Translocation of an endangered endemic Korean treefrog *Dryophytes suweonensis*

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SUMMARY

Endangered species in heavily modified landscapes may be vulnerable to extinction if no conservation plan is implemented. The Suweon treefrog *Dryophytes suweonensis* is an endemic endangered species from the Korean Peninsula. In an attempt to conserve the species, a translocation plan was implemented in the city of Suwon. The receptor site was a specially modified island in a reservoir. Egg clutches were collected from four nearby sites, and were hatched and reared in a laboratory during 2015. One hundred and fifty froglets were released at the new site. In 2016, one year after the translocation, calling male *D. suweonensis*, and newly hatched tadpoles and juveniles were recorded. Juveniles were seen until the last week before hibernation in autumn 2016. However, only a single male was recorded calling in 2017. The population was consequently considered functionally extinct. Failure of the translocation most likely arose from mismanagement of the vegetation surrounding the wetlands, and the resulting inability of the site to fulfil the ecological requirements of the species. The project allowed the development of rearing protocols for the species, and defined its ecological requirements.

BACKGROUND

Due to the threatened status of a high number of amphibian species, in situ and ex situ conservation programmes are being established around the world (Stuart *et al.* 2004). Each type of conservation plan has advantages and drawbacks, but when feasible, the translocation of a few individuals into a restored habitat can enhance the survival of the species (Zippel *et al.* 2011). However, the success rate is often low, and this may be partly due to the lack of reporting on, and therefore knowledge of, unsuccessful attempts by the scientific community (Germano & Bishop 2009).

The Suweon treefrog *Dryophytes suweonensis* is an endangered species endemic to the Korean peninsula (IUCN 2017), although potentially synonymous to *Dryophytes* (or *Hyla*) *immaculatus* from China (Dufresnes *et al.* 2016, Borzée *et al.* 2017a). *Dryophytes suweonensis* is an important evolutionary species as it displays a rare ZW karyotype, or chromosomal sex-determining system, that is not shared with its sister species *D. japonicus* (Dufresnes *et al.* 2015). It is therefore important to develop conservation plans to prevent the extinction of the species. Here, we attempted reintroducing captive-reared *D. suweonensis* froglets at a site in the city of Suwon. The site was modified to meet the ecological requirements of the species, and avoid the shortcomings that led to the extirpation of the species in its eponymous city where it was described (Kuramoto 1980).

ACTION

Translocation site: The translocation site was an island of 6,600 m^2 located at the northwest corner of Ilwol Reservoir in the city of Suwon in the Republic of Korea (37.29° N, 126.97° E; Figures 1 & 2). This site is owned by the Korea Rural Community Corporation, and was chosen for this project as it had been allocated a restoration grant by the Ministry of Environment. Unfortunately, the location of the translocation site was determined without feasibility analyses and risk assessment (IUCN/SSC 2013) prior to the project. Instead the site was selected for opportunistic reasons. The north and west corners of the site were isolated from the surrounding paths by water collection channels, and the south and east corners of the site were facing the waters of the reservoir. This prevented colonisation by other amphibians that could potentially outcompete the released individuals. The site was designed to feature all environmental variables known to be required by *Dryophytes suweonensis* (Roh *et al.* 2014). The island was transformed to encompass a rice paddy covering 580 m^2 and two ponds (Figure 2) with vegetation cover (lotus *Nelumbo nucifera*), and a shallow pond with reed banks (*Typha* spp). Korean willow trees *Salix koreensis* were planted to supplement the tree cover already present at the site (Borzée & Jang 2015). The species was selected based on the only known association between *D. suweonensis* and a tree species. Finally, as the hibernation ecology of the species was not known at the time, and because breeding sites of *D. suweonensis* are often surrounded by forested hills, a low hill planted with pine trees (*Pinus* spp.) was created on the northern edge of the island. Cut wood and stone stacks were also set in the vicinity of the rice paddy to provide shelter during brumation and hibernation, the two successive stages of the over-wintering behaviour. The reedbed at the south of the island was not modified as it was assessed not to be negative to the translocation of *D. suweonensis*.

Collection sites selection: To found a population, Albert *et al.* (2015) recommend mixing animals from multiple sites, while avoiding the most genetically divergent populations. Mixing of lineages has also led to rapid population growth and increased heterozygosity in several amphibian species (Spielman *et al.* 2004a). The collection sites therefore selected for sampling were in Sihung, Yongin, Posung and Oseong (Figure 1). The choice

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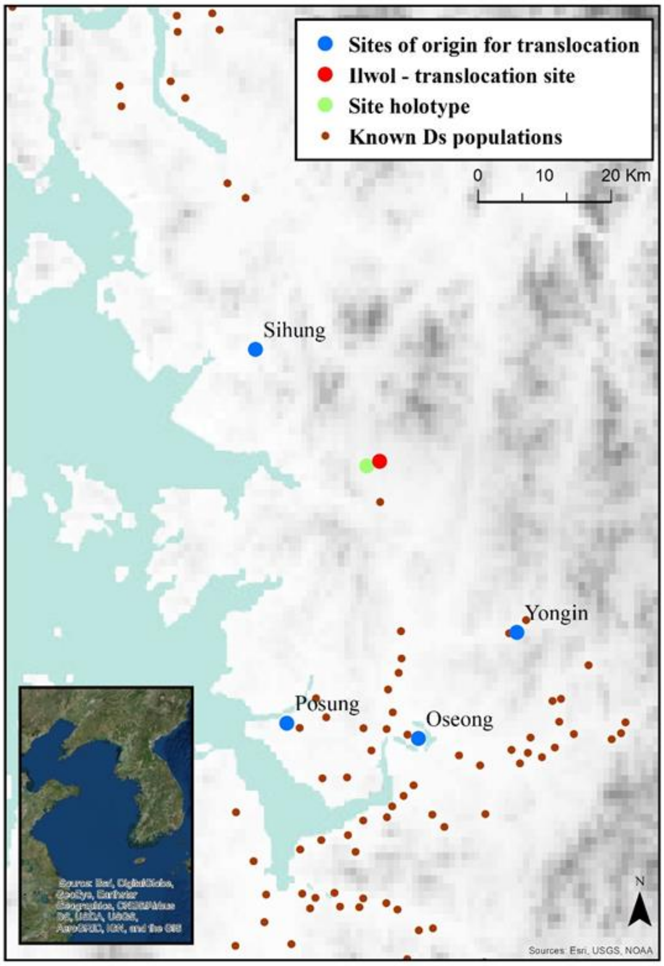

Figure 1. Map of the sites at which egg clutches were collected, the site where individuals were released in Ilwol reservoir in Suwon, and the site where *Dryophytes suweonensis* (Ds) was described.

of collection sites for translocation was based on range-wide surveys of the species (Borzée *et al.* 2016, Borzée *et al.* 2017c) and genetic analyses (Borzée *et al.* 2015). The collection sites selected were close to the translocation site, and displayed high genetic diversity when combined, despite low variation between isolated populations. Genetic analysis was based on the sequences of 578 base pairs of gene CO1, based on the protocol developed by Jang *et al.* (2011). The collection sites selected included 63.4% of all CO1 haplotypes recorded for the species, and 89.7% of haplotypes from the south-Seoul metapopulation, to which the site belongs. Thus, sampling eggs from two unrelated parents from any population had the potential to cover 34.48% of the haplotypes from south-Seoul metapopulation. This was assessed to be adequate, as the highest genetic diversity at a site was 24.1% of haplotypes. This selection maintained the genetic structure of the species, while increasing genetic diversity through cross-population breeding in the F1 generation.

Field surveys at collection sites: In 2015, surveys were conducted at the collection sites on 3 March, 4 and 21 April, plus five surveys at five-day intervals in May, weekly surveys in June, and surveys on 3 and 7 July. This schedule was intended to estimate temporal phenology and population size at all sites. The translocation site in Suwon, and the closest site to the one where the species was described (Kuramoto 1980), were also surveyed on these dates to ensure ecological variables were similar at sites

Table 1. Average values across all sites and surveys for abiotic variables at sites with varying numbers of calling *Dryophytes suweonensis*. We did not record between six and nine individuals calling at any of the sites.

# *D. suweonensis*	1	2	3	4	5	>10
Air temperature (°C)	19.6	19.9	21.2	24.3	21	19.9
Air humidity (%)	50.9	56.9	67.1	49.8	56.5	54.6
Water temperature (°C)	19.5	21.3	21.74	25.8	19.2	21.3
Water acidity (pH)	8.38	8.48	8.56	8.34	8.15	8.6
Rain index (1 to 4)	0.8	0.2	0.8	0.8	0.5	0
Luminosity (lx)	1253	7638	7394	7149	2292	41
Pressure (hPa)	1024	1023	1021	1021	1021	1024
Water conductivity (µS)	2420	1375	1401	2026	2348	1775

where the species was known to occur, and to assess future potential reintroductions (Figure 1).

Monitoring was conducted following the protocol described in Borzée *et al.* (2017b, 2017c). At each site, we recorded abiotic variables including luminosity, air temperature, relative humidity, air pressure, water temperature, water conductivity, water pH and the number of calling individuals of *D. suweonensis* (Table 1).

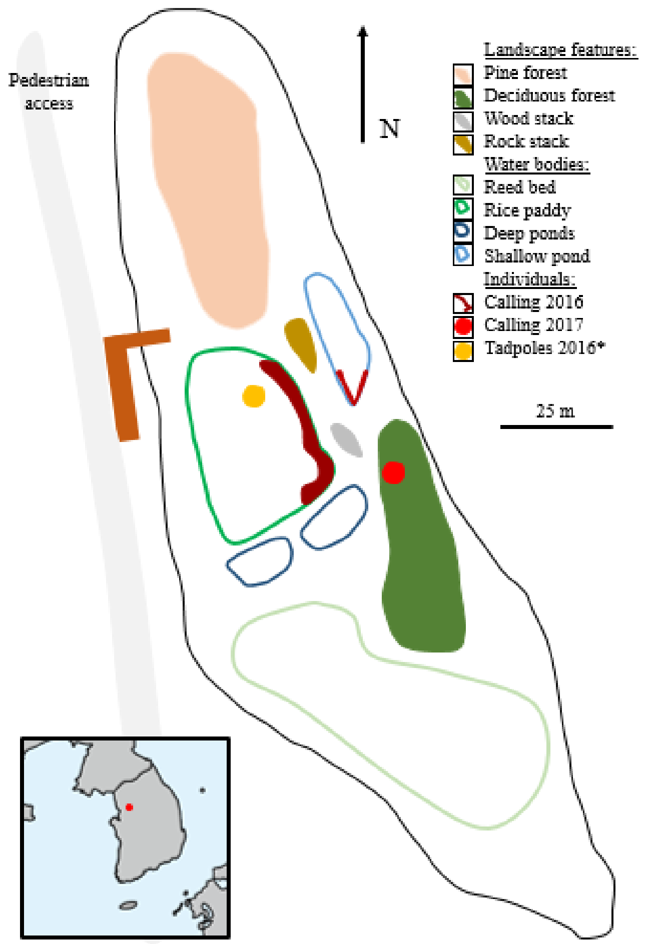

Figure 2. Modified island where 150 *Dryophytes suweonensis* froglets were released. Release took place on the eastern edge of the rice paddy in three separate events between early July and late August 2015. The inset map shows the location of the site within the Republic of Korea. *denotes where the tadpole was caught in 2016, although individuals were seen in the entire rice paddy.

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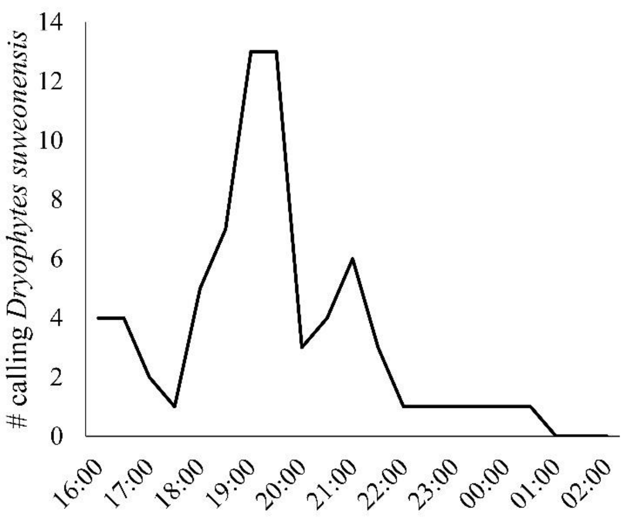

Figure 3. Daily calling activity for *Dryophytes suweonensis* across all sites and all surveys. The line is the number of individuals. The peak is just after sunset.

Dryophytes suweonensis started calling in Posung (Figure 1) in the fourth week of April 2015, and was heard calling at the four collection sites from the second week of May. The highest number of calling individuals (13) was recorded in Oseong, where the calling activity was also the longest, from 12 May until 3 July 2015. Peak calling activity occurred between 19:00 and 19:30 h, with the earliest calls detected at 16:00 h and the latest at 01:30 h the next day (Figure 3). The average number of calling individuals across all sites was 3.8 (±1.2).

We investigated the abiotic factors in relation to the number of calling individuals at a site (Table 1). The highest number of calling individuals was detected below 20 ˚C, although up to four individuals at a time were surveyed at the highest temperature (> 24 ˚C). Air humidity displayed only minor variations through the range of the number of calling individuals, with the lowest points matching with the lowest number of calling individuals. Air pressure displayed the opposite pattern to the air humidity, with the highest values matching with the lowest and highest number of individuals calling. The water temperature was at the highest point when four individuals were calling. The pH and conductivity did not have any visible relationship with the number of calling individuals. Finally, low rain and luminosity were correlated with the highest number of calling individuals.

Egg collection: Collection of egg clutches for translocation was conducted after sunset in spring 2015. We visually searched for amplexed (i.e. mating) pairs at the four collection sites, which was facilitated by listening for advertisement calls. Once found, the amplexed pairs were caught and transferred into aerated plastic boxes (60 x 40 x 40 cm; W x L x H) and left in the rice paddy where they remained until morning. Amplexed pairs were released at the point of capture on the next morning, immediately after the collection of oral and skin swabs, following the protocol from Borzée *et al.* (2017b). One modification was made to the protocol: the two individuals from the pair were considered to be two replicates of a single unit during subsequent tests for disease.

Eggs were brought to the laboratory and put into 30 x 30 x 30 cm glass tanks in the water from the rice paddy where they had been caught. Water was not changed until hatching to facilitate the highest rate of hatching. Each egg cluster was accommodated separately, with a standard aquarium air stone set at the lowest bubbling rate to aerate the water but prevent eggs from drifting. Once hatched, tadpoles were kept together until around stage 26 (Gosner 1960), when they were divided into groups of 50 individuals, and transferred to new 30 x 30 x 30 cm glass tanks.

Tadpole rearing: The tadpoles were accommodated in 30% of water originating from their rice paddy, and 70% of carbon-filtered tap water that had been aged for 72 h in a 50 L bin. A third of the water volume was changed weekly from the bottom to remove decaying matter until metamorphosis of all tadpoles. The water used for changes was also filtered and aged for 72 h. In the tanks, the average conductivity was 230 ± 167 µS (mean ± S.D.), and the pH was 8.3 ± 0.9. The temperature was 16.4 ± 4.2 °C during the first week but 26.3 ± 2.4 °C during the last week, as aquaria were kept at environmental temperature by keeping all doors and windows open. Air stones were provided for each tank to oxygenate water and prevent bacterial multiplication.

Tadpoles were fed *ad libitum* on a boiled and blended mix of Chinese mallow *Malva verticillata* and animal nutrients from NovoTab fish food pellets (43% crude protein; JBL, Neuhofen, Germany). Each tank was illuminated by an LED strip set on the same cycle as the natural photoperiod, and adjusted bi-monthly to follow natural variations. Once an individual had developed both front legs, it was transferred to another tank to finish metamorphosis without risk of predation by siblings. Tanks were checked twice daily during the period of metamorphosis.

During the rearing period, water quality tests did not detect excess nitrates or ammonia. There were five to eight replicate tanks for each family, based on the number of eggs per clutch. Tanks were inspected daily for dead individuals, which seldom happened. A few cases of necrophagy were detected, and in this case the dead individual was not removed and totally consumed by siblings. This decision was taken to enable tadpoles to access any resource that were not provided by their diet. It is unclear whether these cases resulted from cannibalism or whether individuals were dead before being preyed on. Cannibalism was observed, with several individuals preying on a sibling, typically feeding first on the tail, and then on guts once the individual became immobilized.

Froglet rearing: Metamorphosing tadpoles were transferred to terraria (Exo Terra Glass Terrarium 30 x 30 x 30 cm; Colorado, USA) with a lateral opening and mesh ceiling for aeration. Each terrarium was set with a layer of wet towel changed twice a week, a petri dish filled with water, and sprayed daily to maintain > 70 % relative humidity, but no flooded area. Each terrarium was illuminated with a UV-B bulb (PT 2186; Exo Terra Repti-Glo 5.0; Colorado, USA), temporally synchronised with natural sunlight. This was readjusted every second week. Doors and windows of the rearing room were kept open to maintain natural atmospheric variations. The first week was on average 24.3 ± 1.7 °C, with a maximum temperature of 36.7 °C later in the season. A wooden rod (diameter = 5 cm) was placed diagonally from the left front bottom to the back right top in each tank for froglets to climb on.

Individuals transferred from the aquaria were first deposited into petri-dishes, where they usually stayed until tail loss, although some individuals were observed to be at risk. Several deaths due to drying or drowning were reported. Some individuals reaching dry areas were not able to get back to the humid zones and died, and some individuals swam straight against the transparent edge of the plastic petri dishes without moving upwards until death. This was remedied by sticking

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opaque tape on the edge of the petri dishes. Approximately 60 metamorphosing individuals died as the result of these problems. A few cases of abnormal development were detected, such as missing limbs (n < 5 out of > 1,200 individuals) or missing eyes (n < 10 out of > 1,200 individuals).

Terraria were always populated with fewer than 20 individuals, to keep density low, and importantly, to prevent size variation between individuals in order to avoid competition for food items. The youngest individuals were fed daily *ad libitum*, starting with the equivalent of five pinheads or fruit flies per individual. As soon as size allowed, individuals were fed with larger crickets, complemented with small worms. All food items were first powdered with calcium and vitamins (Powder Supplement PT1850 and PT1860; Exo Terra, Colorado, USA).

The survival of froglets was approximately 75%, with a total of about 250 dead individuals retrieved. Death was associated with bloating, lack of activity, lack of appetite, and increasing weakness. Bathing symptomatic frogs in Ringer's solution, which is isotonic to the body fluids of amphibians, did not seem to improve survival but seemed to delay death. All healthy individuals were active and preferentially found on the wooden support in the terraria.

Pre-release screening: Prior to release, all individuals were tested for a set of diseases, including chytrid fungus (*Bd*), ranaviruses and abnormalities. Disease detection was conducted through molecular analyses of skin swabs for every tenth individual randomly picked, and all dead individuals. Swabbing was done with sterile fine-tip swabs (Medical Wire & Equipment Co Ltd; Corsham, UK), wearing a new pair of vinyl gloves for each frog. Frogs were systematically swabbed five times on each toe of the hind legs, each foot, the inner thighs, and both sides of the abdomen. Swabs were stored in individual 1.5 mL tubes at –20 °C until analysis.

Bd detection was conducted according to the protocols, primers, and controls presented in Borzée *et al.* (2017b). Ranaviruses were tested following the protocols set by Mao *et al.* (1997) and the PCR run times described by Greer & Collins (2007). The PCR amplification relied on primers specific for a 500 bp fragment of the ranavirus major capsid protein. Ranavirus had not been detected in Korea at the time of testing, but has since been detected in *Dryophytes japonicus*, *Kaloula borealis* and *Rana huanrenensis* (Park *et al.* 2017, Kwon *et al.* 2017), and is thus likely to be carried by *D. suweonensis*.

Tests for *Bd* and ranavirus were negative for all samples of both randomly picked live as well as dead individuals. A few weak-looking individuals were kept from being released and were instead released with the next wave, once adequately active.

Release: Individuals were released after 20:00 h to ensure minimal predation by birds and reptiles. Individuals were housed in plastic containers in groups of five to ten. The containers were distributed at regular intervals on the eastern edge of the rice paddy (Figure 2), where the vegetation was most abundant. Releases took place in three waves, in order to match the rhythm of metamorphosis of tadpoles in the lab, and to keep metamorphosed individuals for the shortest period possible. All released individuals were old enough to feed on live prey. The first release took place on 15 July 2015, with 20 individuals released. The second release (50 individuals) was on 4 August 2015 and the last release (80 individuals) was on 25 August 2015.

Monitoring: Subsequent to the release in 2015, the site was surveyed every three days until the average temperature dropped below 20 °C. From this point, surveys were conducted weekly until 21 October 2015, when the rice paddy was harvested. The site was also surveyed in 2016 and 2017, with surveys conducted monthly in February and March, twice in April, weekly from the first week of May to the fourth week of August, and then bi-weekly until the first freeze (the second week of November) in 2016 and until the last week of September in 2017. During surveys, the temperature (°C), relative humidity (%), air pressure (hPa), water temperature (°C), water conductivity (µS) and pH were recorded, as well as calling index, or individual count if possible, for all anuran species present at the site.

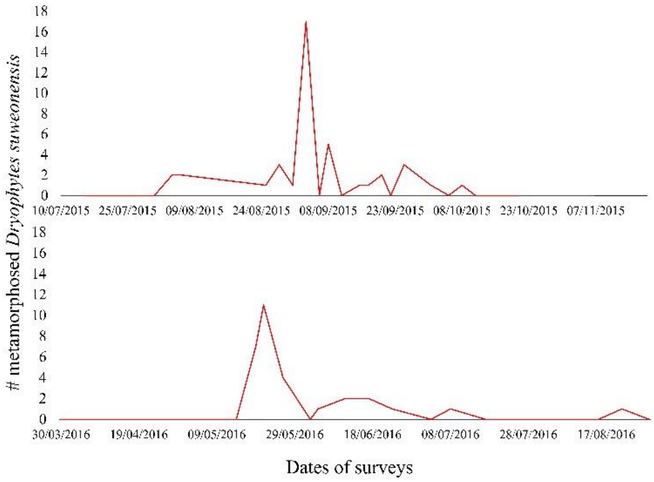

Figure 4. Number of metamorphosed individuals surveyed at translocation release site in 2015 and 2016. Please note different time scale for the two graphs. The individuals surveyed in both years were translocated individuals, surveyed as froglets in 2015 and breeding adults in 2016. Froglets metamorphosed in 2016 are not shown.

CONSEQUENCES

A total of 150 individuals were released at the translocation site, and all remaining froglets were released at their site of origin. The number released at the trial site was the maximum permitted by the permit obtained.

At the translocation site, individuals were surveyed on a regular basis for a year. *D. suweonensis* was reported in 13 of the 23 surveys conducted post-release in 2015 (Figure 4). The maximum number of individuals observed in a survey was 17. The main factor affecting the number of individuals observed appeared to be temperature, with no individuals seen when the temperature dropped below 17.6 °C, and the maximum number of individuals was found between 23 and 30 °C (Figure 5).

In 2016, the year after release, seven calling males were first detected on 19 May. Males were heard calling for seven weeks, and the peak calling activity involved 11 calling males on 21 May. Tadpoles were seen in the rice paddy from the second week of June, and one individual was caught and tail-clipped for molecular identification through CO1 sequencing (protocols from Jang *et al.* 2011). The sequence had a 98.2% match with *Dryophytes suweonensis* from GeneBank, and thus demonstrated breeding post-translocation. Newly emerged froglets were encountered from 17 July 2016, until October in 2015 and September in 2016.

Several management mistakes were made at the translocation site in 2016 due to contrary decision-making and lack of co-ordination between governmental bodies. Grass

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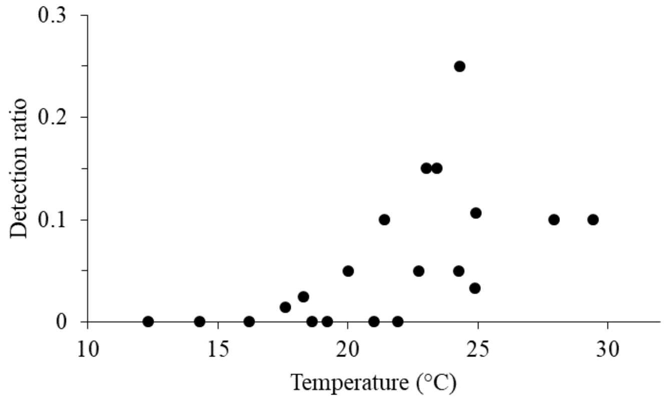

Figure 5. Detection of froglets of *Dryophytes suweonensis* at the release site in relation to air temperature. No individuals were seen at temperatures below 17.6 °C, and the maximum number of individuals were observed between 23 and 30 °C.

around the rice paddy was mowed subsequent to the emergence of froglets in June 2016, where all froglets and metamorphs had been surveyed. This also resulted in the "clean-up" of the site, with the hibernation sites (wood and rock stacks; Figure 2) removed, and the rice straws burnt on top of the "natural compost pile" where individuals were last sighted in autumn 2016. Some dead trees were also replaced for "maintenance" of the site. A single calling male was detected on 25 May 2017, not in the rice paddy but in the flooded forest south of the rice paddy (Figure 2), following heavy rains and abnormally high water levels in the reservoir. No further frogs were recorded throughout 2017.

DISCUSSION

Understanding the ecology and evolution of an endangered species is vital for its effective protection and conservation (Spielman *et al.* 2004b). Here, we demonstrated that translocation of *Dryophytes suweonensis* froglets has the potential to succeed, with both sexes reaching sexual maturity, and males producing advertisement calls within a year.

However, research and management teams need adequate and constant communication, with joint effort and participation to ensure the effectiveness of this type of project. Here, the site no longer fulfilled the ecological requirements of the species following management mistakes, which resulted in the failure to establish a new population. Nevertheless, we argue that this project had other positive impacts, as knowledge of the ecology of the species was improved.

Numerous *in situ* as well as *ex situ* conservation programmes for amphibians exist (Zippel *et al.* 2011), mostly driven by population decline over the last decades (Stuart *et al.* 2004). However, the setting of a conservation programme requires *a priori* knowledge of the genetic landscape of the focal species, in order to conserve genetic diversity and allow for genetic exchange (Yan *et al.* 2013).

In this regard, the selection of the translocation site was considered an adequate choice because it established a stepping-stone population between the disconnected populations south and north of Seoul (Borzée *et al.* 2015), and was within the range of ecological preferences of the species. As another consideration for further projects of this type, we recommend prior assessment of the presence of potential predators. During this project, a ground skink *Takydromus* spp. was seen catching and eating one of the released individuals within 10 seconds of release. It is likely there was additional predation on the released froglets, as tiger keelback snakes, *Rhabdophis tigrinus,* were also found at the site.

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