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Article

Setting Conservation Priorities in a Widespread Species: Phylogeographic and Physiological Variation in the Lake Chub, *Couesius plumbeus* (Pisces: Cyprinidae)

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Abstract: Defining units of conservation below the species level is a widely accepted conservation priority, but is especially challenging for widespread taxa that have experienced diverse geographic histories and exist across heterogenous environments. The lake chub (Pisces: *Couesius plumbeus*) is a widespread freshwater fish in North America and occurs from the southcentral USA to northwestern Alaska and Canada. We used mtDNA sequence analysis to test for divergent lineages predicted to occur as a result of survival of lake chub in distinct glacial refugia. Lake chub consisted of two major mtDNA lineages separated by 3.8% sequence divergence which are probably late to pre-Pleistocene in origin. We combined these data with those consistent with thermal adaptation in fish living in thermal springs versus those living in a lake with wide seasonal temperature variation, and with data on distribution of lake chub in major watershed units. We assessed these data against objective criteria developed to identify conservation units under Canadian endangered species legislation. Our analysis identified twelve major units of conservation within *C. plumbeus* that could be assessed under Canada's *Species-at-Risk Act*. Our study illustrates how different character traits manifested at very different spatial scales can be used to define conservation units within widely-distributed taxa.

Keywords: intraspecific variation; species-at-risk; phylogeography; thermal tolerance; Cyprinidae

1. Introduction

A basic tenet of conservation biology is the desire to conserve phenotypic and genetic variability within species, and the evolutionary processes that generate such variability, to promote the long-term persistence of species across a geographic mosaic of habitats particularly under environmental change (e.g., [1,2]). Geographically-widespread species, however, represent a notable challenge to intraspecific biodiversity conservation. First, their widespread geographical distribution may lead to a false characterization of their being secure in a conservation sense [3]. Geographically widespread species, however, have typically experienced diverse geographic histories and exist across heterogenous environments and they may comprise myriad evolutionary lineages and phenotypes that may be under different levels of threat across the species' range. Hence, the diverse bioheritage and spatial variation in threat status may be underappreciated in widespread species. Second, some of these lineages may represent cryptic species and our current taxonomic understanding may under estimate species level diversity—the so-called "Linnean shortfall" (e.g., [4]). Third, a wide-ranging species may play different functional roles in distinct ecosystems and hence its ecological diversity and threats to such diversity may also be underestimated across its range [5,6]. Finally, intraspecific diversity and threats to such diversity across a broad landscape, often encompassing different jurisdictions, makes the efficient identification, categorization, and conservation prioritization of appropriate conservation units particularly challenging [7].

Perhaps one of the most fundamental developments in conservation policy over the last 40 years has been the recognition of the importance of conserving intraspecific diversity across diverse landscapes and strategies to accomplish the conservation of such diversity. For instance, the *Endangered Species Act* in the United States (1973) [8] specifically recognizes that "distinct population segments" of any species can be legally listable as Threatened or Endangered. More recently, in Canada distinct populations or assemblages of populations can also be legally recognized as Threatened, Endangered, Extirpated, or Extinct under the federal *Species at Risk Act* (SARA) where such populations are denoted as "designatable units" ("DUs"). In the case of Canadian taxa, there are three criteria that can be applied to any population or collection of populations across a landscape to determine if they qualify as a valid DU: (i) if they are recognized as subspecies or varieties, (ii) if they are "discrete" from other members of the same taxon, and (iii) if that discreteness is of some "evolutionary significance" to the taxon in question [9]. Discreteness may take the form of genetic distinctiveness, separation by a major range disjunction, or occupancy of a distinctive biogeographic region [9]. Evolutionary significance can be satisfied if the genetic discreteness signals deep phylogeographic divisions (e.g., typically preglacial) or local adaptations, the putative DU occupies a habitat unique or unusual for the taxon, the putative DU represents the only surviving assemblage within the natural range of the taxon, or evidence that loss of the DU would induce an extensive gap in the range of the taxon in Canada [9]. While satisfaction of any one of these criteria is enough to recognize a DU under

SARA, the more compelling cases are those for which multiple criteria are satisfied. Clearly, the biogeography of the taxon in question, or its attributes, is central to DU recognition and subsequent conservation initiatives.

The lake chub (*Couesius plumbeus*) is a small (typical adult size about 60–100 mm in length) minnow (Pisces: Cyprinidae) endemic to North America. Its distribution includes the Canadian Maritime provinces south to the Delaware River in the northern Atlantic slope rivers in the US, through the Great Lakes region, a single locality in the upper Mississippi River, the Platte and Missouri rivers in the central US, the Hudson Bay and Mackenzie River drainages, and it extends west to the upper Yukon and upper Columbia rivers and throughout the Peace and Fraser rivers in western North America [10]. Historically, three subspecies have been suggested to exist based on morphology [11,12]: *C. p. greeni* (populations in the upper Columbia and Fraser rivers and adjacent Pacific slope rivers), *C. p. dissimilis* (east of the continental divide, the Great Plains in Canada and the US to southwestern portions of lakes Superior and Michigan) and *C. p. plumbeus* (northeastern North America, Atlantic slope rivers). It is possible, therefore, that the lake chub consists of three DUs in Canada based on putative subspecies alone.

Further, and for such a geographically widespread species, it comes as no surprise that the lake chub occurs in a variety of aquatic habitats. One of the more unusual observations is that *C. plumbeus* occurs in a number of geothermal “warm” or “hot spring” habitats in the northwestern portion of its Canadian range. These habitats typically consist of a complex of geothermal warm/hot spring pools and associated streams where water temperatures at the head of the springs can exceed 50 °C. Although the lake chub do not exist directly in such hot water, they are found in areas downstream (a few meters to a few 10s of meters) where annual water temperatures remain well above freezing throughout the winter and may be as high as 25–27 °C year-round despite surrounding winter air temperatures of −30 °C ([13], E.B. Taylor *et al.* unpubl. data). In fact, lake chub occupying such habitats exhibit variation in their tolerance to cold water temperatures and their ability to acclimate to cold temperature by increasing muscle mitochondrial content [14]. Lake chub from Atlin Warmspring demonstrated a significantly higher critical minimum water temperature (CT_{min}; the minimum water temperature at which individual fish demonstrate a loss of equilibrium) at acclimation temperatures of 25 °C than did fish from Green Lake (a lake with “normal” seasonal fluctuations in water temperature) as well as reduced ability to increase mitochondrial enzyme activities as acclimation temperature decreased [14]. Although the genetic basis of such differences still needs to be established, these results may be an adaptive response of Atlin Warmspring lake chub to the constant (25–27 °C) water temperature in their small and spatially simple habitat (a single, circular pool about 11 m in diameter at its widest point and about 1 m deep) compared to fish from populations with seasonally variable water temperatures [13,14].

Finally, the extensive geographic range of the lake chub in North America suggests that it may consist of several distinct phylogeographic lineages because its range overlaps several known Pleistocene glacial refugia (e.g., the Bering, Mississippi, Pacific, and Atlantic refugia) as well as the Rocky and Adirondack mountains in western and eastern North America, respectively, and similarly distributed taxa have been shown to exhibit phylogeographic divisions associated with range fragmentation across these areas (e.g., [15–18]). Whether or not, however, such lineages exist and if they correspond to the proposed subspecies of *C. plumbeus* is unknown.

In this study, we describe the application of the DU concept to lake chub across their distribution in Canada, by (i) applying molecular assays to assess the validity of proposed morphologically-defined subspecies and to test hypotheses about the existence of distinct phylogeographic lineages within the species, their date of evolutionary origins and association with suspected vicariant events, and (ii) applying the COSEWIC criteria for DU recognition by combining the phylogeographic data with the physiological data on temperature tolerance to test for evolutionarily significant subdivisions within the species.

2. Results and Discussion

2.1. Phylogeographic Variation in Lake Chub

After editing of initial unclear portions of sequence from the 630 base pair fragment, a total of 548 base pairs were analyzed in 179 lake chub. Overall, the best model of molecular evolution was found to be the Hasegawa-Kishino-Yano model with a Gamma distribution (G = 0.29) for site variation in evolutionary rates (ranked first using the Bayesian Information and Decision Theoretic criteria, but the TIM2 +I + G model was best supported by the AIC). A total of 40 haplotypes were resolved within the lake chub which differed from each other by an average of 2.1% uncorrected sequence divergence. The average net divergences between lake chub and the cyprinid outgroup taxa redside shiner (*Richardsonius balteatus*) and peamouth chub (*Mylocheilus caurinus*) were 14.2% and 15.7%, respectively. All analyses supported the monophyly of lake chub mtDNA and a subdivision within lake chub comprising two major clades (Figure 1) which differed from one another by a mean 3.8% sequence divergence (or 2.5% after accounting for variation within clades). One clade (88% support) consisted of all samples located from northwestern Ontario and east, while the other clade (80% support) consisted of all samples located west of this area (Figures 1 and 2). Haplotypes within the western group differed from each other more so (average divergence of 1.7%, N = 126 fish, 31 haplotypes) than did the eastern haplotypes (1.0%, N = 53 fish, nine haplotypes). In addition to the major east-west split between haplotype groups, a few subclades were resolved. For instance, a subclade consisting of two fish from the Nahanni River, NWT (86% bootstrap support, Cp35 and Cp36) and another subclade consisting of fish from western tributaries of Hudson Bay and fish from the South Saskatchewan River system (76% bootstrap support) were resolved within the western clade (Figure 1, Cp11, Cp20-25). Similarly, a subclade consisting of two fish from the Lake Superior sample (96% bootstrap support, Cp9 and Cp32) was resolved within the eastern clade.

All sequences from fish sampled from Liard Hotsprings (HS) and Atlin Warmspring (WS) belonged to the western clade (Figure 1); ten fish, six from Atlin WS and four from Liard HS were characterized by the same haplotype (Cp3) which was the most common haplotype (N = 56) in our sample while the another nine, six Atlin WS and three from Liard HS, were characterized a closely-related haplotype (Cp30, ~0.4% divergence from Cp3). There was one fish from Liard HS characterized by a further haplotype (Cp7, 0.2 and 0.6% divergent from Cp3 and Cp30, respectively).

Under an assumption of constant population sizes, the BEAST analysis produced an estimated mean time to most recent common ancestor (TMRCA) between the eastern and western clades of 2.5 million years ago (lower and upper bounds of the 95% highest posterior density interval of estimates were 0.522 and 6.66 million years ago, respectively). Under the expanding population scenario, the estimated mean TMRCA was 3.1 million years ago (lower and upper bounds of the 95% highest posterior density interval of estimates were 0.492 to 8.61 million years ago, respectively).

Figure 1. Phylogenetic relationships among 40 cytochrome *b* mitochondrial DNA haplotypes of lake chub (*Couesius plumbeus*, Cp1-40) and selected outgroup taxa as estimated by Maximum-likelihood clustering of sequence divergence estimates (HKY+ G distance). Numbers at branch points represent bootstrap support levels (N = 1,000 pseudoreplicates). Haplotypes accompanied by closed ovals represent fish both from Atlin Warmspring and Liard Hotsprings. Haplotypes that define the "western" and "eastern" lineages are denoted by the thick vertical bars. Rss = reside shiner (*Richardsonius balteatus*), Pmc = Peamouth chub (*Mylocheilus caurinus*) outgroups.

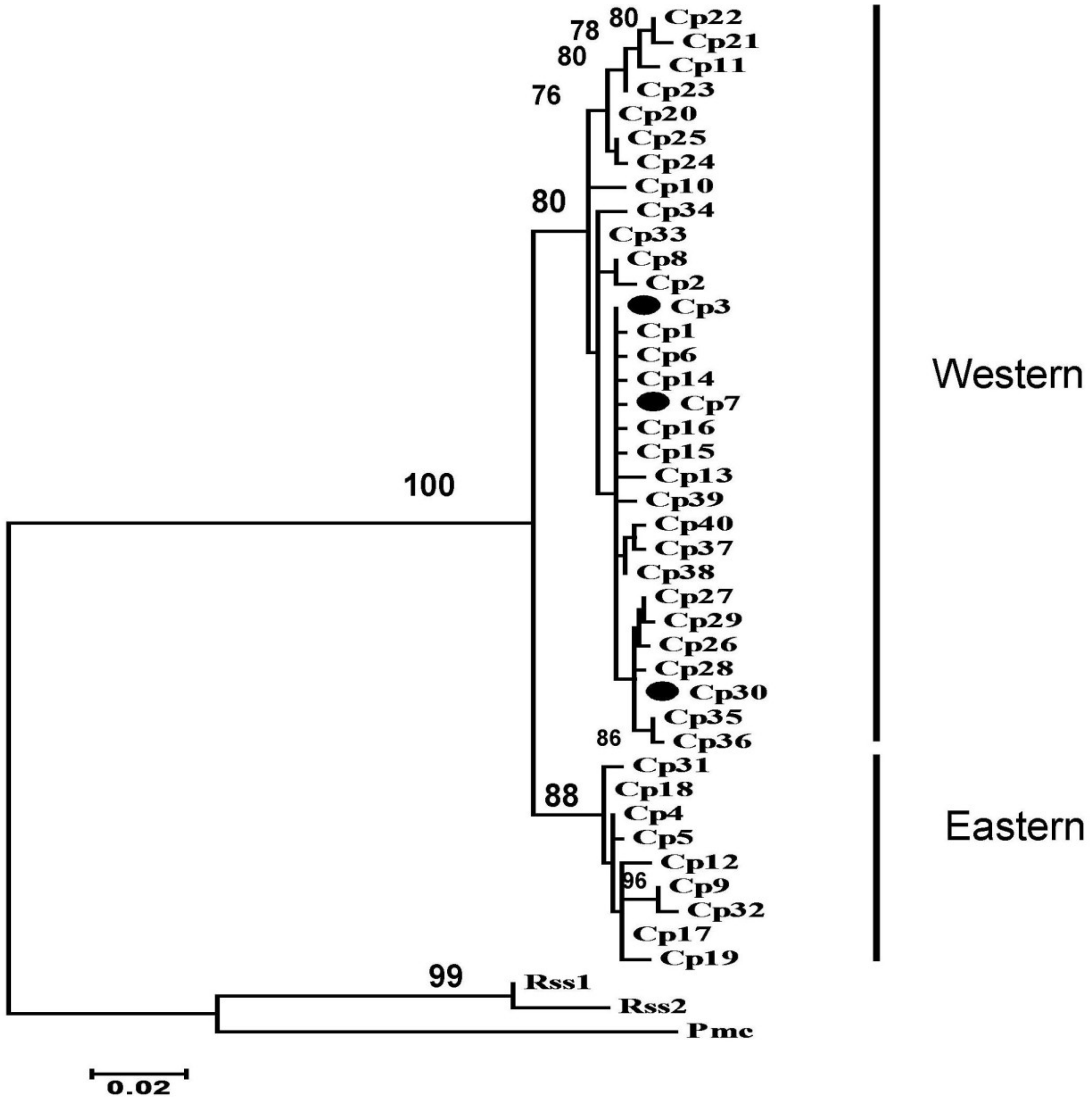
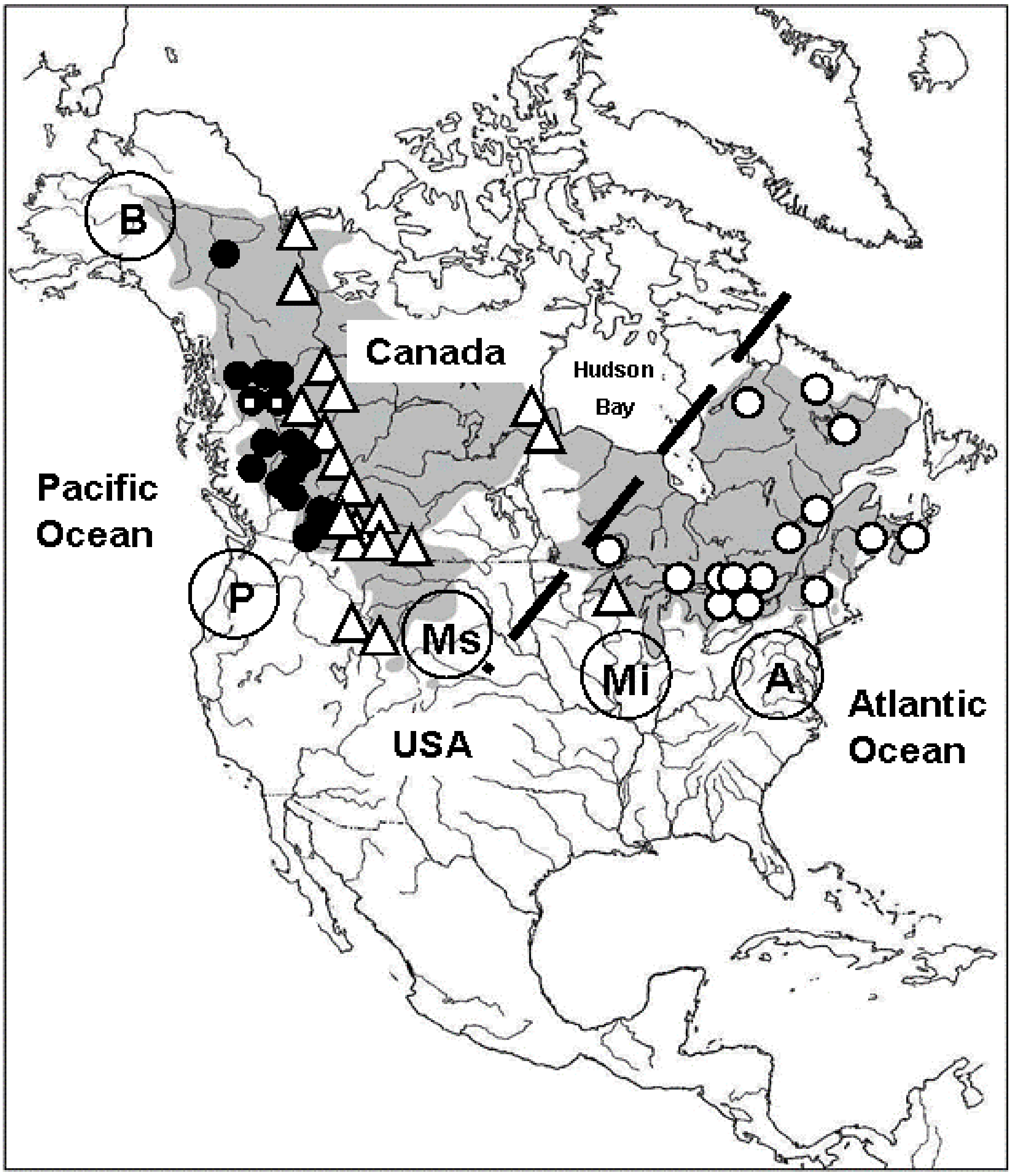


Figure 2. Approximate location of samples of lake chub (*Couesius plumbeus*) in North America. Closed circles represent putative *C. p. greeni*, open circles *C. p. plumbeus*, and open triangles *C. p. dissimilis.* The grey shading represents the geographic distribution of *C. plumbeus*. The thick diagonal dashed line represents the inferred boundary between samples with the western (left of the line) and eastern (right) mitochondrial DNA clades that differed from each other by 2.5% net sequence divergence. The two closed circles with interior white dots represent the Atlin Warmspring (left) and Liard Hotsprings (right) populations, respectively. Areas within established Pleistocene glacial refugia are shown by the encircled letter symbols: B = Bering, P = Pacific, Mi/Ms = Missouri/Mississippi, and A = Atlantic refugia.



There was some association between major mtDNA clade membership and putative subspecies designations. For instance, the western mtDNA clade characterized all of the putative *C. p. greeni*, and all of the putative *C. p. plumbeus* were characterized by the eastern clade (Figure 2. Appendix 1). By contrast, the putative *C. p. dissimilis* was also characterized almost exclusively by the western clade, but the eastern clade was also found in one sample (Figure 2, Appendix 1).

2.2. Physiological Variation in Lake Chub

The detailed physiological results of temperature tolerance trials and enzyme assays are reported in [14] and we briefly summarize them here (Table 1). Atlin WS fish live in a 25–27 °C year round warm spring and these fish showed lower thermal breath, higher values of CT_{min} and lower energy metabolism enzyme activities and lower protein content when challenged with lowered water temperatures than fish from Liard HS which live in a seasonally variable habitat (10–27 °C) influenced by a hot spring, and fish from Green Lake, a "typical" lake which freezes in winter (temperature ranges from <4 °C to 25 °C, Table 1). Liard HS fish showed some evidence of elevated CT_{max} (at 10 °C) and increased energy metabolism enzyme activities at low water temperatures (Table 1). The Euclidean distance summarizing differences across all physiological parameters between each pair of populations varied from a low of 74 between Liard HS and Green Lake, 107 between Atlin WS and Green Lake, to 132 between Atlin WS and Liard HS lake chub.

Table 1. Summary of main thermal physiological differences among three populations of wild-collected lake chub (*Couesius plumbeus*). See [14] for details. CT_{max} = critical maximum water temperature at 5, 10, 20, 25 °C acclimation temperature, CT_{min} = critical minimum water temperature at 5, 10, 20, 25 °C acclimation temperature, ΔCT = thermal breadth (CT_{max}- CT_{min}) at 5, 10, 20, 25 °C acclimation temperature, CS = citrate synthase activity at 5, 10, 20, and 25 °C acclimation temperature, COX = cytochrome oxidase activity at 5, 10, 20, and 25 °C acclimation temperature, LDH = lactate dehydrogenase activity at 5, 10, 20, and 25 °C acclimation temperature, PK = pyruvate kinase activity at 5, 10, 20, and 25 °C acclimation temperature, PC, axial muscle protein content at 5, 10, 20, and 25 °C acclimation temperature. AWS = Atlin Warmspring, LHS = Liard Hotsprings, GL = Green Lake.

Parameter	Summary of differences
CT_{max}	Significant effects of population and acclimation temperature, no interaction, LHS > both AWS and GL at 10 °C
CT_{min}	Significant effects of population and acclimation temperature, no interaction, AWS consistently highest CT_{min} at all temperatures, but significant only at 25 °C compared to GL
ΔCT	Significant effect of population and acclimation temperature, no interaction; AWS with consistently lowest ΔCT, and significantly lower ΔCT at 10 and 25 °C.
CS	Significant effects of population, acclimation temperature, significant interaction; AWS showed no increase in activity with decreasing acclimation temperature, GL and LHS increased activity by 71% and 141%, respectively, significantly greater than AWS at 5 and 10 °C
COX	Significant effects of population, acclimation temperature, significant interaction; AWS showed no increase in activity with decreasing acclimation temperature, GL and LHS increased activity by 86% and 133%, respectively, significantly greater than AWS at 5 and 10 °C (Green Lake only)

Table 1. *Cont.*

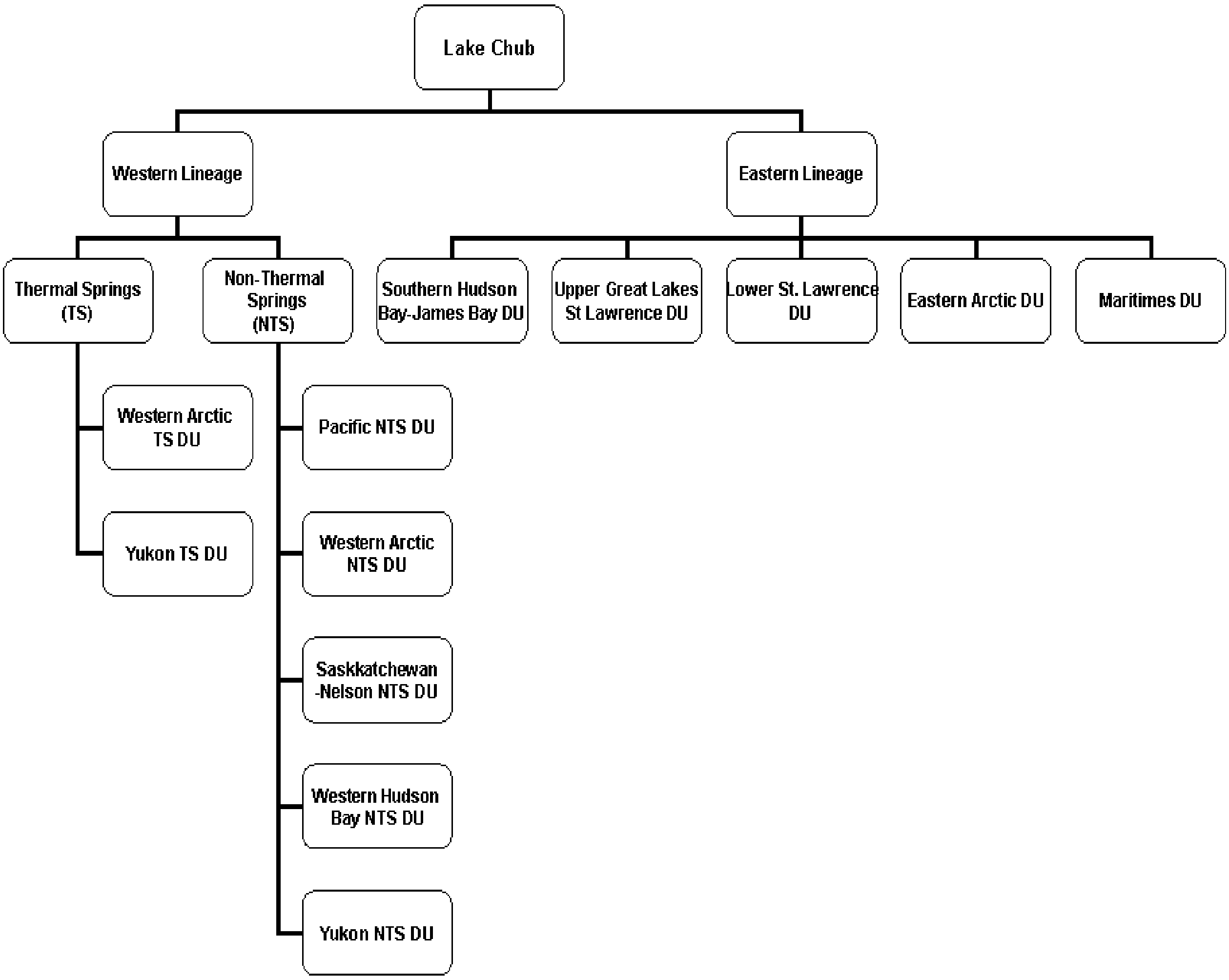
Parameter	Summary of differences
LDH	Significant effects of population, but not of acclimation temperature, no interaction; AWS showed greatest levels of activity at 10 and 25 °C, but not significant differences
PK	No significant effects of population, acclimation temperature, no interaction
PC	Significant effects of population, acclimation temperature, no significant interaction; AWS showed no change in PC with acclimation temperature, GL and LHS increased PC with decreasing acclimation temperature, but no significant pairwise differences.

2.3. Designatable Units in Lake Chub

Our phylogeographic and physiological data were combined in a two-step process to identify putative designatable units in the lake chub. The first step involves considering discreteness and the second the evolutionary significance of that discreteness. In terms of discreteness, the three putative subspecies could be considered as DUs. By definition, the subspecies would seem to satisfy the discreteness criterion for DU recognition because they have been characterized by some morphological distinctiveness [11]. The evolutionary significance of such morphological discreteness is, however, unclear given that the genetic basis to these morphological differences is unknown and subsequent studies established that there is considerable overlap in at least some of the characters used to denote subspecies [12]. If, however, the morphological traits used to identify the three subspecies signal phyletic differences, then one might expect the mtDNA data (as an independent measure of phyletic divergence) to mirror the morphological differences at least broadly. Our data, however, demonstrate considerable sharing of the two major clades of mtDNA amongst at least two of the three putative subspecies (Figure 2, Appendix 1). In addition, putative subspecies identification can be ambiguous owing to the observation of morphological intermediacy in some areas (e.g., in the vicinity of the continental divide [19]), and a lack of morphological characterization in others (e.g., northwestern and northcentral Ontario, northern Québec and Labrador [12]). Rather, evolutionary discreteness within the lake chub seems to be better described by the existence of two divergent lineages (“eastern” and “western”) that differ by an average of about 3.8% sequence divergence (2.5% net sequence divergence) and the geographic division lies somewhere between western Hudson Bay and northwestern Ontario (Figures 1 and 2). The BEAST analysis produced an estimated time of divergence between western and eastern clades that ranged between 2.5 and 3.1 million years ago (*i.e.*, beginning of the Pleistocene to the late Pliocene), but a highest probability density distribution that ranged from 0.491 to 8.6 million years ago (*i.e.*, late Pleistocene to late Miocene). Given that the current geographic distribution of the western and eastern clades overlaps with areas known to have acted as Pleistocene glacial refugia (e.g., Mississippi/Missouri and Atlantic, respectively), the divergence between these two lineages may have resulted from Pleistocene glaciations or perhaps by earlier climate-induced vicariant events in the Pliocene-Miocene [20,21]. These divergence time estimates are not inconsistent with values obtained using a range of molecular clock sequence divergence estimates for cytochrome *b* of about 1.4–2.4% per million years derived for other cyprinid fishes (e.g., [21]), *i.e.*, estimates of divergence between 1.6 and 2.7 million years ago. Although all such estimates are subject to assumptions, are uncertain, and we cannot necessarily tie them to specific vicariant events that impacted lake chub, our results do imply that the divergence is a relatively ancient, pre-Wisconsinan one, and thus it satisfies the evolutionary "significance" criterion for DU recognition [9]. Certainly, corroboration of these eastern and western biogeographic divisions from nuclear DNA assays is desirable (e.g., [22]). Similarities in the geographic distribution of the two lineages of lake chub with that of similarly divergent lineages in several other co-distributed freshwater fishes (see [16]) and other taxa such as the wood frog (*Rana sylvatica*, [18]), however, strongly suggest that the pattern seen in lake chub is part of a general process of vicariant isolation, further heightening the evolutionary significance of the two lineages of *Couesius plumbeus*. Consequently, at least two putative DUs are proposed: "western populations" and "eastern populations" (Figure 3). Within the western populations' DU, however, our physiological data suggest the existence of significant, and potentially adaptive, variation that is strongly associated with unusual environments; lake chub living in thermal springs possess thermal physiological profiles (in terms of CT_{max} and CT_{min}, energy metabolism enzyme activities) that can plausibly be interpreted as adaptations to higher average (Liard HS) and also less variable water temperatures (Atlin WS) (cf. [14]). Consequently, when combining the phylogeographic and physiological data we propose a minimum of three DUs for *C. plumbeus* across at least its Canadian range: "western thermal spring (TS) populations", "western non-thermal springs (NTS) populations" and "eastern populations" (Figure 3).

A further criterion for discreteness of populations that is especially important for freshwater fishes involves the concept of National Freshwater Biogeographic Zones (NFBZ, [9]). These are 14 major watershed groups that have been defined by their varying degrees of historical physical isolation from one another (they are all currently independent watersheds) and the different fish faunal assemblages that characterize each and which are a function, in large part, of their historical isolation. These NFBZ are recognized as potential identifiers of DUs because they represent large-scale and physically discrete assemblages of fishes whose very definition rests in the unique fish communities within them (e.g., each was impacted to varying degrees by Pleistocene glaciations which heavily influenced patterns of postglacial dispersal into each NFBZ and subsequent fish faunal composition; [23]). The two known western thermal spring populations (Liard HS and Atlin WS) are split across two NFBZ: the western Arctic (Liard HS) and Yukon NFBZs (Atlin WS), respectively). Further, the western non-thermal spring populations span five NFBZ: Western Hudson Bay, Western Arctic, Yukon, Pacific, and Saskatchewan-Nelson. Similarly, the eastern populations span five NFBZ: Upper Great Lakes-St. Lawrence River, Lower St. Lawrence, Southern Hudson Bay-James Bay, Eastern Arctic, and Maritimes. Therefore, when combining phylogeographic, physiological, and watershed information, a total of 12 DUs can be rationalized (Figure 3). The existence of such a large number of potential DUs is not without precedent. For instance, based largely on distribution across multiple NFBZ, COSEWIC recognizes eight DUs of the lake sturgeon (*Acipenser fulvescens*), while 14 are recognized in the Atlantic salmon (*Salmo salar*) and 36 have been proposed for the lake whitefish (*Coregonus clupeaformis*) based on genetic, distributional, and life history variation and the status of such DUs ranges from Extirpated to Not at Risk (see [24–26]).

Figure 3. Decision tree illustrating the identification of 12 putative designatable units (DU) in lake chub (*Couesius plumbeus*). The taxon is first subdivided between two major evolutionary (mitochondrial DNA) lineages (western and eastern), then, if applicable, by thermal physiological phenotypes (thermal springs, TS, and non-thermal springs, NTS, populations), then by National Freshwater Biogeographic Zone (NFBZ).



The analyses above provide a useful accounting of biological diversity within single taxon that can be used to monitor species that explicitly accounts for biogeographic variation, and to prioritize such units for conservation assessment and action. The accounting of a large number of DUs does not, however, necessarily present an unwieldy burden on limited conservation resources. The mission of COSEWIC, for instance, involves the assessment of the national conservation status of wildlife species (species, subspecies or other DUs) that are suspected to be *at some level of risk* of extinction or extirpation. In practice, COSEWIC engages in a prioritization process that identifies those DUs most in need of assessment owing to perceived risk using input on geographic range, population and abundance trends, threats, and the potential of rescue effects. Consequently, only those DUs suspected to be of some level of risk (*i.e.*, they could be assigned as status of Special Concern, Threatened, Endangered, Extirpated, Extinct) are assessed. In the case of the lake chub, the existence of only a single known thermal springs population in the Yukon TS DU (Atlin WS) and perhaps two-three in the Western Arctic TS DU (Liard HS and Deer River HS are in the same NFBZ) and their occurrence in a very small area of habitat (each is <0.5 km^2, [13]) suggest that they would be likely candidates for assessment. By contrast, the remaining 10 DUs exist over very large areas and comprise likely hundreds of populations each and, at least until they are better studied, are probably low priorities for assessment.

Our analysis is one of several types of systems that have been employed to characterize and inventory biodiversity at the intraspecific level (e.g., [27–29]). All of these systems have at their core the identification of aspects of biodiversity that are important to the historical legacy of the taxon in question, its evolutionary potential, and its persistence into the future across its natural geographic range. Consequently, they reflect the importance of considering aspects of organismal diversity along a spatial and temporal continuum [30]. For the lake chub, our analysis illustrates the differences in evolutionary processes operating over diverse spatial and temporal scales when generating biodiversity within a taxon and the importance of biogeographic analysis in understanding the origins of such diversity. The major phylogeographic lineages that we resolved in lake chub are present across a continental scale and likely represent divergence in assemblages of populations separated by major vicariant barriers, a common phenomenon in north temperate freshwater fishes impacted by Pleistocene glaciations and orogeny, and are increasingly recognized as important to conserve (e.g., [31,32]). By contrast, physiological differentiation was apparent across relatively small geographic areas (e.g., within and between NFBZ in Atlin WS and Liard HS) at the scale of individual localities and are strongly associated with differences in local thermal environments. As argued in [14] these phenotype-environment associations are consistent with predictions based on the idea that divergent natural selection in contrasting thermal environments can operate across small spatial scales. Of course, it is possible that the diverse environments occupied by lake chub across their range vary in ways other than those relevant to thermal biology and that may drive divergent natural selection. The existence of such geographically and selectively variable environments across is, in fact, part of the rationale for using NFBZ as a basis to recognize DUs in freshwater fishes. Consequently, it is possible that existing DUs based solely on phylogeography and NFBZ could be further partitioned into separate DUs upon finer scale investigation. The recognition of DUs is an example of an explicit attempt to recognize biogeographic variation in conservation prioritization at the intraspecific level. The case of the lake chub, however, illustrates the importance of considering a variety of spatial and temporal scales and different traits, especially those like life-history, morphology, physiology, and behaviour that may evolve more rapidly owing to natural selection, in assessing the significance of intraspecific variation that has arisen both over long and relatively short (e.g., postglacial) timeframes.

3. Experimental Section

3.1. Fish Collections

For DNA analysis, lake chub (*Couesius plumbeus*) were collected from a total of 52 localities from across Canada and the United States (Appendix 1). In general, we attempted to sample a minimum of five to ten fish per locality, but sometimes sample sizes were lower when supplied opportunistically by collaborators. Our principal aim was to assess phylogeographic diversity across the range of the species rather than conduct a detailed analysis of population-level diversity or differentiation across smaller geographic areas, hence we prioritized the sampling of a large and diverse assemblage of localities. Samples consisted of whole fish or, more commonly, fin clips stored in 95% non-denatured ethanol.

Full details of the physiological experiments can be found in [14]. In brief, lake chub collected from two thermal spring populations from northwestern BC: the Liard Hotsprings (a complex of several springs at +59°25'42.69", −126°5'18.32") and Atlin Warmspring (a single pool /stream complex at +59°24'13.98", −133°34'31.21"), and from one typical lake population in the central interior of BC: Green Lake (+51°22'34.60", −121°15'46.97") during July and August 2005 using minnow traps or a dip net. Live fish (80 per population) were transported to the University of British Columbia in temperature controlled containers.

3.2. Molecular Analysis

Genomic DNA was extracted using QiaQuik spin columns, eluted in 150 µL of "AE" buffer supplied by the manufacturer, and stored at −20 °C until analysis. We used the polymerase chain reaction (PCR) to amplify an approximately 630 base pair long fragment of the mitochondrial DNA cytochrome *b* gene using the primers "HD" (5'-GGGTTGTTTGATCCTGTTTCGT-3', [33]) and "GluDG" (5'-TGACTTGAAGAACCACCGTTG-3', [34]. Amplifications were carried out in 50 µL total volumes in the presence of (final concentrations): 1× New England Biolabs ThermoPol Buffer (20 mM Tris-HCl; 10 mM $(NH_4)_2SO_4$; 10 mM KCl; 2 mM $MgSO_4$; 0.1% Triton X-100), 100 µM of each dNTP, 0.2 µM each primer, 1.5 units of Taq polymerase and between 200 and 1000 ng of template DNA. Amplification cycling conditions consisted of an initial denaturation at 95 °C for 3 min, five cycles of 95 °C denaturation, 55 °C annealing, and 72 °C extension for 45 s, 30 s, and 30 s, respectively, 30 cycles of 92 °C denaturation, 52 °C annealing, and 72 °C extension for 45 s, 30 s, and 30 s, respectively, and a final extension step at 72 °C for 10 min. Amplified fragments were checked for quality (e.g., single clear fragment) and quantity on 1.5% agarose gels stained using SYBR® Safe DNA gel stain and viewed under ultraviolet light. Amplified fragments were purified using Qiaquick PCR Purification columns and cycle-sequenced on an Applied Biosystems Inc. 37390S sequencer using dGTP BigDye® Terminator v3.0 chemistry using the "HD" primer.

3.3. Temperature Acclimation Experiments

Full details can be found in [14]. Briefly, upon arrival at the University of British Columbia (UBC), fish were held in four 100 L aquaria (17 °C). Temperature within each aquarium was changed by 1 °C per day until they reached the four acclimation temperatures of 5, 10, 20 and 25 °C (achieved average temperatures were actually 6, 9.5, 19.5 and 24.5 °C) where they were acclimated for two months before initiating measurements. Critical temperature minimum (CT_{min}) and maximum (CT_{max}) tolerance measurements were then performed two weeks apart. Experiments were conducted in 1 L containers that were aerated and submerged in a common reservoir serviced with a re-circulating water bath. Fish were allowed to adjust to the experimental apparatus for 30 minutes before the beginning of the experiment and then temperature was increased or decreased by approximately 0.3 °C per minute. Individual fish were observed until they lost equilibrium, *i.e.*, turned on their backs or sides and unable to swim in a normal upright position. The temperatures at which fish lost equilibrium were our experimental measures of CT_{max} or CT_{min}. For CT_{min} measurements, temperature was reduced by adding ethylene glycol and dry ice pellets to the common reservoir. Two weeks after completion of the CT_{max} experiments, fish were euthanized and a caudal peduncle axial muscle sample was dissected and frozen in liquid nitrogen and stored at −80 °C until enzyme activity and protein content assays as described in [14].

3.4. Statistical Analyses

Cytochrome *b* sequences were aligned using Bioedit (Vers. 7.0.5.3; [35]) and have been deposited in GENBANK under accession numbers KC762976-KC763007. Following sequence alignment, the program jModeltest2 v 2.1.2 [36] was used to estimate the best fit model among 88 models of sequence evolution for the mitochondrial data set using the Akaike Information Criterion (AIC), the Bayesian Information Criterion (BIC), and the Decision Theoretic Framework (DT, [37]). A phylogenetic analysis was subsequently conducted on 40 haplotypes using maximum-likelihood employing the HKY + G model of substitution accompanied by 1,000 bootstrap replicates using MEGA 5.0 [38]. Analyses using model averaging in the maximum likelihood tree, or using Neighbor-joining analyses, maximum parsimony, and network analysis all produced results consistent with the with maximum-likelihood/HKY analysis. Sequences from (*Richardsonius balteatus* GU182814 and GU182876) and the peamouth chub (*Mylocheilus caurinus* AF117169) were used as out-groups. While not sister species of the lake chub, the reside shiner and peamouth chub are part of the same subfamily of cyprinid fishes and previous analyses of these species for cytochrome *b* variation and existing dated fossils of both species facilitated calibrated divergence time estimates (see [21]).

We estimated the divergence dates for major clades resolved within lake chub mtDNA using the Bayesian framework implemented in BEAST (Vers. 1.7.4, [39]). We employed the lognormal relaxed clock model to account to different evolutionary rates amongst lineages and the coalescent constant and expansion growth models to establish a range of priors on the phylogenetic tree. We calibrated the node associated with the divergence between *M. caurinus* and *R. balteatus* based on the first verified *Mylocheilus* fossil in North America (7.0 million years ago, [40]). The fossil calibration was modeled in BEAST using a normal distribution of priors for divergence dates employing a mean date of 7.0 million years with a standard deviation of 1.2. This produced a 95% confidence interval of calibration dates of 4.8–8.7 million years [21]. The MCMC was run for 40,000,000 generations, sampled every 1,000 generations and disregarded the first 4,000,000 steps as a burn-in period which resulted in acceptable effective sample sizes (>200) and acceptance probabilities for all parameters of >0.25 [41].

To summarize variation across all physiological parameters tested at the different acclimation temperatures or assessed in the field, we used the mean value for each parameter obtained from [14] to calculate a Euclidean distance between each pair of populations. The Euclidean distance was calculated as the square root of the sum of the squared differences between the mean values for each parameter between any two populations using the spreadsheet-based statistical software PAST [42].

3.5. Evaluation against COSEWIC DU Criteria

After collection and analysis of mitochondrial DNA and physiological parameters in lake chub, we considered these data within the context of COSEWIC components of the "discrete" and "significance" criteria using the "Guidelines for Recognizing Designatable Units" (Appendix F5 of [9]). This is an informal process that, essentially, consists of asking a series of questions concerning the existence of evidence supporting the discreteness of a population, or assemblage of populations, from other such populations within the taxon. Designatable units can be recognized as: named subspecies or (for plants) variety, or discrete and evolutionary significant populations. Discreteness is indicated by: genetic distinction, a geographic disjunction, or occupancy of different biogeographic regions (National Freshwater Biogeographic Zones for freshwater fishes). Evolutionary significance of discreteness is supported if such discreteness represents: long-term (generally preglacial) neutral differences, occupancy of unusual or unique habitats such that local adaptation to such habitats is likely, it represents the last occurrence of the taxon within its natural range, or loss of the discrete population would introduce a substantial gap in its geographic range [9]. Consequently, a population or assemblage of populations of lake chub was identified as a DU if there was evidence both of discreteness and evolutionary significance of that discreteness [9].

4. Conclusions

Assigning conservation priority to species with broad geographic distributions is challenging because such taxa often comprise multiple, potentially cryptic, evolutionary lineages and divergent selection across variable environments may generate adaptive variation that is important to long-term persistence across a landscape. Our phylogeographic analysis of the lake chub, combined with previous work documenting significant variation in thermal physiology, indicate that this widespread species consists of at least two major evolutionary lineages likely stemming from isolation during Pleistocene or pre-Pleistocene vicariant events in North America: western and eastern lineages.

Using a combination of distribution of the lake chub from the two lineages across Canadian National Freshwater Biogeographic Zones, and evidence for differentiation in thermal physiological performance in three populations that is associated with differing natural thermal environments, we propose that intraspecific variation in lake chub across a continental scale can be approximated by the recognition of 12 designatable units, each of which can be evaluated for their present level of conservation risk.

Our analysis provides an example of a process that can be employed to identify and map the geographic distribution of intraspecific units for conservation assessment that simultaneously accounts for broad spatial and temporal scales of variation.

Supplementary Material

Supplementary materials can be accessed at:

http://www.mdpi.com/1424-2818/5/2/149/s1.

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