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REVIEW

Phylogenetic relationships between three Korean pit viper *Gloydius* (Serpentes: Crotalinae) species using mitochondrial DNA genes

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Abstract

Background Molecular phylogenetic studies of the Asian pit viper genus *Gloydius* have been widely published in Asia, but Korea population have not been conducted till date.

Objective This study aimed to analyze the phylogenetic relationships of three *Gloydius* species (*G. saxatilis, G. brevicaudus,* and *G. ussuriensis*) from Korea with other *Gloydius* species, based on Cytochrome *b* and ND4.

Methods We compared 160 samples representing the three species with those of 17 reference species and their phylogenetic status and genetic diversity were analyzed with concatenated sequences of two mitochondrial DNA.

Results Korean *G. brevicaudus* and *G. saxatilis* showed high haplotype diversity and relatively low and moderate nucleotide diversity, respectively. Although *G. ussuriensis* showed high genetic diversity, it was low in the Baengnyeong Island population. The phylogenetic tree represented two major lineages. One major lineage comprised *G. ussuriensis, G. tsushimaensis, G. blomhoffii*, and *G. brevicaudus*. The Chinese *G. ussuriensis* belonged to the same clade as the Korean *G. ussuriensis* and was closely related to the Baengnyeong Island population. Moreover, *G. tsushimaensis* was closely related to *G. ussuriensis* from southwestern Korean and Jeju Island populations. The other major lineage comprised the remaining 12 species and *G. saxatilis.* Korean *G. saxatilis* was closely related to *G. saxatilis*, *G. shedanoensis*, and *G. intermedius* from China.

Conclusion The phylogenetic status of the Korean *Gloydius* species in comparison with the other *Gloydius* species was identified. We suggesting the conservation management unit for the Baengnyeong Island population, while the current conservation status of Korean *G. saxatilis* is suggested to be revised to a higher level.

Keywords *Gloydius* · Viperidae · Viper · Mitochondrial DNA · Phylogeny

Introduction

The genus *Gloydius* is a venomous pit viper group of snakes, endemic to Asia (Russia, China, Nepal, and the Korean Peninsula) and the three species, *Gloydius saxatilis*, *Gloydius brevicaudus*, and *Gloydius ussuriensis* are widely distributed in Korea. These three species are divided into two groups based on their morphological characteristics (Guo and Zhang 2002): the *brevicaudus* group, comprising *G. brevicaudus* and *G. ussuriensis* generally having 21 rows of dorsal scales and four palatine teeth; and the *intermedius* group with *G. saxatilis* having 23 rows of dorsal scales and three palatine teeth (Gloyd and Conant 1982). These differences in morphological characteristics are also related to the ecological characteristics of the species. *G. brevicaudus* and *G. ussuriensis* prefer humid spaces such as valleys, rivers, and wetlands located at warm and low altitudes. In contrast, *G. saxatilis* prefers dry spaces, such as ridges located in high mountainous areas with relatively low temperatures (Do and Nam 2020; Do 2021).

Phylogenetic studies of the three species have been widely published in Asia and have raised new taxonomic concerns. A phylogenetic relationship study of six *Gloydius* species in China using mitochondrial DNA suggested that *G.*

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shedanoensis, a native species of Shedao Island, is closely related to *G. saxatilis* as a subspecies (Zhou et al. 2000). In addition, molecular phylogenetic studies of the genus *Gloydius* in China using mitochondrial DNA (mtDNA) (ND4 and Cytb) and nuclear DNA (nDNA) (c-mos) indicated that *G. saxatilis* is closely related to *G. shedanoensis* (Yan et al. 2012). Furthermore, a phylogeographic study revealed that *G. brevicaudus*, which inhabits China, was divided into three lineages, and *G. brevicaudus* (one individual) from Korea fell in the same lineage as that in northeastern China (Ding et al. 2011). A phylogenetic study of *Gloydius* using mtDNA (ND4 and Cyt*b*) and nDNA (c-mos) revealed that the Chinese *G. ussuriensis* was closely related to *G. blomhoffii,* which branched into *G. blomhoffii* and *G. ussuriensis* from a common ancestor with *G. brevicaudus* (Yan et al. 2012). A study on the genus *Gloydius* conducted in China using mitochondrial DNA (12SR, 16SR, Cytb, and ND4), showed that Chinese *G. ussuriensis* was most closely related to *G. tsushimaensis,* a Japanese endemic species inhabiting Tsushima Island, followed by *G. blomhoffii*. It was also found that *G. tsushimaensis* and *G. ussuriensis* originated from a common ancestor with *G. blomhoffii* (Shi et al. 2018; Wang et al. 2019).

In Korea, *G. ussuriensis* from Gangwon-do Province and Chungcheongnam-do Province was confirmed to be morphologically similar to *G. tsushimaensis* (Emelianov 1929; Isogawa et al. 1994). However, molecular genetic studies on three *Gloydius* species (*Gloydius saxatilis*, *Gloydius brevicaudus*, and *Gloydius ussuriensis*) inhabiting Korea have never been published, except for one study on the complete mitochondrial genome of *G. saxatilis* (Lee et al. 2021).

Therefore, this study was conducted to: (1) confirm the molecular phylogenetic status of three *Gloydius* species inhabiting South Korea by closely examining their phylogenetic relationship with *Gloydius* species inhabiting Northeast Asia based on the results of previous studies; (2) compare and analyze the genetic diversity of three *Gloydius* species from Korea, and (3) propose a conservation unit to establish conservation strategies for *Gloydius* after confirming the phylogenetically isolated populations.

Materials and methods

Sample collection and DNA analysis

A total of 160 snakes belonging to three species *G. saxatilis*, *G. brevicaudus*, and *G. ussuriensis* were obtained from South Korea in 2020 (Fig. 1, Table 1). All samples were collected from licensed regions (Gangwon-do Province, Gyeonggi-do Province, Incheon Metropolitan city, Chungcheongnam-do Province, Gyeongsangbuk-do Provinces, Gyeongsangnam-do Provinces, jeollabuk-do Provinces, jeollanam-do Provinces Baengnyeong Island, and Jeju Island). Samples were obtained by collecting tail tissue after direct capture or from snakes killed on road. All samples were

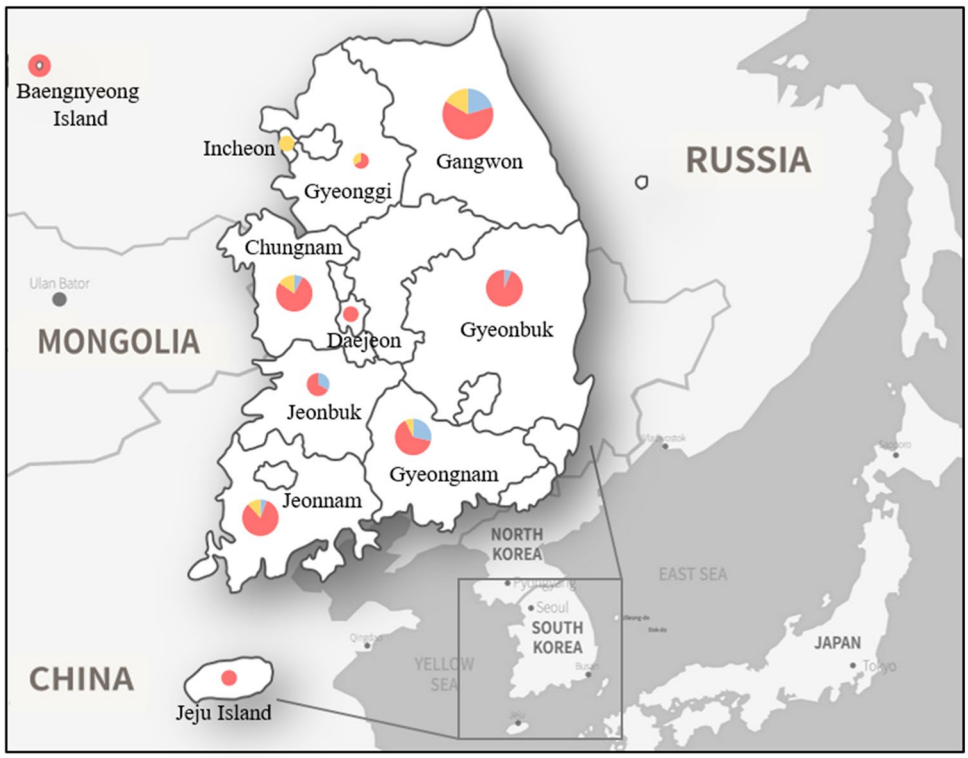

Fig. 1 Geographical sampling location of the three *Gloydius* species. The proportions of circle size and color in each circle reflect the number of samples in each region. The color in each circle indicates *G. saxatilis* (blue), *G. ussuriensis* (red), and *G. brevicaudus* (yellow) (color figure online)

Table 1 Sampling information from Korea and GenBank accession numbers for each species

Species	Locality	*N*	*H*	Haplotype	GenBank accession no	
					Cyt-*b*	ND4
G. saxatilis	Gangwon	11	7	Hap1, Hap4, Hap7-10, Hap12	MZ770876, MZ770879, MZ770882 ~ 770888, MZ770890, MZ770891	MZ771036, MZ771039, MZ771042 ~ 771048, MZ771050, MZ771051
	Gyeongnam	4	2	Hap3, Hap7	MZ770878, MZ770892 ~ 770894	MZ771038, MZ771052 ~ 771054
	Gyeonbuk	1	1	Hap5	MZ770880	MZ771040
	Chungnam	1	1	Hap11	MZ770889	MZ771049
	Jeonnam	1	1	Hap2	MZ770877	MZ771037
	Jeonbuk	1	1	Hap6	MZ770881	MZ771041
G. ussuriensis	Gangwon	34	20	Hap38, Hap51-69	MZ770966 ~ 770999	MZ771126 ~ 771159
	Baengnyeong	26	4	Hap14-17	MZ770896 ~ 770921	MZ771056 ~ 771081
	Gyeonggi	2	2	Hap23, Hap77	MZ771010, MZ771011	MZ771170, MZ771171
	Gyeongnam	9	4	Hap18, Hap40, Hap48, Hap49	MZ770949, MZ770957 ~ 770964	MZ771109, MZ771117 ~ 771124
	Gyeonbuk	15	13	Hap13, Hap29, Hap30-39, Hap50	MZ770895, MZ770936 ~ 770949, MZ770965	MZ771055, MZ771096 ~ 771109, MZ771125
	Chungnam	10	8	Hap21-28	MZ770922, MZ770927 ~ 770935	MZ77108, MZ771087 ~ 771095
	Daejeon	4	2	Hap19, Hap20	MZ770923 ~ 770926	MZ771083 ~ 771086
	Jeonnam	14	12	Hap41-47, Hap70, Hap74-76, Hap78	MZ770950 ~ 770956, MZ771005 ~ 771009, MZ771012, MZ771013	MZ771110 ~ 771116, MZ771165 ~ 771169, MZ771172, MZ771173
	Jeonbuk	2	1	Hap70	MZ771000, MZ771001	MZ771160, MZ771161
	Jeju	3	3	Hap71-73	MZ771002 ~ 771004	MZ771162 ~ 771164
G. brevicaudus	Gangwon	9	5	Hap79, Hap82-84, Hap87	MZ771018 ~ 771024, MZ771026, MZ771031	MZ771178 ~ 771184, MZ771186, MZ771191
	Incheon	7	5	Hap80, Hap81, Hap85, Hap86, Hap90	MZ771015, MZ771016, MZ771025, MZ771028 ~ 771030, MZ771035	MZ771175, MZ771176, MZ771185, MZ771188 ~ 771190, MZ771195
	Gyeonggi	1	1	Hap79	MZ771027	MZ771187
	Gyeongnam	1	1	Hap89	MZ771034	MZ771194
	Chungnam	2	1	Hap79	MZ771014, MZ771017	MZ771174, MZ771177
	Jeonnam	2	2	Hap79, Hap88	MZ771032, MZ771033	MZ771192, MZ771193

N sample size, *H* number of haplotype

frozen at − 70 °C in a deep freezer at the National Institute of Biological Resources (NIBR), Incheon, South Korea, until DNA extraction.

Total genomic DNA was extracted from the tissues using QIAamp® DNA Micro Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol and quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The cytochrome *b* gene (Cyt*b*, 936 bp) was amplified by polymerase chain reaction (PCR) using primers L14910 (5′–GAC CTG TGA TMT GAA AAC CAY CGT TGT–3′) and H16064 (5′–CTT TGG TTT ACA AGA ACA ATG CTT TA–3′) (Burbrink et al. 2000). The PCR reaction conditions were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 60 °C for 1 min (Cyt*b*)/58 °C for 1 min (ND4), and 72 °C for 1 min; and finally, 72 °C for 5 min. The 673 bp fragment of the mtDNA NADH dehydrogenase subunit 4 gene (ND4) was amplified using primers ND4 (5′–CAC CTA TGA CTA CCA AAA GCT CAT GTA GAA GC–3′) and Leu (5′–CAT TAC TTT TAC TTG GAT TTG CAC CA–3′) (Arévalo et al. 1994). The PCR reaction conditions were as follows: 95 °C for 5 min; 35 cycles of 95 °C for 1 min, 58 °C for 1 min, and 72 °C for 1 min; and finally 72 °C for 5 min. Amplification was carried out in 20 µl reaction volumes containing 20–50 ng/template DNA, 2X Bioneer PreMix (100 µM each dNTPs, 1.5 mM $MgCl_2$, 1 unit Taq polymerase), and 10 pmol of each primer. The PCR

products were purified by the Ethanol purification method (Genotech Corp, Korea). The purified PCR products were sequenced using an ABI Prism 3730XL Analyzer (Applied Biosystems, Foster City, CA, USA). The sequencing primers for both mtDNA regions were the same as those used for amplification.

Data analysis

All analyses were conducted with concatenated sequences of two mitochondrial DNA region combined sequences. This study used 1609 bp of combined sequences to analyze the diversity of the three *Gloydius* species. Analysis of genetic distance and phylogenetic tree utilized 1172 bp of combined sequences according to the length of the reference sequence. The Cyt*b* and ND4 sequences obtained from 160 individuals in this study were registered in GenBank (Table 1).

The species of each sequence obtained in this study were identified using BLAST searches (Altschul et al. 1997). Sequences were aligned using Geneious prime v11.0.4 (Kearse et al. 2012). Haplotype diversity (*h*), nucleotide diversity (π), and polymorphic sites (P) for each species were estimated using DNASP version 6 (Rozas et al. 2017). Pairwise genetic distances among species were calculated using MEGA X v10.1.8 (Kumar et al. 2018). To investigate the evolutionary relationships, phylogenetic trees were constructed using three methods: neighbor-joining (NJ) (Saitou and Nei 1987) using Kimura's two-parameter distances (Kimura 1980), maximum parsimony (MP), and maximum-likelihood (ML). The reference sequence data corresponded to 46 individuals of the *Gloydius* species obtained from GenBank (Table 2). *Deinagkistrodon acutus* and *Protobothrops mangshanensis* were used as out-groups for phylogenetic tree construction. NJ, MP, and ML trees were constructed using MEGA X v10.1.8 (Kumar et al. 2018). The MP tree was obtained using tree bisection–reconnection (TBR) branch swapping with 10,000 bootstrap replicates. The most appropriate models of sequence evolution for ML trees were selected using MEGA X v10.1.8 (Kumar et al. 2018). The best-fit model for the ML tree was the Tamura-Nei model (TN93) with gamma distribution (+G) and proportion of invariant sites (+I). The consensus ML trees were found using Nearest-Neighbor-Interchange (NNI) heuristic searches of 1000 bootstrap replicates.

Results

Mitochondrial DNA diversity and genetic distance

The diversity analysis results showed that *G. saxatilis* (19 individuals) and *G. brevicaudus* (22 individuals) had 12 haplotypes, while *G. ussuriensis* (119 individuals) had 66 haplotypes (Table 1). Unique regional haplotypes were observed among 160 individuals of the three *Gloydius* species, with the exception of five haplotypes which shared geographical locations: Hap7 (Gangwon and Gyeongnam), Hap23 (Gyeonggi and Chungnam), Hap38 (Gangwon and Gyeongbuk), Hap70 (Jeonnam and Jeonbuk), and Hap79 (Gyeonggi, Gangwon, Jeonnam, Gyeongnam).

In each species, *G. saxatilis* and *G. brevicaudus* showed high haplotype diversity ($h = 0.936, 0.900$) and relatively low ($\pi = 0.164$), and moderate nucleotide diversity ($\pi = 0.309$), respectively (Table 3). *G. ussuriensis* showed an overall high haplotype and nucleotide diversity. The regional analysis results of *G. ussuriensis* revealed that the genetic diversity of four localities with more than 14 individuals showed high haplotype diversity ($h = 0.981$–0.961) and moderate nucleotide diversity ($\pi = 0.589$–0.298). However, only the Baengnyeong Island population showed low genetic diversity ($h = 0.545$, $\pi = 0.069$). There were some other locations with low genetic diversity (i.e., Jeju, Jeonbuk, and Daejeon) but those could not be considered to be representative due to insufficient sample size.

The pairwise genetic distance analysis of species closely related with three *Gloydius* species showed that *G. saxatilis* in South Korea was genetically similar to *G. saxatilis* (0.013), *G. intermedius* (0.012), and *G. shedaoensis* (0.018) in China (Table 4). However, it was confirmed that the genetic distances between *G. saxatilis* and *G. intermedius* (0.000) and *G. shedaoensis* (0.010) in China were slightly lower than those in South Korea. The Korean *G. brevicaudus* was genetically closer to the Chinese *G. brevicaudus* (0.006), while the Korean *G. ussuriensis* was genetically similar to the Chinese *G. ussuriensis* (0.007). In addition, *G. tsushimaensis* from Tsushima Island, Japan, was slightly closer to *G. ussuriensis* from Korea (0.020) than that from China (0.033).

Phylogenetic analysis of mitochondrial haplotype

To examine the phylogenetic status of the three *Gloydius* species in Korea, phylogenetic trees were constructed, including closely related species. Phylogenetic trees using NJ, MP and ML generated similar patterns of the major branches, and therefore ML tree with three bootstrap values was representatively presented in this study. The phylogenetic trees (NJ, MP, and ML) represent a monophyletic *Gloydius* with two major lineages (Fig. 2). One major lineage (lineage A) consisted of *G. ussuriensis, G. tsushimaensis, G. blomhoffii*, and *G. brevicaudus*. The other major lineage (lineage B) comprised the remaining 12 species and *G. saxatilis.* These two lineages indicated a genetic distance of approximately 0.02.

Lineage A was largely divided into three clades: *G. ussuriensis* from Korea and China and *G. tsushimaensis*

Table 2 Reference sequence information for data analysis from Genbank

Species	Locality	Code	Genbank accession no.		References
			Cyt *b*	ND4	
G. saxatilis	Liaonling, China	G.sax1	JQ687489	JQ687470	Yan et al. (2012)
	Jilin, China	G.sax2	JQ687502	JQ687483	Yan et al. (2012)
G. brevicaudus	Jingzhou, Hubei, China	G.bre1	HQ528467	HQ528346	Ding et al. (2011)
	Jingzhou, Hubei, China	G.bre2	HQ528468	HQ528347	Ding et al. (2011)
	Ningbo, Zhejiang, China	G.bre3	HQ528519	HQ528397	Ding et al. (2011)
	Ningbo, Zhejiang, China	G.bre4	HQ528520	HQ528398	Ding et al. (2011)
	Huanren, Liaoning, China	G.bre5	HQ528442	HQ528311	Ding et al. (2011)
	Huanren, Liaoning, China	G.bre6	HQ528443	HQ528312	Ding et al. (2011)
	Yangju, South Korea	G.bre7	HQ528446	HQ528418	Ding et al. (2011)
G. ussuriensis	Heilongjiang, China	G.uss1	KP262412	KP262412	Yan et al. (2012)
G. intermedius	Zhuanghe, Liaoning, China	G.int1	KY040617	KY040638	Shi et al. (2017)
	Wafangdian, Liaoning, China	G.int2	KX063820	KX063793	Shi et al. (2017)
G. halys	Lingyuan, Liaoning, China	G.hal1	KX063802	KX063775	Shi et al. (2017)
	Xilinhot, Inner Mongolia	G.hal2	KX063803	KX063776	Shi et al. (2017)
	Heilongjiang, China	G.hal3	KY040618	KY040639	Shi et al. (2017)
G. cognatus	Zoige, Sichuan, China	G.cog1	KY040619	KY040640	Shi et al. (2017)
	Sonit Right Banner, Inner Mongolia	G.cog2	KY040621	KY040642	Shi et al. (2017)
	Yinchuan, Ningxia, China	G.cog3	KY040622	KY040643	Shi et al. (2017)
	Wuzhong, Ningxia, China	G.cog4	KX063809	KX063782	Shi et al. (2017)
G. qinlingensis	Xunyangba, Shanxi, China	G.qin1	KY040623	KY040644	Shi et al. (2017)
	Taibai, Shaanxi, China	G.qin2	KF997922	KF997981	Shi et al. (2017)
	Zhouzhi, Shaanxi, China	G.qin3	JQ687490	JQ687471	Yan et al. (2012)
G. liupanensis	Ningxia, China	G.liu1	JQ687491	JQ687472	Yan et al. (2012)
	Ningxia, China	G.liu2	JQ687492	JQ687473	Yan et al. (2012)
	Ningxia, China	G.liu3	JQ687493	JQ687474	Yan et al. (2012)
G. stejnegeri	Tongchuan, Shaanxi, China	G.ste1	KX063817	KX063790	Shi et al. (2017)
	Linfen, Shanxi, China	G.ste2	KX063818	KX063791	Shi et al. (2017)
	Mentougou, Beijing, China	G.ste3	KY040625	KY040646	Shi et al. (2017)
G. strauchi	Kangting, Sichuan, China	G.str1	KY040629	KY040650	Shi et al. (2017)
	Litang, Sichuan, China	G.str2	KY040630	KY040651	Shi et al. (2017)
G. rubromaculatus	Yushu, Qinghai, China	G.rub1	KY040632	KY040653	Shi et al. (2017)
	Yushu, Qinghai, China	G.rub2	KY040633	KY040654	Shi et al. (2017)
G. changdaoensis	Lianyungang, Jiangsu, China	G.cha1	KX063821	KX063794	Shi et al. (2017)
	Changdao, Shandong, China	G.cha2	KX063823	KX063796	Shi et al. (2017)
G. shedaoensis	Lvshun, Liaoning, China	G.she1	KX063819	KX063792	Shi et al. (2017)
	Liaoning, China	G.she2	JQ687498	JQ687479	Yan et al. (2012)
	Liaoning, China	G.she3	JQ687499	JQ687480	Yan et al. (2012)
	Liaoning, China	G.she4	JQ687500	JQ687481	Yan et al. (2012)
G. monticola	Dali, Yunnan, China	G.mon1	KY040635	MG025935	Shi et al. (2017)
	Dali, Yunnan, China	G.mon2	KY040636	MG025936	Shi et al. (2017)
G. blomhoffii	Japan	G.blo1	AY352751	AY352814	Malhotra and Thorpe (2004)
G. tsushimaensis	Japan	G.tsu1	JN870203	JN870211	Fenwick et al. (2012)
G. caraganus	–	G.car1	MF490455	MF490453	Shi et al. (2017)
	–	G.car2	MF490456	MF490454	Shi et al. (2017)
D. acutus	Fujian, China	out1	DQ343647	DQ343647	Yan et al. (2008)
P. mangshanensis	Hunan, China	out2	HM567537	HM567469	Guo et al. (2011)

The code for each reference was used in the phylogenetic tree

Table 3 Genetic diversity estimates of three *Gloydius* species using mitochondrial DNA, cytochrome *b* and ND4, combined sequence (1609 bp)

Species	*N*	Combined sequence		
		ND4 + Cyt-*b* (1609 bp)		
		H	*h*	π (%)
G. saxatilis	19	12	0.936	0.164
G. brevicaudus	22	12	0.900	0.309
G. ussuriensis	119	66	0.970	1.660
Gangwon	34	20	0.961	0.589
Baengnyeong Island	26	4	0.545	0.069
Gyeonggi	2	2	1.000	0.311
Gyeongnam	9	4	0.417	0.235
Gyeonbuk	15	13	0.981	0.381
Chungnam	10	8	0.978	1.670
Daejeon	4	2	0.500	0.062
Jeonnam	14	12	0.978	0.298
Jeonbuk	2	1	0.000	0.000
Jeju Island	3	3	1.000	0.166

The genetic diversity of *G. ussuriensis* was estimated based on sample location

N sample size, *H* number of haplotypes, *h* haplotype diversity, π nucleotide diversity

(clade 1), *G. blomhoffii* from Japan (clade 2), and *G. brevicaudus* from Korea and China (clade 3). In clade 1, the Korean *G. usuriensis* was separated into two groups, Korea main group and southwestern (Jeonnam, Jeonbuk, Jeju) Korea group. The Chinese *G. ussuriensis* belonged to the Korean *G. ussuriensis* main group and was most closely related to the Baengnyeong Island population in particular. Moreover, *G. tsushimaensis* from Tsushima Island was closely related to *G. ussuriensis* from the southwestern Korea group, especially the Jeju Island population. *G. bloomhoffii* (clade 2) diverged from a common ancestor of *G. usuriensis*. The Chinese *G. brevicaudus* (clade 3) was divided into three groups according to geographical location, and the Korean *G. brevicaudus* included the Northeastern China group (G.bre5-7). The phylogenetic tree also showed that *G. usuriensis* has a common ancestor with *G. brevicaudus*, and gradually branches into *G. bloomhoffii, G. tsushimaensis*, southwestern Korea *G. usuriensis*, and Korean and Chinese *G. usuriensis*.

In lineage B, most species formed species-specific clades, except *G. saxatilis*, *G. shedanoensis*, and *G. intermedius* from China. Despite being different species, these three represented a close genetic relationship. In addition, although Korean *G. saxatilis* was closely related to these three Chinese species, it was clearly divided into other clades.

Discussion

Phylogenetic status of the three Gloydius species

Molecular genetic studies on *Gloydius* species in Korea have not been published yet. Until recently, the phylogenetic study of *Gloydius* published in China was the only reference. Therefore, we established the molecular phylogenetic status of three *Gloydius* species inhabiting Korea by closely examining the phylogenetic relationship based on the results of previous studies.

Our results were in agreement with the results of previous studies by Zhou et al. (2000) and Yan et al. (2012), which suggested that *G. saxatilis* was closely related to *G. shedaoensis* from Shedao Island as a subspecies. However, the Korean *G. saxatilis* was differentiated from *G. shedaoensis* and *G. saxatilis* in China. In addition, the Korean *G. saxatilis* originated from a common ancestor with the Chinese *G. saxatilis*, but has the potential to diverge into native species due to its geographical and genetic status.

In a previous study by Ding et al. (2011), *G. brevicaudus* inhabiting China was divided into three lineages, and *G. brevicaudus* from Korea was included in the northeastern China lineage. Our study presented the same results as Ding et al. (2011) and supported previous results on *G. brevicaudus* from Korea. The Chinese *G. brevicaudus* was divided into three groups according to geographical location, while the Korean *G. brevicaudus* included the northeastern China group.

According to previous reports from China, *G. usuriensis* is genetically closely related to *G. bloomhoffii* and *G. tsushimaensis* from Japan (Yan et al. 2012; Shi et al. 2018; Wang et al. 2019). These studies also revealed that the species differentiated gradually into *G. blomhoffii, G. tsushimaensis*, and *G. usuriensis* from a common ancestor with *G. brevicaudus*. However, our study showed slightly different results from previous studies, wherein the Chinese *G. ussuriensis* was closely related to *G. tsushimaensis* and *G. blomhoffii*. The Chinese *G. ussuriensis* belongs to the Korean *G. ussuriensis* and is particularly most closely related to the Baengnyeong Island population. In addition, *G. tsushimaensis* was closely related to *G. usuriensis* from southwestern Korea and Jeju Island. The Jeju Island population has a distinct phylogenetic group and is closely related to *G. tsushimaensis* from Tsushima Island, Japan. It is predicted that the current phylogenetic status can be attributed to the common ancestor of *G. usuriensis*, which existed in Jeju and Tsushima Island in the past, due to geographical isolation and low gene flow. Unusual genetic differentiation on the island has been reported not only in *Gloydius* but also in many other animals (Aquadro and

Table 4 Pairwise genetic distance among *Gloydius* species using mitochondrial DNA, Cytochrome *b* and ND4 combined sequence (1172 bp)

	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	*G. saxatilis* (Korea)	–																			
2	*G. brevicaudus* (Korea)	0.124	–																		
3	*G. ussuriensis* (Korea)	0.099	0.090	–																	
4	*G. saxatilis*	0.013	0.121	0.096	–																
5	*G. brevicaudus*	0.110	0.006	0.073	0.108	–															
6	*G. ussuriensis*	0.108	0.100	0.007	0.105	0.083	–														
7	*G. blomhoffi*	0.107	0.090	0.041	0.104	0.076	0.050	–													
8	*G. caraganus*	0.038	0.124	0.096	0.037	0.109	0.104	0.103	–												
9	*G. cognatus*	0.032	0.111	0.092	0.028	0.098	0.101	0.098	0.039	–											
10	*G. halys*	0.034	0.119	0.106	0.031	0.104	0.115	0.107	0.044	0.029	–										
11	*G. liupanensis*	0.095	0.107	0.102	0.092	0.094	0.115	0.103	0.090	0.086	0.088	–									
12	*G. stejnegeri*	0.028	0.119	0.099	0.027	0.104	0.107	0.103	0.040	0.024	0.028	0.087	–								
13	*G. rubromaculatus*	0.098	0.113	0.096	0.096	0.099	0.104	0.101	0.097	0.088	0.089	0.083	0.092	–							
14	*G. changdaoensis*	0.044	0.120	0.097	0.043	0.105	0.105	0.101	0.044	0.036	0.043	0.093	0.044	0.091	–						
15	*G. shedaoensis*	0.018	0.124	0.100	0.010	0.112	0.109	0.109	0.042	0.032	0.035	0.099	0.033	0.100	0.042	–					
16	*G. intermedius*	0.012	0.117	0.093	0.000	0.104	0.102	0.100	0.034	0.026	0.029	0.089	0.026	0.092	0.039	0.008	–				
17	*G. qinlingensis*	0.096	0.105	0.093	0.090	0.089	0.104	0.092	0.092	0.083	0.088	0.069	0.086	0.074	0.087	0.096	0.087	–			
18	*G. strauchi*	0.096	0.108	0.095	0.093	0.093	0.106	0.091	0.094	0.084	0.088	0.070	0.088	0.078	0.085	0.097	0.090	0.065	–		
19	*G. tsushimaensis*	0.105	0.094	0.020	0.102	0.077	0.033	0.044	0.103	0.099	0.110	0.107	0.104	0.105	0.099	0.105	0.099	0.099	0.100	–	
20	*G. monticola*	0.107	0.116	0.102	0.104	0.102	0.113	0.103	0.103	0.095	0.106	0.083	0.100	0.073	0.095	0.110	0.101	0.073	0.078	0.105	

The lower pairwise genetic distance values are underlined (< 0.015)

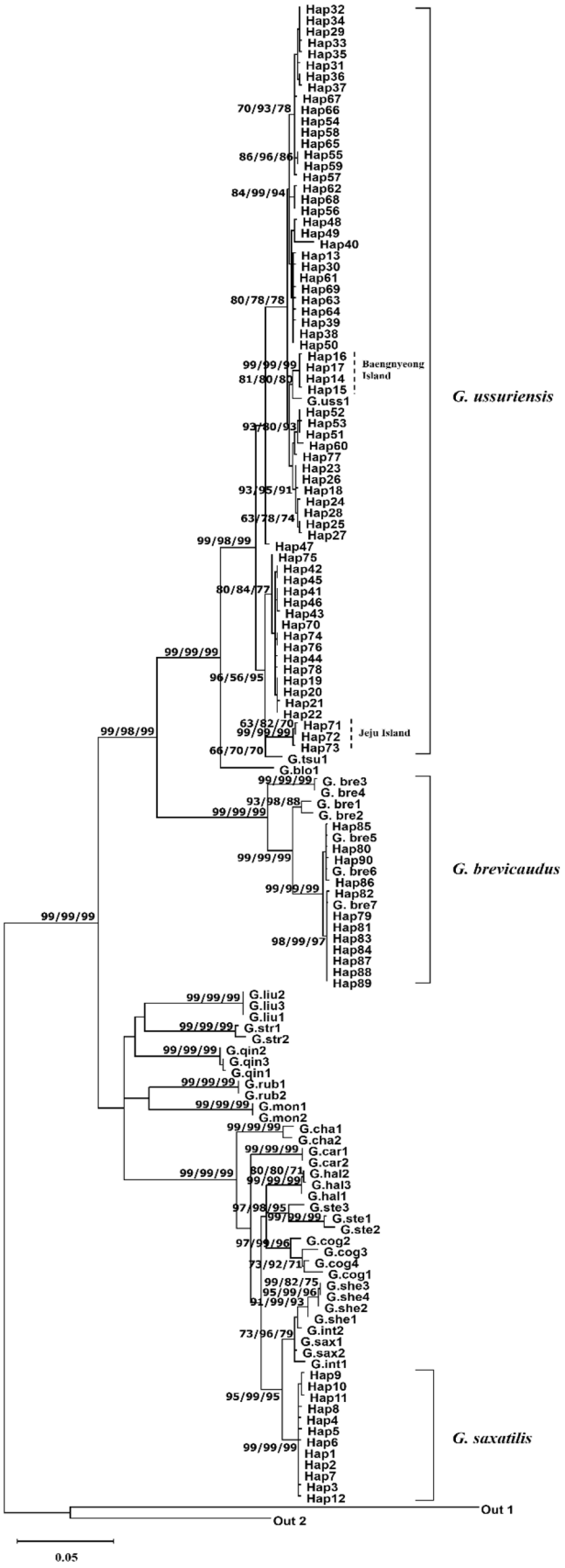

Fig. 2 Phylogenetic haplotype trees [neighbor-joining (NJ), maximum parsimony (MP), and maximum-likelihood (ML)] of *Gloydius* based on mitochondrial DNA (mtDNA) ND4 and cytochrome *b* combined sequences (1172 bp). See Table 1 for composition of haplotype. See Table 2 for the abbreviations of scientific names (code)

Kilpatrick 1981; Barton 1996; Berry 1996; Lee et al. 2015; Funk et al. 2016) for further research, phylogeographic analysis of Jeju Island and Tsushima Island needs to be conducted with sufficient populations.

Conservation unit and strategy for the Gloydius species

Gloydius saxatilis is distributed throughout the inland and several islands of South Korea, except Jeju Island, and is concentrated around the Taebaek Mountains. (Do et al. 2016). It has been reported that the main habitat of this species is forested areas at high elevations (above 400 m) (Do et al. 2017). *G. saxatilis* is distributed widely, but is adversely affected by poaching mainly in Korea and has been registered as an endangered species in the past (Bae 2019). Our genetic research showed that the Korean *G. saxatilis* had low nucleotide diversity and high haplotype diversity. This indicates that most haplotypes have one or two different nucleotides, and also suggests that the Korean *G. saxatilis* population expanded from a small effective population size (Grant and Bowen 1998). The number of *G. saxatilis* is smaller than that of other viper species in Korea, and it is highly likely that the population will decrease sharply due to habitat loss (Bae 2019). Moreover, *G. saxatilis* is a serious ecological threat because it is the most vulnerable to climate change among the three *Gloydius* species and has the highest possibility of extinction (Do et al. 2021). Our results indicate that, although *G. saxatilis* in Korea and China were derived from the same ancestor, the Korean *G. saxatilis* has the potential to diverge into a native species. Therefore, it is necessary to redesignate *G. saxatilis* from the current least concern (LC) category to a higher level due to its low genetic diversity and ecological vulnerability.

Gloydius brevicaudus is distributed throughout the inland regions of Korea, except for Jeju Island, and it also heavily inhabits the Taebaek Mountains (Do et al. 2016). It is known to inhabit forests, rivers, and paddy wetlands at a lower elevation ($\leq$ 500 m) than the highlands. In addition, *G. brevicaudus* is exposed to the risk of population decline due to poaching, similar to other viper species in Korea (Bae 2019). The results of this study revealed that the genetic diversity of *G. brevicaudus* was moderate; therefore, we propose periodic monitoring of poaching, habitat loss, and genetic status.

Gloydius ussuriensis is distributed throughoutthe islands and mainland in Korea, including Jeju Island, and

is concentrated around the Taebaek Mountains (Do et al. 2016). Unlike other vipers inhabiting Korea, they live in various environments, such as forests, paddy wetlands, and rivers at altitudes between 0 and 1300 m, preferring valleys in forests (Do and Yoo 2014). *G. ussuriensis* is designated as a prohibited species rather than an endangered species because it has the largest population among the three *Gloydius* species in Korea. Nevertheless, *G. ussuriensis* is also exposed to the risk of population decline due to poaching, similar to other viper species (Bae 2019). The results of this study showed that *G. ussuriensis* from the inland, which had a large population size, had moderate genetic diversity. Therefore, it appears that the northern part (lineage of the Chinese *G. ussuriensis*) and the southwestern part (lineage closely related to *G. tsushimaensis*) of the Korean Peninsula require continuous monitoring. However, *G. ussuriensis* from the Baengnyeong Island currently has low genetic diversity and is very vulnerable to population decline due to the characteristics of the island. In addition, *G. usuriensis* of the Baengnyeong Island has unique morphological characteristics (longer tail and more abdominal scales) unlike other localities (An 2020). Consequently, this study proposes the designation of the Baengnyeong Island population as a conservation management unit. Similar to the Baengnyeong Island population, *G. usuriensis* from Jeju Island has a distinct phylogenetic group and is closely related to *G. tsushimaensis* from Tsushima Island, Japan. Therefore, it is critical to analyze genetic diversity using sufficient population and establish a conservation strategies for the Jeju Island population based on these results.

In conclusion, this study accurately identified the phylogenetic status of the Korean *Gloydius* species in comparison with the closely related *Gloydius* species. Also this study suggested conservation strategies and further management strategies for genetically vulnerable *Gloydius* species and its isolated population.

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Declarations

Conflict of interest The authors report that they have no conflict of interest.

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