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Evolutionary relationships among several species from the genus *Cricotopus* (Diptera: Chironomidae): What about Turkish representatives of this genus?

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Abstract: The genus *Cricotopus* (Diptera: Chironomidae) has a worldwide distribution and possesses a wide variation in ecology and habitat preference across its distribution. Identification of *Cricotopus* species and distinguishing them from the species of closely related genera (i.e. *Orthocladius* and *Paratrichocladius*) are generally difficult. This study aimed to investigate the evolutionary history of several *Cricotopus* species, with the main focus on *C. bicinctus*, *C. flavocinctus*, *C. triannulatus*, *C. patens*, *C. intersectus*, *C. laricomalis*, *C. ornatus*, *C. reversus*, and *C. sylvestris* from Turkey, using the mitochondrial cytochrome c oxidase subunit I (COI) gene. Molecular classifications of Turkish *Cricotopus* species were full compatible with the morphological species identity, suggesting the adequacy and practicability of existing larval keys for identifying these specimens o species level. The sampled Turkish *Cricotopus* species with two or more representatives were found to produce monophyletic groups. Diversification time estimates showed that the diversification of *Cricotopus* was initiated in the late Cretaceous. Additionally, these estimates suggested a distinct continental structuring in the evolution of some species and a possible origination of the sampled Turkish representatives of this genus from North European representatives. Based on the results of this study, the COI gene is concluded to be useful both for species identification in the genus *Cricotopus* and for estimating their evolutionary relationships. This study provides valuable information on the evolutionary relationships among *Cricotopus* species. However, to obtain more accurate and/or persuasive results, an intensive and targeted sampling from more locations and the inclusion of more sequence data from not only the mitochondrial COI gene but also additional nuclear and/or mitochondrial markers are highly recommended for further studies.

Key words: COI, *Cricotopus,* divergence time, phylogenetic analysis, Turkey

1. Introduction

Insects, the most diverse group of animals on our planet, are found in a wider variety of habitats than most other complex organisms (Bode, 2009). Chironomids (Diptera: Chironomidae), known popularly as non-biting midges, are the freshwater insect family comprising the highest number of species both in lentic and in lotic habitats (Cranston, 1995). As chironomids have undergone extensive adaptive radiation to occupy a wider range of microhabitats at present than any other aquatic insect group (Jacobsen, 2008), they are widely distributed and ecologically adapted to a great variety of environments and occur in all zoogeographical regions, even Antarctica. The larvae generally develop in freshwater, but a limited number of marine and terrestrial species exist as well. Many chironomid species exhibit a wide range of tolerance to harsh environmental conditions, such as hypoxia, desiccation, high salinity, low temperatures, and low pH values (Pinder, 1986; Armitage et al., 1995); however, it is also known that this family has several sensitive species to environmental gradients.

For many years, chironomids have received attention from researchers worldwide due to their outstanding abilities as biological indicators of environmental conditions (Pinder, 1986). The aquatic larvae of the non-biting midges are also of ecological importance and commercial interest, since they hold an important position in the aquatic food chain and are one of the major food sources for fish and other vertebrates and invertebrates (Cranston, 1995).

During the last decades, remarkable advances have occurred in DNA sequencing technologies, bioinformatics, and computational biology, enabling researchers to obtain a great quantity of molecular data and to improve the tools for analysing these data. With the help of these advances, many phylogenetic studies on various groups within the family Chironomidae have been recently conducted to explore the relationships among subfamilies,

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tribes, genera, and species (e.g., Krosch and Cranston, 2013; Sari et al., 2015; Silva and Ekrem, 2016; Lin et al., 2018). Although phylogenetic relationships of different genera of the subfamily Orthocladiinae are addressed in some of these studies (Cranston et al., 2010; Krosch et al., 2011; Sari et al., 2012, 2015; Krosch and Cranston, 2013; Kravtsova et al., 2014), there are comparatively few studies on the evolutionary relationships among *Cricotopus* species whose identification on the basis of morphological characters is generally difficult. However, there are no studies in the literature that deal directly and solely with the evolutionary relationships of the most of *Cricotopus* species including Turkish representatives. As a result of this absence, the origin of Turkish representatives of *Cricotopus* species remains unclear.

The present study aimed to investigate the evolutionary history of several *Cricotopus* species, with a main focus on the species collected from Turkey, using the mitochondrial cytochrome c oxidase subunit I (COI) gene, to test the adequacy of existing larval keys for identifying these specimens to species level, and to probe the importance of Turkey in their distribution.

2. Materials and methods

2.1. Study site and specimen collection

Chironomids are holometabolous insects; their immature larval and pupal stages are generally subservient to aquatic habitats and develop in freshwater (Karima, 2021). Since one of the aims was to test the adequacy of existing larval keys for identifying *Cricotopus* individuals to species level, the larval stage was targeted in this study. Larvae were collected from 10 different locations (i.e. lakes or ponds) across Turkey (Figure 1). For collecting the larvae, two sampling methods were used: (1) picking larvae directly from stones and plants in the water using fine forceps and (2) sieving plant debris in the water such as leaves and branches using a special sieve with a mesh size of 500 µm and picking larvae from the sieve using fine forceps.

All collected larvae were field sorted, transferred immediately to 96% ethanol, and transported to Pamukkale University. Morphological features of all collected larvae, such as segment number, existence of ventral and lateral gills, and morphological characters of gills, were examined under a binocular microscope in Hydrobiology Laboratory for morphological identification. Based on these examinations, species of each examined larva was identified. Their heads were then dissected, and the remaining body sections of the larvae were preserved in 96% ethanol for subsequent DNA extraction. Species identification was validated via examination of head capsule morphology under a binocular microscope using the taxonomic keys of Hirvenoja (1973), Cranston (1979), Cranston et al. (1983), Moller Pillot (1984), and Schmid (1986). All individual dissected heads of the specimens belonging the genus *Cricotopus* were mounted on slides in Euparal to obtain permanent slides as previously described (Brooks et al., 2007), and these slides were stored in the laboratory archives.

2.2. DNA extraction, PCR amplification, and sequencing

Total genomic DNA was extracted from larval body tissues as previously reported (Sari et al., 2015). Fragments of the mitochondrial COI gene were amplified using the primer pair 911 (5'-TTTCTACAAATCATAAAGATATTGG-3') and 912 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') reported by Guryev et al. (2001).

Polymerase chain reactions (PCR) were carried out in a total volume of 25 µL, containing 1X PCR Buffer, 1.5 mM $MgCl_2$, 300 µM dNTP, 0.4 µM of each primer, 5 U of *Taq* polymerase and 6 µL of template DNA. Amplifications for the COI region were performed in a thermocycler with an initial denaturation step of 5 min at 95 °C, followed by 40 cycles of a 45 s denaturation at 94 °C, a 90 s annealing at 53 °C, and a 120 s extension at 72 °C, and a final extension step of 6 min at 72 °C. PCR products were confirmed by electrophoresis on a 1% agarose gel with ethidium bromide and then purified using PureLink Quick PCR Purification Kit (Invitrogen, Germany) for sequencing according to the manufacturer's protocol. The purified PCR products were sequenced by Macrogen Europe (Amsterdam, the Netherlands) in both directions using the same primer pair (911 and 912). All obtained sequences were deposited in GenBank (accession numbers MZ948968-MZ949001). Details of these sequences are outlined in Table 1.

2.3. Data analyses

A total of 34 COI sequences representing nine species from the genus *Cricotopus* were obtained. In addition, 72 COI sequences representing 21 species from the genus *Cricotopus*, which were randomly selected to represent as many species of the genus as possible, and two COI sequences belonging to *Orthocladius* sp., which was selected as outgroup to root generated phylogenetic trees, were retrieved from GenBank for phylogenetic analyses (Table 2). A total of 658-bp nucleotide sequences of the mitochondrial COI region of 108 individuals (including outgroup) were aligned by ClustalW using MEGA 6 (Tamura et al., 2013) and MEGA X (Kumar et al., 2018) and checked by eye. Nucleotide saturation was determined by plotting the absolute number of transitions and transversions against genetic distance values with a GTR model using DAMBE software (Xia and Xie, 2001), and the quality of the phylogenetic information was evaluated. Mean pairwise intra- and inter-specific genetic distances of *Cricotopus* species were calculated in MEGA X using Kimura 2-parameter (K2P) substitution model (Kimura, 1980).

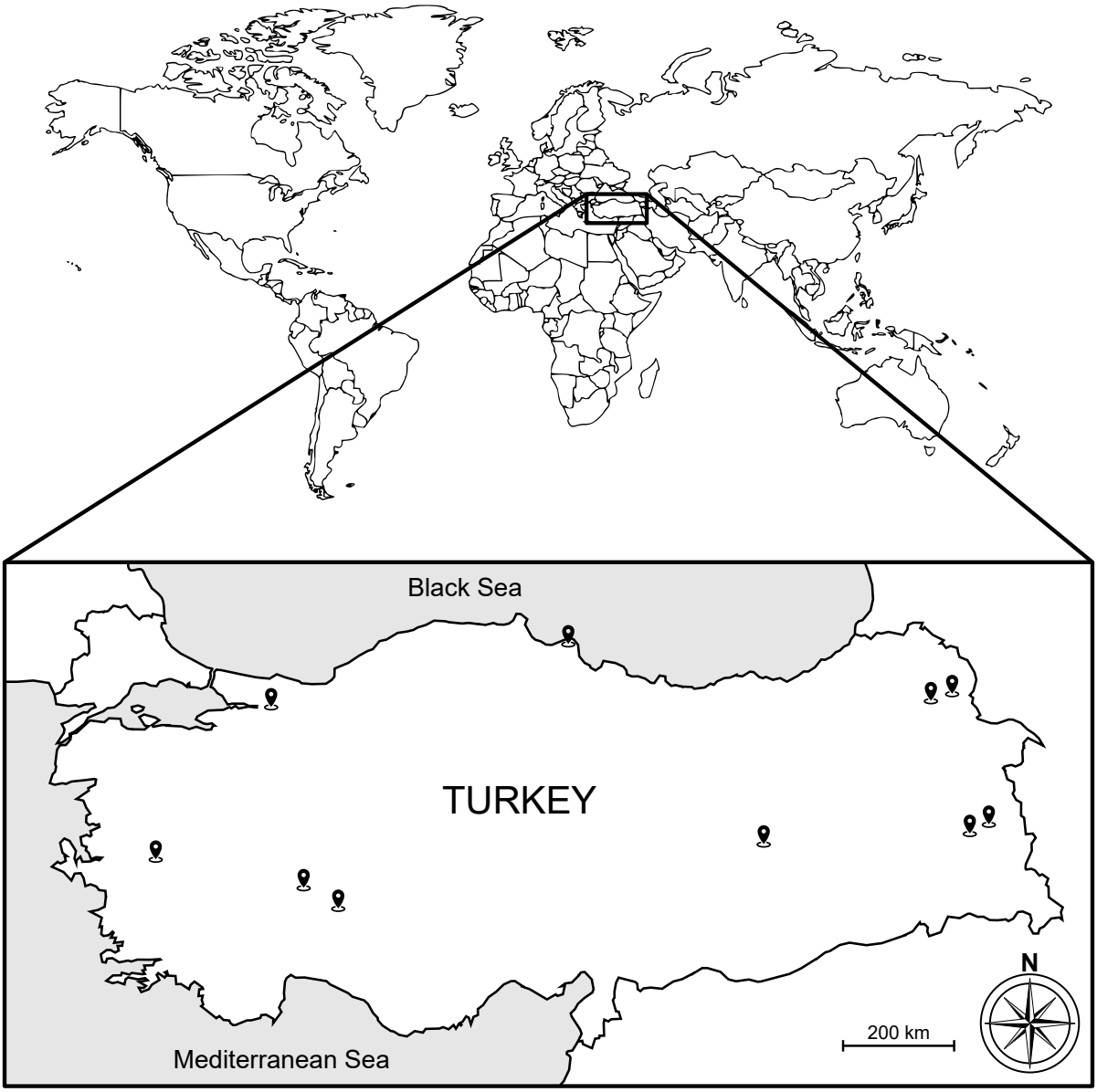

Figure 1. Sampling localities of the collected *Cricotopus* specimens in this study.

For phylogenetic reconstruction, maximum likelihood (ML) and Bayesian inference (BI) were used. The ML reconstruction was performed in RAXMLGUI 1.0 (Silvestro and Michalak, 2012) using general time reversible model with a gamma distribution and proportion of invariable sites (GTR + Γ + I) with 1000 bootstrap replicates. The BI was performed in MrBayes v3.2.5 (Ronquist et al., 2012) under the GTR + Γ + I model of sequence evolution. Two simultaneous runs of four chains were performed for 10 million generations. Convergence of the BI analysis was maximised by ensuring that the average standard deviation of split frequencies fell below 0.01 and that potential scale reduction factors approached 1.0 for all parameters. Produced trees were visualized and edited using FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). Further, SplitsTree4 v4.14.2 (Huson and Bryant, 2006) was used to reconstruct a neighbour-net phylogenetic network for *Cricotopus* species using the default settings in the software with 1000 bootstrap replicates.

Divergence times to most recent common ancestor (tmrca) for relevant nodes of the molecular dataset were estimated using BEAST package v1.8.2 (Drummond et al., 2012). Input files for BEAST v1.8.2 were created with BEAUTi v1.8.2 (part of the BEAST package). The root height of the tree was calibrated following the root height calibration reported by Krosch and Cranston (2013).

Table 1. List of the sequences comprising their GenBank accession numbers and specimen information obtained in the present study.

Genus	Subgenus	Species	Specimen voucher	Location	Accession number
Cricotopus	*Cricotopus*	*Cricotopus bicinctus*	PUHL-405	Konya/Turkey	MZ948968
			PUHL-528	Isparta/Turkey	MZ948969
			PUHL-523	Isparta/Turkey	MZ948970
			PUHL-411	Konya/Turkey	MZ948971
			PUHL-406	Konya/Turkey	MZ948972
			PUHL-518	Isparta/Turkey	MZ948973
			PUHL-421	Konya/Turkey	MZ948974
		Cricotopus flavocinctus	PUHL-823	Samsun/Turkey	MZ948975
			PUHL-832	Samsun/Turkey	MZ948976
		Cricotopus triannulatus	PUHL-476	Konya/Turkey	MZ948977
			PUHL-478	Konya/Turkey	MZ948978
			PUHL-542	Isparta/Turkey	MZ948979
			PUHL-130	Ardahan/Turkey	MZ948980
		Cricotopus patens	PUHL-494	Konya/Turkey	MZ948981
			PUHL-507	Isparta/Turkey	MZ948982
	Isocladius	*Cricotopus intersectus*	PUHL-445	Konya/Turkey	MZ948983
			PUHL-451	Konya/Turkey	MZ948984
			PUHL-581	Isparta/Turkey	MZ948985
			PUHL-590	Isparta/Turkey	MZ948986
		Cricotopus laricomalis	PUHL-463	Konya/Turkey	MZ948987
		Cricotopus ornatus	PUHL-251	Van/Turkey	MZ948988
		Cricotopus reversus	PUHL-135	Ardahan/Turkey	MZ948989
			PUHL-141	Ardahan/Turkey	MZ948990
			PUHL-015	Kars/Turkey	MZ948991
		Cricotopus sylvestris	PUHL-003	Kars/Turkey	MZ948992
			PUHL-115	Ardahan/Turkey	MZ948993
			PUHL-108	Ardahan/Turkey	MZ948994
			PUHL-105	Ardahan/Turkey	MZ948995
			PUHL-317	Elazığ/Turkey	MZ948996
			PUHL-244	Van/Turkey	MZ948997
			PUHL-430	Konya/Turkey	MZ948998
			PUHL-560	Isparta/Turkey	MZ948999
			PUHL-718	Sakarya/Turkey	MZ949000
			PUHL-651	Manisa/Turkey	MZ949001

Accordingly, a lognormal prior was used with the following parameters: a zero offset of 33.7 million years (my) that corresponded to the lower age bound of the oldest fossil *Cricotopus* member (Meunier, 1904); a mean of 121 my, representing the oldest fossil orthoclad (*Lebanorthocladius furcatus*) (Veltz et al., 2007); and a standard deviation of 0.5 so that the 97.5% highest posterior density (HPD) encompassed the oldest fossil chironomid (*Aenne triassica*) (Krzeminski and Jarzembowski, 1999) at 209.6 ± 1 million years ago (mya). The tree prior was set to 'Speciation: Yule Process', and substitution rates were allowed to vary across branches in compliance with a relaxed lognormal molecular clock prior (Drummond et al., 2006). Runs were performed using GTR + Γ model of evolution. The analysis was run for 50 million generations, sampling trees every 10000 generations, from which five million generations

Table 2. List of the sequences obtained from GenBank and used in the present phylogenetic analyses.

Genus	Subgenus	Species	Specimen voucher/isolate	Country	Accession number
Cricotopus	*Cricotopus*	*Cricotopus annulator*	Finnmark125	Norway	HQ551535
			Finnmark231	Norway	JF870877
		Cricotopus bicinctus	NIESH0401	Japan	LC329069
			Finnmark262	Norway	JN275394
		Cricotopus coronatus	Finnmark475	Norway	JN275512
			Finnmark476	Norway	JN275513
			BIOUG<CAN>:10PROBE-10663	Canada	JF875216
			BIOUG<CAN>:10PROBE-10923	Canada	JF875449
			HLC-27203	Canada	KR438004
		Cricotopus curtus	ATNA376	Norway	HM421475
		Cricotopus festivellus	Finnmark472	Norway	JN275510
			Finnmark705	Norway	JN275568
		Cricotopus fuscus	CHARS00247-C01	Canada	MN673037
		Cricotopus gelidus	SV62	Norway	MT047657
			SV69	Norway	MT048184
			SV306	Norway	MT048239
		Cricotopus lestralis	SV215	Norway	MT048052
			SV230	Norway	MT048072
		Cricotopus pilosellus	BJ143	Norway	HM405974
			BJ162	Norway	HM405993
			Finnmark133	Norway	HQ551542
			Finnmark603	Norway	JF870961
			BIOUG03536-A10	Canada	KM994787
			HLC-27222	Canada	KR435425
		Cricotopus polaris	Finnmark471	Norway	JN275509
			BIOUG17940-C08	Canada	KR616403
		Cricotopus pulchripes	Finnmark163	Norway	JF870811
			Finnmark253	Norway	JN275392
		Cricotopus tibialis	SV92	Norway	MT047637
			SV 806	Norway	MT047694
			BJ128	Norway	HM405959
			ATNA258	Norway	HM421368
			Finnmark513	Norway	JN275533
			BIOUG<CAN>:10PROBE-10876	Canada	JF875405
			20117-A10	USA	KF000114
			20117-B3	USA	KF000113
		Cricotopus tremulus	BIOUG05599-A06	Canada	KR767223
			08-SWRC-0238	USA	JF286802
		Cricotopus trifascia	BIOUG<CAN>:10BBDIP-1203	USA	JF871088
			BIOUG24152-G03	Canada	MG305661
			BIOUG23893-F07	Canada	MG306493

Table 2. (Continued).

			BIOUG23658-B02	Canada	MG309065
			10-SCCWRP-0024	USA	HQ938414
			10-SCCWRP-1787	USA	HQ939850
			HT8	Sweden	KC250798
		Cricotopus vierriensis	BIOUG00860-C06	Canada	KR666517
			BIOUG07899-A04	Canada	KR739686
	Isocladius	*Cricotopus glacialis*	SV-pit87	Norway	MT047763
			SV72	Norway	KC130754
			SV319	Norway	KC130767
			IS41	Iceland	KC130781
			IS45	Iceland	KC130792
		Cricotopus obnixus	CHARS00030-F05	Canada	MN666060
			CHARS00044-A10	Canada	MN675692
			BIOUG<CAN>:09PROBE-02177	Canada	GU680193
			BIOUG<CAN>:HLC-26524	Canada	HM860154
			10PROBE-10028	Canada	KR669756
			HLC-27245	Canada	KR425934
			20224-A7	USA	KF000155
		Cricotopus ornatus	Finnmark156	Norway	JF870805
			HI3	Sweden	KC250779
			HJ2	Denmark	KC250781
			Finnmark635	Norway	KC130766
		Cricotopus perniger	Finnmark203	Norway	JF870850
			BIOUG05469-A04	Canada	KM632594
			BIOUG05470-G02	Canada	KM645632
		Cricotopus tricinctus	BIOUG03083-A06	Canada	KM988553
			BIOUG07952-D07	Canada	KR683637
			CHIR_CH102	Canada	KC130751
			BIOUG<CAN>:09BBEDI-2668	Canada	HQ551927
		Cricotopus trifasciatus	NIESH0667	Japan	LC329078
			NIGLAS DYH-4A	China	KP902812
Orthocladius	-	*Orthocladius sp.*	CHARS00026-D12	Canada	MN677482
			CHARS00030-E09	Canada	MN669966

were removed, producing 45 million generations. The results were analysed using Tracer v1.6 (Rambaut et al., 2018) to examine BEAST log file and effective sample sizes (ESSs) for all parameters. A value of ESS greater than 200 was acknowledged as a good indicator of convergence. Finally, chronograms were produced from the stationary distribution by removing 10% of trees as burn-in prior to annotating the obtained tree file in TreeAnnotator v1.8.2 (part of the BEAST package) and visualizing it in FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results and discussion

Thirty-four *Cricotopus* representatives collected from 10 locations across Turkey were vouchered, identified, and sequenced for COI. For an aligned dataset of 658-bp nucleotide sequences of 108 individuals, 397 characters were conserved, 261 were variable, 11 were singleton, and 250 were parsimony informative. Mean base frequencies were A = 0.2694, C = 0.1704, G = 0.1697, and T = 0.3905. In addition, GC and GT percentages were 34.0088 and 56.0238, respectively. The transition/transversion ratio was

estimated to be 2.620, while the transition/transversion ratios for purines and pyrimidines were 4.880 and 4.276, respectively. It was determined that both transitions and transversions increased with increasing genetic distance (Figure 2) and that there was little saturation. Since the nucleotide saturation analysis provided compelling evidence that the selected COI sequences did not undergo substantial saturation, it was concluded that these sequences could be used for phylogenetic analyses in the study.

The intra-specific mutational distances ranged from 0 to 5.24% for the analysed species of the genus *Cricotopus*, with a mean of 1.50%. The number of nucleotide substitutions amongst the species varied from 1.09% to 24.40% (Table 3), with a mean of 17.54%. The intra- and inter-specific genetic distances found in the present study are comparable to similar studies in chironomids. Mean intraspecific divergences of 0.4%–1.4% for all analysed species of the genus *Orthocladius* (Kravtsova et al., 2014), 2.32% for all examined species from the genera *Cricotopus*, *Orthocladius*, and *Paratrichocladius* (Sinclair and Gresens, 2008), and 0.82% for different species from different chironomid genera (Brodin et al., 2013) have been reported. Mean interspecific divergences of 9%–19% for all analysed species of the genus *Orthocladius* (Kravtsova et al., 2014), 14.77% for all examined species from the genera *Cricotopus*, *Orthocladius*, and *Paratrichocladius* (Sinclair and Gresens, 2008), 13.87% for different species from different chironomid genera (Brodin et al., 2013), 14.9% for all analysed *Cricotopus*, *Paratrichocladius*, *Psectrocladius*, and *Rheocricotopus* species from the subfamily Orthocladiinae (Sari et al., 2012), and 19.4% for all studied species from the subfamilies Tanypodinae, Orthocladiinae, Chironominae (Sari et al., 2015). Although these values vary depending on the number of the studied species and the number of the studied individuals per species, it can be suggested that the low values of intra-specific and high values of inter-specific genetic distances are typical for chironomids but that a single threshold value for species identification is unfeasible.

Because the topologies were roughly concordant between two reconstruction methods, only the BI tree is presented, and the relevant nodal support values are shown in Figure 3. In the phylogenetic reconstructions, the subgenera *Isocladius* and *Cricotopus* were well differentiated; however, the group containing the representatives of the subgenus *Isocladius* (PP = 0.77, BS < 50) formed a clade with two specimens of *C. vierriensis* belonging to the subgenus *Cricotopus* (PP = 0.78, BS < 50). For the subgenus *Isocladius*, the consensus-tree topology indicated that the specimens of *C. sylvestris* from Turkey formed a group (PP = 0.77) and was sister to *C. glacialis* (PP = 0.91, BS = 86), with these two combined to form the sister to *C. trifasciatus* (PP = 1.00, BS = 100). This cluster of three was sister to a clade (PP = 0.68, BS < 50) where the specimen of *C. ornatus* from Turkey clustered with the other *C. ornatus* specimens (PP = 1.00, BS = 100) and was nested within a group that also contained *C. tricinctus* (PP = 0.95, BS = 80). The specimens of *C. intersectus* from Turkey formed a well resolved clade (PP = 1.00, BS = 95), and this clade was placed in a group with the specimen of *C. laricomalis* from Turkey (PP = 1.00, BS = 99). *C. reversus* specimens from Turkey formed a well resolved group (PP = 1.00, BS = 100), and this group was closely related to the above-mentioned group containing *C. intersectus* and *C. laricomalis* (PP = 0.98, BS = 93). This cluster of three was sister to a clade (PP = 0.76, BS < 50) containing *C. obnixus*. For the subgenus *Cricotopus*, *C. triannulatus* specimens

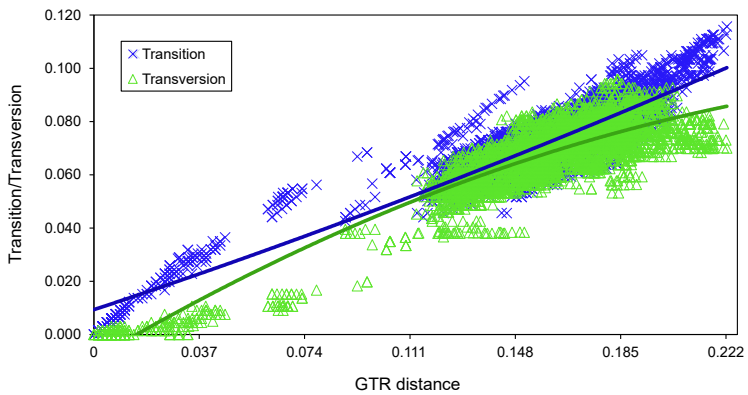

Figure 2. Saturation plot for transitions (blue crosses) and transversions (green triangles). The x axis shows the genetic distance based on the GTR model, while the y axis shows the proportion of transitions and transversions. The lines show the trends of the variance of transitions and transversions with increasing genetic distance.

Table 3. Mean pairwise intra- and inter-specific genetic distances (%) of *Cricotopus* species analysed in the study using K2P substitution model.

Species	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.	11.	12.	13.	14.	15.	16.	17.	18.	19.	20.	21.	22.	23.	24.	25.	26.	27.	28.
1. *C. bicinctus*	**1.69**																											
2. ***C. flavocinctus***	13.50	**0.00**																										
3. *C. intersectus*	15.87	15.45	**0.56**																									
4. *C. laricomalis*	15.13	15.04	2.46	**ND**																								
5. *C. ornatus*	14.00	20.12	14.72	14.62	**0.55**																							
6. *C. patens*	13.99	2.13	16.20	15.60	18.69	**0.15**																						
7. *C. reversus*	18.31	18.60	11.48	10.98	15.11	18.70	**0.51**																					
8. *C. sylvestris*	14.81	17.75	17.04	16.33	13.43	17.85	18.07	**0.84**																				
9. *C. triannulatus*	18.34	18.81	20.96	19.44	19.88	18.55	20.30	19.42	**5.24**																			
10. *C. annulator*	18.80	18.83	18.65	18.43	17.76	18.16	20.07	18.91	17.59	**0.92**																		
11. *C. coronatus*	14.11	9.91	14.50	14.08	18.27	9.53	16.14	19.04	18.11	16.23	**1.92**																	
12. *C. curtus*	19.09	19.54	19.91	18.64	21.27	20.70	20.28	21.23	20.76	16.28	19.87	**ND**																
13. *C. festivellus*	16.70	21.28	17.38	17.30	19.18	21.11	20.68	16.50	22.02	20.65	19.30	20.78	**0.15**															
14. *C. fuscus*	13.21	16.32	17.88	16.80	16.09	15.72	17.77	15.79	19.51	16.08	16.95	16.82	16.57	**ND**														
15. *C. gelidus*	13.52	17.16	17.36	16.08	17.05	16.86	20.49	20.57	22.28	20.18	16.72	17.57	19.99	13.61	**0.62**													
16. *C. lestralis*	15.26	19.35	19.44	17.65	18.43	19.66	20.96	21.61	22.00	20.35	17.91	18.21	20.58	14.49	3.32	**0.00**												
17. *C. pilosellus*	17.37	19.78	20.54	18.58	19.79	19.03	22.76	24.12	23.29	21.75	18.71	23.20	21.38	17.83	13.60	15.67	**4.75**											
18. *C. polaris*	13.99	17.96	18.47	17.36	17.73	17.83	20.65	20.78	23.02	19.50	16.83	19.17	20.10	13.83	3.04	3.49	13.85	**3.42**										
19. *C. pulchripes*	16.49	17.37	20.83	20.07	18.59	18.73	21.20	18.84	21.30	19.49	16.78	19.63	21.28	14.63	16.24	17.04	20.98	17.52	**0.15**									
20. *C. tibialis*	16.67	17.24	18.36	17.94	16.59	17.31	20.16	21.25	22.75	19.60	17.01	21.27	20.82	18.04	13.71	14.44	15.59	13.39	18.40	**2.70**								
21. *C. tremulus*	13.11	17.36	15.41	15.15	16.63	17.35	17.48	17.43	20.08	17.22	15.75	16.86	16.93	12.90	13.73	15.02	17.56	13.08	16.10	16.85	**0.15**							
22. *C. trifascia*	15.20	19.46	18.54	18.36	18.98	20.12	20.05	19.31	18.62	17.22	17.56	18.11	18.30	16.44	19.17	19.63	20.65	19.30	19.88	19.38	16.98	**3.44**						
23. *C. vierriensis*	14.05	15.98	16.32	16.64	15.44	15.96	18.39	16.95	20.20	16.68	15.50	16.16	19.13	15.82	14.79	15.71	19.59	15.02	15.24	16.59	14.23	15.46	**1.56**					
24. *C. glacialis*	15.33	17.67	17.40	16.72	13.67	18.02	18.42	1.09	19.42	19.28	19.28	21.45	16.89	16.35	20.98	21.78	24.40	21.20	19.42	21.67	17.67	19.60	17.42	**0.62**				
25. *C. obnixus*	18.71	17.90	16.43	15.84	17.31	18.81	18.80	17.98	22.93	19.64	17.87	19.39	22.37	19.70	17.85	19.61	21.08	19.17	19.82	20.05	20.34	19.83	15.49	17.99	**2.58**			
26. *C. perniger*	13.95	16.16	15.07	14.31	13.20	17.31	17.80	14.96	18.63	17.41	16.23	16.47	20.95	19.81	16.04	16.68	21.27	16.40	18.97	18.32	13.71	18.88	13.39	14.85	16.82	**2.27**		
27. *C. tricinctus*	15.20	18.52	15.58	15.32	12.85	19.77	16.46	13.92	21.45	18.37	18.68	19.62	19.54	17.24	18.12	18.20	21.56	18.84	21.92	17.58	16.60	20.55	16.12	14.20	18.09	12.91	**2.20**	
28. *C. trifasciatus*	13.67	18.58	17.33	16.85	13.87	19.18	20.45	6.89	19.95	18.60	20.11	20.24	16.05	16.04	19.31	21.02	22.55	20.06	16.45	20.94	16.13	19.28	16.70	7.08	18.15	15.82	13.77	**0.46**

The first nine *Cricotopus* species (in bold) were collected and sequenced in this study. Mean pairwise inter-specific genetic distances are in the lower left section; mean pairwise intra-specific genetic distances are in bold. ND: not detected.

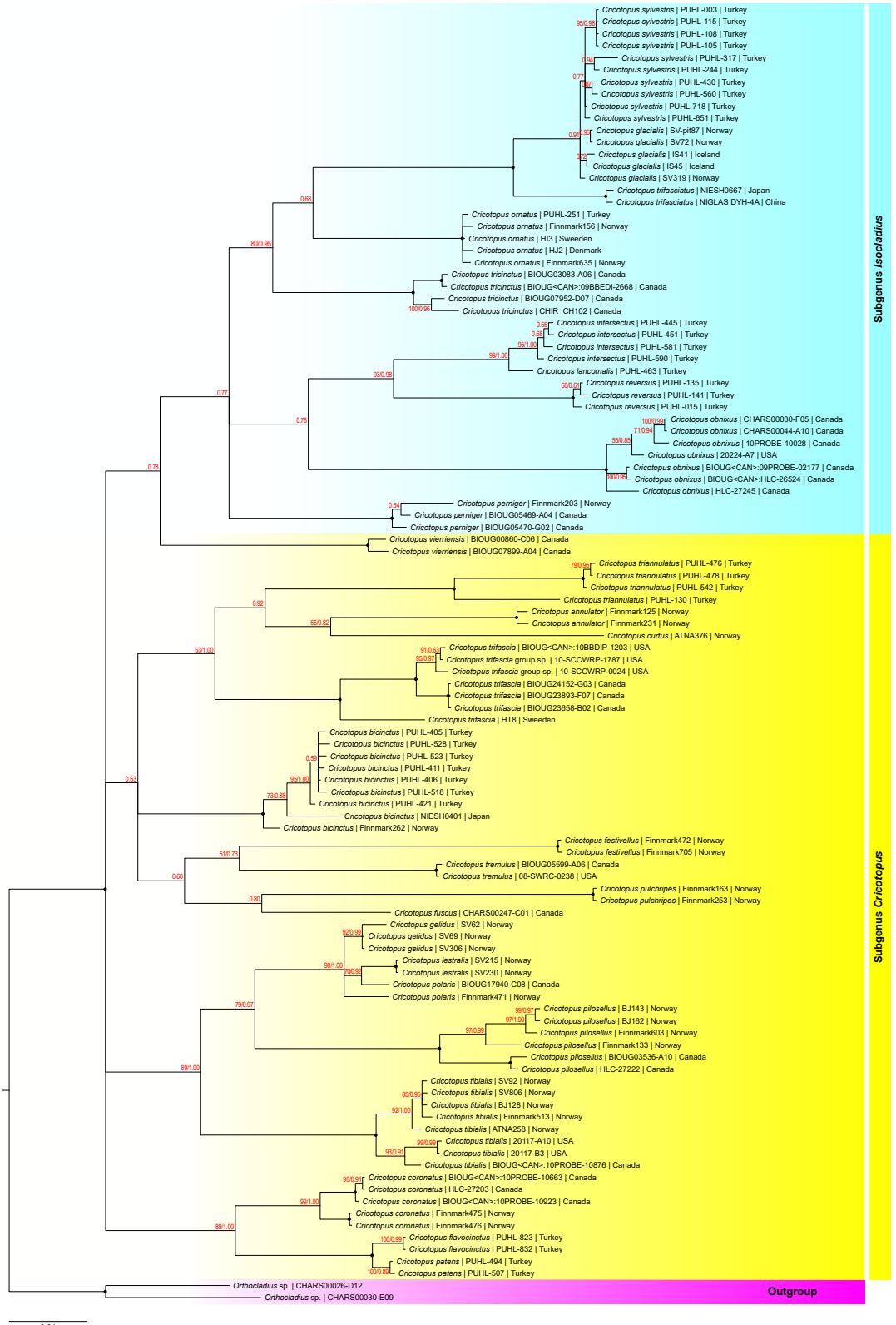

Figure 3. Bayesian tree based on the analysis of COI sequences. Node-associated values (in red) correspond to ML bootstrap support (BS) and BI posterior probabilities (PP), respectively. Full support (100%/1.00) is marked with a bold circle.

from Turkey were nested within a group (PP = 1.00, BS = 100), and this group was sister to *C. annulator* and *C. curtus* (PP = 0.92, BS < 50). This cluster of three was sister to *C. trifascia* (PP = 1.00, BS = 53). The specimens of *C. bicinctus* from Turkey formed a clade (PP = 1.00, BS = 95), and this clade was nested within a well-supported group (PP = 1.00, BS = 100) that also contained *C. bicinctus* specimens from the other countries. *C. patens* and *C. flavocinctus* formed two sister groups (PP = 0.89 and 0.99, respectively, BS = 100 for both species), and these groups were closely related to *C. coronatus* (PP = 1.00, BS = 89). To better visualize relationships among *Cricotopus* species, a neighbour-net phylogenetic network was constructed in the study (Figure 4). The centre of the obtained network was slightly netted. The network indicated well-supported splits and clusters, and these were largely in accordance with the well-supported clades in the topology of the combined tree obtained with the ML and BI analyses (Figure 3).

The present study analysed the phylogenetic relationships of the species from the genus *Cricotopus* using COI sequences, with a main focus on Turkish *Cricotopus* species. COI-based phylogenetic tree showed a good clustering pattern according to species in both reconstruction methods. Molecular classifications of *Cricotopus* species from Turkey were full compatible with the morphological species identity. This finding suggests that the larval keys for these specimens are useful to identify them to species level. As shown in Figure 3, majority of the species with two or more representatives including *C. sylvestris*, *C. ornatus*, *C. intersectus*, *C. reversus*, *C. triannulatus*, *C. bicinctus*, *C. flavocinctus*, and

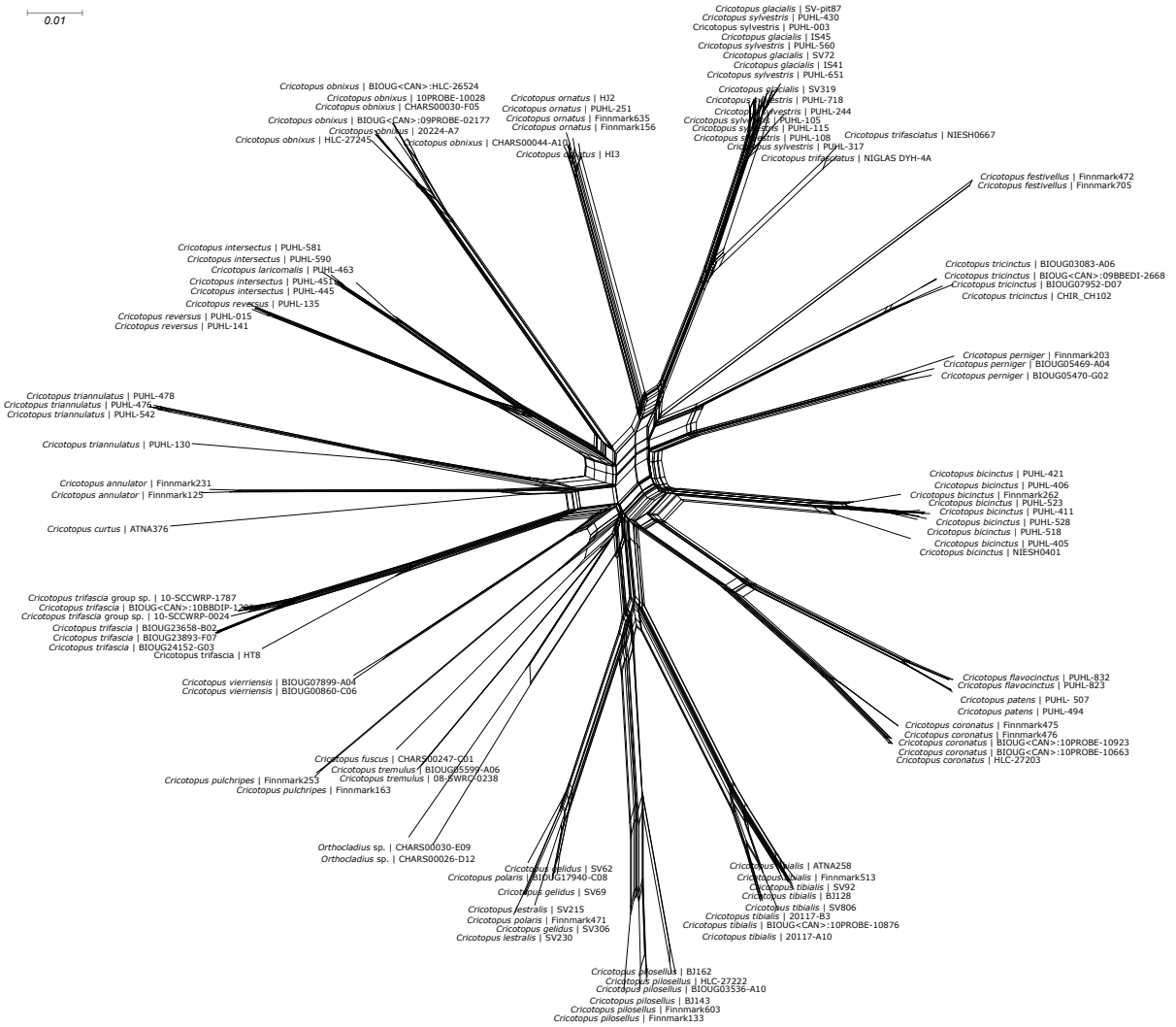

Figure 4. A neighbour-net phylogenetic network constructed using 106 COI sequences of the genus *Cricotopus* and two COI sequences *Orthocladius* sp. (outgroup). For the clarity of the network, bootstrap support values of the nodes are not demonstrated.

C. patens from Turkey produced monophyletic groups. In some studies, *C. bicinctus*, *C. flavocinctus*, *C. patens*, *C. ornatus*, *C. trifascia,* and *C. sylvestris* were found to produce monophyletic groups (Sinclair and Gresens, 2008; Sari et al., 2012, 2015; Brodin et al., 2013), agreeing with the results of the current study. COI sequences have been reported to generally work well for exploring the species relationships in chironomids (Sinclair and Gresens, 2008; Sari et al., 2012, 2015). Similarly, this study indicates that the COI gene is a useful marker for species identification in the genus *Cricotopus* and for estimating their evolutionary relationships. However, in order to comprehensively analyse the evolutionary relationships among *Cricotopus* species not only from Turkey but also from other localities in the world, I here recommend the obtainment of more COI sequences of specimens belonging to the same or different *Cricotopus* species and the inclusion of sequence data from additional nuclear and mitochondrial markers in further studies. If more *Cricotopus* species are sampled from Turkey in future studies, it is possible to reveal potential chironomid biodiversity in Turkey with a unique geographical position and wide variety of habitats. This will undoubtedly contribute to improvement of our knowledge of the phylogenetic relationships among chironomids.

In spite of the fact that majority of 95% HPD ranges were broad, estimates of the tmrca all possessed ESSs greater than 200 (Table 4), indicating a valid support. These estimates suggest that the diversification of the ingroup taxa comprising the representatives of the genus *Cricotopus* was initiated in the late Cretaceous, around 98 mya (47–165 mya; Figure 5). *C. vierriensis* from the subgenus *Cricotopus* diverged from the ancestor of the species of the subgenus *Isocladius* around 82 mya (34–142 mya), while the other species from the subgenus *Cricotopus* last shared a common ancestor in the late Cretaceous, around 90 mya (43–157 mya). All the species from the subgenus *Isocladius* last shared a common ancestor around 73 mya (30–129 mya). Within the subgenus *Isocladius*, the group containing *C. sylvestris*, *C. glacialis*, *C. trifasciatus*, *C. ornatus*, *C. tricinctus*, and *C. perniger* representatives and the group consisting of the representatives of the remaining species last shared a common ancestor around 60 mya (25–104 mya) and 54 mya (24–98 mya), respectively. Within the former group, the divergence of ancestral *C. perniger* was followed by the divergences of the ancestors of *C. tricinctus*, *C. ornatus*, and *C. trifasciatus* around 52 mya (22-91 mya), 44 mya (18-84 mya), and 20 mya (7-36 mya), respectively, and *C. sylvestris* and *C. glacialis* diverged approximately 6 mya (2-11 mya). Within the latter group, the divergence of ancestral *C. obnixus* was followed by the divergence of the ancestors of *C. reversus* and *C. laricomalis* approximately 36 mya (14–66 mya) and 10 mya (3–19 mya), respectively. Within the subgenus *Cricotopus*, the group containing *C. lestralis*, *C. polaris*, *C. gelidus*, *C. pilosellus*, and *C. tibialis* representatives and the group containing *C. coronatus*, *C. patens*, and *C. flavocinctus* representatives last shared a common ancestor approximately 82 mya (33–142 mya), whereas the group comprising *C. trifascia*, *C. triannulatus*, *C. annulator*, *C. curtus*, *C. pulchripes*, and *C. fuscus* representatives and the group comprising *C. bicinctus*, *C. festivellus*, and *C. tremulus* representatives last shared a common ancestor around 78 mya (34–138 mya). The divergence of *C. triannulatus* representatives from the group containing *C. annulator* and *C. curtus* occurred from around 56 mya (25–108 mya), and the age of the most recent common ancestor of these modern *C. triannulatus* individuals was approximately 25 my (8–47 my). *C. bicinctus* representatives diverged from the group containing *C. festivellus* and *C. tremulus* around 63 mya (29–111 mya), and the age of the most recent common ancestor of these modern *C. bicinctus* was approximately 16 my (5–32 my). *C. patens* and *C. flavocinctus* representatives diverged from each other around 7 mya (2–15 mya).

It is known that chironomids have a long evolutionary history. It was reported that chironomids and the biting midges (Diptera: Ceratopogonidae) separated in the Permian-Triassic period, around 269 mya (231-308 mya), and that the divergence of ancestral forms of orthoclads occurred in the Cretaceous (Cranston et al., 2010). It was estimated in this study that the diversification of the representatives of the genus *Cricotopus* had been initiated in the late Cretaceous. The estimated tmrca values for North Europe and North America (Figure 5; nodes V, X, AA, DD, EE, and GG) suggested a distinct continental structuring in the evolution of some species of this genus. For instance, North American representatives of *C. trifascia* diverged from Swedish *C. trifascia* around 26 mya (9–49 mya), and Canadian *C. perniger* diverged from Norwegian *C. perniger* around 10 mya (3–20 mya) (see Figure 5 and Table 4). It is considered that the genus *Cricotopus* may have originated in the Eurasian region (Krosch et al., 2015). Given this consideration and the geological timeline of continental break-up, these divergence events imply the role of trans-Atlantic connections between Eurasia and North America or transoceanic dispersal events in their evolution.

Divergence time analysis also showed that the sampled Turkish representatives of the genus *Cricotopus* (i.e., *C. bicinctus* and *C. ornatus*) may have diverged from its North European representatives. As the ecology and habitat preference of *Cricotopus* species vary widely across the genus, different dispersal routes within the genus are possibly evident. However, the sampling strategy in the study did not allow for accurately determining the dispersal routes for the colonisation history of *Cricotopus* species. To succeed this, a strategy of intensive and targeted sampling

Table 4. Times to most recent common ancestor estimated using BEAST.

Node label	Mean (mya)	95% HPD range (mya)	ESS
A	98.48	47.22-165.33	8364
B	89.65	43.30-156.61	6625
C	82.07	33.33-141.64	4497
D	81.64	33.69-142.26	2080
E	78.24	33.99-137.88	5276
F	72.86	29.98-128.65	1612
G	70.89	28.35-125.17	2321
H	64.51	27.25-113.10	3226
I	63.31	29.17-111.38	3925
J	59.78	24.87-104.38	3187
K	58.52	24.47-102.86	4931
L	55.89	24.58-108.40	2690
M	53.58	23.97-98.44	4696
N	51.76	21.57-91.05	4562
O	47.55	16.21-84.97	4226
P	47.05	19.29-84.04	4580
Q	45.29	14.09-84.78	2501
R	44.03	18.33-83.97	4108
S	43.85	14.12-87.70	2021
T	38.33	13.92-71.28	4311
U	36.20	13.66-65.65	5483
V	26.09	9.29-49.45	5213
W	24.83	8.10-46.63	2551
X	21.04	7.86-38.95	4387
Y	19.72	6.87-36.44	4392
Z	16.25	5.31-31.50	2455
AA	15.45	5.15-29.62	2590
BB	13.59	4.58-25.54	2147
CC	12.65	4.46-24.09	2177
DD	11.82	3.69-24.32	2311
EE	11.08	2.95-22.48	3887
FF	10.35	2.98-20.75	3558
GG	9.76	2.56-20.02	3692
HH	9.74	2.67-19.31	4268
II	7.48	1.81-15.42	4551
JJ	5.77	1.97-11.07	1310
KK	4.49	1.27-9.04	2366
LL	4.09	1.40-9.14	1132
MM	3.45	0.75-9.30	1420
NN	3.37	0.76-7.14	2404
OO	3.31	0.71-7.01	3111
PP	2.51	0.42-5.75	3292
QQ	1.86	0.14-4.53	6001
RR	1.08	0.00-2.98	5697
SS	0.95	0.01-2.68	4456

ESS, effective sample size; HPD, highest posterior density; mya, million years ago

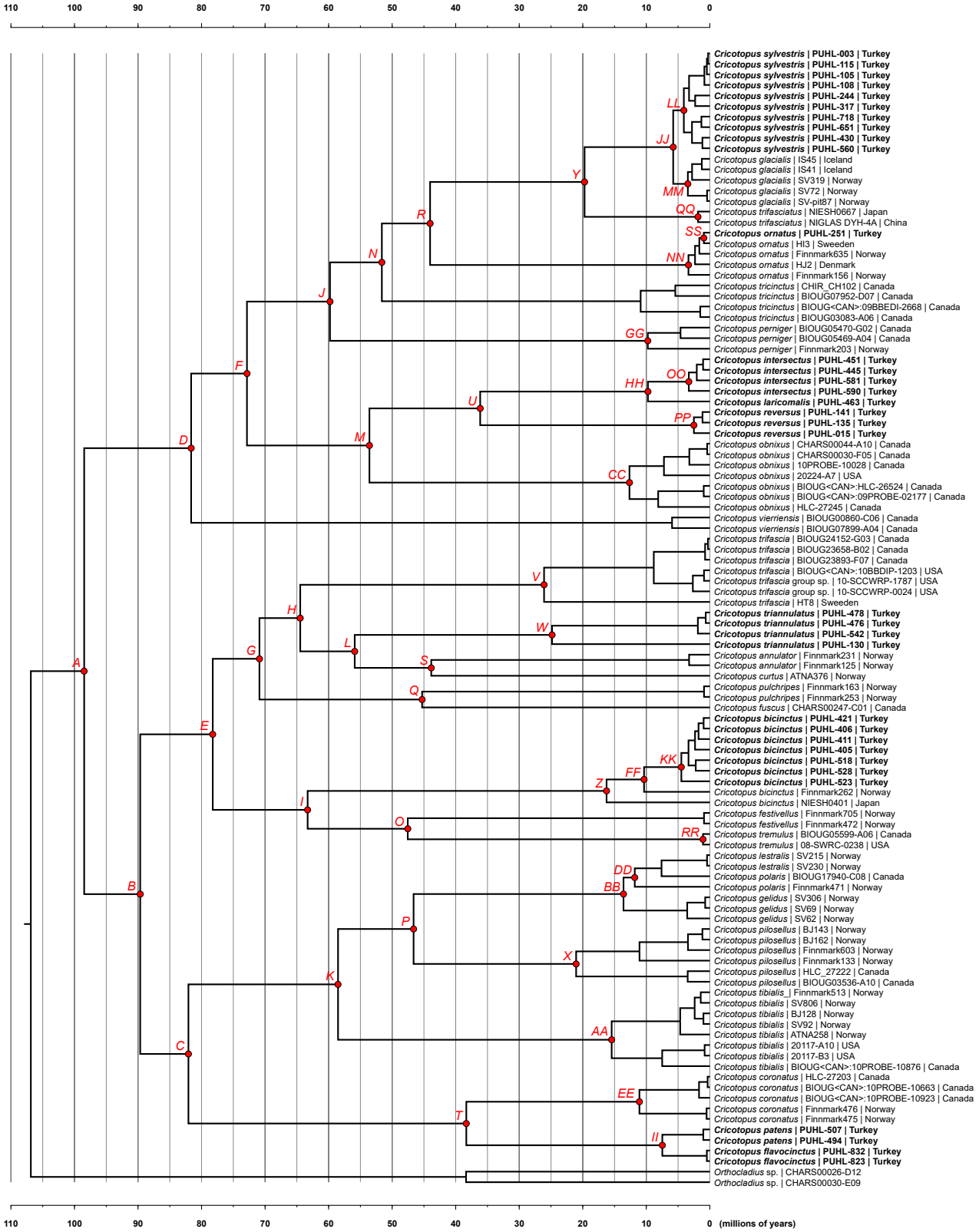

Figure 5. BEAST chronogram of divergence of the representatives of the genus *Cricotopus*. Specimens collected and sequenced in this study are demonstrated in bold. Time to most recent common ancestor (tmrca) was estimated for the lettered nodes (in red) which correspond with those in Table 4. The time scale is in millions of years before present.

from more locations is essential in future studies. It is supposed that the evolution of various species was driven by some natural processes such as endogenous processes taking place on the Earth and global climatic fluctuations particularly in Pleistocene (Hewitt, 1999; Smith et al., 2014). During these processes, Anatolia (Asian part of Turkey) acted as a land bridge for faunal distribution from eastern to western and from northern to southern part of the Palaearctic and as an important refugium for faunal elements (Çıplak, 2004). Therefore, it is possible to expect that Turkish *Cricotopus* species are not likely to share a single common origin and that waves of colonisation into and dispersal out of Turkey are likely to have occurred. In this regard, further phylogeographic studies with greater sampling of *Cricotopus* diversity of Turkey and the surrounding region are highly recommended. Thus, not only the migration routes of *Cricotopus* species but also the importance of Turkey in the *Cricotopus* distribution will be able to be revealed.

4. Conclusion

Molecular classifications of *Cricotopus* species from Turkey were fully compatible with the morphological species identity, suggesting the adequacy and practicability of existing larval keys for identifying these specimens to species. The sampled Turkish *Cricotopus* species with two or more representatives were found to produce monophyletic groups. It was determined in the tmrca estimates that the diversification of the genus *Cricotopus* had been initiated in the late Cretaceous, around 98 mya (47–165 mya). These estimates suggested a distinct continental structuring in the evolution of some species of this genus and a possible origination of the sampled Turkish representatives of the genus *Cricotopus* (i.e., *C. bicinctus* and *C. ornatus*) from North European representatives. The results of this study indicate that the COI gene is a useful tool both for species identification in the genus *Cricotopus* and for estimating their evolutionary relationships. This study provides valuable information on the evolutionary relationships among *Cricotopus* species. However, to obtain more accurate and/or persuasive results, further studies with an intensive and targeted sampling from more locations and with the inclusion of more sequence data from not only the mitochondrial COI gene but also additional nuclear and/or mitochondrial markers are highly suggested. Hence, the potential chironomid biodiversity in Turkey, the migration routes of chironomids, and the importance of Turkey in their distribution could be revealed.

Conflict of interest

There are no conflicts of interest in the current study.

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