Species history masks the effects of human-induced range loss – unexpected genetic diversity in the endangered giant mayfly *Palingenia longicauda*

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Abstract

Freshwater biodiversity in Central Europe has declined dramatically over the past decades. Due to massive habitat pollution and morphological degradation of water bodies, many once widespread species persist in small fractions of their original range. These range contractions are generally believed to be accompanied by loss of intraspecific genetic diversity, due to the reduction of effective population sizes and the extinction of regional genetic lineages. We aimed to assess the loss of genetic diversity and its significance for future potential recolonization of the giant mayfly *Palingenia longicauda*, which experienced approximately 98% range loss during the past century. Analysis of 936 bp of mitochondrial DNA of 250 extant specimen across the current range revealed a surprisingly high level of haplotype diversity. In contrast, historic specimens from the lost range (Rhine catchment) are not differentiated from the extant populations, despite considerable geographic distances and the location in different catchments. These observations can be explained by an overlap of the current with the historic (Pleistocene) refugia of the species. Most likely, the massive range loss mainly affected the range which was occupied by rapid post-glacial dispersal. We conclude that massive range losses do not necessarily coincide with genetic impoverishment and that a species' history must be considered in estimations of genetic diversity loss. The assessment of spatial genetic structures, and prior phylogeographic information seems essential to conserve once wide-spread species.

Introduction

Freshwater biodiversity is declining much faster than marine or terrestrial biodiversity [1]. Morphological degradation and pollution of water and sediments are the major drivers of biodiversity loss in stream systems around the world [2]. In central Europe, industrialization, combined with active channelization of large and small bodies of running waters, led to a dramatic reduction in species diversity of fish,

aquatic invertebrates, and other taxa [3]. While species extinctions have rarely been documented, numerous previously widespread species now only persist as small relict populations in refugia which were not subjected to severe habitat alterations. It is expected that these massive range losses also lead to considerable losses of genetic diversity [4–6], including highly differentiated evolutionary lineages, which can be referred to as cryptic species, or evolutionary significant units (ESU) [7, 8]. Local genetic variants and ESUs might harbor unique evolutionary potential, and provide the source of adaptation to future environmental change [9, 10]. These concerns are particularly relevant in freshwater systems, because several species show high levels of differentiation within their ranges, especially along the main catchments [11–13], and for species with a limited potential for overland dispersal [14–18]. Although existing work suggests that range losses should parallel the loss of genetic diversity [4–6], there is still little empirical information on the coupling of these processes, especially in freshwater species.

In this context we choose the mayfly *Palingenia longicauda* (Ephemeroptera: Palingenidae, Figure 1) to study the effects of severe range contraction on genetic diversity. This lowland riverine species used to be wide-spread and well-known from medium-large rivers in Europe, but today persists on about 2% of its former range (Figure 2) [19,20]. Its dramatic decline coincided with the rapid, intensive hydromorphological alteration and pollution of European rivers and peaked around 1930 [20]. The species became extinct from the Loire (France) in 1922, from the Rhine (Germany) in 1952 and from the Danube (Serbia and Bulgaria) in 1973 [19]. *Palingenia longicauda* is presently restricted to the Tisza and the lower ranges of the river's tributaries in Hungary. It was rediscovered in the Rába river after 40 years of absence of reports [21]. The rapid and almost complete extinction of *P. longicauda* resulted in its listing as one of the few aquatic insects in Appendix II of the Convention on the Conservation of European Wildlife and Natural Habitats (Bern Convention) [22–24]. Current conservation attempts include protecting the species in its remaining native habitats in Hungary, as well as repopulating a Rhine tributary in Germany with Tisza source populations [25]. *Palingenia longicauda* has become a symbol of freshwater conservation efforts in Central Europe, especially in Hungary. This is due to its impressive body size (up to 10 cm) and behaviour, the spectacular synchronized swarming of millions of individuals (Figure 1A-C) [26].

We hypothesized that i) large-scale range loss will lead to high levels of range-wide genetic impoverishment in a once widespread aquatic species, that ii) the original genetic diversity of the species cannot be restored by reintroduction or by active range expansion, and iii) the rediscovery of this species in formerly lost ranges is due to recent range expansion, raising the hope for its re-establishment across Central Europe. We analyzed the haplotypic diversity within the known refugial populations of *P. longicauda*. We compared the present genetic patterns with the haplotype diversity of historic museum specimens collected in the Rhine catchment, where the species is considered extinct. In addition, we compared the genetic structure in the known relict habitats (Tisza catchment) with recently detected populations from the Rába river in Hungary.

Our results provide a striking example that a significant and rapid range loss may not ultimately lead to major losses of genetic diversity and that phylogeographic patterns may overrule even severe anthropogenic effects on species ranges.

Results

We obtained a 936-bp long sequence matrix for 250 extant specimens after combining mitochondrial COI and 16S sequences. We successfully amplified a short fragment of the mtCOI gene in 24 museum specimens (Table S2). This was combined with mtCOI sequences of extant specimens into a 196-bp long matrix. We were not able to amplify either the mtCOI or the 16S fragment for 12 extant specimens and the short mtCOI fragment for 13 museum specimens (Table S2). Amplification success of the museum material varied with the place where the material was deposited, thus we suspect that different preservation conditions are likely to be responsible for the variation in PCR success. Nucleotide sequences are deposited in Genbank [**accession numbers will be provided after MS acceptance**].

We found very high genetic diversity in the populations of both the Tisza and the Rába catchments. The overall haplotype diversity was $Hd = 0.875$. The 250 specimens comprised 87 haplotypes, many of them singletons in both river valleys (Figure 3A). Most of these haplotypes are closely related and form a triple star-like phylogeny with three wide-spread and numerous satellite haplotypes (Figure 3A). Three common haplotypes (separated by 3-4 mutation steps) contained 57% of all sequences. Two of these were recovered exclusively from the Tisza drainage, the third was present in both the Rába and the Tisza systems. There were also numerous haplotypes private to either the Tisza (76/77) or the Rába basins (9/10).

Mitochondrial COI fragments of the specimens collected historically in the Netherlands and Hungary contained the same haplotypes as the extant specimens with the exception of a single individual, collected at an unknown location in Hungary (Figure 3B). The majority of specimens from the Netherlands shared a haplotype characteristic to both Rába and Tisza rivers, and one specimen showed the central Tisza haplotype. The two most common Tisza haplotypes collapsed into a single short mtCOI haplotype, as the short mtCOI fragment contains less informative loci.

Intense demographic changes of the populations were indicated by highly significant deviations from selective neutrality (Tajimas $D = 1.365$, $p = 0.007$; Fu's $Fs = 24.766$, $p < 0.001$). The Bayesian skyline plot (BSP) [27] also showed strong and rapid increase in the population size of about 2.5 orders of magnitude, followed by a plateau in demographic changes (Figure 4). The BSP suggested no recent population decline.

We found significant genetic differentiation between the Tisza and Rába populations (pairwise $Fst = 0.353$, $p < 0.05$) The exact test for population differentiation (ETPD) [28] showed significant differentiation at $p < 0.05$). The "Isolation with Migration" (IMa) [29] analysis showed the highest likelihood for no migration between the two populations (Figure S1). Although the analysis is not time calibrated, the IMa clearly showed the complete divergence of the two populations in the present (Figure S2).

Discussion

We found unexpectedly high levels of genetic diversity in the isolated *P. longicauda* populations of the Tisza and Rába basins. In contrast, we found no genetic differences between the persisting Hungarian (Tisza and Rába), and the extinct Rhine populations, despite of the considerable geographic distance (1200 km between the lower Rhine and the central Tisza), and the distinct river drainages.

Strongly reduced genetic diversity is frequently recorded for species which experienced extensive range losses [4, 5]. Contrary to the expectations, we found very high haplotypic diversity (87 haplotypes in 250 investigated specimens) in the present-day range of *P. longicauda*. All but one of the haplotypes were private to either the Tisza or the Rába river systems. The geographic confinement of both major Tisza haplotypes, and all satellite haplotypes from both the Tisza and the Rába catchments show that the extant populations of these rivers had an independent history. The observed differences can be best explained by at least two distinct glacial refugia in the middle Danube drainage: one for the Rába, and at least one for the Tisza populations. A post-Holocene differentiation of the Rába and Tisza populations is unlikely due to the distance of several satellite haplotypes of the Rába from haplotypes of the Tisza valley (Figure 3A). The IMa also supports that the Rába and Tisza population are no longer intermixing (Figure S1, S2). The presence of the common Rába haplotype in the Tisza basin is most probably the result of incomplete lineage sorting after a past disjunction event, and not of ongoing gene flow. The Rába populations of *P. longicauda* never went completely extinct, and their reappearance after 40 years of absence is the result of cryptic persistence (probably meaning that nobody looked hard enough for them). The observed high genetic diversity in the present ranges is likely the result of a fortunate overlap between several of the species' last glacial maximum (LGM) refugia, and the current ranges. The middle-lower Danube catchment served as an important LGM refugia for numerous European riverine species, e.g. [11–14,30]. Our results thus support the importance of the middle-lower Danube drainage in

the conservation of the European freshwater genetic resources. This region is known to play a similar role for other, biogeographically Danubian taxa sensu Bănărescu [11], such as the Danube Salmon (*Hucho hucho*) (Berne Convention, Appendix III, IUCN endangered) [24, 31], the Carpathian Brook Lamprey (*Eudontomyzon danfordi*), and the Danube Gudgeon (*Romanogobio uranoscopus*) (Berne Convention, Appendix III, IUCN endangered) [24].

In a striking contrast with the high genetic diversity of the presently isolated ranges, all historic *P. longicauda* specimens from the Rhine catchment had haplotypes present today in the Tisza and Rába systems (Figure 3B). This is surprising, especially when considering the large geographic distances between the lower Rhine and the Tisza/Rába. We expected strong genetic differentiation between the Danube and Rhine drainages, given that cryptic taxa of European freshwater species are frequently discovered even in geographically close ranges, e.g. [13–16, 18]. The similarity of the Rhine and Tisza/Rába populations can be explained by a relatively recent, most likely post-LGM colonization of the Rhine from middle-Danubian source populations. Evidence for similar, relatively recent colonization patterns are rare, but not completely unknown [32]. The star-like shape of the haplotype network [33–35], the highly significant deviations from selective neutrality, and the BSP estimates (Figure 4) all suggest important and relatively recent increases in effective population size. The inferred increase is presumably also responsible for the partial formation of the extant genetic diversity, particularly the high numbers of satellite singletons diverged by a single mutation step from one of the central haplotypes. Given the limitations of the museum dataset (relatively few specimens and short nucleotide sequences), we can only approximate the source areas of the Rhine colonization: these were most likely located in the middle Danube drainage. This confirms that the ongoing reintroduction efforts of *P. longicauda* into the upper Rhine catchment [25] use genetically adequate source materials.

Overlapping former LGM refugia and areas relatively unimpacted by recent human activities may account for the preservation of significant intraspecific genetic diversity. The complete extinction of species from large areas may not significantly impact their diversity if the extinction affects only recently colonized areas. On the other hand, even small-scale range reductions may strongly impact intraspecific diversity, if they are affecting former (e.g. glacial) refugia.

The long-term cryptic persistence of *P. longicauda* in the Rába suggests that small populations may still survive on the formerly vast distribution range. Recent observations in distant areas (e.g. the Danube Delta) [36] may also be attributed to cryptic survival instead of recent range expansion. This emphasizes the potential of false absences in the conservation of this formerly wide-spread species, and the importance of extensive surveillance efforts accounting for the opportunities of the unknown unknowns [37]. We suggest that the re-discovery and active local protection of persisting cryptic populations of *P. longicauda* may be just as important for its long-term conservation, as the targeted monitoring of the Tisza and Rába populations [26], and active reintroductions [25].

Materials and Methods

Focal species

Palingenia longicauda is well-known since ancient times due to its size and short, highly synchronized mass swarming. The first written record that likely refers to this species originates from Aristotle in the 4th century B.C. from Greece [20]. The first scientific treatment was given by Clutius in 1635 from Belgium [20]. The species inhabited the probably most vulnerable European freshwaters: lowland navigable rivers. The eroding banks of these rivers were often enforced by riprap or concrete, a practice that destroyed the habitats of the larvae. The larvae develope for 3 years almost exclusively in the continuously eroding steep outer riverbanks composed mostly of clay [38,39]. Mass emergence as subadults and adults after the long larval development lasts only for a few hours. Biogeographically, *P. longicauda* is considered an expansive pontic element, which was present in almost every large European river [36].

After its extinction from the Danube in 1974 [19], the Tisza river in eastern Hungary was considered the last known range where mass swarmings still occur regularly. A small population was discovered in the Rába river in Hungary [21] after about 40 years of absence of reports. The Rába river is now considered the westernmost edge of the present distribution [39]. The recent finding of a few specimens in the Danube is attributed to ongoing range expansion [36].

Field and laboratory methods

We obtained 244 larvae from well-documented *P. longicauda* populations of the Tisza drainage and 18 larvae from the rediscovered habitat on the Rába river (Figure 2, Table S1). We collected larvae from the riverbank with a bager, a conical metal cylinder with an opening of 25 cm, historically used by fishermen for the same purpose. The specimens were preserved in 96% ethanol until DNA extraction, and they were deposited in the Senckenberg Museum (Frankfurt am Main) for long-term storage. We also used one or two legs of 37 historic dried museum specimens from the following collections: Natural History Museum (London), Senckenberg Museum, Natural History Museum (Budapest), and Natural History Museum (Vienna) (for more details see Table S2).

We extracted DNA using the DNeasy Blood & Tissue Kit for the extant specimens, and the QIAamp DNA Investigator Kit for the museum specimens (both Qiagen, Hilden, Germany), according to the manufacturers protocols. Legs of the dried museum individuals were homogenized in a Qiagen TissueLyser II. We extracted DNA from museum specimens in a laboratory dedicated to the pre-PCR processing of non-invasively collected and historic specimens. The laboratory is physically strictly separated from other DNA-laboratories in order to prevent any contamination from samples with high DNA content. Standard routines for the processing of historic samples were considered, such as strict rules for laboratory access, use of filter tips, and regular decontamination of equipment. In addition, negative controls were included in all extraction and amplification steps as contamination check. We amplified an approximately 600 bp long fragment of the mtCOI gene with the primers Jerry [40] and S20 [14], and an approximately 520 bp-long fragment of the 16S ribosomal rRNA with primers 16Sar [40] and 16SB2 [41]. We successfully and repeatedly amplified a short, approximately 200 bp long mtCOI fragment of the museum specimens with newly designed primers (PalJS20Int-1F: 5'TGATTATTGCCGTTCCTACTGG; PalJS20Int-1R: 5'AATGAAAATGGGCTACTACG). We set up PCR reactions on museum specimens in a laboratory dedicated exclusively for the pre-PCR treatment of non-invasively collected or ancient mammalian material. Amplicons were sequenced on an ABI 3730 DNA Analyzer (Applied Biosystems). We manually edited the tracefiles and aligned sequences in BioEdit [42].

Statistical analyses of population structure

We used median-joining networks [43] implemented in Network 4.5.1.6 (Fluxus Technology) to visualize relationships among haplotypes. We calculated Fst values on the basis of pairwise differences, and ran an ETPD [28] to estimate differentiation among sampling sites. We performed 10,000 permutations to estimate the statistical significance of pairwise Fst values. The length of the Markov chain was 100,0000 steps, with 10,000 steps for initial dememorization (burn-in) in the case of the ETPD. We used Tajima's D and Fu's Fs tests for selective neutrality to search for signals of demographic changes. We calculated pairwise Fst values, ETPD, Tajima's D and Fu's Fs in Arlequin 3.11 [44]. We reconstructed changes in effective population sizes using a Bayesian skyline plot [27] implemented in BEAST 1.5.4 [45]. We selected GTR+I+G as substitution model using Akaike's information criterion in Modeltest 3.7 [46]. We used a strict molecular clock model, with timing of events estimated in numbers of substitutions/sites, as no calibration dates were available for the analysis. We used a UPGMA-generated starting tree in our piecewise-constant Bayesian skyline model, with 10 groups. All priors for model parameters and statistics had default distributions. We ran 12 tests in parallel for 50 million generations each, and sampled them for every 5000 generations. We combined the results in LogCombiner 1.5.4 (part of the BEAST program).

The convergence of the tests was assessed in Tracer 1.5 [47] after discarding 10% of the samples as burn-in. We estimated lineage sorting and ongoing gene flow between the Tisza (population 1) and the Rába (population 2) in IMa [29], ensuring the convergence of the results by 5 exploratory runs. We used a single value (10) for all priors for the first exploratory run, then we refined the prior maximum values after each run, until the posterior estimates were fully contained within the bounds of the prior distribution. We established $q1 = 500$, $q2 = 10$, $qa = 10$, $m1 = 0.6$, $m2 = 2$, $t = 2$ as prior maximum values for the final test, according to the outputs of these preliminary runs.

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Figure Legends

Figure 1. Giant mayfly – *Palingenia longicauda*. A – freshly emerged giant mayfly (photo: A. Móra); B – males surrounding a female in a characteristic flower-like structure ("tiszavirág") (photo: A. Orosz); C – the synchronized sunset swarming of millions of adults (photo: L. Polyák).

Figure 2. Former (light) and present (dark) distribution of *P. longicauda*. Approximate collecting locality of the historic Rhine specimens is marked with a star. Subset: collection sites of extant specimens, numbered according to Table S1. The former distribution range of the species was reconstructed after [19, 20].

Figure 3. Relationships of *P. longicauda* haplotypes. A – median-joining networks of combined mtCOI and 16S haplotypes of successfully amplified extant specimens. B – median-joining networks of short mtCOI haplotypes of all extant and 24 historic specimens (Körös, Tisza, Maros and Bodrog marked as belonging to the Tisza catchment). Each circle represents a haloptype. The size of the circle indicates the frequency of the haplotype. Connecting lines represent single nucleotide substitutions.

Figure 4. Demographic changes of *P. longicauda* populations. The black line shows the mean population size, estimated by Bayesian skyline plot. Grey lines show population sizes within the highest and lowest 95% probability density intervals.

Supporting figure S1: Likelihood of ongoing migrations between the Tisza and Rába catchments. The likelyhood of ongoing migration to any directions is 0 (orange: migration from the Tisza to the Rába; blue: migration from the Rába to the Tisza.

Supporting figure S2: Likelihood of timing of the disjunction event between the Tisza and Rába populations. The complete separation of the Tisza and Rába populations happened in the past and an actual connection between the two populations has a likelihood of 0.