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RESEARCHING THE DIVERSITY OF SOME AMPHIBIAN SPECIES AND THEIR FUNGAL INFECTION STATUS IN VIETNAM

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INTRODUCTION

1. The urgency of the thesis

Amphibians are a class of animals with a very diverse number of species with about 8,700 species widely distributed across the world except for the Arctic and Antarctic regions. According to the International Union for Conservation of Nature, as of December 2023, there were 7,486 amphibian species assessed for conservation status, of which 36 species were recorded as extinct and 2 species were extinct in the wild, 722 species rated as Critically Endangered, 1,144 Endangered species, 406 Vulnerable species, 740 Near Threatened species, 3,291 Least of Concern species and 1,145 species rated as Data Deficient.

Vietnam's amphibian fauna is diverse in species composition with many new species described every year. By 2009, the number of recorded amphibian species had increased to 186 species and most recently the number of recorded amphibian species had increased to 295. In particular, there were more than 70 new species described based on samples collected in Vietnam since 2010 until now.

Chytridiomycosis is an infectious disease caused by a fungus with the scientific name *Batrachochytrium dendrobatidis*, known to cause disease in aquatic species and has lead to rapid population decline or extinction of at least about 200 amphibian species. Recently, a new strain of fungus with the scientific name *Batrachochytrium salamandrivorans* was discovered.

It is extremely urgent to provide information about pathogenic fungal infections as well as to be able to provide solutions, forecasts, and prevention, thereby minimizing their consequences in the future. Based on the urgency of the research issues, we carried out the project **"*Researching the diversity of some amphibian species and their fungal infection status in Vietnam*"**

2. Research objectives of the thesis

+ Assess the diversity of amphibian species belonging to the families such as Firebelly Toad (Bombinatoridae), Asian Toads (Megophryidae), Tree Frog (Rhacophoridae) and Newts (Salamandridae) in Vietnam.

+ Evaluate the status of pathogenic fungal infections in amphibians belonging to the Bombinatoridae, Megophryidae, Rhacophoridae and Salamandridae in Vietnam.

3. Main research contents of the thesis:

- Assessing the diversity of amphibian species belonging to the Bombinatoridae, Megophryidae, Rhacophoridae and Salamandridae in Vietnam.

- Determining disease-causing fungal infections in amphibians belonging to the Bombinatoridae, Megophryidae, Rhacophoridae and Salamandridae in Vietnam.

Chapter 1. OVERVIEW

1.1. Brief history of research on amphibian diversity in the world

The number of identified amphibian species in the world increased significantly from 6,300 species in 2010 to 7,480 species in 2015 and nearly 8,700 species at present .

According to statistics from AmphibiaWeb, during the period from 2005 to 2022, there were about 2,749 new amphibian species described to science.

Within the framework of this research, we only briefly outline the research situation in the direction of the topic in countries bordering Vietnam:

In China: Zhao & Adler (1993) recorded 274 amphibian species. Yang & Rao . (2008) described 115 amphibian species recorded in Yunnan province. The number of Chinese amphibian species has increased to 370 species in the publication of the. Currently, 590 species have been recorded in China.

In Laos: The number of amphibian species increased from 58 species in the publication of Stuart et al. (1999) to about 120 species at present.

In Cambodia: Researches focus mainly on the Cardamom Mountains in Southern Cambodia such as: Ohler et al. 2002 recorded 34 species; Grismer et al. (2008) recorded 41 species. Stuart et al. (2006) recorded 30 species in the mountainous area of Eastern Cambodia, bordering Vietnam, and Hartmann et al. (2013) recorded 22 species in the northwestern region of Cambodia. Currently, Cambodia records about 152 amphibian species .

1.2. Brief history of research on amphibian diversity in Vietnam

1.2.1. Studies on species composition

Research by Nguyen Van Sang et al. (2009) on amphibians in Vietnam has a long history. The book *Les Batraciens de l'Indochine* described 171 species and subspecies of frogs in the Indochina region.

In 1977, Dao Van Tien published the identification key for 87 amphibian species. Research by Tran Kien et al. (1981) listed the composition of animal species in Northern Vietnam (1955-1976), including 69 amphibian species. In 1996, Nguyen Van Sang and Ho Thu Cuc recorded 82 species of amphibians. Nguyen Van Sang et al. (2005) reported that there were 162 amphibian species. The most recent and systematic list by Nguyen et al. (2009) recorded a total of 176 amphibian species in Vietnam.

Research since 2015 up to now has recorded and described 38 new amphibian species with standard samples collected in Vietnam.

In the Northwest region: Typical studies in the past 10 years such as: Pham Van Anh et al. (2017) recorded 16 species belonging to 12 genera, 6 families, 1 order in the Pha Din Pass area of Dien Bien and Son La provinces. Nguyen Quang Truong et al. (2017) studied amphibian composition in Muong Bang, Phu Yen, Son La province and recorded 22 species belonging to 15 genera, 6 families, 1 order. Research by Tran Van Huy et al. (2018) recorded two new species of stream frog, bringing the total number of species recorded for Lai Chau province to 24 species. Research by Pham Van Nha et al. (2018) recorded 12 amphibian species belonging to 8 genera, 5 families and 1 order in Tong Lanh forest area, Thuan Chau district, Son La province. Pham Van Anh and Nguyen Quang Truong (2019) announced the composition of 14 amphibian species belonging to 6 families in the Pu Buu Commune Forest area, Song Ma district, Son La province. Pham Van Anh et al. (2019) recorded the composition and distribution characteristics according to habitat of 36 amphibian species in Muong Do commune, Phu Yen district, Son La province. Pham Van Anh et al. (2022) recorded the composition of 43 amphibian species in Sop Cop Nature Reserve, Phu Yen district, Son La province.

In the Northeast region: Studies focus on high mountain areas or protected areas and national parks such as: Pham et al. (2017) recorded two additional frog species (*Rhacophorus kio* and *R. rhodopus*) for Ha Giang province, bringing the total number of amphibian species recorded in this province to 54 species and reptiles to 57 species. Luong Mai Anh et al. (2019) researched in Than Sa-Phuong Hoang Nature Reserve, Thai Nguyen province, recorded 16 amphibian species, including 4 newly distributed

species for this province, increasing the total number of amphibian species recorded for the reserve. This includes 26 amphibian species, ... Pham et al. (2020) recorded 27 amphibian species, of which 10 species were recorded for the first time in the border area with China in Hai Ha district, Quang Ninh province. Luong et al. (2022) researched and recorded 32 amphibian species distributed in Ban Thi-Xuan Lac Nature Reserve, including 8 additional species recorded for Bac Kan province.

In the Northern Delta region: Ziegler et al. (2015) studied the amphibian and reptile fauna at Me Linh Biodiversity Station and recorded 22 amphibian species belonging to 7 families and 2 orders. Le Trung Dung et al. (2016) published a list of amphibian species in Van Long Wetland Nature Reserve, Ninh Binh province with 17 species belonging to 11 genera, 6 families and 1 order. Hoang et al. (2022) recorded a new distribution area of Mangrove Frog (*Fejervarya moodiei*) in Nam Dinh and Thai Binh provinces.

Northern Truong Son region: Dau Quang Vinh et al. (2016) recorded 6 species of 5 tree frog genera in Pu Luong Nature Reserve, Thanh Hoa province. Nguyen et al. (2016) recorded 16 frog species in Bac Huong Hoa Nature Reserve, Quang Tri province. Mr. Vinh An et al. (2016) recorded 9 species of 4 tree frog genera in Pu Huong Nature Reserve, Nghe An province. Do Van Thoai et al. (2017) recorded two additional species of the Megophridae family in Nghe An province. Pham The Cuong et al. (2019) studied in the Dong Chau Protection Forest area, Quang Binh province and recorded 30 amphibian species.

Central Truong Son region: Research by Duong Duc Loi et al. (2016) provides a list of 9 frog species belonging to the Ranidae family in Binh Dinh province. Nguyen Thanh Luan et al. (2016) recorded 5 additional species of Leptobrachella for Bach Ma National Park, Thua Thien-Hue province, bringing the total number of species recorded to 51 species. The most recent study by Do et al. (2018) recorded 8 additional amphibian species, bringing the total number of species in Phu Yen province to 33 species. Pham Hong Thai et al. (2019) updated the list of 19 amphibian species, bringing the total number of species to 52 belonging to 8 families and 2 orders in Ba Na-Nui Chua Nature Reserve, Da Nang province.

Southern Truong Son region: According to Nguyen Thanh Luan et al. (2017) initially published a list of amphibian species in Hon Ba Nature Reserve, Khanh Hoa province with 35 species belonging to 6 families and 12 genera. Cao Tien Trung et al. (2019) recorded 6 species of the Ranidae family in Nui Ong Nature Reserve, Binh Thuan province.

The number of new species providing scientific data and records for Vietnam has increased significantly in recent times, the number of species recorded in 2009 was 176 species, by 2023 there are about 301 species.

Studies on the families of Firebelly Toad Bombinatoridae, Megophryidae, Rhacophoridae and Salamandridae in Vietnam.

Studies on the Bombinatoridae family in Vietnam

The Bombinatoridae family records only one species distributed in Vietnam, *Bombina microdeladigitora* Liu, Hu & Yang, 1960.

It has recently confirmed that the Bombinatoridae species distributed in Vietnam and South China is *Bombina microdeladigitora*, while *Bombina maxima* is considered endemic to central China.

Studies on Megophryidae family in Vietnam

The Megophryidae family has recorded 68 species belonging to 9 genera distributed in Vietnam: *Leptobrachella* , *Leptobrachium, Oreolalax, Atympanophrys, Boulenophrys, Brachytarsophrys, Ophryophryn* , *Xenophrys*.

Research from 2015 up to now has 20 species new to science discovered and described with standard samples in Vietnam including: *Leptobrachella* (13 species), *Boulenophrys* (5 species), *Xenophrys* (1 species).

In addition, there are 9 species recorded with new distribution for Vietnam including: *Leptobrachella aerea, L. eo* , *L. melica*, *L. minima*, *L. niveimontis*, *L. yingjiangensis*, *L. shiwandashanensis*, *Leptobrachium lunatum*, *Atympanophrys gigantica*, *Boulenophrys daweimontis* and *Ophryophryne synoria* .

Orlov et al. (2015) described a new species to science, *Megophrys latidactyla* with standard samples collected in Pu Mat Nature Reserve, Nghe An province. This species has similar morphological characteristics to *Megophrys papebralespinosa*. However, Wu et al. (2019) based on morphological and molecular biological data proposed that *Megophrys latidactyla* should be considered a synonym of *M. papebralespinosa*, changes

in swimming membrane shape may be due to changes in the species' breeding season.

Mahony et al. (2017) divided the genus *Megophrys* into 7 subgenera: *Atympanophrys*, *Brachytarsophrys*, *Megophrys*, *Ophryophryne*, *Xenophrys*, *Pelobatrachus*, *Panophrys*. Among them, Vietnam has representatives of 5 subgenera: *Atympanophrys*, *Brachytarsophrys*, *Ophryophryne*, *Xenophrys*, *Panophrys*. The genus *Megophrys* is only distributed in Indonesia and *Pelobatrachus* is only distributed in Singapore, Malaysia, Indonesia and the Philippines. .

The study of Mahony et al. (2018) using molecular biological analysis of *Xenophrys major* pointed out that the Vietnamese specimens were reclassified as species *X. maosonensis*. *X. major* is distributed only in Northeast India .

The Megophryinae subfamily is one of the most diverse amphibian groups, and has long attracted taxonomic attention. However, taxonomy remains controversial, due to similar morphological characteristics. copper. Based on phylogenetic relationships and morphological data Luy et al. (2023) proposed a new classification for 10 new genera for the Asian horned toad subfamily Megophryinae: *Atympanophrys*, *Brachytarsophrys*, *Grillitschia*, *Jingophrys*, *Megophrys*, *Ophryophryne*, *Pelobatrachus*, *Sarawakiphrys*, *Xenophrys*.

Studies on Rhacophoridae family in Vietnam

Smith's (1924) study evaluated the diversity of amphibians in the Langbian highland area and described 6 new tree frog species for Vietnam, including: *Rhacophorus annamensis*, *R. calcaneus*, *Kurixalus gryllus*, *Theloderma laeve*, *Feihyla palpebralis* and *F. vittata*.

According to Bourret (1937), four new tree frog species were described, including: *Philautus maosonensis*, *Zhangixalus dorsoviridis*, *Gracixalus gracilipes*, *Theloderma bicolor*.

Research on species diversity: Currently, the distribution of 86 species belonging to 13 genera in Vietnam is recorded.

Research from 2015 up to now has 18 species new to science discovered and described with standard samples in Vietnam.

Pyron & Wein (2011) analyzed the genetic relationships of amphibians in the world based on more than 2800 species.

Yu et al. (2019) described the new species *Zhangixalus pachyproctus*

based on the molecular biology of the *Z. smaragdinus* complex group, confirming species *Z. smaragdinus* is not distributed in southern China, Laos and Vietnam.

Li et al. (2019) showed that species in the genus *Rhacophorus* sensu lato are divided into three separate genera including: *Rhacophorus* sensu stricto, *Leptomantis* and *Zhangixalus*. There are two varieties: *Rhacophorus* sensu stricto and *Zhangixalus* distributed in Vietnam.

According to Nguyen et al. (2020) evaluated the genetic relationships of tree frogs in the genera *Kurixalus* and *Gracixalus*, providing information to suggest the species *Gracixalus waza* is the synonym of the species *G. nonggangensis*.

Dubois et al. (2021) reassessed the genetic relationships of amphibian species in the world. The study proposed separating the *Vampyrius genus* from the *Rhacophorus genus* with a single species, *V. vampyrus*.

Studies on Salamandridae family in Vietnam

In Vietnam, two varieties are recorded, *Paramesotriton* and *Tylototriton*, with 9 species; *Paramesotriton* genus includes 2 species: *Tylototriton genus* records 7 species distributed in the North from Ha Giang to Kon Tum : *T. anguliceps*, *T . ngoclinensis*, *T . pasmansi*, *T . sparreboomi*, *T. thaiorum*, *T. vietnamensis* , *T. ziegleri*. Some typical studies are as follows:

Zang et al. (2018) decoded the entire mitochondrial genome of *Paramesotriton deloustali* in Vietnam and recorded the distribution area of this species from China.

Bernardes et al. (2020) based on morphological research and genetic analysis on mitochondrial genes, described two new species named *Tylototriton pasmansi* and *T. sparreboomi* in the *T. asperrimus* complex in Vietnam.

Poyarkov et al. (2021) described the species *Tylototriton thaiorum* in Nghe An, previously mistakenly recorded with *T. notialis* (endemic species of Laos).

In particular, recently Phung et al. (2023) discovered a new species named *Tylototriton ngoclinhensis* in Ngoc Linh mountain area, Kon Tum province.

1.3. Brief history of research on fungal diseases in amphibians

1.3.1. Fungal infections in amphibians around the world

Chytridiomycosis (Chytrid) in amphibians is caused by 2 fungal strains of *Batrachochytrium*: *Batrachochytrium dendrobatidis (Bd)* and *B atrachochytrium salamandrivorans (Bsal)*.

In 2014, another strain of amphibian parasitic fungus besides *Batrachochytrium dendrobatidis* became known from Asia and Europe, which is *Bsal*. Martel et al. (2014) showed that the influence of fungi caused the population decline of *Bd* in the Netherlands in 2013.

According to Rodriguez et al. (2014) hypothesize that the origins of the epidemic were recorded in Latin America.

Bletz et al. (2015) studied the widespread occurrence of the pathogenic fungus *Bd* in wild amphibian populations in Madagascar.

Pet trade and other human activities also contribute greatly to the spread of the fungus *Bd*.

1.3.2. Fungal infections in amphibians in Vietnam

Nguyen et al. (2013) surveyed the danger and impact of the fungus *Batrachochytrium dendrobatidis* on Newt species in Vietnam. Results of analysis for the presence of fungi *Chytridiomycosis* in skin specimens from 19 samples of *T. asperrimus* and 104 samples of *T. vietnamensis* by polymerase chain reaction (PCR). Rowley et al. (2013) studied the incidence of *Batrachochytrium dendrobatidis* fungal infection in amphibians in some populations in Ngoc Linh, Kon Tum province.

The studies of Martel et al. (2014) also discovered that some populations of species of the genus *Tylototriton* in Vietnam were infected with the fungal diseases *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* .

In 2020, Tapley et al. studied the occurrence of fungal strains *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* on amphibians in Hoang Lien National Park, Lao Cai province.

1.3.3. Pathogenic mechanism of fungal strains on amphibians

Fungal spores *Batrachochytrium salamandrivorans* are capable of destroying skin cells of adult toadfish. Techniques used to detect infection can combine electron microscopic observation with quantitative PCR (qPCR).

The fungal strain *Batrachochytrium salamandrivorans* produces two types of infectious spores: those that move flagella and those that are enveloped in spore form.

Chapter 2. RESEARCH OBJECTS AND METHODS

2.1.Research object

Field surveys were conducted in 10 provinces and analysis of specimens collected in 14 provinces is currently stored at the Institute of Ecology and Biological Resources (IEBR) and Vietnam Museum of Nature (VNMN).

Research materials

Based on the results of analyzing 234 samples and referencing documents : Of which 2 samples are newly described for science and 101 new samples have their first distribution areas recorded for the province; ND2 gene sequence of 43 specimens of the genus *Tylototriton*; ND2 gene sequences of 15 specimens and 16S sequences of 9 specimens of the genus *Paramesotriton* used in genetic analysis; 848 fungal samples were collected from amphibians.

2.2. Research Methods

2.2.1. Field survey

The study conducted surveys during the day between 09:00 and 15:00 for firebelly toad and crocodile newt species . For amphibians such as toads and tree frogs, specimens collection and measuremrent were conducted at night between 18:00 and 24:00.

Specimen collection proceeded using a sterile cotton swab tip. Cotton swab is used to sweep and rotate evenly on the skin on the abdomen, underside of the tail (for Newts) and thighs of some amphibians.

2.2.2. Research methods in the laboratory

- 28 morphometric data points were collected on both tailed and tailless amphibians and measured with Mitutoyo calipers to the nearest 0.1 mm with adjustment.

- PAST software Hammer et al. (2001) was used to statistically analyze and compare the level of similarity and difference between species with high morphological similarities in Vietnam.

- ANOVA test was used to compare populations of *P. deloustali* and among species of the genus *Tylototriton* in Vietnam

- Molecular biology analysis was performed at the Department of Immunogenomics, Institute of Genome Research.

- DNA extraction sample was used Qiagen kit, Germany. The real-time PCR process and primers used for each family are different; Specimens used in genetic analysis are cited as reference sources.

- Research methods to identify fungal strains are coordinated with Belgian experts, supported by Project (GOF3816N) of the Research Foundation - Flanders (FWO) and the National Foundation for Science and Technology Development, Vietnam (NAFOSTED) under grant number FWO.106-NN.2015.02.

Chapter 3. RESEARCH RESULTS

3.1. Diversity of species composition in the families, Bombinatoridae, Megophryidae, Rhacophoridae and Salamandridae

3.1.1. List of recorded species

Based on the results of analyzing 234 specimens and consulting references, we have recorded 41 species belonging to 4 families and 2 orders. Among them, 1 species belongs to the Bombinatoridae family, 13 species belong to the Megophryidae family, 19 species belong to the Rhacophoridae family, 8 species belong to the Salamandridae family (Table 3.1).

To evaluate the conservation value of amphibians in the study area, we make statistics of threatened species recorded in locations including species named in Decree 84/2021/ND-CP, CITES 2022 Appendix, Vietnam Red Book (2007), IUCN Red List (2024).

In the list of species composition, the following species are recorded: *Brachytarsophrys* cf. *feae* (according to Poyarkov et al. 2023; He et al. 2024), *Kurixalus* cf. *bisacculus* (according to Nguyen et al. 2020, Mo et al., 2023), *Raorchestes* cf. *parvulus* (according to Du et al., 2024), *Rhacophorus* cf. *napoensis* Li, Liu, Yu & Sun, 2022 (according to Li et al. 2022; Nguyen et al. 2024) are species with a wide distribution in Vietnam and neighboring countries as well as a very complicated subspecies status. . Fellows are continuing to collect additional morphological and molecular biological data, for more in-depth studies of these complex species groups.

Table 3.1. List of amphibian species in the study area.

No	Vietnamese name	Scientific name	Sample collection location
	BỘ KHÔNG ĐUÔI	**ANURA Fischer von Waldheim , 1813**	
	Họ cóc tía	**Bombinatoridae (Gray , 1825)**	
1	Cóc tía	*Bombina microdeladigitora* (Liu, Hu & Yang, 1960)	Lai Chau, Lao Cai
	Họ cóc mắt	**Megophryidae (Bonaparte , 1850)**	
2	Cóc mắt lớn	*Atympanophrys gigantica* (Liu, Hu & Yang, 1960)	Lai Chau
3	Cóc mắt cao bằng	*Boulenophrys caobangensis* (Nguyen, Pham, Nguyen, Luong & Ziegler, 2020)	Cao Bang
4	Cóc mắt jingdong	*Boulenophrys jingdongensis* (Fei & Ye, 1983) *	Lao Cai, Lai Chau
5	Cóc mắt đùi đỏ	*Boulenophrys rubrimera* (Tapley, Cutajar, Mahony, Nguyen, Dau, Nguyen, Luong & Rowley, 2017) *	Lai Chau, Lao Cai
6	Cóc mày phê	*Brachytarsophrys* cf. *feae* (Boulenger, 1887)	Cao Bang
7	Cóc mày na hang	*Leptobrachella nahangensis* (Lathrop, Murphy, Orlov & Ho, 1998) *	Ha Giang
8	Cóc mày đêm khuya	*Leptobrachella nyx* (Ohler, Wollenberg, Grosjean, Hendrix, Vences, Ziegler & Dubois, 2011)	Ha Giang
9	Cóc mày sung	*Leptobrachella sungi* (Lathrop, Murphy, Orlov & Ho, 1998) *	Ha Giang
10	Ếch gai hàm sa pa	*Leptobrachium ailaonicum* (Yang, Chen & Ma, 1983) *	Lai Chau
11	Cóc núi gót	*Ophryophryne gerti* (Ohler, 2003)	Dak Lak, Gia Lai

No	Vietnamese name	Scientific name	Sample collection location
12	Cóc núi han-xi	*Ophryophryne hansi* (Ohler, 2003)	Gia Lai, Kon Tum
13	Cóc núi miệng nhỏ	*Ophryophryne microstoma* (Boulenger, 1903)	Kon Tum, Lao Cai, Gia Lai
14	Cóc mắt mẫu sơn	*Xenophrys maosonensis* (Bourret, 1937)	Vinh Phuc, Lao Cai, Nghe An
	Họ ếch cây	**Rhacophoridae (Hoffman , 1932 (1858))**	
15	Nhái cây chân mảnh	*Gracixalus gracilipes* (Bourret, 1937)	Lai Chau, Cao Bang
16	Nhái cây sa pa	*Gracixalus sapaensis* Matsui, Ohler, Eto & Nguyen, 2017	Lai Chau
17	Ếch cây tay-lơ	*Kurixalus* cf. *bisacculus* (Taylor, 1962)	Lai Chau
18	Nhái cây hải nam	*Kurixalus hainanus* (Zhao, Wang & Shi, 2005)	Cao Bang, Ha Giang
19	Ếch cây hồng kông	*Polypedates megacephalus* (Hallowell, 1861)	Cao Bang, Ha Giang
20	Ếch cây mi-an-ma	*Polypedates mutus* (Smith, 1940)	As tall as
21	Nhái cây tí hon	*Raorchestes* cf. *parvulus* (Boulenger, 1893)	Lai Chau, Cao Bang
22	Ếch cây trung bộ	*Rhacophorus annamensis* Smith, 1924	Dong Nai
23	Ếch cây he-len	*Rhacophorus helenae* Rowley, Tran, Hoang & Le, 2012	Dong Nai
24	Ếch cây ki-ô	*Rhacophorus kio* Ohler & Delorme, 2006 *	Cao Bang, Bac Kan
25	Ếch cây óc-lop	*Rhacophorus orlovi* Ziegler & Köhler, 2001 *	Ha Giang, Bac Kan
26	Ếch cây na pha	*Rhacophorus* cf. *napoensis* Li, Liu & Sun, 2022	Lai Chau
27	Ếch cây sần hà khẩu	*Theloderma hekouense* Du, Wang, Liu & Yu, 2022 *	Cao Bang
28	Ếch cây sần khôi	*Theloderma khoii* Ninh, Nguyen, Nguyen, Hoang, Siliyavong, Nguyen, Le, Le & Ziegler, 2022 **	Ha Giang

No	Vietnamese name	Scientific name	Sample collection location
29	Ếch cây lưng xanh	*Zhangixalus dorsoviridis* (Bourret, 1937)	Lai Chau
30	Ếch cây duy-boa	*Zhangixalus duboisi* (Ohler, Marquis, Swan & Grosjean, 2000)	Lai Chau
31	Ếch cây phê	*Zhangixalus feae* (Boulenger, 1893)	Lai Chau
32	Ếch cây lớn	*Zhangixalus pachyproctus* (Yu, Hui, Hou, Wu, Rao &Yang, 2019)	Bac Kan
33	Ếch cây pueren	*Zhangixalus puerensis* (He, 1999) *	Lai Chau, Cao Bang
	BỘ CÓ ĐUÔI	**CAUDATA Fischer von Waldheim , 1813**	
	Họ cá cóc	**Salamandridae (Goldfuss , 1820)**	
34	Cá cóc tam đảo	*Paramesotriton deloustali* (Bourret, 1934)	Son La
35	Cá cóc quảng tây	*Paramesotriton guangxiensis* (Huang, Tang & Tang, 1983)	Cao Bang
36	Cá cóc gờ sọ mảnh	*Tylototriton anguliceps* Le, Nguyen, Nishikawa, Nguyen, Pham, Matsui, Bernardes & Nguyen, 2015	Son La
37	Cá cóc sần pasmas	*Tylototriton pasmansi* Bernardes, Le, Nguyen, Pham, Pham, Nguyen & Ziegler, 2020	Hoa Binh
38	Cá cóc sần sparreboommi	*Tylototriton sparreboomi* Bernardes, Le, Nguyen, Pham, Pham, Nguyen & Ziegler, 2020	Lai Chau
39	Cá cóc sần thái	*Tylototriton thaiorum* Poyarkov, Nguyen & Arkhipov, 2021	Nghe An
40	Cá cóc việt nam	*Tylototriton vietnamensis* Böhme, Schöttler, Nguyen & Köhler, 2005	Bac Giang
41	Cá cóc zig-lơ	*Tylototriton ziegleri* Nishikawa, Matsui & Nguyen, 2013	Cao Bang

3.1.2. New discovery

- New species for science

Khoi's Mossy Frog *Theloderma khoii*, with holotypes collected in Quan Ba district, Ha Giang.

- New records for provinces

Among the 13 species of the Toad family and 19 species of the Tree Frog family, combining genetic analysis and morphological analysis, we have described and recorded 5 species of the Toad family, recording new distribution areas for the province. Ha Giang and Lai Chau province; Four species of the Tree Frog family were recorded in new distribution zones for Ha Giang province, Bac Kan province and Cao Bang province (Table 3.2).

Table 3.2. Record of new distribution.

	Scientific name	**New distribution area**
Megophryidae	*Boulenophrys jingdongensis*	Lai Chau
	Boulenophrys rubrimera	Lai Chau
	Leptobrachella nahangensis	Ha Giang
	Leptobrachella sungi	Ha Giang
	Leptobrachium ailaonicum	Lai Chau
Rhacophoridae	*Rhacophorus kio*	Bac Kan
	Rhacophorus orlovi	Ha Giang
	Theloderma hekouense	Cao Bang
	Zhangixalus puerensis	Cao Bang

Through analysis of newly collected and archived specimens, we provide new records of tailed amphibians and newly recorded tailless amphibians from the included study areas. In summary, we describe the following new representative species for science:

Khoi's mossy frog *Theloderma khoii* (Ninh, Nguyen, Nguyen, Hoang, Siliyavong, Nguyen, Le, Le & Ziegler, 2022)

Research specimens (n=02): 01 male specimen (VNMN 012757) and 01 female specimen (VNMN 012758) collected in Ha Giang.

Size: Male SVL 52.2 mm, HW 19.7 mm, HL 19.7 mm, ED 4.6 mm; female SVL 59.4 mm, HW 20.7 mm, HL 21.5 mm, ED 5.6 mm

Identifying characteristics: Average body size; Head length equals to head width; The forelimbs do not have a swimming membrane, the finger suckers expand into an attachment plate, there is a large metatarsal tubercle on the thumb, and there are tubercles under the fingers. The hind limbs have a partial swimming membrane, membrane formula I1/3-1II0-1/2III0-1IV1-1/3V, toe tips expand into adhesion plates, the inner metatarsal tubercle is large, oval in shape, with tubercles under the toes.

Skin: The dorsal surface of the back and limbs are rough, with large irregular ridges, arranged symmetrically in the middle vertebrae area, with a large, distinct horn gland behind the X-shaped head; There are small nodules around the eardrum, with 4-5 large nodules behind it.

Live sample color : Dorsal surface is moss green or light olive mixed with moss green; The tips of the ridges and tubercles on the skin are reddish brown; the lateral side has a clear dark brown pattern, the abdomen has a yellow-green strip; black abdomen, irregular in size, yellow-green patterned and with several cream-colored warts; pupil black, iris yellow-green with irregular black grid lines; eardrum dark olive color.

The species is currently only recorded in Ha Giang, Vietnam.

3.1.3. Comparison of morphological correlations between species of the Salamandridae family

+ Morphological comparison between crocodile newt species of the genus Tylototriton

In PCA analysis of the morphology of some crocodile newt species of the genus *Tylototriton* in Vietnam. Specifically, the species *T. ziegleri* has a significantly larger morphology compared to the remaining species due to its larger body size (especially SVL body length). (Figure 3.2). PCA analyses uniformly indicated differences in morphologies and genetic relationships, specifically the species *T. anguliceps* is genetically and morphologically separated from the remaining species clade (according to both Dim1 and Dim2 dimensions in the PCA analysis (Figure 3.1). In addition, the three species *T. sparreboomi* , *T. pasmansi* and *T. thaiorum* are closely related genetically and are grouped into the same morphological

space in PCA analysis. The separation of *T. vietnamensis* in the Dim2 dimension partly explains the genetic divergence from the clade in the phylogenetic tree that does not contain *T. angulices*. Notably, with the population recorded in Kon Tum being recorded as a new species *T. ngoclinhensis*, the morphological data in PCA analysis shows that they have a morphological space similar to that of *T. anguliceps*.

Thereby, the study suggested that the population of *T. ngoclinhensis* is completely distinct from the remaining species previously recorded in Vietnam and ths is a new species to science.

+ Morphological comparison between newt species of the genus *Paramesitriton*

Differences recorded in characteristics such as body length (SVL), tail length (TL), head height (HH), mouth width (MW), limb length (FLL and HLL), and abdomen length (AGS) showed that *P. deloustali* is much larger than the corresponding morphological features of the newt *P. guangxiensis* ($P_{values} < 0.05$) (Figure 3.3).

3.1.4. Genetic relationships of species in the Salamandridae family

3.1.4.1. Genetic relationships among species of the family Salamandridae

+ Crocodile newt (*Tylototriton*)

In this study, we built two phylogenetic tree using the Bayesian Inference (BI) and Maximum Likelihood (ML) methods with similar topologies. The Lnl-values of each model are 3856,384 and 3654,615, the alpha value for the BI tree is 0.361 and the Gamma value for the ML tree is 0.3629.

+ Warty newt (*Paramesotriton*)

The study used two mitochondrial gene segments (16S and ND2) to evaluate the genetic relationship between warty newt populations recorded in Vietnam. The results showed that both ML and BI analyzes gave similar results with the separation of the two branches of the two species, *P. deloustali* and *P. guangxiensis*. Specifically, the warty newt populations recorded in Bac Kan province, Ha Giang province and Son La province, are in the same branch and are closely related to the original population of *P. deloustali* in Vinh Phuc province with genetic differences between populations ranging from 0,00% –1.12%.

3.2. Fungal infections in amphibians in Vietnam

3.2.1. Distribution of fungal strains

The study conducted surveys at 55 sampling points in 15 different provinces. A total of 848 samples were collected from amphibians to check the presence of two fungal strains, *Bd* and *Bsal*, of which 137 samples were collected from the Bombina family, 44 samples were collected from species of the Megophryidae family, 84 samples were collected on species of the tree frog family Rhacophoridae and 583 samples were collected on species of the Newt family Salamandridae.

Distribution of the fungus Bd in Vietnam

The study recorded positive test results of the *Bd fungus strain* in Bao Lac district, Cao Bang province.

Amphibian species composition recorded Bd fungus strain in Vietnam

In this study, only the *Bd fungal strain* was recorded positive for 04 individuals/30 samples collected belonging to only one species of the crocodile newt *Tylototriton ziegleri* in Cao Bang province.

Distribution of Bsal in Vietnam

In this study, this is the first time the fungus *Bsal* has been recorded in Vietnam. Specifically, positive records of the fungus *Bsal* were recorded in the Northern provinces including: Tay Yen Tu Nature Reserve, Bac Giang, Phu Canh Nature Reserve, Hoa Binh, Tam Dao National Park, Vinh Phuc, Ba Be National Park, Bac Kan, Bao Lac district, Cao Bang province, Quang Ba district, Ha Giang, Van Ban district, Lao Cai, Sapa district, Lao Cai.

Amphibian species recorded to be infected with the fungus Bsal in Vietnam

The study recorded the fungus *Bsal* in two amphibian families: the Bombinatoridae family and the Salamandridae family. This is the first time *Bsal* has been recorded in the small-webbed bell toad *Bombina microdeladigitora* in Vietnam.

The fungal strain *Bsal* has been recorded in newt species of both genera: *Paramesotriton* and *Tylototriton* in Vietnam (Table 3.3).

Table 3.3. Amphibian species recorded to be infected with the fungus *Bsal* in Vietnam.

Species name	Sample collection location	Score sample	Sample taken	Positive test	*Bsal* infection rate (%)
B. microdeladigitora	Lao Cai	4	137	18	**13.14%**
Megophryidae family	-	18	44	-	0.00%
Rhacophoridae family	-	29	84	-	0.00%
T. vietnamensis	Bac Giang	11	184	3	**1.60%**
T. vietnamensis	Quang Ninh	2	11	-	0.00%
T. vietnamensis	Lang Son	1	12	-	0.00%
T. ziegleri	Bao Lac, Cao Bang	4	30	2	**6.70%**
T. ziegleri	Nguyen Binh, Cao Bang	1	8	-	0.00%
T. ziegleri	Bac Me, Ha Giang	2	13	-	0.00%
T. ziegleri	Quan Ba, Ha Giang	9	33	2	**6.10%**
T. ziegleri	Bac Quang, Ha Giang	2	26	-	0.00%
T. sparreboomi	Lai Chau	4	17	-	0.00%
T. pasmansi	Son La	1	8	-	0.00%
T. asperrimus	Hoa Binh	7	46	3	**0.65%**
P. guanxiensis	Cao Bang	4	54	-	0.00%
P. deloustali	Bac Kan	2	62	1	**1.60%**
P. deloustali	Lao Cai	4	61	4	**6.60%**
P. deloustali	Vinh Phuc	1	18	2	**11.10%**
8 species		**59**	**720**	**35**	**0.65%–13.14%**

3.2.2. Fungal infections in amphibians

Status of fungal infections in Bombinatoridae

A total of 137 samples were tested for fungal infections in the small-webbed bell toad *Bombina microdeladigitora* in Lao Cai province.

The results of the study showed for the first record of the fungus *Bsal* appearing on Anuran amphibians in Vietnam, specifically on the small-webbed bell toad species *Bombina microdeladigitora* with a low rate of fungal infection.

Status of fungal infections in Megophryidae

A total of 44 samples were taken to test for fungal infections in individuals from 13 species of Megophryidae. The results did not record any samples in this study positive for both fungal strains *Bsal* and *Bd* in the Megophryidae.

Status of fungal infections in Rhacophoridae

A total of 84 samples were conducted to test for fungal infections of 20 species of Rhacophoridae.

No Rhacophoridae treefrogs sample in this study that was positive for either *Bsal* nor *Bd strains*.

Status of fungal infections in Salamandridae

A total of 195 samples were conducted to test for fungal infection in 02 species of warty newts of the genus *Paramesotriton.* The study recorded a total of 07 individuals of *P. deloustali* was positive for the fungal strain *Bsal.*

Table 3.4 . Status of *Bd* and *Bsal* infections in newt species of the genus *Paramesotriton* in Vietnam

Species name	**Sample collection location**	**Number of sampling points**	**Temperature range (^{0}C)**	**Sample taken**	***Bsal* positive samples**	**Number of samples positive for *Bd***	***Bsal* prevalence**	***Bd* prevalence rate**
P. guanxiensis	Cao Bang	4	20,60–22,97	54	-	-	0,00%	0,00%
P. deloustali	Bac Kan	2	21,20–22,47	62	1	-	**1,60%**	0,00%
P. deloustali	Lao Cai	4	20,50–22,07	61	4	-	**6,60%**	0,00%
P. deloustali	Vinh Phuc	first	18,80	18	2	-	**11,10%**	0,00%
02 species	**Total**	**11**	**18,80–22,97**	**195**	**7**	0	**1,60%–11,10%**	0,00%

A total of 388 samples were conducted to test for fungal infections in individuals from 05 newt species of the genus *Tylototriton* (Table 3.5).

Table 3.5 . Status of *Bd* and *Bsal* infections in newt species of the genus *Tylototriton* in Vietnam.

Species name	Sample collection location	Number of sampling points	Temperature range (^{0}C)	Sample taken	*Bsal* positive samples	Number of samples positive for *Bd*	*Bsal* prevalence	*Bd* prevalence rate
T. vietnamensis	Bac Giang	11	22,70–24,80	184	3	-	**1,60%**	0,00%
T. vietnamensis	Quang Ninh	2	23,80–24,90	11	-	-	0,00%	0,00%
T. vietnamensis	Lang Son	1	31,90	12	-	-	0,00%	0,00%
T. ziegleri	Bao Lac, Cao Bang	4	16,07–22,53	30	2	4	**6,70%**	**13,30%**
T. ziegleri	Nguyen Binh, Cao Bang	1	19,57	8	-	-	0,00%	0,00%
T. ziegleri	Bac Me, Ha Giang	2	18,70–19,70	13	-	-	0,00%	0,00%
T. ziegleri	Quan Ba, Ha Giang	9	16,37–20,73	33	2	-	**6,10%**	0,00%
T. ziegleri	Bac Quang, Ha Giang	2	21,60–23,37	26	-	-	0,00%	0,00%
T. sparreboomi	Lai Chau	4	18,53–23,9	17	-	-	0,00%	0,00%
T. pasmansi	Son La	1	20,60	8	-	-	0,00%	0,00%
T. asperrimus	Hoa Binh	7	20,07–26,43	46	3	-	**6,50%**	0,00%
05 species	**Total**	**44**	**16,37–31,90**	**388**	**10**	**4**	**1,60–6,70%**	**13,30%**

Chapter 4. CONCLUSION AND RECOMMENDATIONS

CONCLUSION

Species diversity

- Recorded 41 species belonging to 4 families, 2 orders in the study area: 1 species belonging to the family of Bombinatoridae, 13 species belonging to the family of Megophryidae, 19 species belonging to the family of Rhacophoridae, 8 species belonging to the family of Salamandridae.
- Description and discovery of new species: - *Theloderma khoii*.
- Recorded new distribution of 9 species for the provinces: Ha Giang, Lai Chau, Bac Kan and Cao Bang.
- Described morphological correlation of species belonging to 2 genera *Paramesotriton* and *Tylototriton* belonging to the Salamandridae family.
- Using 2 mitochondrial gene segments 16S and ND2 to evaluate the genetic relationship of 8 species *Tylototriton pasmansi, T. ngoclinhensis, T. sparreboomi, T. thaiorum, T. vietnamensis, T. ziegleri, T. ngoclinhensis* and *T. anguliceps* belonging to the genus Tylototriton; Genetic relationship between 2 populations *Paramesotriton deloustali* and *P. guangxiensis* belonging to the genus Paramesotriton recorded in Vietnam.

* Fungal infection status

- The research results recorded the Bsal fungus strain for the first time in Vietnam, specifically on species belonging to the two families of Bombinatoridae (infection rate 23.75%) and Salamandridae (species belonging to the genus *Paramesotriton* with infection rate from 1.60–11.10%, species belonging to the genus *Tylototriton* with infection rate from 1.60–6.70%).
- A total of 848 samples were collected on amphibians in different environments (on trees, underwater, on land and wet puddles), recorded 2 families of Bombinatoridae (19/137) and Salamandridae (21/583) positive for both *Bd* and *Bsal* fungi; no *Bd* and *bsal* fungi were recorded on the two families of Salamandridae (0/44) and Rhacophoridae (0/84), particularly:

With the genus *Tylototriton*, only *Bd* fungus strain was recorded on 4 individuals of the species *T. ziegleri* in Cao Bang; *Bsal* strain: 3 individuals of *T. vietnamensis* in Bac Giang province; 2 individuals of *T.*

ziegleri in Cao Bang, 2 individuals of *T. ziegleri* in Ha Giang; 3 individuals of *T. asperrimus* in Hoa Binh.

With the genus *Paramesotriton*, *Bsal* strain was recorded on *P. deloustali* species: 1 individual collected in Bac Kan, 4 individuals collected in Lao Cai and 2 individuals collected in Vinh Phuc.

- Research showed that, in the habitat of wet and aquatic amphibians, *Bd* and *Bsal* strains were recorded on 2 families of Bombinatoridae and Salamandridae. These are 2 types of environments with high risk of being sources of *Bd* and *Bsal* strains spreading in nature.

RECOMMENDATIONS

- Due to the many similar morphological characteristics and similarities between individuals in the population, it is necessary to continue to study the homomorphic species, taxonomy of species belonging to the families of Megophryidae, Rhacophoridae and Salamandridae; as well as expand taxonomic research in different families and distribution areas.

- Based on the research results, in addition to the families of Bombinatoridae and Salamandridae corresponding to 2 types of humid environments and aquatic environments, fungal infections were recorded. Continue to study and evaluate the status of fungal infections in the families of Megophryidae and Rhacophoridae, expand research on populations and habitats in different areas in the wild.

- The research results showed that temperature factors affected the susceptibility to fungal infections in Bombinatoridae and Salamandridae populations (the rate of fungal infection decreases as the environmental temperature increases). From there, continue to study environmental conditions: different habitats, microclimate factors affecting the spread of wild fungal strains.

- Study the biological cycle of fungal strains and sequence genes in laboratory conditions in Vietnam.

- Conserve rare and valuable species of the families of Bombinatoridae, Megophryidae, Rhacophoridae and Salamandridae in the Vietnamese Red List, Decree 84/2021/ND-CP, IUCN 2024, CITES 2022, in the research area and expand to other regions in Vietnam;

- Based on the results of the study on the status of fungal infection in the Bombinatoridae are one of the four amphibian families and are species on the list of rare and endemic species that need to be preserved in Vietnam. From there, it is necessary to control the transportation and trade of animals as pets, which is a risk of spreading diseases, directly affecting wild populations.

NEW CONTRIBUTIONS OF THE THESIS

-New discovery and records of distribution:

+ This study discovered a new species of tree frog, namely Khoi's Mossy Frog (*Theloderma khoii*)

+ This study first recorded the infectious fungus of *Batrachochytrium salamandrivorans* (*Bsal*) in some frogs of Bombinatoridae and Salamandridae families.

+ New records of distribution for nine species of two families:

Megophryidae: *Boulenophrys jingdongensis*, *B. rubrimera*, *Leptobrachium ailaonicum* in Lai Chau Province; and *Leptobrachella nahangensis*, *L. sungi* in Ha Giang Province.

Rhacophoridae: *Rhacophorus orlovi* in Ha Giang Province; *R. kio* in Bac Kan Province; and *Theloderma hekouense* and *Zhangixalus puerensis* in Cao Bang Province.

- This study provided data on the infectious status of *Bsal* and *Bd* in four amphibian families species. Especially, this study first recorded the fungus of *Bsal* in Bombinatoridae and Salamandridae families.

LIST OF THE PUBLICATIONS
RELATED TO THE DISSERTATION

1. Tao Thien Nguyen, **Thinh Van Nguyen**, Thomas Ziegler, Frank Pasmans, An Martel (2017). Trade in wild anurans vectors the urodelan pathogen Batrachochytrium salamandrivorans into Europe. *Amphibia-Reptilia*, 38(4):554-556.
2. **Nguyen Van Thinh**, Nguyen Thien Tao, Luong Mai Anh, Pham The Cuong, Nguyen Quang Truong, (2021). New records of three species of treefrogs (Rhacophoridae: Amphibia) from Northeastern Vietnam, *HNUE Jounal of Science*, 66(3): 153-160.
3. Ninh, H. T., Nguyen, T. T., Nguyen, H. Q., Hoang, N. V., Siliyavong, S., **Nguyen**, **T. V**., Le, D. T., Le, Q. K., & Ziegler, T. (2022). A new species of mossy frog (Anura: Rhacophoridae) from Northeastern Vietnam. *European Journal of Taxonomy*, *794*(1), 72–90.
4. **Nguyễn Văn Thịnh**, Ninh Thị Hòa, Nguyễn Thị Ngần, Nguyễn Thiên Tạo, Đỗ Hữu Dũng, Phạm Thế Cường, Phạm Văn Anh, Ngô Ngọc Hải, Nguyễn Quảng Trường, Hoàng Văn Chung (2022). Quan hệ di truyền và đặc điểm hình thái các loài cá cóc thuộc giống *Tylototriton* (Amphibia: Caudata) ở Việt Nam.*Tạp chí khoa học và công nghệ Lâm nghiệp*, 5: 40-49.