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A new perspective on the molecular dating of the brown trout complex with an extended phylogeographic information on the species in Serbia

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Abstract

To elucidate the historical biogeography of a species, the patterns of population divergence must be understood, and the evolutionary history of the species must be accurately known. For brown trout (*Salmo trutta* complex), estimating divergence times remains a challenge due to the lack of well-defined time calibration points and insufficient phylogeographic coverage in previous studies. The present work aims to improve molecular dating of mitochondrial control region sequences by using a multicalibration framework based on the latest paleogeological evidence for dating the origin of Lake Ohrid and two available *Salmo* fossils, including the overlooked *Salmo immigratus*. Our results clearly show that, contrary to common belief, the major divisions within the brown trout occurred in the Late Pliocene, not the Pleistocene. The Pliocene origin suggests that the brown trout lineages did not form because of geo(hydro)morphological changes during glaciation cycles but may be the result of orogeny and drainage evolution. In addition, increased sampling, particularly in Serbia, led to the identification of a new haplogroup (DA-INT) occupying an intermediate position with respect to DA-ES and DA-BS haplogroups. While the control region can delineate brown trout lineages, its phylogenetic resolution is limited, so even extensive sampling could not further resolve the lineage level polytomies.

Keywords

Danubian brown trout lineage – phylogeography – Pleistocene – Pliocene – *Salmo* spp. – time-calibrated phylogeny

Introduction

It is widely accepted among researchers that the first splits in trout of the *Salmo* genus began in the Late Pliocene, but most divisions of the brown trout (BT) complex (all trout except for *Salmo ohridanus* and *Salmo obtusirostris*) observed today are thought to have occurred in the Pleistocene because of climatic, environmental and geomorphological changes that occurred during glaciation events. These events shaped hydrogeography of the major European river systems and led to the isolation of trout populations, which consequently evolved into distinct genetic lineages (Osinov & Bernatchez, 1996; Bernatchez, 2001; Crête-Lafrenière et al., 2012; Alexandrou et al., 2013; Pustovrh et al., 2014; Lecaudey et al., 2018; Ninua et al., 2018; Hashemzadeh Segherloo et al., 2021). As a result, the distribution of the five evolutionary mitochondrial lineages more or less coincides with these principal river systems: Atlantic (AT), Danubian (DA), Mediterranean (ME), Adriatic

(AD), and *marmoratus* (MA) (Bernatchez et al., 1992; Bernatchez, 2001), with the last referring to trout of the northern Adriatic river basin characterized by the marmorated phenotype.

A molecular clock rate of 2% per million years (inferred from mitochondrial (mt) DNA of higher primates; Brown et al., 1979) was initially assumed for estimating mtDNA control region (CR) divergence times (Bernatchez et al., 1992). The Pleistocene origin of BT lineages persisted even when slower molecular clocks (1–2% in Bernatchez, 2001; and 0.75% per million years in Shedlock et al., 1992 and Sanz, 2018) were applied to estimates of net evolutionary divergence from mtDNA CR sequences or when secondary calibration (based on the results of previous molecular dating studies) was used to date the origin of *S. ohridanus* (Pustovrh et al., 2014; Ninua et al., 2018). The latter studies relied on the molecular calibrations of Sušnik et al. (2006), which used the 3 to 5 Ma constrain on the age of Lake Ohrid, an estimate that is no longer valid (Wagner et al., 2019). The existence of BT as early as the Gelasian of the Early Pleistocene (2.58–0.0117 Ma; *sensu* Gibbard & Head, 2009, Gibbard et al., 2010) is evidenced by a *Salmo derzhavini* fossil (Vladimirov, 1946; Rukhkyan, 1989) discovered in Armenia (Caucasus), which lived in the paleo-Vorotan drainage some 2 Ma (Osinov & Bernatchez, 1996; Bernatchez, 2001; Gabrielyan et al., 2004; Pipoyan et al., 2011; Sanz, 2018). However, since it is not clear whether it is a BT crown or stem fossil, this evidence does not exclude the possibility that the BT lineages are even older, such a scenario that was also suggested in some regionally restricted studies – Turkey (Sušnik et al., 2005; Bardakci et al., 2006) and Greece (Apostolidis et al., 1997). In addition, a Salmoninae wide mtDNA (Crête-Lafrenière et al., 2012) as well as RAD-seq phylogeny (Lecaudey et al., 2018) suggest that within BT at least some lineage may have a Pliocene origin. However, as the genus *Salmo* was under represented in both studies and the calibrations relied on fossil records preferentially informative for *Salvelinus* and *Oncorhynchus*, dating of the BT lineages remains unresolved. In addition to *S. derzhavini*, the origin of *Salmo* can also be calibrated with *Salmo immigratus*. This overlooked *Salmo* stem fossil from Croatia near Zagreb, which lived in the Western Parathethys (Pannonian Basin) during the Lower Sarmatian of the Middle Miocene (Gorjanović-Kramberger, 1891; Anđelković, 1989), could provide an informative time point for estimating the divergence between *Salmo salar* and the common ancestor of the other *Salmo* representatives of the genus. Furthermore, when using a single fast evolving marker, such as CR, the effect of substitution saturation on molecular clock estimation can be mitigated by employing multiple independent calibration points spread along the sampled phylogeny (Lukoschek et al., 2012).

Continued phylogeographic research on trout following the break-through publication of Bernatchez et al. (1992) and increased sampling of local populations across the BT natural range has led to the identification of other independent evolutionary lineages (Duero – DU, Suárez et al., 2001; Presa et al., 2002; Hermida et al., 2009; Vera et al., 2010; Tigris – TI, Sušnik et al., 2005; Dades, Snoj et al., 2011; North African – NA, Tougard et al., 2018; Atlantic-Morocco – AT-M, Snoj et al., 2021).

Within the DA lineage, two phylogeographic haplogroups (minor lineages *sensu* Sanz, 2018; i.e., sublineages) have been identified (Bernatchez, 2001; Kohout et al., 2013), of which, Danubian-Black Sea haplogroup (DA-BS) is located mostly in the lower Danube river basin and the southern Black Sea tributaries, while the other, the Eastern Danubian haplogroup (DA-ES or DaDA *sensu* Kohout et al., 2013) is much more widely spread, being found in the Caspian and Aral river basins, in the east Black Sea tributaries and the upper and middle Danube drainage (Kohout et al.,

2013; Sanz, 2018). The haplotypes of both DA haplogroups were found to overlap in the Džepska river system, a tributary of the South Morava River in Serbia, where the two most divergent DA-BS haplotypes were detected along with the DA-ES ones (Marić et al., 2006, 2022; Kohout et al., 2013). Besides the Džepska River, DA-BS haplotypes were also found in the Vrla River (Marić et al., 2022). Both rivers emerge in the Vlasina Plateau in southeast Serbia, which belongs to the oldest landmass in the Balkan Peninsula (Schöffmann, 2021). The specific distributions of DA-BS and DA-ES haplotypes were reported to be due to successive colonizations and secondary contacts during different Pleistocene ages (Sanz, 2018; Schöffmann, 2021), while the apparently discontinuous geographic distribution of DA-BS haplotypes may partly be due to lack of sampling from some crucial areas in the Balkan region and the Caucasus.

The aim of this study is to revise comprehensively the molecular dating of the BT lineages using a relaxed clock framework applied to the available *Salmo* fossils, including the overlooked *Salmo* stem fossil *S. immigratus*, and the latest paleogeological insights into dating the origin of the ancient Lake Ohrid (Wagner et al., 2019), which was often used as a time point to calibrate the molecular clock for *Salmo*. By applying a much larger sample set than previously, we further investigated the phylogenetic patterns within the DA lineage and the distribution of both its haplogroups, DA-BS and DA-ES, in Serbia, where they were reported to co-exist, and integrated our findings within the wider BT phylogeographic and colonization patterns.

Material and methods

Sampling and CR sequences

In the present study, 778 brown trout specimens from 42 localities were sampled throughout the Danube drainage in Serbia and from two hatcheries (i.e., Sokobanjska Moravica and Panjica) during 2016 and 2017 in order to be sequenced for mtDNA CR. Fin clips were collected during regularly conducted surveys. Fish were released at the sampling site immediately after fin clipping; the collected tissue was preserved in 96% ethanol and stored at 4°C. The permits (no. 324-04-59/2016-17, 324-04-15/2017-17) to conduct fieldworks and collect specimens were provided by the Serbian Ministry of Environmental Protection and with the approval of local authorities.

CR sequences of BT in Serbia, originated from the Aegean and Danube drainages, and one hatchery (i.e., Jerma), studied in Marić et al. (2022) were also included. Therefore, the sample-set of Serbian material comprised 1023 BT collected at 55 sampling sites, including 52 localities from the wild and three hatcheries (table 1, fig. 1). To these, 281 unique BT CR sequences mined in GenBank and representing DA, AD, ME, MA, AT, DU, AT-M, NA, TI, and Dades lineages were added. CR sequences of *S. obtusirostris*, *S. ohridanus*, and *S. salar* were used as outgroups (supplementary table S1). The sequence alignment of the CR was trimmed to 985 bp in all analyses except ML, BI, and divergence time estimation, which were reconstructed using the total available length of sequences with the poly T region cut out (1005 bp).

DNA extraction, amplification, and sequencing

Total genomic DNA was extracted from fin clips using the Tissue Genomic DNA Mini Kit (Geneaid Biotech Ltd., Taiwan) and DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturers' protocol.

The complete CR (ca. 1100 bp) was PCR-amplified using primers LRBT-25 and LRBT-1195 (Uiblein et al., 2001) under the following PCR conditions: initial denaturation (94°C, 3 min)

TABLE 1 Sampling locations in Serbia. Details of samples: numerical code, location (asterisks denote ones sequenced in Marić et al., 2022), drainage, geographical coordinates

Location code	Location	Drainage	Coordinates
1	Boranjska River	Drina – Danubian	44°19'23.8"N, 19°09'30.9"E
2	Ljubovidja	Drina – Danubian	44°14'11.4"N, 19°28'26.9"E
3	Rača	Drina – Danubian	43°55'15.8"N, 19°31'50.8"E
4	Gračanica	Drina – Danubian	43°18'08.5"N, 19°40'07.7"E
5	Vapa	Drina – Danubian	43°14'15.1"N, 20°05'52.8"E
6	Povlenska River	Kolubara – Danubian	44°10'28.9"N, 19°45'37.1"E
7	Gradac	Kolubara – Danubian	44°14'59.1"N, 19°53'07.1"E
8	Djetinja	West Morava – Danubian	43°51'47.2"N, 19°37'47.9"E
9	Katušnica	West Morava – Danubian	43°38'43.6"N, 19°52'01.8"E
10	Veliki Rzav	West Morava – Danubian	43°43'59.5"N, 19°55'52.9"E
11	Panjica	West Morava – Danubian	43°38'49.3"N, 20°02'06.5"E
12	Tolišnica	West Morava – Danubian	43°39'51.2"N, 20°26'25.1"E
13	Brvenica	West Morava – Danubian	43°20'45.4"N, 20°34'03.8"E
14	Maglička River	West Morava – Danubian	43°36'07.0"N, 20°33'02.0"E
15	Bresnička River	West Morava – Danubian	43°34'05.9"N, 20°33'06.5"E
16	Studenica	West Morava – Danubian	43°25'28.4"N, 20°22'39.5"E
17	Izubra	West Morava – Danubian	43°25'24.7"N, 20°24'23.7"E
18	Brevina	West Morava – Danubian	43°25'41.3"N, 20°27'22.7"E
19	Gokčanica	West Morava – Danubian	43°27'09.4"N, 20°40'07.9"E
20	Samokovka River	West Morava – Danubian	43°19'32.3"N, 20°45'42.1"E
21	Brzećka River	West Morava – Danubian	43°18'34.6"N, 20°51'09.0"E
22	Štavska	South Morava – Danubian	43°15'42.5"N, 20°58'16.7"E
23	Sokobanjska Moravica	South Morava – Danubian	43°39'31.0"N, 21°50'45.3"E
24	Toplodolska River	South Morava – Danubian	42°35'53.7"N, 22°22'08.4"E
25	Visočica	South Morava – Danubian	43°10'15.6"N, 22°46'48.9"E
26	Dojkinačka River	South Morava – Danubian	43°16'57.4"N, 22°45'55.1"E
27	Vodenička River*	South Morava – Danubian	43°09'51.0"N, 22°55'59.0"E
28	Dursunska River	South Morava – Danubian	42°50'12.0"N, 22°13'39.0"E
29	Bistrička River	South Morava – Danubian	42°57'28.0"N, 22°13'27.0"E
30	Vlasina*	South Morava – Danubian	42°47'07.0"N, 22°19'19.0"E
31	Polomska River	South Morava – Danubian	42°45'43.4"N, 22°19'33.3"E
32	Vučja River	South Morava – Danubian	42°43'24.4"N 22°23'08.8"E
33	Upper Jerma*	South Morava – Danubian	42°46'52.0"N, 22°26'25.0"E
34	Lower Jerma*	South Morava – Danubian	42°53'48.0"N, 22°39'27.0"E
35	Jelašnička River*	South Morava – Danubian	42°37'57.0"N, 22°07'05.0"E
36	Garvanica*	South Morava – Danubian	42°44'47.0"N, 22°11'34.0"E
37	Masurička River*	South Morava – Danubian	42°38'51.0"N, 22°10'50.0"E

TABLE 1 Sampling locations in Serbia. Details of samples: numerical code, location (asterisks denote ones sequenced in Marić et al., 2022), drainage, geographical coordinates (*cont.*)

Location code	Location	Drainage	Coordinates
38	Vrla*	South Morava – Danubian	42°39'07.0"N, 22°17'44.0"E
39	Šaovice	South Morava – Danubian	42°41'07.3"N, 22°19'03.3"E
40	Resava	Great Morava – Danubian	44°06'02.0"N, 21°26'14.0"E
41	Mlava	Danube	44°15'39.3"N, 21°32'58.3"E
42	Krupaja River	Danube	44°11'02.6"N, 21°36'17.5"E
43	Kožica	Danube	44°36'04.0"N, 21°58'06.0"E
44	Vratna	Danube	44°22'42.0"N, 22°20'52.5"E
45	Zlotska River	Timok – Danubian	44°01'40.6"N, 21°57'37.7"E
46	Mirovštica	Timok – Danubian	43°50'02.6"N, 21°53'05.1"E
47	Janjska River	Timok – Danubian	43°25'31.8"N, 22°31'07.2"E
48	Tripušnica River	Vardar – Aegean	42°24'49.0"N, 22°13'20.4"E
49	Lisinska River*	Struma – Aegean	42°34'37.0"N, 22°20'49.0"E
50	Ljubatska River*	Struma – Aegean	42°29'40.0"N, 22°20'59.0"E
51	Dragovištica*	Struma – Aegean	42°27'21.0"N, 22°29'51.0"E
52	Brankovačka River*	Struma – Aegean	42°23'53.0"N, 22°27'28.0"E
53	Hatchery Panjica	–	43°39'34.1"N, 20°04'21.1"E
54	Hatchery Sokobanjska Moravica	–	43°37'54.0"N, 21°59'26.0"E
55	Hatchery Jerma*	–	42°56'33.0"N, 22°36'51.0"E

followed by 35 cycles of denaturation (94°C, 45 s), primer annealing (54°C, 45 s), DNA extension (72°C, 2 min), and the last final extension (72°C, 10 min). Purification of PCR products and both-direction sequencing with the same primers used in the PCR reactions were carried out by Macrogen Service Centre (Amsterdam, Nederland).

DNA sequences were edited with Chromas Lite v.2.6.5 (Technelysium Pty Ltd, Australia; http://technelysium.com.au/wp/chromas/) and aligned by hand. BLASTn (Altschul et al., 1990) was used to compare the sequences with those deposited in the NCBI GenBank (Sayers et al., 2022). The newly observed CR haplotypes were deposited in GenBank (accession numbers in supplementary table S1) following the nomenclature as in Marić et al. (2022).

Phylogenetic analyses and molecular dating

To investigate the genealogical relations between BT lineages, a haplotype network using the Median-joining method (MJ; Bandelt et al., 1999) was constructed with PopART v.1.7 (Leigh & Bryant, 2015). For the MJ network, indels were coded using the simple indel coding method of Simmons & Ochoterena (2000) as implemented in SeqState v.1.4.1 (Müller, 2005). An additional MJ network was constructed to infer the relationships only within the DA lineage; haplotype frequencies were taken from the present study and published sources (Bernatchez et al., 1992; Weiss et al., 2001; Duftner et al., 2003; Marić et al., 2006, 2016; Meraner et al., 2007; Griffiths et al., 2009; Osinov, 2009; Baric et al., 2010; Vera et al., 2011; Hashemzadeh Segherloo et al., 2012; Kohout

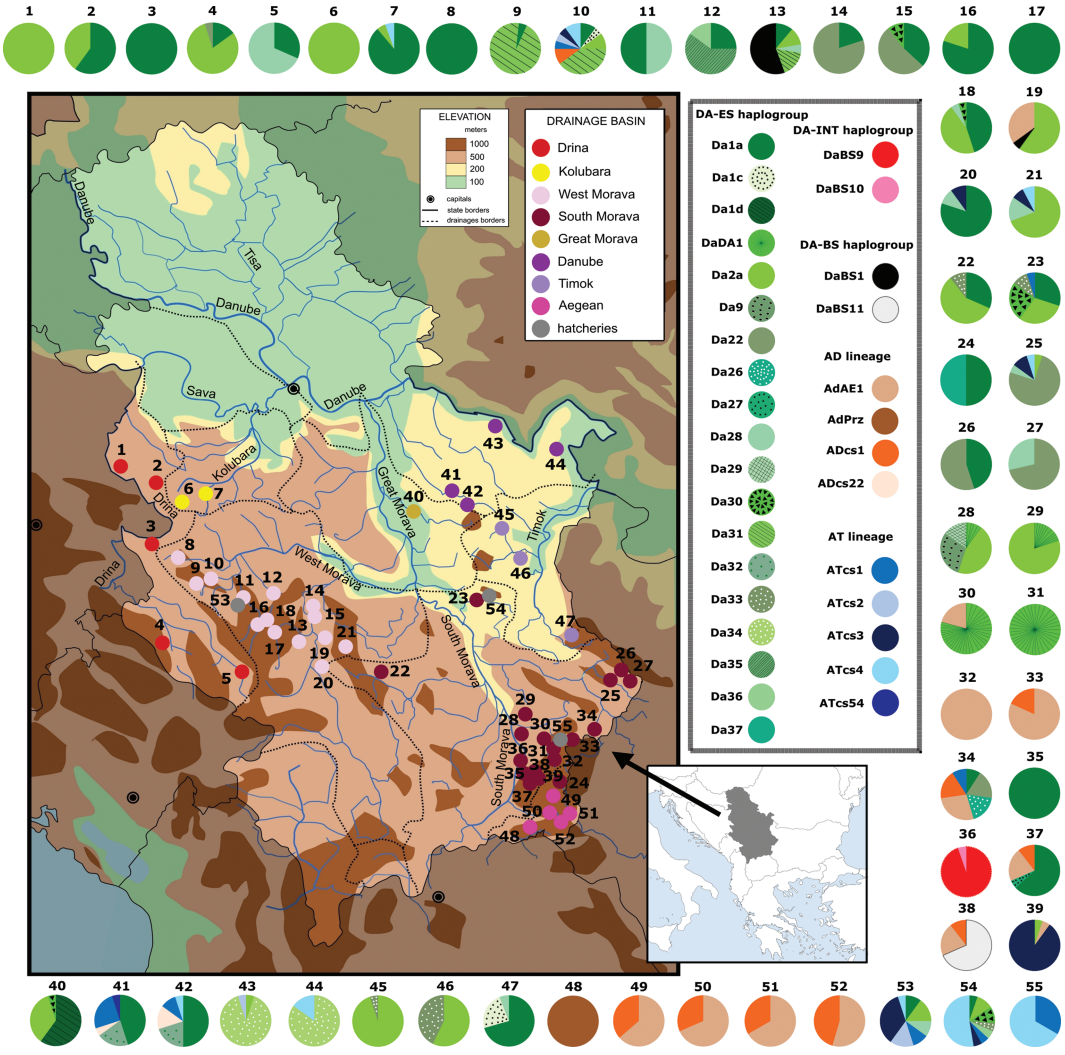

FIGURE 1 Map of sampling locations. The names and codes of the sampling sites are listed in table 1. Dots on the map represent sampling locations, while river drainages are indicated by different colors, as in the legend. Colored pie charts around the map represent the distribution and frequencies of mitochondrial DNA control region haplotypes per site.

et al., 2012, 2013; Özen, 2013; Schenekar et al., 2014; Tošić et al., 2014, 2016; Simonović et al., 2017; Levin et al., 2018; Kanjuh et al., 2020, 2021; supplementary tables S2 and S3). The phylogeographic distribution of DA haplotypes was also investigated with a haplotype distribution map.

For the construction of phylogenetic trees, the best-fit model of nucleotide substitution for the data set was evaluated using the Bayesian information criterion (BIC) in jModeltest v.2.1.10 (Darriba et al., 2012). The selected model of DNA substitution was HKY+I+G (Hasegawa et al., 1985; Yang, 1994; Steel et al., 2000).

The Maximum-Likelihood tree (ML) was constructed using RAxML v.8.2.12 (Stamatakis, 2014) under the GTRGAMMAX model. Phylogenetic confidence was assessed via bootstrap support (BS) with 1000 replicates

(Felsenstein, 1985) using the GTRCATX model (Stamatakis, 2006). In addition to a phylogenetic tree depicting relationships between all haplotypes, a tree with only a subset of haplotypes representing each lineage was reconstructed to account for signal saturation when using bootstrap to evaluate the support among similar sequences. Alternative topologies to the ML tree were investigated by the method of split decomposition (Bandelt & Dress, 1992) as implemented in the SplitsTree software v.5 (Huson & Bryant, 2006) using NeighborNet distance transformation (Bryant & Moulton, 2004), equal angle splits transformation (Dress & Huson, 2004), and with a support of 1000 bootstraps.

MrBayes v.3.2.6 software (Ronquist et al., 2012) was employed for Bayesian inference (BI, Rannala & Yang, 1996) by simultaneously running two independent runs of four Markov chains (Mau & Newton, 1997) for 30 million generations and sampling each 3000th generation. After verifying adequate sampling (ESS > > 500) and convergence of traces of all parameters with Tracer v.1.7.1 (Rambaut et al., 2018), a 25% burn-in was applied before combining the trees into a majority rule consensus tree with posterior probabilities (PP). Inferred trees were visualized using Figtree v. 1.4.3 (Rambaut, 2017).

Divergence time estimation was performed in BEAST 2 v.2.6.7 (Bouckaert et al., 2014) and run online on the CIPRES Science Gateway portal v.3.3 (Miller et al., 2010) and locally on a dedicated computational server. We have calibrated the chronogram using three time points, to which we applied lognormal constrains (table 2). For the age of the *Salmo* genus, we considered *S. immigratus*, found in Croatia and dated to Lower Sarmatian of the Middle Miocene (Gorjanović-Kramberger, 1891; Anđelković, 1989). As this extinct species is a stem lineage and the oldest known fossil for *Salmo*, 10 Ma served as a conservative minimum boundary for the most recent common ancestor (MRCA) of *Salmo* (table 2). To calibrate the age of *S. trutta* complex we considered *S. derzhavini*, the oldest known BT fossil found in Armenia and dated to Upper Pliocene (Vladimirov, 1946; Rukhkyan, 1989). Therefore 1.8 Ma was used as a minimum time constrain to date MRCA of BT. In addition to the fossil data, we used the most up-to-date estimations of the geological origin of the ancient Lake Ohrid, based on the age of the oldest sediments supporting the continued existence of the Lake (1.36 Ma; Wagner et al., 2019); 1.3 Ma was used as a minimum boundary for the MRCA of *S. ohridanus*. Furthermore, groups for which high PP (> 90%) support or high BS values (> 50) was estimated with BI and/or ML were treated as *a priori* monophyletic (supplementary fig. S1). Divergence time estimations were done with uncorrelated lognormal relaxed clock (Drummond et al., 2006) and by applying a birth-death (Gernhard, 2008) and a Yule model (Heled & Drummond, 2012), separately. Calculations were conducted using the BEAGLE library (Ayres et al., 2012) and run in three independent runs for 100 million generations sampling every 10,000th generation. Adequate sampling was verified in Tracer (see above) applying a 10% burn-in, and the tree files were combined with LogCombiner. Finally, TreeAnnotator was used to calculate the maximum clade credibility tree, common ancestor heights, posterior probabilities, and 95% highest posterior density (HPD) intervals. To evaluate the effect of each calibration point on the joint (the three-time point) divergence time estimation, calibration schemes with only two time points were also investigated. The three alternative chronograms, "*S. immigratus* + *S. derzhavini*", "*S. immigratus* + *S. ohridanus*", and "*S. derzhavini* + *S. ohridanus*" were estimated using the same setting as for the three-time point calibration scheme.

TABLE 2 Fossil and geological calibrations used in divergence time estimation. Taxonomic group for which priors for time to most recent common ancestor (T_{MRCA}) are provided along with the sources for calibration and corresponding priors (all are in Ma)

Taxa with priors for T_{MRCA}	Offset	Log (mean)	Log (SD)	Median	5%	95%	Source and additional information
Salmo	10.0	5.0	1.0	13.0	10.6	25.70	*Salmo immigratus* from Sarmatian of Middle Miocene as a stem *Salmo* (Gorjanović–Kramberger, 1891; Anđelković, 1989)
Salmo trutta complex	1.8	1.0	1.0	2.41	1.92	4.94	*Salmo derzhavini* from Upper Pliocene (Vladimirov, 1946; Rukhkyan, 1989)
Salmo ohridanus	1.3	0.6	1.5	1.49	1.32	3.60	Formation of Lake Ohrid, dated at 1.36 Ma according to sediment cores (Wagner et al., 2019)

Results

Relationships among CR sequences and haplotype distribution in Serbia

Haplotype network grouped the samples into 11 groups (fig. 2), which mostly corresponded to the evolutionary lineages identified in previous studies:

- three haplogroups within the DA lineage: (1) DA-ES, (2) DA-BS, and (3) a previously unreported group placed in an intermediate position between the other two and from both it is separated by two mutation steps; tentatively named DA-INT;
- three lineages of the Mediterranean basin: (4) Adriatic (AD), (5) Mediterranean (ME) *sensu stricto* (separated by three mutation steps from AD), and (6) *marmoratus* (MA; separated by three mutation steps from AD);
- three lineages of the Atlantic basin: (7) Atlantic (AT) *sensu stricto*, (8) Duero (DU, separated from AT by two mutation steps), and (9) Atlantic-Morocco group (AT-M; separated from AT by four mutation steps);
- two additional, highly divergent lineages (10) Dades and (11) Tigris (TI), both strongly protruding (ten and eight mutation steps, respectively) from AD.

Examination of the variable nucleotide sites in the control region revealed that the division of the DA lineage into three groups is best defined by three molecular synapomorphies (without observed back mutations) placed at nearby positions in the central part of the control region, between nucleotide positions 540–550 of our alignment, just on the 5' side of the polypyrimidine tract (polyT; supplementary fig. S2). Synapomorphy 542 G → C supported the DA-ES + DA-INT clade, synapomorphy 541 G → A supported the DA-ES + DA-INT (excluding DaBS9) clade, whereas synapomorphy 548 C → T supported the DA-ES clade; the first mutation is one of the only two informative transversions in the alignment of the DA (fig. 3).

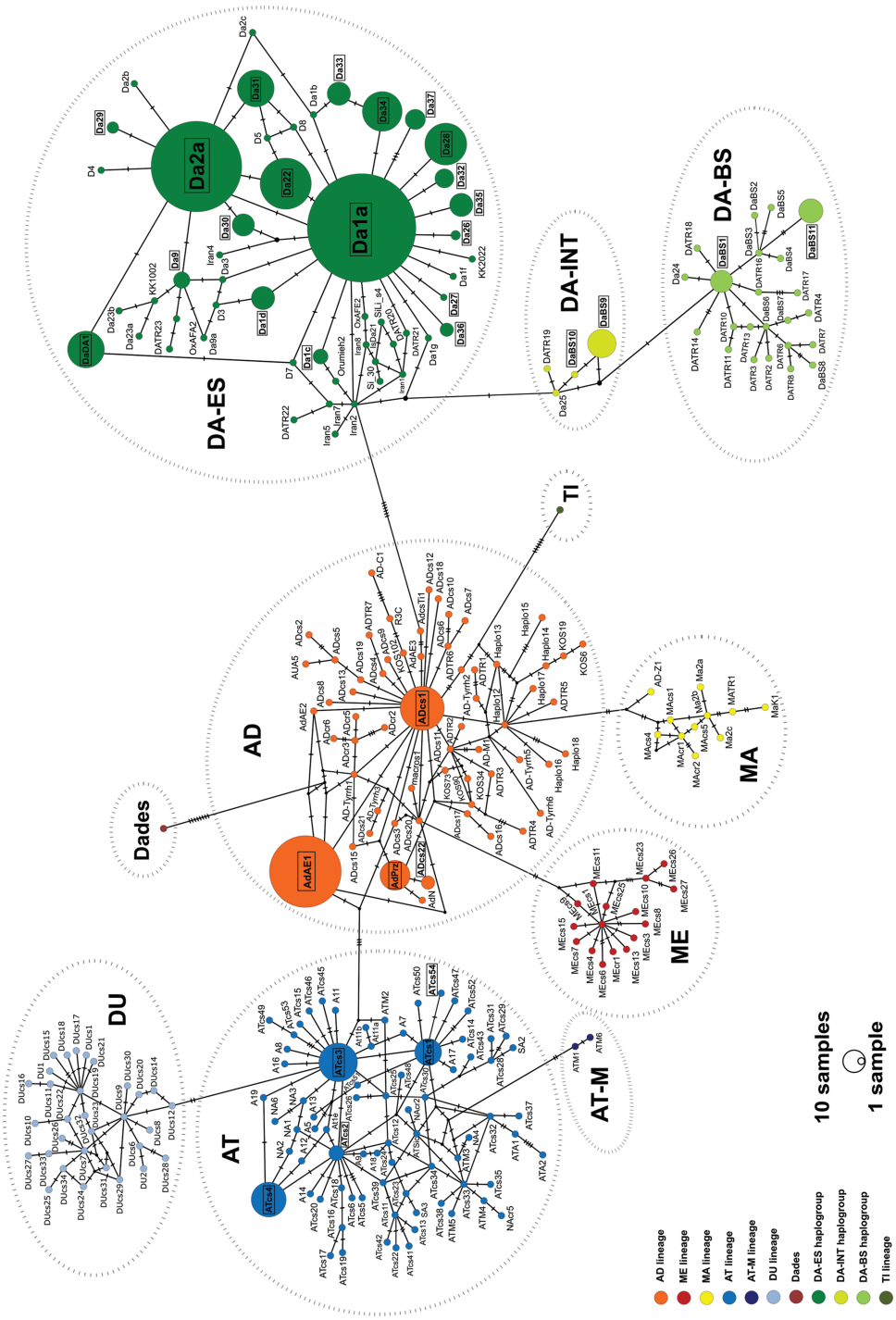

FIGURE 2 Median-joining network of CR mtDNA sequences. Haplotypes are represented by colored circles (supplementary table S1), with individual colors corresponding to the different lineages. The haplotypes found in the present study are framed, and for those, the size of the circles is proportional to the haplotype frequencies in the sample. Mutations are represented by hatch marks on the lines connecting the haplotypes. Missing or theoretical haplotypes are shown as black dots.

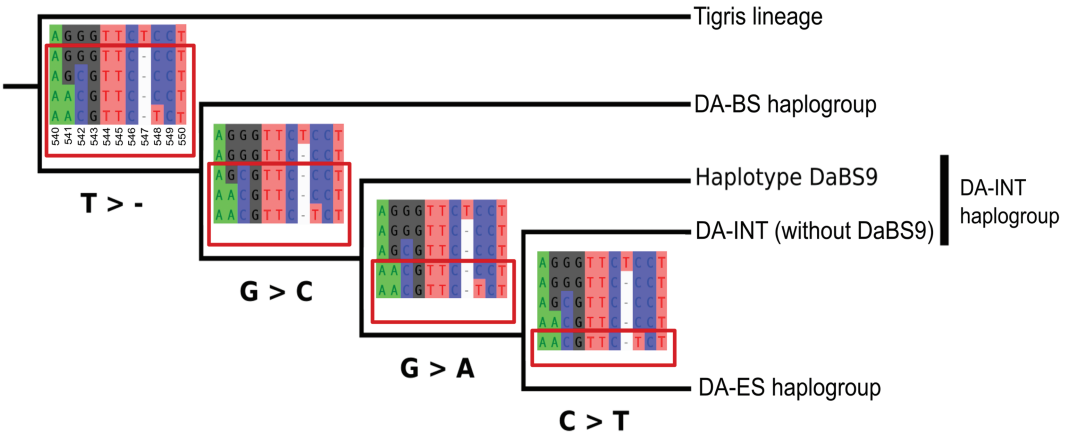

FIGURE 3 Reconstruction of the sequence evolution in the Danubian lineage. Defining variable nucleotide sites in the control region are all placed nearby in the central part of the control region, between the nucleotide positions 540-550 of our alignment. The 542 G → C transversion defines the split of the DA-ES + DA-INT and the DA-BS; 541 G → A split of the DA-ES + DA-INT (excluding DaBS9) from the DaBS9 haplotype; and 548 C → T defines the DA-ES.

In the present study, 32 haplotypes were detected in Serbia, of which 11 have not been previously described (i.e., Da29 to Da37 found in DA-ES, ADcs22 and ATcs54; fig. 1, supplementary table S4). On the haplotype network, the 'Serbian' haplotypes appeared within each of the three Danubian haplogroups as well as in the Adriatic and Atlantic lineages (fig. 2).

Within the DA-ES haplogroup, the most common haplotype was Da1a, followed by Da2a and Da22, while DaBS11 was the most common haplotype within DA-BS, and DaBS9 within DA-INT. The most frequent AD haplotype was AdAE1, followed by ADcs1, both being detected in the Aegean basin as well as in the Danube drainage. Inside the AT lineage, the four haplotypes (ATcs1 to ATcs4) known from previous studies as commonly used in hatchery commercial strains, prevailed. AT haplotypes were identified at 12 Danubian locations, while AD haplotypes were detected at 11; one of the later locations included the newly described ADcs22 haplotype. In general, the five most abundant haplotypes were detected in 64% of Serbian samples (Da1a – 25%, Da2a – 18%, AdAE1 – 11%, Da22 – 6%; ADcs1 – 4%; supplementary table S4). Most haplotypes were found in more than one river drainage (figs. 1, 2). In all three hatcheries, AT haplotypes, characteristic of hatchery-reared BT, were found; in the Panjica and Sokobanjska Moravica hatcheries, DA haplotypes were also detected (in ca. 25 and 30% of the examined specimens, respectively) (fig. 1; table 1, supplementary table S4).

Phylogeography and phylogenetic reconstructions of the brown trout complex

By plotting the DA haplotype data, obtained in the literature and the present study (supplementary tables S2 and S3) onto the map, the phylogeographic distribution of DA haplogroups was revealed (fig. 4). Considering the general distribution of DA haplogroups, DA-ES is the most widely spread. Its geographical distribution ranges from the Upper sections of the Danube drainage and eastwards to Afghanistan rivers draining into the Aral Sea and northern Russian rivers draining into the Caspian Sea. In addition, DA-ES is also present in the most eastern parts of the Black Sea basin and the southern Caspian Sea basin (fig. 4A). DA-BS is predominant in the non-Danubian Black Sea basin and rare in the Danube

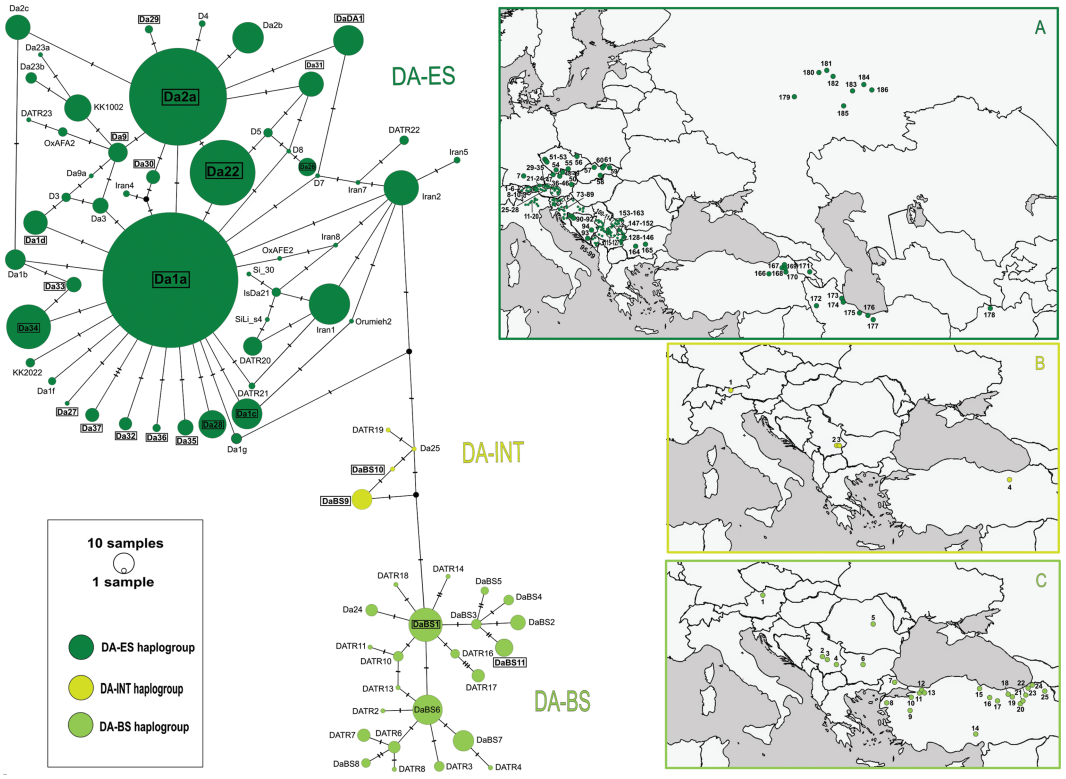

FIGURE 4 Median-joining network of CR mtDNA sequences belonging to Danubian haplogroups. Haplotypes are represented by colored circles whose size is proportional to haplotype frequencies detected and taken from the literature (supplementary tables S2 and S3). Haplotypes from this study are bolded and framed. Mutations are represented by hatch marks on the lines connecting the haplotypes. Missing or theoretical haplotypes are shown as black dots. The maps show the distribution of haplotypes from the network (A – DA-ES haplotypes, B – DA-INT haplotypes, C – DA-BS haplotypes), and their numbering corresponds to that in supplementary table S2.

drainage (one haplotype was recorded in a single population in the Upper Danube, two haplotypes in three populations of the Middle Danube, and five in two populations in lower sections of the Danube) (fig. 4C). DA-INT haplotypes are spread in a very diffuse way and are very rare. Namely, haplotypes DaBS9 and DaBS10 of this haplogroup were recorded only in the Džepska river system (Southern Serbia), while two others have been reported from a tributary to the Upper Danube in Austria and the eastern part of the Black Sea basin in Turkey (fig. 4B). The haplogroups overlap to a certain extent, which is most noticeable in Serbia, where all three were observed. On the contrary, only the DA-ES haplogroup appears in the Caspian River basin.

The topology of ML and BI phylogenetic trees was similar and in line with the patterns observed in the haplotype network, including the presence of the three haplogroups within DA. However, high PP and/or significant BS support was observed in only a few BT lineages (MA, ME, DA, DA-ES, DU) and in several smaller clades present within them (fig. 5, supplementary fig. S1).

The split decomposition approach revealed a similar pattern that clusters the haplotypes into 11 lineages/haplogroups, with splits primarily present near the centre of the tree-like

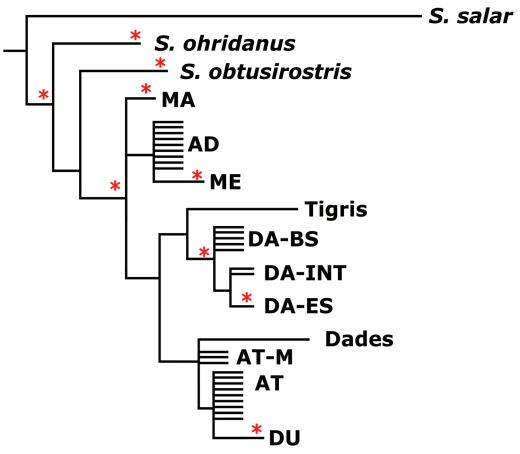

FIGURE 5 Outline of the phylogenetic tree as inferred in this study. Clades with ML bootstrap support > 50% and/or BI posterior probabilities > 90% are indicated with a red star. A detailed phylogeny with ML bootstrap support and BI posterior probabilities is available in supplementary fig. S1.

network (fig. 6). Lineages are further grouped similarly to phylogenetical trees (AD – MA – ME; DA-BS – DA-INT – DA-ES; AT – DU – AT-M). In general, branches leading to the different lineages were comparably long, except the two long branches that connected the divergent TI and Dades to the network center. Alternative arrangements were present within the base of some lineages, with several haplotypes that formed further groups within lineages (e.g., ATM2, and ATM7 within AT).

Time-calibrated molecular phylogeny of Salmo *spp.*

The divergence time estimation analysis based on the BT haplotypes and three other *Salmo* species resulted in a chronogram with similar topology to phylogenetic tree reconstructions. The coefficient of rate variation was moderate ($\bar{x}$ = 0.34) and its 95% credibility interval justified the selection of an uncorrelated relaxed clock as it did not approach zero (Ho et al., 2015). Difference between the chronogram using the birth-death and the Yule model were minimal, therefore only the results from the birth-death model are reported here. Time to most recent common ancestor (T_{MRCA}) of BT, *S. obtusirostris* and *S. ohridanus* was 4.76 Ma, while T_{MRCA} of BT can be dated to 3.97 Ma (fig. 7, supplementary table S5). This was followed by further diversification of the ancestral ME, AT, and DA lineages. The ancestral ME lineage began splitting into modern AD, ME, and MA by 3.44 Ma, while a similar scenario led to the split within the ancestral AT lineage that started to take place approximately at 3.23 Ma, which was followed by the separation between Dades and AT-M (3.03) and between AT and DU (2.62). The ancestral Danubian lineage (DA + TI) began diversifying at about the same time as other ancestral BT lineages, while diversification within the modern DA lineage was more recent. Thus, T_{MRCA} of DA-ES, DA-INT and DA-BS was dated to approximately 2.06 Ma, followed by diversification within DA-BS and the split between DA-ES and DA-INT at about the same time (i.e., ca 1.7 Ma).

Discussion

Time-calibrated molecular phylogeny of the trout Salmo *spp.*

In the present study, a multicalibration framework was used to infer a time-calibrated molecular phylogeny of trout *Salmo* spp. The chronogram was calibrated using two fossil calibration points, one (*S. immigratus*) placed at the base of the *Salmo* genus and the other (*S. derzhavini*) at the base of all the BT, together with a geological calibration point (the geological age of Lake Ohrid) placed at the coalescence of *S. ohridanus* (supplementary table S5). Based on the inferred chronogram, T_{MRCA} of *Salmo* was dated to the Late Miocene (11.39 Ma), while contrary to

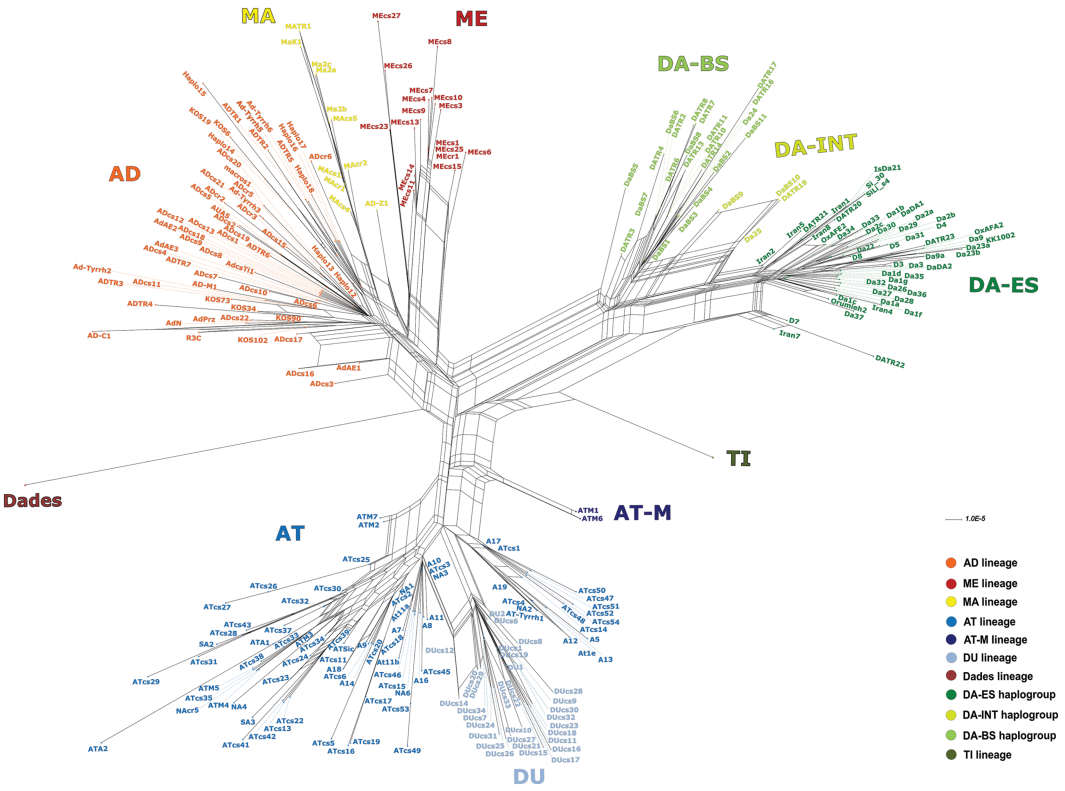

FIGURE 6 Split graph of the Neighbor-Net phylogenetic network analysis of brown trout lineages. The colors of the haplotypes represent different lineages, and the scale bar represents the nucleotide substitutions per site.

common belief, the major divisions within BT occurred in the Late Pliocene (3.44–3.22 Ma) and not in the Pleistocene (*sensu* Gibbard & Head, 2009, Gibbard et al., 2010). The latter was only supported if a two-point calibration scheme was used by excluding the *S. immigratus* fossil from the analysis (supplementary table S5). Moreover, calibration disregarding the *S. immigratus* fossil placed the origin of *Salmo* in the Early Pliocene (4.38 Ma). Obviously, the dating of diversification within BT is strongly influenced by the calibration time of the *Salmo* origin. Similar results were obtained by Crête-Lafrenière et al. (2012) and Lecaudey et al. (2018), who dated Salmonidae phylogeny using *Salvelinus* and *Oncorhynchus* fossils and placed the T_{MRCA} of the *Salmo* to the Middle Miocene (~13 Ma). In the study of Crête-Lafrenière et al. (2012) BT was represented only by *S. platycephalus* (AD lineage) and a lineage-undefined individual, while in the study of Lecaudey et al. (2018) BT was represented by only three lineage-undefined individuals and one *marmoratus*. Both studies dated the T_{MRCA} to the Early Pliocene (~3.4 and ~4.8 Ma, respectively). It should be noted that our time calibrated tree and that of Crête-Lafrenière et al. (2012) were based on mitochondrial DNA, while Lecaudey et al. (2018) based their analyses on nuclear DNA as produced by RAD-sequencing. Thus, two different and mutually independent marker systems gave similar time estimations of *Salmo* origin and BT diversification. Recently, Marić et al.

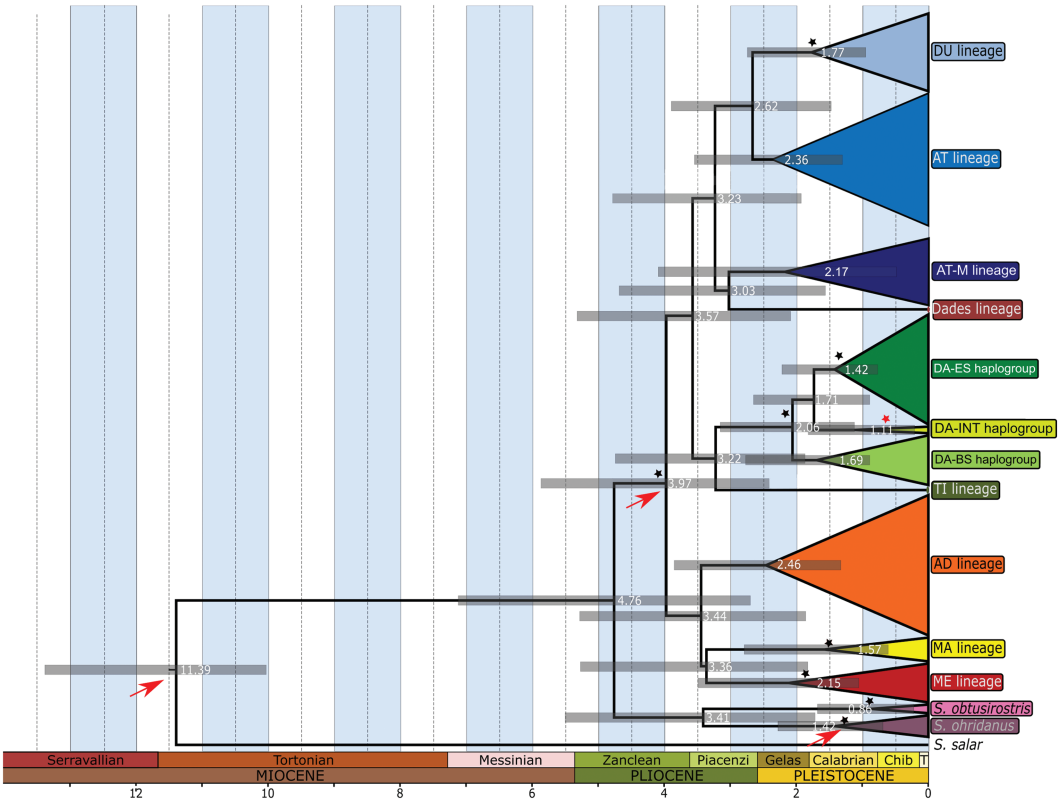

FIGURE 7 Fossil and geologically calibrated chronogram of the genus *Salmo* created with a relaxed clock in BEAST2. 95% highest posterior density (HPD) intervals are shown as gray bars at the nodes. Calibration points are indicated by arrows. Median node ages are shown as node labels. Time estimates are given in millions of years. Clades, that were *a priori* treated as monophyletic are indicated with a black star, while a red star indicates the clade, where posterior probability was > 90% only in BEAST analysis.

(2023) suggested that first divisions within BT could have occurred as early as Late Miocene with the DA lineage branching ~2 Ma, which fully corresponds with our chronogram (2.1 Ma, 95% HPD: 1.12–3.15). In their work, they used the Messinian salinity crisis (MSC) and the Lago Mare stage of the Mediterranean as a single geological calibration for molecular dating of the separation between the AD and DA lineage. This increases confidence in the obtained results and confirms the relevance of the *S. immigratus* fossil as a time calibration point for estimation of *Salmo* divergence times. Moreover, some of the major speciation events that shaped the European cyprinids also occurred in the Pliocene (Zardoya & Doadrio, 1999), as evidenced for *Telestes* (Buj et al., 2017) and *Phoxinus* (Reier et al., 2022). It is expected that many freshwater fishes share a common phylogeographic history and have similar radiation events, as this is tightly related to the biogeographical evolution of the attendant region (Sanz, 2018).

A Pliocene rather than a Pleistocene origin suggests that the major BT lineages may not have formed because of geo(hydro)morphological changes during glacial and interglacial periods, which in the Mediterranean region and Europe occurred between 2.8 and 2.5 Ma (Gibbard et al., 2010), but may be the result of orogeny and drainage evolution. The course and extent of the main systems that drain the Alps changed considerably over time. Between the Late Miocene and Early

Pliocene, the Alps, including the Central Alps (Swiss Alps), were predominantly drained by the Rhône-Aare-Danube rivers, which flowed eastward into the Pannonian Lake. At this time, the proto-Doubs drained the northern Jura and flowed into the Mediterranean, while the proto-Rhine was only a small stream flowing into the North Sea. In the Early Pliocene, the paleo-Aare and paleo-Rhône separated from the Danube and joined the proto-Doubs, draining the Central Alps into the Mediterranean. At this time, the paleo-Danube drained the Eastern Alps, while the Rhine extended its drainage area southward into the Molasse basin. In the Early Pleistocene, the Rhine captured the Aare and Oberrhein and extended its range into the Central Alps (after Khondkarian et al., 2004a, b; Berendsen, 2005; Popov et al., 2006; Reiter et al., 2015).

Assuming a Pliocene origin of the major BT lineages, ancestral BT populations might have originated somewhere in the Parathetys Sea/Pannonian Lake drainage, including paleo-Danube, and crossed into the Mediterranean during the Early Pliocene after the Aare and Rhône separated from the paleo-Danube and joined the Doubs (Aare-Doubs) river system (1 – fig. 8). This is in line with the suggestion of Osinov & Bernatchez (1996) that the Ponto-Caspian basins served as the origin for the BT complex. Alternatively, ancestral BT could have also crossed into the Mediterranean during its Lago-Mare phase, when fresh or low-brackish water was occasionally drained from Paratethys/Pannonian Lake into the Mediterranean (Bianco, 1990; Stoica et al., 2016; van Baak et al., 2017) or even during the Zanclean floods when the Mediterranean Sea was refiled by marine water from the Atlantic via Gibraltar (Van Couverin et al., 2000) and to a lesser extent possibly also with water from the Parathetys/Pannonian basin via the Balkans as proposed by Marić et al. (2023). This would place the split between the ME+AD+MA and AT+DA into Miocene, such a scenario is not contradicted by our molecular dating, where the T_{MRCA} of *S. trutta* complex is dated to 3.97 Ma, but the 95% HPD stretch from 2.34 to 5.88. However, the findings of several authors (Snoj et al., 2011, 2021; Berrebi et al., 2013; Sanz, 2018), along with our own, suggest that the BT lineage originated in the Eastern Mediterranean basin, possibly in the Balkans (2 – fig. 8). According to patristic distances within the phylogenies, AD and some MA haplotypes are closest to *S. obtusirostris* and *S. ohridanus*. Furthermore, the ancestral position of AD is also indicated by the central position of this lineage in the network and in the splits-tree analysis, where some of its haplotypes are closest to the centre. Using a set of nuclear markers, Snoj et al. (2021) recently demonstrated that the basal split within BT was the one that separated Atlantic trout from the Mediterranean, while DA is nested between the ME and AD lineages.

This could mean that BT colonized the Central Alps only after the formation of the Aare-Doubs river system (3 – fig. 8). From here, they could cross into the Atlantic basin when the Rhine reached the Alps in the Early Pleistocene and captured its rivers (4 – fig. 8). Alternatively, colonization of the Atlantic basin along the Mediterranean coastline and via Gibraltar also cannot be dismissed (5 – fig. 8). According to the Mediterranean basin first hypothesis and the phylogeny of Snoj et al. (2021), the DA lineage could have emerged after colonization of the Caspian Sea basin from the Eastern Mediterranean. A such crossing could have been possible via a presumed sea corridor in the upper Euphrates valley (6 – fig. 8; Chepalyga, 1995; Popov et al., 2006), or the crossing occurred in two steps. Firstly, BT colonized the paleo-Euphrates, which was possibly connected to the Mediterranean until the Middle Pliocene (Demir et al., 2007, 2008), and from here,

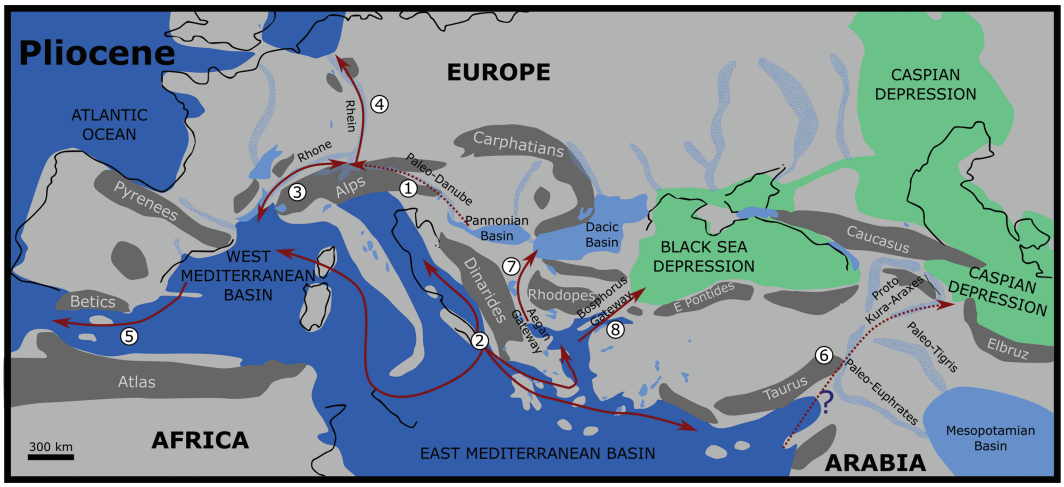

FIGURE 8 Simplified palinspastic map for the Pliocene (compiled and modified after Popov et al., 2004, 2006; Neubauer et al., 2015) with the indication of hypothetic BT colonization routes (arrows). Interrupted lines mark migration routes that are, in our opinion, less likely. Green areas represent brackish environments, light blue freshwater lakes or marshes and rivers, dark blue seas, dark gray mountain ranges, and light gray land mass; all geographic features are tentatively positioned. 1 – Ancestral trout originated in the Ponto-Caspian system and crossed from the paleo-Danube into the Western Mediterranean basin via stream capture of Alpine rivers in Pliocene; 2 – Ancestral trout originated in the Balkans basin and colonized other parts of the Mediterranean Basin from here; 3 – Colonization of the Central Alps took place in the Pliocene after the paleo-Rhône separated from the paleo-Danube and reached the Mediterranean; 4 – Atlantic basin was colonized when the Rhine captured Central Alpine rivers; 5 – Atlantic basin was colonized along the Mediterranean coastline and via Gibraltar; 6 – Ponto-Caspian basin was colonized from the Mediterranean basin following a presumed sea corridor in the upper Euphrates valley or using the paleo-Euphrates, which might have been connected with the Mediterranean until the Middle Pliocene; 7 – Ponto-Caspian basin was colonized via a possible Pliocene gateway that connected the Dacic basin and the Aegean Sea; 8 – Ponto-Caspian basin was colonized through the Bosphorus gateway.

they crossed to the Kura-Araxes river system (Caspian Sea basin) after a presumed stream capture, the Euphrates, Kura, and Araxes all originate in the Armenian Highlands, but their history and origins are largely unknown (Ayvazyan et al., 2019). The crossing into the Caspian Sea basin is also supported by the phylogenetic network, which shows that DA and AD are linked through the Iran2 haplotype, endemic and characteristic for the Caspian Sea (Vera et al., 2011) and the ADcsTi1 haplotype, which occurs in the Vardar basin (Aegean Sea) (Marić et al., 2017). However, the Vardar haplotype suggests another possible colonization root, through a possible Pliocene gateway, which connected the Aegean and the Dacic basin (7 – fig. 8) or through the Bosphorus gateway (8 – fig. 8; Clauzon et al., 2008). Each of these gateways could serve as a route for the crossing of BT from the Eastern Mediterranean into the Ponto-Caspian system, where the DA lineage formed over time.

Since Bernatchez et al. (1992) used variations in CR to identify evolutionary lineages of BT, this marker has become the single most established marker for the study of the phylogeographic history of BT. CR is sufficient to delimitate between major and minor BT lineages, but its resolution is limited, as it does not hold enough informative characters

(Tougard, 2022) to resolve higher hierarchical levels in the BT phylogeny and is also unable to detect hybridizations and reticulate evolution (Sušnik et al., 2007; Snoj et al., 2021). Recently, Sanz (2018) suggested that increasing sampling could be the key for finding intermediate or ancestral haplotypes that clarify obscure aspects of the phylogeny. In the present work, we have demonstrated that while increased sampling can be used to elucidate phylogeographic patterns (distribution of the DA-ES and DA-BS haplogroups) more clearly and even identify groups of intermediate haplotypes within BT lineages (DA-INT), it does not help with resolving lineage level polytomies, as already noted earlier (Snoj et al., 2011; Hashemzadeh Segherloo et al., 2012; Sanz, 2018). At the mtDNA level, those polytomies might be resolved by including additional mtDNA markers (e.g., the cytochrome c oxidase subunit I – *co1*, cytochrome b – *Cytb*; Crête-Lafrenière et al., 2012, Turan et al., 2020) or ideally entire mitogenomes (Guinand et al., 2021; Tougard, 2022). Nevertheless, only sets of nuclear genes (Pustovrh et al., 2014; Snoj et al., 2021) or ideally genome-wide datasets (e.g., RAD-seq, UCE) can also resolve putative hybridization among lineages (Hashemzadeh Segherloo et al., 2021).

Phylogeny and phylogeography of the DA

The complex hydrogeological history of the Ponto-Caspian system shaped the further evolution of the DA. Our phylogeographic results do not contradict the claim of Sanz (2018) that the isolation of the Black and Caspian basins favoured the allopatric divergence of DA-BS and DA-ES, which extended westward and eastward, respectively, and that the presence of DA-ES in the Balkans and the upper Danube is the result of secondary contacts that followed the last glacial maximum in this area. Moreover, our results are not entirely conclusive with respect to the claim (Kohout et al., 2013; Sanz, 2018) that DA-BS is ancestral to DA-ES. This is supported by our phylogenetic tree, chronogram, and split tree, but not by network analysis, according to which the DA-BS is the more divergent within the DA, as the linkage with the other haplogroups is through the DA-ES. These discrepancies could result from low sampling coverage within Turkey, where DA-BS is mostly distributed. Another discrepancy between our analysis and those of Kohout et al. (2013) and Sanz (2018) is that, due to expended geographical coverage, we identified an additional group of DA haplotypes occupying an intermediate position between DA-ES and DA-BS.

Our analysis clearly showed that DaBS9 and DaBS10, together with haplotypes Da25 and DATR19 (Baric et al., 2010; Özen, 2013), form a closely related haplogroup (DA-INT) that lies between the DA-ES and DA-BS (fig. 2). Such midpoint placement was also suggested by the ML and BI phylogenies (fig. 5). Despite low posterior probability values and insignificant bootstrap support, the general topology placing the newly defined haplogroup in an intermediate position was also supported by split decomposition, the method used to study conflicts in phylogenetic signals by drawing a phylogenetic tree-like network (fig. 6).

Emergence of these three haplogroups can be explained by the following reconstruction of the sequence evolution (fig. 3), which suggests that the 542 G → C transversion defined the separation of DA-ES + DA-INT from DA-BS, followed by the 541 G → A transition that separated the DA-ES + DA-INT (excluding DaBS9) clade from the DaBS9 haplotype, and finally by the 548 C → T transition that defined the DA-ES haplogroup. This pattern supports the classification of DA-INT as a transitional haplogroup

in respect to DA-ES and DA-BS, with the DaBS9 haplotype being closest to the DA-BS and most likely the ancestor of other DA-INT haplotypes. The number of observed synapomorphies in CR, defining the DA-INT haplogroup, is small but comparable to that between DU and AT or between ME and AD (Cortey et al., 2004; Vera et al., 2010, 2015). There is only a single synapomorphy (without observed back mutations) separating DU and AT, 713 C → T, whereas 904 G → A and 907 A → G support the same topology, except for one back mutation present in some haplotypes. It is unlikely that the detected molecular synapomorphies supporting division of the DA lineage in three haplogroups are artefacts, as they have been detected by independent researchers (Marić et al., 2006; Baric et al., 2010; Özen, 2013).

It is difficult to draw definite conclusions about the phylogeography of DA-INT because only four localities with haplotypes belonging to this haplogroup have been recorded – Da25 in the Inn river system in the upper part of the Danube drainage (Baric et al., 2010), DATR19 in the Çoruh river system of the Black Sea basin (Özen, 2013), and DaBS9 and DaBS10 in two streams of the South Morava system in the middle part of the Danube drainage (Kohout et al., 2013; Marić et al., 2022; present study). This disjunct distribution could simply be the result of patchy geographic sampling, as full CR sequences are scarce in the lower Danube and other European rivers of the Black Sea basin. Current DA-INT distribution data suggest that it is more abundant in the Danube drainage above the Iron Gates, indicating that this area was colonized in at least two waves, as previously suggested by Marić et al. (2022). Molecular dating suggests that this haplogroup formed in the Early Pleistocene, which roughly coincides with the final arrival of the paleo-Danube in the Black Sea (1.9–1.8 Ma; Matoshko et al., 2019; Krézsek & Olariu, 2021).

Phylogeography and genetic structure of brown trout in Serbia

Our study represents the most comprehensive phylogeographic analysis of BT in the Balkans. The expanded geographic coverage and sampling in Serbia provide new insights into the phylogeny and phylogeography of BT and have yielded nearly twice as many haplotypes overall as previously reported for this country (Marić et al., 2006, 2022; Tošić et al., 2014, 2016; Simonović et al., 2017; Škraba Jurlina et al., 2020; Kanjuh et al., 2021). Haplotype diversity of the DA lineage in Serbia is very high when compared to other regions in the Danube drainage (Duftner et al., 2003; Baric et al., 2010; Kohout et al., 2013; Ősz et al., 2018), which is reflected by the presence of haplotypes from DA-ES and DA-BS, as well as the newly described DA-INT.

The present study confirmed previous reports that BT from the Aegean and Drim-Skadar river system (i.e., Adriatic river basin *sensu lato*) were used for stocking in Serbia probably even before the arrival of the Atlantic domesticated line. This was especially evident in the South Morava river system with the highest concentration of Aegean haplotypes, which is probably a result of stocking in the late eighties with BT originating from the nearby Treklyanska River (Aegean river system) in Bulgaria (for details, see Marić et al., 2022). The same applies to the haplotypes relating the Drim-Skadar river system: these were found in Serbia in some direct tributaries to the Danube as well as in the Aegean river system (i.e., in Tripušnica River), where they were most likely introduced by stocking during the 1970s (Marić et al., 2006, 2017).

In previous studies, AT haplotypes were rarely detected in Serbia (Marić et al., 2006, 2022; Simonović et al., 2017). In the present study, however, we discovered an increase in AT haplotypes, which were found in as

many as a quarter of all examined localities in the wild in the Danube drainage in Serbia. Compared to the high introgression of local BT with Atlantic genes elsewhere in Europe, this seems small. But the finding of concern is the strong prevalence of AT haplotypes in all three hatcheries producing stocking material. Stocking is a common and fast rising practice in Serbia (Marić et al., 2006, 2022), so it is likely that introgression with Atlantic genes will continue to increase unless the current BT management strategy is changed. Since DA haplotypes are also present in the hatcheries, as demonstrated in the present study (supplementary table S4), the actual proportion of locally present haplotypes in the wild is difficult to assess. For example, the native origin of the Da2a haplotype, known as native and widespread in the Upper Danube tributaries in Germany, Slovenia (Bernatchez, 2001) and Austria (Weiss et al., 2001), was previously questioned for Serbia (see Simonović et al., 2017). We found this haplotype to co-occur with highly prevailing AT haplotypes in the hatcheries that breed BT for stocking (supplementary table S4). Nevertheless, Da2a was the second most frequent in Serbia, and was also found in many streams where no AT haplotypes were detected (e.g., in localities 1, 4, 6, 45 and supplementary table S4). This finding does not support a non-indigenous origin of Da2a in Serbia but rather suggests its natural presence there and thus, suggests that the brood-stocks kept at the hatcheries are a mixture of Atlantic and native BT. Other haplotypes (i.e., Da28, Da30, Da33 and Da36), which have been observed only in Serbia, both in wild populations and hatchery brood-stocks (supplementary table S4), further indicate the use of the local Danube BT in artificial breeding.

In Serbia, the DA-ES haplogroup is predominant throughout the entire Danube drainage, deviating from this pattern only in a few locations. In the two locations of the West Morava river system, haplotypes of the DA-BS were detected alongside DA-ES, while in the South Morava system, the Vrla River hosted only DA-BS haplotypes along with the translocated AD haplotypes (Marić et al., 2022). Notably, all three DA haplogroups were recorded in the South Morava river system in the area of the Vlasina Plateau, suggesting successive waves of colonization. This genetic architecture is not surprising given that the Vlasina Plateau appears to be distinct in respect to its geomorphological origin (Dimitrijević, 1995; Knežević-Djordjević & Krstić, 1996, Marić et al., 2023) and very diverse with interesting biogeographic and phylogeographic patterns (Stevanović et al., 1995; Marić et al., 2006; Sotiropoulos et al., 2007; Tot et al., 2015). Due to the limited availability of CR sequences from the lower Danube and other European rivers of the Black Sea basin, it is impossible to say whether DA-BS and DA-INT haplotypes from Vlasina are associated with a larger population or is this genetic signature a remnant of a limited colonization wave. To address this question, future research should focus on the phylogeography of BT in the Eastern Balkans, ideally relying on genome-wide datasets that should be able to detect the exchange of genes between populations that arrived with different colonization waves.

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Supplementary material

Supplementary material is available online at: https://doi.org/10.6084/m9.figshare.22726835

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