


Article

Round-the-World Voyage of the Threespine Stickleback (*Gasterosteus aculeatus*): Phylogeographic Data Covering the Entire Species Range

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Abstract: A total of 205 *COI* sequences and 310 *cyt b* sequences of the threespine stickleback (*Gasterosteus aculeatus*) from basins of all seas throughout the vast range of this species were analyzed. Median networks of haplotypes constructed in this study, combined with the results of reconstruction of paleogeographic conditions, led to the conclusion that the threespine stickleback emerged as a species in the Pacific Ocean basin and spread to Europe from the south, populating the system of water bodies that existed in the Oligocene. The main water body was the Paratethys Ocean (Sea), which existed 5–34 Mya. In the area of the modern North Sea, stickleback populations, part of which later migrated to the eastern and western coasts of North America, gave rise to the group of haplotypes that has the widest distribution in northern Europe. The stickleback populations belonging to the lineage that dispersed along the Arctic and western coasts of North America displaced the carriers of the haplotypes of the ancient phylogenetic lineage that inhabited the Pacific coast. The ancestors of *G. wheatlandi* dispersed from the Pacific to the Atlantic Ocean via the Arctic to meet *G. aculeatus*, which circled the globe from east to west.

Keywords: genetics; threespine stickleback; phylogeography; evolution; barcoding; origin; genetic diversity

1. Introduction

Threespine stickleback (*Gasterosteus aculeatus* Linnaeus, 1758) is widely distributed in various aquatic habitats, including sea, brackish water, and freshwater ecosystems [1–4]. However, the species is better known as a “supermodel” for evolutionary and behavior studies. Many studies have been published on the mechanisms of adaptation of threespine stickleback to various abiotic conditions, including varying salinities, as well as the independent appearance of forms similar in their morphological and ecological characteristics in different parts of the species’ distribution range [5–13] and references therein.

However, evolution in the genus *Gasterosteus* at the species level seems limited, comprising only three species. These are the aforementioned *G. aculeatus*, the vast range of

which encompasses the northern part of the Pacific Ocean basin, eastern North America, almost all of Europe from the Mediterranean to the Barents Sea, the Black and Azov Seas (and their basins), and the Mediterranean coasts of Turkey and Algeria [5]; and two endemic species: *G. wheatlandi* Putnam, 1867, inhabiting the northeastern coast of North America [14], and *G. nipponicus* Higuchi, Sakai & Goto, 2014, found in the Japanese archipelago [15].

How and when the genus *Gasterosteus* dispersed is therefore highly relevant. The earliest fossils of this genus date back to the middle Miocene (about 16 Mya); during this period, threespine stickleback inhabited the Pacific Ocean basin, both in Asia and in North America [5,14,16,17]. The oldest finds of *G. aculeatus* in the Atlantic Ocean basin date to the Pliocene–Pleistocene, and the earliest discovery is about 1.9 Myr old (reviewed in [17]). This suggests that the threespine stickleback entered the Atlantic Ocean basin no later than that time.

The first large-scale study on the genetic diversity of *G. aculeatus*, which employed allozyme analysis [18], revealed two genetically divergent groups of populations in the species range. Later, two large clusters of haplotypes were found in a study wherein a partial sequence of the mitochondrial gene encoding cytochrome *b* (*cyt b*) was used as a marker. Carriers of the haplotypes of one of the clusters have been shown to be dominant in the western Pacific basin and are not found in the Atlantic basin (this group of haplotypes is currently referred to as the *Trans-North Pacific clade*). In contrast, the carriers of haplotypes belonging to the other cluster (referred to as the *Euro-North American clade*) are common in the Atlantic Ocean basin and predominate in the eastern Pacific Ocean basin [19].

Attempts to explain some of the peculiarities of the haplotype distribution over the range were based on the “molecular clock” theory. Calculations using this model showed that the most similar haplotypes identified in the threespine stickleback living in the Atlantic and Pacific basins (both belonging to the *Euro-North American clade*) diverged about 260 kya. Therefore, it was suggested that threespine stickleback migrated to the Atlantic Ocean from the Pacific Ocean via the Arctic during this period [19]. Estimates based on the “molecular clock” theory were also made in a recent study, wherein 8079 SNP loci served as markers [20]. As a result, the authors came to a similar conclusion that “divergence between Pacific and Atlantic lineages occurred 29.5–226.6 kya” [20]. The latter study also dealt with the lineages belonging to the Euro-North American clade.

In spite of agreement between the estimates based on the “molecular clock” theory performed by two independent groups of researchers, the authors of both genetic studies [19,20] encountered a serious problem: there was an obvious discrepancy between recent divergence of the lineages following from their data and the old age of the paleontological finds of threespine stickleback in the Atlantic Ocean basin. As a result, these authors had to assume that the stickleback originally inhabiting the Atlantic basin became extinct and then repopulated the basin about 260 kya: “This confirms the hypothesis put forth by Orti et al. [19], and later corroborated by Fang et al. [21], that extant Atlantic populations originate from a recent re-colonization event from the Pacific, and the Late Pliocene/Early Pleistocene Atlantic populations of *G. aculeatus*—for which there is fossil evidence (. . .)—had gone extinct before the Atlantic Ocean became re-colonized” [20].

The notion that the threespine stickleback populated Europe from the Arctic in a north-to-south direction was formulated in the 19th century and has not been revised since then. The famous ichthyologist Heincke [22] (p. 399) wrote: “Offenbar ist der *Gast. aculeatus* ursprünglich ein nordischer Seefisch, der wahrscheinlich von den Küsten des diluvialen Eismeeres aus in die süßen Gewässer Europas einwanderte” (“Apparently, *Gast. aculeatus* is originally a northern sea fish that probably migrated to the fresh waters of Europe from the coasts of the diluvial Arctic Ocean”); in this context, *diluvialen/diluvial* means *Quaternary*. In line with these ideas, the authors of later studies dated the invasion of stickleback to southern Europe to the glacial or postglacial times [23–26].

The hypothesis that the threespine stickleback spread to the Atlantic coast of Europe and America through the Arctic is also reflected in the interpretation of genetic data on European populations of the species. For example, a number of studies on the phylogeography of the threespine stickleback [20,21,27–31] have shown that there are several separate phylogenetic lineages of the stickleback in southern Europe, specifically in the basins of the Mediterranean and Black seas. The authors believe that these lineages resulted from repeated migrations of representatives of this species from north to south during periods of climate cooling in the Pleistocene.

Thus, we can identify three features of genetic differentiation and related characteristics of the geographical distribution of *G. aculeatus* haplotypes over the range that are poorly explained by the existing hypotheses on the formation of its modern species range: (a) the existence of two strongly divergent phylogenetic lineages in the Pacific basin (the western and eastern parts of this ocean have never been separated by a barrier impervious to stickleback); (b) the discrepancy between paleontological and genetic datings related to the appearance of threespine stickleback in the Atlantic basin; and (c) a number of endemic phylogenetic lineages of stickleback in the Mediterranean and Black Sea basins that have significantly diverged from one another.

The goals of our study are to: (1) clarify the order of origin of various phylogenetic lineages of threespine stickleback; (2) analyze the patterns of distribution of various phylogenetic lineages over the species range; and (3) reconstruct the means by which threespine stickleback dispersal formed the modern range of this species.

2. Materials and Methods

2.1. Sample Collection

Stickleback were caught with traps, hand nets, and minnow seines in fresh waters and with beach seines in the sea [8]. Detailed information on sampling sites and the number of specimens collected in each locality is presented in Supplement S1 (Table S1). Photographs of typical habitats of threespine stickleback are shown in Figure 1. A total of 171 specimens were caught during this study. Whole specimens were fixed in 96% ethanol at a ratio of 1:5, with ethanol replaced the next day.

2.2. DNA Isolation

DNA was isolated from muscle tissue fragments using a DNK-Ekstran-2 commercial reagent kit (Sintol LLC, Moscow, Russia) according to the manufacturer's instructions.

2.3. Selection of Genetic Loci for Analysis

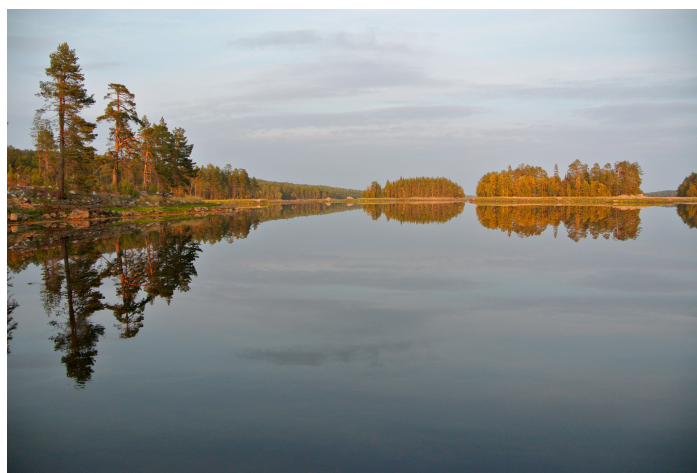
In order to determine the origin of phylogenetic lineages of the threespine stickleback, the loci of two mitochondrial DNA (mtDNA) genes, cytochrome oxidase I gene (*COI*) and cytochrome *b* gene (*cyt b*), were used. We used mtDNA loci because normally, mtDNA does not recombine and therefore accumulates replacements sequentially [27]. Therefore, analysis of mtDNA sequences makes it possible to trace the transformation of these sequences during the dispersal of the species over the range. In the GenBank international database, partial sequences of the *COI* and *cyt b* mitochondrial loci are the most widely represented for *G. aculeatus* from various localities; therefore, we used these partial sequences for analysis in the present study.



(a)



(b)



(c)

Figure 1. Some sites of sample collection: (a) a stream near the Verkhne-Paratunskiye, where thermal springs flow in the Paratunka River basin, Kamchatka (the Pacific Ocean basin); photograph by O.V. Aksenova; (b) the Sukko River, Caucasus (the Black Sea basin); photograph by V.S. Artamonova; (c) the Kandalaksha Gulf, Guba Kislaya Bay, White Sea; photograph by D.L. Lajus.

2.4. Polymerase Chain Reaction (PCR) and Sequencing of PCR Products

Fish F1 (5' TCAACCAACCACAAAGACATTGGCAC 3') and Fish R1 (5' TAGACTTC TGGGTGGCCAAAGAATCA 3') primers were used to amplify a fragment of the gene *COI* [32], and the *cyt b* forward (5' ATGAACTTTGGTTCCCTCC 3') and *cyt b* reverse (5' CGCTGAGCTACTTTTGCATGT 3') primers were used to amplify the fragment of the *cyt b* gene [27]. The amplification conditions were the same for both gene fragments. Amplification was carried out in 25 µL of buffer manufactured by Fermentas (Lithuania, Vilnius) containing 75 mM Tris-HCl (pH 8.8), 20 mM (NH₄)₂SO₄, 0.1% Tween 20, and 2 mM MgCl₂. The amplification mixture contained about 300 ng of total cell DNA, 200 nmol each of four deoxyribonucleotides, 10 pmol of forward and reverse primers, and 0.5–0.7 polymerase units (Bionem, Russia). The amplification program involved a stage of initial denaturation of DNA at +95 °C for 4 min, 31 cycles of DNA fragment amplification at +95 °C for 30 s, +50 °C for 30 s, and +72 °C for 50 s, as well as a final chain elongation stage at +72 °C for 5 min. The obtained PCR products were sequenced from the forward and reverse primers [33].

2.5. Analysis of the Sequenced *COI* and *cyt b* Fragments

The sequenced gene fragments were analyzed using a specialized BioEdit 7.2 editor. As a result of this analysis, sets of the *COI* and/or *cyt b* haplotypes characteristic of the fish collected in each geographical location were identified. The sequences corresponding to these haplotypes were deposited in the GenBank international database (<https://www.ncbi.nlm.nih.gov/genbank/>, accessed on 10 May 2022), where the *COI* sequences were assigned the numbers MZ531736–MZ531778 and OK349512–OK349513, and the *cyt b* sequences were assigned the numbers KR912169–KR912173, MZ584771–MZ584792, and OK482598–OK482649.

We deposited 45 *COI* and 79 *cyt b* sequences in the GenBank database in the course of this study. Details on all these sequences are presented in Supplement S1 (Table S1).

2.6. *COI* and *cyt b* Sequences Deposited in the GenBank by Other Researchers

In addition to our own data, we also used the data on the *COI* and *cyt b* nucleotide sequences of threespine stickleback previously deposited in GenBank by other researchers. We performed a search of the nucleotide section of the website of the National Institutes of Health, United States (<https://www.ncbi.nlm.nih.gov/nucleotide/>, accessed on 10 May 2022), using the keywords *Gasterosteus* and *COI/cyt b*. The *COI* and *cyt b* nucleotide sequences of *G. nipponicus* were excluded from the subsequent analysis, and those of *G. wheatlandi* were used as an outgroup.

A total of 155 *cyt b* sequences and 151 *COI* sequences of *G. aculeatus*, as well as 1 *cyt b* sequence and 11 *COI* sequences of *G. wheatlandi*, were selected in this way. Details on the sequences used in the analysis, including references to the studies in which they were first identified, are presented in Supplement S1 (Table S2).

2.7. Construction of Median Networks of Haplotypes

Median networks of haplotypes for the *COI* and *cyt b* loci were constructed separately with PopART 1.7 and Network 10.2.0.0 software, using a median-joining algorithm. In all cases, the use of both software packages yielded the same results. When constructing the networks for *COI* or *cyt b*, only one sequence of each haplotype from each locality was used for analysis, even if several carriers of a given haplotype were found in a locality. This ensured a unified approach in analyzing our own data together with the data from the GenBank database.

According to an authoritative guidebook on molecular genetics [34] (p. 607), “Standard tree reconstruction is not appropriate for all DNA sequence datasets”. This guidebook lists “problems when ancestral sequences still exist” among the issues that complicate conventional tree building. As clearly observed in the networks presented in our manuscript, the set of sequences studied here included many sequences that are ancestral for other

sequences in the networks. Therefore, we followed the recommendation of the cited guide-book: “One solution to these problems is to use a network rather than a tree to illustrate the evolutionary relationships between the sequences being examined” [34] (p. 608).

3. Results

3.1. Median Networks of Haplotypes and Haplotype Distribution over the Species Range

The lengths of the amplified fragments of the *COI* and *cyt b* genes were 707 and 1069 bp, respectively. For comparison with previously published sequences, we used a 609 bp fragment of the *COI* gene and a 963 bp fragment of the *cyt b* gene.

We used 151 *COI* sequences and 155 *cyt b* sequences available in the GenBank international database. We supplemented the data array on these sequences with our own data on 54 *COI* and 155 *cyt b* sequences identified in this study (45 of these *COI* sequences and 79 *cyt b* sequences were extremely rare: in each local population where any of them was found, only one individual carried it). These data were used to study the distribution of different variants (haplotypes) of the *COI* and *cyt b* sequences over the species range. Tables S1 and S2 in Supplement S1 show the characteristics of all the sequences studied here.

Figures 2 and 3 show the haplotype networks for the partial *COI* and *cyt b* sequences, respectively. The distributions of all variants of the partial *COI* and *cyt b* sequences over the species range are shown in Figure 4a,b and Figure 5a,b, respectively.

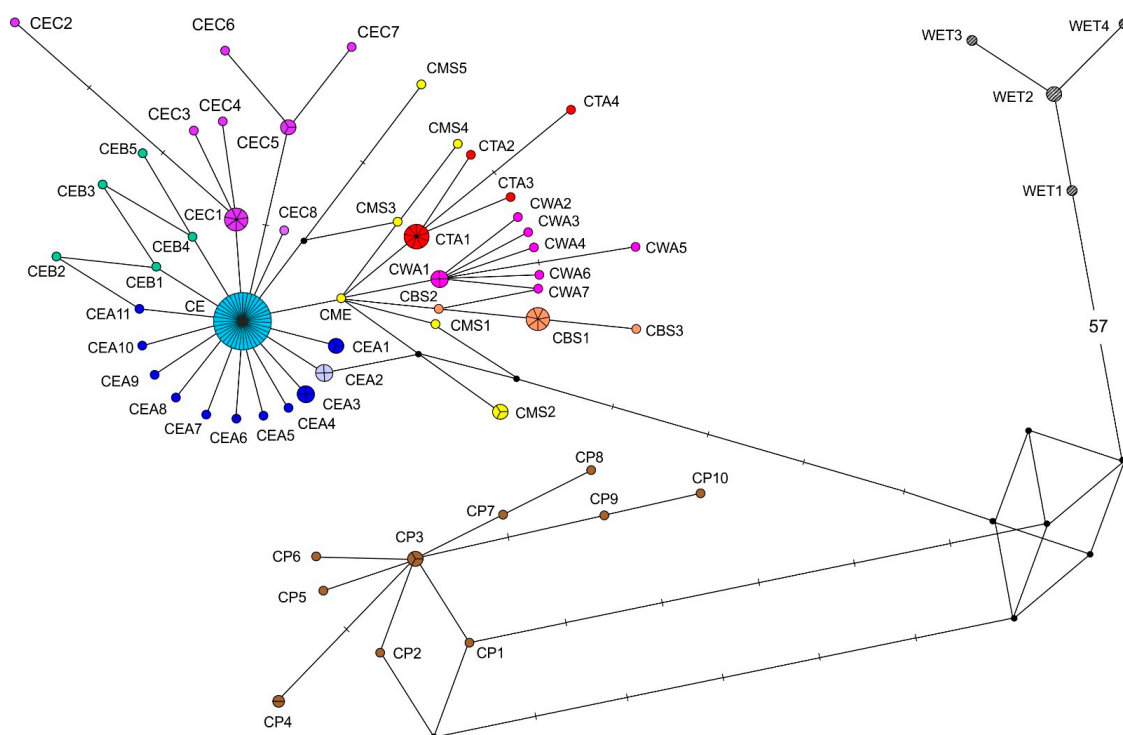


Figure 2. The haplotype network for partial sequences of the *COI* mitochondrial gene. The areas of the circles are proportional to the number of localities where the given haplotype was found. Haplotype color key: gray with shading, haplotypes of *G. wheatlandi* (WET); brown, haplotypes of the Pacific cluster corresponding to the Trans-North Pacific clade (CP); yellow, the group of endemic Mediterranean lineages (CM); orange, the Black Sea lineage (CBS); dark pink, the American lineage (CWA); red, the transatlantic lineage (CTA); different shades of blue and green, phylogenetic lineages of the European cluster (CE). The lineages of the European cluster (CE) form three main subgroups: those associated with the Atlantic and Arctic Ocean basins (CEA, different shades of blue), the Baltic Sea basin (CEB, green), and the Mediterranean Sea basin (CEC, mauve).

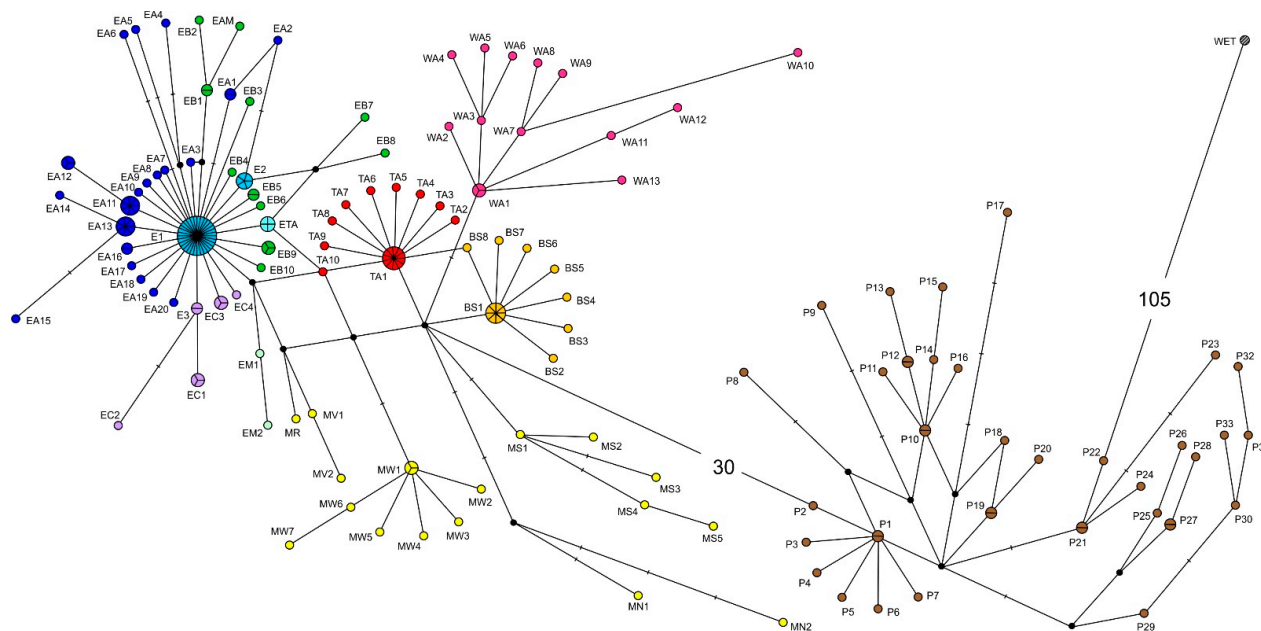


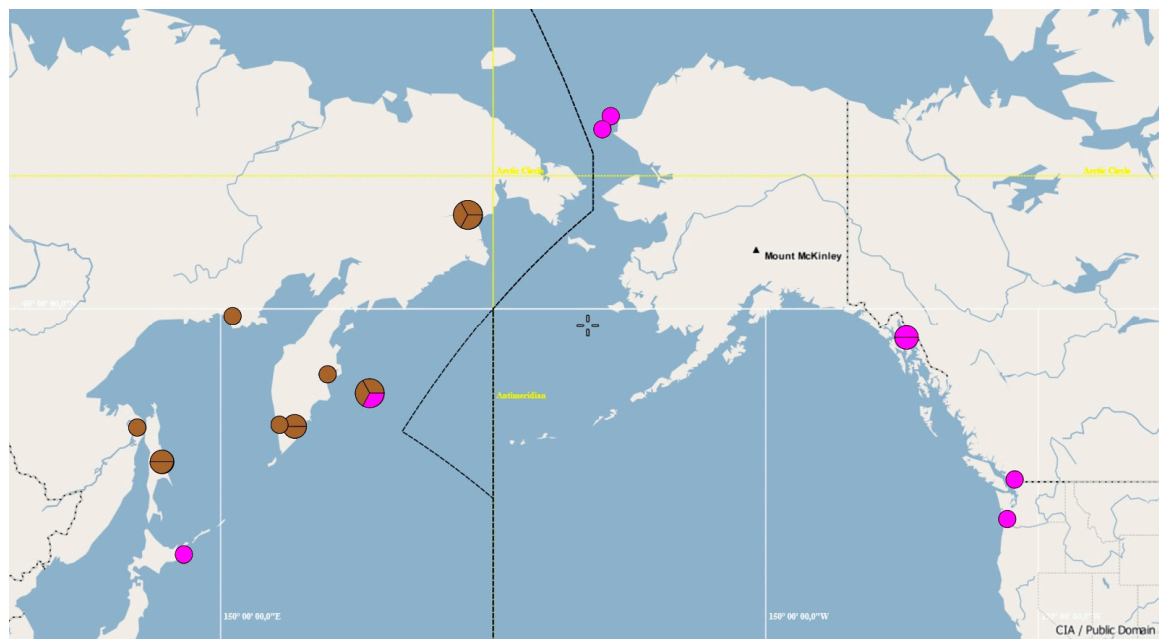
Figure 3. The haplotype network for partial sequences of the *cyt b* mitochondrial gene. The areas of the circles are proportional to the number of localities where the given haplotypes were found. Haplotype color key: gray with shading, haplotype of *G. wheatlandi* (WET); brown, haplotypes of the Pacific cluster corresponding to the Trans-North Pacific clade (P); yellow, the group of endemic Mediterranean lineages (M); pale green, the endemic lineage of the Bay of Biscay (EM); orange, the Black Sea lineage (BS); dark pink, the American lineage (WA); red, the transatlantic lineage (TA); green and different shades of blue, phylogenetic lineages of the European cluster (E). The lineages of the European cluster (E) form three main subgroups: those associated with the Atlantic and Arctic Ocean basins (EA, different shades of blue), the Baltic Sea basin (EB, green), and the Mediterranean Sea basin (EC, mauve). The EAM haplotype found on the Atlantic coast of North America is designated green because it belongs to the lineage included in the EB subgroup.

3.2. Comparison of the Haplotype Networks for the Partial COI and *cyt b* Sequences

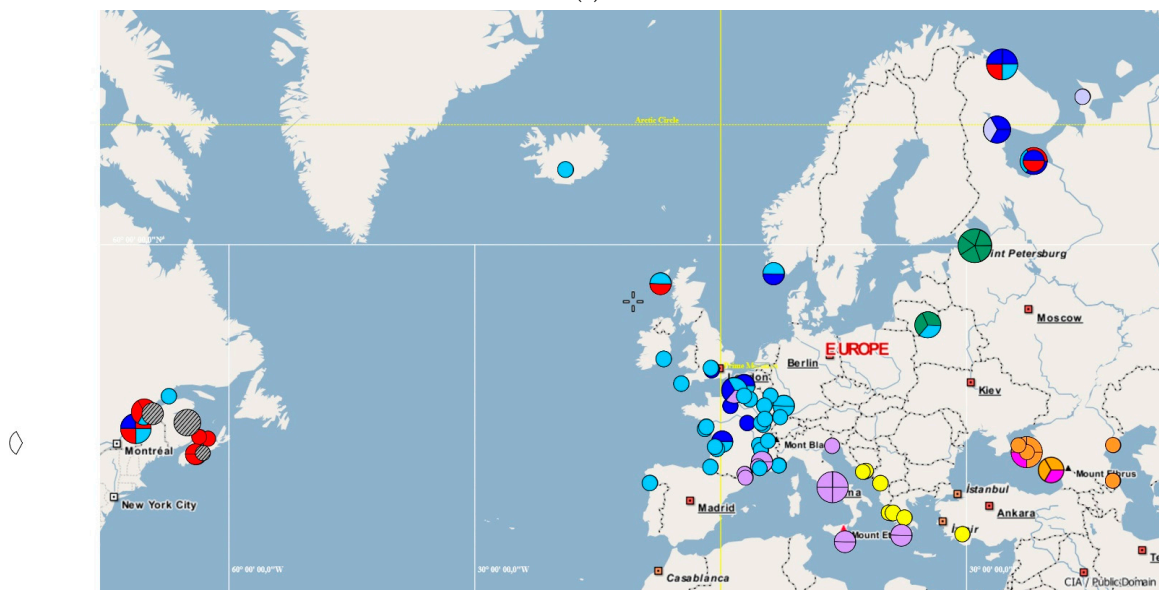
The haplotype networks shown in Figures 2 and 3 have similar structures. In both cases, the most striking feature is two large megaclusters separated by a considerable distance: 13 and 30 nucleotide substitutions, respectively. One of them looks rather homogeneous and is represented only by the variants found in the fish of the Eurasian Pacific coast (the CP and P clusters for the COI and *cyt b* sequences, respectively; these variants are shown in brown in Figures 2 and 3). This cluster corresponds to the *Trans-North Pacific clade*. The second megacluster comprises all other haplotypes, both European and North American (it corresponds to the *Euro-North American clade*).

The latter megacluster consists of separate phylogenetic lineages, most of which originate from a single common point. In the haplotype network for the partial COI sequence (Figure 2), CME haplotype no. KJ553385.1 found in the basin of the ancient Lake Skadar on the Balkan Peninsula [35] is located at this point. It provides an origin for two separate phylogenetic lineages characteristic of threespine stickleback of the Mediterranean region (CMS, the group of lineages marked in yellow in Figure 2), a group of Black Sea haplotypes (CBS, orange), the so-called transatlantic lineage (CTA, red) found on both sides of the Atlantic, and, finally, the American lineage (CWA, pink) typical of the North American coast of the Pacific Ocean. As follows from the data shown in Figure 2, the largest group of haplotypes (CE), which is widespread in Europe and includes three main subgroups associated with the Atlantic and Arctic Ocean basins (CEA), the Baltic Sea (CEB), and the Mediterranean Sea (CEC), also descended from this variant. In Figure 2, the

haplotypes belonging to these groups are marked with different shades of blue, green, and purple (see the caption of Figure 2).



(a)



(b)

Figure 4. The distribution of haplogroups of the partial sequence of the *COI* mitochondrial gene over the threespine stickleback species range: (a) in the Pacific part of the range; (b) in western Eurasia and eastern North America. The areas of the circles are proportional to the number of haplotypes found in fish from the given areas. Haplotype color key: gray with shading, haplotypes of *G. wheatlandi* (WET); brown, haplotypes of the Pacific cluster corresponding to the Trans-North Pacific clade (CP); yellow, the group of endemic Mediterranean lineages (CM); orange, the Black Sea lineage (CBS); dark pink, the American lineage (CWA); red, the transatlantic lineage (CTA); different shades of blue and green, phylogenetic lineages of the European cluster (CE). The lineages of the European cluster (CE) form three main subgroups: those associated with the Atlantic and Arctic Ocean basins (CEA, different shades of blue), the Baltic Sea basin (CEB, green), and the Mediterranean Sea basin (CEC, mauve).

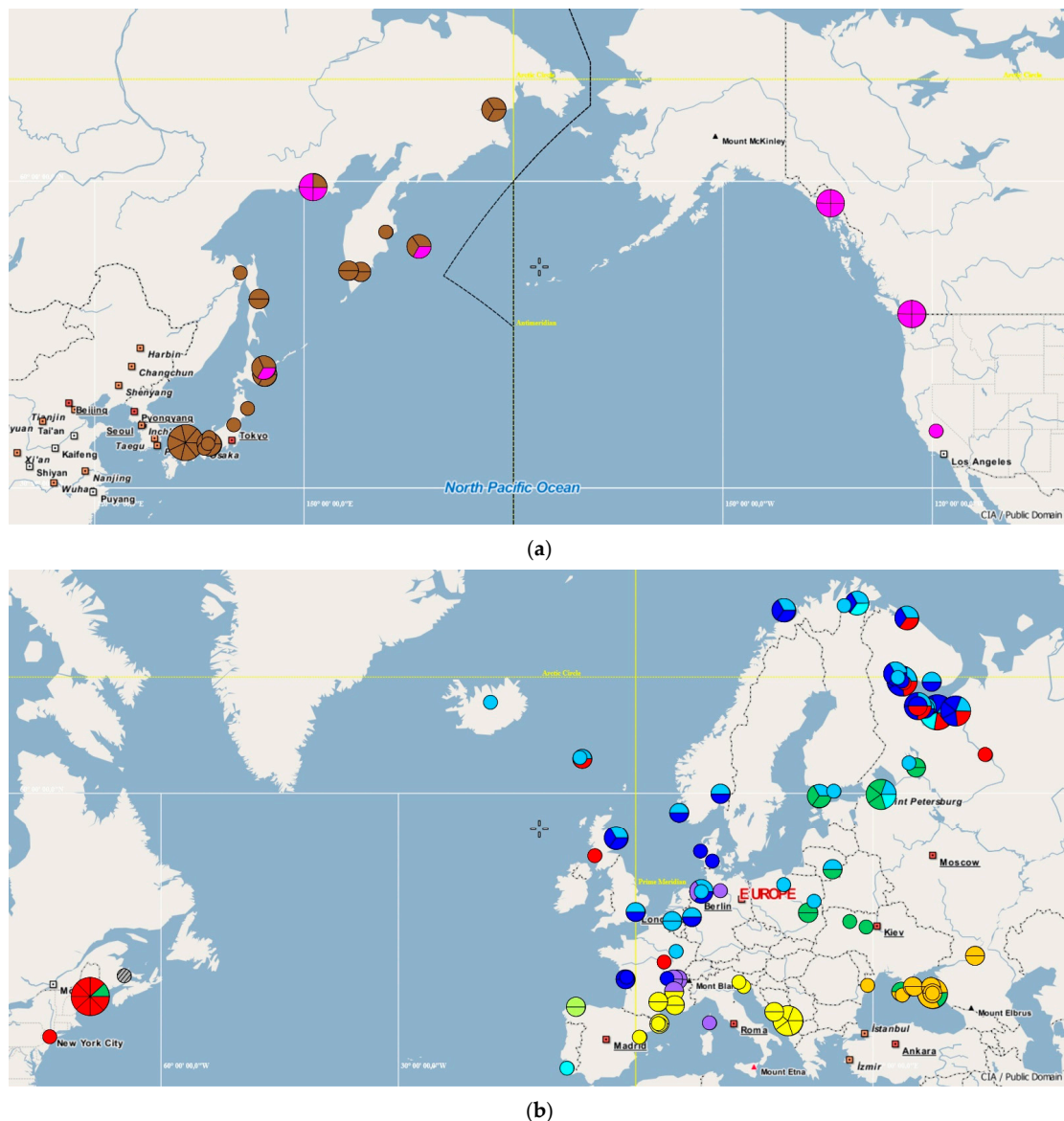


Figure 5. The distribution of haplogroups of the partial sequence of the *cyt b* mitochondrial gene over the threespine stickleback species range: (a) in the Pacific part of the range; (b) in western Eurasia and eastern North America. The groups of haplotypes are marked with the same colors as in Figure 3. The areas of the circles are proportional to the numbers of haplotypes found in fish from the given areas. Haplotype color key: gray with shading, haplotype of *G. wheatlandi* (WET); brown, haplotypes of the Pacific cluster corresponding to the Trans-North Pacific clade (P); yellow, the group of endemic Mediterranean lineages (M); pale green, the endemic lineage of the Bay of Biscay (EM); orange, the Black Sea lineage (BS); dark pink, the American lineage (WA); red, the transatlantic lineage (TA); green and different shades of blue, phylogenetic lineages of the European cluster (E). The lineages of the European cluster (E) form three main subgroups: those associated with the Atlantic and Arctic Ocean basins (EA, different shades of blue), the Baltic Sea basin (EB, green), and the Mediterranean Sea basin (EC, mauve). The EAM haplotype found on the Atlantic coast of North America is designated green because it belongs to the lineage included in the EB subgroup.

The haplotype network for the partial *cyt b* sequence (Figure 3) generally demonstrates similar patterns of evolution of individual lineages distributed outside the Pacific coast of Eurasia from a common ancestral haplotype. However, in contrast to *COI* haplotypes, no ancestral variant has been detected in any study on the *cyt b* sequence. Nevertheless,

as is the case with *COI*, two independent Mediterranean lineages (MS and MN, shown in yellow), one Black Sea group (BS, orange), and one transatlantic group (TA, red), as well as the American phylogenetic lineage, the representatives of which are distributed on the North American coast of the Pacific (WA, pink), descended from the *cyt b* sequence.

Analysis of the haplotype network for the partial *cyt b* sequence allows for more detailed tracing of the origin of the E haplotype group (which includes, by analogy with *COI*, the EA, EB, and EC subgroups, marked with different shades of blue, green, and purple in Figure 3; see the figure caption), which is most widely distributed in Europe. Figure 3 clearly shows that this group is on the periphery of the network and appears to have descended from one of the transatlantic haplotypes.

The other peripheral groups represent a series of strongly differentiated phylogenetic lineages (MW, MV, and MR, marked in yellow) that descended successively from one another and then evolved independently over a long period of time. The carriers of the haplotypes belonging to these lineages live in different areas of the Mediterranean basin, isolated from one another.

The EM phylogenetic lineage (shown in pale green), the representatives of which have been found on the coast of the Bay of Biscay, may also belong to this group rather than having descended from E1. Judging by its position in the network (Figure 3), both possibilities seem equally likely; an unambiguous solution to this question requires additional data.

3.3. Distribution of Representatives of Different *COI* and *cyt b* Phylogenetic Lineages over the Species Range

As shown in Figures 4a and 5a, representatives of only two phylogenetic lineages are found in the Pacific basin: carriers of the haplotypes of the ancient CP/P megacluster (*COI/cyt b*) and of the CWA/WA phylogenetic lineage (*COI/cyt b*). Threespine stickleback with the CP/P haplotypes occur only on the Pacific coast of Eurasia, whereas the CWA/WA lineage is found on the western coast of North America and, occasionally, in some Far Eastern populations.

As evident in Figures 4b and 5b, representatives of all the other phylogenetic lineages inhabit the Atlantic basin. The haplotypes belonging to the Mediterranean phylogenetic lineages (CMS for *COI* and MS, MN, MW, MV, and MR for *cyt b*) only occur in small localities, with the carriers of many haplotypes inhabiting only one freshwater system each (Supplement S1, Table S2). Similarly, the haplotypes belonging to the Black Sea lineage (CBS/BS) are found only in stickleback of the Black Sea basin.

The transatlantic phylogenetic lineage (CTA/TA) and the recent CE/E cluster derived from it stand in complete contrast to the Black Sea and Mediterranean lineages; their carriers are found on both sides of the Atlantic Ocean. A characteristic feature of the transatlantic lineage is that its haplotypes dominate the North American populations of the eastern coast and are common in the stickleback populations of the northern Europe and Russia, inhabiting the basins of the White and Barents Seas. In addition, they occur in stickleback populations of the British Isles and in the basin of the Seine River flowing into the English Channel.

Fish with haplotypes belonging to the CE/E cluster reside predominantly in Europe, but variants of the sequences are distributed unevenly over this part of the threespine stickleback range. The structures of similar clusters for the two mitochondrial loci studied are such that most *COI* haplotypes differ from CE and most *cyt b* haplotypes differ from E1 by only one nucleotide substitution (Figure 3). Therefore, it is not surprising that carriers of the CE and E1 haplotypes are found in the majority of European threespine stickleback populations (Supplement S1, Tables S1 and S2). Characteristically, in the case of *cyt b*, another haplotype, E2, is also widespread and is found in the threespine stickleback populations inhabiting the Northern, Baltic, and White Sea basins (Supplement S1, Table S2).

In Figure 4b, which shows the distribution of *COI* haplotypes of different phylogenetic lineages over the range, the “basic” CE haplotype is shown in blue, as in the network for

this gene (Figure 2), and the gray-blue color indicates the CEA2 haplotype, which, like the Mediterranean CME haplotype, may theoretically be ancestral for the CE cluster.

Similarly, the E1 and E2 haplotypes, which occur in stickleback from different sea basins and cannot be assigned to a specific subgroup, EA, EB, or EC, are shown in blue in the network for *cyt b* (Figure 3) and the map of the distribution of haplotypes from different phylogenetic lineages of this gene over the species range (Figure 5b). In the network (Figure 3) and map (Figure 5b), the ETA haplotype is marked with bright blue color. This haplotype is a link between the transatlantic (TA) lineage and the European (E) cluster. This haplotype has been found in southern Portugal, in the Lake Ladoga basin, and in the White and Barents Sea basin; hence, like the E1 and E2 haplotypes, it cannot be included in the EA, EB, or EC subgroups.

3.4. Reconstruction of the Routes of Threespine Stickleback Dispersal within Its Range

Having studied the characteristics of the haplotype networks for the two mitochondrial genes of the threespine stickleback, as well as the distribution of these haplotypes over the modern range of the species, we attempted to determine in which periods of Earth's history the paleogeographic environment facilitated the formation of this range and which waterways that existed at that time could have served as the routes of dispersal. By comparing all of our own and available published data, we compiled a scheme of the dispersal routes, which is shown in Figure 6. The rationale for this reconstruction is presented in the Section 4.

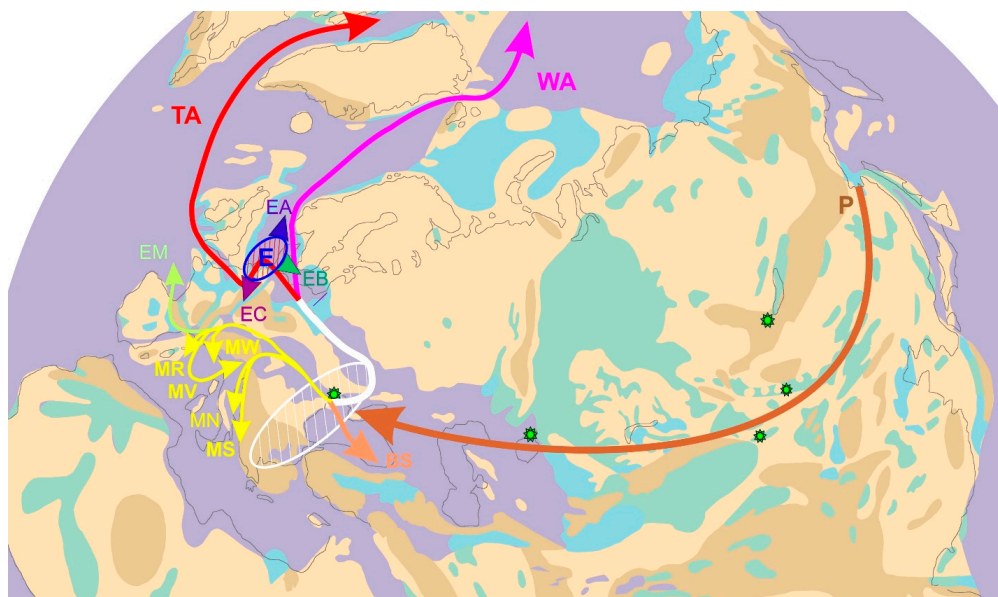


Figure 6. Reconstruction of the dispersal of various phylogenetic groups of the threespine stickleback (an original scheme). The paleogeographic setting corresponds to the period of 30 Mya. Purple, oceans and seas; blue, brackish water reservoirs; turquoise, freshwater reservoirs; yellow, plains; brown, mountains. Dark lines mark the modern boundaries of large water bodies (based on [36]). Green stars indicate the localities of fossil margaritiferid mussels (based on [37]). Shaded areas are the presumed zones of the initial stages of differentiation of phylogenetic lineages. Haplotype color key: brown, haplotypes of the Pacific cluster corresponding to the Trans-North Pacific clade (P); yellow, the group of endemic Mediterranean lineages (M); pale green, the endemic lineage of the Bay of Biscay (EM); orange, the Black Sea lineage (BS); dark pink, the American lineage (WA); red, the transatlantic lineage (TA); green and different shades of blue, phylogenetic lineages of the European cluster (E). The lineages of the European cluster (E) form three main subgroups: those associated with the Atlantic and Arctic Ocean basins (EA, different shades of blue), the Baltic Sea basin (EB, green), and the Mediterranean Sea basin (EC, mauve).

3.5. Haplotypes found in Nontypical Localities

Threespine stickleback with haplotypes belonging to the CE/E cluster are found not only in Europe but also in North America, although less frequently than carriers of transatlantic-lineage haplotypes. Regarding the partial sequence of the *COI* gene, the CE haplotype, which is “basic” for the European cluster, is regularly found in various stickleback populations of the eastern coast of North America. In addition, a unique haplotype, CEA9, differing from CE by one nucleotide substitution, was also detected in one of these populations (Supplement S1, Table S2). Regarding the partial *cyt b* sequence, a unique haplotype, EAM, belonging to a branch of the EB subgroup of the European cluster, was found in stickleback inhabiting the Atlantic coast of North America (Table S2).

Furthermore, *cyt b* haplotypes belonging to the EB group, carriers of which are common in the Baltic Sea basin (EB5 and EB9; Supplement S1, Tables S1 and S2) were detected in at least four threespine stickleback populations of the Black Sea basin. There is also evidence that there was a carrier of the CWA5 haplotype characteristic of the American phylogenetic lineage (KR862861.1) in a population of this basin; analysis of our own data also revealed a haplotype belonging to this phylogenetic lineage in one specimen from the Sukko River (no. OK349512).

4. Discussion

4.1. Direction of Threespine Stickleback Dispersal in the Course of the Formation of Its Modern Range and Irregularity in the Rates of Evolution

As mentioned above, researchers agree that the threespine stickleback, as a species, originated in the Pacific Ocean basin; this viewpoint is based, in particular, on paleontological findings (reviewed in [5,16]). The haplotype networks for partial *COI* and *cyt b* nucleotide sequences constructed in this study also support this hypothesis. In addition, these networks support the notion that the CP/P lineage (related to the *Trans-North Pacific clade*) is the oldest of all currently existing phylogenetic lineages of the threespine stickleback. The results of our study (Figures 4a and 5a) indicate that its carriers currently inhabit the Pacific coast of Eurasia. However, according to published data, representatives of this lineage are preserved in some resident populations on the western coast of North America [19,38–42].

Taking the *COI* and *cyt b* nucleotide sequences of this lineage (the CP and P clusters in Figures 2 and 3, respectively) as a “baseline”, we can thereby determine the direction in which the stepwise transformation of these gene sequences occurred as the species dispersed throughout its modern range.

Considering the patterns of the haplotype networks shown in Figures 2 and 3, the idea of estimating the time of divergence of different threespine stickleback groups on the basis of the “molecular clock” hypothesis mentioned in the Introduction is called into question. The fact that the *COI* haplotype CME, ancestral to both European and American phylogenetic lineages, still exists in the population of the ancient Lake Skadar in the Balkan Peninsula, suggests an uneven rate of evolution of this sequence in different parts of the threespine stickleback range.

4.2. Time and Routes of the Invasion of the Threespine Stickleback into Europe

We further analyzed the haplotype networks for the partial sequences of the two mitochondrial genes, now taking into account that the CP/P cluster is the most ancient. This should inevitably lead us to the conclusion that the differentiation of the threespine stickleback resulting in the formation of the megacluster that corresponds to the *Euro-North American clade* and includes all phylogenetic lineages, except for CP/P, began in a water body covering, among other areas, parts of the modern Balkan Peninsula. It was from this water body that threespine stickleback entered the Atlantic Ocean, spread along the European coast, and then crossed the ocean; that is, a reasonable conclusion is that the threespine stickleback originally invaded Europe from the south rather than the north.

The conclusion with respect to the southern origin of European threespine stickleback contradicts the point of view first published in the literature more than a hundred years ago that has not been revised since then. A number of reputable ichthyologists [22–26] assumed that the threespine stickleback first populated northern Europe and then spread to the south. The notion that *G. aculeatus* spread from the Pacific Ocean to northern Europe via the Arctic Ocean [19] is a further confirmation of this idea. However, only one fact that actually deserves attention supports the hypothesis on the northern origin of European stickleback: the absence of *G. aculeatus* in the Danube basin until the late 19th century.

Thus, the notions of both the northern origin of the threespine stickleback and its dispersal through the Arctic are mere hypotheses. Although they were put forward by prominent scientists and deserved the closest attention, evidence that could definitely prove or disprove these statements was clearly insufficient, even in the mid-20th century. Therefore, it is not surprising that a number of genetic, paleontological, and paleogeographic data contradicting this hypothesis have appeared recently.

We would like to emphasize that our conclusion that the dispersal of stickleback in Europe started from the south, specifically from the Black and Mediterranean Sea basins, is not only based on our own data. For example, this theory is supported by the results of a study wherein the median network for a partial sequence of the *cyt b* gene was constructed using a different set of haplotypes [31]. The results showed that several variants of the sequence found only in stickleback populations of the Adriatic Sea basin were most similar to a stickleback haplotype from Japan. Unfortunately, Vila and coauthors [31] did not discuss this interesting result in their paper.

By summarizing genetic and paleontological data and based on paleogeographic reconstructions [36,43–45], we concluded that the threespine stickleback spread from the Pacific Ocean to southern Europe as early as Oligocene, about 30 Mya, through a chain of water bodies. This chain connected the modern Amur River basin with the ancient Paratethys Sea (Ocean), which, in turn, was connected with the Mediterranean Sea. Precisely during this geological period, the conditions for the migration of the threespine stickleback to the region of modern Europe were formed. The dispersal of hydrobionts via this route is confirmed by paleogeographic data and findings of fossil shells of freshwater pearl mussels (*Margaritifera*) in the area of this chain of water bodies. Together with pearl mussels, salmonid fish, which are hosts of mussel larvae, and, apparently, other hydrobionts, including stickleback, also migrated through these water bodies (reviewed in [37]).

In connection to the dispersal of the threespine stickleback, closest attention should also be paid to the long debates on the place of origin of brown trout (*Salmo trutta* Linnaeus), a coldwater species that, like the threespine stickleback, has been widespread in Europe for a long time (reviewed in [46–48]). Our recent study showed that the most ancient *COI* haplotypes of brown trout are common in the Ponto-Caspian and Mediterranean basins, whereas the populations of central, western, and northern Europe appeared relatively recently, when brown trout managed to enter the Atlantic Ocean through the Strait of Gibraltar and spread from the modern Danube River basin to the modern Rhine River basin through a system of paleo-water bodies [48].

The data reported in this study suggest that the dispersal of the threespine stickleback also occurred from the south to the north of Europe. This conclusion allows us to explain the long-known “zoogeographic anomaly”: in the *G. aculeatus* that inhabit the seas bordering Denmark and northern Germany, the parasite *Magnibursatus caudofilamentosa* (Reimer, 1971) Gibson & Køie (a species of *Digenea*) has been found, which is absent in other areas of northern Europe [49,50]. Considering that other representatives of this genus occur in the Black Sea, Mediterranean Sea, and Bay of Biscay [51,52], it can be assumed that the ancestor of the parasite passed from the modern Mediterranean and/or Black Seas to the north, together with its host, the threespine stickleback. Moreover, the finding of representatives of the genus *Magnibursatus* in the Bay of Biscay confirms the assumption that the EM

phylogenetic lineage (*cyt b*) is related to the Mediterranean phylogenetic lineages rather than having descended from the lineage with the E1 haplotype.

4.3. Differentiation of the Main Phylogenetic Lineages of Threespine Stickleback in the Atlantic Basin and the Spread of the Euro-North American Clade throughout the Range

Analysis of the distribution of different phylogenetic lineages of the threespine stickleback in the Atlantic basin (Figures 4b and 5b) shows that all of them descended, directly or indirectly, from a common ancestor. In general, the nucleotide sequences of one American, one transatlantic, one Black Sea, and at least two Mediterranean lineages directly originated from a single ancestral sequence. The haplotype network for *cyt b* also clearly shows that all other Mediterranean lineages successively descended from one another, and there is a distinct trend toward gradual transformation of the *cyt b* gene sequence through successive nucleotide substitutions in the westward direction, from the modern Black Sea region to the Bay of Biscay. Furthermore, individual phylogenetic lineages of the Mediterranean group became isolated from one another with no further contact.

This situation is reflected in the diagram of the Paleoeuropean dispersal of threespine stickleback (Figure 6): at the time of Paratethys, modern Europe was a group of islands that which were gradually merging with one another. The straits separating them became freshwater lakes and river valleys in which threespine stickleback populations (previously inhabiting salt waters) were isolated. This assumption is supported by the fact that all the stickleback of the Mediterranean group belong to the low-plated morph [25,53]. The stickleback of the Bay of Biscay basin also belongs to this morph [53], which serves as another argument in favor of assigning it to the Mediterranean rather than European group. The data presented in a classic study by Münzing [25] entirely support the concept that stickleback colonized Europe from the south to the north, although the author assumed that the low-plated morph entered the Mediterranean Sea from the Atlantic Ocean and that the fully plated morph entered the Black Sea from the Baltic Sea basin. On the contrary, we assume that Mediterranean stickleback, inhabiting highly desalinated biotopes, lost their armor plates, as happens at present when freshwater populations are formed (reviewed in [5,14]) and therefore could not spread further than the Bay of Biscay. In contrast, the *G. aculeatus* that lived in the area of the modern Black Sea basin spread along the gulf of Paratethys, which, in some periods, was connected with the North Sea in the region where the Baltic Sea was later formed [36] (see Figure 6); therefore, these sticklebacks retained their plates.

The American, transatlantic, and Black Sea lineages originated from the same common ancestor as the group of Mediterranean lineages. However, whereas the endemic lineage that is widespread in the Black Sea basin still inhabits approximately the same area where it originated, the American and transatlantic lineages, which originated independently of each other, as seen from the networks for both mitochondrial genes studied (Figures 2 and 3), reached the western and eastern coasts of North America, respectively.

This distribution of two phylogenetic lineages with a common ancestor suggests that both their *COI* and *cyt b* ancestral haplotypes were widespread in the stickleback populations of the Paratethys. The fish with these haplotypes could inhabit a vast area stretching to the region of the modern watershed between the Dnieper and Vistula River basins, to which one of the largest gulfs of the Paratethys extended. Moreover, in some epochs, particularly 30 Mya, the Paratethys and the Atlantic Ocean were separated only by an area of shallow lagoons periodically flooded by the sea; i.e., Paratethys stickleback had direct access to the Atlantic Ocean in the area of the modern North Sea [36].

Furthermore, we can conclude that the invasion of the American lineage of the three-spine stickleback into the waters of the western coast of the North American continent occurred in the period when Greenland was still part of North America. Only this assumption can explain why the two phylogenetic lineages of stickleback distributed on different sides of the continent have not intermixed. Moreover, the independent origin of the CTA/TA and CWA/WA lineages from a common ancestor inhabiting the gulf of the

North Sea that was once connected with the Paratethys indicates that the dispersal of the threespine stickleback along the eastern and western coasts of North America occurred in different periods.

4.4. Patterns of Geographic Location of the Haplotypes of the Transatlantic CTA/TA Lineage and the European CE/E Cluster Marking the Routes of Spread of *G. aculeatus*

As clearly shown in Figures 4b and 5b, the European threespine stickleback lineage distinctly predominates in the North Sea and Baltic Sea basins, with the “basic” CE and E1 haplotypes of *COI* and *cyt b*, respectively, being the most frequent haplotypes in this area. This allows us to identify this region as the center of origin of the entire European cluster (Figure 6). We believe that the populations of the North Sea and Baltic Sea basins are so weakly differentiated because there was no Baltic Sea in the early Pleistocene, and a large Baltic River flowed into the North Sea [43]; i.e., the two modern water systems were part of a single basin.

The haplotypes of the transatlantic lineage are not found here; they inhabit somewhat more western regions of the European part of the range, including the Faroe Islands, the western coast of Scotland, and a tributary of the Seine River flowing into the English Channel (Figures 4b and 5b). This leads to the conclusion that representatives of the transatlantic lineage came to the Atlantic coast of North America having skirted the area of the modern British Isles from the west, after which they became “trapped” between the modern mainland and Greenland, the northern part of which was connected with North America even 3 Mya [44]. It also cannot be ruled out that carriers of the haplotypes of the transatlantic CTA/TA lineage were once abundant in the North Sea but were then displaced by fish with haplotypes belonging to the CE/E cluster originating from the transatlantic lineage.

In contrast, northeastern Greenland is likely to have been populated by the ancestors of the carriers of the American lineage haplotypes, who, having subsequently passed through the Arctic, spread along the Pacific coast of North America, which apparently occurred even before the transatlantic lineage was formed. This assumption, based on analysis of genetic data, is confirmed by paleontological findings; fossil remains of threespine sticklebacks have been found in the far northeast of Greenland (Kap København) [54]. Bennike [17] (p. 902) notes that the age of sediments where stickleback were found is 2–2.5 Myr, although the author expresses some skepticism about this find: “... but this find is questioned because no more remains of the species have been recovered, in spite of intensive search.”

4.5. Invasion of Carriers of the American Lineage Haplotypes into the Pacific Basin

As mentioned above and as shown in Figures 4a and 5a, two strongly divergent phylogenetic lineages of threespine stickleback are widespread in the Pacific basin. Carriers of the Trans-North Pacific clade haplotypes dominate western coast populations and have been found in some resident populations of the eastern Pacific coast. In contrast, carriers of the haplotypes of a phylogenetic lineage belonging to the *Euro-North American clade* dominate the populations of the eastern Pacific and are rarely found in the western Pacific [19,38–42,55–60].

However, no haplotypes with “intermediate” nucleotide sequences have been found in the Pacific basin, which indicates that neither of the two Pacific phylogenetic lineages descended directly from the other one. Moreover, as evident in Figure 2, the American CWA lineage (its haplotypes are marked in pink) originated from one of the haplotypes found in the Mediterranean stickleback. The same haplotype is, directly or indirectly, ancestral to all the phylogenetic lineages of stickleback, except CP/P (the *Trans-North Pacific clade*).

This structure of the haplotype network makes it improbable that the Atlantic basin stickleback descended from the Pacific stickleback belonging to the *Euro-North American clade*, as suggested by Orti et al. [19]. In contrast to these researchers, we believe that the stickleback of the American lineage invaded the western North American continent 5–2 Mya from the area of modern Europe, which is confirmed by paleogeographic recon-

structions [44]. As noted above, about 30 Mya, one of the largest gulfs of the Paratethys had a direct connection with the Atlantic Ocean in the area of the modern North Sea. Apparently, carriers of the CME haplotype of the *COI* gene, from which, directly or indirectly, all phylogenetic lineages of threespine stickleback originated in the Paratethys, were widespread here. Greenland was part of the North American continent for a long time—until the end of the Tertiary period [44]—and served as a geographical barrier separating the transatlantic and American lineages of *G. aculeatus*.

Paleoclimatological studies have shown that warm waters from the Atlantic Ocean were entering the Arctic Ocean during the mid-Pliocene Warm Period (3.025–3.246 Mya), which led to a significant decrease in the amount of ice in the Arctic [61]. Thus, during this period, the ancestors of the stickleback belonging to the American lineage could freely migrate from the Atlantic Ocean to the Arctic Ocean, together with the waters forming the warm current, reaching the Pacific Ocean. Subsequently, these fish dispersed along the eastern Pacific coast, gradually displacing the stickleback of the ancient *Trans-North Pacific clade*, which had previously inhabited this area. It is not a coincidence that the distribution of the carriers of the *Trans-North Pacific clade* haplotypes on the North American continent is characteristic of relic forms, i.e., they mainly occur in lakes [19,38–42].

It cannot be ruled out that stickleback belonging to the American CWA/WA lineage are currently replacing carriers of the CP/P haplotype (the *Trans-North Pacific clade*) on the Asian coast of the Pacific Ocean.

Our viewpoint is supported by the results of a study on the diversity of the mtDNA gene *COI* of *Gyrodactylus arcuatus* Bychowsky, 1933 parasitizing threespine stickleback, whereas 131 haplotypes of this gene have been identified in European samples, and all samples from both western and eastern coasts of the Pacific Ocean carry the same single haplotype. Lumme, Mäkinen et al. [62] (p. 552) write, “We assume that the parasite species is originally European”. This assumption becomes especially realistic if we suppose that *G. arcuatus* entered the Pacific Ocean from the Atlantic Ocean, together with its host, the threespine stickleback.

Reviews [63,64] have demonstrated that migrations of hydrobionts from the Atlantic Ocean to the Pacific Ocean occurred repeatedly during periods of climate warming in the Arctic region, although they were less frequent than those in the opposite direction. For example, the ancestor of the northern elephant seal (*Mirounga angustirostris* (Gill)) migrated from the Atlantic Ocean to the eastern coast of the Pacific Ocean. Characteristically, the ancestors of this species belonging to the genus *Pachyphoca* inhabited the Paratethys in the Miocene [65].

Thus, threespine stickleback may have circled the globe for millions of years, moving westwards before reached their homeland, the Pacific Ocean basin, which has long been inhabited by carriers of the haplotypes of the *Trans-North Pacific clade*.

4.6. Secondary Contacts between Representatives of Different Phylogenetic Lineages

The distribution of haplotypes belonging to different phylogenetic lineages clearly demonstrates that carriers of the haplotypes of formerly separated lineages have repeatedly contacted one another during the complex geological history of Europe. These contacts were mainly caused by displacement of watershed lines (in this case, parts of some freshwater systems switched between basins, together with the fish populations inhabiting them) and the processes related to glacial retreat (fish from different phylogenetic lineages took part in the colonization of many water systems that were formed in the areas once occupied by the glacier).

Threespine stickleback of the Rhône River. Figure 5b clearly shows that the threespine stickleback populations of the basin of the Rhône River, which flows into the Mediterranean Sea, are characterized by haplotypes of the E group (EC subgroup), although usually, no carriers of the E haplotype are found in other populations of large rivers or lakes of the Mediterranean basin. Furthermore, the haplotypes of the EC subgroup are also found in stickleback from the basins of the Elbe and Weser rivers flowing into the North Sea, from

where they apparently migrated into the Rhône River; paleogeographic reconstructions show that during the period from 15 to 2 Mya, the area north of the Alps belonged successively to several different basins, including those of the Rhône, Danube, and Rhine rivers (reviewed in [66,67]), which provided the conditions for fish migration between rivers belonging to different sea basins.

This hypothesis is supported by finds in the Rhône River basin of typically northern fish from the genera *Salvelinus* and *Coregonus*, which are nontypical of the Mediterranean region [68], as well as finds of COI haplotypes belonging to the same phylogenetic lineage in the brown trout populations of the Danube, Rhine, and Rhône basins [48]. Similarly, fish with COI haplotypes of the North-European clade, which are characteristic of the stickleback populations of the Rhône River and Baltic Sea basins [69], are found in ninespine stickleback (*Pungitius pungitius* (Linnaeus)) populations of the Rhône. In the European grayling (*Thymallus thymallus* (Linnaeus)) population of the Rhône, the haplotypes of the control region of mtDNA similar to the haplotypes identified in the population of this species from the upper Rhine [70] have been found. Although no *cyt b* haplotypes of the EC subgroup have been found to date in the threespine stickleback populations of the Rhine itself, all of the above data suggest that threespine stickleback invaded the Rhône basin from the Rhine basin, as did fish of a number of other species, some of which are nontypical of the Mediterranean ichthyofauna.

Later (probably at the end of one of the glacial periods), when the waters of the Mediterranean Sea were cooler and the inflow of the rivers running from the north considerably increased, threespine stickleback were able to leave the river for the sea, reaching modern Corsica (Figure 5b). After this, the fish spread further, passing along the Apennine Peninsula as far as Sicily and the southern tip of the Peloponnesus, as judged from the finds of stickleback with COI haplotypes belonging to the CEC subgroup of the CE cluster in these regions (Figure 4b).

Haplotypes of the Transatlantic group in threespine stickleback of the White and Barents seas. The transatlantic lineage haplotypes found in stickleback of the western coast of Scotland, the Seine River basin, and the Faroe Islands apparently mark the ancient migration route of *G. aculeatus* across the Atlantic Ocean to the east of the North American continent, which is currently dominated by the carriers of the haplotypes of this phylogenetic lineage.

However, no haplotypes of this lineage have been found along the coast of Norway, although they have been found and are even abundant in the northern European Russia, namely in the populations inhabiting the basins of the Barents and White Seas. We attribute this distribution of haplotypes to the characteristics of fish dispersal in this region during the retreat of the last glacier.

Specifically, we previously showed that, during the postglacial colonization of this region by Atlantic salmon (*Salmo salar* Linnaeus), fish from North American populations were the first to migrate to the area of the modern Kola Peninsula [71]; only later did Atlantic salmon, which had survived the glacial period in the refugium located in the area of the British Isles, appear there and spread along the coast of modern Norway. On this basis, we can conclude that threespine stickleback of the transatlantic lineage could have also come to northwestern Russia from North America. Despite its small size, the threespine stickleback is capable of long sea migrations; live specimens of this species have been found hundreds of kilometers from the shoreline in both the Atlantic and Pacific Oceans [72,73]. Carriers of the European haplotypes are likely to have appeared in this region later, having entered the White Sea through the strait in the area of modern Lake Imandra, which separated the “Kola Island” from modern Scandinavia.

Fish migrations in the opposite direction (from Europe to America) could have facilitated the appearance of haplotypes characteristic of European populations on the eastern coast of North America.

Finds of Baltic haplotypes of the EB group of the E cluster and haplotypes of the American CWA lineage in the Black Sea basin. In the Black Sea basin, *cyt b* haplotypes

belonging to the Baltic EB subgroup were originally found in the threespine stickleback population of the Alma River on the Crimean Peninsula, as well as in the stickleback inhabiting the Teterev and Sluch tributaries of the Dnieper River [27]. The carriers of these haplotypes, nontypical of the Black Sea basin, may have appeared here because the Dnieper, which flows into the Black Sea, is currently connected with Baltic water systems by canals.

In other areas of the Black Sea coast, haplotypes of the European EB cluster of the E subgroup were not found at that time; however, later, mtDNA screening of a large group of fish from the Dyurso River on the Black Sea coast of the Caucasus identified two carriers of haplotypes of this subgroup among 13 anadromous fish, whereas all the 18 fish of the resident form from this river carried typical Black Sea haplotypes (Table S1). Thus, there is reason to believe that the appearance of fish with Baltic haplotypes in the Black Sea basin is related to human activities, and carriers of these haplotypes, which have entered the Dnieper River through the canal system, are now successfully dispersing along the northern Black Sea coast in the eastern direction.

We also cannot ignore a report [74] that a fish with the CWA5 haplotype (no. KR862861.1) belonging to the American lineage was found in the Black Sea basin (the Bzyp River). Our own data confirm that there are representatives of the American lineage in the Black Sea. We found one specimen with the American haplotype CWA1 in the estuary of the Sukko River (no. OK349512). On the other hand, finds of stickleback with CWA haplotypes in the Black Sea basin are so rare that their appearance here seems more likely to be related to human activity. The question requires additional study.

Haplotypes of the Euro-North American clade in threespine stickleback of Lake Kussharo, Japan. To the best of our knowledge, one case of the appearance of CWA/WA haplotypes belonging to the *Euro-North American clade* in populations of the Eurasian Pacific coast can be explained by human activity. As shown in Figure 5a and as previously shown [75], carriers of haplotypes of this lineage have been identified in Lake Kussharo on Hokkaido Island, Japan. However, the threespine stickleback population of this lake is not native, and it is quite possible that it was partly populated by descendants of fish brought there from North America, together with artificially introduced salmonid fish [75].

4.7. Notes on the Origin of *Gasterosteus wheatlandi*

The CP/P phylogenetic lineage, which other researchers relate to the Trans-North Pacific clade, is not only the oldest known haplotype group of *G. aculeatus*, from which all other haplotypes of this species descended, but also the lineage most closely related to the haplotypes of the sticklebacks *G. nipponicus* and *G. wheatlandi*. Specifically, the haplotype network for the partial *cyt b* sequence shown in Figure 3 demonstrates that this is the phylogenetic lineage to which the haplotypes of *G. wheatlandi* are most closely related. The data on the partial COI sequence (Figure 2) do not contradict this conclusion, indicating that the *G. wheatlandi* haplotypes did not directly descended from those of the North Pacific clade of *G. aculeatus*; instead, these two groups of haplotypes share a common ancestral sequence.

No hypotheses on the origin of the species *G. wheatlandi*, which exclusively inhabits northeastern North America, have been published to date (reviewed in [14,76,77]). However, analysis of the median haplotype networks constructed in this study (Figures 2 and 3) allows us to shed some light on the origin of this endemic species. Given that its haplotypes for the partial sequences of the COI and *cyt b* mtDNA genes are related to the haplotypes of the most ancient Trans-North Pacific clade of the threespine stickleback *G. aculeatus*, it is likely that the ancestors of *G. wheatlandi* reached the northwestern Atlantic Ocean from the Pacific Ocean, moving along the Arctic coast of North America (i.e., spreading in the direction opposite to that of the spread of *G. aculeatus*) and that speciation was allopatric.

It should be noted that not only *G. wheatlandi*, but also a number of invertebrate species from the Pacific Ocean inhabit the northwestern part of the Atlantic Ocean but not its other areas [78]. This confined distribution is characteristic of many boreal species that have crossed the Arctic Ocean [79].

The species *G. wheatlandi* apparently underwent both abrupt environmental changes and periods of significant decrease in numbers in the course of its evolution. This assumption explains our finding of a significant acceleration of mtDNA evolution during the formation of this species. Moreover, *G. wheatlandi* is characterized by rapid evolution of sex chromosomes: “After the blackspotted lineage diverged, its sex chromosomes experienced independent and more extensive recombination suppression, greater X–Y differentiation, and a much higher rate of Y degeneration than the other two species” [80] (p. 4403). Apparently, this greater differentiation is the reason why the hybrids of *G. aculeatus* and *G. wheatlandi* are inviable [7], although it should be noted that the amino acid sequences of cytochrome oxidase I and cytochrome b of *G. wheatlandi* have also been changed compared to those of *G. aculeatus*; the *COI* gene has two non-synonymous replacements, whereas the *cyt b* gene has four.

5. Conclusions

Analysis performed in this study has confirmed that the species *G. aculeatus*, as well as the genus *Gasterosteus* in general, originated in the Pacific Ocean basin. The structure of haplotype networks for the *COI* and *cyt b* mtDNA genes, combined with paleogeographic data, convincingly indicates that the *G. aculeatus* spread to Europe by migrating from the modern Far East in the Oligocene via a system of water bodies, the main body of which was the ancient Paratethys Ocean (the present study is the first to posit this hypothesis). Furthermore, the Paratethys stickleback inhabiting the area of the modern Black Sea basin invaded the Mediterranean Sea, and the fish inhabiting a large gulf located in the northwest of the ancient ocean migrated to the basins of the modern North Sea and Baltic Sea. From the northern Atlantic Ocean, *G. aculeatus* spread to the western coast of North America via the Arctic Ocean.

Analysis of our data has also shown that the endemic species *G. wheatlandi* is the most similar to threespine stickleback of the oldest phylogenetic lineage currently found along the Eurasian coast of the Pacific Ocean. This suggests that the ancestors of the endemic species *G. wheatlandi* reached the northwestern Atlantic Ocean by passing through the Arctic in the direction opposite to that of the spread of *G. aculeatus*.

The scheme of stickleback dispersal proposed in this study is consistent with both paleontological and genetic data and, in contrast to the traditional viewpoint, explains (1) the presence of two strongly divergent threespine stickleback lineages in the Pacific Ocean basin, (2) the presence of several strongly divergent phylogenetic lineages of this species in southern Europe, and (3) the fossil stickleback occurrences in Europe (1.9 Mya) and Greenland (2–2.5 Mya). In addition, this hypothesis explains a “zoogeographic anomaly”, namely the finding of *Magnibursatus caudofilamentosa*, a parasite of southern origin, in representatives of *G. aculeatus* inhabiting seas off the coasts of Denmark and northern Germany. This study is the first to propose the hypothesis that *G. wheatlandi* descended from an ancestor that lived in the Pacific Ocean and that the ancestral populations of this species dispersed along the Arctic coast of North America.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/w14162484/s1>. Table S1: Detailed information on sampling sites and the number of specimens collected in each locality. Table S2: The *COI* and *cyt b* sequences of *Gasterosteus* deposited in the GenBank database by other researchers [81–88].

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