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# A morphological and genetic analysis of the European bitterling species complex

SHAMA A. H. ZAKI[1,2], WILLIAM C. JORDAN[2], MARTIN REICHARD[3], MIROSŁAW PRZYBYLSKI[4] and CARL SMITH[1]*

[1]*Department of Biology, University of Leicester, University Road, Leicester, LE1 7RH, UK*
[2]*Institute of Zoology, Zoological Society of London, Regent's Park, London, NW1 7RY, UK*
[3]*Institute of Vertebrate Biology, Academy of Sciences of the Czech Republic, Brno 60365, Czech Republic*
[4]*Department of Ecology & Vertebrate Zoology, University of Łódź, Łódź 90-237, Poland*

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Bitterling fishes lay their eggs on the gills of living freshwater mussels and are valuable models in behavioural and evolutionary ecology. We used morphological and genetic data to resolve the taxonomic relationships of bitterling in Europe. Previous studies have suggested the European bitterling is either a single species with a wide but discontinuous geographic distribution, or a complex of species. Morphometric and meristic data identified differences between three putative species; with a clear distinction between the eastern Asian *Rhodeus sericeus*, western European bitterling *Rhodeus amarus*, and colchian bitterling, *Rhodeus colchicus*. Polymorphism in the mitochondrial DNA control region was predominantly due to insertion/deletion events, making phylogenetic inference difficult, but the single haplotype found in *R. sericeus* populations was detected at low frequency (one of 24 individuals) in *R. amarus* and *R. colchicus* populations. Eight control region haplotypes were found in *R. amarus* populations, which were distinct from the two haplotypes in a *R. colchicus* population. Cytochrome *b* data produced a phylogeny with strongly-supported differentiation between a clade of two *R. sericeus* haplotypes and a clade of six *R. amarus/colchicus* haplotypes. The star-like topology of the *R. amarus/colchicus* haplotypes in a minimum spanning network suggested a rapid radiation in this clade. Our results are consistent with an hypothesis of relatively ancient divergence of *R. sericeus* from *R. amarus*/*colchicus* and more recent and rapid differentiation between *R. amarus* and *R. colchicus*. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **95**, 337–347.

ADDITIONAL KEYWORDS: behaviour – Cyprinidae – morphology – phylogeny – speciation.

## INTRODUCTION

Bitterling are small fishes belonging to the Acheilognathinae, a subfamily of the Cyprinidae, a group comprising approximately 40 species (Arai, 1988) largely restricted to Asia (Nelson, 1984; Okazaki *et al.*, 2001). Bitterling have an unusual spawning relationship with freshwater mussels, which they use for oviposition. During the spawning season, males develop bright nuptial coloration and defend territories around mussels. Female bitterling develop long ovipositors that they use to place their eggs on the gills of a mussel through its exhalant siphon. Males fertilize the eggs by releasing sperm into the inhalant siphon of the mussel. Developing embryos reside in the mussel for approximately 1 month, during which time they develop into actively swimming larvae. Bitterling display remarkable morphological, physiological, and behavioural adaptations for using mussels as spawning sites and represent a valuable model in behavioural, population, and evolutionary ecology (Smith *et al.*, 2004). The value of the bitterling arises from it having a spawning site that can be easily manipulated (Smith *et al.*, 2004).

The habitat of bitterling is linked to the distribution of freshwater unionid mussels. Typical habitats are river backwaters, oxbows, lakes, ponds, and irrigation canals (Holčík, 1999). However, bitterling are

*Corresponding author. E-mail: cs152@le.ac.uk

also found in faster flowing rivers (Przybylski & Ziêba, 2000; Reichard, Jurajda & Matejusová, 2002a), and larval and early juvenile bitterling can constitute the major component of fishes drifting in rivers in the Danube basin (Reichard, Jurajda & Ondračková, 2002b).

Recent research on bitterling has addressed the coevolutionary relationship between these fishes and freshwater mussels (Mills & Reynolds, 2003; Reichard *et al.*, 2006, 2007a, b). Resolution of the taxonomic status of European bitterling is crucial to an understanding of the evolutionary context of these relationships. However, the phylogenetic relationships of bitterling fishes is equivocal (Okazaki *et al.*, 2001). In particular, resolution of the taxonomic status of the European bitterling is challenging. *Cyprinus sericeus*, the Amur bitterling, was first described from the River Amur system in eastern Russia by Pallas (1776). A second species was described from the River Elbe as *Cyprinus amarus* by Bloch (1782). Despite the substantial geographic separation of these two species, they were subsequently considered conspecific (Bogutskaya & Komlev, 2001), and eventually both were designated as subspecies of *Rhodeus sericeus*; the Amur bitterling as *Rhodeus sericeus sericeus* and the European bitterling as *Rhodeus sericeus amarus* (Svetovidov & Eremeev, 1935). These designations persisted, despite some authors considering each as a distinct species (Svetovidov & Eremeev, 1935; Duyvené de Wit, 1955).

In a later study to address the relationship between the two species, the characters used to separate the eastern and western species/subspecies were found to be size- and temperature-dependent and not sufficiently reliable to separate the two (Holčík & Jedlička, 1994). Consequently, the designation *R. sericeus* was retained for both the eastern and western groups (Holčík & Jedlička, 1994). However, in a review of the taxonomy of European freshwater fish, Kottelat (1997) reclassified the western populations as *R. amarus* and the eastern populations as *R. sericeus* on the basis that such a large geographic disjunction between them must necessarily mean the two represent discrete species. In addition, a new species of bitterling, *Rhodeus colchicus*, from West Transcaucasia was recently described, distinguishable from *R. amarus* and *R. sericeus* by fewer scales in the lateral series, deeper and relatively larger infraorbitals, and fewer vertebrae (Bogutskaya & Komlev, 2001).

A recent molecular study by Bohlen, Bogutskaya & Freyhof (2006) concluded that a common ancestor of *R. sericeus s.l.* may have formerly been distributed across Siberia, from East Asia to Europe, with its origin in East Asia. In addition, Van Damme *et al.* (2007) have identified the timing of the spread of bitterling into West and Central Europe from historical documents to be between 1150–1560 AD, and also tentatively linked changes in the western distribution of bitterling to climate change. In the present study, we use a combination of morphological and molecular data for a series of populations of bitterling across Europe and East Asia to clarify the relationship within *R. sericeus s.l.*, comprising *R. amarus*, *R. colchicus* and, *R. sericeus s.s.*

## MATERIAL AND METHODS

### Samples

We collected *R. sericeus s.l.* from 37 populations across Europe, Transcaucasia, Asia Minor, and eastern Russia from 2001–2004 (Table 1). Whole fish were fixed in a 5% formalin solution and stored in glass jars for morphological analysis. Fin clips were collected from subsamples of 10–20 individuals from a subset of populations, fixed in 90% ethanol, and stored in individual eppendorfs before analysis.

### Morphology

Morphometric measurements were made on 757 individuals from 26 populations. Measurements were made of 23 morphometric characters (Fig. 1) by one of us (S.A.H.Z.) to the nearest 0.1 mm using electronic callipers and comprised: BL, body length; HL, head length; POL, pre-opercle length; HD, head depth; POD, pre-orbital distance; PSD, post-orbital distance; ED, eye diameter; UJL, upper jaw length; BD, body depth; CPD, caudal peduncle depth at the posterior end of the anal-fin base; MBD, minimum body depth; PDD, predorsal distance; PVD, pre-pectoral fin distance; PCD, pectoral-pelvic fin distance; PAD, pre-anal fin distance; VAD, pelvic-anal fin distance; DFL, dorsal fin length; DFD, dorsal fin depth; AFL, anal fin length; AFD, anal fin depth; VFL, pelvic fin length; PFL, pectoral fin length; CFL, caudal fin length. The characters POL, HD, POD, PSD, ED, and UJL were standardized by expressing them as a proportion of HL, and BD, CPD, MBD, PDD, PVD, PCD, PAD, VAD, DFL, DFD, AFL, AFD, VFL, PFL, and CFL as a proportion of BL. In addition, all fish were weighed on an electronic balance to the nearest 1 mg. To obtain an objective score that summarized the major components of the variables measured, we used multivariate canonical variate analysis. For 21 populations, counts were made of four meristic characters for 516 individuals: number of dorsal fin branched rays, anal fin branched rays, pelvic fin rays, and pectoral fin rays. In counting fin rays, the two posterior most rays in the dorsal and anal fins, which are connected at the base, were counted as a single fin ray. Analysis of variance (ANOVA) was used to compare

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**Table 1.** Putative species, geographical location, river drainage, sample sizes, and sample codes

| Number | Putative species | Location | Drainage | Country | Control region data | Cytochrome *b* data | Morphometric data | Meristic data | Code |
|---|---|---|---|---|---|---|---|---|---|
| 1 | *Rhodeus amarus* | Moravska Nova Ves | Danube | Czech Republic | – | – | 36 | 36 | Morav-RA |
| 2 | *Rhodeus amarus* | River Kyjovka | Danube | Czech Republic | 2 | 2 | 35 | – | Kyjov-RA |
| 3 | *Rhodeus amarus* | Lake Kociolek | Odra | Poland | – | 1 | 32 | 27 | Kocio-RA |
| 4 | *Rhodeus amarus* | River Liwiec | Vistula | Poland | – | – | 32 | 26 | Liwie-RA |
| 5 | *Rhodeus amarus* | River Pilica | Vistula | Poland | – | – | 33 | 31 | Pilic-RA |
| 6 | *Rhodeus amarus* | River Rawka | Vistula | Poland | 2 | 1 | 30 | – | Rawka-RA |
| 7 | *Rhodeus amarus* | River Vistula | Vistula | Poland | – | – | 23 | – | Wista-RA |
| 8 | *Rhodeus amarus* | Lake Roianh | Bay of Gdansk | Poland | – | – | 33 | 21 | Roian-RA |
| 9 | *Rhodeus amarus* | River Don | Don | Russia | – | 2 | 34 | 18 | DonA-RA |
| 10 | *Rhodeus amarus* | River Kumylga | Don | Russia | – | 1 | – | – | DonD-RA |
| 11 | *Rhodeus amarus* | Tsymlyansk Reservoir | Don | Russia | – | 2 | 22 | 22 | Tysmy-RA |
| 12 | *Rhodeus amarus* | River Kuban | Black Sea | Russia | – | – | 26 | 22 | Kuban-RA |
| 13 | *Rhodeus amarus* | River Karasu | Black Sea | Turkey | 1 | 3 | – | – | Karas-RA |
| 14 | *Rhodeus amarus* | Crimea | Black Sea | Ukraine | – | – | 23 | – | Crime-RA |
| 15 | *Rhodeus amarus* | Danube Delta | Black Sea | Ukraine | 5 | 3 | – | – | Delta-RA |
| 16 | *Rhodeus amarus* | River Al'ma | Black Sea | Ukraine | 3 | 1 | 36 | – | Al'ma-RA |
| 17 | *Rhodeus amarus* | River Kubolta | Dniester | Ukraine | 2 | 1 | – | – | Kubol-RA |
| 18 | *Rhodeus amarus* | Dniester Estuary | Dniester | Ukraine | – | 1 | – | – | Liman-RA |
| 19 | *Rhodeus amarus* | River Tashlychka | Dniester | Ukraine | 2 | 1 | – | – | Tashy-RA |
| 20 | *Rhodeus amarus* | River Obitochnaya | Sea of Azov | Ukraine | 1 | 1 | – | – | Obito-RA |
| 21 | *Rhodeus amarus* | River Berda | Sea of Azov | Ukraine | 1 | 2 | – | – | Berda-RA |
| 22 | *Rhodeus amarus* | River Salgir | Sea of Azov | Ukraine | – | 1 | – | – | Salgi-RA |
| 23 | *Rhodeus amarus* | Wicken Fen | Ouse | UK | – | 1 | 33 | 27 | WickF-RA |
| 24 | *Rhodeus amarus* | South Bug | Vistula | Ukraine | 2 | 1 | – | – | Sbug-RA |
| 25 | *Rhodeus colchicus* | River Kherota | Black Sea | Russia | – | – | 32 | 35 | Khero-RC |
| 26 | *Rhodeus colchicus* | River Hobza | Black Sea | Russia | 3 | 3 | 51 | 49 | Hobza-RC |
| 27 | *Rhodeus sericeus* | Lake Kenon | Amur | Russia | – | 2 | 32 | 21 | Kenon-RS |
| 28 | *Rhodeus sericeus* | Lake Khanka | Amur | Russia | – | 1 | 25 | 23 | Khank-RS |
| 29 | *Rhodeus sericeus* | River Amazar | Amur | Russia | 2 | 2 | – | – | Amaza-RS |
| 30 | *Rhodeus sericeus* | River Bol'shoy | Amur | Russia | 2 | 1 | 22 | 18 | Bolsh-RS |
| 31 | *Rhodeus sericeus* | River Shilka | Amur | Russia | 2 | 2 | 25 | 21 | Shilk-RS |
| 32 | *Rhodeus sericeus* | River Piatigorka | Ussuri | Russia | 2 | 1 | 20 | 19 | Piati-RS |
| 33 | *Rhodeus sericeus* | River Zeya | Amur | Russia | – | – | 21 | 20 | Zeya-RS |
| 33 | *Rhodeus sericeus* | River Spassovka | Khanka | Russia | – | – | 27 | 23 | Spaso-RS |
| 35 | *Rhodeus sericeus* | River Ananievka | Sea of Japan | Russia | – | 2 | 20 | 20 | Anani-RS |
| 36 | *Rhodeus sericeus* | River Amba | Sea of Japan | Russia | – | – | 21 | 20 | Amba-RS |
| 37 | *Rhodeus sericeus* | Unnamed stream | Sea of Japan | Russia | – | – | 33 | 27 | StrmB-RS |

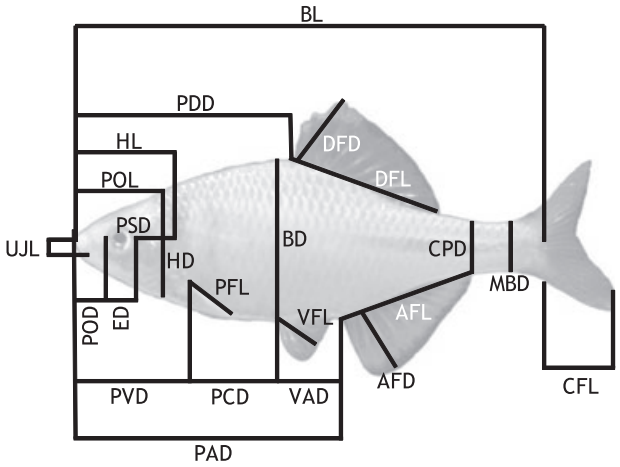


**Figure 1.** Morphometric characteristics measured for three putative bitterling species: BL, body length; HL, head length; POL, pre-opercle length; HD, head depth; POD, pre-orbital distance; PSD, post-orbital distance; ED, eye diameter; UJL, upper jaw length; BD, body depth; CPD, caudal peduncle depth at the posterior end of the anal-fin base; MBD, minimum body depth; PDD, predorsal distance; PVD, pre-pectoral fin distance; PCD, pectoral-pelvic fin distance; PAD, pre-anal fin distance; VAD, pelvic-anal fin distance; DFL, dorsal fin length; DFD, dorsal fin depth; AFL, anal fin length; AFD, anal fin depth; VFL, pelvic fin length; PFL, pectoral fin length; CFL, caudal fin length.

morphological variables among the three species. Finally, weight-length data were fitted to the function: $W = aL^b$, where $W$ is weight (mg), $L$ is standard length (mm), and $a$ and $b$ are growth constants. The length exponent $b$ was compared among putative species using analysis of covariance (ANCOVA).

### MITOCHONDRIAL (MT)DNA ANALYSIS

Total genomic DNA was extracted from fin tissue from fish belonging to 25 populations using the Promega Wizard SV 96 Genomic DNA purification system. Sections of the mtDNA control region and cytochrome *b* (cyt *b*) gene were amplified by the polymerase chain reaction (PCR) from genomic DNA (Gilles *et al.*, 1998). For the control region, the primer pair CR1F 5′-CCGGGCATTCTTTTATATGC-3′ (forward) and PHE1R 5′-ACATCTTCAGTGTTACGCTT-3′ (reverse) was used and for cyt *b* the primer set NEW-FOR 5′-AGCCTACGAAAAACCCACCC-3′ (forward) (Chang, Huang & Lo, 1994) and 34-REV 5′-AAACTGCA GCCCCTCAGAATGATATTTGTCCTCA-3′ (reverse) (Cantatore *et al.*, 1994) was used. Reactions were carried out in a total volume of 25 μL with 50 mM KCl, 20 mM TrisHCl (pH 8.0), 1.5 mM $MgCl_2$, 1.25 mM each dNTP, 0.3 μM each primer, 0.75 U Taq, and 10–100 ng genomic DNA. PCR conditions were: 1 min at 92 °C (one cycle); 15 s at 92 °C, 45 s at 48 °C, 2 min 30 s at 72 °C (five cycles); 15 s at 92 °C, 45 s at 52 °C, 2 min 30 s at 72 °C (30 cycles); and 7 min at 72 °C (one cycle). Purified fragments were directly sequenced using the Applied Biosystems Big-Dye Cycle Sequencing Kit with the sequences resolved on an automated sequencer (Applied Biosystems 3100 Genetic Analyzer). A cyt *b* sequence from an individual from the Saône drainage (France) (Accession number Y10454; Briolay *et al.*, 1998) was downloaded from Genbank. Outgroup cyt *b* sequences for *R. amurensis* (Genbank Accession DQ396627) and *R. sinensis* (DQ396629) were also downloaded (Bohlen *et al.*, 2006).

Phylogenetic analysis was not conducted for the control region sequences. The best fit model of nucleotide substitution for the cyt *b* haplotypes was tested using Modeltest, version 3.7 (Posada & Crandall, 1998). Relationships among cyt *b* haplotypes were reconstructed using Neighbour-joining (NJ) (Saitou & Nei, 1987), maximum likelihood (heuristic search) and maximum parsimony (branch and bound search) methods implemented in PAUP, version 4.10b software (Swofford, 2002). Support for nodes was estimated through 1000 bootstrapping replicates. A minimum spanning network was constructed using ARLEQUIN, version 3.01 (Excoffier, Laval & Schneider, 2005).

The assumption of equal rates of substitution across lineages was tested by calculating likelihoods ($Ln$) of trees based on models which did ($Ln_0$) and did not ($Ln_1$) assume a molecular clock. The significance of the difference between tree likelihoods was determined using a likelihood ratio test where $\chi^2 = -2(Ln_0 - Ln_1)$ with $s - 2$ degrees of freedom, where $s$ is the number of taxa involved (Huelsenbeck & Crandall, 1997). A divergence time between lineages was calculated using a substitution rate of 1–2% per million years previously estimated for teleost cyt *b* and control region sequences (Bowen *et al.*, 2001; Salzburger *et al.*, 2003).

## RESULTS

### MORPHOLOGY

Multivariate canonical variate analysis resulted in two significant canonical axes ($P < 0.001$), that were responsible for 82.7% and 17.3% of variance, respectively (Table 2). Morphologies of fish were separated on the first canonical variate mainly by relative head shape and body shape and fin size, and the second variate by relative caudal depth and caudal and pelvic fin size. Discriminant scores differed significantly among putative species for the first (ANOVA:

**Table 2.** Percentage and cumulative variation explained by canonical variables and loadings of the size-corrected morphometric measurements for *Rhodeus amarus*, *Rhodeus colchicus*, and *Rhodeus sericeus s.s.* collected from 26 populations across Europe and Asia

| | Canonical variate | |
|---|---|---|
| | 1 | 2 |
| Percentage of variation explained | 82.7 | 17.3 |
| Cumulative percentage variation | 82.7 | 100.0 |
| Variables | Loadings | |
| POL, pre-opercle length | –0.271 | –0.096 |
| HD, head depth | **0.456** | 0.227 |
| POD, pre-orbital distance | –0.188 | 0.245 |
| PSD, post-orbital distance | 0.143 | 0.056 |
| ED, eye diameter | 0.038 | –0.313 |
| UJL, upper jaw length | 0.079 | 0.246 |
| BD, body depth | –0.229 | 0.287 |
| CPD, caudal peduncle depth | –0.009 | **0.359** |
| MBD, minimum body depth | 0.121 | 0.074 |
| PDD, pre-dorsal distance | 0.301 | –0.083 |
| PVD, pre-ventral distance | **–0.330** | 0.295 |
| PCD, pectoral-ventral distance | –0.154 | 0.263 |
| PAD, pre-anal distance | –0.262 | –0.130 |
| VAD, ventral-anal distance | 0.011 | –0.219 |
| DFL, dorsal fin length | –0.053 | 0.164 |
| DFD, dorsal fin depth | **–0.347** | –0.048 |
| AFL, anal fin length | **–0.614** | –0.292 |
| AFD, anal fin depth | –0.325 | 0.089 |
| VFL, pelvic fin length | 0.142 | **–0.804** |
| PFL, pectoral fin length | **1.210** | –0.053 |
| CFL, caudal fin length | **0.423** | **0.753** |

Variables with the highest loadings are shown in bold.

$F_{(2,518)} = 285.3$, $P < 0.001$) and second variate (ANOVA: $F_{(2,518)} = 59.8$, $P < 0.001$). Post-hoc tests showed significant differences among all species for both factors (Scheffe test: $P < 0.05$). A plot of the first and second canonical variates (Fig. 2) showed clear separation of *R. amarus* from *R. colchicus* and *R. sericeus* along the first variate, and *R. colchicus* and *R. sericeus* on the second variate. The separation of the three putative species was further demonstrated by the fact that 80% of individuals were classified into correct species groups (Table 3), indicating that the morphological variables used for analysis were appropriate for distinguishing among the species groups.

We detected significant differences in three meristic characters among species (Table 4). Mean counts of dorsal and anal fin branched rays, and pectoral fin rays were significantly different among all three species (Scheffe test: $P < 0.006$). Pelvic fin ray counts were uninformative because they showed no variation in *R. amarus* and *R. colchicus*.

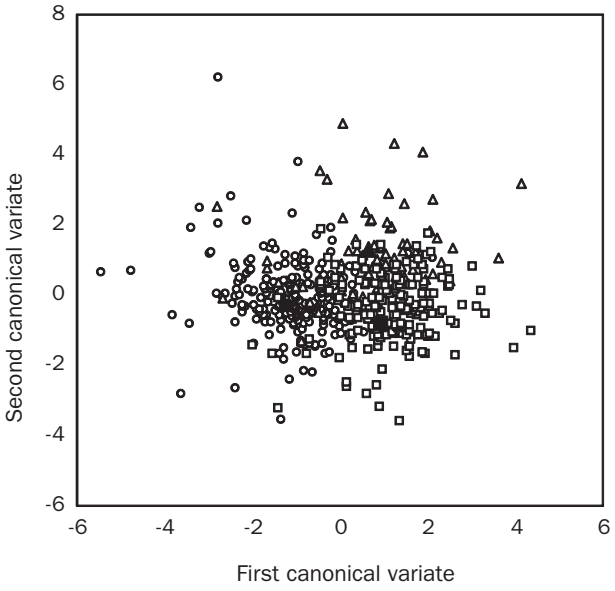


**Figure 2.** Plots of values for the first and second canonical variates generated from size-corrected morphological measurements of *Rhodeus amarus* (○), *Rhodeus colchicus* (Δ), and *Rhodeus sericeus* (□). Filled symbols indicate centroids of respective groups.

We further detected a significant difference in the length exponent *b* in the length-weight relationships among species (ANCOVA: $F_{2,530} = 39.16$, $P < 0.001$). A post-hoc test among species showed differences between *R. amarus* and *R. sericeus* (Scheffe test: $P < 0.001$) and *R. colchicus* and *R. sericeus* (Scheffe test: $P < 0.001$), but not between *R. amarus* and *R. colchicus* (Scheffe test: $P = 0.146$).

## MTDNA ANALYSIS

### *Control region*

Data on a 329-bp section of the control region were obtained for 32 individuals from 15 populations. Twenty-four polymorphisms defined 12 haplotypes (Table 5). Most (14 of 16) of these polymorphisms were single base pair insertion/deletion events (indels). One haplotype (*ConReg-Rs1*) was shared by all the individuals from the four *R. sericeus* populations sampled and one individual from a putative *R. amarus* population (Table 6). The three individuals from putative *R. colchicus* populations displayed two haplotypes not found elsewhere, with one haplotype (*ConReg-Rc2*) being shared by two individuals (Table 6). The remaining eight haplotypes were distributed among *R. amarus* populations.

**Table 3.** Predicted and observed species group membership based on multivariate canonical variate analysis for *Rhodeus amarus*, *Rhodeus colchicus*, and *Rhodeus sericeus s.s.* collected from 26 populations across Europe and Asia

| | Predicted group membership | | | | |
|---|---|---|---|---|---|
| | Species | *Rhodeus amarus* | *Rhodeus colchicus* | *Rhodeus sericeus* | Total |
| Observed group membership | *Rhodeus amarus* | 230 (87) | 12 (5) | 22 (8) | 264 |
| | *Rhodeus colchicus* | 7 (10) | 46 (67) | 16 (23) | 69 |
| | *Rhodeus sericeus* | 15 (9) | 31 (18) | 126 (73) | 172 |

Overall, 80% of individuals were classified into the correct species group. The percentage in each group is shown in parentheses.

**Table 4.** Meristic characteristics (mean ± SD) and analysis of variance for *Rhodeus amarus*, *Rhodeus colchicus*, and *Rhodeus sericeus s.s.* collected from 26 populations across Europe and Asia

| | *Rhodeus amarus* | *Rhodeus colchicus* | *Rhodeus sericeus* | $F_{(2,513)}$ | *P* |
|---|---|---|---|---|---|
| Sample size | 220 | 84 | 212 | | |
| Dorsal fin soft rays | 9.6 ± 0.50 | 9.4 ± 0.49 | 9.9 ± 0.36 | 36.84 | < 0.001 |
| Anal fin rays | 9.6 ± 0.48 | 9.4 ± 0.49 | 9.9 ± 0.33 | 40.24 | < 0.001 |
| Pelvic fin rays | 7.0 ± 0.00 | 7.0 ± 0.00 | 7.6 ± 0.49 | | |
| Pectoral fin rays | 12.4 ± 0.49 | 13.3 ± 0.69 | 13.7 ± 0.47 | 352.91 | < 0.001 |

**Table 5.** Polymorphic positions in bitterling control region sequences

| | Base position | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | 32 | 37 | 54 | 69 | 75 | 80 | 118 | 153 | 178 | 182 | 206 | 214 | 282 | 289 | 314 | 326 |
| *ConReg-Ra1* | A | C | – | – | – | – | A | – | – | T | – | – | T | T | G | – |
| *ConReg-Ra2* | · | · | · | · | · | · | · | · | · | · | · | · | C | · | · | · |
| *ConReg-Ra3* | · | · | · | · | · | · | · | · | · | · | · | · | · | · | · | T |
| *ConReg-Ra4* | · | · | · | · | · | · | · | · | · | · | C | C | C | – | – | · |
| *ConReg-Ra5* | · | · | · | · | · | · | · | · | · | · | C | C | · | – | – | · |
| *ConReg-Ra6* | – | – | · | A | G | A | · | A | · | · | · | · | · | – | – | · |
| *ConReg-Ra7* | – | – | C | · | · | · | · | · | · | · | · | · | · | – | – | · |
| *ConReg-Ra8* | – | – | C | · | · | · | C | · | · | · | · | · | C | – | – | · |
| *ConReg-Rc1* | · | · | · | · | · | · | · | · | C | · | C | C | C | – | – | · |
| *ConReg-Rc2* | – | – | C | · | · | · | · | · | C | – | · | · | C | – | – | · |
| *ConReg-Rs1* | · | · | · | · | · | · | · | · | C | – | · | · | C | G | · | · |

·, identity with the top reference sequence; –, gaps introduced to improve sequence alignments.

*Cyt* b

We determined 227-bp sequences of the cyt *b* gene for 39 individuals from 25 populations plus a sequence from Genbank for one individual from a putative *R. amarus* population (Briolay *et al.*, 1998). Among populations, we detected a total of seven haplotypes, defined by 12 polymorphic sites (Table 7). Two haplotypes were found only in samples of *R. sericeus* (*CytB-Rs1* and *CytB-Rs2*; Table 8). Six haplotypes (*CytB-Ra1-5* and *CytB-Rc1*) were found in putative *R. amarus* and *R. colchicus* individuals. Two *R. colchicus* individuals shared one haplotype (*CytB-Rc1*), whereas another shared a haplotype (*CytB-Ra1*) with several putative *R. amarus* individuals. It is notable that no single haplotype was shared between *R. amarus* and *R. sericeus*.

The best-fit model of nucleotide substitution was Hasegawa–Kishino–Yano plus gamma (gamma shape distribution parameter = 0.3943) as assessed by both likelihood ratio tests and the Akaike Information Criterion; this model was used to reconstruct the cyt *b* haplotype phylogeny using NJ and maximum likeli-

**Table 6.** Control region haplotypes for individual bitterling samples with location

| Haplotype | Population/individual | Location |
|---|---|---|
| *ConReg-Ra1* | Al'ma-RA2, Al'ma-RA3 | River Al'ma |
| | Berda-RA4 | River Berda |
| | Delta -RA2 | Danube Delta |
| | Obito-RA1 | River Obitochnaya |
| | SBug-RA1, SBug-RA2 | River South Bug |
| | Tashy-RA2 | River Tashlychka |
| *ConReg-Ra2* | Delta-RA1, Delta-RA4 | Danube Delta |
| | Rawka-RA2 | River Rawka |
| *ConReg-Ra3* | Al'ma-RA4 | River Al'ma |
| *ConReg-Ra4* | Kyjov-RA1, Kyjov-RA2 | River Kyjovka |
| *ConReg-Ra5* | Rawka-RA3 | River Rawka |
| *ConReg-Ra6* | Kubol-RA2 | River Kubolta |
| | Tashy-RA3 | River Tashlychka |
| *ConReg-Ra7* | Delta-RA3, Delta-RA6 | Danube Delta |
| *ConReg-Ra8* | Karas-RA4 | River Karasu |
| *ConReg-Rc1* | Hobza-RC4 | River Hobza |
| *ConReg-Rc2* | Hobza-RC1, Hobza-RC2 | River Hobza |
| *ConReg-Rs1* | Amaza-RS1, Amaza-RS3 | River Amazar |
| | Bolsh-RS2, Bolsh-RS3 | River Bol'shoy |
| | Kubol-RA3 | River Kubolta |
| | Piati-RS2, Piati-RS3 | River Piatigorka |
| | Shilk-RS3, Shilk-RS4 | River Shilka |

Population codes are as shown in Table 1 with the numerical suffix indicating the individual sample for that population.

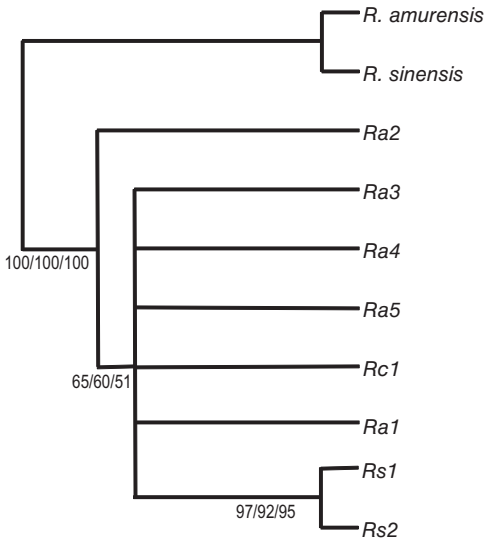


**Figure 3.** Phylogenetic relationships among cytochrome *b* haplotypes from *Rhodeus amarus* (*Ra1-Ra5*), *Rhodeus colchicus* (*Rc1*), and *Rhodeus sericeus* (*Rs1*, *Rs2*) individuals, with *Rhodeus amurensis* and *Rhodeus sinensis* included as an outgroup. The topology shown is that obtained using Neighbour-joining (NJ), but numbers under lines give the bootstrap support (% of all replicates) for adjacent nodes from the different methods of phylogeny reconstruction (NJ/maximum likelihood/maximum parsimony). Only bootstrap values > 50% are shown and nodes with bootstrap values < 50% have been collapsed.

hood methods. Results from all methods were highly consistent and each revealed only three groups with bootstrap support > 50%; for simplicity, only the NJ topology is displayed here (Fig. 3). The *R. amarus/colchicus/sericeus* sequences formed a monophyletic group with 100% bootstrap support in all methods. Within this group, relationships among most *R. amarus* and *R. colchicus* haplotypes, with the exception of haplotype *CytB-Ra2*, were unresolved. However, the two *sericeus* haplotypes formed a strongly-supported monophyletic group. A minimum spanning network showed two distinct haplotype groups corresponding to *R. amarus/colchicus* and *R. sericeus* haplotypes (Fig. 4). The *R. amarus/colchicus* group comprised a high frequency haplotype (*CytB-Ra1*) surrounded by a number of lower frequency haplotypes.

A likelihood ratio test did not detect significant deviation from a constant rate of substitution in cyt *b* sequences across lineages ($Ln_0 = 588.74$, $Ln_1 = 593.11$; $\chi^2 = 8.74$, d.f. = 6, $P = 0.189$). Based on the average pairwise divergence between *R. amarus/colchicus* and *R. sericeus* haplotypes of 2.5%, commonly used rates of divergence for cyt *b* in fishes of 1–2% per million years (Bowen *et al.*, 2001; Salzburger *et al.*, 2003) gave a divergence time estimate of 1.25–2.50 Mya.

## DISCUSSION

We used genetic and morphological data to investigate the relationships of the European bitterling, *R. sericeus s.l.*, comprising three putative species; *R. amarus*, *R. colchicus*, and *R. sericeus s.s*. Both morphometric and meristic data discriminated among all three groups. mtDNA control region data, although suggesting genetic differentiation among the three putative species, could not unequivocally differentiate among them due to a high frequency of indel polymorphism and consequent difficulties in sequence alignment. However, cyt *b* data demonstrated a clear distinction, supported by high bootstrap values, between *R. sericeus* and *R. amarus/R. colchicus* (Fig. 3). Together, these data suggest that the

**Table 7.** Polymorphic positions in bitterling cytochrome *b* sequences

| | Base position | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Haplotype | 24 | 78 | 90 | 93 | 94 | 105 | 120 | 129 | 132 | 195 | 219 | 227 |
| *CytB-Ra1* | A | T | T | T | T | T | A | C | A | C | C | A |
| *CytB-Ra2* | · | · | · | · | · | · | · | · | · | T | · | · |
| *CytB-Ra3* | · | · | · | · | · | · | · | · | G | · | · | · |
| *CytB-Ra4* | · | · | · | · | · | · | G | · | · | · | · | · |
| *CytB-Ra5* | · | C | · | A | A | · | · | · | · | · | · | · |
| *CytB-Rc1* | · | · | · | · | · | · | · | · | · | · | · | T |
| *CytB-Rs1* | G | · | C | · | · | C | · | T | · | · | A | · |
| *CytB-Rs2* | · | · | C | · | · | C | · | T | · | · | A | · |

·, identity with the top reference sequence.

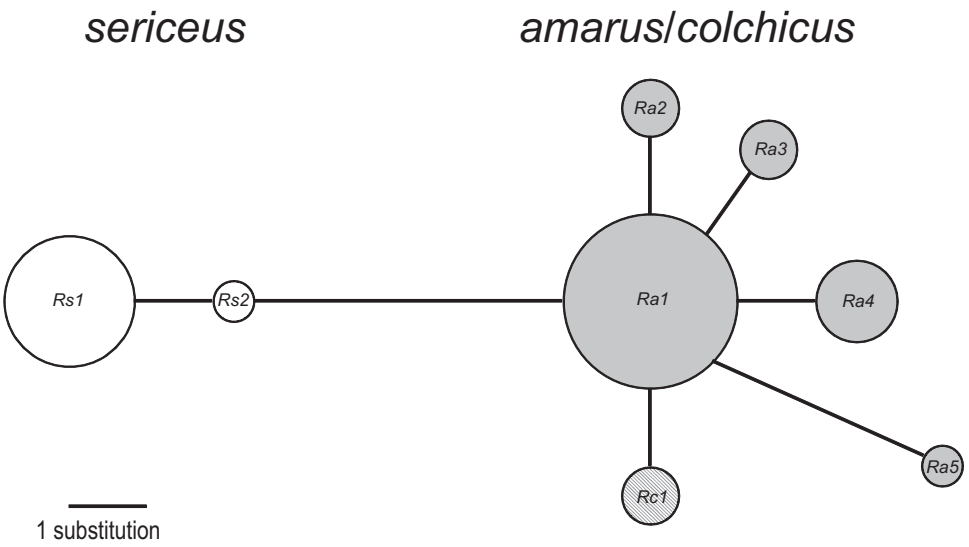


**Figure 4.** Minimum spanning network of *Rhodeus amarus* (shaded grey), *Rhodeus colchicus* (hatched), and *Rhodeus sericeus* (no shading) cytochrome *b* haplotypes. The area of the circle representing each haplotype is proportional to the frequency of the haplotype in the total sample.

European bitterling (*R. amarus*) is sufficiently distinct from the Amur bitterling (*R. sericeus s.s.*) to warrant separate designation.

Our morphological data support the separation of *R. amarus* from *R. sericeus*; both morphometric and meristic data and the form of their length–weight relationships showed significant differences between the two putative species. However, these data were ambiguous in explaining the relationship of *R. colchicus* with the other two groups. *Rhodeus colchicus* was distinct from both *R. amarus* and *R. sericeus* on the basis of morphometric data, but could only be separated from *R. sericeus* using meristic data. Thus, morphological data mirrored those from genetic analyses, indicating a distinct difference between *R. sericeus* and *R. amarus*/*R. colchicus*.

Bogutskaya & Komlev (2001) used a suite of taxonomic characters to separate *R. amarus* (i.e. European populations of *R. sericeus s.l.*) from Asian *R. sericeus s.s.* Some differences were evident, with *R. sericeus s.s.* characterized by a longer caudal region and predorsal abdominal region, although no characters clearly confirmed the specific status of *R. amarus*. From within populations of *R. amarus*, Bogutskaya & Komlev (2001) also described *R. colchicus* as a new species, distinguished by fewer scales in the lateral row, fewer vertebrae, and differences in infraorbital shape.

For genetic analysis, we initially concentrated our efforts on obtaining data for control region sequences as the control region is generally the most polymorphic section of vertebrate mtDNA. However, the vast

**Table 8.** Cytochrome *b* haplotypes for individual bitterling samples with location

| Haplotype | Population/individual | Location |
|---|---|---|
| *CytB-Ra1* | Berda-RA5 | River Berda |
| | Delta-RA1, Delta-RA2 | Danube Delta |
| | DonA-RA1, DonA-RA2, | River Don |
| | DonD-RA1 | River Kumylga |
| | Hobza-RC3 | River Hobza |
| | Kocio-RA1 | Lake Kociolek |
| | Kubol-RA1 | River Kubolta |
| | Kyjov-RA2 | River Kyjovka |
| | Liman-RA1 | Dniester Estuary |
| | Obito-RA1 | River Obitochnaya |
| | Rawka-RA1 | River Rawka |
| | Salgi-RA1 | River Salgir |
| | Tashy-RA1 | River Tashlychka |
| | Tysmy-RA1, Tysmy-RA2 | Tsymlyansk Reservoir |
| | WickF-RA2 | Wicken Fen |
| *CytB-Ra2* | Berda-RA4 | River Berda |
| | Delta-RA3 | Danube Delta |
| *CytB-Ra3* | Al'ma-RA1 | River Al'ma |
| | SBug-RA3 | River South Bug |
| *CytB-Ra4* | Karas-RA1, Karas-RA2, Karas-RA3 | River Karasu |
| | Kyjov-RA1 | River Kyjovka |
| *CytB-Ra5* | Y10454* | River Saône |
| *CytB-Rc1* | Hobza-RA1, Hobza-RA2 | River Hobza |
| *CytB-Rs1* | Amaza-RS1, Amaza-RS2 | River Amazar |
| | Anani-RS1, Anani-RS2 | River Ananievka |
| | Bolsh-RS1 | River Bol'shoy |
| | Kenon-RS1, Kenon-RS2 | Lake Kenon |
| | Khank-RS1 | Lake Khanka |
| | Piati-RS1 | River Piatigorka |
| | Shilk-RS1 | River Shilka |
| *CytB-Rs2* | Shilk-RS2 | River Shilka |

Population codes are as in Table 1 with the numerical suffix indicating the individual sample for that population. *From Briolay *et al.* (1998).

majority of the polymorphism we found consisted of single base pair indels in mononucleotide tracts. Indels represent a problem for phylogeny reconstruction and are often treated as missing data (Gilles *et al.*, 2001) although they may be useful in refining phylogenies based on base substitutions (Kawakita *et al.*, 2003) and models of sequence evolution have been developed to allow integration of indel data into phylogeny reconstruction methods (Thorne, Kishino & Felsenstein, 1991; McGuire, Denham & Balding, 2001; Cartwright, 2005; Lunter *et al.*, 2005). However, our control region data violate the assumptions of these methods because the indels were not rare in relation to substitution events and were not randomly distributed across the sequence, but were concentrated in repetitive DNA (mononucleotide tracts) that are often hotspots for such mutations. Therefore, we did not proceed with phylogenetic reconstruction using control region data. Based on sharing of control region haplotypes among putative species, it appeared that *R. sericeus s.s.* individuals were genetically distinct, although they shared only a single haplotype at low frequency with *R. amarus* populations. Similarly, individuals from a putative *R. colchicus* population did not share haplotypes with either *R. sericeus* or *R. amarus* individuals. However, conclusions on the genetic discreteness of the groups based on haplotype sharing depends on the accuracy of alignment of indels across haplotypes; the haplotype apparently shared by *R. sericeus* and *R. amarus* may be different haplotypes that are identical not by descent, but through homoplasy.

Cyt *b* polymorphism, in contrast to that for the control region, was exclusively due to base substitutions and, therefore, was more amenable to phylogenetic analysis and afforded more robust conclusions. Although relationships among *R. amarus* and *R. colchicus* haplotypes were generally unresolved, the *R. sericeus* haplotypes formed a distinct and well-supported monophyletic group. The star-like topology of the *R. amarus* and *R. colchicus* haplotypes, radiating from the basal and most common haplotype (*CytB-Ra1*) in a minimum spanning network, indicates a rapid expansion and radiation of this clade. Shared cyt *b* haplotypes between *R. amarus* and *R. colchicus* populations suggest relatively little genetic differentiation between these two groups.

Several hypotheses have been proposed to explain the disjunct distribution of members of the *Rhodeus* genus (Holčík & Jedlička, 1994; Bogutskaya & Komlev, 2001; Bohlen *et al.*, 2006; Van Damme *et al.*, 2007). These hypotheses involve scenarios of divergence, in various temporal sequences, among *sericeus/amarus/colchicus* from a common ancestor in East Asia with possible recolonization of East Asia by *R. sericeus*. Our results are consistent with an hypothesis of relatively ancient divergence of *R. sericeus* in East Asia from *R. amarus/colchicus* in Europe, with more recent and rapid morphological differentiation between *R. amarus* and *R. colchicus*, in broad agreement with Bohlen *et al.* (2006). Our sampling scheme did not allow any comparison of within-population levels of variation; *R. sericeus* as a group displays relatively low levels of genetic variability in relation to *R. amarus*. The level of divergence between *R. sericeus* and *R. amarus/colchicus* argues against *R. sericeus* being recently derived from *R. amarus* and, instead, for data derived from cyt *b* at least, suggests

a bottleneck that has reduced genetic variability in modern-day *R. sericeus* populations. Thus, the most likely scenario for the colonization of Europe by bitterling is of the invasion of an ancestral *R. sericeus s.l.*, probably in the late Pliocene, to the present region of the Black and Caspian Seas. Following the isolation of East Asian populations from those in Europe during glacial events of the Pleistocene, recent divergence of isolated populations within the derived *R. amarus* lineage has occurred to varying degrees, typified by the emergence *R. colchicus* and *R. meridionalis* Bohlen *et al.* (2006).

In terms of the timing of divergence of *R. sericeus* and *R. amarus/colchicus*, our estimate of 1.25–2.50 Mya corresponds broadly with that of Holčík & Jedlička (1994), who estimated 2–4 Mya based on geological and geographical events, and Bohlen *et al.* (2006) who estimated 2.36 Mya based on a molecular clock estimate. However, estimates of the divergence of lineages using molecular clocks are notoriously imprecise (Pulquério & Nichols, 2007) and estimates should be treated with caution. The expansion of *R. amarus* from its original distribution around the Black and Caspian seas is recorded in historical documents and field surveys, indicating the recent arrival of *R. amarus* in west and central Europe (Kozhara *et al.*, 2007; Van Damme *et al.*, 2007).

In summary, on the basis of morphological and genetic data, we have identified a significant discontinuity between *R. amarus* (the European bitterling) and *R. sericeus s.s.* (the Amur bitterling). We tentatively propose these be treated as separate species in accordance with Kottelat (1997) and Bohlen *et al.* (2006). However, we have failed to identify a consistent pattern of discontinuity, from both morphological and genetic data, between *R. amarus* and *R. colchicus* within Europe, although differences in taxonomic characters between these groups have been described (Bogutskaya & Komlev, 2001).

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## REFERENCES

**Arai R. 1988.** Fish systematics and cladistics. In: Uyeno T, Okiyama M, eds. *Ichthyology currents 1988*. Tokyo: Asakura Shoten, 4–33.

**Bloch ME. 1782.** *Economische naturgeschichte der fische Deutschlands*, Vol. 1. Berlin: Schlesinger Verlag.

**Bogutskaya NG, Komlev AM. 2001.** Some new data to morphology of *Rhodeus sericeus* (Cyprinidae: Acheilognathinae) and a description of a new species, *Rhodeus colchicus*, from west Transcaucasia. *Proceedings of the Zoological Institute* **287:** 81–97.

**Bohlen J, Bogutskaya V, Freyhof NJ. 2006.** Across Siberia and over Europe: phylogenetic relationships of the freshwater fish genus *rhodeus* in Europe and the phylogenetic position of *r. Sericeus* from The River Amur. *Molecular Phylogenetics and Evolution* **40:** 856–865.

**Bowen BW, Bass AL, Rocha LA, Grant WS, Robertson DR. 2001.** Phylogeography of the trumpetfishes (*Aulostomus*): Ring species complex on a global scale. *Evolution* **55:** 1029–1039.

**Briolay J, Galtier N, Brito RM, Bouvet Y. 1998.** Molecular phylogeny of Cyprinidae inferred from cytochrome *b* DNA sequences. *Molecular Phylogenetics and Evolution* **9:** 100–108.

**Cantatore P, Roberti M, Pesole G, Ludovico A, Milella F, Gadaleta M, Saccone C. 1994.** Evolutionary analysis of cytochrome *b* sequences in some Perciformes: evidence for a slower rate of evolution than in mammals. *Journal of Molecular Evolution* **39:** 589–597.

**Cartwright RA. 2005.** DNA assembly with gaps (Dawg): simulating sequence evolution. *Bioinformatics* **21** (Suppl. 3)**:** 31–38.

**Chang Y, Huang F, Lo T. 1994.** The complete nucleotide sequence and gene organisation of carp (*Cyprinus carpio*) mitochondrial genome. *Journal of Molecular Evolution* **38:** 138–155.

**Duyvené de Wit JJ. 1955.** Some observations on the European bitterling Rhodeus amarus. *South African Journal of Science* **1:** 249–251.

**Excoffier L, Laval G, Schneider S. 2005.** Arlequin ver. 3.0: an integrated software package for population genetics data analysis. *Evolutionary Bioinformatics Online* **1:** 47–50.

**Gilles A, Lecointre G, Faure E, Chappaz R, Brun G. 1998.** Mitochondrial phylogeny of the European cyprinids: Implications for their systematics, reticulate evolution, and colonization time. *Molecular Phylogenetics and Evolution* **10:** 132–143.

**Gilles A, Lecointre G, Miquelis A, Loerstcher M, Chappaz R, Brun G. 2001.** Partial combination applied to phylogeny of European cyprinids using the mitochondrial control region. *Molecular Phylogenetics and Evolution* **19:** 22–33.

**Holčík J. 1999.** *Rhodeus sericeus*. In: Bănărescu PM, ed. *The freshwater fishes of Europe 5/1. Cyprinidae*. Wiebelsheim: AULA-Verlag, 1–32.

**Holčík J, Jedlička L. 1994.** Geographical variation of some taxonomically important characters in fishes: the case of the bitterling *Rhodeus sericeus*. *Environmental Biology of Fishes* **41:** 147–170.

**Huelsenbeck JP, Crandall KA. 1997.** Phylogeny estimation and hypothesis testing using maximum likelihood. *Annual Review of Ecology and Systematics* **28:** 437–466.

**Kawakita A, Sota T, Ascher JS, Ito M, Tanaka H, Kato M. 2003.** Evolution and phylogenetic utility of alignment gaps within intron sequences of three nuclear genes in bumble bees (*Bombus*). *Molecular Biology and Evolution* **20:** 87–92.

**Kottelat M. 1997.** European freshwater fishes. *Biologia (Bratislava)* **52** (Suppl. 5)**:** 1–271.

**Kozhara AV, Zhulidov AV, Gollasch S, Przybylski M, Poznyak VG, Zhulidov DA, Gurtovaya TYu. 2007.** Range extension and conservation status of the bitterling, *Rhodeus sericeus amarus* in Russia and adjacent countries. *Folia Zoologica* **56:** 97–108.

**Lunter G, Miklós I, Drummond A, Ledet Jensen J, Hein J. 2005.** Bayesian coestimation of phylogeny and sequence alignment. *BMC Bioinformatics* **6:** 83.

**McGuire G, Denham MC, Balding DJ. 2001.** Models of sequence evolution for DNA sequences containing gaps. *Molecular Biology and Evolution* **18:** 481–490.

**Mills SC, Reynolds DC. 2003.** The bitterling-mussel interaction as a test case for co-evolution. *Journal of Fish Biology* **63** (Suppl. S1)**:** 84–104.

**Nelson JS. 1984.** *Fishes of the world*. New York, NY: Wiley.

**Okazaki M, Naruse K, Shima A, Arai R. 2001.** Phylogenetic relationships of bitterlings based on mitochondrial 12S ribosomal DNA sequences. *Journal of Fish Biology* **58:** 89–106.

**Pallas PS. 1776.** *Reise durch verschiedene Provinzen des Russisches Reichs,* Vol. 3. St Petersburg: Kaiserlichen Academie der Wissenschaften.

**Posada D, Crandall KA. 1998.** Modeltest: testing the model of DNA substitution. *Bioinformatics* **14:** 817–818.

**Przybylski M, Ziêba G. 2000.** Microhabitat preferences of European bitterling, *Rhodeus sericeus* in the Drzewiczka River (Pilica basin). *Polish Archives of Hydrobiology* **47:** 99–114.

**Pulquério MJF, Nichols RA. 2007.** Dates from the molecular clock: how wrong can we be? *Trends in Ecology and Evolution* **22:** 180–184.

**Reichard M, Jurajda P, Matejusová MI. 2002a.** Size-related habitat use by bitterling (*Rhodeus sericeus*) in a regulated lowland river. *Ecology of Freshwater Fishes* **11:** 112–122.

**Reichard M, Jurajda P, Ondračková M. 2002b.** Inter-annual variability in seasonal dynamics and species composition of drifting young-of-the-year fishes in two European lowland rivers. *Journal of Fish Biology* **60:** 87–101.

**Reichard M, Liu H, Smith C. 2007a.** The co-evolutionary relationship between bitterling fishes and freshwater mussels: insights from interspecific comparisons. *Evolutionary Ecology Research* **9:** 1–21.

**Reichard M, Ondračková M, Przybylski M, Liu H, Smith C. 2006.** The costs and benefits in an unusual symbiosis: experimental evidence that bitterling fish (*Rhodeus sericeus*) are parasites of unionid mussels in Europe. *Journal of Evolutionary Biology* **19:** 788–796.

**Reichard M, Przybylski M, Kaniewska P, Liu H, Smith C. 2007b.** A possible evolutionary lag in the relationship between freshwater mussels and European bitterling. *Journal of Fish Biology* **70:** 709–725.

**Saitou N, Nei M. 1987.** The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* **4:** 406–425.

**Salzburger W, Brandstätter A, Gilles A, Parson W, Hempel M, Sturmbauer C, Meyer A. 2003.** Phylogeography of the vairone (*Leuciscus souffia*, Risso 1826) in Central Europe. *Molecular Ecology* **12:** 2371–2386.

**Smith C, Reichard M, Jurajda P, Przybylski M. 2004.** The reproductive ecology of the European bitterling (*Rhodeus sericeus*). *Journal of Zoology* **262:** 107–124.

**Svetovidov AN, Eremeev GK. 1935.** On the European and Amur bitterling (*Rhodeus sericeus*). *Doklady Akademii Nauk SSSR* **1:** 582–587.

**Swofford DL. 2002.** *PAUP*. Phylogenetic analysis using parsimony (*and other methods)*, Version 4. Sunderland, MA: Sinauer Associates.

**Thorne JL, Kishino H, Felsenstein J. 1991.** An evolutionary model for maximum likelihood alignment of DNA sequences. *Journal of Molecular Evolution* **33:** 114–124.

**Van Damme D, Bogutskaya NG, Hoffmann RC, Smith C. 2007.** The introduction of the European bitterling (*Rhodeus amarus*) to West and Central Europe. *Fish and Fisheries* **8:** 79–106.