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Circumpolar phylogeography of the northern pike (*Esox lucius*) and its relationship to the Amur pike (*E. reichertii*)

Anna Skog^{1,2}, L Asbjørn Vøllestad¹, Nils Chr Stenseth¹, Alexander Kasumyan³ and Kjetill S Jakobsen^{1*}**Abstract**

Background: Freshwater fishes of the genus *Esox* are found throughout the Holarctic region. The northern pike (*E. lucius*) has a circumpolar distribution whereas the assumed sister species the Amur pike (*E. reichertii*) is only found in the Amur region. The genetic structure and post-glacial dispersal of these species are not well known. Here, we use sequence variation at two mitochondrial DNA regions (*cytb* and D-loop) to investigate the phylogeography, infer location of glacial refugia and investigate the time of divergence and potential demographic expansion of the various clades detected.

Results: The two species did not share haplotypes implying long-term isolation with no gene flow, and divergence of the taxa were estimated at 4.55 Myr. The northern pike mtDNA haplotypes revealed three main lineages. One of the northern pike mtDNA lineages was found throughout the entire Holarctic region suggesting transcontinental dispersal from a single refugium. The three lineages exhibited a star phylogeny, indicating population expansion following isolation in separate glacial refugia. Estimated time of divergence of these lineages was 0.18 – 0.26 Myr.

Conclusions: The precise location of the glacial refugia is uncertain, but our data suggests an Asian origin. The expansion of the circumpolar lineage is estimated to be around the end of the second glacial, implying that the current distribution is due to a recent recolonization from an east-Asian refugium. All three northern pike mtDNA lineages occurred sympatrically in Europe, possibly due to secondary contact. Two of the lineages probably originated from different European refugia, one in the Danube-region and one in Western Europe, the latter seems to be the primary source for recolonization of northern Europe.

Keywords: *cyt b*, D-loop, Glacial refugia, Phylogeny

Background

Present-day distribution of temperate species of plants and animals are strongly influenced by geological and climatic processes such as glacial cycles. During glaciation periods populations were displaced and population size reduced, and as a consequence genetic diversity was lost [1,2]. During interglacial periods aquatic species such as freshwater fishes had access to large lakes formed by meltwater, providing a diversity of dispersal opportunities. There are a number of phylogeographic studies investigating the geographic distribution of genealogical lineages of

freshwater fish, but patterns differ between species [3], and there are many potential biases.

Freshwater fishes of the genus *Esox* are found throughout the Holarctic region. The northern pike (*E. lucius*) is the only species within the genus with a circumpolar distribution, whereas *E. americanus*, *E. masquinongy* and *E. niger* are only found in North America and two species only in Eurasia (*E. reichertii*, and *E. cisalpinus* (synonymous with *E. flaviae*)) [4-6]. The geographic origin of the genus *Esox* is unclear. The relatively higher number of species in North America, together with fossil evidence [7], has led to the suggestion that the genus originated on this continent [8].

While northern pike is one of the freshwater fish species with the widest Holarctic distribution range, the closely related Amur pike (*E. reichertii*) [9,10] has a narrow

* Correspondence: ks.jakobsen@ibv.uio.no

¹Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biosciences, University of Oslo, P. O. Box 1066 Blindern, N-0316 Oslo, Norway
Full list of author information is available at the end of the article

distribution essentially being restricted to the Amur River basin in northeastern Asia. Some recent genetic evidence suggests that the northern pike originated in North American subsequently dispersing to Eurasia over the Bering land bridge [8]. This is contrary to what has been suggested earlier [11]. Further, studies indicate that North American northern pike populations have less genetic diversity and structure than the European populations [8,12-19]. Lack of genetic diversity and structure are signs often associated with recently colonized regions, while more variability and genetic structuring are seen in populations that have been existing for longer periods of time [20]. During the different glacial periods Holarctic freshwater fishes survived in a number of geographically separated refugia, and this isolation and the subsequent dispersal from these refugia have been important for shaping the intraspecific genetic variability currently seen for many freshwater fish species in the Holarctic [1,3]. Generally, the geographic distribution of a species and the association with distinct phylogeographic groups (clades) are useful data for identifying glacial refugia [21-23].

Here, we assess the level and geographic distribution of genetic variation of the northern and the Amur pike. Amur pike has so far only been investigated in context of higher level phylogeny [9,10]. Maes et al. [24] have suggested, on the basis of short fragments of mitochondrial DNA, that the northern pike experienced a recent expansion across the Holarctic from a single refugium. The geographic location of this refugium could not be inferred. A later study [25] found more structuring (in the distribution of haplotypes) based on a longer mitochondrial DNA fragment, implying that larger fragments are needed to test this hypothesis. In this study, we used two mitochondrial DNA markers, a 1133 bp sequence of the cytochrome *b* (*cyt b*) region and a 443 bp sequence of the D-loop, to evaluate the origin and dispersal of northern pike and Amur pike using a circumpolar dataset for a northern pike.

Results

Mitochondrial diversity in northern and Amur pike

We obtained samples from 24 northern pike populations representing a total of 12 drainages across the Holarctic, and three Amur pike populations from the Amur River (sampling information and GenBank accession numbers are provided in Additional file 1). Details on diversity is given in Additional file 2, but is summarized here. The final mtDNA alignments included 443 bp of the D-loop region, and 1133 bp of the *cyt b* region for both northern and Amur pike. There were, respectively, 39 and 17 segregating sites in the *cyt b* and D-loop region for northern pike. Nucleotide diversity for this species ranged from 0.0052 (D-loop) to 0.0031 (*cyt b*). The alignments of Amur pike showed 10 segregating sites for

cyt b and 3 for D-loop. The nucleotide diversity ranged from 0.0012 (D-loop) to 0.0020 (*cyt b*).

Haplotype distribution

The phylogenetic tree of concatenated D-loop and *cyt b* sequences revealed 38 haplotypes, 28 in northern pike and 10 in Amur pike (Figure 1). The northern and Amur pike haplotypes each formed two strongly supported clades and the two species did not share any haplotypes. Thus, the minimum spanning network revealed two separate, unconnected clusters (Figure 2).

Three groups of northern pike sequences were detected in all analyses (Figures 1 and 2) and genetic differentiation among the three lineages ranged from 0.4-0.6%. One lineage (seen for both markers) of northern pike consisted

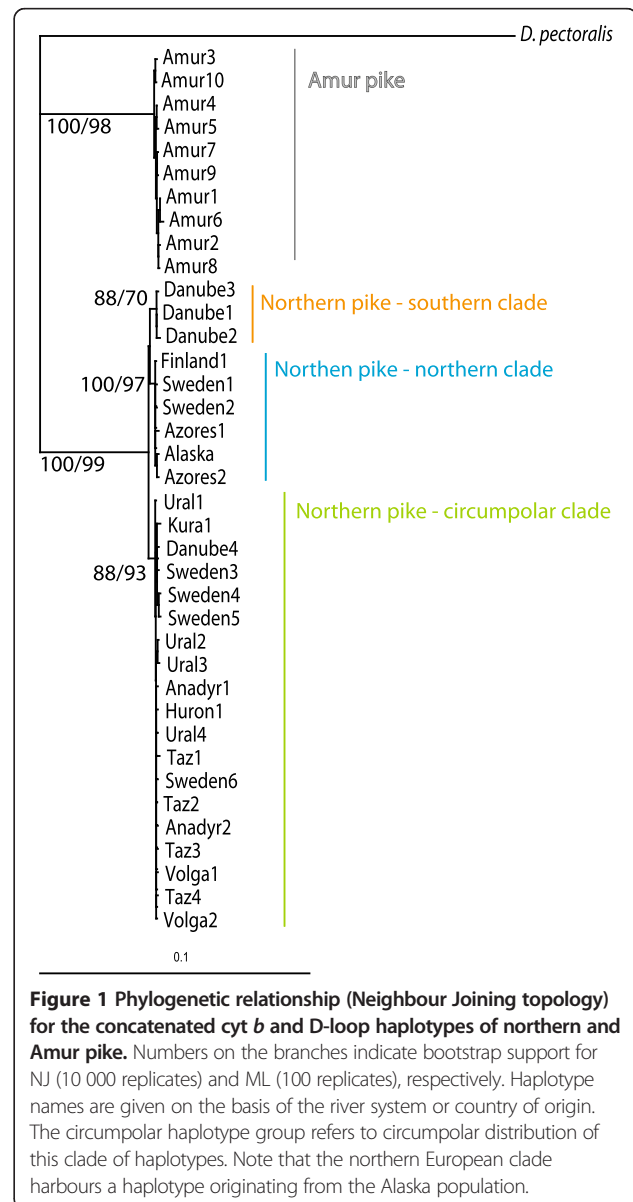
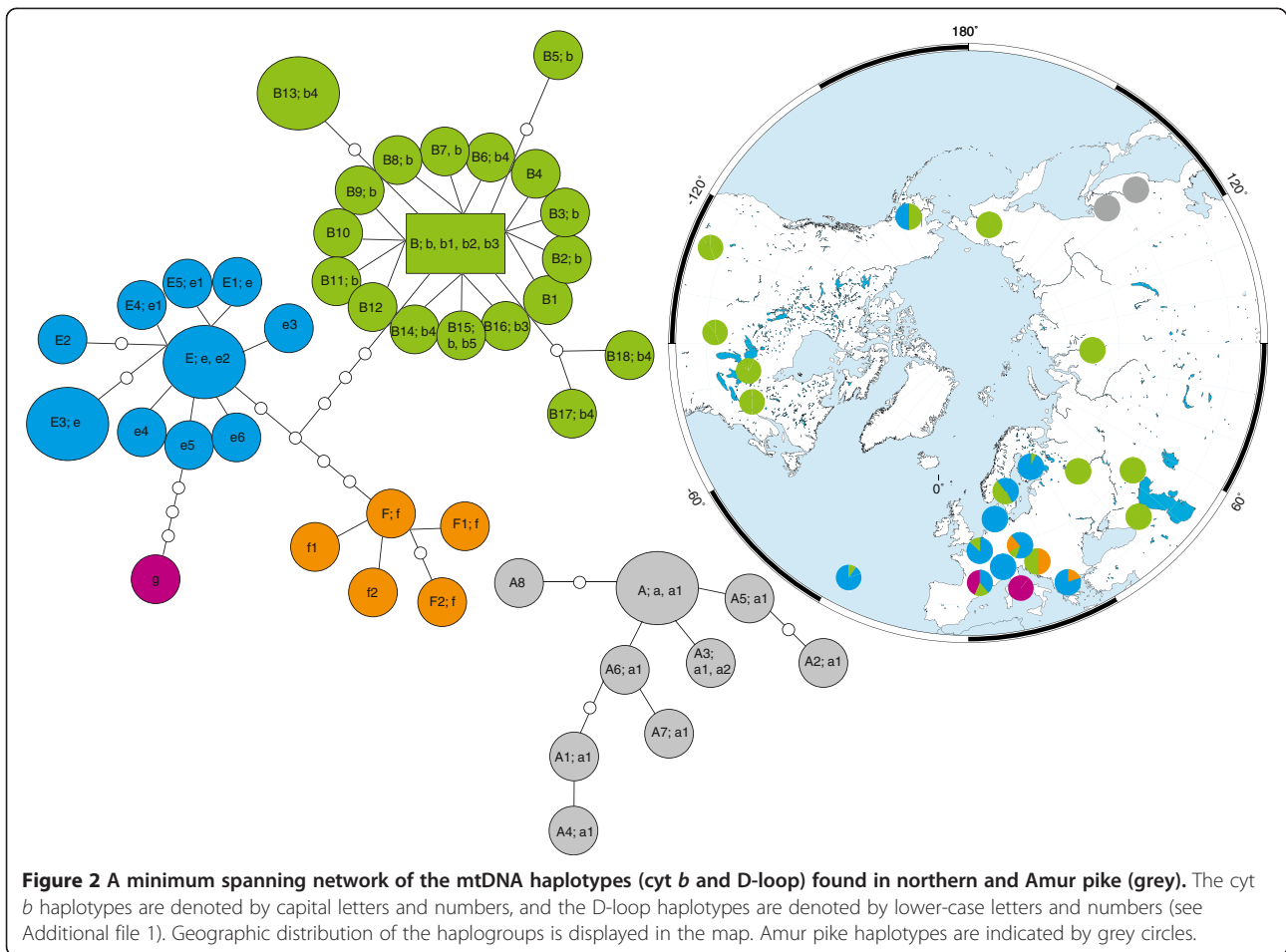


Figure 1 Phylogenetic relationship (Neighbour Joining topology) for the concatenated *cyt b* and D-loop haplotypes of northern and Amur pike. Numbers on the branches indicate bootstrap support for NJ (10 000 replicates) and ML (100 replicates), respectively. Haplotype names are given on the basis of the river system or country of origin. The circumpolar haplotype group refers to circumpolar distribution of this clade of haplotypes. Note that the northern European clade harbours a haplotype originating from the Alaska population.



of closely related haplotypes with a circumpolar distribution implying recent dispersal across the Holarctic (Figure 2, lineage B). Notably, within this lineage the same haplotype showed a Holarctic distribution as it could be detected in Europe, Asia and North America. Almost all populations of northern pike also displayed site-specific haplotypes (Additional file 1). The presence of site-specific haplotypes in most populations indicates limited present gene flow between northern pike populations inhabiting different freshwater bodies. The diversity of haplotypes was clearly larger in Eurasia than in North America, with clade E(e) and F(f) common in southern Europe. Further, a highly divergent haplotype (g) appeared in Mediterranean drainages (Italy, Switzerland; [25]). The Cyt *b* haplotype diversity (Hd, Additional file 2) was slightly higher than the D-loop diversity.

The split between Amur and northern pike

The Amur – northern pike diversification was estimated to be ~ 4.6 Myr ago in the early Pliocene (Table 1). The further differentiation of the northern pike into the three different lineages was estimated to have taken place

much later, 0.2-0.3 Myr ago (Table 1). There was no evidence for multiple lineages within the Amur pike (Figure 2).

Historical demography

The star pattern seen in the minimum spanning network for northern pike mtDNA haplotypes (Figure 2) may be a sign of potential recent population expansion, indicating that the haplotypes diverged simultaneously from a

Table 1 Time of divergence

Comparison	τ	Time of divergence * Myr	P	T	Time of divergence ** Myr
<i>E. reichertii</i> – <i>E. lucius</i>	103	4.55	0.53	6.7	4.73
B – F	5.9	0.26	0.32		
B – E	5.1	0.22	0.53		
E – F	4.1	0.18	0.28		

Estimated time of divergence of *E. reichertii* and *E. lucius*, and clades of *E. lucius*, based on cyt *b* data and the corresponding percentile value (P), using a mutation rate (μ) of 1.133×10^{-5} substitutions per year. B, E and F denote the *E. lucius* cyt *b* mt DNA lineages.

* $\tau = 2 \mu$ [62], ** $T = t_1/N_{ef}$ [63].

common ancestor. Analyses showed that all *cyt b* lineages could recover the signal of sudden expansion - i.e.; none were significantly different from the expectation of sudden expansion (Table 2). However, additional tests (Tajima's D and Fu's F) were not consistent with historic expansion for the Danube population (the F-lineage in Figure 2). Estimated time of expansion for the Amur pike and the B and E lineages of the northern pike was around 0.1 Myr (Table 2). The estimated expansion of the B and E lineages was considerably later than the estimated split between these two lineages (0.22 Myr, Table 1). The overall time of expansion for the northern pike, based on *cyt b*, was 0.41 Myr (Table 2).

Discussion

The circumpolar northern pike is monophyletic and clearly differentiated from the Amur pike. Based on the observed haplotype diversity in the northern pike we infer that present day distribution is due to expansion from three different glacial refugia. One lineage was found throughout the Holarctic suggesting dispersal from a common refugium, probably in Asia. An Eurasian origin of most of present day northern pike diversity is also supported by the higher overall diversity in Eurasia relative to Northern America.

Amur pike – an old species with restricted distribution

The northern pike and Amur pike haplotypes grouped as two monophyletic lineages with high support suggesting complete divergence (see Figure 1). A similar conclusion was also reached by Grande et al. [9]. We found no shared haplotypes between Amur pike and northern pike, and limited geographic structuring of haplotypes within Amur pike (Figure 2). Our data suggest that the Amur and northern pike split in the Pliocene (see Table 1). All Amur pike haplotypes formed a monophyletic group. The limited distribution of this species compared to northern pike may be explained by the mountain ranges Yablonovy and Stanovoy enclosing the Amur River and separating it from other eastern Siberian rivers [26].

Recently, two new species of pike were described from Italy - with a limited distribution somewhat parallel to that of the Amur pike. In two publications these species

have been described as *E. cisalpinus* [5] and *E. flaviae* [4]. These species are with high probability the same, with a suggested vernacular name of southern pike [4]. The type locality of *E. flaviae* is Trasimeno [4], the same location where we have samples of northern pike belonging to the D-loop g clade [25]. Based on the minimum spanning network (Figure 2) the g clade is closely related to the e clade; the e clade has a wide distribution in Western Europe. The g clade seems to be present in drainages to the Mediterranean Sea, with related genotypes and phenotypes observed also in Switzerland and in the Danube [4,25]. Because of the limited data on the g clade, we could not estimate time of divergence. *E. flavia* is suggested to be a junior synonym of *E. cisalpinus* (see www.fishbase.org), and the distribution of the species is not clarified. More detailed genetic work is clearly needed to resolve the taxonomy of the European *Esox*.

Low levels of genetic diversity in northern and Amur pike

The genetic diversity of the northern and Amur pike was low. Low levels of genetic diversity are expected for freshwater fishes at northern latitudes as a result of bottlenecks and reduced effective population sizes within refugia during glaciation periods [1]. Our data obtained from sequencing *cyt b* ($\pi = 0.0031$) and D-loop ($\pi = 0.0052$) for northern pike are in the low end of what has been reported for other freshwater fish species in northern latitudes [1,21,22,27]. The estimates are, however, in agreement with previous reports of low genetic variability in northern pike based on investigations of populations belonging to regions such as Europe and North America [16,24,25,28]. Here, we demonstrate that this is the case for the entire Holarctic distribution of pike.

Several reasons for low genetic variation in northern pike have been put forward, including low effective population size as is common for top predators [13], bottlenecks during glaciations in combination with subsequent founder effects during postglacial re-colonization [28], and restricted gene flow among populations [24,28]. The same arguments may hold for the Amur pike and other pike species. We used published *cyt b* sequences for 10 muskellunge (*E. masquinongy*) individuals from Canada

Table 2 Estimated time of expansion

Comparison	τ	Time of expansion Myr	95% CI Myr	Tajima's D	Fu's F
<i>E. reichertii</i> (A)	2.5	0.11	0.13 – 0.18	-0.65	-2.6
<i>E. lucius</i> (B, E, F)	9.2	0.41	0.01 – 4.20	-1.59	-11.7
B-lineage	1.5	0.07	0.00 – 0.16	-2.19	-17.5
E-lineage	2.3	0.10	0.00 – 0.21	-0.82	-10.6

Estimated time of expansion based on *cyt b* data and the corresponding 95% confidence intervals (95% CI), using a mutation rate (μ) of 1.133E-05 substitutions per year. The *cyt b* lineages representing *E. reichertii* (A) and *E. lucius* (B, E, F), respectively are denoted in parenthesis. Significant values of Tajima's D and Fu's F are indicated in bold.

and USA to compare level of genetic variation for the two related and similar species [9,29]. The estimated genetic variation for the muskellunge ($\pi = 0.0078 \pm 0.0010$) was larger than for the northern pike, but similar to what is observed for other freshwater fishes. This indicates that the top-predator hypothesis probably cannot explain the low level of genetic variation in northern and Amur pike.

We also detected a northern pike haplotype (Supplement 1, cyt *b* haplotype B and D-loop haplotype b) that was distributed throughout Eurasia and North America. Closely related haplotypes distributed across vast geographical areas (Europe, Asia and North-America) have also been shown for burbot (*Lota lota*) [30] and arctic char (*Salvelinus alpinus*) [22]. Although such data are consistent with rapid postglacial expansions from a single refugium (for all three species) it is difficult to envision how such events – involving the Eurasian and the North American continents, could have taken place. Possibly, we are here facing ancient colonization processes at a point in time where the circumpolar distributed mtDNA haplotypes (for unknown reasons) were highly uniform.

Geographic origins of the northern pike mtDNA lineages

The phylogenetic tree of the concatenated cyt *b* and D-loop haplotypes shows that the northern pike haplotypes are divided into three lineages. The northern pike phylogeny was not consistent with deep-branching clades indicating long-term reproductive isolation, which is usually caused by isolation in multiple refugia (see Figure 1). The three clades were also identified in the minimum spanning networks for the northern pike cyt *b* and D-loop sequences (see Figure 2). Although the genetic differentiation among lineages was low (0.4 – 0.6%), compared to lineages traced to different refugia in other Holarctic freshwater fishes [21,30], the lineage distribution most likely point towards disjunctive refugia (see Figure 2), rejecting the suggestion of one single refugium for northern pike put forward by Maes et al. [24].

The localization of glacial refugia is often indicated by higher gene diversity in the refugial regions [20]. For the circumpolar distributed lineage (see Figure 2, B-lineage), the highest gene and nucleotide mtDNA diversities were found in the Ural River population. This river drains into the Caspian Sea, and the Ponto-Caspian region is a known and important aquatic refugium [1,31]. However, we cannot rule out the alternative possibility of a Beringian refugium for the circumpolar lineage, as the Anadyr River population also showed high diversity indices. Beringia has previously been implicated as a northern pike refugium by Bânârescu [26]. In addition, a glacial refugium in West Siberian ice lakes has been suggested for whitefish (*Coregonus lavaretus*) [32]; this might also possibly be a refugium for the northern pike B-lineage.

Two of the lineages primarily displayed a European distribution (see Figure 2, lineage E and F). The oldest and most diverse northern pike lineage is most likely the haplo-group found in the Danube watershed (the F-lineage). The high diversity and restricted distribution indicates most probably presence of a refugium in this region. Two earlier studies on northern pike detected distinct branches connected to southern Europe in general [16,25], and one to Danube in specific [19]. Several studies on European freshwater fishes have shown that the Danube region was an important refugium during the Pleistocene glaciations [21,26,30,31]. However, recolonization of northern Europe by northern pike from this refugium seems improbable. The second European pike lineage might be derived from a central European refugium located between the northern and alpine ice caps as previously suggested [26,31]. A western European refugium is also suggested for other freshwater fish species [33-35]. However, the precise location of this refugium for northern pike is uncertain [19,25]. Notably, Scandinavia has probably been colonization from the central European refugium as well as from an eastern refugium by the circumpolar haplogroup, as also implied for the Eurasian perch (*Perca fluviatilis*) recolonization of Scandinavia [31,36]. Thus, northern Europe has most likely been recolonized from at least two different northern pike refugia and not one as earlier suggested [16,24].

A recent circumpolar expansion of northern pike?

The northern pike network revealed two star-like clusters of haplotypes, reflecting demographic expansions (Figure 2). Diversification events can often be attributed to a period of time when changes in the environment were particularly dramatic. Terminations of glacial periods are likely to be such events. The second glacial termination was initiated 130 kyr ago [37], and both lineage B and E expanded around that time. A late Pleistocene Holarctic expansion has earlier been suggested for the northern pike [24], and our results support this for the circumpolar lineage. However, the total northern pike variation seen today cannot have accumulated after the last glaciation (ended 14 kyr ago; [37]), as this would result in a divergence rate ($2\mu = \tau/t$) at 58% per Myr for the cyt *b* region. In total, our data and analyses support the existence of multiple refugia for northern pike in the Holarctic during the last glaciation, followed by expansions from these refugia leading to separate star phylogenies.

Conclusions

The northern pike and the Amur pike did not share any haplotypes and are clearly differentiated taxa. The divergence time of the two taxa was estimated to 4.55 Myr. The northern pike showed three well-defined evolutionary lineages, each defined by a star phylogeny indicating

population expansion following isolation in separate glacial refugia. One of these lineages has a present-day Holarctic distribution suggesting transcontinental dispersal from a single refugium. The observation of higher genetic diversity in Europe indicates that this lineage probably originated in Europe (east-Asia). Overall, there seems to be a need for further detailed phylogenetic studies of the genus *Esox*, particularly so for the Eurasian populations and species.

Materials and methods

Populations sampled and use of existing GenBank data

Samples from 24 northern pike populations representing a total of 12 drainages across the Holarctic, and three Amur pike populations from the Amur River were sequenced for variation in the mtDNA cytochrome *b* (*cyt b*) and D-loop regions. In addition to the samples sequenced here, 12 *cyt b* sequences (9 northern pike and 3 Amur pike) and 11 D-loop sequences (derived from altogether 108 European northern pike samples) were downloaded from GenBank (see [9,25] for more sampling information). Sampling information and GenBank accession numbers are provided in Additional file 1.

DNA extraction, amplification and sequencing

DNA was isolated using chelex extraction [38]. The *cyt b* region was amplified using the forward and the two reverse primers developed for esocid species by Grande et al. [9] according to the PCR protocol described therein – producing a PCR fragment of 1133 bp. A 443 bp fragment of the D-loop region was amplified using the primers CDL-D and PIDL and protocol described in [25]. The Anadyr samples of northern pike amplified poorly and only weak bands of the *cyt b* region could be detected (using different annealing temperatures, concentrations of DNA, primer, dNTP and MgCl₂). Due to a substantial level of heteroplasmy in both the Amur and northern pike all PCR products were subsequently cloned employing a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA, USA). Five positive clones for each individual were then sequenced using M13 forward and reverse primers (Invitrogen, Carlsbad, CA, USA). Polymorphic sites detected in cloned products were verified by direct sequencing of PCR amplified clone inserts. For sites (single base) displaying heteroplasmy only the major variant was used in the subsequent analyses. DNA sequencing was performed with an ABI3730 system (Applied Biosystems, Foster City, USA) using the same primers for sequencing as in the PCRs. All sequences were aligned and visually inspected using the alignment explorer in MEGA 3.1 [39]. Sequences are available to GenBank (accession numbers KM281447-KM281494; Additional file 1).

Genetic analyses

Phylogenetic analyses were performed on the concatenated mtDNA alignments to assess whether we could detect distinct phylogenetic groups within the northern pike or Amur pike, and to investigate the relationship between these two species. This was done using maximum likelihood (ML) and distance (Neighbour-Joining, NJ [40]) phylogenetic methods as implemented in PAUP 4.0 [41] employing the model of evolution selected by Modeltest [42]. The optimal model selected by Akaike's information criterion [43] was an extended HKY model [44] including gamma distributed rates and rate variation across sites. *Dallia pectoralis* was used as outgroup based on the phylogenetic analysis done by López et al. [10,45]. Other *Esox* species (*E niger* and *E americanus*) were also tested as outgroups and provided essentially the same topology. All PAUP and Modeltest analyses were performed at the Bioportal website at University of Oslo (<http://www.bioportal.uio.no>) The maximum likelihood analysis was done in PhyML [46].

Estimates of the net average divergence, D_A [47] between sequences were calculated in MEGA 3.1 [39]. Standard errors were estimated by 10 000 bootstrap replicates. Two different nucleotide diversity estimates were used; one based on the number of segregating sites (K) between the sequences, $\theta_W = K/a$ [48] and another based on the amount of diversity (Π) between two sequences, $\pi = \Pi/L$ [47]. For each phylogenetic lineage (supported by the bootstrap values), we calculated the nucleotide and haplotype diversity using DnaSP 4.0 [49].

We estimated the most parsimonious haplotype minimum spanning network (MSN) with 95% probability using the program TCS 1.21 [50]. Furthermore, a median-joining network (MJ) comprising both the northern pike and Amur pike haplotypes was constructed by the program NETWORK 4.2.0.1 (<http://www.fluxus-engineering.com/> [51]). The northern pike haplotype networks created by the TCS program were nested by hand according to the rules of Templeton et al. [52] and Templeton and Sing [53] to reveal the grouping of haplotypes.

Both the haplotype networks and earlier reports [24] have indicated recent population expansion of northern pike. To test for this, we calculated the mismatch distribution of the different *cyt b* phylogenetic lineages and compared the distributions to models of population expansion [54,55]. As more reliable mutation rate estimates are available for the coding *cyt b* compared to non-coding D-loop, the mismatch distribution was calculated only for the *cyt b* lineages, and not for the concatenated mtDNA or the D-loop dataset. For the expanding populations (defined by the mismatch distributions) we estimated the time of expansion (t) using $\tau = 2\mu t$, where τ (time to expansion) is estimated from the mismatch distribution and μ is the mutation

rate. We assumed a 2% divergence rate per Myr in cyt *b*, based on published estimates of 2% in bony fishes [56], and 0.9–3.0 in freshwater fish [33,57,58]. Recent population expansion was also tested using Tajima's *D* [59] which utilizes the difference between π and θ_W (as defined above) to detect recent demographic changes (as these two parameters are differently affected by population bottlenecks and expansions). Population expansion was additionally tested using Fu's F_S [60] which is particularly sensitive to population demographic expansion resulting in negative F_S -values. AMOVA, mismatch distribution, τ , Tajima's *D* and Fu's F_S were calculated using Arlequin v. 3 [61]. Confidence intervals were calculated by a parametric bootstrap approach as implemented in Arlequin v. 3.

We estimated divergence times between northern and Amur pike by the method of Gaggiotti and Excoffier [62] as implemented in Arlequin v. 3. This model assumes two populations diverging at time *T* and no further contact after time *T*. Furthermore, the size of the two populations may be different, but their sum equals the ancestral population size. The time of divergence (based on $\tau = 2T\mu$), the size of the ancestral population ($\theta_0 = 2N_0\mu$), and the relative population sizes of the two diverged populations are estimated, and their significance (H_0 : undifferentiated populations) tested by a permutation procedure. Divergence time was additionally calculated using MDIV [63], which estimates posterior distributions of divergence time ($T = t_1 / N_{ef}$; t_1 is number of generations since divergence for the first ancestral population/species, whereas N_{ef} is the effective population size of this first population/species), and other demographic parameters using MCMC. Both these methods for estimating divergence time scale the parameters by mutation rate (μ). Furthermore, they assume that population size does not change over time. The MDIV analyses were carried out using the resources of the Computational Biology Service Unit from Cornell University (<http://cbsuapps.tc.cornell.edu/im.aspx>) - partially funded by Microsoft Corporation.

Additional files

Additional file 1: Sampling of northern and Amur pike. Sampling drainage, geographic location, sample sizes, haplotype names and Genbank accession numbers are indicated.

Additional file 2: Diversity estimates. Gene diversity and nucleotide diversity for *cytb* and D-loop in the phylogeographic units identified (see Figure 2 and Additional file 1) in northern and Amur pike.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AS, NCS and KSJ designed the study. AS and AK organised and analysed data. AS, KSJ and LAV wrote the paper and all authors read and approved the final manuscript.

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Author details

¹Centre for Ecological and Evolutionary Synthesis (CEES), Department of Biosciences, University of Oslo, P. O. Box 1066 Blindern, N-0316 Oslo, Norway.

²Cancer Registry of Norway, P.O. Box 5313 Majorstuen, N-0304 Oslo, Norway.

³Department of Ichthyology, Faculty of Biology, Moscow State University, Leninskie gory, 119991 Moscow, Russia.

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