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Pseudo but actually genuine: *Rhodeus pseudosericeus* provides insight into the phylogeographic history of the Amur bitterling

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Geographic isolation and colonization events have been considered complimentary historical processes in shaping the contemporary structure of regional biotas and taxonomic diversity. Accordingly, insight regarding vicariance and geodispersal might be gained by the analysis of phylogenetic pattern of related but geographically isolated taxa. Here, we examined endangered *Rhodeus pseudosericeus* in the Korean Peninsula based on mitochondrial cytochrome *b* sequences to provide information regarding the evolutionary origin of Amur-European bitterlings, *Rhodeus sericeus* and *Rhodeus amarus*. Our data and the contemporary distribution of *R. pseudosericeus* indicate that founding individuals have colonized the western coast of the Korean Peninsula via the paleo-Huang He River and since been subdivided into populations isolated by drainage formation. Our phylogenetic analyses indicated that it was likely *R. pseudosericeus* had a sister relationship to the *R. sericeus*–*amarus* complex, indicating that the *R. sericeus*–*amarus* clade originated from the dispersal of either *R. pseudosericeus* or its ancestral lineage. The geodispersal scenario based on our phylogenetic analyses supports the previous hypothesis that the Amur River likely created confluences with some tributaries of the paleo-Huang He River. Overall, the present study offers new insight into the taxonomic entity of *R. pseudosericeus* and a more comprehensive understanding of the phylogeographic history of *R. sericeus* and European bitterling lineages.

Keywords: phylogeography; *Rhodeus pseudosericeus*; bitterling; Acheilognathinae; cytochrome *b*

Introduction

Acheilognathinae (bitterlings) is a taxonomic subfamily of approximately 60 small freshwater fish species that are divided into three genera: *Acheilognathus*, *Tanakia*, and *Rhodeus* (Arai & Akai 1988; Nelson 2006; Kitamura et al. 2012). Over the last several decades, this subfamily has attracted scientific interest among evolutionary biologists due to the unique breeding behavior of its members, which includes obligate spawning in the gill cavities of living freshwater unionid and margaritiferid mussels (Smith et al. 2004; Spence & Smith 2013). The majority of bitterling species are found in lotic and lentic freshwater systems throughout East Asia (Nelson 2006), though some are currently endangered (Ohta et al. 2001; Kubota & Watanabe 2003; Kitamura et al. 2009).

A single valid species, *Rhodeus amarus*, is present throughout Europe (Reichard et al. 2007; Zaki et al. 2008; Bryja et al. 2010). A phylogeographical study based on a cytochrome *b* (cyt *b*) gene indicated that there are four lineages of *R. amarus* (Figure 1; Bohlen et al. 2006; Bryja et al. 2010), among which two were recently distinguished from *R. amarus* as separate species, *Rhodeus meridionalis* (from the Vardar River in Greece; Bohlen et al. 2006) and *Rhodeus colchicus* (from an isolated region in central Caucasus; Bohlen et al. 2006). All four lineages have traditionally been identified as *R. sericeus*, which is native to northeast Asia (Holcík & Jedlicka 1994), or as a

subspecies, *R. s. amarus*, until they were validated as discrete species by taxonomic reevaluation (Bohlen et al. 2006). The cyt *b* gene has also demonstrated that the common ancestor for the four European lineages originated from the dispersal of *R. sericeus* (Figure 1; Bohlen et al. 2006).

Rhodeus pseudosericeus, an endemic species in the Korean Peninsula (Figure 1), was also identified as *R. sericeus* when it was first recovered (Chae & Yang 1993). Despite their morphological similarities, *R. pseudosericeus* is clearly distinct from *R. sericeus* as well as from the European *R. amarus* lineages based on several characteristics, justifying its status as a separate species (Arai et al. 2001). This species is currently classified as endangered under the Protection of Wild Fauna and Flora Act of the Korean Ministry of Environment. Our study was designed to provide novel data for the relationship between the *R. pseudosericeus* and *R. sericeus*–*amarus* complex and their phylogenetic placement in the subfamily. Given that they are recovered as a monophyletic clade, *R. pseudosericeus* may provide a critical model system for the investigation of the origin and historical dispersal of *R. sericeus* and European bitterling lineages. Two exclusively alternative dispersal scenarios could be envisaged (Figure 1); namely, either *R. pseudosericeus* was colonized by *R. sericeus* or its ancestral lineage or *R. sericeus* originated from the dispersal of *R. pseudosericeus* or its ancestral lineage.

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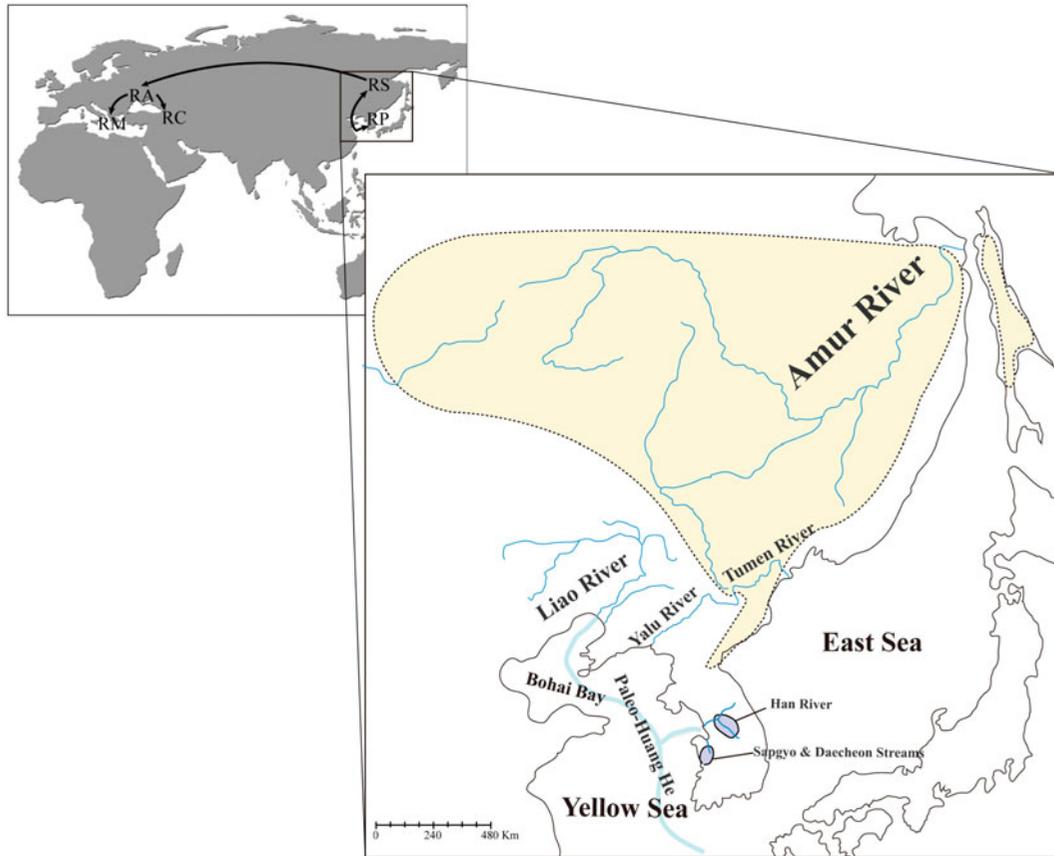


Figure 1. Two alternative scenarios regarding the geographic isolation and colonization of *R. pseudosericeus* (RP), *R. sericeus* (RS), and European bitterling lineages (RA: *R. amarus*; RM: *R. meridionalis*; and RC: *R. colchicus*) on the basis of Bohlen et al. (2006; the above part of the figure) and the structure of freshwater systems among and within the Korean Peninsula (the below part of the figure).

Materials and methods

Sampling and sequencing

R. pseudosericeus is only found in three western-flowing drainages in the Korean Peninsula (Figure 1). For this study, individuals were collected during March–September of 2011 from six localities (Table 1; Figure 2). Collection and preservation were conducted under the permission of the local administrative institutions of the Ministry of Environment in South Korea. Total genomic DNA was extracted from caudal peduncle tissue using the Wizard Genomic DNA purification kit (PROMEGA, Fitchburg, WI, USA) in accordance with the manufacturer’s protocol. The mitochondrial *cyt b* gene was used for the analysis of genetic variation within and among populations. Each amplification was conducted in a 50-μL reaction volume composed of ~75 ng genomic DNA extract, 0.25 mM of each deoxynucleotide, 0.4 μM of each primer (forward: Brito et al. 1997; backward: Perdices et al. 2002), 3 mM MgCl₂, 1× PCR buffer, and 0.25 units of Taq DNA polymerase (SOLGENT, Daejeon, South Korea). GenePro (BIOER, Hangzhou, P.R. China) was used to amplify the *cyt b* gene at an annealing temperature of 54°C. Amplified products were directly

sequenced in both directions using the PCR primers with an ABI PRISM BigDye terminator system in an ABI3700 automatic sequencer (GENOTECH, Daejeon, South Korea).

Data analysis

The nucleotide sequences of the *cyt b* gene were confirmed through Basic Local Alignment Search Tool

Table 1. List of the six *R. pseudosericeus* populations used in the present study.

Drainage system	Population	N	Location
Han River	Jucheon	10	N37°, 25', 53"/E128°, 10', 50"
	Heuk	10	N37°, 30', 43"/E127°, 39', 04"
	Seom	10	N37°, 32', 26"/E127°, 57', 56"
	Jojong	10	N37°, 45', 53"/E127°, 26', 50"
Saggyo stream	Muhan	10	N36°, 33', 51"/E126°, 46', 53"
Daecheon stream	Daecheon	10	N36°, 23', 25"/E126°, 39', 49"

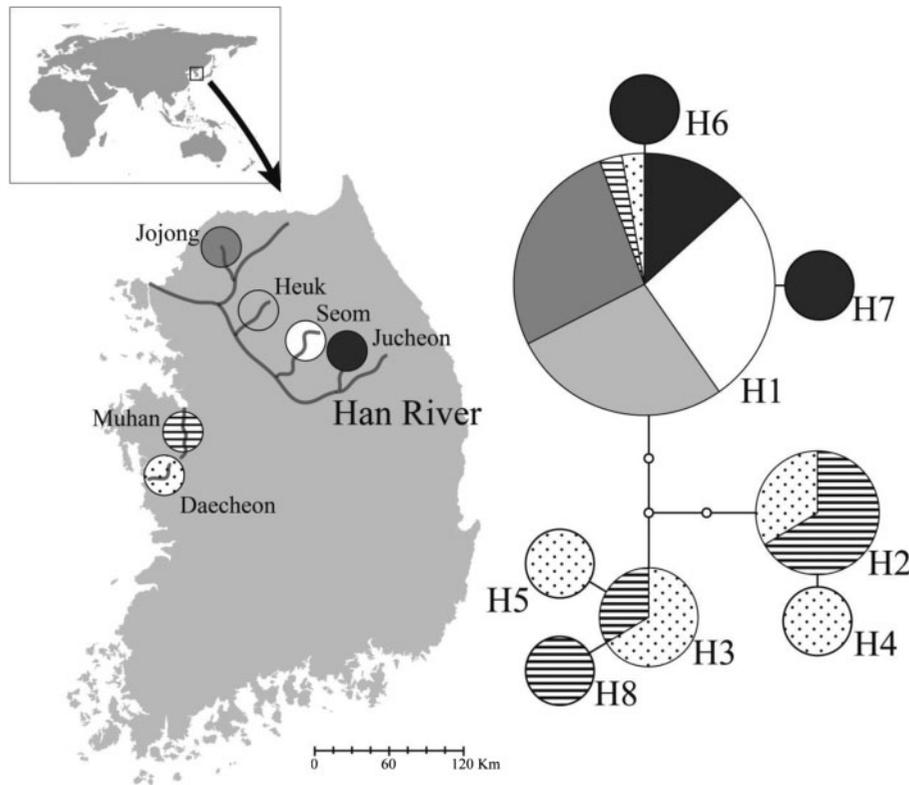


Figure 2. Statistical parsimony network model drawn based on the *cyt b* sequence data obtained from 60 *R. pseudosericeus* individuals. Each circle represents a different haplotype with its diameter proportional to the haplotype frequency. Each line between haplotypes represents a nucleotide substitution. The six sampling localities are indicated with a relative frequency in each haplotype circle.

(BLAST) searches, edited with GENEIOUS, ver. 5.4, and aligned with the nucleotide sequences from other bitterling species and out-group taxa (Table 2) retrieved from GenBank in the program MEGA, ver. 5.1 (Tamura et al. 2011). Genetic diversity at the intrapopulation level was quantified by estimating the number of haplotypes, haplotype diversity (H_d), and nucleotide diversity (π) using DnaSP, ver. 5.10 (Librado & Rozas 2009). An unrooted network was constructed to depict the relationships among haplotypes based on the 95% statistical parsimony method using TCS, ver. 1.21 (Clement et al. 2000). Genetic structure was estimated with a hierarchical analysis of molecular variance (AMOVA) implemented in ARLEQUIN, ver. 3.1 (Excoffier et al. 2005) to examine whether all populations were regarded as a single group (Table 3).

For the phylogenetic analysis, out-group taxa included *Tanichthys albonubes* from other subfamily within Cyprinidae and *Kichulchoia brevifasciata* from Cobitidae, Cypriniformes (Table 2). The GTR+I+G model was selected as the most appropriate nucleotide substitution model with MrModeltest, ver. 2.3 (Nylander 2004). Phylogenetic relationship and divergent time estimation were inferred in BEAST, ver. 1.7.4 (Drummond et al. 2012), enforcing a relaxed molecular clock under models

of uncorrelated lognormal distribution rate and a Yule speciation. Two independent runs of 1×10^8 generations were conducted, sampling parameters every 1000 generations and utilizing the fossil calibration point of 23 Mya (early Miocene) at the stem of Acheilognathinae lineage (Yang et al. 2011) and a *cyt b* substitution rate of 0.76% (Zardoya & Doadrio 1999). Lognormal distributions were applied for the fossil calibration point with a mean of 23.0, SD of 0.2, and an offset of 0.0, and for the rate prior for *cyt b* with a mean of 0.76, SD of 0.49, and an offset of 0.0. LOGCOMBINER, ver. 1.7.4 (Drummond et al. 2012), was used to combine two independent BEAST analyses, which converged on the same parameter space. The tree was annotated with TREEANNOTATOR, ver. 1.7.4 (Drummond et al. 2012) and was visualized in FIGTREE, ver. 1.4.0.

Results

In the present study, *cyt b* gene sequences of 860 nucleotides from 60 individuals yielded eight distinct haplotypes (deposited in GenBank under accession nos. KJ028096–028103; Table 2), with one of them (H1) occupying a wide geographic distribution (Figure 2). The remaining haplotypes were of low frequency, with each one represented by just one or two populations (Figure 2).

Table 2. GenBank accession numbers and the references of the haplotypes used in the BEAST species-tree reconstruction based on fossil calibration at the stem of Acheilognathinae lineage and a substitution rate for the *cyt b* gene (Figure 2).

Species	Accession nos.	Reference
<i>Kichulchoia brevifasciata</i>	KC524521	Kim et al. (2013)
<i>Tanichthys albonubes</i>	EU241475	Fang et al. (2009)
<i>Acheilognathus tabira nakamurae</i>	AB620146	Kitamura et al. (2012)
<i>Acheilognathus yamatsutae</i>	NC013712	Unpublished
<i>Acheilognathus cyanostigma</i>	AB620134	Kitamura et al. (2012)
<i>Acheilognathus melanogaster</i>	AB620136	Kitamura et al. (2012)
<i>Acheilognathus rhombeus</i>	AB620135	Kitamura et al. (2012)
<i>Acheilognathus macropterus</i>	NC013711	Unpublished
<i>Acheilognathus typus</i>	NC008668	Saitoh et al. (2006)
<i>Acheilognathus chankaensis</i>	DQ396628	Bohlen et al. (2006)
<i>Tanakia lanceolata</i>	EF483933	Unpublished
<i>Tanakia koreensis</i>	NC013704	Hwang et al. (2013)
<i>Tanakia somjinensis</i>	FJ515921	Hwang, Byeon, et al. (2014)
<i>Rhodeus ocellatus</i>	HQ113265	Yang et al. (2011)
<i>Rhodeus ocellatus kurumeus</i>	NC008642	Saitoh et al. (2006)
<i>Rhodeus notatus</i>	NC013709	Hwang, Lee, et al. (2014)
<i>Rhodeus sinensis</i>	EF483937	Unpublished
<i>Rhodeus amurensis</i>	DQ396627	Bohlen et al. (2006)
<i>Rhodeus colchicus</i>	DQ396678	Bohlen et al. (2006)
<i>Rhodeus sericeus</i>	DQ396683–DQ396686	Bohlen et al. (2006)
<i>Rhodeus meridionalis</i>	DQ396679–DQ396682	Bohlen et al. (2006)
<i>Rhodeus amarus</i>	DQ396620	Bohlen et al. (2006)
	DQ396630–DQ396635	
	DQ396637–DQ396649	
	DQ396651–DQ396677	
<i>Rhodeus pseudosericeus</i> H1	KJ028096	The present study
<i>Rhodeus pseudosericeus</i> H2	KJ028097	The present study
<i>Rhodeus pseudosericeus</i> H3	KJ028098	The present study
<i>Rhodeus pseudosericeus</i> H4	KJ028099	The present study
<i>Rhodeus pseudosericeus</i> H5	KJ028100	The present study
<i>Rhodeus pseudosericeus</i> H6	KJ028101	The present study
<i>Rhodeus pseudosericeus</i> H7	KJ028102	The present study
<i>Rhodeus pseudosericeus</i> H8	KJ028103	The present study

Table 3. Summary of the AMOVA results that partition genetic variation among six *R. pseudosericeus* populations from the Korean Peninsula to two different geographic levels (among groups: the Han River vs. Muhan-Daechon).

Source of variation	df	Sum of squares	Variance components	Percentage of variation
Among groups	1	30.458	1.11992	71.44*
Among populations	4	2.375	0.01623	1.04
Within populations	54	23.3	0.43148	27.52*
Total	59	56.133	1.56763	100

* $P < 0.001$.

Three populations (Heuk, Jojong, and Seom) from the Han drainage harbored only a single haplotype, H1 (Figure 2). Only seven variable nucleotide sites were found among 860 bp, as were the corresponding haplotype diversity ($H_d = 0.512$) and nucleotide diversity ($\pi = 0.0022$). Two distinct genetic clusters, the Han River populations and Muhan-Daechon, were identified from the

minimum parsimony network (Figure 2), and the results of AMOVA indicated that more genetic variance occurred among clusters than among populations or individuals within populations (Table 3).

In our phylogenetic analysis, *R. sericeus* and *R. amarus* lineages formed a monophyletic group. Eight haplotypes of *R. pseudosericeus* were recovered as the

most likely sister group of *R. sericeus*–*amarus* clade by a strong statistical value (Figure 3), indicating that the *R. sericeus* and European bitterlings originated from the dispersal of either *R. pseudosericeus* or its ancestral lineage or vice versa. The three genera of Acheilognathinae formed complete and independent monophyletic clades, with *Acheilognathus* being placed at the ancestral position (Figure 3). *Tanakia* was recovered as a sister to the monophyly of *Rhodeus* species (Figure 3). Within *Rhodeus*, the remaining species were basal and sister to *R. pseudosericeus* plus *R. sericeus*–*amarus* complex with high statistical supports (Figure 3). The root nodes for Acheilognathinae and *Rhodeus* were estimated to be about 17.27 Mya (CI: 10.32–25.33) and 9.20 Mya (4.60–14.37), respectively. An age estimate of 5.02 Mya (1.90–8.94) was assigned to the node leading to the separation between the *R. pseudosericeus* and *R. sericeus*–*amarus* complex (Figure 3).

Discussion

Low to moderate levels of intrapopulation genetic diversity were shown across the six populations investigated.

Surprisingly, more diverse haplotypes were found from the much smaller Daecheon and Sapgyo streams than those observed from the Han River, one of the major freshwater systems in the Korean Peninsula. The current distribution of *R. pseudosericeus* and the population genetic structure may bear the mark of drainage formation and colonization dating back to when sea levels were much lower than at present. It is well known that the Huang He River, which is the major drainage system running through China and discharging eastward into the Yellow Sea, likely created confluences with the present-day western-flowing drainages along the western coast of the Korean Peninsula during the Quaternary glacial periods (Figure 1; Nishimura 1974). Thus, it is conceivable that a refugial unit of *R. pseudosericeus* (or its lineage) has colonized along the current western coastal areas of the Korean Peninsula via the paleo-Huang He River and has been subdivided into populations that were isolated by the disappearance of paleo-river systems and drainage formation. Following colonization events, many populations of *R. pseudosericeus* might have been depleted, which could be attributed to the contemporary

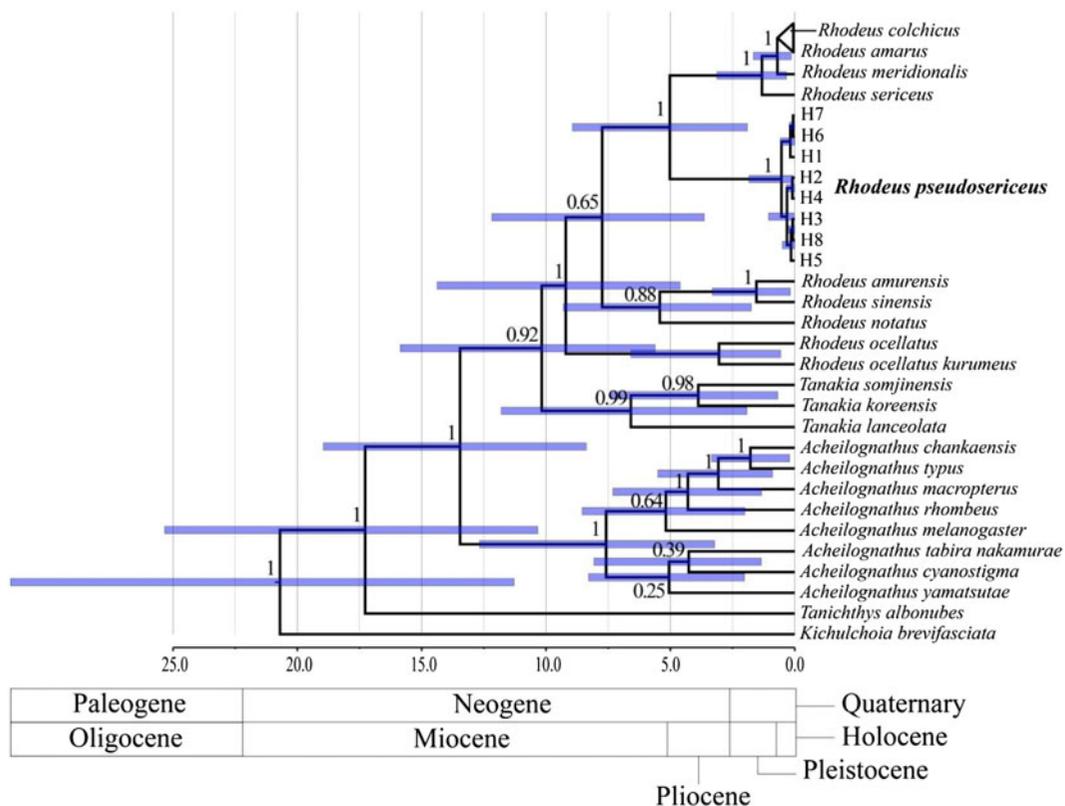


Figure 3. BEAST species-tree reconstructed to infer the relationship among the species within the subfamily Acheilognathinae and divergence time estimates based on fossil calibration at the stem of Acheilognathinae lineage and a substitution rate for the *cyt b* gene. Bars and numbers on nodes represent 95% credibility intervals and node-supporting values (posterior probabilities), respectively. The information (GenBank accession nos. and references) of haplotypes and species used were listed in Table 2. Multiple haplotypes were used for *R. sericeus*, *R. amarus*, and *R. meridionalis* in the tree (Table 2), and a single haplotype of *R. colchicus* was recovered to be in the *R. amarus* clade.

scattered and limited distribution. Alternatively, the Han drainage and Daecheon and Sapgyo streams may have been independently colonized by different founding populations, which might have led to the genetic subdivision between the Han River and Daecheon-Muhan group. In addition to geographic isolation and colonization patterns, the genetic structuring might be at least partially due to genetic drift arising from repeated population bottlenecks.

Insight regarding geodispersal processes of *R. sericeus* and *R. amarus* lineages could be obtained by our analyses of the relationship with *R. pseudosericeus* and the phylogenetic position within Acheilognathinae. While *R. sericeus* and *R. amarus* lineages formed a monophyletic group, *R. pseudosericeus* could be treated as a sister of the *R. sericeus*–*amarus* complex by strong node-supporting values. Given that most of *Rhodeus* bitterlings are currently distributed in the paleo-Huang He River with being placed at the basal position in the phylogenetic tree, our results indicated that the *R. sericeus*–*amarus* complex was derived from the dispersal of either *R. pseudosericeus* or its ancestral lineage. Solely based on this scenario, it is conceivable that the founding individuals of *R. sericeus* originated from the paleo-Huang He River colonized the Amur River. However, the paleo-Huang He River might not appear in the present Yellow Sea area at the estimated time of divergence between *R. pseudosericeus* and *R. sericeus*–*amarus* complex, since no glacial periods have been required around that time. Those two phylogenetic groups might have been differentiated from each other before their dispersal into the Amur region.

It is unclear how this bitterling dispersed from the paleo-Huang He River to the geographically isolated Amur River. The Amur River likely created confluences with the Liao River, a tributary of the paleo-Huang He River (Figure 1), as those two rivers contain freshwater fauna with a high degree of similarity (Sakai et al. 2006). More detailed inferences regarding their processes of speciation and colonization require additional extensive sampling of *R. sericeus* and the related species throughout their distribution range. In addition, the more complete resolution of the colonization history suggested in the present study could be obtained by the investigation of other bitterling species. For example, *Rhodeus amurensis*, native to the Amur River, is closely related to *Rhodeus sinensis* occurring in the rivers originated from the paleo-Huang He River (see also Figure 3).

Gil et al. (2007) reported that *R. sericeus* individuals occurred in eastern-flowing freshwater systems – probably originated from the paleo-Amur River – from North Korea. In addition, Arai et al. (2001) investigated some North Korean *R. sericeus* samples based on morphological characteristics and found the overall similarity to *R. pseudosericeus*. Considering that *R. pseudosericeus* populations exist along the eastern coast in North Korea, it may

also be possible to infer that the divergence between *R. pseudosericeus* and *R. sericeus*–*amarus* complex had happened in the Amur region and the *R. pseudosericeus* populations examined in the present study had been colonized by North Korean populations. From this idea, however, it should be proved how these two geographically separated rivers could have created confluences via the Korean Peninsula. Direct comparison with North Korean samples may provide more concrete solution for the origin and the historical dispersal pattern of *R. pseudosericeus*.

Taken together, the present study provides new insight into the taxonomic entity of *R. pseudosericeus* and more comprehensive understanding of the phylogeographic history of *R. sericeus* and European bitterlings. In summary, our results indicated that (1) founding individuals of *R. pseudosericeus* have colonized the western coast of the Korean Peninsula via the paleo-Huang He River, (2) *R. sericeus* and *R. amarus* shared the common ancestor with *R. pseudosericeus* or might originate from the dispersal of its ancestral lineage, and (3) the Amur River likely created confluences with the paleo-Huang He River.

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