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Shallow phylogeographic structuring of *Vimba vimba* across Europe suggests two distinct refugia during the last glaciation

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Genetic variation and geographical structuring of vimba *Vimba vimba* were analysed across 26 sites (80 individuals) by means of mtDNA sequences (cyt *b* gene, mitochondrial control region) to localize hypothesized glacial refugia and to reconstruct postglacial recoloniation routes. Although genetic diversity among sequenced individuals was low, a combined analysis of the two sequenced fragments revealed a western (central and northern Europe: Danube, Elbe and lakes of Sweden) and an eastern clade (eastern Europe: Dnieper–South Bug, Don, Neman). Furthermore, a number of divergent ancestral haplotypes distributed around the Black and Caspian Seas became apparent. Mismatch analyses supported a sudden expansion model for the populations of the western clade between 50 and 10 000 BP. Overall, the study provides strong evidence for a northward and westward expansion of *V. vimba* from two refugial regions located in the Danubian drainage and the northern Pontic regions respectively.

Key words: freshwater fish; phylogeography; Pleistocene; recolonization; refugia; *Vimba vimba*.

INTRODUCTION

During the Phanerozoic, speciation as well as extinction was affected by repeated fluctuations of the climate due to recurrent Milankovitch oscillations (Hays *et al*., 1976). These shifts in climate and associated geomorphological and hydrographic conditions repeatedly changed the geographical distribution of species (Jannson & Dynesius, 2002). The changes in landscape through glaciers and melting water were particularly important for freshwater organisms that often cannot disperse between catchments. Species were forced to contract their range in times of adverse climatic conditions. Range contractions were followed by range expansions when climate

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conditions were more favourable and modified river systems provided new opportunities for dispersal. In Europe, the most recent fluctuations of the climate date back to the Pleistocene (1·8–0·01 million BP). It is suggested that in particular the last glacial maximum (approximately 20 000 BP) had the strongest influence on the contemporary distribution of the Central European biota. In the last 20 years, numerous phylogeographic studies analysed the Pleistocene biogeography of European biota (Hewitt, 1999, 2002). In fresh waters, phylogeographic studies on fishes revealed two important findings. While some cold-adapted species survived the last glacial maximum in northern refugia (Hänfling & Brandl, 1998; Hänfling *et al*., 2002; Sediva *et al*., 2008), most European freshwater species recolonized central Europe and northern Europe from southern refugia (*e.g.* Koskinen *et al*., 2002; Taberlet & Cheddadi, 2002; Triantafyllidis *et al*., 2002; Kotlik *et al*., 2004; Mesquita *et al*., 2005; Bohlen *et al*., 2007; Reyjol *et al*., 2007; Kotlik *et al*., 2008; Sediva *et al*., 2008). Broadly summarized, three important regions of southern refugia have been identified: (1) a western (Atlantic), (2) a central (Danube) and (3) an eastern (north-east Pontic–Caspian) refuge (Durand *et al*., 1999; Nesbo *et al*., 1999; Culling *et al*., 2006). Typically such species show high levels of genetic diversity in south-eastern located populations with a gradual decline of genetic diversity towards the western range edge. Low genetic diversity on range edges has been attributed to founder events during range expansion when populations are founded by a low number of individuals from a homogenous and small source population (Rogers & Harpending, 1992).

Previous research on European freshwater fishes mostly found distinct phylogeographic structuring which suggests multiple refugia during a number of glacial cycles (Durand *et al*., 1999; Nesbo *et al*., 1999; Engelbrecht *et al*., 2000; Kotlik & Berrebi, 2001; Hänfling *et al*., 2002; Gum *et al*., 2005; Espanhol *et al*., 2007). For some species, however, only low genetic variation and population structuring was found (Triantafyllidis *et al*., 2002; Bohlen *et al*., 2007). This is usually interpreted as the result of a recent colonization from a common refugium but can also point to strong effects of human-induced translocations or purifying selection. In general, the number of refuges defines the number of recent genetic major lineages and the geographic distribution of these lineages provides hints for the routes of recolonization (Culling *et al*., 2006). The relative contributions of different refugial populations to the recolonization of northern, eastern and central Europe vary considerably among species. For instance, the recolonization of western Scandinavia (Sweden and Norway) proceeded in some species purely across an eastern route from a Ponto–Caspian refugium (Durand *et al*., 1999; Culling *et al*., 2006). In contrast, in other species' descendants of both the north-east Pontic–Caspian and the Danubian refuge contributed to the recolonization of western Scandinavia (Nesbo *et al*., 1999; Kontula & Väinölä, 2001; Gum *et al*., 2005).

The cyprinid vimba *Vimba vimba* (L.) is a migratory fish that occurs in fresh waters as well as brackish waters. The semi-migratory form enters fresh water only for reproduction in spring. After spawning, it migrates to estuaries and brackish waters to feed until the next reproductive season. Landlocked lake populations migrate to lake tributaries for spawning, as do the recently established populations from dam lakes and electric power-plant reservoirs such as Tsymlyansk and Krasnodar reservoirs in Russia. The natural distribution of *V. vimba* ranges from the Rivers Weser (North Sea basin) and Danube (western Black Sea basin) in the west to

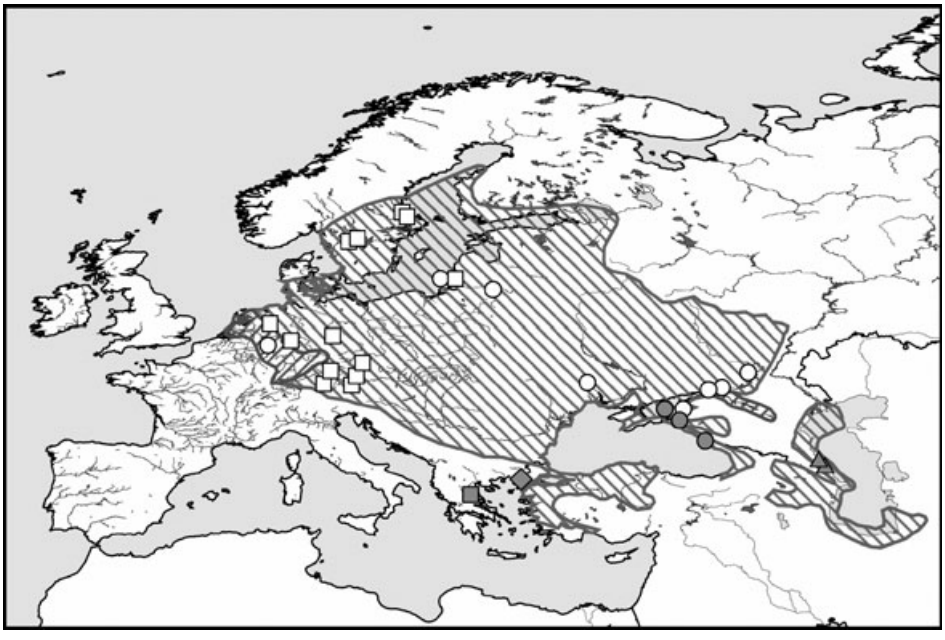

FIG. 1. Sampling locations and distribution of combined haplotype lineages. Symbols follow the demarcation of the identified lineages in Fig. 3. *Vimba vimba*: ○, eastern clade; □, western clade; ⬭, Sea of Azov and Western Caucasus; △, Caspian clade. *Vimba melanops* samples are indicated by grey squares and diamonds. The hatched areas represent the distributional range of *V. vimba* and described subspecies. The introduced range of the species is indicated as left-hatched (western) area. See Appendix for further details.

Volga and Atrek in the east. In the north, it occurs in Scandinavia and the southern Ladoga Lake basin up to about 60° N and makes migrations further northwards in coastal waters of the Baltic Sea, and southwards to the Marmara Sea basin and the southern Caspian basin eastwards to Atrek River (Fig. 1). Recent records west of its natural distribution such as in the Rhine drainage are most likely due to introductions (Freyhof, 1999). Stock translocations and introductions were also carried out in the former U.S.S.R. Within the genus *Vimba*, two valid species are recognized (Kottelat & Freyhof, 2007), *V. vimba* (originally described as *Cyprinus vimba* L. from lakes of Sweden, North Sea and Baltic Sea basins) and dark vimba, *Vimba* (*Abramis) melanops* (Heckel), originally described from the Maritsa (Evros) River, occurring in the northern Aegean basin southwards to the Pinios drainage. Historically a number of controversial local taxa, now synonymized with *V. vimba*, however, have been established (Rábová *et al*., 2003). For example, morphological studies (Uiblein & Winkler, 1994; authors' unpubl. data) failed to support the existence of a distinct lineage [*Vimba elongata* (Valenciennes)] in subalpine lakes (Bănărescu *et al*., 1970; Kottelat, 1997).

In the present study, mtDNA sequences were used (cyt *b*, D-loop) to examine the biogeography of the North Sea and Baltic Sea *V. vimba* populations applying a phylogeographic approach. On the basis of the current distribution pattern of the species, previous research suggested a postglacial recolonization of central, northern

and eastern Europe from the Danubian and the Caspian region (Thienemann, 1950; Bănărescu, 1992). *Vimba vimba* had and still has in some regions, especially in the Sea of Azov basin, a significant economic value. Thus, genetic compositions of populations might have been altered to a certain degree by human-induced translocations (see Freyhof, 1999). Long-distance transport of stocks is, however, less likely. Therefore, relatively unbiased phylogeographic patterns that unravel the species postglacial recolonization were expected. In particular the study addresses two questions: (1) do *V. vimba* populations show a distinct geographic structuring? (2) What are the most likely refugia of *V*. vimba during the last glaciation and how did these refugia contribute to the recolonization of central and northern Europe?

MATERIALS AND METHODS

SAMPLING AND SEQUENCING

A total of 80 individuals were collected from 26 sites across the entire range of *V. vimba* (Fig. 1 and Appendix) using angling, electrofishing and gillnets. Furthermore, six individuals of *V. melanops* were collected from two sites. Fish were killed and a sample of muscle or fin tissue was preserved in 98% ethanol for DNA analysis. DNA was extracted from ethanol preserved tissue using the Gentra Puregene DNA purification kit (www.qiagen.com). A 660 base pair (bp) fragment of the mitochondrial cyt *b* gene was amplified using the primers Glu-F and Cytb-R (Zardoya & Doadrio, 1998). A fragment of approximately 500 bp was amplified from the mitochondrial control region using the primers H355 and L16473 (Imsiridou *et al*., 1998).

Polymerase chain reaction (PCR) was performed using a 20 μl reaction volume containing 1·5 mM $MgCl_2$, 0·2 mM of forward and reverse primer, 0·2 mM of dNTPs and 1 unit of Bioline Taq, in the supplied ammonium buffer. Cycling conditions included an initial denaturation step stage at 94° C for 3 min, followed by 35 cycles consisting of 30 s denaturation at 94° C, 50 s at primer annealing temperature and 1 min extension at 72° C. Primer annealing temperature was 46° C during the first five cycles followed by five cycles at 48° C and 25 cycles at 50° C. A final step of 10 min at 72° C was added to complete extension of all fragments. PCR products were sequenced directly in both directions using the PCR primers. Sequencing was performed on a Beckman Coulter multicapillary sequencer using their DTCS Quick Start sequencing kit (www.beckmancoulter.com). The software CodonCode Aligner (Ewing *et al*., 1998) was used to edit individual sequences and to align forward and reverse sequence of each individual. The resulting consensus sequences of all individuals were aligned using ClustalW (Thompson *et al*., 1997) in combination with MEGA version 3.0 (Kumar *et al*., 2003) and all haplotypes were submitted to GenBank (Accessions GQ279734–GQ279765). Alignments of both datasets were concatenated for combined phylogenetic analyses of both

TABLE I. Results of the R_2 simulations to test the null hypothesis of *Vimba vimba* constant population size. R_2 = observed values, P = probability to reject the null hypothesis of constant population size. In addition, time estimates for clade expansion t (in years) were calculated based on two different mutation rates. See text for further details. n.a. = not available

	Tau	R_2	R_2 simulations (95% CI)		P	$t(\mu = 10{-}8)$	$t(\mu = 5 \times 10{-}8)$
Pontic clade	1·871	0·070	0·071	0·209	0·047	81 000	17 000
West	1·124	0·110	0·115	0·272	0·036	50 000	10 000
East	2·067	0·125	0·092	0·241	0·245	n.a.	n.a.

fragments. Unique haplotypes were identified in the alignments of individual sequences using the programme DnaSP version 4.5 (Rozas *et al*., 2003) and transferred into the separate dataset.

PHYLOGENETIC INFERENCE

Phylogenetic relationships of cyt *b* and D-loop haplotypes were reconstructed independently using both maximum likelihood (ML) methods and neighbour-joining (NJ). Gaps in the alignment of D-loop haplotypes were treated as non-informative using a pair-wise deletion. The programme ModelGenerator v0.82 (Keane *et al*., 2006) was used to choose the most appropriate substitution model for cyt *b* (TrN + G) and D-loop (HKY + G) data, respectively, which was subsequently used for phylogenetic analysis to calculate pair-wise genetic distances among haplotypes. These distances were also used to calculate mean net distances (D_A) between phylogenetic clades. An NJ-tree was constructed from pair-wise genetic distances using the programme MEGA 4.1. Branch support of the NJ-tree was obtained using non-parametric bootstrapping as per cent of 1000 repeats. The ML analysis was carried out using TREEFINDER version of June 2008 (Jobb, 2004). Parameter optimization was carried out for each codon position independently using the data partition option. An approximate bootstrap method (LR-ELW edge support) implemented in TREEFINDER was used as a measure of confidence topologies adjacent to a particular edge. Furthermore, an ML analysis was carried out on the combined dataset as above using fragments as additional partitions. Because both cyt *b* and D-loop are mitochondrial regions they are expected to have undergone identical stochastic processes during the lineage sorting process. A combination of fragments is therefore appropriate and expected to increase the resolution of the phylogenetic reconstructions.

In addition, a median joining network (Bandelt *et al*., 1999) of the combined haplotypes of *V. vimba* was constructed using the software Network 4.510 (Fluxus Engineering 2008). Single deletions/insertions were coded as individual characters, but a 38 bp deletion in haplotype Con4 was coded as a single mutation. Transitions were weighted according the predicted Ts:tv ratio from the ML analysis. Otherwise the default options of the programme were used.

DEMOGRAPHIC HISTORY

Mismatch distribution (Slatkin & Hudson, 1991; Rogers & Harpending, 1992) was used to analyse the demographic history of populations. For this purpose, we defined populations as individuals from allopatric lineages as identified in the phylogenetic analysis. The analysis was carried out for both cyt *b* and combined dataset. Substitution differences between pairs of individuals were calculated and compared with a fit to a unimodal Poisson distribution as expected under sudden expansion from a small population using the programme DnaSP (Rozas *et al*., 2003). The expected distribution was simulated assuming a large final population size ($\theta_1 = 1000$) and values of initial population size (θ_0) and expansion parameter τ which were estimated by the programme. Statistical tests directly based on the distribution of pair-wise sequence differences such as raggedness statistics (Harpending, 1994), however, are not very powerful to reject the null hypothesis of constant population size (Ramos-Onsins & Rozas, 2002). Here, we have used the R_2 statistics (Ramos-Onsins & Rozas, 2002), which is based on the difference between the number of singleton mutations and the average number of nucleotide differences. Ramos-Onsins & Rozas (2002) have shown that in particular for small sample sizes R_2 performed better in detecting population expansion than a number of commonly used neutrality tests. Values of R_2 are expected to be lower under a scenario of sudden expansion compared with constant population size. The hypothesis of population growth was tested comparing the observed value of R_2 with a test distribution generated by coalescent simulations assuming a constant population size using DnaSP. The null hypothesis was rejected when the observed value fell outside the 95% CI of the simulated distribution. The time of population expansion t was calculated using the equation $t = \tau/2(k\mu)$ (Slatkin & Hudson, 1991) with k representing the length of the investigated DNA region and μ the yearly mutation rate per site.

RESULTS

SEQUENCE ANALYSIS

Initially, both cyt *b* and D-loop fragments of 44 individuals from 28 locations were sequenced. This dataset included all sampled individuals from South-eastern and Southern Europe but only a subset of individuals from Northern and Central Europe. A total of 15 cyt *b* haplotypes, 15 D-loop haplotypes and 21 combined haplotypes were found. A preliminary analysis, however, showed that the number of haplotypes and sequence diversity was low among the northern and central European samples (D-loop haplotypes: six positions 1·3% polymorphic, three positions 0·6% parsimony informative; cyt *b* haplotypes: nine positions 1·3% polymorphic, three positions 0·5% parsimony informative). In order to explore whether increasing sample sizes would increase the power of the phylogenetic analysis, the D-loop of an additional 42 individuals from seven northern and central European populations was sequenced, which revealed only two more haplotypes which were closely related to the previously identified haplotypes [Fig. 2(b)]. Because the aim of this study was not to attempt a population genetic analysis, no further individuals were sequenced.

PHYLOGENETIC INFERENCE

Both cyt *b* and D-loop trees showed a broadly similar topology and geographical distribution of haplotypes (Fig. 2). In the D-loop tree, however, basal branches are short and only poorly supported. This may be due to a number of large deletions between those groups. The inclusion of GenBank sequences to the cyt *b* dataset additionally shows three interesting results. The samples from the River Evros cluster together with the published sequences from *V. melanops* from northern Greece (Volvi Lake: AY026403, Durand *et al*., 2002; River Strymon: AF090778; Zardoya & Doadrio, 1999) and haplotype Cy1 is identical to sequence AY026404. Surprisingly one individual sampled in the Curonian Lagoon was highly divergent from all other

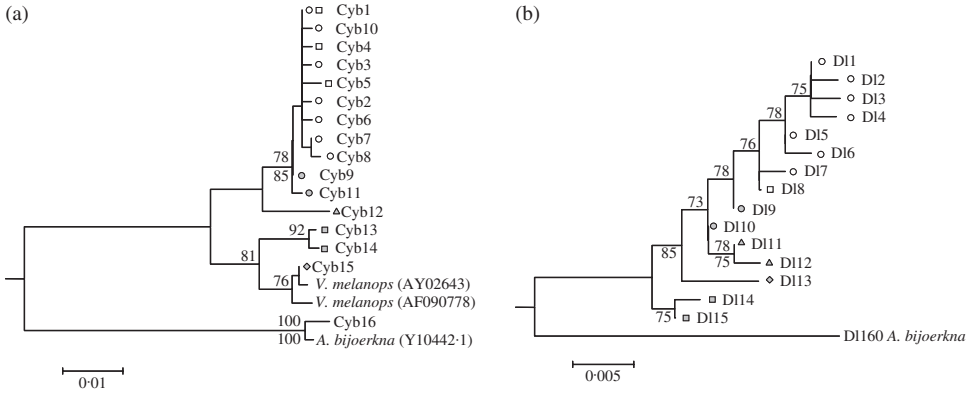

FIG. 2. Phylogenetic relationship of (a) cyt *b* and (b) D-loop haplotypes based on ML analysis. Values above branches indicate LR-ELW edge support from the ML analysis. Values below branches are from non-parametric bootstrapping of the NJ-tree. Values are only given if at least one of the two support values was >70%.

haplotypes and a GenBank-Blast search revealed a close match with a published sequence of silver bream, *Abramis bjoerkna* (L.) (Y10442). This was confirmed by subsequent phylogenetic analysis (Fig. 2). This individual was morphologically not distinguishable from individuals containing *V. vimba* haplotypes (unpubl. data). Mitochondrial introgression is an attractive explanation for this finding.

The combined analysis showed improved phylogenetic resolution compared with the individual gene trees (Fig. 3). A number of distinct clades and clear geographical patterns became apparent that were also supported by the median joining network (Fig. 4). First, the two main clades are formed by haplotypes of *V. vimba* and *V. melanops*, respectively. Interestingly the haplotypes found in individuals from the River Pinios were highly divergent from the *V. melanops* haplotype of the River Evros. The net cyt *b* sequence divergence between those two groups was $0{\cdot}013 \pm 0{\cdot}004$ (mean $\pm$ S.D.). Second, there are two main clades among the *V. vimba* haplotypes. The first clade includes the haplotypes from the Caspian Sea (Caspian clade), whereas the second clade comprises all haplotypes from northern, central and eastern Europe and was also found in the Sea of Azov and will subsequently be referred to as the main Pontic clade. The net cyt *b* sequence divergence between those two groups was $0{\cdot}015 \pm 0{\cdot}005$ (mean $\pm$ S.D.). Furthermore, there are three haplotypes that appear to be ancestral to the Pontic clade and are found in the Sea

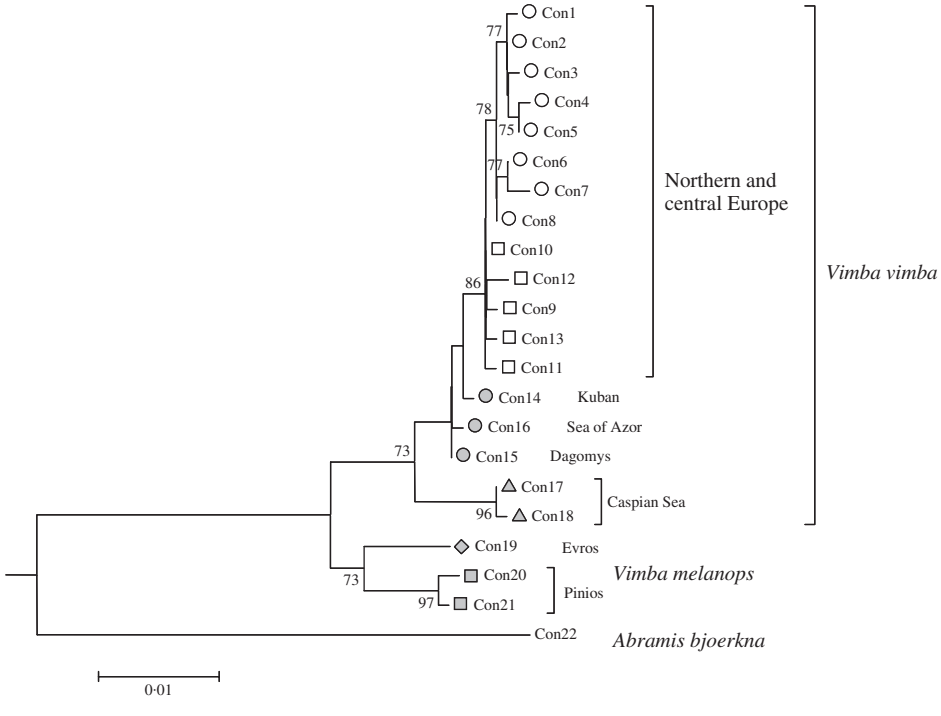

FIG. 3. Phylogenetic relationship of haplotypes from a concatenated dataset of cyt *b* and D-loop-based ML analysis. Values above branches indicate LR-ELW edge support from the ML analysis. Values are only given if the support value was >70%. Symbols indicate haplotype lineages displayed in Fig. 1. *Vimba vimba*: ○, eastern clade; □, western clade; ◍, Sea of Azov and Western Caucasus; △, Caspian clade; ▣, ◇, *Vimba melanops*.

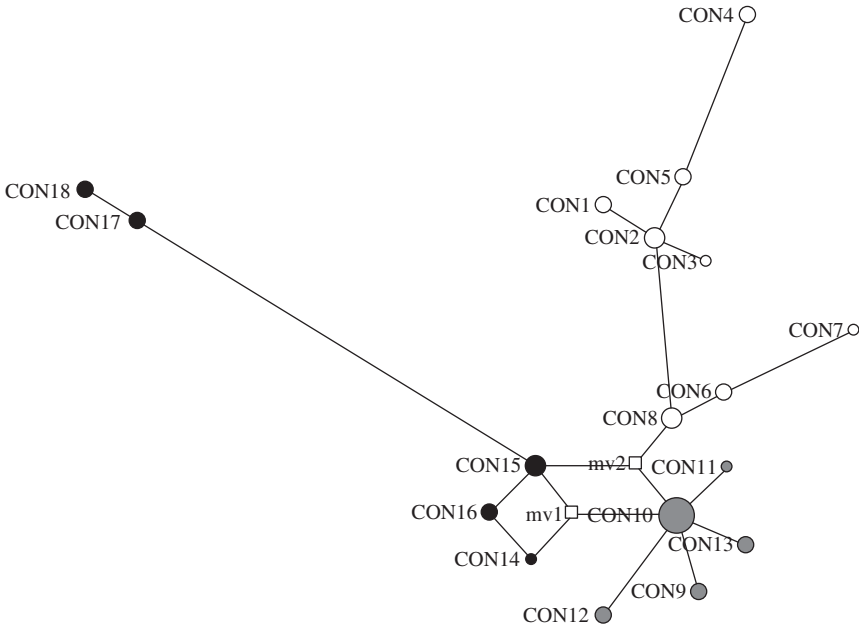

FIG. 4. Median joining network of combined *Vimba vimba* haplotypes. Size of circles is proportional to the frequency of haplotypes. Median vectors are indicated as quadrats. Distance between haplotypes is proportional to the number of mutations. ○, Eastern lineage, ◌, Western lineage; ●, haplotypes found in the Caspian Sea, Sea of Azov and the drainages of the western Caucasus.

of Azov and the drainages of the western Caucasus. The low bootstrap support of the basal branches allows no clear conclusions about the position of the root of the *Vimba* clade. There is also a significant geographic substructure within the main Pontic clade. Haplotypes Con9–Con13 occurred only in the western part of the distribution including all North Sea drainages, the Danube and two alpine lakes and in Scandinavia but also in the Curonian Lagoon (southern Baltic) and will subsequently be referred to as the western lineage (Figs 1 and 3, square symbols). Haplotypes Con1–Con8 form a monophyletic clade which was found mainly in Eastern Europe including the Baltic Sea drainages and the River Don, southern River Bug and the Sea of Azov (eastern lineage). The only exception to the eastern distribution of this lineage was one individual from the River Sieg (Rhine drainage) which constitutes a non-indigenous population.

DEMOGRAPHIC HISTORY

The mismatch distribution of the Pontic clade revealed a unimodal distribution closely matching a Poisson distribution as expected under a sudden expansion model (Fig. 5). Furthermore, the value for the R_2 statistic were significantly lower than expected under a model of constant population growth which is also expected under the expansion model (Table I). Assuming a widely accepted cyt *b* divergence rate of 1% M years (Durand *et al*., 2002), the expansion time would be around 81 000 BP during the last interglacial period. The control region that constituted almost 50%

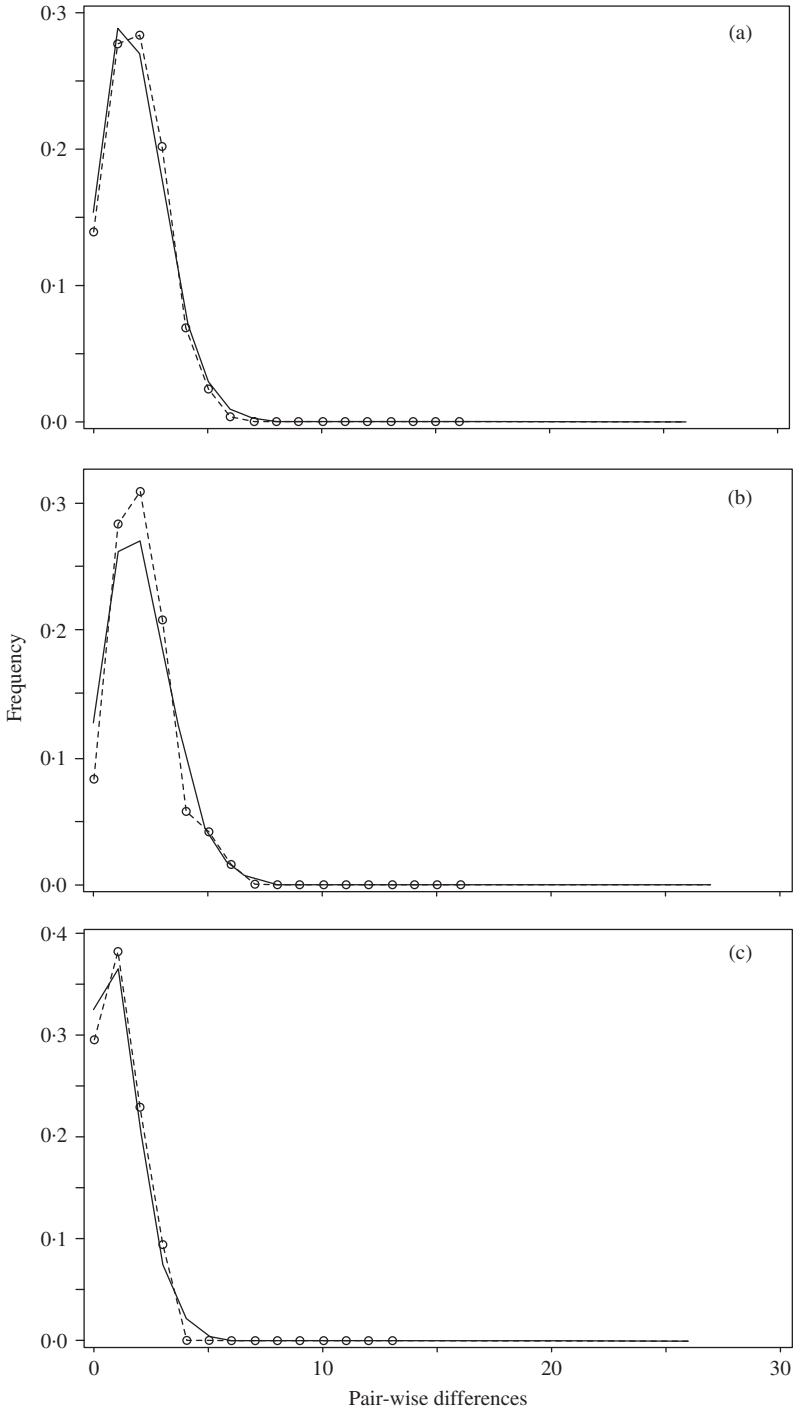

FIG. 5. Mismatch distribution for the combined dataset of (a) Pontic clade (b) eastern lineage (c) and western lineage of *Vimba vimba*. Dotted lines represent the observed distribution, whereas solid lines represent the distribution expected under a sudden expansion model as estimated using DnaSP.

of the combined fragment usually, however, evolves considerably faster in fishes (*e.g.* 9% in the bullhead *Cottus gobio* L.; Volckaert *et al.*, 2002). On the basis of a considerably faster rate of 5% M years for the combined fragment, the expansion time would have been around 17 k years, close to the last glacial maximum. The significant geographic substructure (eastern and western lineage) within the main Pontic clade which is seen in the combined data suggests that the two lineages had independent demographic histories. Therefore, the analysis was carried out for both lineages independently. A close match to the expected mismatch distribution under the expansion model was found. The R_2 statistics, however, indicated that only in the western clade the model of constant population size could be rejected with confidence, whereas such a possibility cannot be excluded for the eastern clade. The calculated expansion time for the western lineage was between 50 and 10 k years applying the two different mutation rates.

DISCUSSION

PHYLOGEOGRAPHIC PATTERNS

Two important phylogeographic patterns emerged from this study. First, the sequences obtained from the drainages of the Caspian basin are phylogenetically distinct from those of the remaining *V. vimba* individuals (Pontic clade). Second, the existence of two independent lineages of *V. vimba* within the main Pontic clade which diverged during the last interglacial period. We propose a recolonization scenario from two classical refugia: the Danubian basin and the eastern Pontic region. These findings match suggestions derived from recent distribution pattern of the species (*e.g.* Thienemann, 1950; Bănărescu, 1992). The two refugia, however, contributed differently to the recolonization of Europe. While eastern Europe (eastern lineage) was colonized from the north-eastern Pontic region (*e.g.* Rivers Dnjepr and Don), central Europe and the western parts of Scandinavia (western lineage) were colonized from the Danubian basin (Fig. 1). The Caspian region and the rivers of the western Caucasus did not contribute to the postglacial recolonization of Europe. Note that these phylogeographical patterns are very similar to those reported for bitterlings, genus *Rhodeus* (Bohlen *et al.*, 2006).

Applying a standard molecular clock for cyt *b* in cyprinid fishes of approximately 1% per M years (Durand *et al.*, 2002) the divergence between Caspian and Pontic clade would date back 1–2 M years to the beginning of the Pleistocene. Such a divergence is considerably younger than the initial separation of the Black and Caspian Sea basins that took place about 5 M million BP (Popov *et al.*, 2004). A number of more recent transgressions (*e.g.* Akchagylian–Kuyanician and Apsheronian) that lead to re-establishment of the connection between the two basins through the Kumo–Manych depression coincide much better with the observed divergences of Pontic and Caspian clades (Reid & Orlova, 2002; Popov *et al.*, 2004). Such a pattern of Pleistocene-aged phylogenetic divergences among the Black and Caspian Sea basins is also found in other fishes (black sea roach, *Rutilus frisii* Nordmann; Kotlik *et al.*, 2008) and some aquatic invertebrates (Cristescu *et al.*, 2003, 2004; Stepien *et al.*, 2003; Audzijonyte *et al.*, 2006, 2008).

Recolonization routes of European freshwater fishes have been interpreted considering the hydrographical and geographical settings during the retreat of the glaciers (Culling *et al*., 2006). The large amount of melt water provided connections between drainages across large ice-dammed lakes (Marks, 2002; Mangerud *et al*., 2004). Such ice lakes may have facilitated the expansion of the eastern lineage from its Pontic refugium towards the Baltic basin. The distribution of western lineage haplotypes in the River Elbe, however, indicates that the Elbe drainage must have been separated from the proglacial spillway before the arrival of the expansion front of the eastern lineage. The data of this study clearly support the suggestion that they were two distinct hydrographic dispersal routes. This supports earlier paleogeological data on development of the periglacial hydrographic network (Kvasov, 1975). Kvasov's schemes show that the Dnieper catchment included the present valleys of the Neman and Daugava (Zapadnaya Dvina) and was sporadically connected to the South Baltic periglacial lake which overlapped the present drainage areas of the western Baltic including the Odra River. This system of the periglacial lakes, however, was separated from other catchments located in the west of the Oder even during the maximum glaciation. For colonization of central and northern Europe, a postglacial northward expansion from the River Danube across the River Elbe as it was shown for the weather loach *Misgurnus fossilis* (L.) (Bohlen *et al*., 2007) is suggested. The occurrence of closely related haplotypes in the Danube and the Elbe as well as their tributaries (Saale) together with the mismatch analyses supports this scenario. An alternative possible dispersal across the drainages of the Black Sea is less likely. Haplotypes of the Danubian lineage were not found in the other drainages of the Black Sea. Considering that at the end of the last glaciation melt water from the retreating Scandinavian ice sheet filled the Baltic basin with fresh water forming a large freshwater sea (the Ancylus sea; Thienemann, 1950), the migration of freshwater fishes from central to northern Europe was facilitated. Therefore, a colonization of the Swedish populations across this freshwater sea is likely. The sympatric occurrence of western and eastern lineage haplotypes in the Curonian Lagoon and the high genetic diversity of this population (Fig. 1) most likely represent postglacial secondary contact. *Vimba vimba* can tolerate salinity of up to 8% and even current salinity levels in the western Baltic would not be an obstacle for gene flow across the Baltic Sea. An admixture of different phylogeographic clades across the Baltic Sea has also been reported for the perch *Perca fluviatilis* (Refseth *et al*., 1998; Nesbo *et al*., 1999).

For most of the European freshwater fishes, the Danubian basin acted as a refugiaum and provided pathways for recolonization (Durand *et al*., 1999; Nesbo *et al*., 1999; Bernatchez, 2001; Kotlik & Berrebi, 2001; Weiss *et al*., 2002; but see Culling *et al*., 2006) and *V. vimba* is no exception. Genetic diversity, however, in the western lineage is lower than diversity of the eastern lineage (Fig. 3). This supports the findings of the mismatch analyses which points to an earlier expansion of the eastern lineage. Thus, the populations from the northern Black Sea basin are presumably older. Older populations are in general characterized by higher levels of population differentiation, whereas in recently founded populations a homogeneous gene pool dominates (Hutchison & Templeton, 1999).

TAXONOMIC IMPLICATIONS

The present study also has some important taxonomic implications. First, the species status of *V. elongata* is not supported by our data. Two samples from subalpine lakes, Lake Starnberg and Lake Mondsee, showed no or only little divergence to other individuals sampled in central and northern Europe. This supports the morphological analyses of Uiblein & Winkler (1994) that *V. elongata* does not represent a valid distinct species. Reported morphological differences of subalpine lake populations are probably caused by a number of ecological and abiotic factors such as differences in resource utilization or differences in water movement. Preliminary morphological analyses of the individuals from this study, however, showed no distinct differences among populations from lentic and lotic water bodies (unpubl. data). Second, the data of this study do not contradict the suggestion to rank the Caspian populations as a separate species *Vimba persa* (caspian vimba Bănărescu, 1992) or a subspecies *Vimba vimba persa* (Pallas) (*e.g.* Berg, 1949; Bănărescu *et al*., 1970). Given the unresolved relation between the basal haplotypes from the western Caucasus and the Pontic and Caspian clade, however, further research and more comprehensive sampling of the Caspian and western Caucasian populations is needed to provide full support for such conclusions. Third, surprisingly high genetic diversity was found within *V. melanops*. Moreover, a distinct and old subdivision between northern (River Evros, River Strymos and Lake Volvi) and southern Greece (River Pinios) became apparent. The most likely explanation for this strong structuring is that the southern Balkan was relatively unaffected by geological events during the Pleistocene. Hence, high levels of genetic diversity are conserved in this region (Durand *et al*., 2002). This study therefore adds to the increasing body of literature showing that topographical complexity of the Balkans promoted allopatry and isolation on a small geographical scale during interglacial periods (Krystufek *et al*., 2007).

Overall, the results of this study strongly support a northwards and westwards expansion of *V. vimba* from two refugial regions located in the Danubian drainage and the northern Pontic regions. It is important to note that only the use of the combined sequence dataset revealed this finding. Hence, the authors strongly emphasize the use of multiple sequence information to improve phylogenetic resolution in phylogeographic studies. Furthermore, the study found large genetic diversity in the eastern lineage. This finding underlines the importance of the Pontic regions as hot spots of genetic diversity for the European freshwater fish fauna. The high genetic divergence between the *V. melanops* populations highlights the importance of the southern Balkans as a reservoir for intraspecific genetic diversity.

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APPENDIX. Sampling localities, haplotypes of cyt *b* (Cyb), D-loop (Dl) and the concatenated sequences (Con) for the sequences obtained for *Vimba vimba*. Numbers within parenthesis indicate frequency of haplotypes. *, subalpine lake populations; #, *Vimba melanops* populations.

River sites	Catchment	Country	Latitude (° N)	Longitude (° E)	Haplotypes
Saale I	Elbe, North Sea basin	Germany	51·475	11·967	Cyb6 (2×), Dl8 (4×), Con9 (2×)
Saale II	Elbe, North Sea basin	Germany	51·475	11·967	Cyb1, Dl8, Con10
Sieg	Rhine, North Sea basin	Germany	50·801	7·183	Cyb1, Dl5, Con8
Eder	Weser, North Sea basin	Germany	51·155	8·902	Cyb2, Dl8, Con13
Ems	Ems, North Sea basin	Germany	52·408	7·334	Cyb1, Dl8, Con10
Vltava	Elbe, North Sea basin	Czech Republic	49·5	14·187	Cyb1, Dl8 (3×), Con10
Nemunas	Nemunas, Baltic Sea basin	Lithuania	54·892	23·903	Cyb1 (3×), Dl2 (2×), Dl5, Con1, Con8
Curonian Lagoon	Curonian Lagoon, Baltic Sea basin	Lithuania	55·705	20	Cyb1, Cyb10, Cyb16, Dl1 (7×), Dl2 (2×), Dl4, Dl7 (3×), Dl8 (2×), Dl16, Con3, Con10, Con22
Testeboån	Baltic Sea basin	Sweden	60·68	17·127	Cyb1, Dl8, Con10
Olandsan	Baltic Sea basin	Sweden	60·34	17·522	Cyb4, Dl8, Con11
Tidan	Lake Vänern	Sweden	58·709	13·833	Cyb1, Dl8, Con10
Lidan	Lake Vänern	Sweden	58·502	13·165	Cyb1, Dl8, Con10
Danube I	Danube, Black Sea basin	Germany	48·89	11·815	Cyb5 (2×), Dl8 (2), Con12 (2×)
Danube II	Danube, Black Sea basin	Germany	48·505	13·732	Cyb1, Dl8, Con10
Mondsee*	Danube, Black Sea basin	Germany	47·828	13·384	Cyb2, Dl8, Con13
Starnberger See*	Danube, Black Sea basin	Germany	47·911	11·311	Cyb1, Dl8, Con10
Dagomys	Dagomys, northeastern Black Sea basin	Russia	43·667	39·652	Cyb9 (2×), Dl10 (2×), Con15 (2×)
Southern Bug	Southern Bug, northwestern Black Sea basin	Ukraine	48·034	30·874	Cyb1 (2×), Dl1, Dl5 Con8, Con2
Kuban	Kuban, Sea of Azov basin	Russia	45·199	37·715	Cyb9, Cyb11, Dl9, Dl10, Con14, Con15

APPENDIX. Continued

River sites	Catchment	Country	Latitude (° N)	Longitude (° E)	Haplotypes
Tuzlov	Don, Sea of Azov basin	Russia	49·983	42·018	Cyb3 (2×), Dl1, Dl3, Con2, Con5
Seversky Donetz	Don, Sea of Azov basin	Russia	47·627	40·888	Cyb1, Cyb3, Dl1 (2×) Con2, Con5
Tsymlyansk Reservoir I	Don, Sea of Azov basin	Russia	47·7656	42·8219	Cyb3, Dl1 (6×), Dl3 (4×), Con4
Tsymlyansk Reservoir II	Don, Sea of Azov basin	Russia	47·7656	42·8219	Cyb1, Dl1 (7×), Dl3 (3×), Con2
Sea of Azov	Sea of Azov basin	Ukraine	46·064	36·615	Cyb7 (2×), Cyb8, Cyb11 (2×), Dl5 (2×), Dl6, Dl10 (2×), Con6, Con7 (2×), Con16 (2×)
Bashly-chai	Bashly-chai, Caspian Sea basin	Russia	42·3436	48·0919	Cyb12 (2×), Dl11 (2×), Dl12 (2×), Con18 (2×)
Samur	Samur, Caspian Sea basin	Russia	41·8741	48·5597	Cyb12 (2×), Dl11 (2×), Con17 (2×)
Pinios#	Pinios, Aegean Sea basin	Greece	39·666	22·234	Cyb13, Cyb14 (3×), Dl14, Dl15 (3×), Con20, Con21 (3×)
Evros#	Evros, Aegean Sea basin	Greece	40·845	26·023	Cyb15 (2×), Dl13 (2×), Con 19 (2×)

