eDNA-based detection of a vulnerable crocodile newt (*Tylototriton uyenoi*) to influence government policy and raise public awareness

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**Abstract**

**Aim:** Although at least five *Tylototriton* species were recorded in Thailand, only *Tylototriton verrucosus* is registered as a protected species under the Wildlife Preservation and Protection Act in Thailand. Populations of *T. uyenoi* are now severely declining, caused by anthropogenic activities. As intense human pressure is having profound effects on the diminishment in *T. uyenoi* populations, a conservation plan is needed. Information such as the abundance and distribution of a species is necessary. Yet, current established survey methods are either time-consuming or labour-intensive. Here, eDNA-based detection was developed for tracking the presence of the *T. uyenoi*.

**Location:** Thailand.

**Methods:** We target the surveillance of *T. uyenoi* using eDNA. Primers and a probe specific to *T. uyenoi* were designed and tested for their specificity and sensitivity. Water samples were collected once a month from August to January at three sites in Doi Suthep and at three extra sites within the range of the species. Three hundred ml samples of water were collected and filtered. Environmental DNA was extracted and then subjected to qPCR assay in an attempt to detect *T. uyenoi*.

**Results:** The qPCR assay was found to be species-specific to *T. uyenoi*. Both PCR and qPCR did not result in any positive detection of three congeners or other non-target species. The LOD and the LOQ of the assay were determined by an analysis of the standard curve, and it was found that the LOD and the LOQ were 7.99 and 9.0 copies per reaction, respectively. Environmental DNA was detected in water samples from all sites where *T. uyenoi* has been known to occur, but detection varied among sites and sampling times. In addition, low amounts of eDNA were detected in three sites with unknown occupancy of newts, but within the species’ range.

**Conclusions:** Our findings suggest that eDNA survey is a powerful tool for tracking *T. uyenoi* throughout the year regardless of the sampling site conditions. Similar to other amphibians, *T. uyenoi* is severely declining due to anthropogenic factors. In order to have an effective conservation plan, knowledge of a species’ distribution is needed. To our knowledge, this was the first study that used eDNA to track the crocodile newt in Thailand.

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INTRODUCTION

Amphibians are facing massive population declines (Stuart et al., 2004; Wake & Vredenburg, 2008). Amphibians play a major role in ecosystems, and they are critical components of both aquatic and terrestrial communities. Thus, anthropogenic factors that negatively affect amphibians could influence entire ecosystems. Both direct and indirect anthropogenic activities such as habitat modification, overexploitation, exotic species introductions, emerging pathogens, chemical pollutants and climate change are all proposed causes for amphibian declines (Anderson 2019; Wake & Vredenburg, 2008). As with other amphibians, humans are the main reason behind an unparalleled decline in newt populations. Illegal trading, habitat alteration (loss) and hunting for food or medicine are major causes of local declines of the *Tylototriton* newts. The genus *Tylototriton* currently contains at least 19 species/subspecies of newts native to Southern and Southeast Asia (Bernardes et al., 2020; Nishikawa, Khonsue et al., 2013; Nishikawa, Matsui et al., 2013; Phimmachak et al., 2015). Many of them are now recognized as threatened species by the International Union for Conservation of Nature (IUCN) Red List. In Thailand, *Tylototriton verrucosus* has long been documented as the only newt. Recently, three new *Tylototriton* were described: *Tylototriton uyenoi*, *Tylototriton panhai* and *Tylototriton phukhaensis* (Nishikawa, Khonsue et al., 2013; Pomchote et al., 2020). Here, we focus on the *T. uyenoi* or Chiang Mai crocodile newt.

Although three *Tylototriton* are listed in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), only *T. verrucosus* is registered as a protected species under the Wildlife Preservation and Protection Act in Thailand. Unofficial surveys of *T. uyenoi*, the Chiang Mai crocodile newt, in Doi Suthep-Pui and Doi Inthanon National Park, reveal that over the past few years *T. uyenoi* have been severely declining, with causes ranging from habitat loss to being hunted for food by locals. As only *T. verrucosus* is protected by law, the risk of *T. uyenoi* disappearing from the areas is high.

In order to develop effective conservation interventions, information on the abundance and distribution of imperilled species is necessary. Species monitoring is therefore needed. Yet, currently established survey methods are either time-consuming or labour-intensive. Detecting a species’ occurrence, decline and extinction is considerably challenging due to the high level of effort and cost associated with the assessment and monitoring of biodiversity. The advancement of molecular genetic methods has dramatically improved the reliability, portability and widespread application of biomonitoring with environmental DNA (eDNA) in the past decade. The eDNA analysis is based on the retrieval of genetic material naturally released by organisms in their environments such as water, soil and air (Ficetola et al., 2008). Two main applications of eDNA use are (1) to gain an insight of species richness, estimates of relative abundance and quantification of biodiversity using next-generation sequencing and (2) to target surveillance of a single-specific species using conventional PCR, quantitative PCR (qPCR) or digital PCR (ddPCR). The second application, targeted surveillance, is useful for our focal species. Environmental DNA-based species detection has already proven valuable in tracking various organisms including rare, elusive, endemic and endangered species (e.g., Dejean et al., 2012; Fukumoto et al., 2015; Katz et al., 2020; Pilliod et al., 2013; Preißler et al., 2018). As with many other amphibians, life history characteristics of salamanders and newts, including small population sizes and variation in annual reproductive success, can have an effect on detectability. Thus, different sampling techniques are found to have varying effectiveness (Bevelhimer et al., 2008; Farmer et al., 2009; Gunzburger, 2007; Liner et al., 2008; Parris et al., 1999; Smith et al., 2006). In addition, conditions at the sampling sites also affect the choice of sampling method (Smith et al., 2006). Recent studies have demonstrated the potential of eDNA for detecting salamanders and newts, which reduce those limitations of other survey methods. Some work has demonstrated superior sensitivity and effectiveness of the eDNA survey over physical capture or visual observation (Katano et al., 2017; McKee et al., 2015; Rees et al., 2014). Environmental DNA is useful for detecting not only adults but also larvae (Bevelhimer et al., 2008; Preißler et al., 2018). Here, the eDNA survey and monitoring of Chiang Mai crocodile newt (*T. uyenoi*) were developed, which can be incorporated into a conservation plan for monitoring of the species.

MATERIALS AND METHODS

2.1 Ethics Statement

This study was approved by the Animal Care and Use Committee Review (the Laboratory Animal Center Chiang Mai University) under Protocol Number: 2561/FA-0001.

2.2 qPCR assay

To design primers and a probe specific to Chiang Mai crocodile newt (*T. uyenoi*), the partial NADH dehydrogenase subunit 2 region (ND2) of the mitochondrial DNA (mtDNA) region of six individuals was sequenced. *Tylototriton uyenoi* were caught from Doi Suthep (three individuals) and from Doi Inthanon (three individuals). All the DNA analysed originated from the mucus of the individual.
T. uyenoi. The animals were released back into nature after mucus collection. Total DNA was extracted from the mucus sample using the DNeasy Blood and Tissue Kit (Qiagen) according to the manufacturer’s protocol. Mucus DNA concentrations were determined using the Qubit dsDNA HS Assay (Invitrogen). Extracted DNA was first used as a template for sequencing with the primers Sal_ND2_F1: 5’-AAGCTTTGGGCCCCATACC-3’ and Sal_ND2_R2: 5’-GGTGGCATCCAGAAGATGTG-3’ both of which were from previously reported primers (Nishikawa, Khonsue et al., 2013).

Species-specific primers and a minor-groove binding (MGB) probe incorporating a 5’ FAM reporter dye and a 3’ non-fluorescent quencher were then designed based on the consensus sequences generated from this study and the public database to amplify a 116-bp amplicon targeting the ND2 region for the T. uyenoi, using Primer Express (V3.0, Life Technologies; Table 1). Probe and primer sequences were matched against the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) nucleotide database with BLASTn (Basic Local Alignment Search Tool) to confirm the species’ specificity for the T. uyenoi in in silico assays.

The qPCR assay was deployed using Environmental Master Mix (Applied Biosystems) on the mucus samples from the T. uyenoi and non-target species including Amolops marmoratus, Limnonectes taylori, Limnonectes kuhlii and Megophrys parva to ensure that the assay only amplified the T. uyenoi. Also, the qPCR assay was carried out using synthetic fragments of T. anguliceps, T. panhai and T. verrucosus. As we had not yet been able to obtain these vulnerably Tylototriton species, the synthesised target DNA was designed based on sequences retrieved from GenBank, Accession Numbers LC505611, MK097271 and KT304276 for T. anguliceps, T. panhai and T. verrucosus, respectively. In addition, water containing DNA extracted from mucus (concentration 0.5 ng/µl) was included as a positive control for the presence of amplifiable eDNA in water samples.

All eDNA qPCR amplifications were performed using a Rotor-Gene Q (Qiagen) in three replicates in a final volume of 20 µl, using 10.0 µl of 2× TaqMan Environmental Master Mix 2.0 (Thermo Fisher Scientific), 2.0 µl of DNA template, 900 nM each of the F/R primers and 125 nM of the probe. Samples were run under the following conditions: an initial 10 min of incubation at 95°C followed by 50 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Negative controls with all PCR reagents but no template (three replicates) were run in parallel to assess potential contamination. The quantification cycle (Cq) was converted to quantities per unit volume using the linear regression obtained from the synthesised target gene standard curve (Integrated DNA Technologies Pte. Ltd.). The T. uyenoi eDNA concentrations were then reported as copies/ml. The limit of detection (LOD) and the limit of quantification (LOQ) were also measured using the standard dilution series of synthesised target gene fragment with known copy numbers. The concentrations of the standards were adjusted to 15,000, 1,500, 150, 15 and 1.5 copies per reaction with 13 technical replicates used for each of the dilution steps. The calculation of LOD and LOQ was done using published R script by Klymus et al. (2020).

### 2.3 | eDNA field collection

Water samples were collected in 2017 and 2018, once a month from August to January at three sites in Doi Suthep including site (1) artificial pond, site (2) artificial pond and site (3) natural pond. In addition, water from three extra sites with an unknown occupancy of T. uyenoi but located within the species’ range was collected (August to October). Each site was sampled in triplicate, and 300 ml samples of water were collected and filtered on GF/F filter (0.7 µm; Whatman International Ltd.). Filtering control (300 ml of distilled water) was filtered as a last sample on each day of sample filtration. All filters were folded inwards, placed in 2-ml tubes and stored at −20°C until DNA extraction, which took place within 48 hr. To avoid contamination, all field equipment was sterilized using 10% bleach, UV crosslinker or autoclaved and sealed prior to transport to the study site, and a separate pair of nitrile disposable gloves was used for each sample.

### 2.4 | DNA extraction from the filters

DNA trapped on the filters obtained from field collections was extracted using Qiagen DNeasy Blood and Tissue Kit (Qiagen) using a protocol modified from the manufacturer’s protocol with the following changes: the DNA from all samples was eluted twice with 25 µl AE buffer, in a total volume of 50 µl to obtain a more concentrated eDNA solution. The volumes of ATL buffer (360 µl), proteinase K (40 µl), AL buffer (400 µl) and ethanol (400 µl) were doubled.

### 2.5 | Statistical analysis

An ANOVA was used to determine whether the collection site or sampling month had had any statistically significant effects on DNA concentration. Statistical analyses were conducted in R version 3.3.1 (R Core Team 2017).

| Primer name | Type | Length (bp) | Primer sequence 5′-3′ |
|-------------|------|-------------|----------------------|
| Tuy-F       | Forward primer | 25 | CTAGGCTTTTCTTCTACCTACGCG |
| Tuy-R       | Reverse primer  | 23 | GGTTGTTGGTTATAGGTTGGGTTG |
| Tuy-P       | Probe          | 13 | CCACCGCGCCAAA |

**TABLE 1** Details of species-specific primers and the probe designed to amplify a 116-bp fragment of the ND2 region of T. uyenoi
3 | RESULTS

3.1 | Species-specific primer design

The partial ND2 region sequences of the T. uyenoi from two locations in Chiang Mai were generated and deposited in GenBank (Accession Numbers MG018993 and MG018994). The sequences obtained from this study and GenBank were aligned and analysed (Table 2). There are seven variable sites found within 497 bp analysed ND2 fragments of T. uyenoi. Nucleotide variations were also found between the ND2 fragment of the individual from Doi Suthep and Doi Inthanon of this study. Regions of intraspecific variation were then avoided when designing the specific primers and probe.

Primers that amplified a short region of 116 bp specific to the T. uyenoi were designed. To determine specificity, the designed primers and probe were tested in silico and in vitro. The specificity of the generated primers and probe was checked against the NCBI database by comparing the similarity of primers to related species (T. uyenoi, T. podichthys, T. lizhenchang, T. notialis, T. shanjing, T. shanorum, T. verrucosus, Amolops marmoratus, Duttaphrynus melanostictus and Polypedates leucomystax). The homology of Tytlotriton sequences to the specific forward and reverse primers of T. uyenoi was around 80-85% with at least one mismatch on the forward and four mismatches on the reverse primers (Table 3). When comparing with other amphibian species found in Doi Suthep, the homology was found to be lower (51%-62%). The primers designed were found to be specific to T. uyenoi after testing with other Tytlotriton found in Thailand (T. anguliceps, T. panhai and T. verrucosus) and other non-target species (Table 4). The same result was achieved when the probe was added for use in qPCR (Table 4) and resulted in the single detection of the target species, T. uyenoi.

3.2 | qPCR assay

The assay designed in this study was found to be species-specific to T. uyenoi. Both PCR and qPCR did not result in any positive detection of the non-target species. The LOD and the LOQ of the assay were determined by an analysis of the standard curve (slope = −3.568, Y inter = 42.376, R² = 0.988, Eff% = 90.664) generated from a dilution experiment under laboratory conditions. The LOD was 7.9 copies per reaction, and the LOQ was 9.0 copies per reaction.

3.3 | Field collection samples

Previous traditional surveys of T. uyenoi in Doi Suthep, Chiang Mai, report the presence of the Chiang Mai crocodile newt in two artificial ponds (sites 1 and 2) and one natural pond (site 3). Thus, water samples were collected from the three reported sites once a month from August 2017 to January 2018. However, during the eDNA sampling, the T. uyenoi were only observed at site 1 throughout the sampling period. On the other hand, none of the T. uyenoi were found at sites 2 and 3, although the eDNA of the T. uyenoi was detected in water samples from all three collection sites using the qPCR assay. No amplification in the negative controls was detected.

eDNA concentration of the T. uyenoi was found to vary among the sites and sampling months (Figure 1). An average eDNA concentration of 2.07 copies/ml at site 1 (range: 0.43–4.79), 2.10 copies/ml at site 2 (range: 0.68–4.80) and 2.05 copies/ml at site 3 (range: 0.30–4.95) with no difference during the sampling month (Figure 1). In contrast, a significant difference was found in measured eDNA concentrations between the months. Concentration of the T. uyenoi found in collecting sites ranges from around 3.42 to 4.95 copies/ml in August, 2.65 to 3.52 copies/ml in September, 1.04 to 3.84 copies/ml in October, 0.30 to 1.27 copies/ml in November, 0.49 to 3.19 copies/ml in December and 0.43 to 0.93 copies/ml in January. It was found that the respective sampling month significantly affected average DNA concentrations of the water samples, and the interaction of collecting site and month was also statistically significant (month: p < 0.01, site: p = 0.92, month × site: p = 0.47). Low amounts of T. uyenoi eDNA were also detected in water taken from the three extra sites with unknown occupancy of the T. uyenoi.

| TABLE 2 | Nucleotide variable sites found in ND2 sequences of T. uyenoi from different localities |
|----------------|----------------|----------------|----------------|
| **Species** | **Locality** | **GenBank** | **Nucleotide variable site** |
| | | | C | G | G | A | C | C | T |
| T. uyenoi | Doi Suthep, Chiang Mai, Thailand | MG018994 | • | • | • | • | • | • | This study |
| T. uyenoi | Doi Suthep, Chiang Mai, Thailand | AB830733 | • | • | • | G | • | • | Nishikawa et al. (2013) |
| T. uyenoi | Doi Suthep, Chiang Mai, Thailand | AB830732 | • | • | • | G | • | • | Nishikawa et al. (2013) |
| T. uyenoi | Doi Inthanon, Chiang Mai, Thailand | MG018993 | T | A | • | • | • | T | A | This study |
| T. uyenoi | Doi Inthanon, Chiang Mai, Thailand | AB830730 | T | A | • | • | • | T | • | Nishikawa et al. (2013) |
| T. uyenoi | Doi Inthanon, Chiang Mai, Thailand | AB830731 | T | A | • | • | T | T | • | Nishikawa et al. (2013) |
4.1 eDNA survey

As eDNA is thought to rapidly degrade into short fragments, PCR primer sets for eDNA studies should be designed to yield short amplicons (100–180 bp) (Dejean et al., 2011). Also, it is noteworthy that short-amplicon PCR assays have high detection sensitivity (Huver et al., 2015). The specific primers for the *T. uyenoi* generated in this study were a desirable length of 116 bp and thus suitable for an eDNA assay. Also, as intraspecific nucleotide variation was found, the primers and probe were designed outside those variable regions. This would make sure that the eDNA of *T. uyenoi* will be amplified across its range.

*Tylototriton uyenoi* eDNA was found to be varied between sampling months. Highest eDNA concentrations were found in August with a reduction throughout the other sampling months (from August to January), being lowest in January. Breeding season and larvae abundance may also affect on the eDNA concentration (Buxton et al., 2017). Increased eDNA levels of great crested newts (*Triturus cristatus*) were found at the end of breeding season (early June). Similarly, in our study, eDNA was also detected at high concentration in July–August when the sampling ponds have high occupancy by larvae.

Previously published work on *T. uyenoi* in Thailand is limited to systematic and diet studies. Because amphibian detectability can vary among conventional, capture-based survey methods due to life history and population size, alternative survey methods are needed (Bevelhimer et al., 2008; Liner et al., 2008; Smith et al., 2006; Strain et al., 2008). The findings here show that eDNA-based survey is useful for tracking the presence of the Chiang Mai crocodile newt. To our knowledge, this was the first study, which had been carried out in Thailand. In this study, eDNA was proved to be an alternative method for rapid and sensitive surveys.

### TABLE 3
Homology of the query to the forward and reverse primers and probe, and percentage identity as a function of the number of matching base sites divided by 61 (total number of base sites across the primer pair and probe). Base site homology between the query and the primer is shown as a dot.

| Species                  | Forward                                      | Reverse                                      | Probe                                      | Identity (%) | GenBank      |
|--------------------------|----------------------------------------------|----------------------------------------------|--------------------------------------------|--------------|--------------|
| *Tylototriton uyenoi*    | ..................................................  | ..................................................  | .................................................. | 100          | AB830732     |
| *Tylototriton panhai*    | ........................G..........................  | ..................................................  | .................................................. | 85           | AB830737     |
| *Tylototriton podichthys*| .........A........A..........................  | .........A........A..........................  | .........A........A.......................... | 80           | KT304280     |
| *Tylototriton lizhenchang* | .........G.......................... | .........G.......................... | .........G.......................... | 85           | KY800882     |
| *Tylototriton notialis*  | .........A.......................... | .........A.......................... | .........A.......................... | 80           | KY800884     |
| *Tylototriton shanjing*  | .........A.......................... | .........A.......................... | .........A.......................... | 82           | KY800890     |
| *Tylototriton shanorum*  | C.......................... | C.......................... | C.......................... | 84           | AB922823     |
| *Tylototriton verrucosus* | .........A.......................... | .........A.......................... | .........A.......................... | 82           | AB830738     |
| *Amolops marmoratus*     | .........A.......................... | .........A.......................... | .........A.......................... | 51           | FH17205      |
| *Duttaphrynus melanostictus* | .........A.......................... | .........A.......................... | .........A.......................... | 62           | KY555677     |
| *Polypedates leucomystax* | .........A.......................... | .........A.......................... | .........A.......................... | 61           | DQ287056     |

### TABLE 4
Results of PCR and qPCR using the primers and probes targeting the ND2 region of *Tylototriton uyenoi* on seven different species.

| Species                  | PCR | qPCR |
|--------------------------|-----|------|
| *Tylototriton species*   |     |      |
| *T. uyenoi*              | ✓   | ✓    |
| *T. anguliceps*          | x   | x    |
| *T. panhai*              | x   | x    |
| *T. verrucosus*          | x   | x    |
| *Non-target species*     |     |      |
| *Amolops marmoratus*     | x   | x    |
| *Limnonectes taylori*    | x   | x    |
| *Limnonectes kuhlii*     | x   | x    |
| *Megophrys parva*        | x   | x    |

4.2 Where could the eDNA-based survey stand in the conservation action plan?

Responding to the Amphibian Conservation Summit’s response to the amphibian crisis worldwide, the Amphibian Conservation Action Plan (ACAP) was designed and first announced in 2007 (Gascon et al., 2007). Eleven relevant actions for global amphibian conservation were included in ACAP. Since then, although updated in 2015, questions and gaps remain that should be taken into consideration for effective conservation (Wren et al., 2015). For example, for reintroduction, one of the eleven proposed actions to take place, it is essential that species are carefully appraised for their suitability for reintroduction so that status and distribution of the species are required. However, due to highly diverse range of habitat requirements and life history traits displayed by amphibians, conventional monitoring methods such visual
observation are commonly ineffective. In addition, to prevent further habitat loss, the effects of anthropogenic perturbations on amphibian populations are another big question that needs to be addressed. Traditional field survey cannot provide up-to-date information, which would normally help to answer the question. Environmental DNA poses potential in filling these gaps and also could provide answers to these questions.

In Thailand, studies of Tylototriton species population dynamics, abundance, geographical distribution and the collection of monitoring data are all seriously lagging behind and there is a lack of standardised investigation and monitoring procedures. To date, there are not many studies on the Tylototriton, of which only one focusses on distribution (Hernandez et al., 2019). As mentioned earlier, although three Tylototriton species are listed in CITES, only T. verrucosus is gaining attention in Thailand right now. Notably, T. uyenoi have been severely declining, with causes ranging from habitat loss to being hunted for food by locals. Using eDNA-based monitoring of the species seems necessary for striving towards policy impact by awareness raising, as many studies have shown that eDNA could be faster and more cost-effective in assessing biodiversity. The Wildlife Preservation and Protection Act B.E. 2535 (1992) has been in operation for nearly 30 years, yet is ineffective as a low fine is the only penalty incurred for the possession and hunting of wildlife. Until late 2019, three new acts were published in the Government Gazette to update existing laws and introduce new regulations related to the protection and conservation of the environment, which came into effect on November 2019. Under the new act, the Wildlife Preservation and Protection Act B.E. 2562 (2019), wildlife trading, possessing and hunting are punishable by substantially increased prison terms, fines or both. However, none of additional Tylototriton species (only T. verrucosus is protected by law since the previous act) were added to the list of preserved wildlife. All Tylototriton species found in Thailand are locally rare and occur in low abundance in their natural environments. Therefore, the possibility of monitoring the species composition and distribution in near real-time using conventional approaches is low. Lack of this information could be the reason why other Tylototriton were still not added to the list. Environmental DNA provides previously unattainable insight into communities and ecosystems, aiding our understanding of them and has the capacity to bolster current monitoring techniques. Therefore, results or data from an eDNA survey would be informative and of particular use in influencing government policy.

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DATA AVAILABILITY STATEMENT
The data that support the findings of this study were derived from the following resource available in the public domain: GenBank www.ncbi.nlm.nih.gov/genbank

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REFERENCES
Anderson, R. B. (2019). Human traffic and habitat complexity are strong predictors for the distribution of a declining amphibian. PLOS ONE, 14(3), e0213426. https://doi.org/10.1371/journal.pone.0213426.
Dejean, T., Valentini, A., Duparc, A., Pellier-Cuit, S., Pompanon, F., Buxton, A. S., Groombridge, J. J., Zakaria, N. B., & Griffiths, R. A. (2017). Evaluation of seven aquatic sampling methods for amphibians at Okefenokee National Wildlife Refuge. *American Midland Naturalist*, 175(1), 149–161. https://doi.org/10.1674/0003-0031.

Parris, K. M., Tony, W. N., & Cunningham, R. B. (1999). A comparison of techniques for sampling amphibians in the forests of South-East Queensland, Australia. *Herpetologica*, 55(2), 271–283.

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., & Gough, K. C. (2014). REVIEW: The detection of aquatic animal species using environmental DNA—A review of eDNA as a survey tool in ecology. *Journal of Applied Ecology*, 51(5), 1450–1459. https://doi.org/10.1111/1365-2664.12306.

Smith, L. L., Blust, R., Rees, H. C., and Minamoto, T. (2015). Amphibian Conservation Action Plan. IUCN SSC Amphibian Specialist Group. Retrieved from https://www.iucn-amphibians.org/resources/acap/.

Gunzburger, M. (2007). Evaluation of seven aquatic sampling methods for amphibians and other aquatic fauna. *Applied Herpetology*, 4, 47–63. https://doi.org/10.1163/15705740777966750.

Hernandez, A., Escoriza, D., Pomchote, P., and Hou, M. (2019). New localities of *Tylototriton uyenoi* and *T. anguliceps* in streams of Maryland. *Environmental Monitoring and Assessment*, 156(1-4), 1-16. https://doi.org/10.1007/s10661-008-0459-3.

Strain, G. F., Raesly, R. L., and Hilderbrand, R. H. (2008). A comparison of techniques to sample salamander assemblages along highland streams of Maryland. *Environmental Monitoring and Assessment*, 161(1), 1-16. https://doi.org/10.1007/s10661-008-0459-3.

Preißler, K., Watzal, A., Vences, M., and Steinfartz, S. (2018). Detection of elusive fire salamander larvae (*Salamandra salamandra*) in streams via environmental DNA. *Amphibia-Reptilia*, 40(1), 55–64. https://doi.org/10.1163/15685381-18000007.

R Core Team. (2017). *R: A Language and Environment for Statistical Computing*. Retrieved from https://www.R-project.org/.

M., Piaggio, A. J., Stokdyk, J. P., Wilson, C. C., & Richter, C. A. (2020). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, 2, 271–282.

McKee, A. M., Calhoun, D. L., Barichivich, W. J., Spear, S. F., Goldberg, C. S., & Glenn, T. C. (2015). Assessment of environmental DNA for detecting presence of imperiled aquatic amphibian species in isolated wetlands. *Journal of Fish and Wildlife Management*, 6(2), 498–510. https://doi.org/10.3897/jfwm.042014/FWJM-034.

Nishikawa, K., Khonsue, W., Pomchote, P., and Matsu, M. (2013). Two new species of *Tylototriton* from Thailand (Amphibia: Urodela: *Salamandridae*). *Zootaxa*, 3737, 261–279. https://doi.org/10.11646/zootaxa.3737.3.5.

Nishikawa, K., Matsu, M., and Nguyen, T. T. (2013). A new species of *Tylototriton* from Northern Vietnam (Amphibia: Urodela: *Salamandridae*). *Current Herpetology*, 32(1), 34–49. https://doi.org/10.5358/hsj.32.34.
**BIOSKETCHES**

**Maslin Osathanunkul** keens on using molecular techniques as tool for study in various fields, for example biodiversity, agriculture, environment, alternative medicine and industry.

**Toshifumi Minamoto** mainly research on the ecology and eco-physiology in aquatic environments by combining the conventional and new techniques such as animal physiology and environmental DNA.

Author contributions: M.O. conceived the project, designed the experiments, collected samples, performed the experiments and analysed the data. M.O. and T.M. participated in data interpretation and discussed the experiment. M.O. wrote the paper with contributions from all authors, and all authors approved this manuscript.

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