

## **COI-Based Genetic Structure of an Exotic Snapping Turtle *Chelydra serpentina* Imported to South Korea**

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### **ABSTRACT**

A common snapping turtle *Chelydra serpentina* inhabiting North America is internationally protected as an endangered species. It is known that the individuals of common snapping turtles were imported to South Korea as pets, and after being abandoned, some inhabit the natural ecosystem of South Korea like wild animals. No genetic survey has yet been performed for the common snapping turtles imported to South Korea. Hereby, cytochrome *c* oxidase subunit I (*COI*) information, which is 594 bp long, was determined for a total of 16 *C. serpentina* individuals, of which one was found in nature, twelve legally imported and their descendants, and the other three were provided from the Kansas Herpetological Society, USA. The obtained data were combined with thirteen *COI* sequences of *C. serpentina* retrieved from NCBI GenBank for the subsequent population genetic analyses. The results showed that there exist five haplotypes with high sequence similarity (only three parsimoniously informative sites). In the TCS and phylogenetic analyses, all the examined *C. serpentina* samples coincidentally formed a strong monoclade with those collected mostly from Kansas State, USA, indicating that the imported ones to South Korea are from the central North America. In addition, there found the amino acid changes and the high degree of nucleotide sequence differences between *C. serpentina* and *C. rossignoni* with some important morphological characters. It is expected that the present results could provide an important framework for systematic management and control of exotic snapping turtles imported and released to nature of South Korea.

**Keywords:** *Chelydra serpentina*, *Chelydra rossignoni*, snapping turtle, endangered species, *COI*, genetic diversity

### **INTRODUCTION**

The common snapping turtle, *Chelydra serpentina* (Linnaeus, 1758), is a species of freshwater turtle widely distributed from southern Canada to the southeastern United States (Honda et al., 2002; Ernst and Lovich, 2009). This species commonly found around static freshwater areas, such as brackish marsh-

es, freshwater lakes, and rivers (Ernst et al., 1994). The size varies considerably, but in nature, individuals over 40 cm in length have been recorded (Gibbons and Lovich, 1990). Common snapping turtles are known as one of the highest predators in freshwater ecosystems throughout their range that eat fish, amphibians and even small mammals such as rodents, though they are omnivores that often consume plants around

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freshwater (Hammer, 1972). It is known that the related species of *C. serpentina* are *C. rossignoni* from Central America and *C. acutirostris* from South America (Rhodin et al., 2010). The Florida population of *C. serpentina* is classified as a subspecies *C. serpentina osceola* (Legler and Vogt, 2013).

Common snapping turtles can be purchased or imported as pets in many countries according to legal procedures (Ceballos and Fitzgerald, 2004). Collection of common snapping turtles from the wild and captive production in turtle farms particularly for export to East Asia has increased tremendously in recent years; in contrast to about 10,000 exports in 1999, over 300,000 were exported annually outside the United States in 2005 (Schlaepfer et al., 2005). In fact, it was estimated that “348,529” snapping turtles were harvested from 11 states in the United States for export to Asian countries from 1998 to 2013 (Colteaux and Johnson, 2017). The common snapping turtles are a legally protected species in the United States and Canada, and only with permission would it be possible to capture natural individuals (Schlaepfer et al., 2005). This species is currently assigned to the Class III International Endangered Species (CITES) and the Least Concern (LC) in the IUCN Red List (IUCN, 2012).

It is known that the common snapping turtles are bigger and more aggressive than any native turtle species (Ernst and Barbour, 1989). Because their potential for being a hostile destroyer in the East Asian ecosystems is great (Honda et al., 2002), they are required a special management and tracking. The biggest problem is that imported snapping turtles are likely to be abandoned and introduced into the natural ecosystem, making them more likely to become exotic species (Kato and Etoh, 2012). In Japan, it is often reported that abandoned snapping turtles live as natural species in local ecosystems since mid-1990s (Kobayashi, 2000; Kato and Etoh, 2012). Moreover, young snapping turtles that have just hatched have been seen several times in ponds around Tokyo (Kobayashi et al., 2006).

The primary purpose of this study is to identify the exact origin and genetic diversity of *C. serpentina* imported to South Korea with one released to nature on the basis of cytochrome *c* oxidase subunit I (*COI*) sequences. In addition, the *COI* haplotypes of *C. serpentina* were compared with that of *C. rossignoni*. This study is designed to make a cornerstone of building a database for tracking management in the future, by grasping the genetic diversity of traded snapping turtles.

## MATERIALS AND METHODS

### Sample collection

A total of 16 individuals of a common snapping turtle *C. serpentina* were used to determine the *COI* sequences, among

which the ten of IM1-10 were imported to South Korea by an amphibian reptile breeder in a legal way, the two of BR1 and BR2 were ones bred in South Korea, and the three of KA1-3 were the tissue samples kindly provided by the Kansas Herpetological Society, United States, and the last one of AB1 was the individual that was found in the natural freshwater ecosystem, located in Gongju city, Chungcheongnam-do, South Korea (Appendix 1). The collection localities of the examined samples listed in Appendix 1 are depicted on the map in Appendix 2.

### DNA extraction and PCR amplification

Total genomic DNA was isolated from bloods or the ethanol-preserved tissues using DNeasy Blood & Tissue kit (Qiagen, Valencia, CA, USA) in accordance with the manufacturer's protocol. The mitochondrial cytochrome *c* oxidase subunit I gene (*COI*) was amplified by thermal cycling and sequenced using the primers LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3') and HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA G-3') (Folmer et al., 1994). The PCR amplifications were performed in a 20- $\mu$ L final reaction volume containing 1  $\mu$ L of template DNA (100 ng/ $\mu$ L), 2  $\mu$ L of PCR buffer (Solgent Co., Daejeon, Korea), 2.5 mM of each dNTP, 10 pM of each primers, and 2.5 unit of *Taq* DNA polymerase (iNtRON Biotechnology, Seongnam, Korea) under the thermal cycling profile being composed of a denaturation at 95°C for 3 min, 35 cycles of a denaturation at 95°C for 30 s, an annealing at 50–53°C for 30 s and an extension at 72°C for 30 s and a final extension at 72°C for 5 min. The PCR products were sequenced directly using ABI PRISM BigDye terminator system on an ABI3700 automatic sequencer (Genotech, Daejeon, Korea). The newly obtained *COI* sequences in this study were deposited in GenBank with the accession numbers of MT181176 to MT181191 (Appendix 1).

### Genetic diversity and phylogenetic analysis

Each of the *COI* sequences obtained from 16 *C. serpentina* individuals was confirmed through BLAST searches, and edited with BioEdit software ver. 7.09 (Hall, 1999). With addition of 13 *COI* sequences retrieved from the Genbank (Table 1), the present data were aligned using Clustal X (Thompson et al., 1997) under the default options implemented in Mega7 (Kumar et al., 2016). Genetic diversity was quantified by estimating the indices of haplotype diversity ( $H_d$ ) (Nei, 1987) and nucleotide diversity ( $\pi$ ) (Nei, 1987) with DnaSP ver. 5.10 (Librado and Rozas, 2005). Haplotype networks were constructed in PopART (Leigh and Bryant, 2015) using TCS network (95% connection limit).

### Phylogenetic analyses

Phylogenetic analyses were conducted with the *COI* se-

**Table 1.** The genetic diversity indices estimated with the five *COI* haplotypes obtained from 28 individuals of *Chelydra serpentina* and the distribution of the haplotypes along the localities

Area	Population	N	h	$H_d \pm SD$	$\pi \pm SD$
East Asia	South Korea	13	H2/4/5	0.295 ± 0.156	0.00078 ± 0.0016
	Japan	1	H4	–	–
	China	1	H1	–	–
North central North America	Ontario, Canada	1	H1	–	–
	Illinois, USA	2	H1/4	1.000 ± 0.500	0.00168 ± 0.0016
	Indiana, USA	1	H1	–	–
Central North America	Kansas, USA	6	H4	0.000 ± 0.000	0.00000 ± 0.0000
Eastern North America	New York, USA	2	H1/2	1.000 ± 0.500	0.00505 ± 0.0000
	North Carolina, USA	1	H3	–	–
Total		28	H1–5	0.526 ± 0.104	0.00121 ± 0.0017

*COI*, cytochrome *c* oxidase subunit I; *N*, the number of individuals analyzed;  $\pi$ , nucleotide diversity; *h*, the number of haplotypes;  $H_d$ , haplotype diversity.

quence alignment, which contains the *COI* sequences from 28 individuals of *C. serpentina* and one individual of a central snapping turtle *C. rossignoni* as an outgroup. Since no *COI* of *C. acutirostris* has published yet, it was not included in the present study. The maximum likelihood (ML) tree was reconstructed using the *GTR+I+G* model in IQ-tree online site (<http://iqtree.cibiv.univie.ac.at>). The neighbor-joining (NJ) tree was performed by MEGA version 7 (Kumar et al., 2016) using the Kimura-2-parameter model.

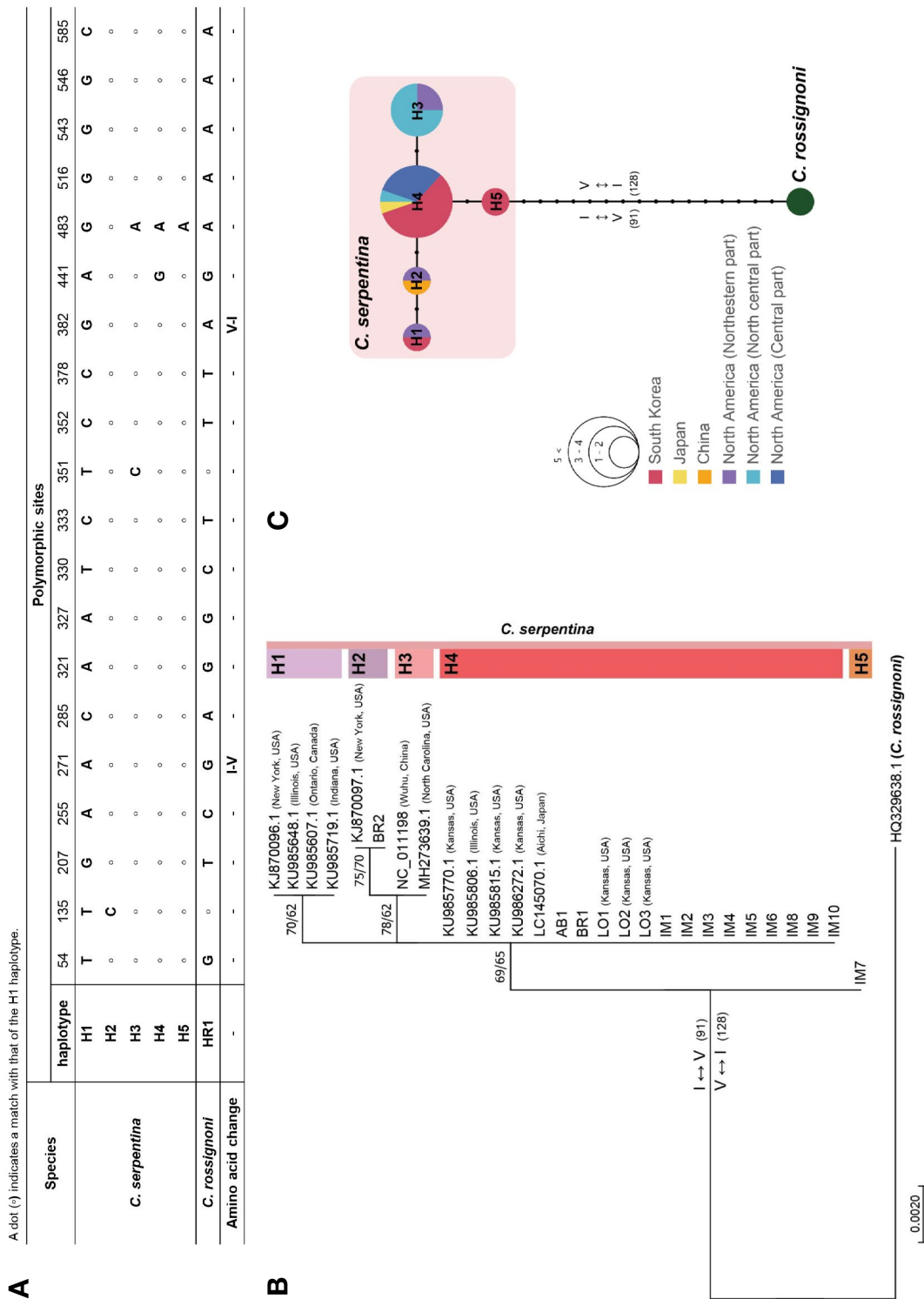
## RESULTS AND DISCUSSION

A total of five *COI* haplotypes were observed from 28 examined individuals of *C. serpentina* (Appendix 1). Among the five haplotypes (Fig. 1A), there were only one or two pairwise sequence differences on the four variable sites. Only 3 parsimoniously informative sites were observed and any sign of insertion or deletion was not found. The mean nucleotide diversity ( $\pi$ ) and mean  $H_d$  were low, with  $0.00121 \pm 0.00173$  (0.00000–0.00505) and  $0.526 \pm 0.104$  (0.000–0.295), respectively (Table 1).

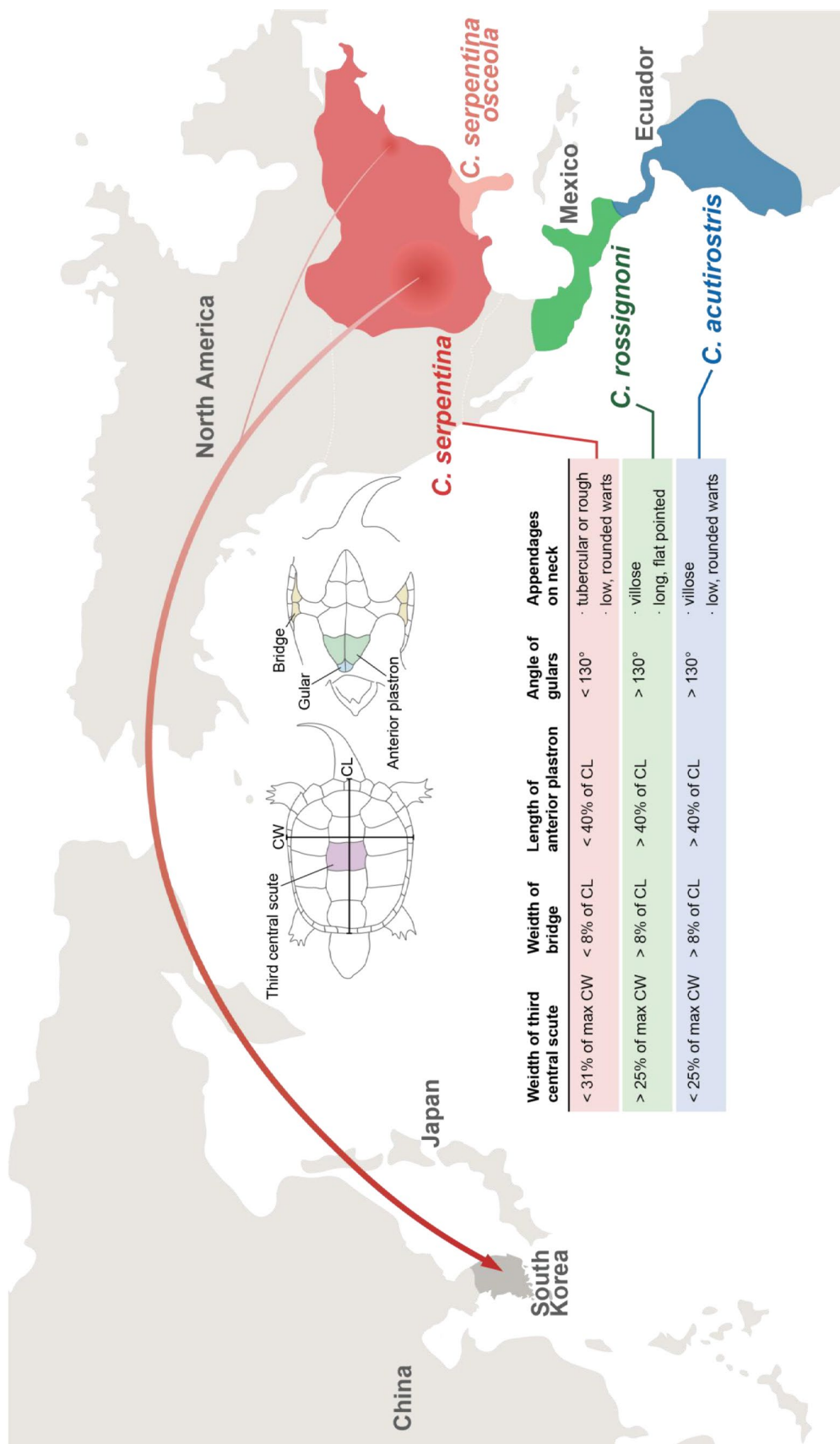
In both ML and NJ phylogenetic trees (Fig. 1B), all the examined individuals of *C. serpentina* formed a strong monophyletic group with those collected mostly from Kansas State, USA. The bred individual of BR2 (H2) showed closer genetic affinity to those from the eastern part of North America including New York and North Carolina. Interestingly, IM7 (H5) was apart from all the others of *C. serpentina* investigated in this study. However, this haplotype only differs from other haplotypes in a single nucleotide sequence. Since there are so few nucleotide variations among the sequences, it seemed to

be separated from the clade even with such a small difference. As shown in Fig. 1C, the five haplotypes were also identified from the TCS network drawn by Popart (Leigh and Bryant, 2015). Except for BR2 (H2) and IM7 (H5), the remaining 14 individuals of *C. serpentina* have commonly H4 like those in Central North America (Fig. 1). The results are most likely to indicate that *C. serpentina* imported to South Korea are mainly from the central North America. The point of view may be coincided with the fact that the largest number of snapping turtles has been harvested annually in central North America such as Iowa and Arkansas States for export to Asian countries (Colteaux and Johnson, 2017).

The genus *Chelydra* contains the three species of *C. serpentina* from North America, *C. rossignoni* from Central America, and *C. acutirostris* from South America (Rhodin et al., 2010). In addition, the Florida population of *C. serpentina* is classified as a subspecies, *C. serpentina osceola* (Shaffer et al., 2008). When we compared *COI* data of *C. serpentina* with that of *C. rossignoni* (Fig. 1A), twenty variable nucleotide sequence alignment sites (3.36%) were identified. In *C. rossignoni*, isoleucine (I) on the 91 aa site of the alignment is replaced by valine (V), and valine (V) is also replaced by isoleucine (I) on the 128 aa site (Fig. 1). The high degree of nucleotide sequence differences as well as the two significant amino acid residue changes can strongly support species differences between *C. serpentina* and *C. rossignoni* with some important morphological characters (Legler and Vogt, 2013) such as the length ratio of plastron and carapace, the height of body depth, and the appendages of neck. In Fig. 2, the key morphological characteristics of *C. serpentina* were well summarized in comparison with those of Central American snapping turtle *C. rossignoni* and South American snapping



**Fig. 1.** Genetic diversity, TCS network, and phylogenetic analyses based on the cytochrome c oxidase subunit I (COI) sequences from 28 *Chelydra serpentina* and one *C. rossignoni*. A, The polymorphic sites found in the five COI haplotypes of H1–H5 of *C. serpentina* and the HR1 of *C. rossignoni* and the amino acid changes of COI between *C. serpentina* and *C. rossignoni*; B, Maximum likelihood (ML) tree reconstructed with 29 COI sequences, among which 16 were directly sequenced with the collected samples of *C. serpentina* in this study, and 13 were retrieved from NCBI GenBank. The numbers above the internal branches on the tree indicate the bootstrap values for ML and neighbor-joining (NJ) obtained with 1,000 bootstrap replicates, in order. The ML tree was reconstructed using the IQ-Tree tree building program with the GTR+I+G best evolutionary model. The NJ tree construction was performed by MEGA version 5.0 with the Kimura-2-parameter model; C, TCS network in which each circle represents a different haplotype, with its diameter proportional to the haplotype frequency. The 29 samples of snapping turtles used in this study are listed in Appendix 1.



**Fig. 2.** The major imported route of *Chelydra serpentina* to South Korea inferred from the cytochrome c oxidase subunit I (COI) sequences and morphological differences of the three *Chelydra* species. The arrow indicates the routes of *C. serpentina* imported to South Korea from Central North America. As depicted with different colors on the map, *C. serpentina* is distributed mainly in North America (light red), a subspecies *C. serpentina osceola* in Florida (red), *C. rossignoni* in Central America (green), and *C. acutirostris* in South America (blue). The details of geographical distribution of the five COI haplotypes of *C. serpentina* refer to Table 1. In addition, morphological differences of *C. serpentina*, *C. rossignoni*, and *C. acutirostris* are summarized with the illustration of the general body plan of snapping turtles. CW, carapace width; CL, carapace length.

turtle *C. acutirostris*.

As *C. rossignoni* from Central America and *C. acutirostris* from South America are adapted to relatively hot regions, they are likely not to tolerate the winter environment in South Korea. On the other hand, *C. serpentina* has occupied most of snapping turtles imported to South Korea, which were from the central United States where the climatic conditions are similar to that of South Korea. *C. serpentina* from the central United States are able to settle naturally in South Korea, and are likely to reproduce in large quantities in an environment rich in food (Ernst and Barbour, 1989). Thereby, these are required special tracking and management, which leads great implications for conservation of freshwater ecosystem in South Korea. We expect that the present results may provide the cornerstone for establishing the contemporary migration network of *C. serpentina* and may be used for preparing an effective management plan for such exotic reptilian species.

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## CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

## ACKNOWLEDGMENTS

We thank to Dr. Curtis J. Schmidt, Fort Hays State University for providing the tissue samples of *C. serpentina* by the Kansas Herpetological Society, United States. This study was supported by the grant No. NIBR201905202 to UWH funded by the National Institute of Biological Resources (NIBR), Ministry of Environment, South Korea.

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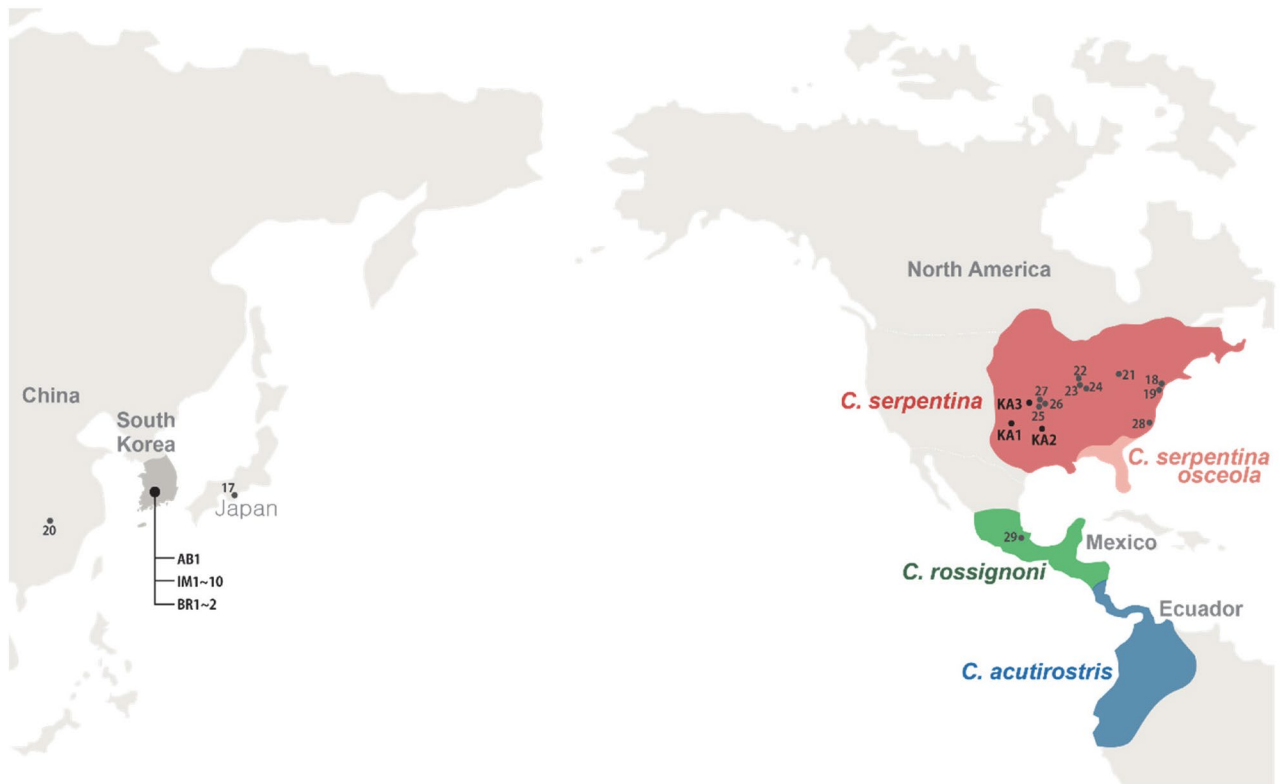
Received June 17, 2020  
Revised October 7, 2020  
Accepted October 7, 2020

**Appendix 1.** Sample numbers, sampling localities, and GenBank accession numbers of the *COI* sequences from 28 *Chelydra serpentina* and one *C. rossignoni* samples used in this study

No.	Sample No.	Accession No.	Locality	Reference
1	AB1	MT181181	South Korea (Gongju, Chungnam)	Present study
2	BR1	MT181182	South Korea	Present study
3	BR2	MT181183	South Korea	Present study
4	IM1	MT181176	South Korea	Present study
5	IM2	MT181177	South Korea	Present study
6	IM3	MT181178	South Korea	Present study
7	IM4	MT181179	South Korea	Present study
8	IM5	MT181180	South Korea	Present study
9	IM6	MT181184	South Korea	Present study
10	IM7	MT181185	South Korea	Present study
11	IM8	MT181186	South Korea	Present study
12	IM9	MT181187	South Korea	Present study
13	IM10	MT181188	South Korea	Present study
14	KA1	MT181189	Harper, Kansas, USA	Present study
15	KA2	MT181190	Neosho, Kansas, USA	Present study
16	KA3	MT181191	Shawnee, Kansas, USA	Present study
17	NCBI data	NC_011198	Wuhu, China (pet shop)	Nie and Yan (unpublished)
18	NCBI data	KJ870096.1	Cornwall, New York, USA	Drzewicki (unpublished)
19	NCBI data	KJ870097.1	Cornwall, New York, USA	Drzewicki (2014)
20	NCBI data	LC145070.1	Higashiyama Zoo, Aichi, Japan	Suzuki-Matsubara and Moriyama (unpublished)
21	NCBI data	KU985607.1	Ontario, Canada	Chambers and Hebert (2016)
22	NCBI data	KU985648.1	Chicago, Illinois, USA	Chambers and Hebert (2016)
23	NCBI data	KU985806.1	Chicago, Illinois, USA	Chambers and Hebert (2016)
24	NCBI data	KU985719.1	Gary, Indiana, USA	Chambers and Hebert (2016)
25	NCBI data	KU985770.1	Lawrence, Kansas, USA	Chambers and Hebert (2016)
26	NCBI data	KU985815.1	Lawrence, Kansas, USA	Chambers and Hebert (2016)
27	NCBI data	KU986272.1	Lawrence, Kansas, USA	Chambers and Hebert (2016)
28	NCBI data	MH273639.1	Hyde, North Carolina, USA	Mulcahy (unpublished)
29	NCBI data	HQ329638.1	<i>C. rossignoni</i>	Reid et al. (2011)

AB, Abandoned into South Korean ecosystems; BR, Breeding in South Korea after imported; IM, Imported to South Korea; KA, Direct collection from Kansas State, USA.





**Appendix 2.** The 29 collection sites of *Chelydra serpentina* and *C. rossignoni* used in this study, and the geographic distribution of the three *Chelydra* species. The collection sites mark with dots, the details of which are explained in Appendix 1. As depicted with different colors on the map, *C. serpentina* is distributed mainly in North America (red), a subspecies *C. serpentina osceola* in Florida (light red), *C. rossignoni* in Central America (green), and *C. acutirostris* in South America (blue). AB, Abandoned into South Korean freshwater ecosystems; BR, Breeding in South Korea; IM, Imported into South Korea; KA, Direct collection from Kansas State, USA.