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High cryptic diversity of bitterling fish in the southern West Palearctic

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ABSTRACT

South-east Europe, along with the adjacent region of south-west Asia, is an important biodiversity hotspot with high local endemism largely contributed by contemporary continental lineages that retreated to southern refugia during colder Quaternary periods. We investigated the genetic diversity of the European bitterling fish (*Rhodeus amarus*) species complex (Cyprinidae) across its range in the western Palearctic, but with a particular emphasis in the region of Balkan, Pontic and Caspian refugia. We genotyped 12 polymorphic microsatellite loci and a partial sequence of mitochondrial gene cytochrome *b* (*CYTB*) for a set of 1,038 individuals from 60 populations. We used mtDNA sequences to infer phylogenetic relationships and historical demography, and microsatellite markers to describe fine-scale genetic variability and structure. Our mtDNA analysis revealed six well-supported lineages, with limited local co-occurrence. Two lineages are distributed throughout central and western Europe (lineages "A" and "B"), with two zones of secondary contact. Another two lineages were restricted to the Ponto-Aegean region of Greece (lineages "C" and "D") and the final two lineages were restricted south of the Caucasus mountains (lineage "E" from the Black Sea watershed and lineage "F" from the Caspian watershed). A signal of recent expansion was revealed in the two widespread lineages and the Ponto-Aegean lineage "C". The geographic distribution of clusters detected by nuclear microsatellites corresponded well with mitochondrial lineages and demonstrated finely sub-structured populations. A profound population structure suggested a significant role of genetic drift in differentiation among lineages. Lineage divergence in the Ponto-Aegean and Caspian regions are substantial, supporting the validity of two described endemic species (*Rhodeus meridionalis* as lineage "D" and *Rhodeus colchicus* as lineage "E") and invite taxonomic evaluation of the other two southern lineages (Thracean "C" and Caspian "F").

1. Introduction

Contemporary species distributions and intraspecific diversity are largely driven by Pleistocene climatic oscillations, with climatic dynamics of the Holocene having major impacts on the species and intraspecific diversity of many Palearctic taxa (Hewitt, 1999). In cold periods, thermophilic species retreated to thermal refugia; in the west Palearctic they were primarily located in the Mediterranean peninsulas (Iberian, Apennine, Balkan) and Caspian-Caucasian region (Stewart et al., 2010). Some species retreated to smaller cryptic refugia at higher latitudes (Stewart and Lister, 2001; Stewart et al., 2010) with sheltered topography and suitable microclimates (Stewart and Lister, 2001). The contribution of disparate refugia to the current diversity of the European biota was uneven, with an overrepresentation of lineages expanding from the Balkan refugium where the geography favoured dispersal (Hewitt, 1999). The Ponto-Caspian refugia from the region along the Black Sea coast and on the north-western slopes of the Caucasus Mountains (Adams and Faure, 1997), also supported re-colonisation of Europe, but to a much lesser extent (Culling et al., 2006). These areas may have been relatively fragmented, supporting genetically diverse

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populations, termed 'refugia-within-refugia' (Gómez and Lunt, 2007), rather than representing a single continuous refugium with large and interconnected populations. A possible outcome is that these former refugial areas may represent hotspots of biodiversity as a consequence of two different proximate mechanisms (Dufresnes et al., 2016); long-term persistence of large refugial populations or, alternatively, a set of small, geographically isolated populations with limited gene flow. These two scenarios are predicted to bear unique genetic signatures. In the case of large continuous refugial populations with substantial effective population sizes over successive glacial cycles, genetic diversity should be high but homogenous across the refugium. In the case of refugia-within-refugia, in contrast, spatially restricted and highly structured refugial distributions predict well-defined population genetic structure within the greater refugium region, but with signals of population expansion and genetically rich populations in secondary contact zones.

South-east Europe and adjacent West Asia (northwestern Middle East: Asia Minor, Caucasian and Caspian regions) are important hotspots of genetic diversity for a number of taxa (e.g. Kramp et al., 2009; Dufresnes et al., 2016; Jablonski et al., 2016) with many locally endemic lineages (Geiger et al., 2014). A combination of the effects of historical climate, topography and dramatic changes in sea level during the last 5 million years resulted in variable patterns of continental colonization from this refugium (e.g. Durand et al., 1999; Kotlík and Berrebi, 2007), which is reflected in the complex structure of the Balkan biotic assemblage (Economidis and Banarescu, 1991). In particular, the Aegean Sea was approximately 120 m lower than it is today during the Plio-Pleistocene (Perissoratis and Conispoliatis, 2003), redisposing rivers, streams, wetlands, and their connections (Hewitt, 2000). Until the early Holocene (11,500 BP), the Black Sea basin was an extensive freshwater lake (Degens and Ross, 1972), and likely to have supported, rather than limited, dispersal of freshwater taxa. A connection between the Black and Caspian Seas established periodically during the Pleistocene, with termination of the last connection estimated at 17,000–16,000 years BP (Reid and Orlova, 2002).

We investigated genetic diversity of bitterling fishes in the West Palearctic (*Rhodeus amarus* species complex), with special attention on the relative contribution of refugial populations to the current bitterling diversity in the region. Bitterling belong to a distinct subfamily of cyprinid fishes (Cyprinidae: Acheilognathinae) (Chang et al., 2014) that parasitize freshwater molluscs by ovipositing in their gill chambers (Smith et al., 2004). The global centre of distribution of bitterling is in East Asia (China, Japan, Korea and adjacent countries) (Chang et al., 2014; Kawamura et al., 2014). One bitterling lineage colonized the western Palearctic (Bohlen et al., 2006; Chang et al., 2014) and three species of the genus *Rhodeus* have been formally named in region (Kottelat and Freyhof, 2007). In addition to widespread *Rhodeus amarus* (Bloch, 1782), *Rhodeus colchicus* Bogutskaya and Komlev, 2001 was described from the foothills of the western Caucasus on the basis of osteological characters and confirmed as a separate lineage by mitochondrial genetic data (Bohlen et al., 2006; Zaki et al., 2008). Populations in the River Vardar were described as *Rhodeus sericeus meridionalis* Karaman 1924 and were proposed as representing a valid species, *R. meridionalis*, by Kottelat and Freyhof (2007) on the basis of its genetic divergence. However, bitterling diversity in other areas of the West Palearctic distribution is poorly explored and their phylogenetic relationships and intraspecific structure are unresolved, perhaps as a result of relatively rapid diversification, incomplete lineage sorting and/or gene flow among populations/species (Bohlen et al., 2006; Bryja et al., 2010; Chang et al., 2014).

The natural distribution of bitterling in the West Palearctic covers a large part of continental Europe, excepting the Iberian and Apennine Peninsulas, Fennoscandia, Denmark and the region east and north of the Dnieper basin (Kottelat and Freyhof, 2007). Recent introductions have expanded its range to Great Britain, Denmark, Italy and several basins in European Russia (Kozhara et al., 2007; Bartáková et al., 2018). Bitterling are thermophilic (Smith et al., 2004; Van Damme et al., 2007) and former studies established that their populations survived the Pleistocene glacial periods in refugia located in the Balkans, Black Sea region, lower Danube, and southern Caucasian region (Bohlen et al., 2006; Zaki et al., 2008; Bryja et al., 2010; Bektas et al., 2013). Some of these populations repeatedly expanded during warmer climatic conditions and colonized large parts of Europe (Kozhara et al., 2007; Van Damme et al., 2007; Bryja et al., 2010). Populations from the lower Danube spread throughout the River Danube basin and colonised most of Central and West Europe, while a population from a putative refugium in the northern Black Sea region colonized north-eastern Europe (Bohlen et al., 2006; Bryja et al., 2010), with the area of secondary contact in Central and West Europe (Bryja et al., 2010). Preliminary evidence suggested that other populations remained isolated in their refugia and differentiated in allopatry (Bryja et al., 2010).

To investigate the patterns of genetic differentiation of the West Palearctic bitterling species complex, we used dense and fine-scale sampling of bitterling populations in the region, with a particular focus on the Balkan region and other parts of the greater Mediterranean region with the reported presence of bitterling populations. We specifically concentrated on the analysis of (1) genetic differentiation among bitterling lineages to describe their phylogenetic patterns, (2) genetic diversity within the lineages and populations to understand their demographic history and (3) geographic aspects of the distribution of particular lineages to characterise past and recent connections between the lineages and their contribution to the current expansion of the bitterling in the West Palearctic region.

2. Materials and methods

2.1. Sampling and DNA extraction

We analysed a total of 1038 fish from 60 sampling sites, with particular attention to the bitterling distribution in the southern part of its range in the West Palearctic. Reference to zoogeographical regions follows terminology for freshwater fishes from Economidis and Banarescu (1991). On the basis of preliminary analysis of an individual-based Bayesian clustering procedure, implemented in the STRUCTURE software, we pooled some populations for the final analyses. The pooled samples came from adjacent sites within the same streams, with the exception of the IRAZ population that was composed of pooled samples from several bitterling populations from the Caspian region (Azerbaijan, Iran) that lacked a precise geo-reference (Azerbaijan) and were too small for most population genetic analyses. All individuals in the IRAZ sample belonged to the same mitochondrial haplogroup. Fish were collected between 2004 and 2015. Sampling sites, along with details on the number of analysed individuals and haplotype composition are listed in Appendix A. DNA extraction was performed from small fin clips taken from the caudal fin and stored in 96% ethanol, using the DNeasy Blood and Tissue Kit (Qiagen) following a standard protocol. Extracted DNA was stored at −20 °C.

2.2. Genotyping

All bitterling individuals were genotyped at 12 microsatellite loci in three multiplex PCR sets (Table B.1). Primer names and sequences were taken from Dawson et al. (2003) and Reichard et al. (2008). A detailed genotyping protocol is provided in Bartáková et al. (2018). The length of DNA fragments was analysed manually using GeneMapper v. 5.0 (Applied Biosystems).

A partial sequence of mitochondrial gene cytochrome *b* (*CYTB*) was amplified by primers Thr-H (5′-ACCTCCRATCTYCGGATTACA-3′) and Glu-L (5′-GAAGAACCACCGTTGTTATTCAA-3′) in a subset of individuals (Appendix A) following the protocol of Bohlen et al. (2006) with conditions described in Bartáková et al. (2018). PCR products were commercially Sanger-sequenced by Macrogen Europe. Sequence

editing was performed in SeqScape V.2.5 (Applied Biosystems) and aligned in BioEdit v.7.0.9.0 (Hall, 1999), producing a final alignment of 914 bp. All sequences have been deposited in GenBank (accession numbers MH041650–MH041876).

2.3. Phylogenetic analysis and haplotype distribution based on mitochondrial DNA

The most appropriate substitution model for the *CYTB* dataset was the Generalised time-reversible model with a gamma-distributed rate variation across sites (GTR + G), which was selected on the basis of BIC in TOPALi v. 2.5 (Milne et al., 2009). Three unique sequences of *Rhodeus sericeus* (the sister lineage of the western Palearctic bitterling complex (Chang et al., 2014)) from Lake Kenon (River Amur basin) were used as outgroups in all phylogenetic analyses. Phylogenetic relationships were inferred by maximum likelihood (ML) and Bayesian (BI) approaches. ML analysis was performed in RAxML 8.2.10 (Stamatakis, 2014), applying the GTR + G model (option -m GTRGAMMA). The robustness of the nodes was assessed by the default rapid bootstrap procedure with 1,000 replications (option -# 1000). Bayesian analysis was performed by Markov Chain Monte Carlo (MCMC) simulation using MrBayes 3.2.6 (Ronquist and Huelsenbeck, 2003). Two independent analyses were initiated from random trees. Three heated and one cold chain were run for 10 million generations per run, sampling every 1000 generations. For each run, 25% of trees were discarded as burn-in. Bayesian posterior probabilities were used to evaluate branch support of the consensus tree. All phylogenetic analyses were performed on Cipres Science Gateway webserver (Miller et al., 2010). The final trees were edited in FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree).

To analyse the phylogeny in a time-calibrated framework, we estimated the relative time to the most recent common ancestor (TMRCA) for all unique sequences in BEAST v. 2.4.6 (Bouckaert et al., 2014). To avoid unrealistic assumptions of a strict molecular clock, a relaxed molecular clock model (uncorrelated lognormal) was used for the analysis (Drummond et al., 2006). We performed two runs (10 million generations each) under the GTR + G model with parameters sampled every 1000 iterations. We discarded the first 20% as burn-in, based on likelihood stationarity visualized using Tracer 1.5 (Rambaut et al., 2018). The effective sample sizes (*ESS*) of all parameters sampled from MCMC were > 300. The resulting parameter and tree files from the two runs were combined in LogCombiner 2.4.6 (Bouckaert et al., 2014) and a maximum clade credibility tree was calculated in TreeAnnotator 2.4.6 (Bouckaert et al., 2014). Because there are no reliable tools for absolute calibration of the *Rhodeus* molecular clock (no suitable fossils or well-dated biogeographic events), we used this analysis only for relative comparison of divergence events.

The haplotypes and their frequencies were identified using DnaSP v. 5.10.01 (Librado and Rozas, 2009). The relationships among haplotypes were also visualised as a median-joining (MJ) network in Network 5.0.0.1 (Bandelt et al., 1999) using an equal transition/transversion ratio. All sequences were geo-referenced and the distribution of the haplogroups was inspected visually. The matrix of mean p-distances within/between *CYTB* clades (using 101 unique haplotypes of 914 bp) was calculated in MEGA v. 6 (Tamura et al., 2013) and standard errors were estimated with 1000 bootstraps.

2.4. Historical demography based on mtDNA variation

To analyse historical demography we identified six genetic lineages (see Appendix A for assignment to lineages) based on the phylogenetic analyses, the haplotype network of mtDNA and the geographical distribution of haplotypes. We used the reduced dataset of 208 sequences (914 bp) (Appendix A). We excluded three populations (RHI2, STR2, MER1) because they represented secondary contacts of differentiated lineages and contained individuals with haplotypes from two lineages. Diversity estimates for the six lineages; i.e. number of polymorphic sites (*Np*), number of haplotypes (*Nh*), haplotype diversity (*Hd*), nucleotide diversity (*p*), and average number of nucleotide differences (*k*) were computed in DnaSP 5.00.04 (Librado and Rozas, 2009).

To infer demographic histories, we estimated the neutrality indices (Tajima's D and Fu's Fs statistic) in DnaSP v. 5.10.01 (Librado and Rozas, 2009). These indices are sensitive to population size change and return significantly negative values in the case of recent population expansion. As an additional test of demographic expansion, the distribution of pairwise nucleotide differences in each lineage (mismatch distribution; MD) was calculated in DnaSP. We used the sum of square deviations (SSD) between the observed and expected mismatch as a test statistic for the validity of the estimated stepwise expansion model (Schneider and Excoffier, 1999). Parameter τ (the moment estimator of time to the expansion) was estimated with DnaSP using the moment method of Rogers (1995) assuming the infinite sites model (IFM) and, additionally, in ARLEQUIN using the method of Schneider and Excoffier (1999) to relax the IFM assumption. Confidence intervals were obtained by a parametric bootstrap approach based on 1000 replicates performed in ARLEQUIN. Under the assumption of the (sudden) demographic expansion model the MD also permits estimation of the time of onset of population expansion τ ($\tau = 2 * t * \mu$; t = time in years, μ = mutation rate per locus of 914 bp). We converted the parameter τ, calculated from the mismatch distribution, to estimate the time since the expansion (t) using the equation $t = \tau/2\mu$, assuming the commonly accepted substitution rates of 0.76% per million years for *CYTB* in cyprinid fishes (Zardoya and Doadrio, 1999) and an average generation time of one year for the bitterling (Smith et al., 2004; Konečná and Reichard, 2011).

We reconstructed historical population size dynamics of the main lineages backward in time using the coalescent-based Bayesian skyline plot (BSP) in BEAST 2.4.6. (Drummond and Rambaut, 2007). Analyses were run twice for each lineage and the model of sequence evolution for each lineage was selected with the jModelTest (Posada, 2008) under the BIC criterion. The MCMC simulations were run with 50 million iterations with a sampling increment of 5000 and 25% burn-in. Results were checked for convergence and stationarity of different runs in Tracer 1.6 and the outputs from two runs were combined in the LogCombiner 2.4.6 module. The BSPs were produced in Tracer 1.6.

2.5. Analysis of genetic variability and structure based on microsatellite data

To analyse interpopulation and intrapopulation genetic variability on nuclear markers, the proportion of null alleles (*NA*) at each locus and population was estimated in FreeNA (Chapuis and Estoup 2007). The number of alleles (*A*), and observed (H_O) and expected (H_E) heterozygosities were calculated in GENETIX. The rarefaction method in FSTAT 2.9.3.2 (Goudet, 2001) was used to calculate allelic richness (*AR*) for each population standardized for a minimum sample size of 7 individuals. Deviations from Hardy-Weinberg equilibrium (*HWE*) were tested for each locus and population using the Markov chain method in the software GENEPOP and the correction for multiple testing was performed using the false discovery rate (FDR) approach (Benjamini and Hochberg, 1995) in QVALUE (Storey, 2002). For these analyses populations with a low number (< 10) of sampled individuals were not used (see Table B.2). Analyses of intrapopulation variability were computed only from 10 microsatellite loci; locus *Rser13* (D05) was excluded due to high polymorphism (110 alleles) and *Rser09* (D12) was excluded given a high level of null alleles (mean of 5.64% per population). Genetic differentiation among study populations was quantified by F_{ST} (Weir and Cockerham, 1984) and their significance was tested by 1000 permutations in GENETIX 4.05 (Belkhir et al., 1996–2004).

An individual-based Bayesian clustering procedure implemented in STRUCTURE 2.3.4 (Hubisz et al., 2009) was used to detect the best genetic structure among sampled individuals (n = 1002 individuals

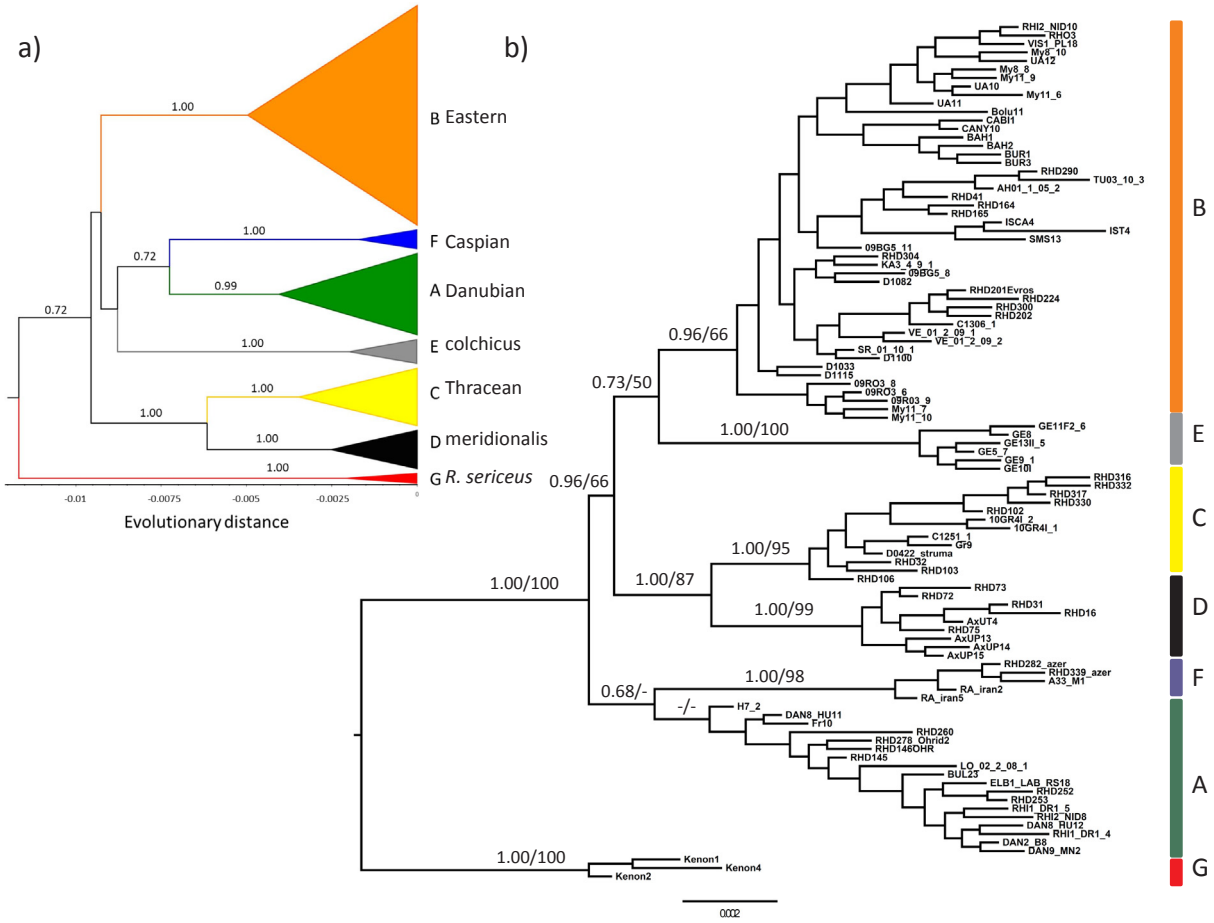

Fig. 1. Mitochondrial phylogeny of the European bittering complex based on 98 ingroup and 3 outgroup haplotypes of the mitochondrial gene *CYTB*. (a) Ultrametric tree with relative dating from BEAST 2.4.6. Posterior probabilities (/bootstrap support for maximum likelihood tree from RAxML in b) are shown above branches (only values higher than 0.70/50 are shown). Putative taxonomic names or a label of geographic distribution are shown for each lineage. (b) Bayesian reconstruction from MrBayes 3.2.6 with Bayesian inference posterior probabilities/bootstrap support from the maximum likelihood analysis for each node.

from 52 populations). The program was run with 20 independent simulations for each of *K* from 1 to 30, with 1 million iterations for each simulation, following a burn-in period of 100,000 iterations. The computation was realised using an admixture ancestry model and correlated allele frequencies model (with $\lambda = 1$). The web-based software STUCTURE HARVESTER (Earl and vonHoldt, 2012) was used for parsing and summarizing output data from STRUCTURE. It reformatted data for downstream programs and produced the likelihood of *K*; i.e. Ln Pr(X|K), for inferring the best number of real populations in the datasets using the method of Evanno et al. (2005). The results of 20 replicate runs for each *K* were combined using the Greedy algorithm of CLUMPP 1.1.1 (Jakobsson and Rosenberg, 2007) and summary outputs for each *K* were displayed using DISTRUCT v. 1.1 (Rosenberg, 2004).

2.6. Specific testing of isolation/gene flow

Based on the comparison of results from *CYTB* and microsatellite analyses, we specifically tested the origin of the MER1 population with respect to possible gene flow between genetic groups "C" and "D" using Approximate Bayesian Computation (ABC; Beaumont et al., 2002) implemented in DIYABC 2.0.4 (Cornuet et al., 2014). This analysis models population histories by combining population divergences, admixtures and population size fluctuations and compares alternative evolutionary scenarios by estimating their relative support and quantifying parameters for particular scenarios (Cornuet et al., 2014). Three "populations" were generated according to the Bayesian assignment of their genetic structure and haplotype distribution. The populations MER1 (35 individuals) and MER3 (26 individuals) were formed exclusively by those single population samples. The SSTR (i.e. "South Struma/Strymon") population was composed of STR4, STR5 and STR6 population samples (total of 57 individuals). Effective population size, timing of events (merging, splitting or change in effective population size) and rates of admixture in the case of merging events, were used to describe seven scenarios (Table B.3, Fig. C.1). The range of uniform priors is provided in Table B.4. The generalized stepwise model was used as a mutation model (GSM; Estoup et al., 2002). All microsatellites used have regular motifs (motif lengths of 2 bp, except of a 4 bp motif in Rser11) and all microsatellite mutation parameters were at default settings. An average generation time of one year was used (Smith et al., 2004).

We simulated 1 million data sets per scenario. For each simulation, a set of summary statistics was computed to compare with the observed dataset for the best model selection. The relative posterior probability (95% credible intervals) of each scenario was determined using the 1% of simulated data sets closest to the observed data (Euclidean distances) and logistic regression was used to select the most likely evolutionary scenario. The posterior parameter distributions were estimated from the closest 1% of simulated data sets of the most likely model through a local linear regression procedure (Cornuet et al., 2008). The assessment of goodness-of-fit of the best model was checked by evaluating consistency of the observed data with the posterior predicted distribution of the model for the best scenario. We carried out model checking using all summary statistics, including those that had not been used in the initial ABC analyses for model selection (Cornuet et al., 2010).

3. Results

Genotyping success of microsatellites was high (97.14%), resulting in multilocus genotypes of 1002 individuals from 52 populations. All analyses of genetic structure were based on a complete dataset of all 12 loci. The analyses of intrapopulation genetic variability were performed using a reduced dataset of 10 loci and 924 individuals from 38 populations; excluding populations with < 10 individuals (Appendix A). Analysis of mitochondrial variability was based on 227 *CYTB* sequences (914 bp; 101 haplotypes) from 57 sites (Appendix A).

3.1. Analysis of mitochondrial variability

Phylogenetic analysis of 101 unique *CYTB* sequences revealed a highly structured tree of West Palaearctic bitterling (Figs. 1, C.2) with six well-supported phylogenetic lineages, referred to as lineages "A"-"F". However, relationships among these lineages were not well resolved, except the sister position of lineages "C" and "D". The topologies of the trees differed between BEAST and MrBayes (Fig. 1a vs. b), suggesting a rapid radiation that produced the current diversity of mitochondrial lineages. This view is further strengthened by similar estimates for the age of the first divergence within particular lineages (Fig. 1a).

A haplotype network supported the same pattern (Figs. 2, C.3). Two lineages occur throughout Central and West Europe (Fig. 3a). Lineage "A" (Danubian, green on Figs. 1–3a) was the only lineage distributed in the River Danube basin and was dominant across Central Europe. Surprisingly, lineage "A" was also found in the ancient lakes of Prespa and Skadar in the western Balkans (south Adriatic-Ionian division). Lineage "B" (Eastern, orange on Figs. 1–3a) was widely distributed around the Black Sea coast (excluding the eastern shore) and extended to north-eastern Europe (Fig. 3a). Its distribution included East Bulgaria and Thracean-East Macedonia subdivisions of the Balkans (the Rivers Mesta/Nestos and Marmaras, Lake Vistonis). It was also present in the Mediterranean region of southern France (the River Rhône), a case of a disjunct distribution. One middle Rhine population (RHI2, River Nida, central Germany) represented a likely secondary contact between the "A" and "B" lineages; two individuals possessed haplotype h8 of lineage "B" while three individuals had haplotypes h1 and h9 of lineage "A". Another case of sympatry between "A" and "B" lineages was located in the upper River Struma/Strymon (STR2, southern Bulgaria, Aegean watershed); four individuals had the h1 haplotype commonly found in lineage "A" and three individuals possessed haplotype h22 of the "B" lineage.

Two other lineages were restricted to the western part of the Ponto-Aegean division of the Balkans in Greece (Fig. 3a). Lineage "C" (Thracean, yellow on Figs. 1–3a) occurred in the Thracean-East Macedonian subdivision (the River Struma/Strymon basin, including Lake Volvi, upper River Mesta/Nestos and River Macropotamos). Lineage "D" (meridionalis, black on Figs. 1–3a) was found only in Macedonia-Thessaly subdivision of the Ponto-Aegean region (Rivers Vardar/Axios, Aliakmon/Haliacmon and Pinios/Pineios) and represents the putative *R. meridionalis*. Interestingly, the "C" and "D" lineages coexist in the River Aliakmon/Haliacmon (MER1); one individual had haplotype h47 of the lineage "C" and three individuals had haplotypes of the "D" lineage (h46, h77). No sympatric coexistence between "B" and "C" lineages was detected despite their interwoven geographical distribution in the Thracean-East Macedonia subdivision along the northern Aegean coast (Fig. 3a).

Finally, two lineages were exclusive to the eastern part of the study area, southern Caucasian region. Lineage "E" (colchicus, grey on Figs. 1–3a) occurred in the Transcaucasian part of the Black Sea region (Georgia; described as *R. colchicus*). Lineage "F" (Caspian, blue in Figs. 1–3a) included samples from southern Caspian region (Azerbaijan and Iran). Mean p-distances within and between the *CYTB* lineages are provided in Table B.5.

3.2. Demographic changes in mtDNA lineages

Demographic history was analysed separately for all six mitochondrial lineages. The summary of mtDNA variation and outcome of neutrality tests are detailed in Table 1. The two lineages with a continental distribution displayed a lack of neutrality (suggestive of a recent expansion); the Eastern lineage "B" in both estimates (Tajima's and Fu's Fs tests) and Danubian lineage "A" in Fu's Fs test only (Table 1). However, all lineages demonstrated unimodal mismatch distribution curves of population growth (Fig. C.4). None of the sums of squared deviations (SSD) of the mismatch distribution was significant, indicating that the curves fitted the sudden expansion model (Table 1). The mismatch distribution was similar among lineages (Fig. C.4), with the lowest

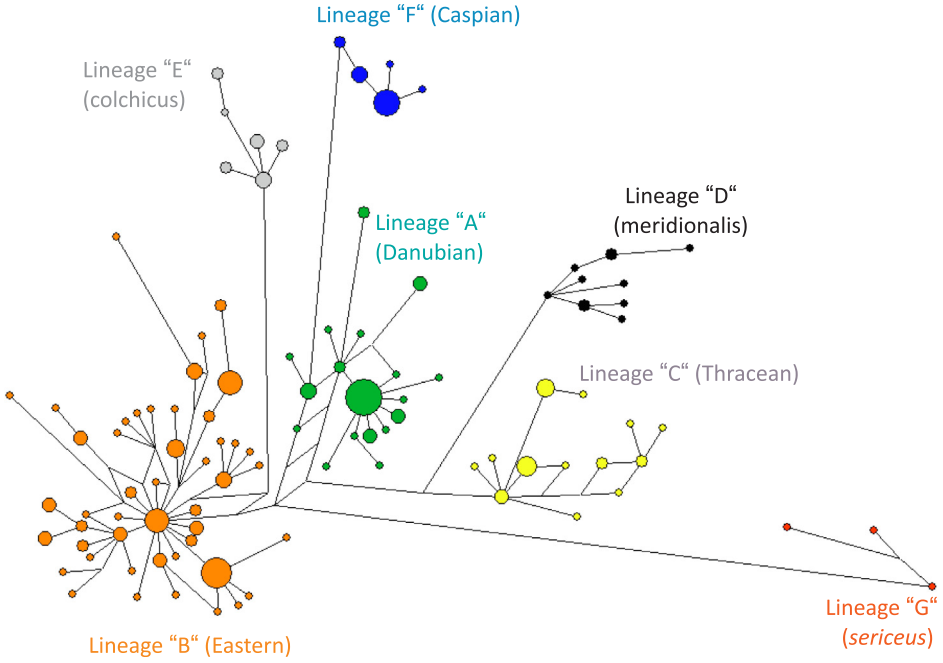

Fig. 2. Haplotype network of 101 haplotypes (914 bp) from 227 *CYTB* sequences (including *R. sericeus* as the outgroup) of the European bitterling complex. Length of branches in the network is proportional to the number of substitutions along a given branch. Circle size is proportional to haplotype frequency. Further information on haplotype data is provided in Appendix A and Fig. C.2.

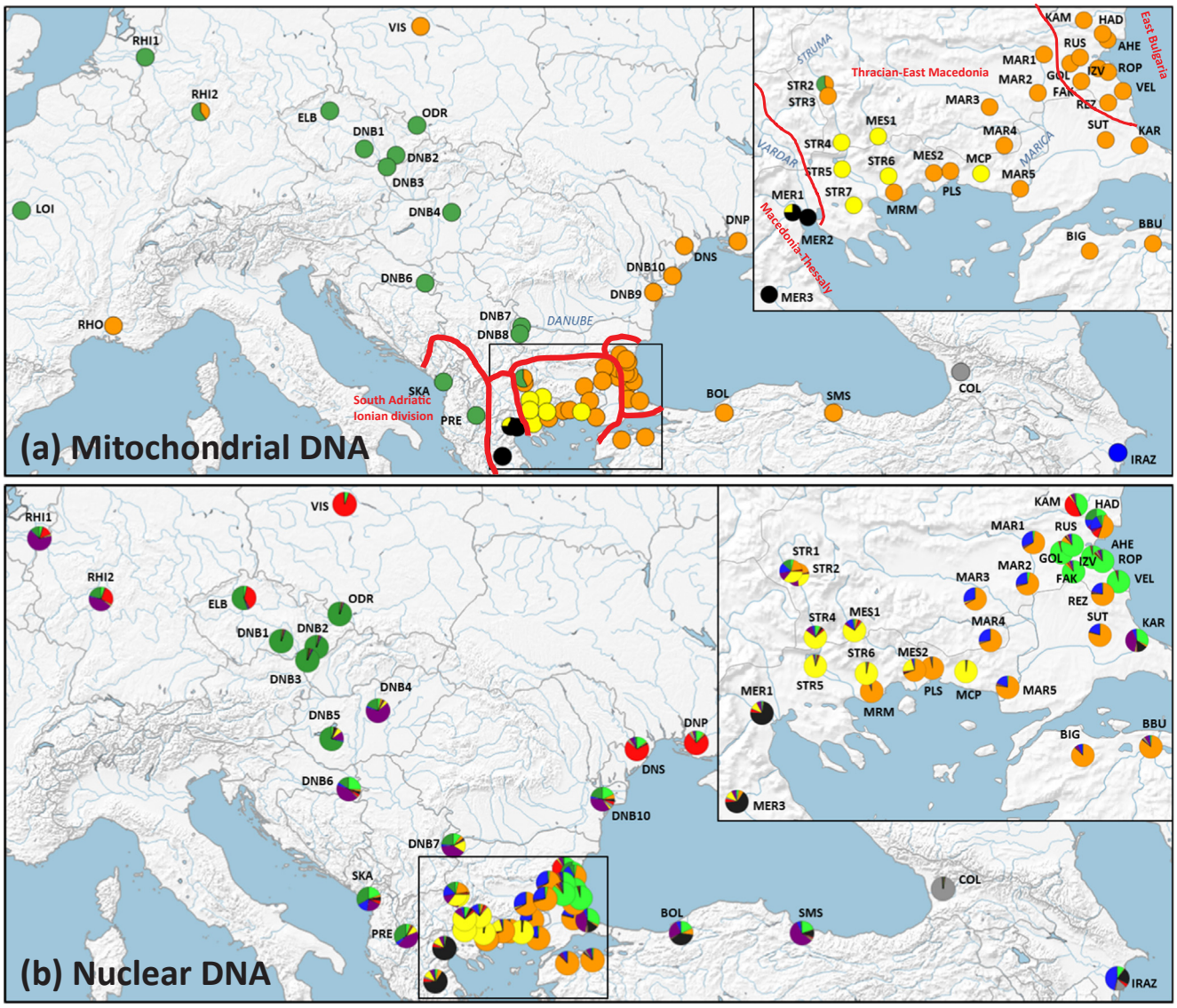

Fig. 3. Geographic distribution of (a) mitochondrial lineage diversity and (b) genetic diversity from nuclear microsatellites based on assignment to 9 clusters following STRUCTURE analysis across study area. (a) The colours correspond to the lineages as defined in Fig. 1 and indicate the relative proportions of lineages at a particular locality. The inset details the distribution in the most diverse part of the Balkan region. Names of localities correspond to those in Appendix A. Borders of freshwater zoogeographical regions (divisions and subdivisions) *sensu* Economidis and Banarescu (1991) are shown by red lines. (b) Pie chart colours represent the proportional membership of individuals to microsatellite-based clusters inferred from the models selected using the approach of Evanno et al. (2005) (for further details and barplots for all models se C, for geographic distribution of the diversity assigned to K = 7 and K = 18 clusters, see Figs. C.6 and C.7, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mean number of differences in the lineages "F" (Caspian) (0.865; age expansion parameter $\tau = 0.984$), "A" (Danubian) and "E" (colchicus) (1.879; $\tau = 1.930$) (Table 1), suggesting relatively recent demographic expansions. Mean number of differences, τ values and estimated timing of the most important demographic expansion for each group indicate relatively older expansions of the lineages "B" (Eastern) and "C" (Thracean) (Table 1). Based on BSP, population growth was detected in the most widespread lineages ("A", "B", "C") but no change in population size was detected in lineages "D", "E" and "F" (Fig. C.5), in agreement with the neutrality test results (Table 1).

3.3. Intrapopulation variability – microsatellites

Expected (H_e) and observed (H_o) heterozygosity, allelic richness (*AR*), and tests of HWE for all 38 population samples are given in Table B.2. Significant departure from HWE was detected in 13 out of 38 populations. After FDR correction, deviations from HWE were mostly limited to a single locus, suggesting locally increased frequency of null alleles. Only the population IRAZ showed deviance from HWE on six loci and population STR2 on four loci. The deficit of heterozygotes in the IRAZ population was likely due to pooling individuals from several localities (Wahlund effect and different allele frequencies in each population), and in the STR2 population it was possibly caused by admixture of two genetic (mitochondrial) lineages and a subpopulation structure. The range of *AR* was 2.23–6 (rarefaction estimate for the lowest sample size N = 7). The populations with the highest genetic diversity were from the lower reaches of large rivers of the Black Sea region – the Danube (DNB8; mitochondrial lineage "A", DNB10; lineage "B") and Dnieper and Dniester (DNP, DNS; lineage "B"), and the population from the River Pinios/Pineios (MER3, lineage "D"). In contrast, the lowest AR was detected in the populations from small streams in the European part of Turkey (REZ, SUT; western Black Sea watershed; lineage "B"), the River Vistula in Poland (VIS, lineage "B") and in Lake Skadar (SKA, lineage "A").

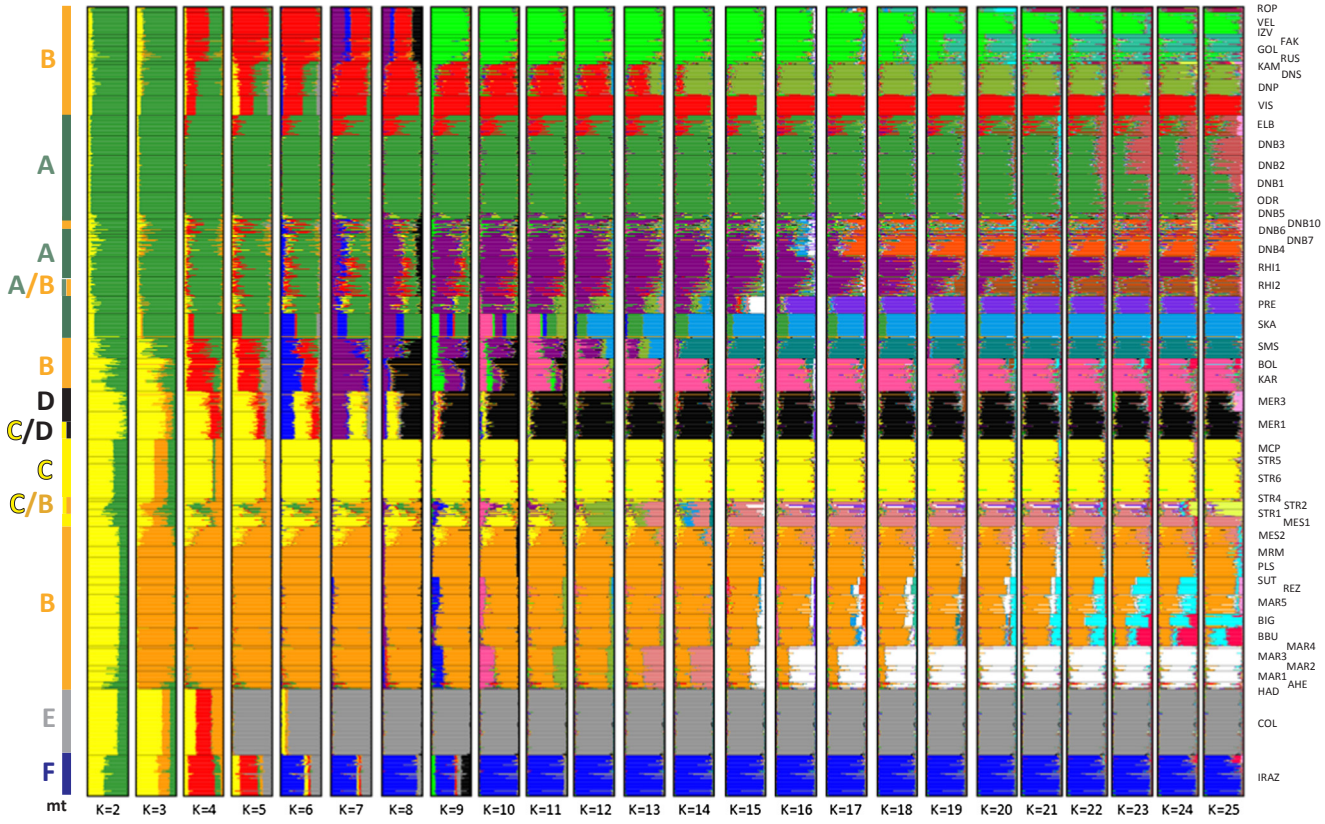

Fig. 4. Bayesian analysis of genetic similarity among *Rhodeus* populations performed in STRUCTURE for 1002 individuals from 52 populations for $K = 2$–25. Assignment to a particular mitochondrial lineage ("A" to "F") is indicated for each population.

3.4. Genetic structure inferred from nuclear markers

Populations were highly structured, indicating a significant role for genetic drift. Non-significant estimates of F_{ST} were only found between two pairs of geographically adjacent populations (DNB2 and DNB3 in the River Morava and MAR1 and MAR2 in the River Maritsa) (Table B.6). The best model in STRUCTURE separated genetic variation into 25 clusters; other acceptable models were for 2, 4, 6, 7, 9 and 18 populations (Fig. C.6). The distribution of mitochondrial lineages corresponded well with the geographic distribution of clusters detected by microsatellite data. The geographic distribution of individual clusters for $K = 9$ rather than $K = 6$ is shown on Fig. 3b, because the populations from the most genetically diverse region were further separated into well-supported clusters in that model. Arrangement of individuals into 2–30 clusters, along with their assignment to particular mitochondrial lineages, is visualised in Fig. 4, with geographic distribution of individual clusters for $K = 7$ and 18 in Appendix C (Figs. C.7 and C.8, respectively).

The populations from the Ponto-Aegean division (the most genetically diverse region) were separated into four main clusters in the model for $K = 9$. The first is composed of populations from a Macedonia-Thessaly subdivision (MER1, MER3), where most individuals had mitochondrial haplotypes from lineage "D". The second cluster is composed of populations from the River Struma/Strymon

Table 1
Analysis of historical demography within six clades based on 914 bp of CYTB.

Lineage	*N*	*S*	*H*	*Hd*	*Pi (%)*	*k*	*Tajimás D*	*Fús Fs*	*τ Arl* (95% CI)	*τ DnaSP*	*SSD*	*P(SSD)*	*Obs. mean*	*t* (95% CI)
"A"	42	21	17	0.858 ± 0.047	0.0026 ± 0.0004	2.334	−1.721	−9.496*	1.156 (0.295–4.936)	1.533	0.0014	0.81	2.334	83,225 (21,238–355,364)
"B"	100	52	46	0.965 ± 0.008	0.0047 ± 0.0003	4.316	−1.898*	−38.564*	4.605 (3.123–5.424)	4.316	0.0016	0.29	4.336	331,533 (224,838–390,497)
"C"	25	12	12	0.9 ± 0.037	0.0036 ± 0.0003	3.327	−0.116	−3.451	4.484 (1.818–6.721)	2.983	0.0279	0.10	3.327	322,822 (130,886–483,873)
"D"	8	7	7	0.964 ± 0.077	0.0023 ± 0.0004	2.071	−1.107	−4.418	2.273 (0.291–3.783)	2.071	0.0192	0.45	2.071	163,643 (20,950–272,354)
"E"	14	6	6	0.868 ± 0.054	0.0021 ± 0.0004	1.879	−0.014	−1.136	1.930 (0.938–3.156)	1.879	0.0102	0.45	1.879	138,949 (67,531–227,214)
"F"	19	4	5	0.637 ± 0.105	0.001 ± 0.0002	0.866	−0.718	−1.555	0.984 (0.424–1.885)	0.865	0.0056	0.52	0.865	70,842 (30,526–135,709)
Total	208						$p < 0.05$ *	$p < 0.01$ *						

N = number of individuals; *S* = number of variable sites; *H* = number of haplotypes; *Hd* = haplotype (gene) diversity ± SD; *Pi* = nucleotide diversity (in %) ± SD; *k* = average number of nucleotide differences; *τ* = onset of population expansion assuming the stepwise growth model ($\tau = 2 * t * \mu$; t = time in years, μ = mutation rate per locus); *t* = time in years computed from τ Arl; *SSD* = sum of squared deviations; *P(SSD)* is the probability of observing a less good fit between the model and the observed distribution by chance; *Obs. mean* = the mismatch observed mean. Fu's Fs significance $p < 0.01$ is marked by *. Tajima's D significance $p < 0.05$ is marked by *.

(STR1-STR2, STR4-STR6), in close geographic proximity on the upper River Mesta/Nestos (MES1) and River Macropotamos (MCP), and corresponding closely to mitochondrial lineage "C". In population STR2, a sympatric occurrence of mitochondrial "A" and "C" lineages was detected (compare Fig. 3a and b).

The next two clusters represent a subdivision of populations belonging to the widespread mitochondrial lineage "B" and closely follow the division between Thracean-East Macedonia and East Bulgaria subdivisions. One cluster (orange-blue in Fig. 3b) is formed by populations from Thrace-East Macedonia (the River Maritsa; MAR1-MAR5), both sides of the Marmara Sea in western Turkey (REZ, SUT, BIG, BBU), River Marmaras (MRM), the lower River Mesta/Nestos (MES2) and Lake Vistonis (PLS). Another cluster (light green in Fig. 3b) consists of populations of the East Bulgaria subdivision – the rivers emptying into the Black Sea (RUS, GOL, IZV, ROP, FAK, VEL, KAM). These two clusters possess a finer substructure at higher K (Figs. C.6 and C.7). Higher values of K ($K = 18$, Fig. C.8) also reveal some other populations to be discrete, including from the lakes Skadar (SKA) and Prespa (PRE) in the South Adriatic-Ionian subdivision, and River Yeşilırmak on the southern coast of the Black Sea (SMS).

In accord with the mitochondrial dataset, populations from Southern Caucasian region (mitochondrial lineages "E" and "F") formed consistent and unique clusters. The structure of bitterling populations from Central Europe has been addressed elsewhere (Bryja et al., 2010; Bartáková et al., 2018) and current data confirmed former conclusions; two lineages ("A" and "B" colonized the European continent via north-eastern and Danubian routes, with secondary contacts in Central Europe.

3.5. Testing reproductive isolation of R. meridionalis

We used ABC to test alternative scenarios for the origin of MER1, in which two distinct mitochondrial lineages ("C" and "D") co-occur. The best model suggested that the MER1 population derived from MER3 (i.e. meridionalis from the Pinios/Pineios drainage) without immigration from the SSTR population, where mitochondrial lineage "C" predominates (Table B.3, Fig. C.1). This indicates that the lineage "C" haplotype present in the MER1 population has a different origin to that in the SSTR population or, alternatively, that the level of introgression in MER1 is negligible. Model checking (Fig. C.9) demonstrated that the observed dataset falls within the cloud of simulated parameter estimates. Estimates of the posterior parameter distribution are shown in Table B.7. The analysis suggests that the MER1 and SSTR populations arose in the same period but from different sources. Admixture was not supported; the best model had a support of 37.6%, while the model with admixture between MER1 and SSTR was supported by only 6.5%.

4. Discussion

4.1. Continental perspective

The genetic structure of bitterling populations across Europe illustrates how postglacial expansions have led to the loss of genetic variation (Bernatchez and Wilson, 1998). The overall phylogenetic pattern was concordant between mitochondrial and nuclear DNA markers. Deeply divergent lineages, signals of expansion and admixture events suggested persistence in multiple small isolated populations during climatically unfavourable periods. This finding supports predictions from the refugia-within-refugia model (Goméz and Lunt, 2007) that has been established in other animal (e.g. Dufresnes et al., 2016; Jablonski et al., 2016) and plant taxa (e.g. Kramp et al., 2009). The range of bitterling in Europe north of the Balkans is dominated by two lineages that expanded from refugia in south-eastern Europe. Lineage "A" colonized Central and West Europe from the lower Danube refugium via the River Danube system (lineage WEST *sensu* Bohlen et al., 2006) and lineage "B" (EAST lineage *sensu* Bohlen et al., 2006) colonized eastern and northern Europe from an area in the northern part of the Black Sea via river systems east of the Carpathians (Bryja et al., 2010). This colonization pattern is congruent with those recorded in many other freshwater fishes (e.g. Durand et al., 1999; Nesbø et al., 1999). Two other European lineages ("C" and "D") are restricted south of the Balkans and have not contributed to the contemporary continental colonization. Two West Asian lineages from regions south of the Caucasus mountain range also remained endemic and have not expanded. Lineage "E" from the Black Sea basin (Georgia) has been formally described as *R. colchicus* (Bogutskaya and Komlev, 2001), while lineage "F" from the Caspian basin (Azerbaijan and northern Iran) has yet to be taxonomically investigated.

Interestingly, we have confirmed the presence of lineage "B" in the River Rhône in the Mediterranean region of southern France, an apparent mismatch of a predictable spatial distribution of the lineages "A" and "B" across continental Europe. Similarly, Bohlen et al. (2006) reported the presence of the same mitochondrial lineage from the River Saone from central France, a tributary of the Rhône. Relatively divergent haplotypes in the River Rhône population (Bryja et al., 2010) suggest that this region contains a relict population from continental colonisations in previous interglacials that survived the last glacial maximum in local refugia, similarly to the pattern hypothesised for the European barbel, *Barbus barbus* (Kotlík and Berrebi, 2001). A region of secondary contact between the lineages "A" and "B" in the River Elbe and the River Rhine basins in Central Europe is a pattern repeatedly found in other freshwater (Durand et al., 1999; Bernatchez, 2001; Kotlík and Berrebi, 2001) and terrestrial taxa (Hewitt, 1999). The results of the present study, analysed at a finer resolution than previous studies, confirmed previous conclusions on the geography and dynamics of postglacial colonization (Bohlen et al., 2006; Zaki et al., 2008; Bryja et al., 2010; Bektas et al., 2013) and add further support to the emerging evidence that Danubian and Black Sea refugia were the almost exclusive sources of contemporary postglacial colonization of continental Europe by freshwater fishes (Durand et al., 1999; Nesbø et al., 1999; Bernatchez, 2001).

4.2. Diversity of southern populations

The current study primarily focused on the genetic diversity in the region of putative bitterling refugia during glacial cycles in the Balkans, around the Black Sea and in adjacent Caspian region. Bitterling are generally thermophilic, with a predominantly subtropical distribution in the Far East (Chang et al., 2014; Kawamura et al., 2014). Only a single species complex is present in Europe, apparently following a single dispersal event to Europe dated to the late Pliocene (< 3 Mya) with subsequent rapid diversification (Bohlen et al., 2006; Chang et al., 2014).

The Ponto-Aegean region of the Balkans was found to support a diverse geographic mosaic of populations, though the three genetic lineages present in that region were relatively well geographically separated. The lineage "D" of the Macedonia-Thessaly region, has been suggested to represent a valid species, *Rhodeus meridionalis* Karaman 1924, by Kottelat and Freyhof (2007). Bryja et al. (2010) contested the validity of *R. meridionalis* by demonstrating the presence (albeit rare) of a widespread haplotype of the lineage "A" in *R. meridionalis* populations. Indeed, tributaries of the Danube and Vardar/Axios rivers were hypothesised to be connected via river captures by Economidis and Banarescu (1991). Our current analysis does not demonstrate any evidence of the connection between the lineages "A" (Danubian) and "D" (meridionalis). Instead, we found that two endemic mitochondrial lineages "D" and "C" co-occur in the lower River Aliakmon/Haliacmon (MER1). Data from nuclear microsatellites analysed by ABC modelling did not detect any admixture between the nuclear genomes of "C" and "D", indicating a case of ancestral polymorphism. We acknowledge that our limited sample size cannot exclude the role of recent translocation.

The lineage "C" (Thracean) represents a second unique Balkan

lineage that did not contribute to the continental expansion of the bitterling. It has a mosaic distribution with lineage "B" in the Thracean-East Macedonia region. There was a connection between the Black and Aegean Seas via the former River Aegeopotamos (Economidis and Banarescu, 1991) that likely supported dispersal of the lineage "B" to the Thracean region. Lineage "C" is prevalent in the western part of the Thracean-East Macedonia region, while lineage "B" populations are more common in the east of the region and replace lineage "C" in the River Maritsa basin and eastwards (Fig. 3a). The pattern is repeated on nuclear genetic markers, with an indication of some admixture of both gene pools in one Mesta/Nestos population (MES2) (Fig. 3b). We hypothesise that the mosaic distribution of the "B" and "C" lineages is contingent on paleogeographic patterns during the lowered level of the Aegean Sea when dispersal via the River Aegeopotamos was possible.

The River Struma/Strymon harbours the highest bitterling genetic diversity. The upper Struma/Strymon in the north contains a southern secondary contact of the two continental lineages ("A" and "B") that are also admixed in Central Europe. While it could be a consequence of past connections between tributaries of the River Danube and Struma/Strymon basins (Banarescu, 1990), a recent introduction by anglers that commonly use the bitterling as a baitfish in that region (Bogoev, 1999; Kozhara et al., 2007) is a more plausible explanation. The dominant "B" lineage is replaced by the "C" lineage in the middle and lower Struma/Strymon, with no record of their sympatric occurrence. The same pattern is reported for *Cobitis* loaches in the River Struma/Strymon basin (Choleva et al., 2008), another frequently used commercial baitfish in Bulgaria (Bogoev, 1999). In other regions, the mitochondrial and nuclear genetic structure of the European bitterling was found to mismatch boundaries of watersheds, with lineage "A" (Danubian) present in the River Oder (Baltic watershed) in the north-eastern part of the Czech Republic and a signal of admixture from the Danube basin in the bitterling populations from the River Elbe basin (North Sea watershed) (Bartáková et al., 2018). These departures from otherwise congruent fine-scale genetic structure in Central Europe were also attributed to human-mediated translocations, related to aquaculture trade or game-fish management (Bartáková et al., 2018).

The bitterling range includes two lake systems of ancient origin (> 5 Mya), Lake Prespa (part of the Lake Ohrid system) and Lake Skadar, which harbour diverse freshwater lineages, including many endemic species (Albrecht and Wilke, 2008; Pešić and Glöer, 2013). Unexpectedly, our data suggest that bitterling populations in the lakes are of recent origin, with depauperate genetic diversity. Both lakes support lineage "A" populations that are closely related to geographically proximate populations from the River Danube basin, suggesting that baitfish introduction to those lakes might also explain their presence and low genetic diversity.

The region around the Black Sea (except the eastern part) is dominated by lineage "B" populations that are finely sub-structured at nuclear markers. In the south-western part of that region (where our sampling was particularly dense), the populations divide according to suggested regional division between Thracean-East Macedonia and East Bulgaria subdivision of Economidis and Banarescu (1991). Thracean-East Macedonian populations form cluster with the populations from the southern coast of the Marmara Sea, suggesting recent gene flow. Elevated freshwater discharge from the Black Sea across the Bosporus to the Marmara and Aegean Seas led to low salinity conditions in the northern Aegean Sea approximately 16–8500 BP (Aksu et al., 1999), permitting recent connection across the Marmara Sea. A similar pattern was observed in other taxa, for example in *Triturus* newts (Taberlet et al., 1998). Populations from many coastal streams of southwest Black Sea region form a separate genetic group. Further genetic divergence is apparent in distant populations from the Danube delta, lower River Dnieper and Dniester, as well as from Asia Minor along the northern coast of the Black Sea (Figs. 4, C.6 and C.7).

The west Transcaucasian (Georgian) region of the Black Sea basin is inhabited by lineage "E" that has been described as a separate species, *Rhodeus colchicus* by Bogutskaya and Komlev (2001) on the basis of osteological characters. Its uniqueness was confirmed at a mitochondrial marker by Bohlen et al. (2006) and we corroborated its distinction using nuclear microsatellite markers. Bitterling populations from the Caspian watershed of the southern Caucasian region (Azerbaijan, Iran) formed a separate, well-characterised lineage ("F"). Distinct Caucasian and Caspian lineages have been reported in amphibians (e.g. Dufresnes et al., 2016), a semi-anadromous fish (*Rutilus frisii*) (Kotlík et al., 2008) and in freshwater fishes of the genus *Alburnoides* (Stierandová et al., 2016). Caspian bitterling populations were not included in previous analyses of the European bitterling complex (Bogutskaya and Komlev, 2001; Bohlen et al., 2006; Zaki et al., 2008; Bryja et al., 2010; Bektas et al., 2013), though a subfamily level phylogeny suggested its distinctiveness (Chang et al., 2014). A finer-scale analysis of the two Asian bitterling lineages in the West Palearctic would require denser sampling in the area.

5. Conclusions

Describing fine-scale genetic variability and structure, we demonstrate the plausibility of the refugia-within-refugia model for European bitterling populations, a freshwater fish species with a limited dispersal capacity. West Palearctic bitterling persisted during periods of less favourable climatic conditions in several isolated populations along the southern margin of their current distribution. During recent interglacials, only two lineages colonized much of continental Europe, with secondary contact in West and Central Europe. A diverse mosaic of populations was detected in the Aegean region, with limited local coexistence. Recent admixture of separate lineages in the south could be a consequence of ancestral polymorphism or recent introductions related to the use of bitterling as baitfish rather than natural secondary contact zones in that region, in contrast to natural secondary contact in Central Europe. Divergence of populations in the Aegean and Caspian regions, and in the region east of the Black Sea, reflect their different origins, genetic distinction and failure to contribute to the recent continental expansion of the West Palearctic bitterling. In the present study, we refrain from raising any taxonomic implications of the findings until morphological samples of divergent lineages are available, but we acknowledge that such a high level of genetic divergence coupled with geographic and genetic isolation requires taxonomic examination of the species complex and demonstrates that the Caspian and Thracean lineages represent unique evolutionary units.

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Author contributions

M.R. and J.B. conceived the idea; R.Š., Y.B., T.S., L.C., C.S. and M.R. collected the material, V.B. produced genetic data, V.B. and J.B. analysed the data, V.B., J.B. and M.R. wrote the first version of the

manuscript that was commented and approved by all authors.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2018.12.025.

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