Complete Mitochondrial Genomes of the Cherskii’s Sculpin Cottus czerskii and Siberian Taimen Hucho taimen Reveal GenBank Entry Errors: Incorrect Species Identification and Recombinant Mitochondrial Genome

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ABSTRACT: The complete mitochondrial (mt) genome is sequenced in 2 individuals of the Cherskii’s sculpin Cottus czerskii. A surprisingly high level of sequence divergence (10.3%) has been detected between the 2 genomes of C czerskii studied here and the GenBank mt genome of C czerskii (KJ956027). At the same time, a surprisingly low level of divergence (1.4%) has been detected between the GenBank C czerskii (KJ956027) and the Amur sculpin Cottus szanaga (KX762049, KX762050). We argue that the observed discrepancies are due to incorrect taxonomic identification so that the GenBank accession number KJ956027 represents actually the mt genome of C szanaga erroneously identified as C czerskii. Our results are of consequence concerning the GenBank database quality, highlighting the potential negative consequences of entry errors, which once they are introduced tend to be propagated among databases and subsequent publications. We illustrate the premise with the data on recombinant mt genome of the Siberian taimen Hucho taimen (NCBI Reference Sequence Database NC_016426.1; GenBank accession number HQ897271.1), bearing 2 introgressed fragments (approx. 0.9 kb) from 2 lenok subspecies, Brachymystax lenok and Brachymystax lenok tsinlingensis, submitted to GenBank on June 12, 2011. Since the time of submission, the H taimen recombinant mt genome leading to incorrect phylogenetic inferences was propagated in multiple subsequent publications despite the fact that nonrecombinant H taimen genomes were also available (submitted to GenBank on August 2, 2014; KJ711549, KJ711550). Other examples of recombinant sequences persisting in GenBank are also considered. A GenBank Entry Error Depositary is urgently needed to monitor and avoid a progressive accumulation of wrong biological information.

KEYWORDS: Cherskii’s sculpin Cottus czerskii, Amur sculpin Cottus szanaga, erroneous taxonomic identification, Siberian taimen Hucho taimen, introgression, recombinant mitochondrial genome, GenBank entry errors monitoring, GenBank Entry Error Depositary

Introduction
The Cherskii’s sculpin Cottus czerskii Berg 1913 is an amphidromous fish inhabiting the Sea of Japan’s inland coast rivers. The range of the species is limited by the North Nandai River (North Korea) on the South and the Serebryanka River (Primorye Region, Russia) on the North.1–6 Recently, Han et al7 have published the complete mitochondrial (mt) genome of allegedly C czerskii from the Sungari River (the Amur River basin, Heilongjiang Province, China; 47°03’ 39”N, 128°59’ 33”E). The previously described range of C czerskii did not, however, include the Amur River basin. There were only 2 described sculpin species in the Amur basin, Cottus szanaga and Macroscopus baiji.8 Consequently, we were interested in a comparative genetic analysis of C czerskii specimens collected from the Primorye Region, where this species was described originally1 and the sample from the Amur River basin investigated by Han et al.7

The Siberian taimen Hucho taimen Pallas is another fish species, which is considered here in relation to GenBank entry errors. Hucho taimen is the world’s largest salmonid fish, reaching up to 2 m in length and 105 kg in weight.9 The unique biological features and severe decline of taimen populations have stimulated intensive genetic investigations of the species (Balakirev et al10 and references therein). We previously revealed that the GenBank reference sequence of the H taimen mt genome (NC_016426.1; accession number HQ897271.1)10 is recombinant bearing 2 introgressed fragments (around 0.9 kb) from 2 lenok subspecies, Brachymystax lenok and Brachymystax lenok tsinlingensis.10 We sequenced and submitted to GenBank (August 2, 2014; KJ711549, KJ711550) 2 mt genomes of H taimen from natural populations of the Amur River basin without introgressions13; yet, the recombinant sequence still serves as the GenBank reference sequence of the H taimen mt genome.

We describe here GenBank entry errors for 2 fish species, C czerskii and H taimen. In the case of C czerskii, there is reasonable doubt on correct species identification; the data show that...
the accession number KJ956027 should be listed as C. szanaga instead of C. czerskii. In the case of H. taimen, the mt genome sequence HQ897271.1 appears to be a recombinant sequence including a big chunk of mitochondrial DNA (mtDNA) from 2 lenok subspecies (genus Brachymystax) leading to significant biases in phylogenetic inferences.

Our results are of consequence concerning the GenBank database quality, highlighting the potential negative consequences of entry errors, which once they are introduced tend to be propagated among databases and subsequent publications. Taking into account that GenBank entry errors are not rare, a GenBank Entry Error Depositary (EED) is urgently needed to monitor and avoid a progressive accumulation of wrong biological information.

Materials and Methods

The C. czerskii specimens were collected from the Barabashevka River (43° 11′51″N, 131° 29′54″E), Primorye Region, Russia. A complete morphological description of C. czerskii has been performed by one of the authors of this work (P.A.S.).\textsuperscript{13} Cottus czerskii differs from all other Palearctic Cottinæ by the following complex of features: the presence of teeth on the palatines, a long internal ray of the ventral fin (73.4%-96.0% of the length of the largest ray of the ventral fin), full body seismosensoric canal of 39 to 44 pores passing through the midline of the body, high total number of vertebrae (38-40), and large body size (up to 25 cm in total length).

We have also analyzed the GenBank mt control region (CR) sequences investigated by Yokoyama et al\textsuperscript{14} who collected samples of C. czerskii from the Barabashevka River. The multiple entries of C. czerskii (GenBank accession numbers AB308533, AB308534, AB308535, AB308536, AB308537, and AB059350) investigated by Yokoyama et al\textsuperscript{14} are important to visualize the range of intraspecific diversity in the species.

The specimens (Ht5 and Ht16) of the Siberian taimen H. taimen were collected from the Amur River basin; a specimen of blunt-nouted lenok Brachymystax tumenis Mori was collected from the Bikin River (see Balakirev et al.\textsuperscript{10} for sampling locations and procedures). In addition, we used full mt genomes from GenBank (Table S1), which were selected based on previous molecular evidence of close relationship to families Cottidae and Salmonidae and screening of nucleotide sequences available in GenBank.

Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) from 96% ethanol-preserved muscle tissue. The procedures for DNA amplification and direct sequencing have been described previously.\textsuperscript{10,15} The mt fragments were amplified with primers designed with the program mitoPrimer, v. 1.\textsuperscript{16} The polymerase chain reaction details and primers are presented in Text S1 and Table S2 (online supporting information). The C. czerskii mt genomes were annotated with the program DOGMA\textsuperscript{17} and deposited in GenBank under accession numbers KY783659 and KY783660.

The mt genomes were assembled using the program SeqMan (Lasergene, DNASTAR, Inc., Madison, Wisconsin, USA). Multiple sequence alignment was conducted using MUSCLE\textsuperscript{18} and MAFFT, v. 7\textsuperscript{19} and manually curated. DnaSP, v. 5\textsuperscript{20} and PROSEQ, v. 2.9\textsuperscript{21} were used for intra- and interspecific comparisons; MEGA, v. 7\textsuperscript{22} was used for basic phylogenetic analyses.\textsuperscript{10,15} For all reconstructions, the best-fit model of nucleotide substitution was chosen with the Akaike information criterion and the Bayesian information criterion in MEGA and jModelTest, v. 2.\textsuperscript{23} The alignments were analyzed for evidence of recombination using various recombination detection methods implemented in the program RDP3.\textsuperscript{24}

Results and Discussion

The size of the mt genome of our 2 samples of C. czerskii is 16 560 bp (base pairs) and the gene arrangement, composition, and size are very similar to the sculpin fish genomes published previously.\textsuperscript{25-27} There were only 6 single nucleotide differences and no length differences between the haplotypes CCZ2-14 and CCZ5-14; total sequence divergence ($D_s$) was $0.0004 \pm 0.0001$. The comparison of the 2 mt genomes now obtained with other complete mt genomes available in GenBank for the genera Cottus, Mesocottus, and Trachidermus reveals a close affinity of C. czerskii to other Cottus species (Figure 1A). However, a surprisingly high level of sequence divergence ($D_s = 0.1033 \pm 0.0030$) is detected between the C. czerskii samples now studied (CCZ2-14 and CCZ5-14) and the C. czerskii mt genome from GenBank (KJ956027). The average level of mt genome divergence ($D_s$) between all 8 Cottus available in GenBank (excluding the GenBank C. czerskii, KJ956027 and our 2 samples), which include C. bairdii, C. dzungaricus, C. hangiongensis, C. koreanus, C. reinitii, C. szanaga, C. volki, and C. amblytomopis, is $0.0097 \pm 0.0017$. The difference (0.1033) between the mt genomes of C. czerskii studied here and the previously published GenBank C. czerskii (KJ956027\textsuperscript{27}) is within the range of interspecific level of divergence observed between the 8 listed Cottus species. Thus, the mt data indicate that the C. czerskii sample from the Primorye Region, where this species was described originally, and the sample from the Sungari River and the Amur River basin are not the same species.

Figure 1A shows the C. czerskii (KJ956027) from the Sungari River clusters with the Amur sculpin C. szanaga (KX762049, KX762050) with a surprisingly low level of divergence ($D_s = 0.0135 \pm 0.0008$), which is in the range of intraspecific mt genome variability in sculpins (about 0.0342 in, eg, C. volki)\textsuperscript{26}. Thus, we may conclude, on one hand, that the GenBank entries for C. czerskii (KJ956027) and C. szanaga (KX762049, KX762050), despite their different species names, actually represent the same biological species. On the other hand, 2 entries with the same species name, the previously published GenBank C. czerskii (KJ956027) and the C. czerskii now studied, show a surprisingly high level of sequence divergence (0.1033),
comparable with the average divergence between other *Cottus* species, clearly indicating that they are different biological species.

The phylogenetic inconsistency we have detected might reflect hybridization event(s) between *C. czerskii* and *C. szanaga*, which might have resulted in interspecific recombination of their mtDNA (as it has been found for other organisms including fishes\(^{10,15}\)), or it could be due to incorrect taxonomical identification if it is the case that a specimen of *C. szanaga* was erroneously identified as *C. czerskii*. We therefore analyzed the mt genome alignments for evidence of recombination using various recombination detection methods implemented in the program RDP3.\(^ {24}\) All methods failed to reveal any signal of recombination between the GenBank mt genomes of *C. czerskii* (KJ956027) and *C. szanaga* (KX762049, KX762050) (\(P > .05\)), thus rejecting hybridization as a possible explanation of the anomalous similarity between the GenBank *C. czerskii* (KJ956027) and *C. szanaga* (KX762049, KX762050) mt genomes. Thus, the obvious discrepancy in the level of divergence between the mt genome sequences obtained by us and the one downloaded from GenBank is a result of mistaken species identification of KJ956027, so that the specimen investigated by Han et al.\(^ {7}\) actually represents *C. szanaga* erroneously identified as *C. czerskii*. This conclusion is in accordance with the ichthyologic data describing only 2 sculpin species in the Amur basin, *C. szanaga* and *M. haitej*\(^ {8}\); the Cherskii’s sculpin *C. czerskii* does not inhabit the Amur River basin.

One more argument supporting incorrect taxonomical identification of KJ95607 as *C. czerskii* comes from the analysis of the GenBank nucleotide sequences (nt CR) investigated by Yokoyama et al.\(^ {14}\) who collected *C. czerskii* samples from the Primorye Region. Figure 1B shows very close similarity (\(D_{xy} = 0.0024 \pm 0.0011\)) between our 2 specimens of *C. czerskii* (CCZ2-14 and CCZ5-14) and the specimens investigated by Yokoyama et al.\(^ {14}\). Moreover, the data sets show 32.9 times higher divergence (\(D_{xy} = 0.0789 \pm 0.0089\)) between the GenBank mt genome of the misidentified *C. czerskii* (KJ956027) and the other *C. czerskii* listed in Figure 1B, confirming the analysis based on the complete mt genomes. The intraspecific level of divergence detected between the GenBank CR sequence of the misidentified *C. czerskii* (KJ956027) and *C. szanaga* (KX762049, KX762050) is \(D_{xy} = 0.0170 \pm 0.0039\). Thus, once again the data show an entry error in the GenBank database so that the accession number KJ956027 should be listed as *C. szanaga* instead of *C. czerskii*.

Our observations concerning the GenBank database quality highlight a case of potential entry errors, which, once they first appear, tend to be propagated among public databases and subsequent publications (see discussion in the work by Pool and Esnayra\(^ {28}\)). We illustrate this potential error propagation with the salmonid fish Siberian taimen *H. taimen* hybrid mt genome below.

We have recently sequenced a portion (8141 bp) of the mt genome in 28 specimens of *H. taimen* from 6 localities in the Amur River basin.\(^ {10}\) A comparison of the data with the GenBank *H. taimen* mt genome (HQ897271.11\( ^ {11}\)) revealed significant differences between them despite the fact that the fish specimens come from neighboring geographical areas. The distribution of divergence was nonuniform, with 2 highly pronounced divergent regions centered on 2 genes, ND3 and ND6.
We have found that the first and second divergent regions are identical between the GenBank Hucho taimen and 2 lenok subspecies, Brachymystax lenok and Brachymystax lenok tsinlingensis, respectively. Therefore, both divergent regions represent introgressed mtDNA (~0.9 kb) resulting from intergeneric hybridization between the 2 lenok subspecies and Hucho taimen. The 2 recombination events were highly significant ($P = 2.984 \times 10^{-25}$ and $8.528 \times 10^{-42}$ for the first and second recombination events, respectively\textsuperscript{10}) with all 7 methods implemented in the program RDP3.\textsuperscript{24} Introgression is, however, not detected in our Hucho taimen specimens (Figure 2B).

Consequently, we sequenced 2 complete mt genomes of Hucho taimen from natural populations (the Amur River basin) without introgressions (KJ711549, KJ711550\textsuperscript{12}). Yet, the recombinant sequence (HQ897271.1\textsuperscript{11}) is used to represent the GenBank mt genome of Hucho taimen. It is actively used in phylogenetic inferences,\textsuperscript{29–37} which in turn have been cited in at least 48 subsequent publications (Google Search, July 7, 2017). It is worth noting that the phylogenetic inferences based on recombinant genes and genomes are significantly biased.\textsuperscript{10,15}

Figure 3 illustrates sharply discordant phylogenetic signals between recombinant genome of Hucho taimen (HQ897271) and Brachymystax subspecies. As a consequence, the position of Hucho taimen (HQ897271) was sharply different, depending on the fragments used for tree reconstruction. The trees based on first and second introgressed fragments separately showed Hucho taimen (HQ897271) identical to Brachymystax lenok (JQ686730) or to Brachymystax lenok tsinlingensis (JQ686731), respectively (Figure 3A and B). The tree based on both introgressed fragments displayed Hucho taimen (HQ897271) between the 2 lenok species (Figure 3C). On the tree excluding the introgressed fragments, Hucho taimen (HQ897271) was within the same cluster as the other Hucho taimen specimens (Ht5 and Ht16; Figure 3D). Thus, most of the mt genome of Hucho taimen (HQ897271) has obvious similarity to the Hucho taimen specimens obtained in our study (the specimens Ht5 and Ht16), whereas the introgressed fragments have unexpected similarity to Brachymystax subspecies and could be explained by introgression of mtDNA resulting from hybridization between lenok and taimen. Other salmonids included in this analysis (Salmo salmo, Salmo trutta, Salvelinus fontinalis, and Salvelinus alpinus) did not show any visible discordance in the level of divergence between the introgressed fragments and the rest of the mt genome (Figure 3).

Instances of interspecific mtDNA recombination have been occasionally detected in hybridizing conifers, salmonids, Salmo and Salvelinus,\textsuperscript{39,40} and primates.\textsuperscript{41} We, however, conjecture that the number of recombinant sequences persisting in GenBank could be higher if they are mostly indistinct with basic phylogenetic analysis. For instance, we previously detected unrecognized ("cryptic") recombinant COI genes in 2 brown algae, Saccharina latissima (EU681420) and Cystophora retorta (GQ368259).\textsuperscript{15} These cryptic recombinants were not detected in the original publication.\textsuperscript{42} However, we showed\textsuperscript{15} that the recombinant sequences have drastic consequences in phylogenetic inferences. Figure 4 shows an example for Saccharina latissima phylogenetic reconstructions based on recombinant COI sequences. The position of Saccharina latissima on 5′-COI and 3′-COI–based trees are sharply different; on the 5′-COI–based tree, Saccharina latissima is
within the order Laminariales (Figure 4A). On the 3′-COI tree, S. latissima is significantly different from Laminariales algae and clusters with some species of the order Ectocarpales (Figure 4B). On the full-length COI tree, S. latissima is within the order Laminariales (Figure 4C) but significantly different from other Saccharina species. Using various recombination detection methods implemented in the program RDP3, 24 we showed that the COI sequence in S. latissima is recombinant with the parental COI sequences of S. latissima coming from different algae orders, Ectocarpales and Laminariales. 15
recombinant COI sequences are from a highly cited paper, 138 citations; Google Search, July 7, 2017), which potentially might introduce significant biases in subsequent phylogenetic analyses. Our results are relevant concerning the DNA “bar-coding” for algae and possibly other organisms. The 5'-COI “barcode” region is not representative and might be even misleading (at least in case of S latissima and C retorta) in resolving taxonomic relationships between algal species.

Thus, entry errors are indeed progressively multiplied, thus propagating incorrect biological information. We may note that entry errors are not easy to remove from the GenBank database. Even if an error is corrected, it may have been already multiplied in computational analyses by GenBank users, who have already downloaded the erroneous data. One possible approach to reduce the flow of incorrect information is to establish a GenBank EED, where all known entry errors should be collected. The EED should then be first visited by GenBank users so as to identify possible entry errors that may have been reported earlier concerning the genes and/or species of interest, which would avoid their continuing propagation.

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Author Contributions

ESB designed the study, carried out the molecular genetic studies, performed the sequence assembling and alignment, statistical analysis, and drafted the manuscript. PAS collected fish samples and contributed to write the manuscript. FJA participated in the design of the study and contributed to write the manuscript. All three authors read and approved the final manuscript.

Disclosure Statement

The funders had no role in the study’s design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors alone are responsible for the content and writing of the paper.

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