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Short Communication

Evidence of Ranavirus in a green tree frog (*Litoria caerulea*) in captive zoo

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Abstract

Ranavirus is one of the causative agents responsible for the rapid decline in amphibian populations worldwide. The objectives of this study were to detect and to genetically characterize ranavirus infection in 36 liver tissues obtained from dead imported zoo amphibians in Thailand. Polymerase chain reaction and molecular phylogenetic analysis was performed based on nucleotide sequences containing major capsid protein gene. Results showed 1 positive sample (~531 bp) from a green tree frog (*Litoria caerulea*). The virus was highly homologous (98%) and closely related to ranavirus. Our results extend the risk and evidence of ranavirus infection in zoo amphibian in Thailand.

Keywords: green tree frog, ranavirus, zoo amphibian

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Introduction

In recent decade, there has been a rapid global decline in amphibian populations. Many important factors including the destruction and alteration of the physical habitats of the animals and the emergence of infectious diseases are known to be closely associated with the decline (Harp et al., 2006; Gray et al., 2009; Kim et al., 2009). Emerging infectious diseases such as chytridiomycosis, saprolegniasis and ranavirus infection are increasingly recognized as the cause of the high mortality rate in farmed and wild amphibian populations worldwide (Bletz et al., 2015; Warne et al., 2016). Ranavirus has affected 3 different taxonomic classes of cold-blooded vertebrates: teleost fish, amphibians, reptiles and turtles (Mazzoni et al., 2009; Chinchar and Waltzek, 2014; Winzeler et al., 2015). This virus causes serious systemic infection in amphibians, resulting in economic losses in many countries in different continents including Australia (Speare and Smith, 1992), Europe (Hyatt et al., 2000; Miaud et al., 2016), Asia (Marschang et al., 1999), South America (Speare et al., 1991; Galli et al., 2006), and North America (Bollinger et al., 1999; Green et al., 2002; Une et al., 2009).

Ranavirus, the large enveloped virus, contains a linear double stranded DNA genome and belongs to family *Iridoviridae*. Viral size ranges from 120 to 300 nm in diameter with icosahedral symmetry cytoplasmic DNA (Mazzoni et al., 2009). Amphibians are one of the primary reservoirs of ranaviruses. According to the biphasic life cycle of many amphibians, amphibian reservoirs could occur in aquatic or terrestrial environments. In Thailand, the disease has occurred in frog culture farms located in central Thailand since early 1998. The disease affects 20-100% of the frog population in affected farms (Kanchanakhan et al., 2002). Ranavirus infection was also reported in a private fish farm in Srakeaw Province in January 2004. The frogs were imported from Cambodia for feeding the carnivorous fish (Kanchanakhan et al., 2008). The prevalence or severity of the disease outbreaks have been reported in association with seasonal variations, with both being greater during the warmer months. Therefore, temperature is considered a likely factor influencing the disease outbreaks, but no experimental data are available.

According to the rapid decline in amphibian populations worldwide, ranavirus infection has been considered to be the major cause of the severe mortality and morbidity rates. In recent years, a number of exotic amphibian species from many countries have been imported-exported to captive zoos in Thailand, and the prevalence and epidemiological studies of ranavirus infections in zoo amphibians in Thailand have never been recorded. The objectives of this study were to detect and to genetically characterize the ranavirus infection in zoo amphibians in Thailand.

Materials and Methods

Thirty-six liver tissues were collected from dead amphibians from zoos in Nakornratchasima province, in the north-eastern region (28 samples), and Chonburi province, in the eastern region (8 samples), of Thailand during 2010-2013 (Table 1). These dead frogs were found in frog aquariums in the zoos without clinical records. The frogs were kept individually in each aquarium. High mortality was observed in imported frogs during the first few months after introducing them to the aquariums. The liver tissues were fixed in alcohol for preservation and kept at -20°C for DNA analysis. The amphibians were imported from different countries (unknown data). According to the investigation of a veterinarian of Nakornratchasima Zoo, the frogs had high morbidity and mortality rate (up to 80%). Total DNA extractions were conducted according to manufacturer's protocol (QIAamp DNA Mini kit, QIAGEN, UK). Total DNAs were quantified using Nanodrop® Lite (Thermo Fisher Scientific Inc., Massachusetts, USA) at absorbance A260/A280 and stored at -20°C until polymerase chain reaction (PCR) processing. A specific primer pair targeting major capsid protein (MCP) gene were 5'-GTCTCTGGAGAAGAAGAA-3' (forward primer) and 5'-GACTTGGCCACTTATGAC-3' (reverse primer) (Mao et al., 1997). Reaction was composed of 5x buffer, 25mM $MgCl_2$, 0.2 mM dNTP, DNA polymerase, 10 mM each primer, and 5 µl of DNA sample. Finally, dH_2O was added up to 25 µl for each PCR reactions. Cycling conditions consisted of initial denaturation at 95°C for 1 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. Final extension was done at 72°C for 5 min. The amplified PCR product (~531bp) was electrophoresed on 1.5% agarose gel and visualized by 10% ethidium bromide staining. Negative control was run in parallel.

For sequence analysis, positive PCR product was purified using a commercial purification kit (NucleoSpin Extract II™ PCR purification kit, Macherey-Nagel, Germany) and submitted for genetic sequencing (1st BASE Pte Ltd., Singapore). Derived sequence was deposited in GenBank with an assigned accession number KU669288 (isolate 055-01). Sequence similarity was achieved by using BLAST program compared with available published ranavirus strains in GenBank. Maximum likelihood phylogenetic tree was constructed using MEGA6 software. Standard error was calculated by the bootstrap method using 1000 replicates.

Results and Discussion

Evidence of ranavirus infection was observed in a green tree frog (*Litoria caerulea*) from Nakornratchasima Zoo. Only one out of the 36 liver samples was PCR positive by presenting distinct band of MCP gene at 531 bp. Sequence analysis showed 98% identities with the MCP gene of frog virus 3. Phylogenetic analysis revealed that our sequence (KU669288) belonged to the genus *Ranavirus* of family Iridoviridae (Fig 1).

Ranavirus infection induces acute mortality in tadpoles (98-100%), and can induce skin ulceration, necrotic changes in visceral organs and eventual death in adults (Greer and Collins, 2007; Cunningham et al., 2008). In the present study, high mortality was observed in the imported amphibians in Nakornratchasima Zoo according to the veterinarian's

investigation. Our result suggested that ranavirus was unlikely the causative agent responsible for high mortality of frogs in captive zoo. As suggested by the study of Forzan and Wood (2013), green frogs are a poor reservoir for FV3 ranavirus and may not be the ideal species for sampling when determining the disease status in an area as free of ranavirus. Low detection of ranavirus DNA was detected (2/72) in wild postmetamorphic green frog, *Rana (Lithobates) clamitans*, liver tissues despite previous or concurrent tadpole mortality (Forzan and Wood, 2013). Moreover, the sample size in the present study was quite limited and, therefore, unable to represent the overall prevalence and epidemiological data of ranavirus infection in zoo amphibians in Thailand. Establishing molecular technique for ranavirus disease surveillance and monitoring will be a crucial step to control the widespread outbreak of new emerging disease introduced into captive amphibian zoos in Thailand. Understanding of the genetic characteristic of the virus is also beneficial for tracking the original source of transmission and the relationship of genetic distribution of virus worldwide. Large scale surveillance study should be further carried out. The importance of zoo amphibians as sources of virus replication and spread to the environment as well as the potential threat to wild amphibian species should be taken into account in future works.

Table 1 Details of thirty-six zoo amphibians in north-eastern and eastern Thailand during 2010-2013

No	Common name	Scientific name	Isolate	Location*
1	Green tree frog	*Litoria caerulea*	055-01	NEZ
2	Nongkhor Asian tree frog	*Chirixalus nongkhorensis*	KK01-01	EZ
3	Marbled sucker frog	*Amolops marmoratus*	KK12-01	EZ
4	Marbled sucker frog	*Amolops marmoratus*	KK12-02	EZ
5	Marbled sucker frog	*Amolops marmoratus*	KK12-03	EZ
6	Hainan small tree frog	*Rhacophorus bisacculus*	KK04-01	EZ
7	Spiny-breasted giant frog	*Eripaa fasciculispina*	KK06-01	EZ
8	Hose's frog	*Rana hosii*	KK07-01	EZ
9	Lekagul’s horned frog	*Xenophrys lekaguli*	KK09-01	EZ
10	Red eyed tree frog	*Agalychnis callidryas*	020-04	NEZ
11	Red eyed tree frog	*Agalychnis callidryas*	020-05	NEZ
12	Smith's litter frog	*Leptobrachium smithi*	021-01	NEZ
13	Long-nosed horned frog	*Megophrys nasuta*	023-01	NEZ
14	Long-nosed horned frog	*Megophrys nasuta*	025-01	NEZ
15	Concave-crowned horned toad	*Megophrys parva*	031-01	NEZ
16	Doria's frog	*Limnonectes doriae*	031-02	NEZ
17	Kokarit frog	*Rana lateralis*	035-01	NEZ
18	Limborg's frog	*Taylorana limborgi*	035-02	NEZ
19	Big-eyed tree frog	*Leptopelis vermiculatus*	036-03	NEZ
20	Wallace's flying frog	*Rhacophorus nigropalmatus*	037-03	NEZ
21	False ornate narrow-mouthed frog	*Micryletta inornata*	038-02	NEZ
22	Red eyed tree frog	*Agalychnis callidryas*	039-01	NEZ
23	Red eyed tree frog	*Agalychnis callidryas*	039-02	NEZ
24	Red eyed tree frog	*Agalychnis callidryas*	039-03	NEZ
25	Green tree frog	*Litoria caerulea*	040-01	NEZ
26	Barking tree frog	*Hyla gratiosa*	042-01	NEZ
27	Argentine horned frog	*Ceratophrys ornata*	043-03	NEZ
28	Common tree frog	*Polypedates leucomystax*	043-04	NEZ
29	Crested toad	*Bufo divergens*	044-01	NEZ
30	Large-headed frog	*Limnonectes macrognathus*	046-01	NEZ
31	Berdmore s chorus frog	*Microhyla berdmorei*	049-02	NEZ
32	Dark-sided frog	*Rana nigrovittata*	049-03	NEZ
33	Green tree frog	*Litoria caerulea*	052-02	NEZ
34	Large-crested toad	*Bufo cristatus*	053-01	NEZ
35	Large-crested toad	*Bufo cristatus*	054-01	NEZ
36	Green tree frog	*Litoria caerulea*	056-01	NEZ

*Location: NEZ = zoo located in Nakornratchasima province, north-eastern Thailand; EZ = zoo located in Chonburi province, eastern Thailand

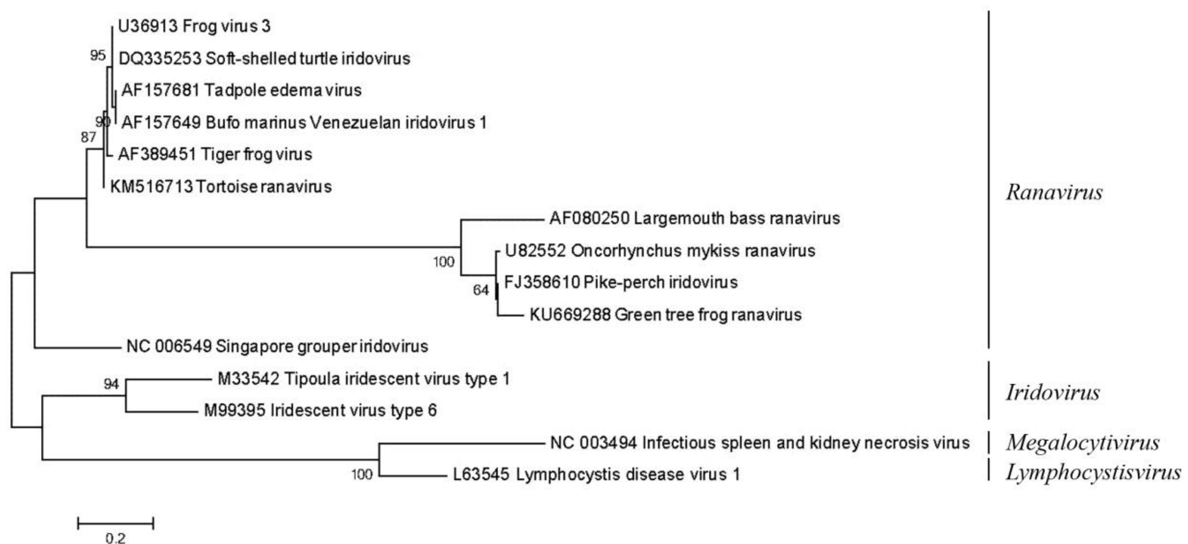

Figure 1 Phylogenetic tree depicting the relationship of KU669288 green tree frog ranavirus, isolate 055-01 to other selected ranavirus and viruses in other genuses (*Iridovirus*, *Megalocytivirus*, *Lymphocystisvirus*) within family *Iridoviridae*

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บทคัดย่อ

การตรวจพบไวรัสรานาในกบต้นไม้สีเขียว (*Litoria caerulea*) ที่เลี้ยงในสวนสัตว์

นพดล พิษารัตน์[1] อังคณา สมนัสทวีชัย[2] สมพร เตชะงามสุวรรณ[1,3*]

ไวรัสรานาเป็นหนึ่งในสาเหตุของการลดจำนวนประชากรของสัตว์สะเทิ้นอย่างรวดเร็วทั่วโลก วัตถุประสงค์ของการศึกษาครั้งนี้ เพื่อตรวจสอบการติดเชื้อและจำแนกลักษณะทางพันธุกรรมของไวรัสรานาในชิ้นเนื้อตับจำนวน 36 ตัวอย่างของสัตว์สะเทิ้นที่นำเข้าจากต่างประเทศเข้ามายังสวนสัตว์ในประเทศไทย ด้วยวิธีปฏิกิริยาลูกโซ่โพลิเมอเรสและการวิเคราะห์แผนภูมิต้นไม้วิวัฒนาการต่อจีนเมเจอร์แคปซิดโปรตีน การศึกษาพบตัวอย่างที่ให้ผลบวกเพียง 1 ตัวอย่าง ที่ 531 คู่เบส จากกบต้นไม้สีเขียว (*Litoria caerulea*) จากการวิเคราะห์ลำดับเบสพบว่ามีความใกล้เคียงกับไวรัสรานาถึง 98% ผลการศึกษาครั้งนี้บ่งชี้การเกิดโรคติดเชื้อไวรัสรานาในสัตว์สะเทิ้นในประเทศไทย ซึ่งควรเฝ้าระวังการเกิดโรคต่อไป

คำสำคัญ: กบต้นไม้สีเขียว ไวรัสรานา สัตว์สะเทิ้นในสวนสัตว์

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