

# Pliocene-Pleistocene dispersal bring along low inter species diversity between *Vimba* species based on multilocus analysis

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## Abstract

This study investigates phylogenetic and phylogeographic relationships of *Vimba* species using mitochondrial cytochrome b (cyt b) (1023 bp) and cytochrome c oxidase subunit I (COI) barcoding region (652 bp) genes. Ninety-one samples from 36 populations for the cyt b gene and 67 samples from 20 populations for the COI were analyzed. We identified 29 haplotypes and calculated overall haplotype diversity as  $Hd: 0.907 \pm 0.015$  for cyt b. We also identified 13 COI haplotypes and calculated overall haplotype diversity as  $0.826 \pm 0.026$  for this marker. The phylogenetic analysis of *Vimba* species reveals the presence of four clades, based on concatenated cyt b and COI sequences. The first and second clade consist of *Vimba vimba* Western lineage, and *Vimba vimba* Caspian lineage, while the third and fourth clade consist of *Vimba mirabilis* and *Vimba melanops*. Based on haplotype network analyses and phylogeographic inferences, the *Vimba* genus is monophyletic, and its species dispersed in the Pleistocene era.

## Key Words

cyt b, genetic diversity, Phylogeography, *Vimba* bream, *Vimba mirabilis*

## Introduction

As a member of the Leuciscidae family, the genus *Vimba* is distributed throughout almost all Eurasia and consists of three species: *Vimba vimba*, *Vimba melanops*, and *Vimba mirabilis*. *V. vimba* was initially described as *Cyprinus vimba* L. from several Swedish lakes in Scandinavia, the North Sea, coastal waters of Baltic Sea basins, and, subsequently, after the description, it was also found in the Caspian, Black Sea, Marmara Sea basins, and the Rhine River. In Anatolia, *V. vimba* is distributed from the Marmara basin up to Büyük Menderes, Eğirdir Lake, Köprüçay and Eşen rivers in the south, and Kızılırmak in the east. *V. melanops* was described initially from the Meriç (Evros) River and its distribution now extends within the borders of Turkey, Greece, Bulgaria, and Macedonia in the North Aegean basin from Meriç to the Pinios River. The Anatolian endemic *V. mirabilis* was detected only in the type locality Büyük Menderes and two individuals in Bafa Lake in Southwest Anatolia (Bogutskaya 1997). According to Crivelli (1996),

*V. mirabilis* is under threat of extinction due to the water intake from the basin for drinking and irrigation.

Since then, a few taxonomic developments of the genus *Vimba* have occurred, like the subalpine *Vimba* lineage previously identified as *Vimba elongata*, but now considered a synonym of *Vimba vimba*. *Acanthobrama mirabilis* was synonymized with *Vimba vimba tenella* by Durand et al. (2002), as *A. mirabilis* belongs to the *Vimba* clade. But, Perea et al. (2010) found *Acanthobrama mirabilis* is the synonym of *V. mirabilis* based on genetic evidence. Also, in some literature, *V. vimba*, distributed in the Caspian Sea and Caspian bream, has been identified as *Vimba persa* (Hänfling et al. 2009; Naseka and Bogutskaya 2009; Chachi et al. 2011a, 2011b; Mohamadian et al. 2012).

Globally, *Vimba* genus has been well studied in terms of growth parameters and its parasites, but genetic studies are more limited (Zardoya and Doadrio (1999), Durand et al. (2002), Hänfling et al. (2009), Perea et al. (2010), Triantafyllidis et al. (2011), Geiger et al. (2014), Schönhuth et al. (2018). In particular, Hänfling et al. (2009) discovered

the phylogeographic origin and colonization factor of *V. vimba*, but other studies have quite limited samples.

Phylogeographic and phylogenetic studies have been made on some European freshwater fishes such as *Alburnoides*, *Barbus*, *Capoeta*, *Gobio*, *Squalius*, *Telestes* and typically uncovered distinct patterns according to mitochondrial cytochrome b (cyt b) and cytochrome c oxidase I (COI) genes. In order to study systematics and phylogeny of Leuciscidae and its congeners, cyt b and COI sequence analysis have been demonstrated to be useful DNA markers. (Zardoya and Doadrio 1999; Durand et al. 2000, 2002; Tsigenopoulos et al. 2002; Levin et al. 2012; Bektas et al. 2017; Turan et al. 2018; Aksu and Bektaş 2019).

In European rivers, freshwater fishes have largely colonized the Black Sea upstream, up rivers such as the Dnieper and the Danube. Freshwater fishes contain numerous lineages with genetic divergences representing separation over the past 2 Myr in this region. Several of these have clear geographic distributions and provide evidence of older Black Sea-Caspian Sea divergence. Interglacial and postglacial expansions also indicate colonization of Western Europe from numerous major refugia, particularly the Black Sea, Dnieper-Volga, Danube, Rhine– Rhone, Elbe, and other rivers. The influence of older water bodies such as the Ponto-Caspian Sea and recent great periglacial lakes and floods is also apparent (Hewitt 2004).

While it frequently highlighted that the Pleistocene increased speciation rates, molecular data have recently revealed that species diverged in the Pleistocene and Pliocene in Europe (Hewitt 2000).

In the current study, mtDNA sequences were used (cyt b, COI barcoding) to examine the biogeography of *Vimba* populations, applying a phylogeographic approach. The objective is to reveal the phylogenetic relationships and genetic diversity of *Vimba* species whose populations are currently decreasing.

## Materials and methods

### Sample collection, DNA extraction, and sequencing

All currently recognized taxa of *Vimba* were included in our dataset. We sequenced 68 *Vimba* specimens collected from their distribution ranges in Turkey and further included sequences of 23 specimens from NCBI GenBank. *Vimba* species of Turkey were collected from 14 sampling sites of drainages of the Black and Aegean and Marmara seas, comprising type localities or type basins (Fig. 1, Table 1). The animals were experimented with as per the Republic of Turkey animal welfare laws, guidelines, and policies, and was approved by the Republic of Turkey Recep Tayyip Erdogan University Local Ethics Committee for Animal Experiments (Decision No: 2014/72). For faunal surveys, fishes were collected, surgical procedures were performed only for excision of fin clips after anesthesia with MS222,

and fin clips were further preserved in 70% ethanol for genetic studies. In our experiments, none of the fishes were distressed by the experimental conditions. Specimens and tissue vouchers from Turkey were kept in DNA Collection and Zoology Museum, Faculty of Fisheries, Recep Tayyip Erdoğan University, Rize. Total DNA was extracted from fin clips via Qiacube automated DNA/RNA purification system by using Qiagen DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany). Both the quality and quantity of DNA were checked on a NanoDrop 2000/c spectrophotometer (Thermo Scientific, Rockford, IL, USA) and 0.8% agarose gel electrophoresis. Cyt b (1023 bp) gene was amplified by the newly designed primer set B-cytbF (5'-GAAGAACCACCGTTGTWVTTCAAC-3') and the B-cytbR (5'-CGGATTACAAGACCGATGC-3'), and COI barcoding gene (652 bp) was amplified by the FishF1 (5'-TCAACCAACCACAAAGACATTGGCAC-3') and FishR1 (5'-TAGACTTCTGGGTGGC-CAAAGAATCA-3') (Ward et al. 2005). PCR reactions were performed in a 50 µL reaction volume containing 5 µL 10x PCR buffer, 100 ng template DNA, 0.5 mM dNTPs mix, 3 mM MgCl<sub>2</sub>, 0.5 mM of each primer, and 1 µL Taq DNA polymerase (New England Biolabs). The polymerization was carried out under the following conditions: initial denaturation at 95 °C for 30 s, denaturation at 95 °C for 30 s, annealing at 55 °C for 50 s for cyt b and 58 °C for 45 s for COI, extension at 68 °C for 1 min through 35 cycles, and a final extension at 68 °C for 5 min using Biorad T100 (Bio-Rad, Hercules, CA, USA) thermal cycler. The PCR products were run and visualized under UV Quantum-Capt ST4 system (Vilber Lourmat, France) also, purified and sequenced at MacroGen Europa Inc. (Amsterdam, Netherlands) (Turan et al. 2018).

### Genetic structure and phylogenetic analysis

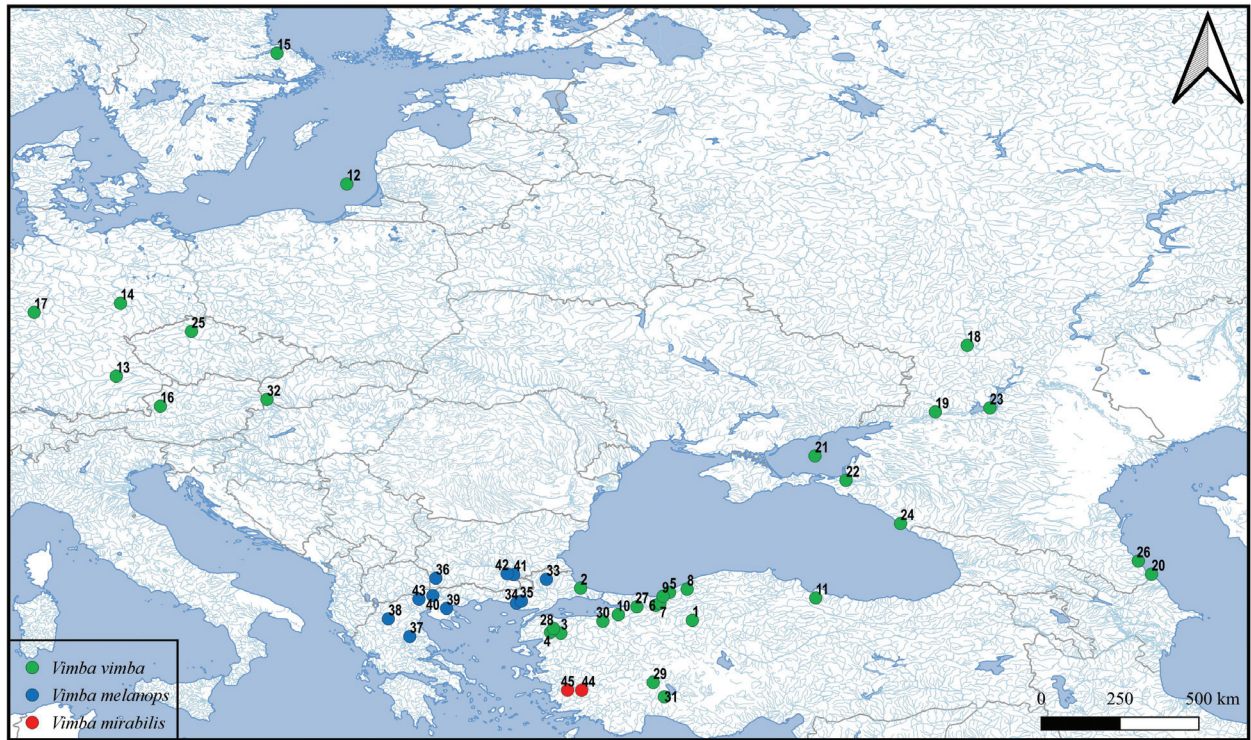
Clustal W (Thompson et al. 1994) algorithm was implemented in Bioedit v7.2.5 (Hall 1999) software to align cyt b and COI barcoding regions. Haplotype number (H), polymorphic and variable sites, haplotype diversity (Hd), and nucleotide diversity ( $\pi$ ) were computed for each species by DnaSP version 6.12.03 (Rozas et al. 2017) program. Nucleotide frequencies and transition/transversion rate were calculated by MEGA X (Kumar et al. 2018). Molecular Variance (AMOVA) was analyzed using the Arlequin v3.5.1.2 (Excoffier and Lischer 2010) software to calculate genetic variation among and within the groups. The haplotypes were submitted to the NCBI GenBank with accession numbers **OK493404–OK493416** for cyt b **OK444819–OK444823** for COI barcoding region. Cyt b and COI sequences were concatenated (1675 bp) for further phylogenetic inferences. The TrN+G model:ln = 2905.1890 was the best-fit nucleotide substitution model for the concatenated dataset according

**Table 1.** List of sequences analyzed in this study with information on drainage, GenBank no, haplotype no, and country of origin.

Locality no	Species	Locality	Coordinate	cyt b			COI			Reference
				N	Genbank no	Haplotype no	N	Genbank no	Haplotype no	
1	Vimba vimba	Kırmır stream, Ankara, Turkey	40°14'10.5"N, 32°15'41.2"E	7	OK493404	H1	3	OK444821	H7	This study
2	Vimba vimba	Binkılıç stream, Black sea, Istanbul, Turkey	41°22'48.3"N, 28°17'46.0"E	9	OK493404, OK493407, OK493408	H1,H4,H5	8	OK444820	H6	This study
3	Vimba vimba	Koca stream, Balıkesir, Turkey	39°46'55.2"N, 27°35'46.2"E	4	OK493404, OK493406, OK493409	H1,H3,H6	4	OK444820, OK444821	H6, H7	This study
4	Vimba vimba	Koca çayı stream, Çanakkale, Turkey	39°48'52.9"N, 27°13'46.1"E	3	OK493405, OK493406	H2,H3	3	OK444820, OK444821	H6, H7	This study
5	Vimba vimba	Aydınlı stream, Zonguldak, Turkey	41°13'47.1"N, 31°27'11.2"E	2	OK493410	H7				This study
6	Vimba vimba	Aksu stream, Düzce, Turkey	40°45'49.0"N, 30°57'43.0"E	3	OK493411	H8				This study
7	Vimba vimba	Büyük Melen stream, Düzce, Turkey	40°50'08.0"N, 31°06'35.0"E	4	OK493411	H8				This study
8	Vimba vimba	Yenice stream, Zonguldak, Turkey	41°20'27.6"N, 32°04'40.8"E	5	OK493410	H7				This study
9	Vimba vimba	Çayagzı stream, Düzce, Turkey	41°05'27.2"N, 31°13'18.5"E	3	OK493410, OK493411	H7,H8				This study
10	Vimba vimba	Iznik lake, Kocaeli, Turkey	40°26'18.1"N, 29°38'03.5"E	10	OK493406	H3				This study
11	Vimba vimba	Suat Ugurlu Dam lake, Samsun, Turkey	41°01'52.4"N, 36°38'33.4"E	2	OK493412, OK493413	H9,H10				This study
12	Vimba vimba	Curonian Lagoon, Baltic Sea basin, Lithuania	55°42'18.0"N, 20°00'00.0"E	1	GQ279763	H11				Hänfling et al. 2009
13	Vimba vimba	Danube I, Black Sea basin Germany	48°53'24.0"N, 11°48'54.0"E	1	GQ279762	H12				Hänfling et al. 2009
14	Vimba vimba	Elbe, North Sea basin, Germany	51°28'30.0"N, 11°58'01.2"E	1	GQ279761	H13				Hänfling et al. 2009
15	Vimba vimba	Olandsan, Baltic Sea basin, Sweden	60°20'24.0"N, 17°31'19.2"E	1	GQ279756	H14				Hänfling et al. 2009
16	Vimba vimba	Mondsee, Danube, Black Sea basin, Germany	47°49'40.8"N, 13°23'02.4"E	2	GQ279755, AY026405	H18				Hänfling et al. 2009 Durand et al. 2002
17	Vimba vimba	Eder, Weser, North Sea basin, Germany	51°09'18.0"N, 8°54'07.2"E	1	GQ279755	H18				Hänfling et al. 2009
18	Vimba vimba	Tuzlov, Don, Sea of Azov basin, Russia	49°58'58.8"N, 42°01'04.8"E	1	GQ279751	H15				Hänfling et al. 2009
19	Vimba vimba	Seversky, Donetsk Don, Sea of Azov basin, Russia	47°37'37.2"N, 40°53'16.8"E	1	GQ279751	H15				Hänfling et al. 2009
20	Vimba vimba	Samur, Caspian Sea basin Russia	41°52'26.8"N, 48°33'34.9"E	1	GQ279765	H16				Hänfling et al. 2009
21	Vimba vimba	Sea of Azov, Sea of Azov basin Ukraine	46°03'50.4"N, 36°36'54.0"E	2	GQ279754, GQ279752	H1 H3				Hänfling et al. 2009
22	Vimba vimba	Kuban, Sea of Azov basin, Russia	45°11'56.4"N, 37°42'54.0"E	1	GQ279753	H10				Hänfling et al. 2009
23	Vimba vimba	Tsymlyansk Reservoir, Don, Sea of Azov basin, Russia	47°45'56.2"N, 42°49'18.8"E	1	GQ279751	H15				Hänfling et al. 2009
24	Vimba vimba	Dagomys, northeastern Black Sea basin, Russia	43°40'01.2"N, 39°39'07.2"E	1	GQ279750	H3				Hänfling et al. 2009
25	Vimba vimba	Libechovka river, Elbe basin, Czech Republic	50°28'45.0"N, 14°29'07.5"E, (predict)	1	HM560237	H17	1	HM560383	H6	Perea et al. 2010
26	Vimba vimba	Bashly-chai, Caspian Sea Basin, Russia	42°20'37.0"N, 48°05'30.8"E	1	GQ279765	H16				Hänfling et al. 2009
27	Vimba vimba	Lake Sapanca, Sakarya, Turkey	40°43'14"N, 30°17'41"E				20	KC501853-KC501872	H13	Keskin & Atar 2013
28	Vimba vimba	Gönen drainage, Çanakkale, Turkey	39°56'45.6"N, 27°20'13.2"E				2	KJ554799, KJ554924	H11, H12	Geiger et al. 2014
29	Vimba vimba	Egirdir Lake, Isparta, Turkey	38°02'00.9"N, 30°52'24.3"E (predict)				2	MW940905, MW940906	H10, H6	Eren,H. (unp.)
30	Vimba vimba	Biga drainage, Bursa, Turkey	40°12'18.0"N, 29°05'13.2"E				1	KJ554609	H6	Geiger et al. 2014
31	Vimba vimba	Koeprue drainage, Isparta, Turkey	37°31'40.8"N, 31°16'08.4"E				2	KJ554606, KJ554754	H6	Geiger et al. 2014
32	Vimba vimba	Danube R., Slovakia	48°04'04.2"N, 17°09'53.2"E (predict)	1	AY026404	H1				Durand et al. 2002
33	Vimba melanops	Inece stream, Kırklareli, Turkey	41°41'34.0"N, 27°04'59.0"E	4	OK493415, OK493416	H27 H28	2	OK444819	H1	This study
34	Vimba melanops	Evros, Aegean Sea basin, Greece	40°50'42.0"N, 26°01'22.8"E	1	GQ279757	H26				Hänfling et al. 2009



Locality no	Species	Locality	Coordinate	cyt b			COI			Reference
				N	Genbank no	Haplotype no	N	Genbank no	Haplotype no	
35	<i>Vimba melanops</i>	Biserska River, Greece	40°55'08.5"N, 26°11'48.0"E (predict)	1	MG806725	H25	1	MG806910	H1	Schonhuth et al. 2018
36	<i>Vimba melanops</i>	River Strymon, Greece	41°43'53.5"N, 23°09'30.9"E (predict)	2	AF090778, HM560236	H24 H21	1	HM560382	H4	Zardoya & Doadrio, 1999 Perea et al 2010
37	<i>Vimba melanops</i>	Pinios, Aegean Sea basin, Greece	39°39'57.6"N, 22°14'02.4"E	2	GQ279758, GQ279759	H23 H22				Hänfling et al. 2009
38	<i>Vimba melanops</i>	Aliakmon R. Kaloneri, Greece	40°17'26.5"N, 21°28'17.9"E (predict)	1	HM560235	H21				Perea et al. 2010
39	<i>Vimba melanops</i>	Volvi lake, Greece	40°39'36.0"N, 23°32'24.0"E	1	AY026403	H29	3	HQ600801-HQ600803	H1	Triantafyllidis et al. 2011
40	<i>Vimba melanops</i>	Kerkini lake, Greece	41°06'36.0"N, 23°03'00.0"E				3	HQ600804-HQ600806	H1, H3, H4	Durand et al. 2002
41	<i>Vimba melanops</i>	Biserska R., Evros drainage, Bulgaria	41°51'18.0"N, 25°55'22.8"E				3	KJ554935, KJ554568, KJ554722	H1	Triantafyllidis et al. 2011
42	<i>Vimba melanops</i>	Charmanlijskaja drainage, Bulgaria	41°53'20.4"N, 25°41'13.2"E				1	KJ554876	H2	Geiger et al. 2014
43	<i>Vimba melanops</i>	Vardar drainage, Greece	40°59'16.8"N, 22°33'28.8"E				2	KJ554926, KJ554576	H5	Geiger et al. 2014
44	<i>Vimba mirabilis</i>	Akçay stream, Büyük Menderes, Aydın, Turkey	37°45'34.0"N, 28°20'07.0"E	9	OK493414, AY026410	H20 H19	4	OK444822, OK444823	H8, H9	This study, Durand et al. 2002
45	<i>Vimba mirabilis</i>	Çine stream, Büyük Menderes, Aydın, Turkey	37°45'43.8"N, 27°50'13.1"E	4	OK493414	H20	1	KJ554739	H8	This study, Geiger et al. 2014



**Figure 1.** Map showing the analyzed population stations.

to Akaike information criterion (AIC) and Bayesian information criterion (BIC), as implemented in jModeltest v. 0.0.1 (Posada, 2008). Phylogenetic relationships among haplotypes and species were estimated by the maximum likelihood (ML) method using PhyML (Guindon et al. 2010) with 1000 bootstrap. Similarly, the Bayesian inference (BI) analysis was run in the MrBayes 3.1.2 software (Ronquist and Huelsenbeck 2003), using the Metropolis-coupled Markov chain

Monte Carlo (MCMC) algorithm from randomly generated starting trees for five million generations with sampling taking place in every 1000 generations. The initial 25% of saved trees sampled in each MCMC run were discarded as burn-in. In all phylogenetic analyses, *Blicca bjoerkna* (AP009304) was selected as outgroup taxa. Haplotypes' network inference was constructed through a median-joining (MJ) algorithm (Bandelt et al. 1999) implemented in Network 5.0.0.1 software

([www.fluxus-engineering.com](http://www.fluxus-engineering.com)). MEGA X (Kumar et al. 2018) software was used to calculate pairwise genetic distance among the species using the Kimura 2-parameter substitution model (Kimura 1980).

We estimated divergence times using StarBEAST (Ogilvie et al. 2017), which was implemented in BEAST 2.6.0 (Bouckaert et al. 2014). Compared with standard BEAST, StarBEAST better accounts for species trees vs. gene trees and intraspecific vs. interspecific events. Species were delimited according to the individual grouping recovered by previous phylogenetic analyses. Thus, the analysis was conducted with 5 groups of individuals consisting of *V. vimba* (Caspian), *V. vimba* (Western), *V. mirabilis*, *V. melanops*, and outgroup *Blicca bjoerkna*. The molecular clock calibration was based on the divergence rate of cyt b gene in Leuciscinae of 0.4% per lineage per million years, as determined by Perea et al. (2010), and this rate was used by Buj et al. (2019) and Viñuela Rodríguez et al. (2020). Based on a Yule speciation prior and a strict clock model, branch rate estimates were calculated. Partitioned cyt b and COI dataset were used for the molecular clock analysis and the partitions were linked except for substitution models. The substitution models were used the TrN+G:-ln = 1878.5334 (Tamura and Nei 1993) and the HKY:-ln = 1063.2973 (Hasegawa et al. 1985) for cyt b and COI barcoding region, respectively, with Gamma site heterogeneity for both markers. The number of MCMC steps (chain length) was ten million, with parameters logged every 1000 generations. The residual parameters were default parameters of the software. Tree results were summarized in TreeAnnotator v.2.6.0 software with 10% burn-in to get a maximum clade credibility tree. Node bars, height median with height 95% HPD, and node labels were mapped on the tree with FigTree v1.4.4 (Rambaut 2018). Geological scale was plotted using the geoscalePhylo function in R package strap (Bell and Lloyd 2015). Effective sample size (ESS) and convergence of parameters was estimated using Tracer v.1.6 (Rambaut and Drummond 2013). The effective sample sizes for all parameters of interest were greater than 200.

## Results

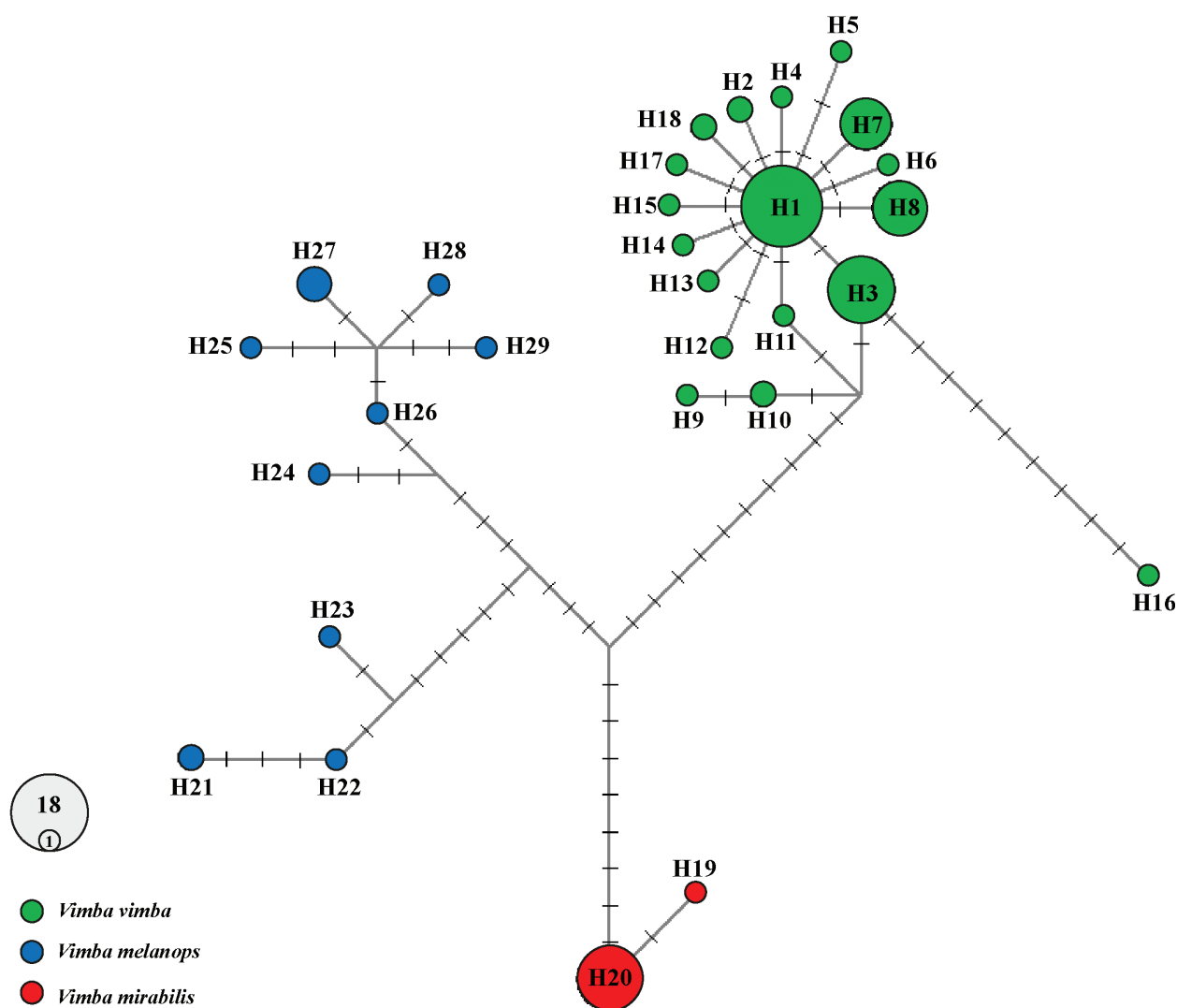
### Genetic diversity and species divergence

The nucleotide sequences of the cyt b gene region (1023 bp) were studied in 91 specimens from three species of *Vimba* (Additional Table 1). The average nucleotide frequencies were estimated as 26.9% A, 29.7% T, 28.0% C, and 15.4% G. The transition/transversion rate  $k_1 = 115.99$  (purines),  $k_2 = 34.563$  (pyrimidines) and the overall transition/transversion bias were estimated as  $R = 31.458$ . The haplotype number was identified as 29: 18 from *V. vimba* (N = 66), nine from *V. melanops* (N = 12), and two from *V. mirabilis* (N = 13) (Additional Table 1). We identified 65 variable sites, of which 36 were parsimony-informative. Species-specific nucleotide (SSN) positions in the mitochondrial DNA cyt b gene sequences provide evidence for

genetic differentiation of three *Vimba* species, as in *V. vimba*, *V. mirabilis*, and *V. melanops* are determined: 7, 7, and 1, respectively. The haplotype and nucleotide diversity are  $H_d = 0.855 \pm 0.025$  and  $\pi = 0.00175 \pm 0.00029$  for *Vimba vimba*,  $H_d = 0.939 \pm 0.00333$  and  $\pi = 0.00783 \pm 0.00119$  for *V. melanops*, and  $H_d = 0.154 \pm 0.01590$  and  $\pi = 0.00015 \pm 0.00012$  for *V. mirabilis*. The overall haplotype and nucleotide diversity are  $H_d = 0.907 \pm 0.015$  and  $\pi = 0.00994 \pm 0.00104$ . According to the AMOVA, 88.34% of all genetic variation occurs among three species. In the haplotype network analysis, the 29 distinct haplotypes are separated by one up to nine mutations. The most common haplotype was H1, shared by many populations belonging to *V. vimba*. Many haplotypes were distributed in either one or two populations (Fig. 2). Average pairwise genetic distances between species were estimated as 0.0212 (*V. vimba* and *V. melanops*), 0.0198 (*V. vimba* and *V. mirabilis*), and 0.0171 (*V. melanops* and *V. mirabilis*). Average pairwise genetic distance was estimated as 0.0101 between *V. vimba* Caspian (H16) and West lineages. Average intraspecific variations within *V. vimba*, *V. mirabilis*, and *V. melanops* were estimated as 0.002, 0.000, and 0.008, respectively. Nucleotide sequences of the COI barcoding region (652 bp) were obtained in 67 specimens from three species of *Vimba* (Additional Table 1). The nucleotide frequencies were calculated as 25.28% A, 29.00% T, 27.71% C, and 18.01% G. The transition/transversion rate  $k_1 = 3.588$  (purines),  $k_2 = 1.848$  (pyrimidines), and the overall transition/transversion bias was calculated as  $R = 1.27$ . The haplotype number was identified as 13: six from *V. vimba* (N = 46), five from *V. melanops* (N = 16), and two from *V. mirabilis* (N = 5) (Table 1). A total of 21 variable sites were recognized, and 10 of them were parsimony-informative. The haplotype and nucleotide diversity with the standard deviation are  $H_d = 0.680 \pm 0.00156$  and  $\pi = 0.00308 \pm 0.00021$  for *V. vimba*,  $H_d = 0.608 \pm 0.01695$  and  $\pi = 0.00204 \pm 0.00065$  for *V. melanops*, and  $H_d = 0.400 \pm 0.237$  and  $\pi = 0.00123 \pm 0.00073$  for *V. mirabilis*. The overall haplotype and nucleotide diversity are  $H_d: 0.826 \pm 0.026$  and  $\pi = 0.00457 \pm 0.00041$ . According to the AMOVA, 58.52% of all observed genetic variations in three species are occurring within species. Haplotype network analysis has 13 unique haplotypes with at least one mutational step, and no haplotype is shared between species that are separated from each other by at least one mutational step. The most common haplotypes were H13, represented by 20 specimens sampled by İznik Lake. All haplotypes were distributed in either one or two populations (Fig. 3). Average pairwise genetic distances between species were estimated as 0.0054 (*V. vimba* and *V. melanops*), 0.0093 (*V. vimba* and *V. mirabilis*), and 0.0088 (*V. melanops* and *V. mirabilis*).

### Phylogenetic relationships and divergence times

Based on ML and Bayesian phylogenetic inferences, four mitochondrial clades of *Vimba* species are observed. The first and second clades include distinct *V. vimba*



**Figure 2.** Median-joining network of the cyt b haplotypes. Circle size corresponds to sample size; one bar indicates an additional mutational step. Each small line represents one nucleotide difference.

populations, while the other clades comprise *V. mirabilis* and *V. melanops*. *V. vimba* is not monophyletic because the Western (Pontic) and Caspian basin haplotypes of *V. vimba* are located in two different clades. As shown in Fig. 4, the maximum likelihood and Bayesian inference analyses of concatenated data of mitochondrial cyt b and COI barcoding region trees were supported by high bootstrap values ( $>75$ ) for the distinction of species and *Vimba vimba* lineages. According to the divergence time between the two main clades, the divergence among the *Vimba* species starts in 2.27 (0.57–4.02) mya, early Pleistocene (Gelasian), and differentiates into four clades in early Pleistocene (Calabrian) based on StarBEAST analysis (Fig. 5).

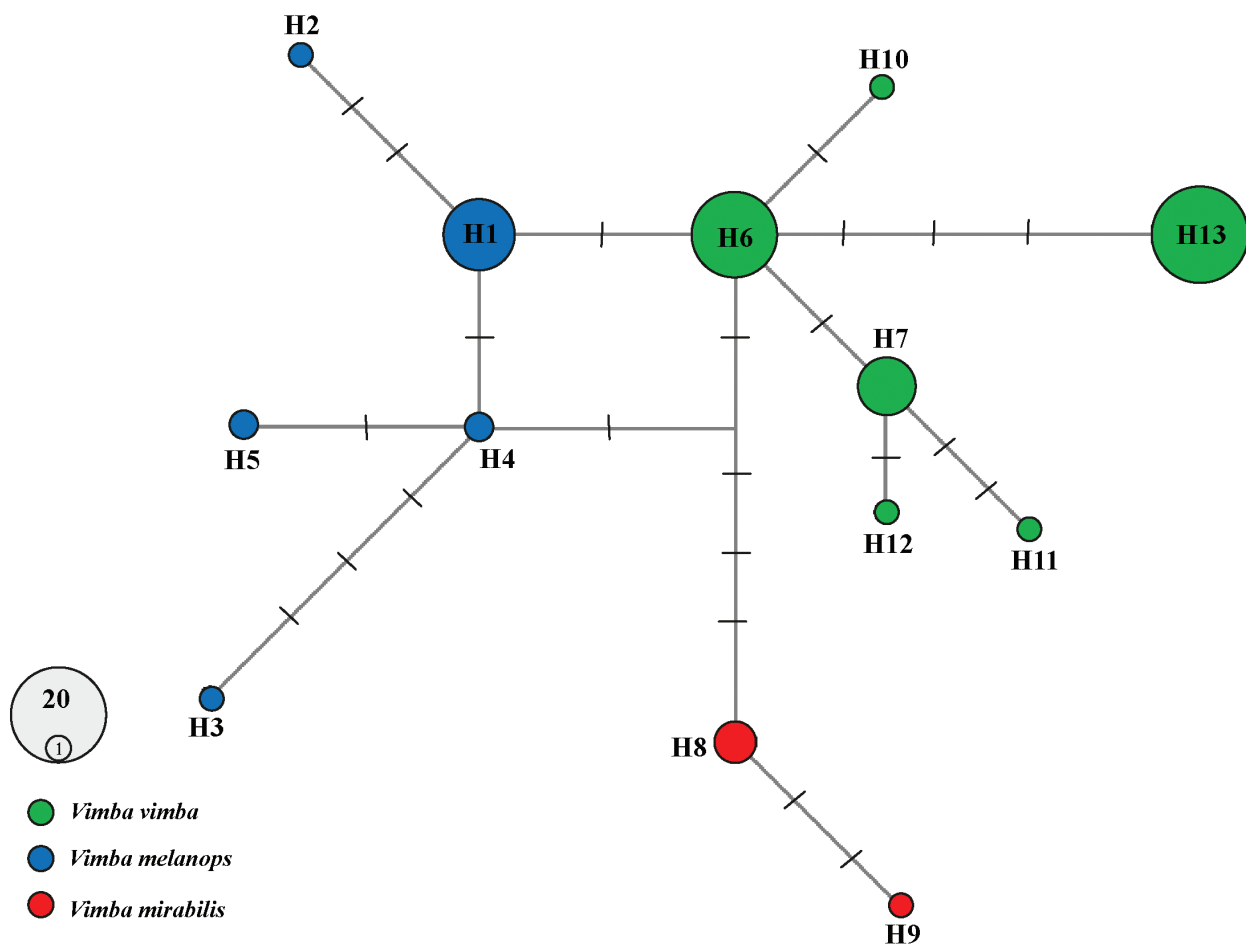
## Discussion

The present study investigates the phylogeny and phylogeography of three *Vimba* species to provide the first comprehensive molecular study on the genus. Although some work has been conducted on *Vimba* species, there

are no comprehensive studies on their phylogeography, which is provided in the present study.

Here, the phylogeny constructed by ML and BI approaches is based on concatenated mtDNA cyt b and COI sequences of three *Vimba* species. The phylogenetic tree topology revealed that species were clustered into four well-supported clades (*V. vimba* (Western), *V. vimba* (Caspian), *V. melanops*, and *V. mirabilis*) with high-reliability BI Posterior probability value (BI = 0.98) although low ML bootstrap value (ML  $< 50$ ). In *V. vimba*, two lineages are observed, the Caspian and the Western, monophyly of both group supported by high BI (0.98) value. *V. melanops* and *V. mirabilis* belong to different clades with high BP (97) and BI posterior probability (1.00) (Fig. 4). According to tree topology, a clear geographical pattern emerged, which is also supported by the median joining network. However, variability at cyt b and COI markers varied, the AMOVA analysis found almost all (88.34%) of the genetic variations observed for the cyt b gene occurred between species, while this value drops to 58.52% for the COI barcoding region.

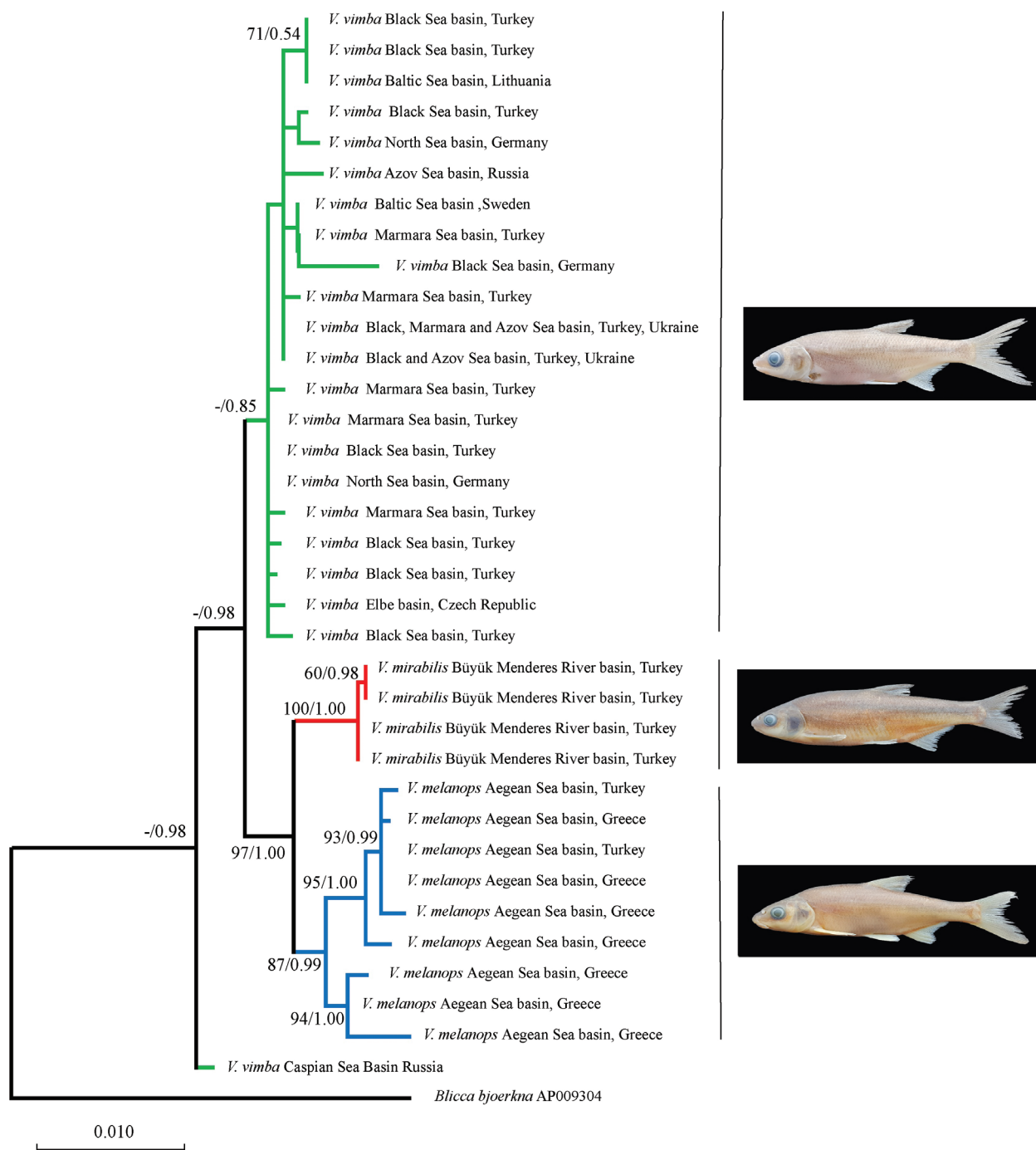




**Figure 3.** Median-joining network of the COI haplotypes. Circle size corresponds to sample size; one bar indicates an additional mutational step. Each small line represents one nucleotide difference.

Geiger et al. (2014) similarly reported that *V. vimba*, *V. melanops*, *V. mirabilis* are closely related species, with relatively low interspecific genetic distances, but they can be distinguished from each other by morphological characters. Species with high haplotype diversity and low nucleotide diversity are usually thought to originate through small founder populations during the expansion period, in accordance with previous analyses of Anatolian freshwater fish species (Hrbek et al. 2004; Bektas et al. 2017). A small independent evolution of similar origin (originating from the Pliocene/Pleistocene) may account for the low intraspecific diversity and corresponds with tectonic or glacial activity in the area. Anatolia and Central Europe were connected from the late Oligocene to the late Miocene (23–5.33 mya) (Popov et al. 2004). The estimated separation time of *Vimba* from sister genus *Blicca* was calculated as 6.16 (1.42–13.11) mya, a timeframe consistent with a dispersal from the Danube basin to Anatolia via river capture during this period (Levy et al. 2009). Similarly, Hänfling et al. (2009) estimated the time of separation of *Vimba* genus in Pleistocene as well. The divergence time between the two main clades shows that the divergence among the *Vimba* species started in 2.27 (0.57–4.02) mya, early Pleistocene (Gelasian) (Fig. 5).

According to Hänfling et al. (2009), *V. vimba* lineages were defined as Pontic and also Caspian clade, including Caspian Sea haplotype which is highly divergent from the other haplogroup. This suggests *V. vimba* originated from two refugial regions located in the Danubian drainage and the northern Pontic regions. Moreover, Hänfling et al. (2009), Naseka and Bogutskaya (2009), Jouladeh-Roudbar et al. (2015), and Esmacili et al. (2018) stated that the Caspian *Vimba* should be considered as a distinct species corresponding to *V. persa*, which is anadromous and endemic to the Caspian Sea. Similarly, in this study, it is supported that Caspian *Vimba* should be classified as *V. persa*, not *V. vimba* considering intra and interspecies divergence of *Vimba* species. Furthermore, both phylogenetic and StarBEAST analyses support Caspian *Vimba* corresponding to a different lineage from the Western lineage of *V. vimba*. However, advanced research and comprehensive sampling of Caspian and Western populations are required to clarify this. *V. vimba* in other regions has relatively low intraspecific diversity, except for the Caspian haplogroup. Natural distribution records for *V. vimba* in Europe, the former USSR, are most likely due to stock translocations and introductions from other areas due to their economic value (Freyhof 1999).



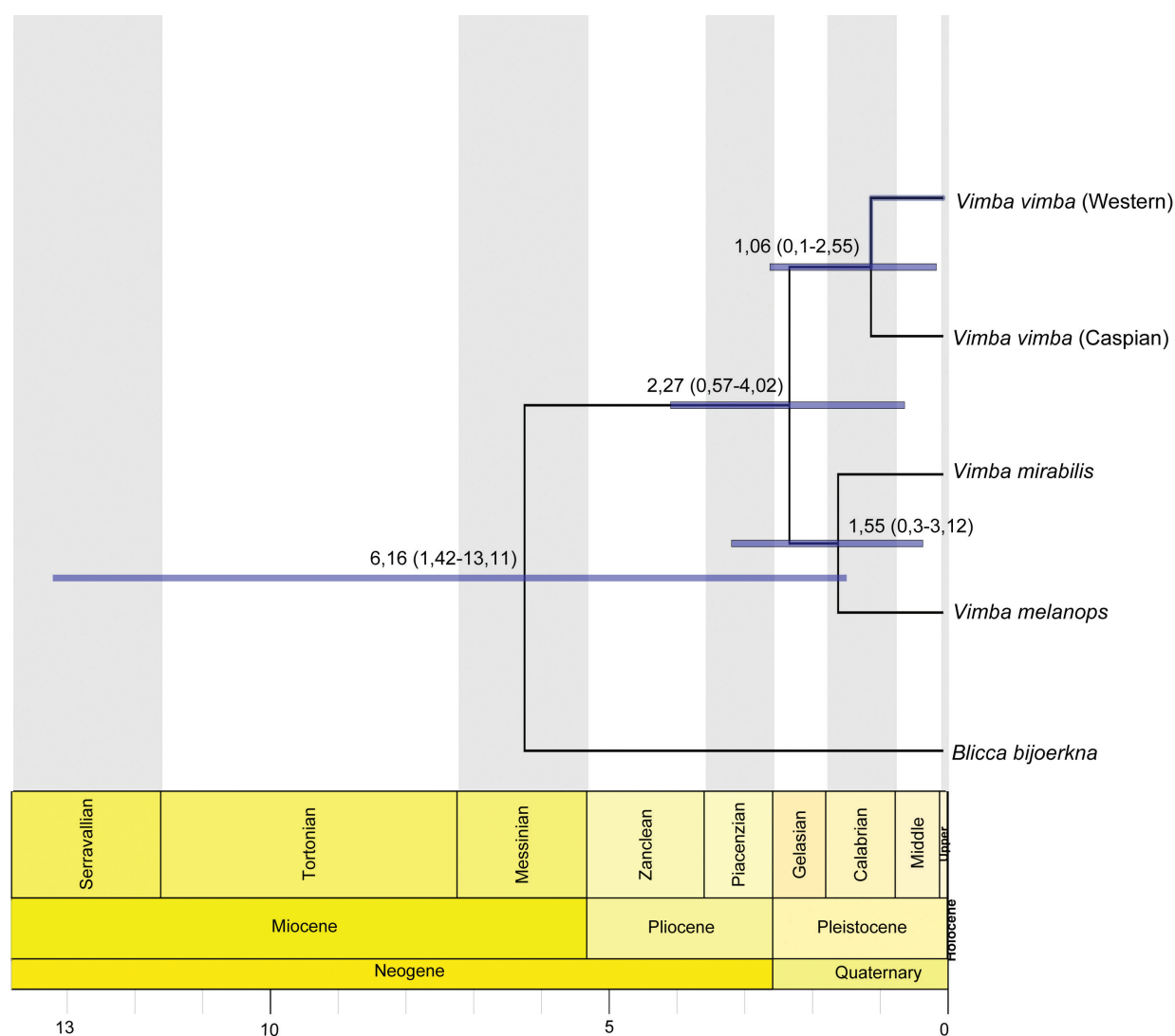
**Figure 4.** Maximum likelihood tree based on the two concatenated mitochondrial genes (cyt b and COI) (1675 bp) sequences of *Vimba* species. Maximum likelihood and Bayesian inference analyses resulted in congruent trees. Bootstrap and posterior probability values are shown above nodes on a tree if 50% or higher.

Phylogeography of *V. vimba* was investigated by Hänfling et al. (2009), and it reflected the presence of two refugia and recolonization of *V. vimba* as Caspian sea, and Western or Pontid clade, dating back 1–2 mya during the early Pleistocene. According to the present study, the separation of *Vimba*, dated in Pleistocene, occurred at 1.06 (0.2–2.55) mya. Correspondingly, in view of the timing, Pleistocene events must have played a central role in structuring the Balkan's marbled goby populations (Van-hove et al. 2012). In addition, multiples fish groups in

the Black and Caspian Sea basins display similar patterns of Pleistocene divergence such as the Black Sea roach, *Rutilus frisii*, salmon, barbell, and dreissenid mussels (Stepien et al. 2003; Kotlík et al. 2008; Ninua et al. 2018; Bartáková et al. 2019; Levin et al. 2019). Due to the migration of *Vimba* using brackish waters, it spread all over Eurasia also in Northern Germany and Netherlands, using the Danube River and old canal system (Freyhof 1999).

In the Early Pleistocene, the depression of the Marmara Sea and the uplift of the Aegean mountains contributed





**Figure 5.** Divergence timescale for the *Vimba* species inferred under Bayesian strict clock method from two concatenated mitochondrial genes (cyt b and COI) (1675 bp) sequences. Numbers in front of the node represent divergence times in million years (Ma) and their HPD 95% credibility intervals.

greatly to the separation of *V. vimba* and *V. mirabilis*. Furthermore, while the water of the Büyük Menderes River was flowing in the north-south direction because of the western Anatolian Mountains barrier, fractures and foldings which occurred in the Early Pleistocene caused the river to turn west and take its present form. *V. mirabilis* is present in Bafa Lake because of alluvium brought in by the Menderes River that blocked the old sea gulf and separated it from the sea, creating Bafa Lake (Akçer-Ön et al. 2020).

Por, (1989) specified that a line drawn from west to east in the middle of Turkey could be considered as a major suture, which leads to distinct species of *Vimba* between the Büyük Menderes and northern Aegean. The differences between the Balkan and Anatolian species can primarily be associated with the formation of the Aegean in the late Pliocene (Kosswig 1955; Bilgin 2011). Also, Bektas et al. (2019) discovered that dispersal of *Alburnoides symrnae* and *Alburnoides economui* was dated in 5.42–2.31 Ma (Early Pliocene), when the former Aegeopotamus River

was a very large river that discharged the waters of Paratethys into the Aegean Sea. Divergence time estimated between *V. melanops* and *V. mirabilis* as 1.69 (1.07–2.38) mya is consistent with this event. Durand et al. (2000), Hewitt (2000), and Hewitt (2004) further highlighted that the Danube was an essential component of the Chub (*Squalius cephalus*) evolutionary history, especially during the Pleistocene glaciations as it acted as a refugium for this species. Our molecular data confirm this influence on the divergence of *V. melanops* and *V. mirabilis*.

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