)

Table S5 Estimations of the posterior distributions of parameters revealed from the approximate Bayesian computation (ABC, Beaumont *et al.* 2002) for the best scenario for the second step (scenario 2.1 in Table 3)

Table S6 Estimations of the posterior distributions of parameters revealed from the approximate Bayesian computation (ABC, Beaumont *et al.* 2002) for the best scenario for the third step (scenario 3.1 in Table 3)

Table S7 Estimations of the posterior distributions of parameters revealed from the approximate Bayesian computation (ABC, Beaumont *et al.* 2002) for the two best scenarios for the fourth step (scenarios 4.3 and 4.1 in Table 3)

Table S8 Haplotypes of cytochrome *b* (425 bp) analysed in this study

Table S9 Distribution of haplotypes of partial cytochrome b (425 bp) at localities under study. *Bohlen *et al.* (2006) indicated the corresponding haplotypes. ** For abbreviations of populations – see Table 1

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