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Exploring the Genetic Structure and Phylogeographic Patterns of the Copepod Genus *Eurytemora* in Europe

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Abstract: The genus *Eurytemora* is a diverse group of copepods found in coastal, estuarine, brackish, and freshwater environments. The main research has focused on *Eurytemora affinis* (Poppe, 1880) and revealed it to be a species complex. *Eurytemora velox* (Liljeborg, 1853) has only recently been characterized in the Scheldt estuary but never within a global phylogenetic context. This study integrated nearly all European *Eurytemora* species sequences available, along with original ones from the Scheldt. A total of 351 sequences were analyzed using one mitochondrial gene (*CO1*) and one nuclear gene (*nITS*), with sequencing performed specifically on the *CO1* gene. Phylogenetic reconstructions were performed using the Maximum Likelihood method, along with haplowebs and genetic diversity indices. A significant subdivision between six European species was observed. The monophyletic clade status of the *E. affinis* complex was confirmed alongside the existence of three geographically isolated lineages of the *E. affinis* species, the East Atlantic, the North Sea/English Channel, and the Baltic lineages, each exhibiting pronounced genetic differentiation. The population of *E. velox* from the Urals differs significantly from the European ones. These results provide both an overview of the genetic structure of the genus in geographic Europe as well as new insights on *E. velox*.

Keywords: copepoda; cytochrome oxidase I (*CO1*); phylogenetics; biogeography

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1. Introduction

The genus *Eurytemora* (Giesbrecht, 1881) is a group of coastal, estuarine, brackish, and/or fresh water copepods (Crustacea) that includes at least 21 species [1]. Most studies on this genus focus on *Eurytemora affinis* (Poppe, 1880) and concern various fields such as genetics, ecology, and physiology [2–5]. This species is widely distributed in the northern hemisphere, with habitat types ranging from hypersaline salt marshes to freshwater, and, as a result, has high intra-specific morphological variability [6–8].

The global phylogeography of *E. affinis* s.l. was first investigated by Lee [8], who showed a polytomy of highly divergent clades (15–20%) in the Palearctic using the mitochondrial genes *CO1* and *16sRNA*. At the same time, morphological searches did not reveal significant differences [9]. This finding suggests that *E. affinis* is a complex of cryptic species, which is also supported by eco-physiological differences [10]. Nuclear ribosomal Internal Transcribed Spacer (*ITS1–ITS2* = *nITS*) gene analysis also supports these

clades [1]. Although no published work has demonstrated reproductive isolation between European and North American species, preliminary results (Lee and Souissi, personal communication) seem to show an absence of hybridization. However, the monitoring of coexisting European and invasive North American *Eurytemoras* in the Gulf of Finland has shown that they are still genetically and morphologically isolated [11]. Following detailed morphological analyses and molecular validation, the *E. affinis* complex is now represented by three valid species: Atlantic North American *Eurytemora carolleeae* Alekseev and Souissi, 2011, *Eurytemora caspica* Sukhikh and Aleskeev, 2013, and European *E. affinis* (Poppe, 1880). This highlights the importance of revising the genus using both morphological and genetic approaches.

Considering the highly divergent clades of *E. affinis* in North America found by Lee, similar studies were conducted in Europe [8,12]. They showed congruent results with three geographically separated lineages: (1) the Baltic ("BS") lineage, including specimens from the Baltic Sea and its drainage basin situated in Sweden, Finland, Russia, and Estonia; (2) the East Atlantic ("EAt") lineage, including specimens from the Loire and Gironde (France); and (3) the North Sea/ English Channel ("NSEC") lineage, including specimens from the Seine (France), the Scheldt (Belgium), the Elbe (Germany), and the Tamar (UK) (Figure 1).

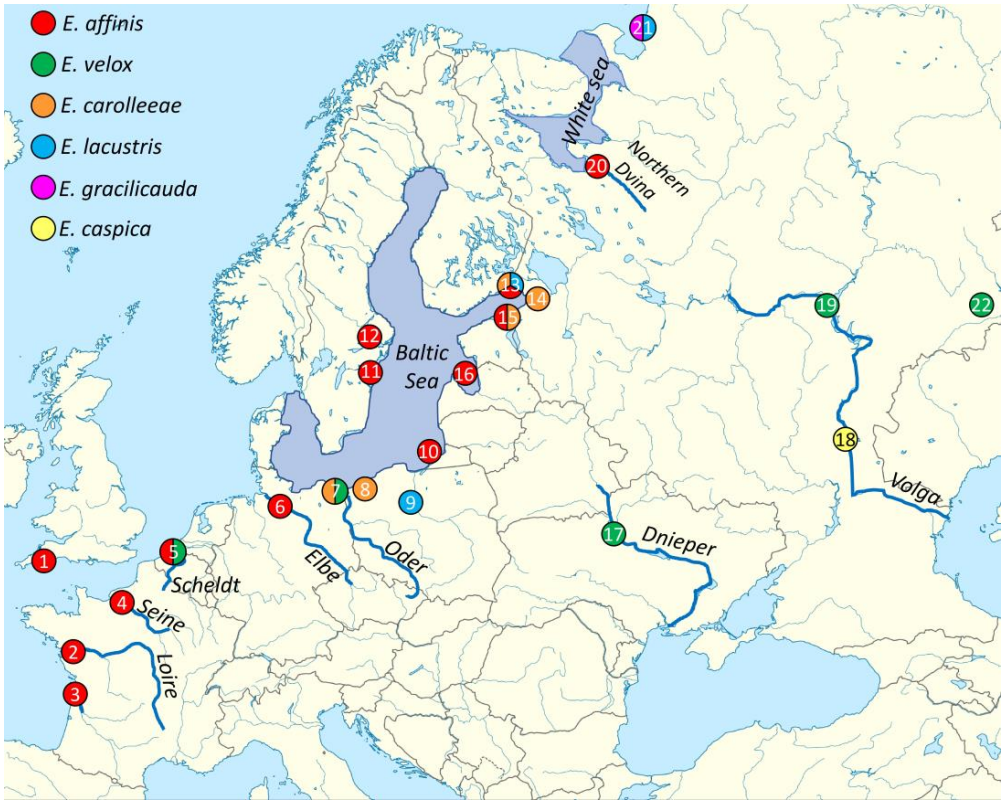

Figure 1. Sampling locations of the geographic European *Eurytemora* species used in this study: (1) Tamar estuary (UK); (2) Loire, (3) Gironde, and (4) Seine (France); (5) Scheldt (Belgium, The Netherlands), and (6) Elbe estuaries (Germany); (7) Oder river, (8) Dzwina channel, and (9) Cieszecino lake (Poland); (10) Vistula lagoon (Poland/ Russia); (11) Swedish coast; (12) Himmerfjarden (Sweden); (13–15) Gulf of Finland (13—Gulf of Vyborg, 14—Neva, and 15—Luga estuaries); (16) Gulf of Riga (Latvia); (17) Feofania ponds (Ukraine); and (18) Volga river, (19) Cheboksary reservoir, (20) Northern Dvina river, (21) Pechora Bay, and (22) Smolino lake (Russia). Sampling locations in the Baltic Sea and White Sea are represented by shapes due to a lack of precise GPS coordinates. A color code is assigned to each species. The sampling localities of the species occurring outside of geographic Europe (*Eurytemora bilobata* Akatova, 1949, *Eurytemora foveola* Johnson M.W., 1961, *Eurytemora herdmani* Thompson I.C. and Scott A., 1897, *Eurytemora americana* Williams, 1906, *Eurytemora arctica* Wilson M.S. and Tash, 1966, and North American *E. carolleeae*) are not represented on the map.

Until the beginning of the 21st century, the species *Eurytemora gracilicauda* Akatova, 1949 was recorded only in the following regions: the Kruzenshtern Lagoon in Alaska [13], Lake Lakhtak in Kamchatka, and its typical habitat—a tributary of the Kolyma River in eastern Siberia [14]. Only in recent decades has the species been discovered in the central and European parts of the Arctic [15]. Studies based on genetic (parts of *CO1* and ITS1 genes) and morphological analyses of its populations from different points of Arctic waterbodies have shown that the species likely dispersed sequentially along the Arctic coast [16]. The White Sea population, which is the most widely represented in the currently available genetic analysis, exhibits a low level of genetic variability. This, along with significant tolerance to fluctuations in salinity in the rock pools where it lives, may indicate its recent introduction to the region. According to existing studies [16], the inferred phylogeny of the species lacks a clear topology with high bootstrap values. Molecular clock dating using part of the *CO1* gene and paleontological calibration has revealed that the speciation of *E. gracilicauda* from *E. arctica* occurred at the Paleocene–Late Cretaceous boundary.

Among all *Eurytemora* species investigated in the frame of the present paper, *Eurytemora lacustris* (Poppe, 1887) has been less thoroughly researched genetically. *Eurytemora lacustris* is a stenothermic glacial relict found in Europe [17] with narrow environmental requirements, making it an indicator species for good ecological conditions [18]. Few sequences of populations from the Pechora Sea, water bodies of Poland, and the Gulf of Finland have been published as comparative material. According to the *CO1* phylogenetic tree in [1], *E. lacustris* is grouped with the Arctic species *Eurytemora bilobata* Akatova, 1949.

Recent studies of the *E. affinis* population structure in the Scheldt estuary and its tributaries (Belgium, The Netherlands) has revealed a complex and dynamic polymorphism using highly variable genetic markers (Inter-Simple Sequence Repeat) within the species between localities [19,20]. Several hypotheses have been advanced, including the coexistence of two *Eurytemora* species that were never properly identified [19,20]. Recently, *Eurytemora velox* (Liljeborg, 1853) was also reported to occur in the Scheldt estuary. *Eurytemora velox* is an euryhaline species, nowadays widely distributed in fresh and brackish waters of Northern and Western Europe [21]. The Caspian region is thought to represent its source of distribution [22]. To date, only a few studies have presented the results of a molecular genetic analysis of *E. velox* [23–25].

In the Scheldt, Mouth and coauthors [26] revealed distinct distributional ranges of *E. affinis* and *E. velox* that correspond to different environmental conditions. *Eurytemora affinis* thrives in the tidal Scheldt tributaries (i.e., tributaries with tides), where there is more Suspended Particulate Matter, more Particulate Organic Matter, and hence less dissolved oxygen, whereas *E. velox* is found in the non-tidal ones with a higher phytoplanktonic biomass and higher oxygen concentrations. So, whilst *E. velox* has been morphologically characterized and shown to have a different environmental distribution to *E. affinis* in the Scheldt tributaries, molecular identification has never been carried out.

The recent identification of both *E. affinis* and *E. velox* in the Scheldt estuary and its tributaries [26] has led us to consider the genetic distribution/structure of both species in Europe. A considerable number of studies on various *Eurytemora* species are available [1,6,12,18,27–30]. Although Sukhikh and coauthors [1] presented an overview of the genetic structure of the *Eurytemora* genus in the Palearctic as a whole, no comprehensive overview of all species represented in Europe was provided. The present work used a larger dataset by integrating European *Eurytemora* sequences from individuals originating from all localities to date sequenced with 22 new sequences from two species (i.e., *E. affinis* and *E. velox*) that had never been studied before within a global phylogenetic context.

Thus, this study aimed at (1) analyzing the genetic structure of the *Eurytemora* genus in geographic Europe (that stretches west to east from the Atlantic coast to the Urals region in Russia and north to south from the Pechora Bay in Russia to the Gironde estuary in France) and (2) describing the phylogeographic and demographic structure of *E. affinis* and *E. velox* based on the analysis of mitochondrial (mtDNA) and nuclear (nDNA) genes with sequencing performed specifically on the *CO1* gene.

2. Materials and Methods

2.1. Sampling Strategy

Specimens of *E. velox* and *E. affinis* were collected during the monthly OMES ("*Onderzoek Milieu Effecten Sigmaplan*"; "research on the effects of the Sigmaplan") monitoring campaign of May 2022 at the Dendermonde station of the Scheldt river (51°02′10″ N 4°06′23″ E) and in the Dender tributary (51°02′13″ N 4°04′38″ E) approximately 1 km from its confluence. Zooplankton was collected from a pontoon by filtering 50 L of sub-surface water through a 50 µm mesh plankton net using bucket hauls and fixed in 70% alcohol (for genetic purposes). Individuals were identified after dissection procedure [31] under a stereo microscope (90× magnification) and light microscope (960× magnification).

2.2. Molecular Analysis

DNA was extracted individually from whole individuals using the DNeasy Blood and Tissue extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. More information on the extraction protocol can be found in Appendix A. DNA concentration was determined by spectrophotometry (Nanodrop ND-1000, Waltham, MA, USA) coupled with Qubit 2.0 (Invitrogen, Waltham, MA, USA). Considering the low concentrations obtained, the extracts were concentrated using the SpeedVac RC1010 (Jouan, Saint-herblain, France) vacuum concentrator to reach 30 ng/µL.

PCR amplifications were performed in a 50 µL reaction volume containing a GoTaq G2 Hot Start Green Master Mix (1X; Promega, Madison, WI, USA), 0.5 µM of LCO1490/HCO2198 [32] primer mix, and 2 µL of template DNA. The cycling conditions were as follows: initial denaturation at 95 °C for 5 min and 38 cycles of denaturation at 94 °C for 30 s, annealing at 51 °C for 30 s, and elongation at 72 °C for 1 min 30 s, followed by a final extension at 72 °C for 10 min. All PCR were performed on a C1000 Touch Thermal Cycler instrument (Biorad, Hercules, CA, USA). For samples with no/poor amplification, 40 cycles were performed. DNA contamination was controlled by analyzing blanks at the same time as samples during extraction (Extraction Blank Control, C−) and PCR. Electrophoresis was carried out with 8 µL of amplified products on a 1% agarose gel for 20 min at 75 V. The gel was read under UVs with the Genosmart instrument (VWR, West Chester, PA, USA).

2.3. DNA Sequencing

Sequencing analysis was performed by Sanger DNA sequencing services of Eurofins Genomics (Nantes, France) with the same set of primers used to obtain amplicons. The forward and reverse sequences of each PCR product were analyzed on the Geneious v.9.05 software (https://www.geneious.com). Subsequently, sequences were aligned using the CLUSTAL W algorithm [33] implemented in BIOEDIT v.7.2.5 [34] with manual editing of ambiguous sites.

A total of 373 sequences were analyzed (303 sequences of the 531 bp *CO1* [32] and 70 sequences of the 842 bp nITS-5.8SrRNA-ITS2 (*nITS* = nuclear ribosomal DNA internal transcribed spacer) [35]). The vast majority of sequences (351) were retrieved from GenBank and 22 *CO1* sequences were obtained from our molecular analyses (2 *E. affinis* and 20 *E. velox* individuals) and deposited in the GenBank database under the accession numbers PP971656 to PP971674 and PP973821 (*E. velox*) and PP968572 and PP968573 (*E. affinis*) (Table 1; Figure 1).

Table 1. *Eurytemora* sequences with GenBank accession numbers as used in this study.

Species Name	*CO1* Accession Numbers	nITS Accession Numbers	Sources
E. affinis	MT653564, MT653565, MT653567	KX401005-KX401023	[1]
	HM473958-HM473961, HM473963-HM474010 HM474013, HM474015-HM474018 HM474020-HM474023, HM474025-HM474028	MT667429-MT667431	[1]
	JF72731-JF727331, JF72734-JF727349 JF727376-JF727395, JF727402-JF727405 JF727410, JF727411, JF727417 JF727423-JF727430, JF727432-JF727446 JF727449-JF727454, JF727496-JF727525 JF727527, JF727529-JF727532, JF727534-JF727548 JF727550-JF727552, JF727555, JF727556		[12]
	PP968572, PP968573		Present paper
E. velox	OR578619-OR578621 MZ373318-MZ373322	MZ400499-MZ400504 OR583025-OR583031	[24,25]
	MT146445, MT146446	MT787212-MT787214	[36]
	PP971656-PP971674, PP973821		Present paper
E. caspica	MN271657-MN271659	MT667435-MT667438	[1]
E. carolleeae	HM474003, HM474011, HM474012, HM474029	MT752961-MT752963	[30], unpublished
	MT151289-MT151293	KX401024-KX401038	[30]
	MT653566-MT653568	MN541395-MN541397	[1]
E. lacustris	MH316160, MH316161	MT787215, MT787216	[25,36]
	HM474030-HM474035		[37]
	MN256864, MN256865		[15]
E. gracilicauda	MN256855, MN256862		[15]
	MT653572-MT653582		[1]
E. americana	MN542728, MN542729, MN542731, MN542733		[15]
E. arctica	MN256859-MN256861	MT667432-MT667434	[1]
E. foveola	MN256857, MT653591		[1]
E. bilobata	MN256858, MT653589, MT653590		[1]
E. pacifica	AY145427, KR048961- KR048963		unpublished
E. herdmani	OP876964, MG321072, HQ967229		[38], unpublished
Temora longicornis	KT209513		[39]
Centropages typicus	GU132316	GU125729	[40]

2.4. Phylogenetic Reconstruction, Haplowebs, and Genetic Diversity

Phylogenetic reconstruction was carried out for the *CO1* and *nITS* loci independently, as well as on a concatenated *CO1-nITS* dataset using a Maximum Likelihood (ML) method. The best-fit models for each locus were estimated on IQTree ModelFinder according to the BIC. Due to the large number of samples and the few informative sites of the loci, a General Time-Reversible (GTR) (with Gamma rate distribution (+G) and with a proportion of invariable sites (+I)) model with RAxML v.8 [41] was applied for each dataset. For the *CO1* locus, one sequence of *Temora longicornis* (Müller O.F., 1785) was used as outgroup. For the *nITS* gene and concatenated dataset, one sequence of *Centropages typicus* Krøyer, 1849 was used as outgroup. A 1000-replicate bootstrapping to obtain a measure of tree topology robustness was applied and branches with less than 60% bootstrap support were collapsed. The *nITS* and concatenated *CO1-nITS* trees are presented in Supplementary Materials

(Figures S1 and S2). The level of nucleotide substitutions on the CO1 gene between species was calculated using the Tamura-Nei model in MEGA 11 and this analysis is presented in Supplementary Materials (Table S1).

Using the Arlequin 3.0 software [42], the fixation index (F_{ST}) for each species and for each pair of populations of *E. affinis* and *E. velox*, defined a priori according to geographic locality groups (lineages), was calculated, as was the number of migrants (Nm) [43]. The significance of F_{ST} values was tested on R-Studio (version 2023.06.2) with the {Ape} package. The fixation index and number of migrants were also calculated for species present at the same localities in order to gain more insight on genetic differentiation at a more local scale. This analysis is presented in Supplementary Materials (Table S2).

In order to determine inter- and intra-population genetic structure and variability in the different localities for *E. affinis* and *E. velox*, a non-parametric analysis of molecular variance (AMOVA) was carried out using the same software.

Genetic diversity on the *CO1* gene was compared across *E. affinis* and *E. velox* populations on the DNASP6 v.6 software [44], including haplotype diversity (Hd, probability that two randomly chosen haplotypes are different in the sample, as well as their relative frequencies; [45]) and nucleotide diversity (π, average number of nucleotide differences per location between two sequences; [45]). This analysis is presented in Supplementary Materials (Table S3).

In order to obtain a more comprehensive insight of population dynamics across a more fine-scale organization, median-joining (MJ) haplotype networks of the *CO1* locus for *E. affinis* and *E. velox* were constructed with PopArt v.17 [46]. These allow to illustrate the relationships between individual haplotypes. The homoplasy level parameter (ε) was set at the default value ($\varepsilon = 0$). The haplotype network of the *nITS* locus for the two species is presented in Supplementary Materials (Figure S3).

3. Results

3.1. Phylogenetic Reconstruction and Genetic Diversity of the Eurytemora Genus in Geographic Europe

Overall, the three ML inferred trees (*CO1*, *nITS*, and concatenated *CO1-nITS*) were congruent and exhibited similar topologies for the deeper nodes (Figures 2, S1 and S2).

In the *CO1* tree, all studied species diverged into five clades: (1) the *E. affinis* group (*E. affinis*, *E. carolleeae*, and *E. caspica*), (2) *E. velox*, *E. lacustris*, *E. foveola*, *E. bilobata*, and *E. arctica*, and (3–5) three separated species (*E. gracilicauda*, *E. americana*, and *E. pacifica*) (Figure 2).

The phylogenetic analyses conducted in this present study confirmed the existence of three geographically well-separated *CO1* lineages of *E. affinis* in Europe with strong node support, as stated in previous studies [7,12]. In the *CO1* tree, sequences from the Scheldt estuary were grouped within the North Sea "NSEC" lineage, consisting of sequences from populations in the Tamar, Elbe, Seine estuaries, and, unexpectedly, the remote Northern Dvina river. The East Atlantic "EAt" lineage consisted of individuals from the Loire and Gironde estuaries and the Baltic "BS" lineage of sequences from the Gulf of Finland (Gulfs of Riga, Vygorg, and the Luga estuary), the Swedish coast, and Himmerfjarden.

Eurytemora velox CO1 sequences were structured into two lineages: the Trans-Ural "TU" lineage and the European "EU" lineage, consisting of sequences from populations in the Scheldt estuary, the Feofania lake, the Oder river, and the Cheboksary reservoir (Figures 2 and 4).

The significant subdivision between geographic European species observed in the ML trees (Figures 2, S1 and S2) was further validated by the extremely high genetic pairwise distances between them ($F_{ST} > 0.9$; Table 2), highlighting their substantial genetic divergence. Additionally, the low level of genetic flow ($Nm < 0.06$; Table 2) among these species indicates minimal genetic exchange.

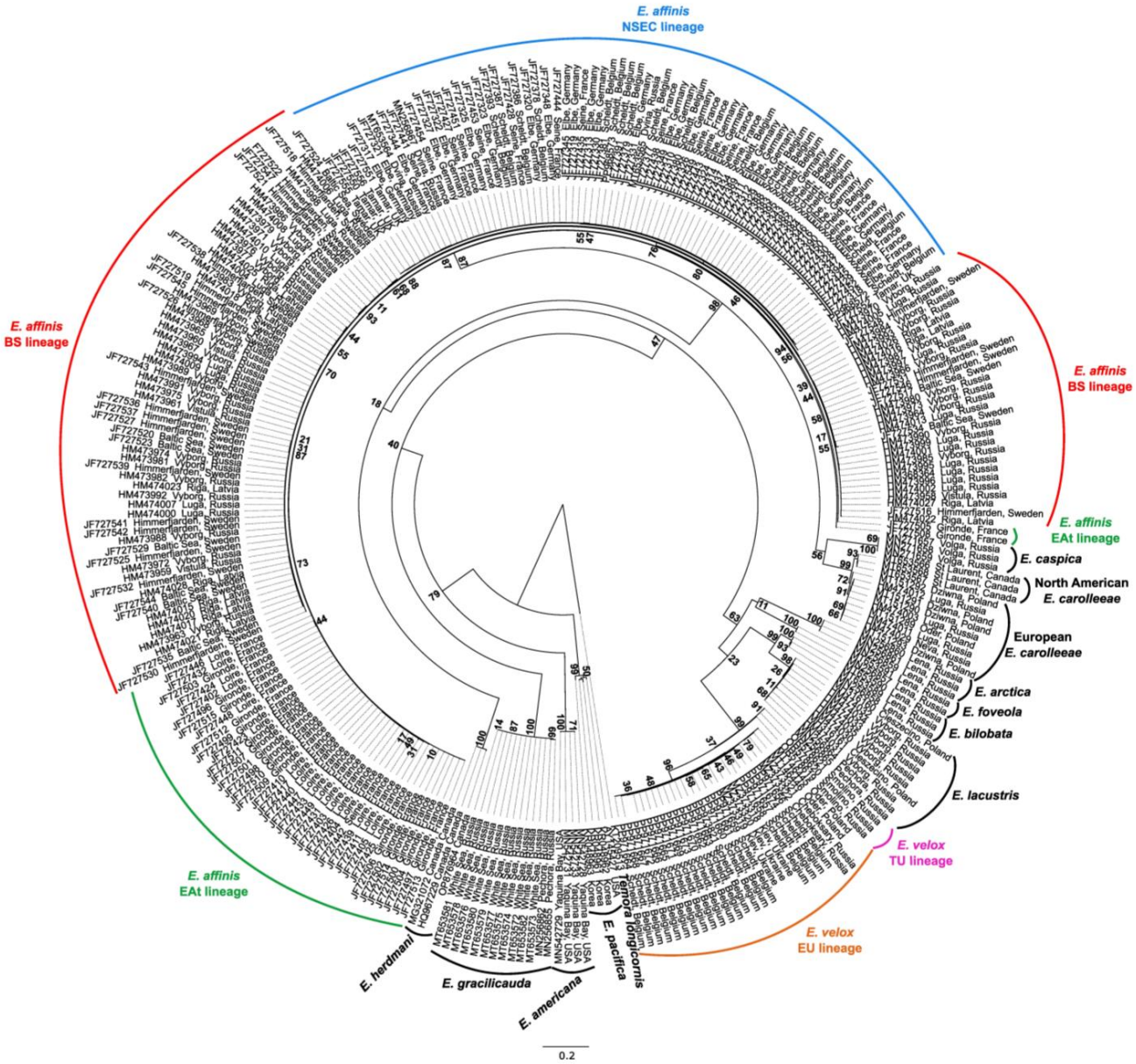

Figure 2. Maximum Likelihood phylogenetic tree of *Eurytemora* species based on 302 sequences of *CO1* (531 bp). *Temora longicornis* (Müller O.F., 1785) is used as outgroup. Nodes indicate bootstrap support (values of <10% are not shown). A color code is assigned to each lineage for *E. affinis* and *E. velox*.

Table 2. Pairwise species F_{ST} values (lower diagonal) and Nm (upper diagonal) of geographic European *Eurytemora* species based on the *CO1* locus.

	E. affinis	*E. velox*	*E. lacustris*	*E. gracilicauda*	*E. caspica*	*E. carolleeae*
E. affinis		0.037	0.037	0.038	0.052	0.057
E. velox	0.931 ***		0.026	0.018	0.028	0.024
E. lacustris	0.930 ***	0.950 ***		0.006	0.009	0.012
E. gracilicauda	0.928 ***	0.964 ***	0.988 ***		0.002	0.007
E. caspica	0.904 ***	0.947 ***	0.981 *	0.994 ***		0.014
E. carolleeae	0.897 ***	0.953 ***	0.975 ***	0.985 ***	0.971 *	

* Estimated pairwise F_{ST} *p*-values are indicated with asterisks. Significance levels: <0.001 '***'; <0.01 '**'; <0.05 '*'; 0.1 ' '.

3.2. Haplotype Genetic Diversity and Network of *E. affinis* and *E. velox*

To further substantiate the existence of *E. affinis* and *E. velox* lineages, haplotype networks using the median-joining (MJ) method were constructed (Figures 3 and 4).

For *E. affinis*, 51 distinct haplotypes were found among the sequenced 215 individuals (Figure 3; Table S2). The haplotype network analysis corroborated the results obtained by the *CO1* and concatenated ML trees, showing that *E. affinis* is divided into three geographical haplogroups (Figure 3). The three lineages were separated by three mutational steps each (BS-NSEC and BS-EAt).

The BS and NSEC lineages were star-like, centered around the dominant haplotype, whereas the haplotype network for the EAt lineage was less star-like and more dispersed. In the latter, two closely related dominant haplotypes were observed. In addition, one Seine haplotype (individual) belonged to the EAt rather than NSEC lineage.

The dominant haplotypes within lineages were mainly one mutational step away from the less frequent haplotypes (Figure 3).

For *E. velox*, nine distinct haplotypes were found among the 30 sequenced individuals (Figure 4; Table S2). Two haplogroups were observed, also corroborating the *CO1* and concatenated ML results (Figures 2 and S3). *Eurytemora velox* haplotypes were not distributed in a star-like manner but more dispersed and typically separated by one or two mutational steps from each haplotype (Figure 4). Between the two lineages, 17 mutational steps were observed.

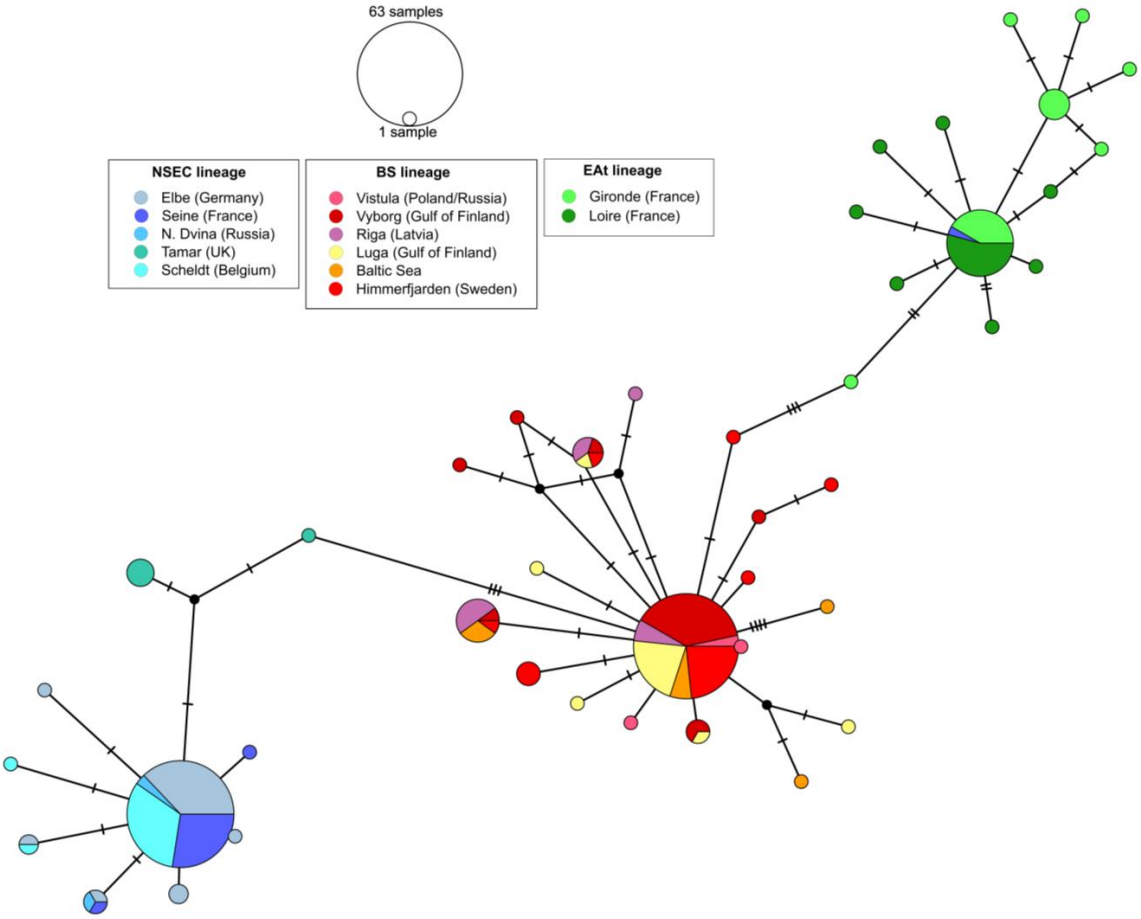

Figure 3. Median-joining mtDNA (*CO1*) network for *E. affinis* populations. The sizes of the circles are proportional to haplotype frequency. The smallest and biggest circles represent 1 and 63 individuals, respectively. The numbers of mutations between haplotypes are indicated on the branches by small bars and the dark unlabeled circles represent inferred ancestral nodes. Black circles represent missing intermediate haplotypes. A color code to the locality of origin is given.

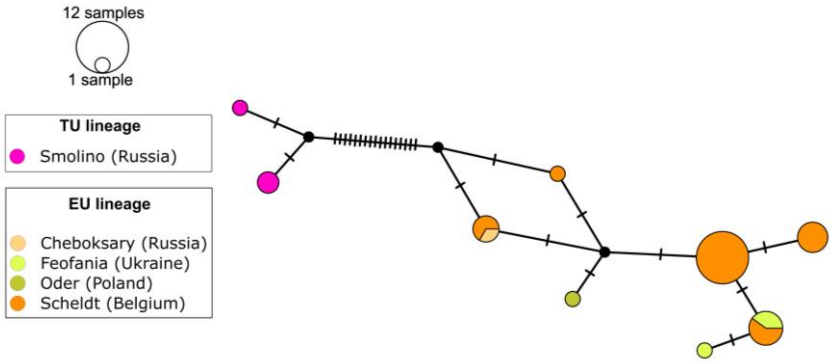

Figure 4. Median-joining mtDNA (*CO1*) network for *E. velox* populations. The sizes of the circles are proportional to haplotype frequency. The smallest and biggest circles represent 1 and 12 individuals, respectively. The numbers of mutations between haplotypes are indicated on the branches by small bars and the dark unlabeled circles represent inferred ancestral nodes. Black circles represent missing intermediate haplotypes. A color code to the locality of origin is given.

The AMOVA analysis revealed a strong genetic subdivision among lineages for both *E. affinis* and *E. velox* wherein most of the genetic variance was distributed among lineages (80.57% and 87.36%, respectively). For *E. affinis*, 1.66% of the variance was distributed among the populations within the lineages and 17.76% within the populations (Table 3A). Conversely for *E. velox*, 7.9% of the variance was distributed among the populations within the lineages and 4.73% within the populations (Table 3B).

Table 3. Analyses of molecular variance (AMOVA) as calculated by Arlequin v.3.0 on the *CO1* locus for (A) *E. affinis* populations and (B) *E. velox* populations.

	Source of Variation	Sum of Squares	Variance Components	% Variation	*p*-Value
A	Among lineages	588.361	4.299	80.575	<0.0001
	Among populations within lineages	22.984	0.089	1.666	<0.0001
	Within populations	191.042	0.948	17.759	<0.0001
	Total	802.387	5.336	100	
B	Among lineages	54.177	9.285	87.363	<0.0001
	Among populations within lineages	11.169	0.839	7.901	<0.0001
	Within populations	12.428	0.503	4.736	<0.0001
	Total	77.774	10.627	100	

The significant subdivision between lineages was confirmed by the large genetic pairwise distances between them (F_{ST}; Tables 4 and 5) and low level of genetic flow (Nm; Tables 4 and 5).

For *E. affinis*, within the BS lineage, there was no to very little genetic differentiation between populations as indicated by extremely low F_{ST} (<0.111) and Nm values (<0.1). Within the EAt lineage, there was high genetic differentiation with little genetic flow (F_{ST} = 0.221, Nm = 0.283). In the NSEC lineage, the Tamar population was the most differentiated with F_{ST} values ranging from 0.514 (Tamar–Seine) to 0.790 (Tamar–Scheldt). The rest of the populations showed no to little differentiation as indicated by extremely low F_{ST} and Nm values (Table 4).

For *E. velox*, the TU lineage was genetically extremely distant from the populations within the EU lineage (F_{ST} from 0.922 to 0.959) and exhibited the lowest level of genetic flow (Nm from 0.024 to 0.042). Populations within the EU lineage exhibited moderate to high genetic differentiation, with the populations of Cheboksary and Oder showing no evidence of genetic subdivision as indicated by an F_{ST} of 0 and no genetic flow (Table 5).

Table 4. Pairwise population F_{ST} values (lower diagonal) and Nm (upper diagonal) of *E. affinis* on the *CO1* locus.

Populations	Vistula	Vyborg	Riga	Luga	Baltic Sea	Himmerfjarden	Gironde	Loire	Elbe	N. Dvina	Tamar	Scheldt	Seine
Vistula		0.089	0.079	0	0	0	7.059	5.434	11.096	6.607	6.336	15.520	5.156
Vyborg	0.082		0.100	0.041	0.079	0.027	8.359	7.272	9.402	7.754	7.039	10.485	6.322
Riga	0.074	0.091 *		0.012	0	0.078	6.880	5.758	8.945	5.414	5.186	10.234	5.191
Luga	−0.111	0.039 **	0.012		0	0.012	1.499	1.437	1.899	0.763	0.802	1.713	1.359
Baltic Sea	−0.026	0.073 *	−0.016	−0.014		0.043	5.038	4.376	6.549	2.809	2.886	6.919	3.804
Himmerfjarden	−0.001	0.026	0.072 *	0.012	0.041		6.045	5.313	7.195	4.850	4.548	7.692	4.727
Gironde	0.876 ***	0.893 ***	0.873 ***	0.599 ***	0.834 ***	0.858 ***		0.283	15.908	11.989	9.918	17.832	9.325
Loire	0.845 ***	0.879 ***	0.852 ***	0.590 ***	0.814 ***	0.841 ***	0.221 ***		12.455	8.355	6.974	13.248	7.486
Elbe	0.917 ***	0.904 ***	0.899 ***	0.655	0.867 ***	0.878 ***	0.941 ***	0.926 ***		0	2.555	0.001	0.014
N. Dvina	0.868 *	0.886 ***	0.844 ***	0.433	0.737 *	0.829 ***	0.923 **	0.893 **	−0.016		2.104	0.117	0
Tamar	0.864 **	0.876 ***	0.838 ***	0.445 ***	0.743 *	0.819 ***	0.908 ***	0.874 ***	0.719 ***	0.678 *		3.772	1.056
Scheldt	0.939 ***	0.913 ***	0.911 ***	0.631 ***	0.874 ***	0.885 ***	0.947 ***	0.929 ***	0.001	0.104	0.790 ***		0.004
Seine	0.837 ***	0.863 ***	0.838 ***	0.576 ***	0.792 ***	0.825 ***	0.903 ***	0.882 ***	0.014	−0.114	0.514 ***	0.004	

* Estimated pairwise F_{ST} *p*-values are indicated with asterisks. Significance levels: <0.001 '***'; <0.01 '**'; <0.05 '*'; 0.1 ' '.

Table 5. Pairwise population F_{ST} values (lower diagonal) and Nm (upper diagonal) of *E. velox* on the *CO1* locus.

Populations	Cheboksary	Feofania	Oder	Scheldt	Smolino
Cheboksary		0.076	0	0.217	0.024
Feofania	0.868		0.259	0.545	0.026
Oder	0	0.659		0.352	0.042
Scheldt	0.697 **	0.479 **	0.587 *		0.021
Smolino	0.953	0.949	0.922	0.959 ***	

* Estimated pairwise F_{ST} *p*-values are indicated with asterisks. Significance levels: <0.001 '***'; <0.01 '**'; <0.05 '*'; 0.1 ' '.

4. Discussion

This study investigated the genetic structure of the genus *Eurytemora* in geographic Europe based on a more extensive sampling strategy than in previous studies. The results of this study are congruent with previous findings [1,7,12,23,24] but also further elucidate the differentiation patterns at both species and population levels. This is particularly striking when examining the fixation index (F_{ST}) and the level of gene flow (Nm), which have not been previously estimated for populations of *E. velox*.

4.1. Global Phylogeny of the Eurytemora Genus in Geographic Europe

To date, only a few nuclear and mitochondrial markers have been used to successfully elucidate phylogenetic relationships in copepods [47,48]. More specifically, for the *Eurytemora* genus, only three mitochondrial markers (*CO1*, *cytb*, and *16sRNA*) and three nuclear markers (ITS1, ITS2, and *18sRNA*) have been used [1]. The mitochondrial COI gene has been extensively adopted as a DNA barcode for copepod identification [49–51]. It is considered to be a powerful tool for this purpose [52]. This marker has proven its high resolution within the *Eurytemora* genus at the species level [1,7,8,12]. Sukhikh and coauthors [1] carried out a comparative analysis of the genetic differentiation of the *Eurytemora* genus using the *CO1*, *nITS*, and *18sRNA* genes. They both showed a congruence between the *CO1* and *nITS* genes as well as demonstrated that the *18sRNA* gene was uninformative within the *E. affinis* complex. Similar results from *18sRNA* analysis have been shown for several other copepod species [53]. Due to the limited number of available sequences for the *cytb*, *16sRNA*, and *18sRNA* genes, the present study relied solely on the *CO1* and *nITS* loci.

The present work integrated nearly all published European *Eurytemora* sequences with 22 new *CO1* sequences from two species (i.e., *E. affinis* and *E. velox*), which had never been studied before within a global phylogenetic context. The study area extended from the Atlantic coast to the Urals region in Russia and comprised six *Eurytemora* species.

The phylogenetic reconstruction of the studied *Eurytemora* species and populations generally yielded high statistical levels of node support (Figure 2).

All three ML trees showed a clear differentiation between the studied species with levels of nucleotide differences ranging from 20 to 26% (Table S1). Crustaceans exhibit

higher sequence variation in the *CO1* region compared to other animal groups. For instance, divergence values between species of lepidopterans show 6.1% variation [54], those in birds 7.93% [55], and those in fishes 9.93% [56]. For crustaceans, the average sequence divergence is 17% between species of the same genus and 0.46% within species [57,58]. However, the results in this study indicate higher genetic distances between *Eurytemora* species, as also shown by [18]. Furthermore, Bucklin and coauthors [59] showed that congeneric species of calanoid copepods (Calanidae and Clausocalanidae) differed by 9 to 24% in the *CO1* gene, which was more in line with the results for the *Eurytemora* genus. The range of nucleotide differences between species was similar with the *nITS* locus, where average nucleotide differences varied from 3.9 to 27.6% within the *E. affinis* complex [1].

Our findings based on both mtDNA (*CO1*) as well as nDNA (*nITS*) confirmed the monophyletic clade status of the *E. affinis* complex, comprising *E. affinis*, *E. carolleeae,* and *E. caspica* [7,12]. Within this complex, nucleotide differences ranged from 15 to 16.4% (Table S1), similar to findings by [28].

Apart from the *E. affinis* species complex, all other species are genetically distinct. *E. velox*, *E. lacustris*, *E. bilobata*, *E. foveola,* and *E. arctica* belong to a monophyletic clade (Figure 2). Sukhikh and coauthors [23] also showed the close topology of these species.

The ML trees (Figures 2, S1 and S2) reveal a significant subdivision between species. This finding is corroborated by high genetic pairwise distances (F_{ST}; Table 2). Moreover, the low level of genetic flow (Nm; Table 2) indicates minimal genetic exchange between the species. Given the low Nm rates and high F_{ST} values, interspecific genetic homogenization—and, therefore, a high hybridization rate—is unlikely. When examining pairwise F_{ST} and Nm values among species in the same localities (Table S1), we observe a consistent pattern of high genetic pairwise distances and low genetic flow. This suggests that the species do not intermix despite sharing the same habitat. Furthermore, Sukhikh and Fefilova [24] note that no reliable evidence of hybrids among the species of the genus *Eurytemora* has been identified. However, the results in Table S1 should be interpreted with caution considering the small sampling size in the Luga Bay (*E. carolleeae* n = 2), the Oder river (*E. velox* n = 2, *E. carolleeae* n = 1), and the Pechora Bay (*E. lacustris* n = 2 and *E. gracilicauda* n = 2). Very little information on the coexistence/interaction of *Eurytemora* species is available [11,26]. In the Baltic Sea, the cohabitation of the American invasive species *E. carolleeae* and the European *E. affinis* has been quite well documented [28,60]. Despite their coexistence, the species have not mixed with each other for at least 20 years of observations and hybrids have only rarely been observed [11]. Typically, hybridization cases among copepods are rare and have been observed primarily within marine groups [61,62]. In fresh water habitats, hybridization is also rare [63]. The case of *E. carolleeae* in the Baltic Sea should be further investigated as far as interspecific competition with *E. affinis* is concerned, considering that its larger body size, coupled with different reproductive traits, could enable it to outcompete the *E. affinis* native species [11].

4.2. Eurytemora affinis Population Structure

This study corroborated previous findings concerning the genetic heterogeneity between populations of *E. affinis*. The species is divided into three geographically isolated geographic European lineages: the Baltic (BS), comprising the populations of the Gulf of Finland and Baltic Sea; the East Atlantic (EAt), comprising populations of the Gironde and Loire estuaries; and the North Sea and English Channel (NSEC), comprising the populations of the Tamar, Seine, Scheldt, and Elbe and the remote Northern Dvina (Figures 2 and 3).

Phylogenetic analyses of the *nITS* locus allowed clear differentiation between the studied species but bared less resolution at the population level (Figure S1).

Among the *E. affinis* lineages, pairwise nucleotide differences ranged from 1.5 to 2.5% (Table S1). These lineages remained geographically isolated. Only one exception was found wherein one Seine haplotype was found in the EAt lineage (Figure 3). According to [12], sequences from the Riga Bay were revealed among the EAt and NSEC lineages. This could be explained by human-mediated transport as a potential vector of dispersal among the

Seine, Riga Bay, and EAt lineages. Another possible explanation, considering that the sequences from the EAt and the Seine estuary were both sequenced by the same laboratory, could be human error in labelling the sample, which remains difficult to assess.

Baek and coauthors [64] showed that percentage differences within species of copepods ranged from 0.62 to 2.42% in the *CO1* gene, which was congruent with the results obtained in this study between the lineages (Table S1). The genetic heterogeneity observed through the percentage of pairwise nucleotide differences could be indicative of the subspecies level [15,65]. Furthermore, significant morphological differences were observed among the *E. affinis* populations in measures of the genital somite, the fourth pair of legs (P4), and the caudal rami [7]. However, additional studies on the stability of said morphological differences among these populations are needed for subspecies description.

The BS and NSEC lineages are star-like, centered around a dominant haplotype, whereas the EAt lineage demonstrates a more dispersed distribution (Figure 3), reflecting its limited occurrence across only two localities (Loire and Gironde estuaries). The NSEC lineage displays low haplotype and nucleotide diversity, contrasting with the higher haplotype diversity observed in the EAt and BS lineages (Table S2). The BS lineage shows high nucleotide diversity while the NSEC lineage shows the lowest (Table S2). These diversity patterns suggest recent population bottlenecks or founder events for lineages with low diversity and rapid population growth following periods of low effective size for those with high haplotype diversity [66–68]. These results align with previous studies on the genetic heterogeneity of *E. affinis* populations [7,12]. Furthermore, Winkler and coauthors [12] observed evidence of recent demographic expansion across the lineages, with the BS and the EAt lineages showing indications of population growth, while the NSEC lineage was consistent with a stable population size. This interpretation is in line with the results in the present study.

The AMOVA analysis revealed a strong genetic subdivision among lineages among which most of the variance was distributed (Table 3A). Within each lineage, little to no genetic differentiation between populations was shown, given the low F_{ST} and Nm values. This was particularly the case for the BS lineage ($F_{ST} < 0.111$ and $Nm < 0.1$ across populations; Table 4). This suggests that the populations are genetically similar but geographically or ecologically separated. This suggestion is corroborated by the fact that only 1.66% of the variance was distributed among populations (Table 4).

Within the NSEC lineage, the westernmost population of the Tamar estuary showed rather high and significant F_{ST} values with the other populations, suggesting another gene pool in this region. Rather high Nm values with the other estuaries were also observed, which may seem counterintuitive with F_{ST} values (Table 4). However, the low sampling size (n = 5) might have biased estimations. There are several possible explanations for this phenomenon. While gene flow typically inhibits genetic divergence and speciation, intense divergent selection can lead to allele frequency divergence, even in the presence of gene flow [69]. There is considerable literature to support this suggestion [70–73], which suggests that strong divergent selection can shield closely linked loci from gene flow [74]. So, whilst rather high Nm values (Table 4) were observed between the Tamar and the other estuaries, if populations are subjected to distinct selective pressures, they can evolve independently, resulting in genetic differentiation. Another possible explanation may be genetic drift, which is more pronounced in smaller populations and can increase genetic differentiation between them [75]. Hence, despite a substantial level of gene flow, F_{ST} values indicate considerable population divergence, possibly driven by the influence of genetic drift over gene flow.

Finally, within the EAt lineage, the two localities showed intermediate significant divergence and a low level of genetic flow ($F_{ST} = 0.221$, $Nm = 0.283$; Table 4). In the Gironde estuary, *E. affinis* is predominantly found in the upstream oligohaline zone [76], so natural dispersal between the Gironde and Loire estuaries via the Atlantic Ocean may be restricted. Furthermore, no natural connectivity between the two estuaries exists, apart from the ocean. However, previous studies revealed that this clade is found in the Bois-de-Boulogne lake

(Paris, France), which is connected to the Seine River [7]. More data will be needed to test whether central France might be a sympatric area or a geographic border for NSEC and EAt populations or the occurrence of specimens of the EAt lineage might be the result of human activity. A similar picture was observed for *E. carolleeae* in the Pacific Northwest and the Northeast Atlantic along the North American continent [6].

According to Winkler and coauthors [12] and Sukhikh and coauthors [7], the East Atlantic (EAt) lineage was separated earlier than the other two lineages. This is also supported by primitive features observed in the Loire and Gironde estuaries specimens (e.g., seta segmentation) described in *E. carolleeae* and *E. capsica* [68,69]. Furthermore, only one haplotype was shared between the EAt and the NSEC region, suggesting no participation of the EAt in the most recent range expansion to the northern areas. An interesting result is the position of the Northern Dvina individuals within the NSEC lineage. The present Baltic basin is a Quaternary phenomenon that is post-Holsteinian in age (0.4 Mya, [77]). During the Eem stage, 0.12 Mya, there was a broad connection between the Baltic basin and the ocean, as well as a supposed connection with the White Sea basin [78,79]. At ~10,000 BP, the contraction of the ice cap led to the Ancylus lake episode where the connection to the Skagerrak closed [80]. The final seawater incursion seems to have taken place at about 7500 BP, when the Littorina Sea came into being, once again connecting the Baltic basin to the North Sea. A possible explanation for the Northern Dvina's lineage affiliation could be that there was an original single genotype covering the whole northern region that was then isolated during the Ancylus lake episode. In this context, population mechanisms, such as genetic drifts and bottlenecks, may have enabled the genetic differentiation of the Baltic populations, thus explaining the Northern Dvina's inclusion in the NSEC lineage. The low dispersal ability of *Eurytemora* and different drainage basins may have prevented populations from meeting again, thus maintaining/increasing the genetic differentiation acquired during geographical isolation. Further investigations on the evolutionary history of the species are needed in order to confirm this hypothesis.

4.3. Eurytemora velox Population Structure

This study confirmed previous results about the genetic heterogeneity between populations of *E. velox* [23,24]. There are two known lineages: the European lineage (EU), comprising the populations of the Scheldt estuary, the Feofania lake, the Oder river, and the Cheboksary reservoir; and the Trans-Ural lineage (TU), comprising the Smolino lake population (Figures 2 and 4). The two lineages are more widespread than previously observed for *E. affinis,* and 17 mutational steps are counted between the two, which is much more than between *E. affinis* populations (Figure 3) This goes in line with the higher nucleotide differences observed in the *CO1* gene. Previous studies have shown the close relationship between the "strictly" European populations (Feofania lake and Oder river) and Central Russia (Cheboksary reservoir) with just a few nucleotide substitutions between the two [23–25]. This study corroborated these results with the addition of a new population, that of the Scheldt. Furthermore, Sukhikh and coauthors [23] highlighted that Southern Ural populations differed by 4% in the *CO1* gene with the European populations, which was also the case here, corresponding to the subspecies level in Copepoda [65,81]. Our results are congruent with those of Sukhikh and coauthors [23], who extended their investigation all the way to the Ob Bay in the Yamal region (Russia) and the lake in Magnitogorsk (the Urals). Their results indicate that the *E. velox* individual of the Ob Bay is identical to the ones in the Chelyabinsk population.

Eurytemora velox is believed to have a Ponto-Caspian origin [22] and exhibits a significant range extension in recent decades that is supposedly due to high species invasiveness. The euryhaline species occupies fresh and brackish waters and has been found in the brackish reach of rivers of the North Baltic, Caspian, Black, and Azov Seas as well as in the fresh water of rivers flowing into these said seas [82,83].

In a time-calibrated phylogeny based on the *CO1* locus (~530 bp), Sukhikh and coauthors [23] estimated that the European (Ponto-Caspian) and Ural-Siberian lineages

diverged ~0.397 Mya (0.133–0.927 Mya), which could coincide with Middle Pleistocene glaciation events. However, it remains necessary to interpret current *Eurytemora* phylogenies with caution considering (1) the limited molecular data at hand (less than 1.5 kbp), (2) the poor taxonomic coverage for some loci (only six species have data for nDNA), and (3) the absence of species-specific substitution rates. Nevertheless, it is plausible to assume that one lineage spread towards Europe and the other along the eastern part of the Ural region during the interglacials. The cyclical advances and retreats of the Pleistocene glaciers can be implicated in the initial divergence between the two lineages. As the ice sheets acted as strong barriers to gene flow, glacial retreats led to the formation of dispersal pathways [84,85]. Photogeological and dating studies suggest that Pleistocene ice sheets expanding onto continental Russia blocked the northward flow of rivers and created paleo-lakes west of the Ural Mountains [86]. The subsequent rerouting of drainage outlets toward the Ponto-Caspian Basin may have provided additional passageways that facilitated colonization [87]. These two lineages' common origin can be confirmed by the *nITS* haplotype network structure (Figure S3) wherein some Smolino haplotypes are dispersed within the EU lineage. Haplotype diversity (Hd) values in the two lineages are high and quite similar, with the EU lineage exhibiting a slightly higher Hd (Table S2), whilst nucleotide diversities (π) were also similar between them and rather low. As stated above, high haplotype and low nucleotide diversity can indicate rapid population growth after a period of low effective size. These results are consistent with the rapid expansion of the species. Furthermore, the species was only recently detected in Western Siberia [88,89] whereas it was not observed throughout the 1979–2008 monitoring period of the lower Ob [90], indicating a recent colonization of the area.

For *E. velox*, the genetic subdivision between lineages was higher than for *E. affinis*, where 87.36% of genetic variance was distributed among lineages and 7.9% distributed among the populations (Table 3B). These results are congruent with the F_{ST} and Nm values obtained (Table 5). Indeed, two lineages of *E. velox* are genetically very distant from one another (F_{ST}; Table 5) and exhibit an extremely low level of genetic flow (Nm; Table 5). These results reinforce the subspecies hypothesis. Conversely to *E. affinis*, a higher genetic variance is observed among the populations. Where *E. affinis* seems to be geographically well structured, *E. velox* populations within the EU lineage exhibit moderate to high differentiation, except for the Cheboksary and Oder populations, which show no evidence of genetic subdivision. However, this result has to be interpreted with caution due to the small sampling sizes of the Cheboksary (n = 2) and Oder (n = 2) populations.

A more comprehensive sampling scheme should be considered in order to better understand *E. velox* genetic and diversity structure. Overall, only ten sequences were available throughout four localities (Cheboksary reservoir, Feofania ponds, Oder river, and Smolino lake). This study allowed to enrich the database by adding 20 sequences from the Scheldt estuary. Whilst the species is widely distributed in Northern and Western Europe and in the Western Mediterranean region [21], genetic sampling is lacking.

5. Conclusions

In conclusion, this study investigated the genetic structure of the *Eurytemora* genus in geographic Europe based on a more extensive dataset than previous research. The results of this study align with prior findings but provide deeper insights into differentiation patterns at both species and population levels. Particularly noteworthy are the fixation index (F_{ST}) and level of gene flow (Nm) estimations, previously unexplored for *E. velox*, revealing a substantial subdivision between species and high genetic differentiation among geographically isolated lineages. Our findings further emphasize the need for more extensive genetic sampling and comprehensive analyses to elucidate the evolutionary history and population dynamics of *E. velox*, particularly in light of its ecological significance and potential invasive nature.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d16080483/s1, Figure S1: Maximum Likelihood phylogenetic tree of *Eurytemora* species based on 69 sequences of *nITS* (842 bp). *Centropages typicus* Krøyer, 1849 is used as an outgroup. Nodes indicate bootstrap support (values of <10% are not shown); Figure S2: Maximum Likelihood phylogenetic tree of *Eurytemora* species based on the concatenation of *CO1* and *nITS* for 46 terminals. *Centropages typicus* Krøyer, 1849 is used as an outgroup. Nodes indicate bootstrap support (values of <10% are not shown). A color code is assigned to each lineage for *E. affinis* and *E. velox*; Figure S3: Median-joining nDNA (*nITS*) network for (A) *E. affinis* populations and (B) *E. velox* populations. The sizes of the circles are proportional to haplotype frequency. For (A), the smallest and biggest circles represent 1 and 14 individuals, respectively. For (B), the smallest and biggest circles represent 1 and 7 individuals, respectively. The numbers of mutations between haplotypes are indicated on the branches by small bars. Black circles represent missing intermediate haplotypes. A color code to the locality of origin is given; Table S1: Mean nucleotide differences based on CO1 sequences, among (A) *Eurytemora* species, (B) *E. affinis* populations and (C) *E. velox* populations; Table S2: Pairwise species F_{ST} values (lower diagonal) and Nm (upper diagonal) for *Eurytemora* species found in the same localities, based on the *CO1* locus; Table S3: Patterns of mtDNA (*CO1*) haplotype variation of (A) *E. affinis* and (B) *E. velox* populations at each location.

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Appendix A

DNA was extracted individually from whole individuals using the DNeasy Blood and Tissue extraction kit (QIAGEN) according to the manufacturer's protocol. A chemical lysis was performed and samples were incubated overnight at 56 °C with 180 µL ATL buffer and 20 µL proteinase K under constant mixing. AL buffer and absolute ethanol were added and supernatants were loaded onto the "DNeasy spin columns" and then washed with buffers AW1 and AW2. Elution was carried out with 70 µL of AE buffer heated at 56 °C and incubated for 3 min on the column before centrifugation.

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