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Pleistocene Glacial Refugia in the Don River Basin: Witness from the Endangered Depressed River Mussel

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Abstract: The depressed river mussel *Pseudanodonta complanata* (Rossmässler, 1835) is an endangered freshwater bivalve with sparse distribution across Europe. Despite its high conservation priority, available data on the current population genetics and biogeography of *P. complanata* are very limited. In this paper, we studied the genetic diversity and differentiation of *P. complanata* based on newly sequenced COI and ND1 gene fragments, and on available sequences from the NCBI GenBank. We discovered a localized and highly divergent lineage in the Don River basin and a widespread but low diversified lineage distributed from the Volga River basin to the Oise River in France. A star-like haplotype network, results of mismatch analysis, and significant negative values of neutrality tests reveal that the pan-European lineage went through a bottleneck event. The discovery of the separate lineage in the Don River basin is and additional evidence of the existence of Pleistocene glacial refugia in the south of the Russian Plain. Moreover, this finding confirms the significance of the Azov Sea drainage as a freshwater biodiversity hotspot of great conservation importance.

Keywords: *Pseudanodonta complanata*; freshwater ecosystems; genetic lineage; mitochondrial DNA; COI; ND1; population genetics

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1. Introduction

The last glacial maximum (LGM, i.e., 25–17 kya) is the most recent global cooling event in the past that has shaped the extant European biota. During the glaciations, most of the terrestrial and freshwater species retreated to lower latitude areas, where climatic conditions were less extreme [1–3]. Long-term isolation benefits intraspecific diversification due to restricted gene flow and preservation of initial genetic diversity in isolated populations [4]. During the interglacial periods and after the ice sheet retreat, these areas, called refugia, become sources of recolonization of deglaciated regions [5–8].

Detecting glacial refugia is often based on genomic data of certain taxa and reconstruction of their demographic history [6,7,9]. The Iberian, Italian, and Balkan peninsulas of Southern Europe, as well as the Azov Lowland, have been described as such refugia for freshwater biota [2,9–11].

Among the aquatic invertebrates, freshwater mussels (order Unionida) are the most imperiled group worldwide [12]. Being a keystone and umbrella species of benthic communities in freshwater ecosystems, mussels provide important ecosystem functions that benefit aquatic communities [13]. Moreover, mussel functions in ecosystems can in turn be framed as the ecosystem services that they provide or contribute towards, and that humans derive from ecosystems [14].

The depressed river mussel *Pseudanodonta complanata* (Rossmässler, 1835) is one of the three European representatives of the tribe Anodontini Rafinesque, 1820 (Unionidae: Unioninae) and phylogenetically neighbors to the swan mussel *Anodonta cygnea* (Linnaeus, 1758) and the duck mussel *Anodonta anatina* (Linnaeus, 1758) [15]. The distribution range of *P. complanata* is the smallest among the European Anodontini and is greatly overlapped with the two *Anodonta* species [15,16]. Populations of depressed river mussel were recorded across Europe from the British Isles to the Volga River basin [15,17–19]. This mussel is one of the 16 European species listed under the risk categories in the IUCN Red List [20] and legally protected at national levels [16,17,21].

The sparse distribution, low population density of *P. complanata*, which is rare in most of the range, and a small number of recorded populations [16] raises the question on its actual biogeography patterns. The depressed river mussel inhabits a narrow range of suitable habitats in lotic environments, including lowland and mountainous rivers, large drains, and canals [22]. *P. complanata* is also reported as a bivalve species that is highly sensitive to unfavorable conditions such as habitat disturbance [18] or alien species invasions [19] and it disappears first from mussel communities [23].

Molecular data are a keystone tool for mussel conservation management [24,25]. Molecular markers that are able to infer intraspecific population diversity and differentiation are especially important because they help to define units for prioritizing conservation. Previous work attempted to use mitochondrial DNA to examine *P. complanata* population structure in Britain but failed to reveal useful levels of intraspecific variability [26–28]. A significant part of the depressed river mussel range, situated in the east of species distribution, remains unstudied in many aspects but especially in terms of population genetics. However, previous evidence of the Azov glacial refugium allows us to hypothesize that the level of genetic divergence in this area can be higher than in the other part of the species range. In this case the genetic hotspot, which is significant for species conservation, can be overlooked.

In this study, we aimed to check the hypothesis that *P. complanata* may possess a high level of genetic diversity in the Don River basin and that the population in this basin can be highly divergent from others because of Pleistocene glacial refugium recovered in the region. For this purpose, we (1) established the most comprehensive set of newly obtained mtCOI and ND1 sequences to date and combined it with previously obtained available data; (2) based on this combined dataset we determined the genetic diversity and differentiation of *P. complanata* populations; and (3) studied intraspecific demographic processes.

2. Materials and Methods

2.1. Sample Collection, DNA Extraction, PCR and Sequencing

The main body of the material was collected under a series of field surveys on rivers and streams of the Baltic Sea, Volga River, and Don River basins (Figure 1, Table S1). Soft tissue samples of *P. complanata* were snipped from the mussel's foot by a non-lethal procedure [29,30] and immediately stored in 96% ethanol. After snipping, all mussels were carefully returned to the habitat. Soft tissue samples were deposited in the collection of the Russian Museum of Biodiversity Hotspots (RMBH) of the N. Laverov Federal Center for Integrated Arctic Research of the Ural Branch of the Russian Academy of Sciences (Arkhangelsk, Russia).

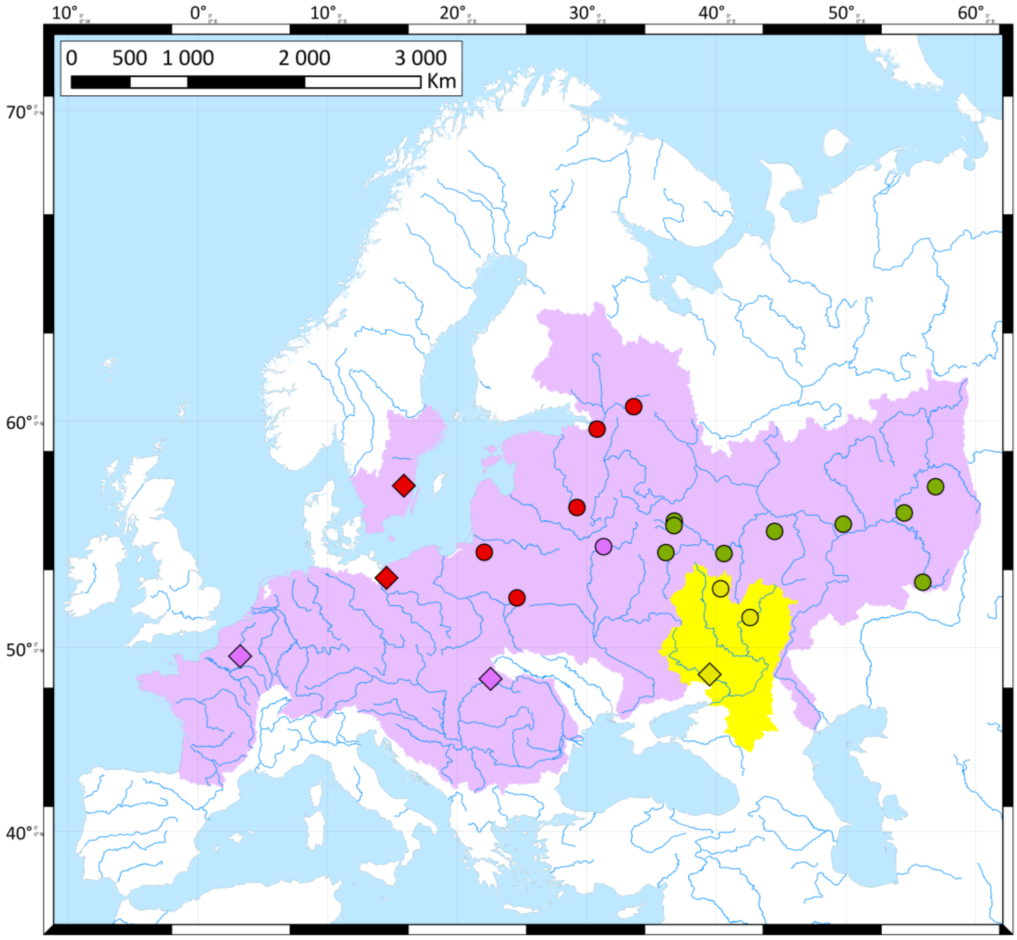

Figure 1. Sampling localities of *Pseudanodonta complanata* in freshwater basins of Europe (our data (dots) and samples from NCBI GenBank with available georeferenced data (diamonds)). Point colors represent the following groups of haplotypes: yellow—DON, green—VOL, red—BS, and violet—EUR. Filling colors represent the range of the following lineages across freshwater basins: yellow—DON lineage, and violet—panEUR lineage (Table S1). The map was created using ESRI ArcGIS 10 software (https://www.esri.com/arcgis, accessed on 8 May 2023). The topographic base of the map was compiled with Natural Earth free vector and raster map data (http://www.naturalearthdata.com, accessed on 8 May 2023), and the HydroSHEDS database (http://www.hydrosheds.org, accessed on 8 May 2023) [31].

Total genomic DNA was extracted from the tissue snips using the NucleoSpin Tissue Kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany), following the manufacturer's protocol. For molecular analyses, we generated sequences of the COI ($n = 105$) and ND1 ($n = 19$) genes. The sequences were amplified and sequenced using the primers pair LCO1490 and HCO2198 [32] for COI, LeuF and LoGlyR [33] for ND1. The PCR mix contained approximately 100 ng of total cellular DNA, 10 pmol of each primer, 200 µmol of each dNTP, 2.5 µL of PCR buffer (with 10 × 2 mmol $MgCl_2$), 0.8 units of Taq DNA polymerase (SibEnzyme Ltd., Russia), and ddH_2O which was added up to a final volume of 25 µL. Thermocycling included one cycle at 95 °C (4 min), followed by 28–32 cycles of 95 °C (50 s), 48–57 °C (50 s), and 72 °C (50 s), and a final extension at 72 °C (5 min). Forward and reverse sequencing was performed on an automatic sequencer (ABI PRISM3730, Applied Biosystems) using the ABI PRISM BigDye Terminator v.3.1 reagent kit [34]. The resulting sequences were checked manually using BioEdit v. 7.2.5 [35]. In addition, 17 COI sequences of *P. complanata* were obtained from the NCBI GenBank (Table S1). The sequences were aligned using the MUSCLE algorithm implemented in MEGAX [36]. For the subsequent analyses, each COI and ND1 sequence of the aligned datasets was trimmed, leaving a 624-bp and 901-bp fragment correspondingly.

2.2. Genetic Diversity, Genetic Differentiation, and Demographic History

The COI dataset under investigation mainly covers the *P. complanata* range in the East-European (Russian) Plain and includes available sequences from the rest of the range in Europe. For the following analysis, we structured our dataset into the four subsets corresponding to certain groups of populations: Don River (DON; $n = 9$), Volga River (VOL; $n = 47$), Baltic Sea (BS; $n = 31$), and European rivers (EUR; $n = 18$). The DON, VOL, and BS groups of populations were delineated by corresponding watersheds. The EUR group includes populations from the rest of the species range. The ND1 dataset contains 19 sequences and was used only to build haplotype network and to calculate pairwise Fst's values and p-distances to verify results obtained from the COI dataset. All population genetic statistics were performed using the latter one.

Genetic diversity was estimated through haplotype diversity (H_d) and nucleotide diversity (π) calculations. Genetic differentiation was estimated through calculations of pairwise Fst's by the method of Tajima and Nei and mean genetic distances under the Kimura 2-parameter model (K2p) [37]. To detect deviation from mutational-drift equilibrium in the studied groups, we calculated Fu's F and Tajima's D neutrality tests. In the case of significance in at least one neutrality test we examined the frequency distributions of pairwise mismatch between sequences (MMD). The observed mismatch distribution was compared with that obtained under models of spatial expansion and population expansion for the evidence of model fit by calculating the sum of squared deviations (SSD) of the observed data relative to the model and Harpending's raggedness statistic (HRag). Genetic diversity indices, Fst's values, neutrality tests, and MMD were calculated using Arlequin v. 3.5.1.2 [38] all with 10,000 permutations. Genetic distances under K2p model were calculated using MEGAX [36]. To estimate the phylogeographic structure of haplotypes obtained from groups of populations, we applied a TCS haplotype network [39] using POPArt (http://popart.otago.ac.nz, accessed on 10 May 2023).

3. Results

3.1. Genetic Diversity

A total of 23 haplotypes were identified from the COI dataset ($n = 105$) and 12 haplotypes from the ND1 dataset ($n = 19$) of *P. complanata* sequences (Table S1). The COI haplotype diversity varies in a wide range among groups of populations. The highest Hd value was observed in the DON group (0.94 ± 0.07) and the lowest in the BS group (0.24 ± 0.1) (Table 1). The level of nucleotide diversity varied in the same way.

3.2. Genetic Differentiation and Genetic Structure

TCS COI and ND1 haplotype networks demonstrate the existence of highly divergent and isolated intraspecific genetic lineage in the Don River basin and the widely distributed lineage composed of multiple closely related haplotypes that derived from populations across the species range (Figure 2). We defined this lineage as pan-European (panEUR). DON and panEUR lineages are divided by 11 mutational steps in the COI network and by 15 mutational steps in the ND1 network. Haplotype ordering in both COI lineages represents star-like structures, though significant differences occur. The ancestral haplotype (H4) in the DON lineage is recorded in two individuals from Hoper and Voronezh populations as well as haplotype H5. Five other haplotypes are unique, and the most divergent H6 haplotype was discovered in the Hoper population. The most abundant haplotype in the European lineage (H1) is shared between all delineated groups of populations. Most of the recorded haplotypes are divided from the central one in one mutational step, being singletons. Three of the most distant haplotypes H14, H15, and H16 are diverged in two, three and four mutational steps correspondingly and belonged to Ukrainian populations, two of which had undefined locations and one (H14) belonging to the Dnieper River Basin.

ND1 data return almost the same picture of haplotype ordering, considering a much smaller sample size. Two of the most distant haplotypes in the DON lineage are diverged by two mutational steps and recorded from the Voronezh River. In the panEUR lineage, the most distant haplotype is observed in the Velikaya River from the BS group of populations and diverged by three mutational steps from the central haplotype (H6).

Table 1. Summary of molecular diversity indices estimated from the COI sequences of *Pseudanodonta complanata*: sample size (n), number of haplotypes (N_H), haplotype diversity (H_d) and nucleotide diversity (π) with standard deviation values; results of deviation from mutational-drift equilibrium by different tests, and statistics of mismatch distribution analysis (MMD); statistically significant values are in bold ($p < 0.05$ for Tajima's D, SSD and r-raggedness; and $p < 0.02$ for Fu' Fs).

Population Group	n	N_H	$H_d \pm$ SD	$\pi \pm$ SD	Fu's F-Test		Tajima's D-Test		MMD under Spatial Expansion Model			
					FS	p-Value	D	p-Value	SSD	p-Value	r	p-Value
BS	31	4	0.24 ± 0.1	0.0004 ± 0.0005	−2.77	**0.003**	−1.54	**0.027**	0.000	0.057	0.324	0.705
DON	9	7	0.94 ± 0.07	0.004 ± 0.003	−3.37	**0.009**	−1.44	0.080	0.012	0.423	0.068	0.473
EUR	18	7	0.57 ± 0.14	0.002 ± 0.002	−2.75	0.02	−2.28	**0.001**	0.003	0.86	0.063	0.898
VOL	47	7	0.28 ± 0.09	0.001 ± 0.001	−7.23	**0.0001**	−2.02	**0.003**	0.000	**0.015**	0.273	0.690
panEUR (BS + EUR + VOL)	96	16	0.32 ± 0.06	0.001 ± 0.001	−21.39	**0.0001**	−2.59	**0.0001**	0.000	0.15	0.230	0.877

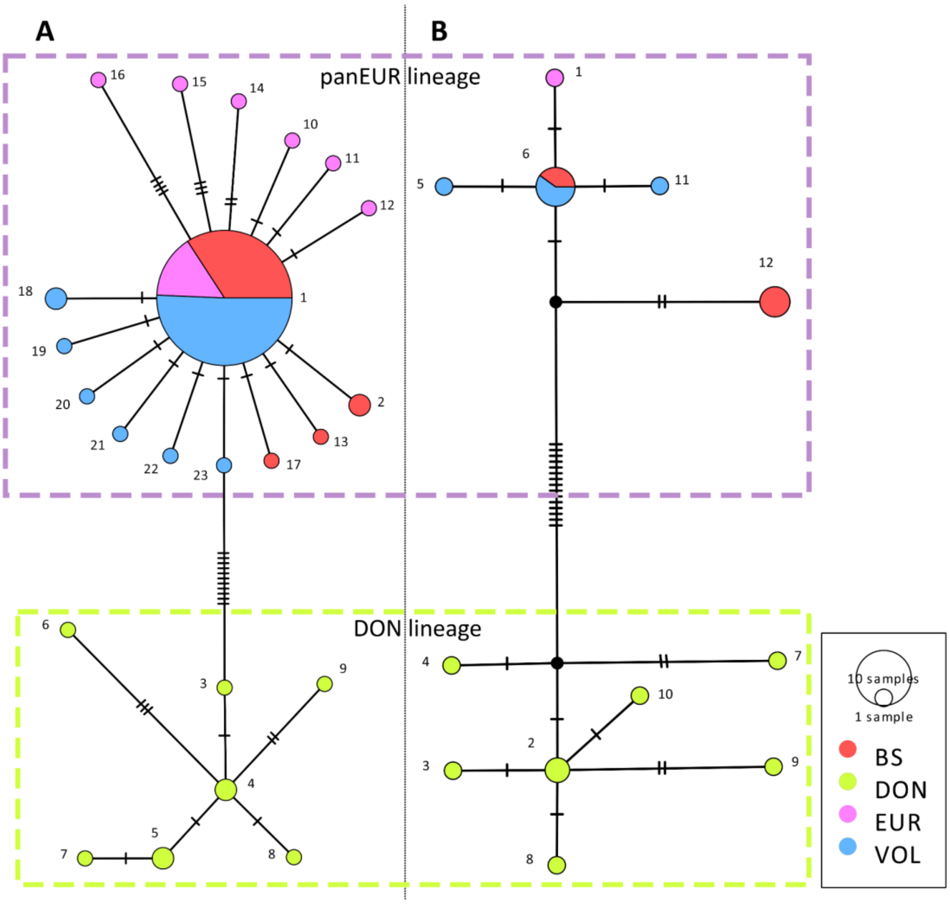

Figure 2. TCS haplotype network of the COI (**A**) and ND1 (**B**) sequences of *Pseudanodonta complanata* datasets (COI, $n = 105$; ND1, $n = 19$) delineated in four groups of populations. Dashes on branches indicate nucleotide substitutions between haplotypes. The list of sequences is presented in Table S1.

Pairwise differentiation, estimated using the Fst's fixation index calculated by Tajima and Nei method and mean genetic distances under the K2p model, support the pattern illustrated by haplotype networks (Table 2). The COI and ND1 divergence of the DON lineage is exceptionally high in the range of 0.86–0.95 of Fst's values and exceeds 2% by p-distances. The genetic divergence between groups within the panEUR lineage is almost absent by means of p-distances. Fst's fixation index, calculated using the COI dataset, returns very low but significant values within EUR–BS and EUR–VOL pairs, and insignificant value between VOL and BS.

Table 2. Mean genetic divergences by Tajima and Nei Fst's values (statistically significant values with $p < 0.05$ are in bold) below diagonal and mean genetic distances under the Kimura 2-parameter model (K2p) ± SD above diagonal for the COI and ND1 datasets between groups of *Pseudanodonta complanata* populations.

Molecular Marker	Group of Populations	BS	DON	EUR	VOL
COI	BS		0.023 ± 0.006	0.001	0
	DON	**0.951**		0.024 ± 0.006	0.023 ± 0.006
	EUR	**0.022**	**0.888**		0.001
	VOL	0.010	**0.957**	**0.032**	
ND1	DON	n/a		0.021 ± 0.005	0.021 ± 0.005
	EUR	n/a	**0.86**		0.002 ± 0.001
	VOL	n/a	**0.89**	−0.38	

3.3. Demographic Trends

The deviation from the mutation-drift equilibrium was detected for all delineated groups of populations (Table 1). BS and VOL groups show deviation by both Fu's Fs and Tajima's D tests while EUR deviate only by Tajima's D and DON by Fu's Fs. The observed mismatch distributions for panEUR lineage (Figure 3A), as well as for BS and EUR subsets (not shown), show a unimodal pattern with the prevalence of closely related samples with zero mismatch. This kind of unimodal distribution illustrates a founder effect in the most recent past that can be a consequence of the bottleneck event. The observed mismatch distribution for DON is also unimodal but much more uniform, with a slight peak at 1 bp (Figure 3B). This kind of picture illustrates a stable population that started to expand in the most recent past. We rejected the hypothesis of population expansion for the VOL group because SSD values deviate significantly from the model ($p = 0.015$).

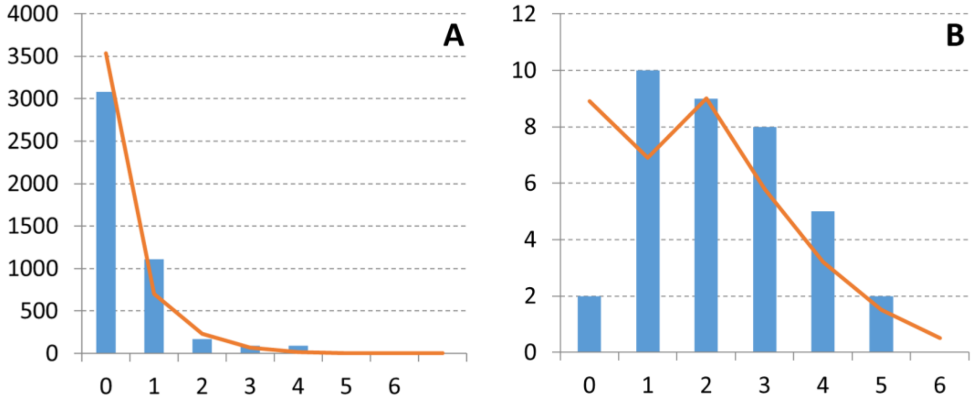

Figure 3. Diagram of the mismatch distribution (MMD) of pairwise differences of the COI sequences of *Pseudanodonta complanata* from the panEUR (**A**) and the DON (**B**) lineages. Simulated MMD under expectation of spatial expansion is marked by the orange line.

4. Discussion

Combining newly sequenced COI and ND1 gene fragments of *P. complanata* with available data from the NCBI GenBank, we identified the divergent genetic lineage in the Don River basin (Figure 1) and showed that populations from the rest of the range belong to one genetic lineage that passed through a bottleneck event in the past. Sample sizes are unequal across the studied population groups. The main cause of this is the rareness of the mussel and low densities in many populations. However, we believe that this sample bias did not significantly affect the results of population statistics. The smallest DON group demonstrates the highest H_d, while much bigger VOL and BS groups show smaller values of H_d. The only concern is the EUR group, the genetic diversity of which could be overestimated due to the insufficient sample volume of this widely distributed group. Nonetheless, a broad-scale sampling allowed us to show that the intraspecific mitochondrial diversity of the depressed river mussel is much higher than was considered earlier. Previous studies attempted to assess the population genetics of the species at the narrow spatial scale [26–28] and sampled populations fell within the low-diverged pan-European lineage. The existence of widely distributed but weakly diversified European lineage was shown earlier for *A. anatina* [9]. The duck mussel and depressed river mussel are closely related species belonging to the same tribe. Despite their environmental preferences being different [16]), the biogeographic patterns of widely distributed lineages of both species seem to be similar. Both species are host generalists with overlapped host-fish composition [15 and references herein]. The reproductive biology of both species is also very similar, with an overwintering brooding period and glochidia discharge in spring-summer time. Similar biology features can facilitate mussels' distribution by the same migration pathways, but different habitat preferences allowed them not to compete with each other in colonized river systems.

Besides the widely distributed lineage of *A. anatina*, the presence of localized and divergent lineages of this species in several glacial refugia has been described. Two of them are related to peninsular populations from Iberia and Apennines, and one was reported in the Azov Lowland [9,40]. The latter includes the Kuban River basin, the lower part of the Don River basin, and several separate rivers that drain into the Azov Sea. We found that the COI p-distances between DON and panEUR lineages of *P. complanata* and AZOV and EUR lineages of *A. anatina* are almost equal (2.3% vs. 2.35%, correspondingly). The level of intraspecific mitochondrial divergence of *P. complanata*, and the topology of haplotype networks, unambiguously show the existence of isolated genetic lineage in the Don River basin. This finding is an additional confirmation of the existence of the Pleistocene glacial refugium in the Azov Sea drainage (Kuban and Don rivers). Based on the analysis of populations that were sampled from the upper and middle parts of the Don River basin, we can suppose that the entire river system served as a refugium.

The level of genetic diversity of the DON lineage agrees well with the theoretical expectations about maximum diversity in areas where a species exists for enough time to accumulate mutations [41,42]. The output of MMD analysis confirms that populations in the Don River basin can be considered stable and contain diverse COI gene variants. At the same time, MMD indicates that the observed distribution does not deviate significantly from that predicted under the population expansion model. Together with significant negative neutrality tests, the observed MMD can be a sign of an expansion event in the past, since we observe a very low peak of mismatches at 2 bp.

A classical concept of glacial refugium is a large region that harbors a subset of genetically diverse populations from which modern extensive populations are derived [43,44]. At first glance, our results, i.e., haplotype distribution, levels of genetic diversity and differentiation, neutrality tests, and MMD analysis, agree well with this model. However, the absence of common haplotypes between DON and panEUR lineages and large genetic distance may indicate a long-term isolation of both groups that prevents us from concluding that European populations of *P. complanata* originated from the Don River basin. Alternatively, the small sample size of the DON lineage did not allow us to find haplotypes shared with the panEUR lineage. If so, additional sampling efforts in the Don River basin can help to find the link between two lineages. Considering the biogeographic pattern of *A. anatina*, we suppose the latter case is the most probable.

Recently it has been shown that multiple freshwater taxa penetrated river systems of Northeastern Europe using the Volga River as a migration corridor [45]. Considering the complex hydrological periglacial network during the LGM [46,47] and reconstructed *A. anatina* distribution during the Late Quarternary [8], we can speculate that multiple connections between rivers across Europe also occurred. Extremely low genetic differentiation between groups of populations within panEUR lineage indicates high gene flow and supports our suggestion about migration flows across the species range. A recent wide-scale study of *A. anatina* biogeography discovered a separate sub-lineage within the trans-Eurasian lineage of the duck mussel [8]. This sub-lineage is distributed in Southeastern Europe—the region where three of the most divergent haplotypes (H14, H15, and H16) within the panEUR lineage of *P. complanata* are recorded. This finding may reveal that similar biogeographic patterns may also occur in *P. complanata*, though additional sampling efforts are necessary to validate this hypothesis.

The discovery of a separate genetic lineage of *P. complanata* in the Don River basin confirms the significance of the Azov Sea drainage as a freshwater biodiversity hotspot of great conservation importance. Harboring the unique part of the genetic diversity of the depressed river mussel and duck mussel, this region potentially may also serve as a refugium for *Unio crassus* Retzius, 1788, *U. pictorum* (Linnaeus, 1758), and *U. tumidus* Retzius, 1788, but no relevant population genetic studies have been performed.

The Don and Kuban River basins are highly populated areas with developed agriculture and significant usage of freshwater resources. Moreover, freshwater ecosystems of the region are subject to aquatic invasions [48–50]. These threats underline that freshwater biodiversity of the region require intensive study and effective conservation management based on the delineation of conservation units.

5. Conclusions

The broad-scale dataset of mitochondrial gene fragments of the depressed river mussel *P. complanata* revealed that the species has a more complicated biogeographic pattern than was considered earlier. We discovered the divergent and isolated genetic lineage of *P. complanata* in the Don River basin. Populations from the rest of the range were determined as belonging to the widespread and low-diversified pan-European lineage. Phylogenetic pattern of the pan-European lineage represents one widespread ancestral COI haplotype and numerous singleton haplotypes. This pattern clearly illustrates the founder effect and is commonly observed for postglacial dispersal events. Haplotypes from populations of the Don River basin also form a star-like structure, though the network is more distributed, and the central haplotype occurs only in two populations. The level of mitochondrial diversification between two lineages of *P. complanata* is comparable to those from closely related species *A. anatina*, where a geographically restricted lineage from the Azov lowland and widely distributed European lineage were discovered earlier. Genetic diversity in studied populations of *P. complanata* is much higher in the lineage from the Don River basin than in the pan-European lineage. Mismatch distribution indicates a pattern of a long-term and stable population in the Don River basin, while the pan-European lineage passed through a bottleneck event. Extremely low genetic differentiation within the pan-European lineage and signs of a bottleneck event in the past support the hypothesis about the recent distribution of *P. complanata* across Europe after glaciation using a periglacial hydrological network. We suppose that our discovery is additional evidence for the existence of the Pleistocene glacial refugium in the Azov Sea drainage (Kuban and Don rivers). Our finding reveals that the group of populations from the Don River basin can be considered as a key evolutionary significant unit of the species as it contains a unique part of the genetic diversity of *P. complanata*. The discovery of a separate genetic lineage of *P. complanata* in the Don River basin confirms the significance of the Azov Sea drainage as a freshwater biodiversity hotspot of great conservation importance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/d15070829/s1, Table S1: List of sequences used in this study, including the location, the haplotype numbers, NCBI's GenBank accession numbers and references [51–54].

Author Contributions: I.V.V., O.A.Y. and A.V.K. developed the concept of this study. I.V.V., O.A.Y., A.V.K., G.V.B., E.S.K., D.V.K., A.A.L., A.A.S. and I.N.B. collected samples. A.V.K., O.A.Y. and E.P.P. designed and carried out molecular analyses. I.V.V. created the images, performed population genetics and statistical data analyses. I.V.V. wrote the paper, with input from A.V.K. and I.N.B. All authors have read and agreed to the published version of the manuscript.

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References

1. Hewitt, G. The Genetic Legacy of the Quaternary Ice Ages. *Nature* **2000**, *405*, 907–913. [CrossRef]
2. Consuegra, S.; García De Leániz, C.; Serdio, A.; González Morales, M.; Straus, L.G.; Knox, D.; Verspoor, E. Mitochondrial DNA Variation in Pleistocene and Modern Atlantic Salmon from the Iberian Glacial Refugium. *Mol. Ecol.* **2002**, *11*, 2037–2048. [CrossRef] [PubMed]
3. Svenning, J.C.; Skov, F. Could the Tree Diversity Pattern in Europe Be Generated by Postglacial Dispersal Limitation? *Ecol. Lett.* **2007**, *10*, 453–460. [CrossRef]
4. Hewitt, G.M. The Structure of Biodiversity—Insights from Molecular Phylogeography. *Front. Zool.* **2004**, *1*, 234. [CrossRef]
5. Makhrov, A.A.; Bolotov, I.N. Dispersal Routes and Species Identification of Freshwater Animals in Northern Europe: A Review of Molecular Evidence. *Russ. J. Genet.* **2006**, *42*, 1101–1115. [CrossRef]
6. Schmitt, T. Molecular Biogeography of Europe: Pleistocene Cycles and Postglacial Trends. *Front. Zool.* **2007**, *4*, 1–13. [CrossRef] [PubMed]
7. Sworobowicz, L.; Mamos, T.; Grabowski, M.; Wysocka, A. Lasting through the Ice Age: The Role of the Proglacial Refugia in the Maintenance of Genetic Diversity, Population Growth, and High Dispersal Rate in a Widespread Freshwater Crustacean. *Freshw. Biol.* **2020**, *65*, 1028–1046. [CrossRef]
8. Lyubas, A.A.; Tomilova, A.A.; Kondakov, A.V.; Konopleva, E.S.; Vikhrev, I.V.; Gofarov, M.Y.; Eliseeva, T.A.; Aksenova, O.V.; Bovykina, G.V.; Kryuk, D.V.; et al. Phylogeography and Genetic Diversity of Duck Mussel Anodonta Anatina (Bivalvia: Unionidae) in Eurasia. *Diversity* **2023**, *15*, 260. [CrossRef]
9. Tomilova, A.A.; Lyubas, A.A.; Kondakov, A.V.; Vikhrev, I.V.; Gofarov, M.Y.; Kolosova, Y.S.; Vinarski, M.V.; Palatov, D.M.; Bolotov, I.N. Evidence for Plio-Pleistocene Duck Mussel Refugia in the Azov Sea River Basins. *Diversity* **2020**, *12*, 118. [CrossRef]
10. Froufe, E.; Alekseyev, S.; Alexandrino, P.; Weiss, S. The Evolutionary History of Sharp- and Blunt-Snouted Lenok (Brachymystax Lenok (Pallas, 1773)) and Its Implications for the Paleo-Hydrological History of Siberia. *BMC Evol. Biol.* **2008**, *8*, 40. [CrossRef]
11. Bernatchez, L. The Evolutionary History of Brown Trout (*Salmo trutta* L.) Inferred from Phylogeographic, Nested Clade, and Mismatch Analyses of Mitochondrial DNA Variation. *Evolution* **2001**, *55*, 351–379. [CrossRef] [PubMed]
12. Böhm, M.; Dewhurst-Richman, N.I.; Seddon, M.; Ledger, S.E.H.; Albrecht, C.; Allen, D.; Bogan, A.E.; Cordeiro, J.; Cummings, K.S.; Cuttelod, A.; et al. The Conservation Status of the World's Freshwater Molluscs. *Hydrobiologia* **2021**, *848*, 3231–3254. [CrossRef]
13. Vaughn, C.C.; Nichols, S.J.; Spooner, D.E. Community and Foodweb Ecology of Freshwater Mussels. *J. N. Am. Benthol. Soc.* **2008**, *27*, 409–423. [CrossRef]
14. Vaughn, C.C. Ecosystem Services Provided by Freshwater Mussels. *Hydrobiologia* **2017**, *810*, 15–27. [CrossRef]
15. Bolotov, I.N.; Kondakov, A.V.; Konopleva, E.S.; Vikhrev, I.V.; Aksenova, O.V.; Aksenov, A.S.; Bespalaya, Y.V.; Borovskoy, A.V.; Danilov, P.P.; Dvoryankin, G.A.; et al. Integrative Taxonomy, Biogeography and Conservation of Freshwater Mussels (Unionidae) in Russia. *Sci. Rep.* **2020**, *10*, 59867. [CrossRef] [PubMed]
16. Lopes-Lima, M.; Sousa, R.; Geist, J.; Aldridge, D.C.; Araujo, R.; Bergengren, J.; Bespalaya, Y.; Bódis, E.; Burlakova, L.; Van Damme, D.; et al. Conservation Status of Freshwater Mussels in Europe: State of the Art and Future Challenges. *Biol. Rev.* **2017**, *92*, 572–607. [CrossRef]

17. Mcivor, A.L.; Aldridge, D.C. The Reproductive Biology of the Depressed River Mussel, Pseudanodonta Complanata (Bivalvia: Unionidae), with Implications for Its Conservation. *J. Molluscan Stud.* **2007**, *73*, 259–266. [CrossRef]
18. Ćmiel, A.M.; Zając, K.; Lipińska, A.M.; Zając, T. Is Pseudanodonta Complanata the Most Vulnerable of Widespread European Species of Unionids? An Intense Stress Test Leading to a Massive Die-Off. *Aquat. Conserv. Mar. Freshw. Ecosyst.* **2019**, *29*, 2185–2192. [CrossRef]
19. Ożgo, M.; Urbańska, M.; Hoos, P.; Imhof, H.K.; Kirschenstein, M.; Mayr, J.; Michl, F.; Tobiasz, R.; Von Wesendonk, M.; Zimmermann, S.; et al. Invasive Zebra Mussel (Dreissena Polymorpha) Threatens an Exceptionally Large Population of the Depressed River Mussel (Pseudanodonta Complanata) in a Postglacial Lake. *Ecol. Evol.* **2020**, *10*, 4918–4927. [CrossRef]
20. Van Damme, D. *Pseudanodonta complanata*. Available online: https://doi.org/10.2305/IUCN.UK.2011-2.RLTS.T18446A8279278.en (accessed on 20 April 2023).
21. Zając, K. Pseudanodonta Complanata (Rossmässler, 1835). Available online: http://www.iop.krakow.pl/pckz/opis6113-2.html?id=129&je=pl (accessed on 20 April 2023).
22. Bonk, M. A New Locality of the Depressed River Mussel Pseudanodonta Complanata (Rossmässler, 1835) (Bivalvia: Unionidae) in the Wisłok River (Carpathian Mountains). *Folia Malacol.* **2019**, *27*, 71–74. [CrossRef]
23. Ollard, I.; Aldridge, D.C. Declines in Freshwater Mussel Density, Size and Productivity in the River Thames over the Past Half Century. *J. Anim. Ecol.* **2023**, *92*, 112–123. [CrossRef]
24. Geist, J. Strategies for the Conservation of Endangered Freshwater Pearl Mussels (*Margaritifera margaritifera* L.): A Synthesis of Conservation Genetics and Ecology. *Hydrobiologia* **2010**, *644*, 69–88. [CrossRef]
25. Geist, J.; Kuehn, R. Genetic Diversity and Differentiation of Central European Freshwater Pearl Mussel (*Margaritifera margaritifera* L.) Populations: Implications for Conservation and Management. *Mol. Ecol.* **2005**, *14*, 425–439. [CrossRef] [PubMed]
26. Muller, S.J. Population Genetics, Ecology and Waterway Management in the Conservation of Pseudanodonta Complanata (Rossmassler). Ph.D. Thesis, University of Cambridge, Cambridge, UK, 1999.
27. Källersjö, M.; Von Proschwitz, T.; Lundberg, S.; Eldenäs, P.; Erséus, C. Evaluation of ITS RDNA as a Complement to Mitochondrial Gene Sequences for Phylogenetic Studies in Freshwater Mussels: An Example Using Unionidae from North-Western Europe. *Zool. Scr.* **2005**, *34*, 415–424. [CrossRef]
28. Skidmore, R.; Leach, C.; Hoffman, J.; Amos, W.; Aldridge, D. Conservation Genetics of the Endangered Depressed River Mussel, Pseudanodonta Complanata, Using Amplified Fragment Length Polymorphism (AFLP) Markers. *Aquat. Conserv.* **2010**, *20*, 560–567. [CrossRef]
29. Karlsson, S.; Larsen, B.M.; Eriksen, L.; Hagen, M. Four Methods of Nondestructive DNA Sampling from Freshwater Pearl Mussels *Margaritifera margaritifera* L. (Bivalvia:Unionoida). *Freshw. Sci.* **2013**, *32*, 525–530. [CrossRef]
30. Berg, D.J.; Haag, W.R.; Guttman, S.I.; Sickel, J.B. Mantle Biopsy: A Technique for Nondestructive Tissue-Sampling of Freshwater Mussels. *J. N. Am. Benthol. Soc.* **1995**, *14*, 577–581. [CrossRef]
31. Lehner, B.; Verdin, K.; Jarvis, A. New Global Hydrography Derived from Spaceborne Elevation Data. *Eos Trans. AGU* **2008**, *89*, 93–94. [CrossRef]
32. Folmer, O.; Black, M.; Hoeh, W.; Lutz, R.; Vrijenhoek, R. DNA Primers for Amplification of Mitochondrial Cytochrome c Oxidase Subunit I from Diverse Metazoan Invertebrates. *Mol. Mar. Biol. Biotechnol.* **1994**, *3*, 294–299.
33. Serb, J.M.; Buhay, J.E.; Lydeard, C. Molecular Systematics of the North American Freshwater Bivalve Genus Quadrula (Unionidae: Ambleminae) Based on Mitochondrial ND1 Sequences. *Mol. Phylogenet. Evol.* **2003**, *28*, 1–11. [CrossRef]
34. Bolotov, I.N.; Kondakov, A.V.; Vikhrev, I.V.; Aksenova, O.V.; Bespalaya, Y.V.; Gofarov, M.Y.; Kolosova, Y.S.; Konopleva, E.S.; Spitsyn, V.M.; Tanmuangpak, K.; et al. Ancient River Inference Explains Exceptional Oriental Freshwater Mussel Radiations. *Sci. Rep.* **2017**, *7*, 2312. [CrossRef]
35. Hall, T.A. BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
36. Kumar, S.; Stecher, G.; Li, M.; Knyaz, C.; Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Mol. Biol. Evol.* **2018**, *35*, 1547–1549. [CrossRef] [PubMed]
37. Kimura, M. A Simple Method for Estimating Evolutionary Rates of Base Substitutions through Comparative Studies of Nucleotide Sequences. *J. Mol. Evol.* **1980**, *16*, 111–120. [CrossRef] [PubMed]
38. Excoffier, L.; Lischer, H.E.L. Arlequin Suite Ver 3.5: A New Series of Programs to Perform Population Genetics Analyses under Linux and Windows. *Mol. Ecol. Resour.* **2010**, *10*, 564–567. [CrossRef] [PubMed]
39. Clement, M.; Snell, Q.; Walke, P.; Posada, D.; Crandall, K. TCS: Estimating Gene Genealogies. In Proceedings of the 16th International Parallel and Distributed Processing Symposium, Kyoto, Japan, 15–19 April 2002; p. 7.
40. Froufe, E.; Sobral, C.; Teixeira, A.; Sousa, R.; Varandas, S.; Aldridge, D.C.; Lopes-Lima, M. Genetic Diversity of the Pan-European Freshwater Mussel Anodonta Anatina (Bivalvia: Unionoida) Based on CO1: New Phylogenetic Insights and Implications for Conservation. *Aquat. Conserv.* **2014**, *24*, 561–574. [CrossRef]
41. Vavilov, N.I. *The Origin, Variation, Immunity and Breeding of Cultivated Plants*; Chester, K.S., Translator; Ronald Press: New York, NY, USA, 1951.
42. Hewitt, G.M. Some Genetic Consequences of Ice Ages, and Their Role in Divergence and Speciation. *Biol. J. Linn. Soc.* **1996**, *58*, 247–276. [CrossRef]

43. Jones, E.L. What Is a Refugium? Questions for the Middle–Upper Palaeolithic Transition in Peninsular Southern Europe. *J. Quat. Sci.* **2022**, *37*, 136–141. [CrossRef]
44. Bennett, K.D.; Provan, J. What Do We Mean by "Refugia"? *Quat. Sci. Rev.* **2008**, *27*, 2449–2455. [CrossRef]
45. Artamonova, V.S.; Bolotov, I.N.; Vinarski, M.V.; Makhrov, A.A. Fresh-and Brackish-Water Cold-Tolerant Species of Southern Europe: Migrants from the Paratethys That Colonized the Arctic. *Water* **2021**, *13*, 1161. [CrossRef]
46. Bolotov, I.N.; Konopleva, E.S.; Chan, N.; Lunn, Z.; Win, T.; Gofarov, M.Y.; Kondakov, A.V.; Tomilova, A.A.; Vikhrev, I.V. A Riverine Biodiversity Hotspot in Northern Myanmar Supports Three New and Narrowly Endemic Freshwater Mussel Species. *Aquat. Conserv.* **2022**, *23*, 3850. [CrossRef]
47. Mangerud, J.; Jakobsson, M.; Alexanderson, H.; Astakhov, V.; Clarke, G.K.C.; Henriksen, M.; Hjort, C.; Krinner, G.; Lunkka, J.P.; Möller, P.; et al. Ice-Dammed Lakes and Rerouting of the Drainage of Northern Eurasia during the Last Glaciation. *Quat. Sci. Rev.* **2004**, *23*, 1313–1332. [CrossRef]
48. Son, M.O.; Prokin, A.A.; Dubov, P.G.; Konopacka, A.; Grabowski, M.; Macneil, C.; Panov, V.E. Caspian Invaders vs. Ponto-Caspian Locals—Range Expansion of Invasive Macroinvertebrates from the Volga Basin Results in High Biological Pollution of the Lower Don River. *Manag. Biol. Invasions* **2020**, *11*, 178–200. [CrossRef]
49. Artaev, O.; Pashkov, A.; Vekhov, D.; Saprykin, M.; Shapovalov, M.; Levina, M.; Levin, B. Fish Occurrence in the Kuban River Basin (Russia). *Biodivers. Data J.* **2021**, *9*, e76701. [CrossRef]
50. Barinova, S.; Wink, M.; Makhrov, A.A.; Artamonova, V.S.; Sun, Y.-H.; Fang, Y.; Pashkov, A.N.; Reshetnikov, A.N. New Records of the Alien Chinese Ricefish (Oryzias Sinensis) and Its Dispersal History across Eurasia. *Diversity* **2023**, *15*, 317. [CrossRef]
51. Mezhzherin, S.V.; Yanovich, L.M.; Zhalay, E.I.; Vasilieva, L.A.; Pampura, M.M. Genetic and morphological variability and differentiation of freshwater mussels (Bivavia, Unionidae, Anodontinae) in Ukraine. *Vestn. Zool.* **2014**, *48*, 99. [CrossRef]
52. Prié, V.; Puillandre, N. Molecular phylogeny, taxonomy, and distribution of French Unio species (Bivalvia, Unionidae). *Hydrobiologia* **2014**, *735*, 95–110. [CrossRef]
53. Riccardi, N.; Froufe, E.; Bogan, A.E.; Zieritz, A.; Teixeira, A.; Vanetti, I.; Varandas, S.; Zaccara, S.; Nagel, K.O.; Lopes-Lima, M. Phylogeny of European Anodontini (Bivalvia: Unionidae) with a redescription of *Anodonta exulcerata*. *Zool. J. Linn. Soc.* **2020**, *189*, 745–761. [CrossRef]
54. Soroka, M. Characteristics of mitochondrial DNA of unionid bivalves (Mollusca: Bivalvia: Unionidae). I. Detection and characteristics of doubly uniparental inheritance (DUI) of unionid mitochondrial DNA. *Folia Malacol.* **2010**, *18*, 147–188. [CrossRef]