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Verification of the breeding periods of two clawed salamander species (*Onychodactylus koreanus* and *O. sillanus*) using environmental DNA analysis and field surveys

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Abstract

Determining the breeding frequencies and breeding periods of species that spend part or all of their lives underground is challenging because of limited population surveys. Environmental DNA (eDNA) analysis can be performed to address this issue. eDNA analysis and population surveys were combined to verify and compare the breeding frequencies and breeding periods of *Onychodactylus koreanus* and *O. sillanus* in mountain valley streams, which are their typical habitats in the Republic of Korea, between April 2024 and May 2025. Both species externally fertilize their eggs. Adult *O. koreanus* appeared at the sites during two periods, from April to June and from October to early December, whereas adult *O. sillanus* was not detected despite similar survey efforts. eDNA concentrations detected in the upper, middle, and lower streams differed; however, the major eDNA detection patterns were largely consistent. In *O. koreanus*, two major eDNA detections occurred in early May and early December, whereas in *O. sillanus*, eDNA detections occurred between January and June. These results suggest that the two species have different breeding frequencies and breeding periods. However, whether these differences are species- or habitat-specific needs to be further explored.

Key Words

External fertilization, Hynobiidae, mountain stream, subterranean

Introduction

Because of climate change and human disturbances, amphibians are facing a high risk of extinction. Approximately 41% of amphibian species are threatened worldwide (Stuart et al. 2008; Luedtke et al. 2023; Borzée et al. 2025). The order Caudata, which includes salamanders and newts, is a group whose members have low mobility and limited habitats, rendering them particularly prone to population decline (Cushman 2006; Guirguis et al. 2023; Wang et al. 2026). Understanding the life history and seasonal activities of these organisms is essential to effectively conserve them. In particular, an understanding of the frequency and duration of breeding, which are key aspects of population dynamics, is necessary (Nanglu et al. 2023). However, determining the breeding frequencies and breeding periods of species that spend part or all of their lives underground is challenging because of limited population surveys (Kuzmin 1995; Gorički et al. 2017; West et al. 2020). This lack of information leads to dif-

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ficulties in applying various conservation measures, such as establishing protection periods and habitat conservation areas, as well as investigating the impacts of environmental changes. Environmental DNA (eDNA) analysis, which has been widely applied in recent years in various ecological studies, can be performed to overcome these limitations associated with underground species (West et al. 2020; van der Heyde et al. 2023; Park et al. 2026a, 2026b).

eDNA refers to the DNA of organisms that has been released into the environment through skin cells, mucus, and feces, among other sources (Beng and Corlett 2020). Recently, eDNA analyses have been used to track the presence of cryptic and invasive species and to investigate the patterns of their habitat use (van der Heyde et al. 2023; Ongkulee et al. 2025; Park et al. 2026a, 2026b). In fish species such as *Ctenopharyngodon idella*, *Micropterus salmoides*, and *Petromyzon marinus*, eDNA detection was positively correlated with the number of individuals at the site (Bracken et al. 2019; Hayer et al. 2020; Wu et al. 2023). Additionally, eDNA analyses are currently applied to investigate the spatiotemporal activity of populations (Troth et al. 2021). In urodeles, such as *Onychodactylus koreanus*, *Triturus cristatus*, and *Hynobius kimurae*, seasonal eDNA fluctuations are related to changes in the number of individuals and their seasonal activities (Buxton et al. 2017; Jo et al. 2020; Park et al. 2026a). However, additional reliable information about the reproductive ecology of a species may be obtained by combining eDNA analyses with traditional population surveys (Moss et al. 2022; Svenningsen et al. 2022).

The genus *Onychodactylus* inhabits Northeast Asia, and its reproductive ecology is not fully understood because of its underground breeding characteristics (Kuzmin 1995; Maslova and Rogashevskaya 2023). The genus consists of 12 species: one species (*O. fischeri*) in the Russian Federation; two species (*O. zhaoermii* and *O. zhangyapingi*) in the People's Republic of China (hereafter referred to as China), the Democratic People's Republic of Korea, or both; two species (*O. koreanus* and *O. sillanus*) in the Republic of Korea; and seven species (*O. fuscus*, *O. intermedius*, *O. japonicus*, *O. kinneburi*, *O. nipponoborealis*, *O. pyrrhonotus*, and *O. tsukubaensis*) in Japan (Poyarkov Jr et al. 2012; Yoshikawa and Matsui 2013, 2014; Yoshikawa et al. 2013; Borzée et al. 2022). Due to their degenerated lungs, these species mainly inhabit streams in high-altitude mountain valleys and surrounding deciduous broadleaf forests, where the temperature is low and dissolved oxygen is abundant (Kuzmin 1995). Larvae spend 2–3 years in the valley stream before metamorphosing and can be observed throughout the year in the stream (Hayase and Yamane 1982; Lee et al. 2008). Adults are observed only sporadically and are detected mainly during the breeding season. Since their reproduction, which involves external fertilization, mainly occurs in underground habitats, such as in caves, in subterranean streams, or beneath the streambed where spring water emerges (Akita 1982; Park 2005; Yoshikawa et al. 2013; Maslova and Rogashevskaya 2023), the timing and frequency of breeding periods remain unclear across species and habitats. During mating, females lay egg sacs, and males grasp the sacs and release sperm onto them (Park 2005). Two breeding events per year, in spring and late autumn, have been suggested based on observations of adult and larval *O. japonicus* and *O. fischeri* in valley streams (Akita and Miyazaki 1991; Griffin and Solkin 1995). Conversely, Serbinova and Solkin (1992) suggested that breeding may occur once per year because of the low temperatures in late autumn. Recently, Park et al. (2026a) combined eDNA analysis and population surveys to investigate an *Onychodactylus koreanus* population in a cave over the course of one year in the Republic of Korea, revealing that these organisms breed twice, from April to June and from November to December. The study was conducted in a special population, where laid eggs can be observed and the population can be monitored throughout the year. However, whether the same breeding pattern occurs in mountain valleys, which are more frequently inhabited by *Onychodactylus* species, is unclear. Moreover, whether differences exist among the different species remains unknown.

Two *Onychodactylus* species (*O. koreanus* and *O. sillanus*) inhabit the Korean Peninsula (Poyarkov Jr et al. 2012; Borzée et al. 2022). *Onychodactylus koreanus* is observed in most areas of the Korean Peninsula, whereas *O. sillanus*, which was newly reported in 2022, is distributed only in the southeastern region of the Korean Peninsula (Borzée et al. 2022). The distribution range of *O. sillanus* is restricted by natural barriers such as the Nakdong River and the Geumho River (Kim et al. 2026). In this study, eDNA analysis and population surveys were combined to verify and compare the breeding frequencies and breeding periods of *O. koreanus* and *O. sillanus* in mountain valley streams, which represent their typical habitats.

Materials and methods

Field survey

This study was conducted in two mountain valley streams: Mount (Mt.) Daeryong for *O. koreanus* (37°55.28'N, 127°49.58'E; 410 m asl) in Chuncheon, Gangwon Province (Fig. 1A), and Mt. Biseul for *O. sillanus* (35°42.80'N, 128°30.36'E; 400 m asl) in Dalseong, Daegu Metropolitan City (Fig. 1B), Republic of Korea. Both study sites are located in the upper regions of the mountain valleys. Three eDNA sampling sites were established along the upper, middle, and lower sections of each valley. On Mt. Daeryong, the sites were 50 and 120 m apart (Fig. 1A), whereas on Mt. Biseul, they were 80 and 100 m apart (Fig. 1B). Based on the results of preliminary surveys, sites where eDNA sampling could be conducted year-round and where water flow rates were slow, thus possibly allowing for a longer duration of eDNA sampling, were selected. eDNA sampling was conducted biweekly

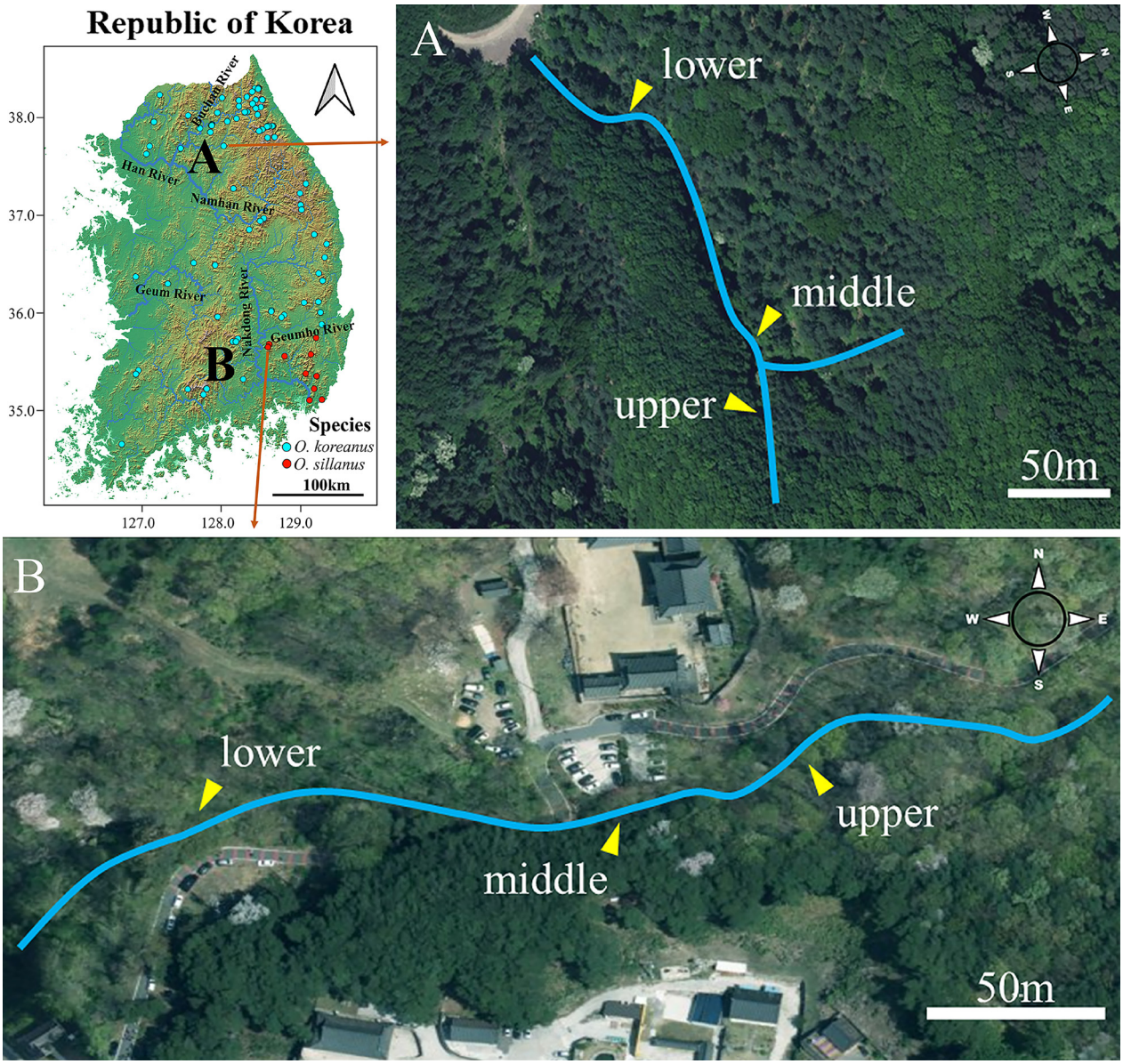

Figure 1. Study sites on Mount (Mt.) Daeryong. **A.** In Chuncheon, Kangwon State, and Mt. Biseul; **B.** In Dalseong, Daegu Metropolitan City, Republic of Korea, with three biweekly eDNA sampling sites (upper, middle, and lower; indicated by arrowheads) on each mountain. Population surveys were conducted along an approximately 40 m stretch of stream between the middle and lower sites on Mt. Daeryong and an approximately 30 m stretch of stream above the upper sampling site on Mt. Biseul between April 2024 and May 2025. Major rivers, such as the Nakdong, Geumho, Geum, Namhan, Buchan, and Han Rivers, are presented on the map of the Republic of Korea. The blue and red dots indicate populations of *Onychodactylus koreanus* and *O. sillanus*, respectively, which were confirmed by the authors between 2023 and 2025. The satellite images were acquired from the Spatial Information Open Platform, Vworld (MOLIT, Republic of Korea; https://map.vworld.kr/map/ws3dmap.do).

within three hours for each stream during the day. Thirty sampling events were conducted on Mt. Daeryong between 8 April 2024 and 25 May 2025, and 27 sampling events were conducted on Mt. Biseul between 24 May 2024 and 24 May 2025.

After eDNA sampling, two researchers conducted population surveys for approximately 30 min. Considering the physical structure of the valley stream on Mt. Daeryong, a 40 m stretch of stream between the middle and lower eDNA sampling sites was surveyed, except from 17 December 2024 to 27 February 2025, because of ice formation. Tree debris, stones, and boulders were searched for individual organisms. After the survey, these structures were repositioned. The collected larvae and adults were maintained separately until the survey was completed. In accordance with the methods reported by Park et al. (2026a, 2026b), the larvae were classified as 1 year old if their total length was less than 5 cm and as 2 years old or older if their total length was greater than 5 cm. If the total length of metamorphosed individuals was less than 10 cm, they were classified as subadults, which may not have participated in breeding events. If the

total length was more than 10 cm, the individuals were considered full-grown adults. The sex of the adults was determined based on cloacal morphology (Kuzmin 1995). Females were further classified as ovulated females when ovulated eggs were visible inside the abdomen; otherwise, the females were classified as oviposited females (Park et al. 2026a, 2026b). In externally fertilizing salamanders, only breeding females emerge at breeding sites; thus, this classification is reasonable (Lee and Park 2008). After the survey, all individuals were released at their respective collection sites. A 30 m stretch of stream on Mt. Biseul, located above the upper eDNA sampling site, was surveyed throughout the study period. Surveys could not be performed in the other sections on this mountain because of the presence of large rock clumps. The larvae and adults were classified using the same method as that used for larvae and adults collected on Mt. Daeryong.

All animal sampling and handling procedures were conducted according to the field use guidelines for amphibians of the Society for the Study of Amphibians and Reptiles (SSAR) and the American Society of Ichthyologists and Herpetologists (ASIH). For the study on Mt. Daeryong, permission was obtained from Chuncheon City (Permit No. 2024-1) and the Northern National Forest Service (Permit No. 10284). For the study on Mt. Biseul, permission was obtained from Dalseong-gun (Permit No. 2024-8). All other animal handling procedures were approved by the Kangwon National University Institutional Animal Care and Use Committee (KW-240603-1).

eDNA sampling and extraction

eDNA sampling was conducted by collecting stream water in a sterile plastic container. Afterward, 1 L of water was filtered through a Sterivex filter (pore size: 0.45 µm, Millipore, Burlington, MA, USA) using a 50 mL syringe; this approach was successfully used in a previous study (Park et al. 2026a, 2026b). eDNA sampling was repeated twice at each site. After filtering, the filter was filled with 99.5% EtOH, sealed with Parafilm, transported to the laboratory, and stored at –20 °C. New gloves, water bottles, syringes, and sampling kits were used at each site to prevent cross-contamination between sampling sites. After eDNA sampling was completed in each mountain valley, 1 L of commercially available water was filtered on-site using the same procedure as that used for the field samples and processed as a field control.

Following the methods of Kawato et al. (2021) and Park et al. (2026a, 2026b), DNA was extracted from all eDNA samples and field controls using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany). The extracted DNA was quantified using a NanoDrop spectrophotometer (NanoDrop Lite Plus, Thermo Fisher Scientific, Waltham, MA, USA) and stored in a –80 °C ultralow-temperature freezer (VS-90DF-C Series, Vision Scientific, Bucheon, Republic of Korea) until quantitative polymerase chain reaction (qPCR) analysis was conducted.

Development of a TaqMan probe for *O. sillanus* qPCR primers

For *O. koreanus* qPCR, mtDNA COI primers (OkeDNA-COI F/R, product size: 93 bp) and a probe (OKeDNA-COI probe) that had previously been developed and successfully used were employed (Park et al. 2026a). These primers also amplified the mtDNA *COI* sequence of *O. sillanus*; however, the sensitivity of the probe was low (Park et al. 2026a). Therefore, a new probe was designed using Primer3 in Geneious Primer v.21.0.4 (https://www.geneious.com), as described by Rodríguez et al. (2015). The developed probe (5'-FAM-GGGGCCGGTACAGG-GTGAAC-BHQ1-3'; OSeDNACOI probe) and the predeveloped primer set (OkeDNACOI F/R) were used to conduct *in silico* tests with the COI sequences (n = 168) of 11 amphibian and seven reptile species (Suppl. material 1: table S1), which may cohabit with *O. sillanus*, using the PrimerMiner package (ver. 0.22) in R (ver. 4.3.3; Elbrecht and Leese 2017).

The primer and probe sets were subsequently tested with tissue DNA obtained from 10 amphibian and five reptile species (Suppl. material 1: table S2), which may coexist with *O. sillanus*, by performing qPCR analysis. Additionally, the optimal ratio of primer-to-probe concentrations was determined using ratios of 500 nM primer to 150, 200, and 250 nM probe. The ratio that produced the lowest cycle threshold (CT) value was selected and applied in subsequent experiments. A gBlock based on the *O. sillanus* sequence was produced to determine the limit of detection (LOD) and limit of quantification (LOQ) of the developed eDNA primer-probe set (Suppl. material 1: table S4). The LODs and LOQs of the primer and probe sets were determined using the drc package (ver. 3.0.1) according to the methods of Klymus et al. (2020). A concentration range of 10^8 copies/µL to 10^{-2} copies/µL of gBlock in a 10-fold serial dilution was used (Suppl. material 1: table S5).

eDNA analysis

The qPCR analysis of mtDNA *COI* was performed using the QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The experimental conditions of Park et al. (2026a) were followed. The following reaction mixture was used for qPCR: 500 nM forward primer (1 µL), 500 nM reverse primer (1 µL), 200 nM (*O. koreanus*) or 150 nM (*O. sillanus*) TaqMan probe (0.4 µL), 10 µL of TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific, Waltham, MA, USA), 4 µg of molecular biology-grade bovine serum albumin (BSA; Thermo Fisher Scientific, Waltham, MA, USA), and 4 µL of eDNA template. The total volume was adjusted to 20 µL with molecular biology-grade water (HyClone, Waltham, MA, USA). BSA was included in the amplification solution because the addition of 4 µg

of BSA improved the performance of the qPCR analyses (Park et al. 2026a).

The following temperature conditions were used for qPCR: 10 min at 95 °C and 40 cycles of 15 s at 95 °C, 30 s at 64 °C, and 30 s at 60 °C. The OSeDNACOI probe for *O. sillanus* had an annealing temperature (60 °C) identical to that of the OKeDNACOI probe for *O. koreanus*. Each qPCR run included gBlock standards (10^4,10^3,10^2, and 10 copies/µL) for quantification, a field control, and a no-template control as a laboratory control. All PCRs for each sample were performed in triplicate.

The qPCR results were analyzed using Design & Analysis Software (ver. 2.7.0; Thermo Fisher Scientific, Waltham, MA, USA). Samples with eDNA concentrations between the LOD and LOQ were subjected to sequencing. If the acquired sequence was correct for the targeted species, it was considered a detection. For the analysis of DNA concentration in a sample on a specific sampling day, the average amount of eDNA in six replicates (duplicate samples × triplicate qPCR runs) was calculated. In addition, sequencing confirmed the accuracy of *O. koreanus* and *O. sillanus* eDNA detection in the 31 and 25 experimental eDNA samples, respectively, with detected concentrations above the LOQ.

Statistical analysis

The eDNA detection rates between Mt. Daeryong and Mt. Biseul, as well as among the three eDNA sampling sites within each valley, were analyzed using the chi-square test. The numbers of 1-year-old and 2-year-old larvae captured in the two valleys were also compared using the chi-square test. Whether the eDNA concentration varied significantly over the study period and whether a difference in the detected eDNA amounts was observed between the two valleys and among the three sampling sites in each valley were analyzed using a repeated-measures general linear model (GLM). If the difference was significant, post hoc analysis was conducted within the GLM. The daily relationships between the numbers of larvae and adults and the amount of detected eDNA were not analyzed. The study area was located in the upstream section of a mountain stream with a high flow rate. Therefore, the amount of eDNA detected on a given day may not reflect the number of individuals surveyed on the same day. All statistical analyses were conducted using SPSS (ver. 29.0.2.0). The data are presented as the mean ± 1 standard deviation, unless indicated otherwise.

Results

Field survey

On Mt. Daeryong, a total of 153 *Onychodactylus koreanus* larvae (5.1 ± 5.6, n = 30) were observed (range: 0–25 larvae; Fig. 2C; Suppl. material 1: table S6). Overall, 84 1-year-old larvae (2.8 ± 4.0) were detected, in addition to 69 2-year-old larvae (2.3 ± 3.7). On Mt. Biseul, a total of 288 *O. sillanus* larvae (10.7 ± 5.1, n = 27) were observed (range: 3–22 larvae; Fig. 3B, C; Suppl. material 1: table S7). Overall, 118 1-year-old larvae (4.4 ± 4.7) were detected, and 170 2-year-old larvae (6.3 ± 5.5) were observed. A significantly higher proportion of 2-year-old larvae than 1-year-old larvae was captured on Mt. Biseul (59.0%) than on Mt. Daeryong (45.1%; X^2 = 7.81; df = 1; p < 0.01). More than 10 1-year-old larvae were observed at two different times on Mt. Daeryong: on 7 June 2024 (n = 12) and on 8 October 2024 (n = 17). On Mt. Biseul, the maximum numbers of larvae were detected on 14 August (n = 9) and 25 August (n = 8).

Breeding adults were observed only on Mt. Daeryong (Fig. 2D). A total of 63 adults were observed, including 47 males, one ovulated female, and 15 oviposited females. Adult emergence occurred from 8 April to 17 June 2024, from 24 September to 19 November 2024, and from 25 March to 25 May 2025. On Mt. Biseul, only three subadults were observed, with two detected on 10 October 2024 and one detected on 24 October 2024 (Suppl. material 1: table S7).

eDNA analysis

The mtDNA *COI* target probe for *O. sillanus* and the previously developed primers (Park et al. 2026a) did not detect any species other than *O. koreanus* and *O. sillanus* in the *in silico* or tissue qPCR tests (Suppl. material 1: tables S1, S2). Only *O. sillanus* is observed on Mt. Biseul (Kim et al. 2026); thus, the primer and probe set developed in this study was used. According to the results of the qPCR analysis, the LOD of the set was 0.962 copies/µL, and the LOQ was 2 copies/µL (slope: –3.61; intercept: 38.88; R^2 = 0.999; efficiency: 89.3%; Suppl. material 1: figs S1, S2). The LOD and LOQ of the previously developed *O. koreanus* primer and probe set were both 4.52 copies/µL (Park et al. 2026a). Among the eight samples from Mt. Biseul with eDNA concentrations above the LOD but below the LOQ, all were identified as *O. sillanus* through sequencing, and their data were used in the analyses (Suppl. material 1: table S8). The species identity in all tested experimental samples was also confirmed by sequencing (Suppl. material 1: table S9).

The overall eDNA detection frequency was significantly higher on Mt. Daeryong (457/540, 84.6%) than on Mt. Biseul (198/486, 43.8%; X^2 = 187.9; df = 1; p < 0.01). On Mt. Daeryong, the detection rates differed significantly among the upper (95.0%), middle (88.9%), and lower (77.8%) sections (X^2 = 47.01; df = 2; p < 0.01; Suppl. material 1: table S10). On Mt. Biseul, these values also differed significantly (upper section, 65.4%; middle section, 41.4%; and lower section, 24.7%; X^2 = 55.2; df = 2; p < 0.01; Suppl. material 1: table S11).

Three major eDNA detection periods were identified on Mt. Daeryong. In the upper section, eDNA was de-

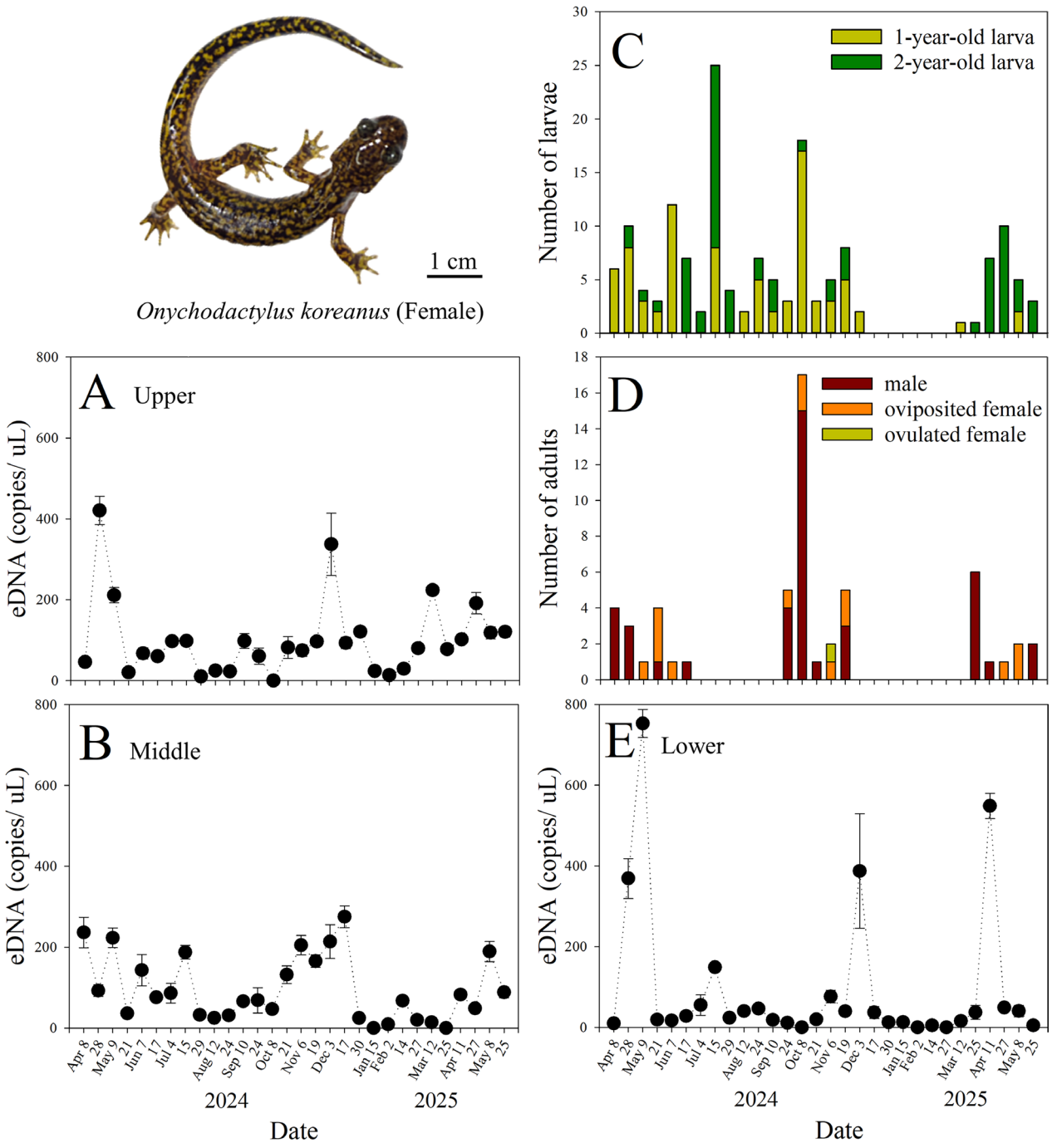

Figure 2. Results of population surveys of larvae (**C**) and adults (**D**) and amounts of eDNA of *Onychodactylus koreanus* detected at the upper: **A.** Middle; **B.** Lower; **E.** Sites in a mountain valley stream on Mt. Daeryong between 8 April 2024 and 25 May 2025. A population survey was conducted along a 40 m stretch of stream between the middle and lower sampling sites. The photograph was taken by MW Park.

tected on 28 April 2024, 3 December 2024, and 12 March 2025, whereas in the middle section, eDNA was detected on 8 April 2024, 17 December 2024, and 8 May 2025 (Fig. 2A, B). In the lower section, eDNA was detected on 9 May 2024, 3 December 2024, and 11 April 2025 (Fig. 2E). The major detection periods were most pronounced at the lower sampling site. Two major detection periods were identified on Mt. Biseul. In the upper section, eDNA was detected on 19 June 2024 and 13 March 2025; in the middle section, eDNA was detected on 24 May 2024 and 26 March 2025; and in the lower section, eDNA was detected on 4 June 2024 and 8 April 2025 (Fig. 3C–E). The major detection periods were most distinctive at the middle sampling site.

The concentration of eDNA changed significantly over time at both study sites (Mt. Daeryong: $F_{29} = 50.3$, $p < 0.01$; Mt. Biseul: $F_{26} = 19.4$, $p < 0.01$). The interaction between time and concentration was significant (Mt. Daeryong: $F_{58} = 18.9$, $p < 0.01$; Mt. Biseul: $F_{52} = 13.3$, $p < 0.01$). The eDNA concentrations did not differ among the upper, middle, and lower sampling sites on Mt. Daeryong over time ($F_{2,15} = 0.439$, $p = 0.653$; Fig. 2). In contrast, the

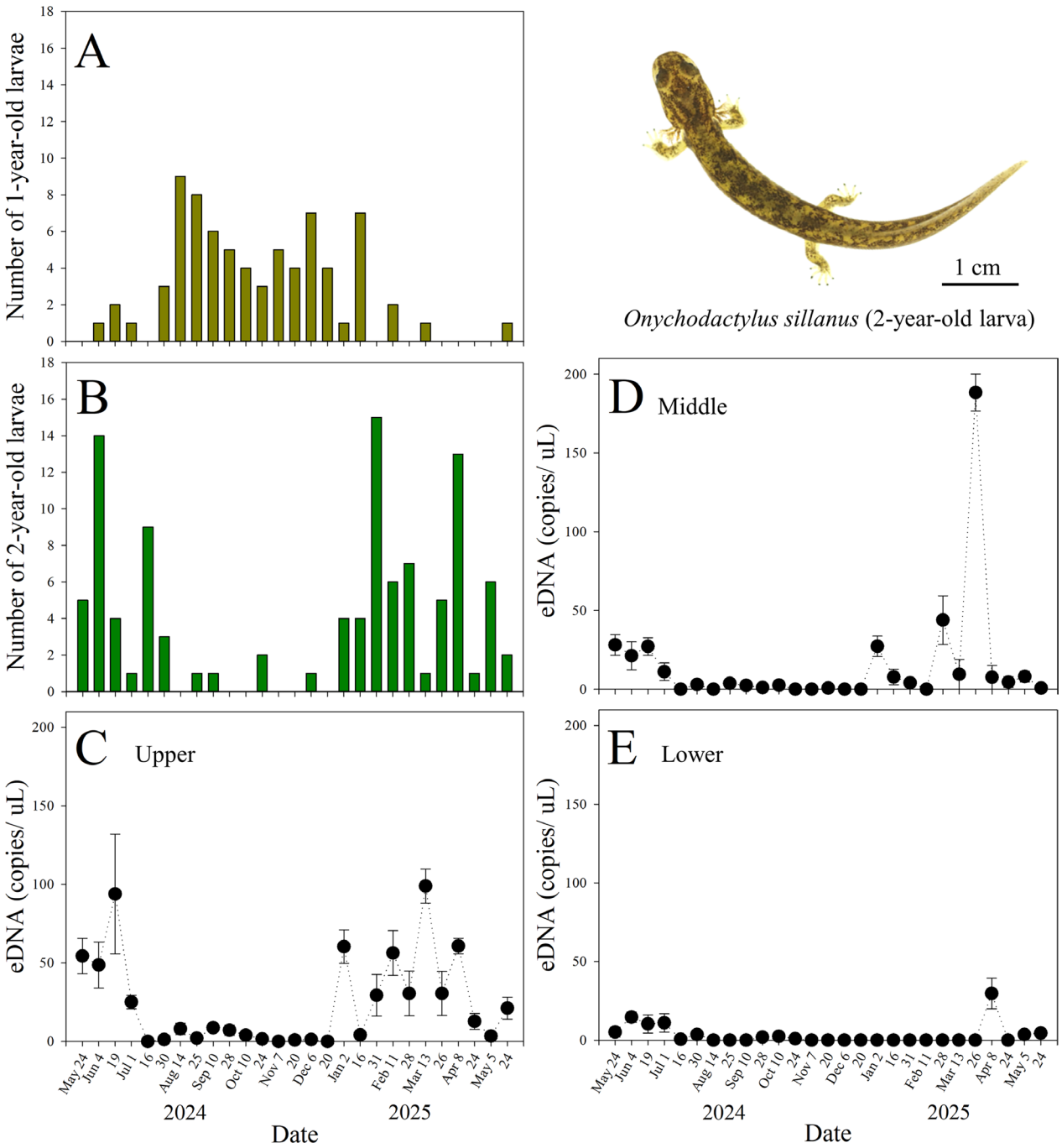

Figure 3. Results of population surveys of 1-year-old (**A**) and 2-year-old (**B**). Larvae and amounts of eDNA of *Onychodactylus sillanus* detected at the upper: **C.** Middle; **D.** Lower; **E.** Sites in a mountain valley stream on Mt. Biseul between 24 May 2024 and 24 May 2025. The population survey was conducted along a 30 m stretch of stream above the upper sampling site. The photograph was taken by MW Park.

difference was significant on Mt. Biseul ($F_{2,15} = 48.8$, $p < 0.01$; Fig. 3). According to the results of the post hoc tests, the concentrations differed significantly among the upper, middle, and lower sampling sites (Tukey's test, $p < 0.05$).

Discussion

eDNA analyses and population surveys were conducted over the course of one year in typical mountain valley habitats of the genus *Onychodactylus* to verify and compare the breeding frequencies and breeding periods of *O. koreanus* and *O. sillanus* in the Republic of Korea. *Onychodactylus koreanus* was predicted to breed twice per year, in April–June and November–December, based on the results of both eDNA analyses and population surveys, whereas *O. sillanus* was predicted to breed once between late winter and early summer, mainly based on the results of eDNA analyses. No full-grown breeding adults of *O. sillanus* were observed during the field surveys. These results suggest that the two species may have different breeding ecologies.

The emergence of adults and larvae at the study sites differed between *O. koreanus* and *O. sillanus*. Adult *O. koreanus* emerged twice per year, in spring and late autumn. These periods are comparable to the known emergence periods of May–June and September–October for *O. fischeri* in the Russian Federation (Griffin and Solkin 1995), as well as April–June and October–November for *O. japonicus* in Japan (Akita and Miyazaki 1991). The periods also overlap with the May–June and November–December emergence periods reported in a recent study of *O. koreanus* from a cave in the Republic of Korea (Park et al. 2026a). Conversely, despite similar sampling effort during population surveys, no full-grown adult *O. sillanus* was observed at the study site. These findings suggest that adult *O. sillanus* may use microhabitats during the breeding season that differ spatially from those of adult *O. koreanus*. During the surveys, breeding adults of *O. sillanus* may have remained in locations that were inaccessible. Given that their habitat is located in the southern region of the Korean Peninsula, where temperatures are relatively high (Kim et al. 2026), breeding adults of *O. sillanus* may remain deeper underground, exhibiting more subterranean and cryptic activity. Future research should determine whether these findings are characteristic of the Mt. Biseul study site or whether they are common among populations in the southern regions of the Korean Peninsula.

The survey results for the larvae also differed between *O. koreanus* and *O. sillanus*. On Mt. Daeryong, a major recruitment of 1-year-old *O. koreanus* larvae into the streams occurred twice, in early June and early October. Conversely, on Mt. Biseul, recruitment occurred once or twice between early August and late December. The separation of the two periods in *O. sillanus* was not evident, unlike the separation observed in *O. koreanus*. The emergence pattern of *O. koreanus* is similar to the results of a recent study conducted in a cave, in which recruitment occurred in June and early November (Park et al. 2026a). On Mt. Biseul, many large larvae that were 2 years old or older tended to be observed. Specifically, Joo et al. (2025) reported *Onychodactylus* larvae with an SVL of 75.4 mm during this study on Mt. Biseul, which is larger than the previously known maximum SVL of larvae and suggests that this species may have a longer larval period. The timing of the emergence of 1-year-old larvae into a mountain valley stream after hatching may vary depending on the time needed for hatching and absorption of the egg yolk, the microhabitat of the underground spawning sites, and the potential for larvae to be transported by the water current from the subterranean spawning sites (Hayase and Yamane 1982; Yoshikawa et al. 2013; Park et al. 2026a). Therefore, determining the breeding period based on the emergence of 1-year-old larvae is not recommended, although this information may be useful for determining the breeding frequency (Park et al. 2026a, 2026b). Additionally, urodele larvae may release small amounts of eDNA. *Salamandrella keyserlingii* larvae release only one-fifth as much eDNA as adults (Takeshita et al. 2020). In a previous study, the amount of detected eDNA was not related to the number of larvae (Park et al. 2026a). These results suggest that determining the breeding periods based on the eDNA released by larval *Onychodactylus* is difficult with the current primer and probe set. Nevertheless, *Onychodactylus* larvae can be good indicators of population dynamics, as they can be observed in the valley throughout the year. In particular, considering the relatively large size of *O. sillanus* larvae on Mt. Biseul (Joo et al. 2025), the possibility that eDNA released from large *O. sillanus* larvae substantially contributes to the overall amount of eDNA detected in the stream cannot be excluded. Studies focusing on the development of more sensitive or larval-specific primers, such as eRNA primers, and investigations of the relationship between the number of larvae and the amount of released eDNA are necessary.

The amounts of eDNA detected along the upper, middle, and lower sections within a mountain valley stream exhibited both section-specific characteristics and overall consistency across the study ranges. In mountain streams, the amount of eDNA in a specific section is influenced by various factors, such as the number and activity of individuals in that area, the amount of eDNA released, the degree of adsorption onto the substrate, and the rate of degradation (Strickler et al. 2015; Tsuji et al. 2017; Breton et al. 2022; Park et al. 2026a, 2026b). Despite the higher LOQ and LOD of the primer for *O. koreanus* than for *O. sillanus*, the detection rate was higher on Mt. Daeryong, which is inhabited by *O. koreanus*. The higher population density of *O. koreanus* adults may have fundamentally led to this result (Breton et al. 2022). Additionally, the lower habitat temperature associated with the higher latitude and dense canopy of Mt. Daeryong may have reduced the rate of eDNA degradation (Strickler et al. 2015). When comparing major eDNA detections along the upper, middle, and lower sections, the detection rate was highest in the upper section on Mt. Daeryong and Mt. Biseul, whereas the lower section presented the lowest detection rate. If the eDNA in the upper section directly moved to the lower section, the lower section would exhibit the highest eDNA concentration. These results suggest that a considerable amount of eDNA released in each section may have been adsorbed onto the substrate of the streambed or dispersed by various algae and aquatic plants before it reached the next sampling site within the mountain valley streams (Pont 2024). Despite the different amounts of eDNA detected across the sites, the major eDNA detection patterns were largely consistent across the three sites. On Mt. Daeryong, three major detections commonly occurred in April–May 2024, November–December 2024, and April–May 2025. On Mt. Biseul, one or two major detections occurred from early January to late June regardless of the location of the eDNA sampling site. These results indicate that the pattern of major eDNA detections may reflect overall major population dynamics in the valley stream habitat. Although breeding adults were not directly observed on Mt. Biseul, these findings suggest the necessity of performing eDNA sampling in at least several sections along the stream.

The breeding periods of *Onychodactylus* species can be determined by combining the results of population surveys and eDNA analyses. In externally fertilizing salamanders, adult females typically appear at breeding sites only during the breeding season (Lee and Park 2008). The observation of males, ovulated females, and oviposited females during a specific period suggests that breeding activity occurred during the survey. The survey results indicate that on Mt. Daeryong, *O. koreanus* breeds twice per year, with one breeding event occurring between early April and early June and another event occurring between early October and early December, as previously reported (Park et al. 2026a, 2026b). The period of major eDNA detection at the lower eDNA sampling site, where the eDNA patterns were most distinct, also closely aligned with the breeding periods observed between late April and late May and between early November and mid-December. According to the results of the eDNA analysis, breeding may occur once per year on Mt. Biseul over a long period from January to June. The distribution of *O. sillanus* is limited to the southeastern part of the Korean Peninsula, where the temperature is relatively high (Kim et al. 2026). Therefore, these climatic conditions may be responsible for the different breeding frequencies and breeding periods. Despite these results, the exact breeding period of *O. sillanus* remains tentative, unlike that of *O. koreanus*, because breeding adults of *O. sillanus* were not directly observed in this study. More intensive field surveys, including nocturnal surveys, are necessary to determine the exact breeding period of this species. Moreover, compared with Mt. Biseul, some populations of *O. koreanus* are located in the more southwestern part of the Korean Peninsula (Suk et al. 2018). Comparative studies of similar latitudinal populations of *O. koreanus* and *O. sillanus* may provide clues as to whether these differences are species- or habitat-specific.

The results from the combined eDNA analysis and population surveys demonstrate that *O. koreanus* breeds twice per year, in spring and late autumn, in its typical mountain valley habitats. Conversely, *O. sillanus* may experience a long breeding season extending from late winter to early summer, when the temperature is relatively low in southern geographical locations. Additional comparative studies in more southern regions or among similar latitudinal populations of *O. koreanus* and *O. sillanus*, including intensive field surveys to observe breeding adults, particularly those of *O. sillanus*, could reveal more information about their adaptive breeding strategies and resilience to changing climate conditions.

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

No ethical statement was reported.

Artificial Intelligence (AI) use

The authors accept full responsibility for the content of the manuscript, including the disclosure of any use of AI.
Regarding the use of AI in the preparation of this manuscript, the authors declare the use of QuillBot for language, style and writing. We used an AI-assisted editing tool, not a generative AI tool.

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Author contributions

Conceptualization: JK, DP, MWP, JP. Data curation: DP, NJ, HN, JP. Formal analysis: DP, HN, JP, NJ. Funding acquisition: DP. Investigation: YGK, JK, JJ, HN, NJ, MWP, JP. Methodology: MWP, JK, DP, JP. Project administration: DP. Resources: DP. Software: MWP. Supervision: DP, MWP. Validation: DP, JP, JK. Visualization: MWP. Writing – original draft: MWP, DP. Writing – review and editing: MWP, DP.

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Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

Supplementary material 1

Supplementary information

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Explanation note: **table S1.** List of amphibian and reptile species and GenBank accession numbers used for the *in silico* tests of the *COI* primer-probe sets developed in this study to detect *Onychodactylus sillanus* using eDNA samples. **table S2.** List of amphibian and reptile species used for the qPCR specificity test conducted using tissue DNA and the *COI* primer-probe set (OSeDNACOI probe and OKeDNACOI F/R primer) developed to detect *Onychodactylus sillanus* using eDNA samples, with the amplification outcomes. **table S3.** Optimization of the probe and primer concentrations for the *COI* primer-probe set (the OSeDNACOI probe and OKeDNACOI F/R primer) used to detect *Onychodactylus sillanus* in eDNA samples. qPCR was performed with three probe concentrations and muscle tissue-derived DNA from *O. sillanus* (1 ng/μL). The primer-probe concentration combination with the lowest mean CT value was selected for subsequent analyses. **table S4.** Sequences of the gBlocks used in the mtDNA *COI* eDNA amplification assays for *Onychodactylus koreanus* and *O. sillanus* in this study. **table S5.** Serially diluted gBlock concentrations used to determine the LOD and LOQ of the *COI* primer-probe set developed to detect *Onychodactylus sillanus* using eDNA samples. CT values are shown for five replicates at each concentration. **table S6.** Numbers of adults and larvae of *Onychodactylus koreanus* observed on Mt. Daeryong during 30 surveys conducted between 8 April 2024 and 25 May 2025. Adult individuals are categorized as males, ovulated females, or oviposited females, and larvae are categorized as 1-year-old or 2-year-old individuals. **table S7.** Numbers of adults and larvae of *Onychodactylus sillanus* observed on Mt. Biseul during 27 surveys conducted between 24 May 2024 and 24 May 2025. Subadults found during the surveys were considered only in the total number of adults. Larvae are categorized as 1-year-old or 2-year-old individuals. **table S8.** Verified sequences of the eDNA samples that exhibited eDNA amounts below the LOQ but above the LOD after amplification of samples collected on Mt. Biseul. Information on the site, sampling date, CT value, acquired eDNA sequence, percentage identity in NCBI BLAST, and identified species is included. **table S9.** Verified sequences of the experimental eDNA samples that exhibited eDNA amounts above the LOQ after amplification of samples collected on Mt. Daeryong and Mt. Biseul. Information on the site, sampling date, CT value, acquired eDNA sequence, percentage identity in NCBI BLAST, and identified species is included. **table S10.** Amounts of eDNA from *Onychodactylus koreanus* detected using the *COI* primer-probe set at three sampling sites (upper, middle, and lower sampling sites) on Mt. Daeryong between 8 April 2024 and 25 May 2025. For each sampling date, the amount of extracted eDNA, the success of qPCR-dependent detection, and the estimated eDNA concentration (copies/μL) are presented for two replicates at each sampling site. **table S11.** Amounts of eDNA from *Onychodactylus sillanus* detected using the *COI* primer-probe set at three sampling sites (upper, middle, and lower sampling sites) on Mt. Biseul between 24 May 2024 and 24 May 2025. For each sampling date, the amount of extracted eDNA, the success of qPCR-dependent detection, and the estimated eDNA concentration (copies/μL) are presented for two replicates at each sampling site. **fig. S1.** Amplification plot generated from serially diluted gBlocks (10^8 copies/μL to 10^{-2} copies/μL) used to calculate the LOD and LOQ of the *COI* primer-probe set developed to detect *Onychodactylus sillanus* using eDNA samcples. **fig. S2.** Standard curve of the *COI* primer-probe set developed to detect *Onychodactylus sillanus* using eDNA samples; the curve was generated using serially diluted gBlocks (10^8 copies/μL to 10^{-2} copies/μL) according to the method of Klymus et al. (2020). The calculated LOD was 0.962 copies/μL, and the LOQ was 2 copies/μL.

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