ความหลากหลายทางพันธุกรรมและสัณฐานวิทยา

ของกระดูกกะท่างน้ำสกุล *Tylototriton* ในประเทศไทย

นายปรวีร์ พรหมโชติ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต

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ปีการศึกษา 2550

GENETIC VARIATION AND BONE MORPHOLOGY OF NEWTS
GENUS *Tylototriton* IN THAILAND

Mr. Porrawee Pomchote

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Zoology

Department of Biology

Faculty of Science

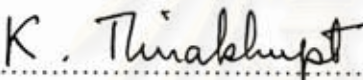Chulalongkorn University

Academic Year 2007

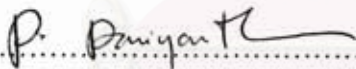|	
Thesis Title	GENETIC VARIATION AND BONE MORPHOLOGY OF NEWTS GENUS *Tylototriton* IN THAILAND
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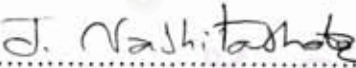
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ปรวีร์ พรหมโชติ: ความหลากหลายทางพันธุกรรมและสัณฐานวิทยาของกระดูกกะท่างน้ำ สกุล Tylototriton ในประเทศไทย. (GENETIC VARIATION AND BONE MORPHOLOGY OF NEWTS GENUS Tylototriton IN THAILAND) อ. ที่ปรึกษา: ผศ. ดร. วิเชฏฐ์ คนซื่อ, อ. ที่ปรึกษาร่วม: รศ. ผุสตี ปริยานนท์, 184 หน้า.

การศึกษาความหลากหลายทางพันธุกรรมและสัณฐานวิทยากระดูกของกะท่างน้ำชนิด Tylototriton verrucosus ในประเทศไทย ได้ทำการสำรวจในภาคสนามตั้งแต่เดือนธันวาคม พ.ศ. 2544 ถึงเดือนกันยายน พ.ศ. 2549 จำนวน 14 สถานที่จาก 7 จังหวัด พบกะท่างน้ำ 2 แบบ คือ แบบลำตัวสีส้มถึงเหลืองและแบบสีคล้ำ โดยมีการกระจายตามแนวเทือกเขาภาคเหนือและภาค ตะวันออกเฉียงเหนือตอนบนตามลำดับ และขนาดของเพศเมียของแบบสีส้มถึงเหลืองมีขนาดใหญ่ กว่าแบบสีคล้ำอย่างชัดเจน นอกจากนั้นได้ทำการศึกษาข้อมูลด้านแหล่งที่อยู่อาศัย การสืบพันธุ์ และลักษณะสัณฐานวิทยาภายนอกด้วย

การตรวจสอบความหลากหลายทางพันธุกรรม โดยใช้ยีน 16S ribosomal RNA และ D-loop ของไมโทคอนเดรียลดีเอ็นเอจากกะท่างน้ำ 7 ประชากรจำนวน 21 ตัว และการศึกษาสาย สัมพันธ์ทางวิวัฒนาการด้วยวิธี distance และ maximum likelihood พบว่ากะท่างน้ำ T. verrucosus มี 2 เชื้อสายทางพันธุกรรมที่แยกกันอย่างชัดเจน ซึ่งสัมพันธ์กับการกระจายทาง ภูมิศาสตร์และรูปแบบของสี

การศึกษากระดูกของกะท่างน้ำจำนวน 12 ตัวจาก 6 ประชากร พบว่ามีความแตกต่าง ทางด้านรูปร่างของกระดูกอย่างชัดเจน โดยข้อมูลด้านรูปร่างของกระดูกสนับสนุนความแตกต่าง ของประชากรภาคเหนือและภาคตะวันออกเฉียงเหนือตอนบน จากข้อมูลทั้งด้านรูปแบบการ แพร่กระจาย ข้อมูลด้านชีวโมเลกุล และสัณฐานวิทยาของกระดูก แสดงให้เห็นว่ากะท่างน้ำ T. verrucosus ในประเทศไทยมี 2 แบบ

ภาควิชา	ชีววิทยา	ลายมือชื่อนิสิต	ปรวีร์ พรหมโชติ
สาขาวิชา	สัตววิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา	
ปีการศึกษา	2550	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม	

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KEY WORD*: TYLOTOTRITON VERRUCOSUS* / DISTRIBUTION / ECOLOGY / GENETIC VARIATION / BONE MORPHOLOGY

PORRAWEE POMCHOTE: GENETIC VARIATION AND BONE MORPHOLOGY OF NEWTS GENUS *Tylototriton* IN THAILAND. THESIS ADVISOR: ASST. PROF. WICHASE KHONSUE, PH. D., THESIS COADVISOR: ASSOC. PROF. PUTSATEE PARIYANONTH, 184 pp.

The genetic variation and bone morphology of the Himalayan newt (*Tylototriton verrucosus*) in Thailand were examined through field surveys, which were carried out at 14 locations in 7 provinces of Thailand, from December 2001 to September 2006. It was observed that, in Thailand, this species can be divided into two morphotypes based upon the body coloration (orange to yellow and dull colorations), and female sizes (orange to yellow type was noticeably bigger than dull type). The two morphotypes as described also coincided with their current distribution ranges (northern and northeastern mountain ranges). The current local distributions within Thailand of both two types of *T. verrucosus* were evaluated with new localities discovered and are reported. Other information including habitat, reproduction and external morphology were also recorded.

Among 7 populations, genetic variation of 21 individuals was examined using DNA sequence analysis of two mitochondrial DNA gene fragments: 16S ribosomal RNA (498-500 base pairs) and D-loop (729-730 base pairs). Phylogenetic relationships were established using distance and maximum likelihood methods. The clear findings revealed the existences of two distinct genetic lineages, which are related to their geographic distributions and color patterns.

Osteological investigation of 12 specimens, from 6 populations, revealed obvious differences between northern and northeastern populations of *T. verrucosus*. These data showed that there are two morphotypes of *T. verrucosus* in Thailand, based on the distribution patterns, molecular characters, and bone morphology.

Department………………….....   Biology   Student's signature……………………………...

Field of study……………….....   Zoology   Advisor's signature……………………….…...

Academic year………………….   2007   Co-advisor's signature…………………….…...

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To all of my teachers and friends at the Department of Biology, Faculty of Science, Chulalongkorn University for their help and support for many years.
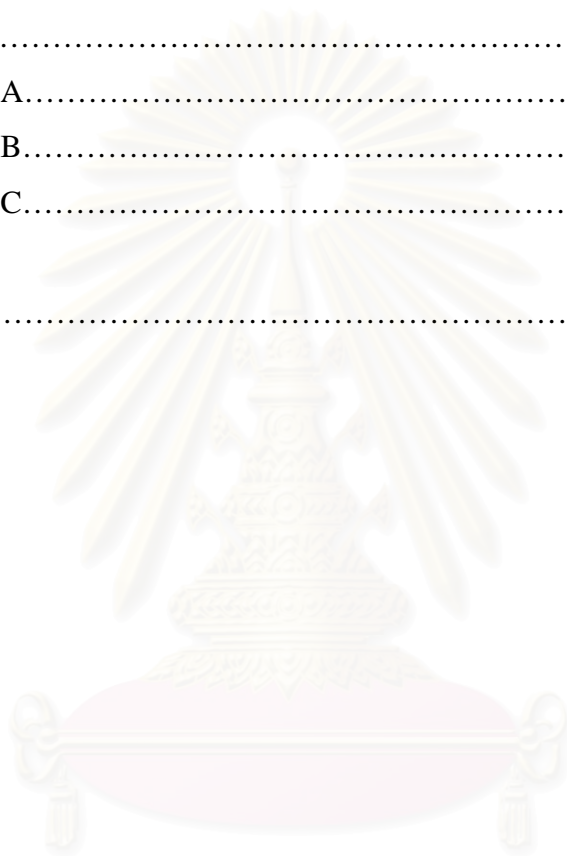
To my beloved family in the Pomchote and Noium for their understanding, love and great supports through my life.

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LIST OF ABBREVIATIONS

A, G, C, and T	The nitrogenous bases Adenine, Guanine, Cytocine and Thymine, respectively, as ribonucleotide bases in DNA or RNA (for A, G and C only) polymers
AIC	Akaike Information Criterion
asl	above mean sea level
ATPase	adenosine triphosphatase
bp	base pair
°C	degree Celcius
cm	centimeter
cyt *b*	cytochrome *b*
D-loop	displacement loop
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
e.g.	exempli gratia (for example)
EDTA	ethylene diamine tetra-acetic acid
EtBr	ethidium bromide
g	gram
gen. nov.	genus novem (new genus)
i.e.	id est (that is)
KOH	potassium hydroxide
m	meter
M	molar
mg	milligram
MgCl$_2$	magnesium chloride
min	minute
ml	milliliter
ML	maximum likelihood
mm	millimeter
mM	millimolar
mRNA	messenger ribonucleic acid
MS222	ethyl 3-aminobenzoate methanesulfonic acid
mtDNA	mitochondrial deoxyribonucleic acid
Mya	million year ago
N/A	not available
NaCl	sodium chloride
ng/µl	nanogram per microliter
NJ	neighbor-joining
no	number
NP	national park
PCR	polymerase chain reaction
rpm	revolutions per minute
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate, sodium lauryl sulphate
sec	second
sp., spp.	species
sp. nov.	species novem (new species)

TBE buffer	tris-HCl, boric acid, and EDTA buffer
TBR	tree-bisection-reconnection
TEN buffer	tris-HCl, EDTA, and sodium chloride buffer
Thymol	5-methyl-2-(1-methylethyl)phenol
Tris	tris (hydroxyl methyl) aminomethane
tRNA	transfer ribonucleic acid
UV	ultraviolet
V	volt
v/v	volume per volume
w/v	weight per volume
WS	wildlife sanctuary
μl	microliter
μM	micromolar

CHAPTER I
GENERAL INTRODUCTION

Currently some 5,948 species of amphibians have been described and are found in all terrestrial and freshwater habitats except the coldest and driest regions or the oceans (Frost et al., 2006). They are composed of three orders; Gymnophiona, Caudata and Anura, with examples from all three orders being found in Thailand and specifically; six species from Gymnophiona, 134 species from the order Anura and only one species from the order Caudata, namely *Tylototriton verrucosus* or the Himalayan newt in the genus *Tylototriton* (Chan-ard, 2003).

The genus *Tylototriton* has been reported to be distributed in eastern and western China, Nepal, Sikkim and Darjeeling in India, Burma, northern Thailand, northern Vietnam and Okinawa in Japan (Zhao et al., 1988). This taxon presently is recognized to consist of eight species; *T. verrucosus*, *T. asperrimus*, *T. kweichowensis*, *T. taliangensis*, *T. hainanensis*, *T. wenxianensis*, *T. shanjing* and *T. vietnamensis*.

T. verrucosus is the sole known representative salamandrid species found in Thailand (Taylor, 1962; Beaver, 1982; Wongratana, 1984; Chan-ard et al., 1999; Nabhitabhata, Chan-ard, and Chuaynkern, 2000; Nutphund, 2001; Sanguansombat, 2001; Chan-ard, 2003). It normally found in small pools, hill streams, under logs or wood debrises in the northern and northeastern mountain ranges at an altitude of more than 1,000 m (Pomchote, 2004). The great majority of records are from the Chiang Mai and Loei Provinces (Wongratana, 1984; Nabhitabhata et al., 2000; Nutphund, 2001; Pomchote, 2004; Nabhitabhata and Chan-ard, 2005). Moreover, this species is reported from eastern Nepal, Bhutan, east to north-eastern India, southern China, northern Myanmar, northern Thailand and may occur in northern Vietnam (Dutta, 1997; Frost, 1985). From the above records, Thailand is the lowest latitude area that this species is recorded in.

Almost all previous reports of *T. verrucosus* in Thailand (i.e., Sanguansombat, 2001; Chan-ard, 2003) have never mentioned the existence of an obvious color pattern polymorphism of this species in Thailand, but rather have either offered no descriptions or only that of the established morphology of the species in its archetypal northern range. The exceptions are then interesting, the reports of Nutphund (2001)

and Pomchote (2004). Pomchote (2004) presented basic data that the Himalayan newt in Thailand may be divided into two different types based on difference in morphometry, body coloration and distribution. The color form of newt populations from northern mountain range is similar to *T. shanjing* found in Yunnan Province, China. It is therefore possible that this taxon in Thailand may be comprised of more than one species, or if not is potentially in the process of speciation.

Molecular methods have been widely used to examine variation within and between salamander populations (i.e., Hedgecock, 1976; Weisrock et al., 2006). The sequencing of significant fragments of DNA, usually either rRNA or gene encoding, is the most detailed method to detect genetic variability (Domingo-Roura et al., 2001). In amphibians, like in most but not all animals, the mitochondrial genome is maternally inherited and moreover is haploid (e.g., Li and Graur, 1991; Macdonald, 2003). As such, the evolutionary rate at the nucleotide sequence level is rapid and faster than nuclear DNA (e.g., Brown et al., 1979; Vawter and Brown, 1986). Thus, mtDNA is useful and often employed for the study of the evolutionary process and in particular for assessing relationships among populations or species that are closely related (Brown et al., 1979; Li and Graur, 1991). However, as reviewed in Tan and Wake (1995), mtDNA analysis alone is insufficient to define species boundaries, as it cannot distinguish paternal introgression and recent speciation events and is prone to foundress bottleneck effects especially if gene flow is unequal between females and males.

Morphological comparisons and descriptions have been basically used to elucidate the relationships in several animal groups such as amphibians (Parra-Olea, Canesco-Marquez, and Garcia-Paris, 2004), reptiles (Glaw, Franzen, and Vences, 2005) and birds (Chiappe and Bertelli, 2006). The osteological characters are considered for the object of mainly taxonomic studies on many salamander groups (Noble, 1928; Titus and Larson, 1995; Evans et al., 2005).

Nevertheless, the basic data of *T. verrucosus* in Thailand are scanty. Most of the published studies have only focused on its distribution or habitat (e.g., Nutphund, 2001; Chan-ard, 2003), despite the fact that this species is a sole representative of order Caudata recorded in Thailand and is placed in the near threatened species category of the IUCN Red List Categories (Nabhitabhata and Chan-ard, 2005). Moreover, it has been considered a sensitive species, based on its patchy distribution and isolated populations. It was threatened severely from extensive deforestation and

agricultural development which cause altered and destroyed habitats (i.e., Beaver, 1982; Wongratana, 1984).

As mentioned previously, there are at least two morphological forms, based upon external features, found in Thailand. This study questioned, what are the differences between *T. verrucosus* populations within its range in Thailand? To address this, this study specifically studied the degree of variation between these populations using; 1) genetic variation, assayed by mtDNA sequence analysis, 2) bone morphology polymorphism, to clarify relationships, and 3) a more extensive variation of their range distribution within Thailand. Other information regarding habitat, reproduction, and external morphology were also recorded as available. The knowledge gained from this study can be used in the implementation of future conservation plans.

CHAPTER II

DISTRIBUTION OF NEWTS GENUS *Tylototriton* IN THAILAND

INTRODUCTION

The order Caudata or Urodela (composes of salamanders and newts) has many distinct characteristic differences from the other amphibian orders Gymnophiona (caecilians) and Anura (frogs). They have an elongated body, two pairs of equal sized limbs (except in the family Sirenidae); present tails in all stages of development including adults, and have differences in several characters of skeleton and muscle morphology (Larson, Wake, and Devitt, 2006).

Salamanders of the family Salamandridae are divided into two groups. The true salamanders, means a "fire-lizard", includes the genera *Chioglossa, Lyciasalamandra, Mertensiella* and *Salamandra*, are distributed almost entirely in Europe except for some populations located in the Middle East, northwest Africa, southern Turkey, and some Aegean islands. The newts, including all remaining extant genera, have a widespread distribution covering most of Europe, southeastern China, northern Laos, Vietnam, the Middle East, northwestern Africa, and North America (Griffiths, 1996; Larson et al., 2006).

Newts, urodelan amphibians, belong to the family Salamandridae, which includes 18 genera and 73 described species in North America and Eurasia (Stuart and Papenfuss, 2002; Frost et al., 2006). The five genera in Asia are *Cynops*, *Echinotriton*, *Pachytriton*, *Paramesotriton*, and *Tylototriton* (Stuart and Papenfuss, 2002). *Tylototriton* has been reported from eastern and western China, Nepal, Sikkim and Darjeeling in India, Burma, northern Thailand, northern Vietnam, and Okinawa in Japan (Zhao et al., 1988). This genus currently consists of eight species: *Tylototriton verrucosus*, *T. asperrimus*, *T. kweichowensis*, *T. taliangensis*, *T. hainanensis*, *T. wenxianensis*, *T. shanjing*, and *T. vietnamensis*.
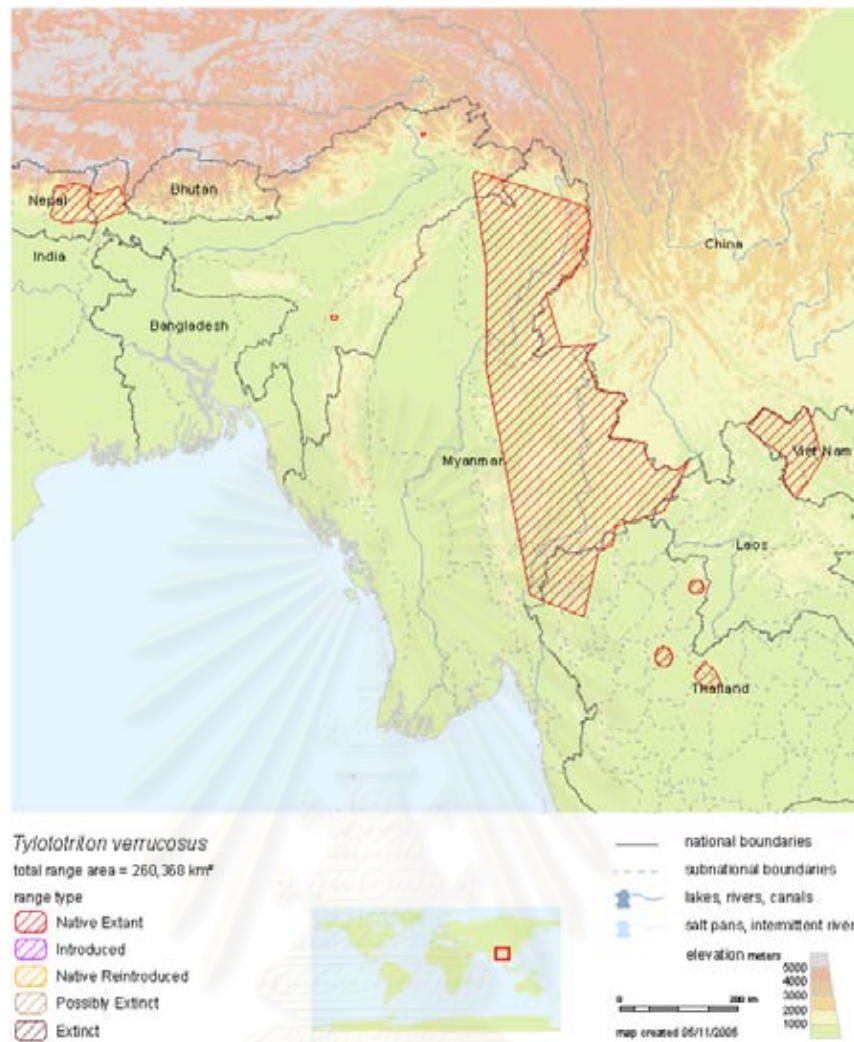
Figure 2.1   The geographic range of *T. verrucosus* (www.globalamphibians.org).

The Himalayan newt, *T. verrucosus*, is the only Urodelan species currently recorded in Thailand despite fairly comprehensive investigation (Taylor, 1962; Beaver, 1982; Wongratana, 1984; Chan-ard et al., 1999; Nabhitabhata, Chan-ard, and Chuaynkern, 2000; Nutphund, 2001; Sanguansombat, 2001; Chan-ard, 2003). It is usually found in still-water ponds, streams (Chan-ard et al., 1999; Nutphund, 2001; Chan-ard, 2003; Pomchote, 2004), or under logs or wood debrises (Sanguansombat, 2001) in the northern mountain ranges (Sanguansombat, 2001; Chan-ard, 2003): at Chiang Mai (Smith, 1924; Suvatti, 1965; Beaver, 1982; Wongratana, 1984; Matsui et al., 1996; Nabhitabhata et al., 2000; Nutphund, 2001; Pomchote, 2004; Nabhitabhata and Chan-ard, 2005), Mae Hong Son (Nabhitabhata et al., 2000; Pomchote, 2004; Nabhitabhata and Chan-ard, 2005), and Nan Provinces (Nabhitabhata and Chan-ard, 2005), and in the northeastern mountain ranges (Sanguansombat, 2001; Chan-ard,

2003): at Loei Province (Wongratana, 1984; Chan-ard et al., 1999; Nabhitabhata et al., 2000; Nutphund, 2001; Pomchote, 2004; Nabhitabhata and Chan-ard, 2005) at an altitude more than 1,000 m (Smith, 1924; Taylor, 1962; Wongratana, 1984; Matsui et al., 1996; Chan-ard et al., 1999; Nutphund, 2001; Pomchote, 2004; Nabhitabhata and Chan-ard, 2005).

Interestingly, Pomchote (2004) presented basic data that this taxon in Thailand may be divided into two different types based on differences in morphometry, body coloration and distribution.

Salamandrid species have many natural enemies because they have a relatively small size and slow movement. Correspondingly, many of them have toxic skin-gland secretions (Zug, 1993; Griffiths, 1996; Hofrichter, 2000; Halliday and Adler, 2002). Lai et al. (2002) found that one toxin, a component of the secretion from the toxic gland of *T. verrucosus*, is protein which is harmful to both mice and bacteria, and contains both proteolytic and trypsin inhibitory activities. However, neither hemolytic nor hemorrhagic activities were found. Brodie Jr. (1977) and Brodie Jr., Nussbaum, and DiGiovanni (1984) studied anti-predator mechanisms of salamanders including the genus *Tylototriton*. This taxon exhibited a great diversity of defensive postures and especially by displaying the regions that consists of the glandular or toxic glands in response to a threatening stimulus. These behaviors serve as warnings of its toxicity and may be used in defensive display.

The Himalayan newt, *T. verrucosus*, displays sexual dimorphism in the breeding season with larger sized females than males (Roy and Mushahidunnabi, 2001; Pomchote, 2004). This species also has a nuptial dance as a form of the courtship behavior (Roy and Mushahidunnabi, 2001). The number and features of fertilized eggs and its egg laying sites were reported by Kuzmin, Dasgupta, and Smirina (1994), Anders, Schleich, and Shah (1998), Roy and Mushahidunnabi (2001), Pomchote (2004), and Hegde and Deuti (2007). Moreover, the correlation of parental care and egg size of this species by Ferrier (1974) was reviewed by Nussbaum (1987).

For food patterns of this species, the data were recorded by Nussbaum (1987), Kuzmin et al. (1994), Anders et al. (1998), and Pomchote (2004) suggest that larvae normally forage on small crustaceans, invertebrates or smaller siblings. Adults consumed both small invertebrates and vertebrates.

There is an extensive biological study of *T. verrucosus* from East Nepal by Anders et al. (1998). They compared data between individuals from five localities of

East Nepal and reported details including their distribution, habitats, morphometry, external and internal morphology, coloration, and ecology.

T. verrucosus is considered a sensitive species in Thailand, based on its known patchy distribution and isolated populations (Wongratana, 1984). Because of extensive deforestation and agricultural development, much of their potential habitats have either been altered to reduced suitability, or destroyed (Beaver, 1982; Wongratana, 1984; Kuzmin et al., 1994; Seglie et al., 2003; Hegde and Deuti, 2007). Thus, it is likely that their distribution pattern is now different from previously reported, and given its threatened status, any local extinctions and loss of occupied habitat is of interest and concern.

Therefore, field surveys will be carried out in an attempt to assess the present distribution pattern and status of the Himalayan newt (*Tylototriton verrucosus*) in Thailand. The field sites were mountainous areas in the northern and northeastern Thailand. In addition to the distribution and status, information regarding habitat, reproduction, and morphology were recorded. The knowledge gained from this study can be used in the implementation of future conservation efforts.

MATERIALS AND METHODS

In this study, data regarding the distribution of *T. verrucosus* were obtained by 1) reviewing the existing published literature (both scientific and non-scientific), 2) examining available museum specimens and reference collections, and 3) conducting field surveys in the northern and northeastern parts of Thailand.

The preliminary data in this study were gathered from the published (Smith, 1924; Taylor, 1962; Suvatti, 1965; Beaver, 1982; Wongratana, 1984; Matsui et al., 1996; Chan-ard et al., 1999; Nabhitabhata et al., 2000; Nutphund, 2001; Chan-ard, 2003; Pomchote, 2004; Nabhitabhata and Chan-ard, 2005) and from the non-scientific literature i.e., nature magazines, photographs taken by tourists or park rangers, and Thai websites.

Additional information was obtained by examining preserved specimens kept at the Chulalongkorn University Museum of Zoology (CUMZ). Field surveys were conducted at 14 locations within seven northern and northeastern provinces of Thailand, as follows:

1. Watershed management station, Doi Ang Khang, Chiang Mai Province
2. Phuping Rajanives Palace, Doi Suthep, Chiang Mai Province
3. Doi Chang Kien, Doi Pui, Chiang Mai Province
4. Royal Garden Siribhume, Doi Inthanon, Chiang Mai Province
5. Watershed management station, Doi Inthanon, Chiang Mai Province
6. Doi Inthanon NP, Chiang Mai Province
7. Doi Lahnga, Khun Chae NP, Chiang Rai Province
8. Namtok Mae Surin NP, Mae Hong Son Province
9. Doi Phuka NP, Nan Province
10. Phu Suan Sai NP, Loei Province
11. Phu Reua NP, Loei Province
12. Phu Luang WS, Loei Province
13. Phu Hin Rong Kla NP, Phitsanulok Province
14. Nam Nao NP, Phetchabun Province

The visual encounter survey method (Heyer et al., 1994) was used. Surveys were conducted during December 2001 to September 2006. The previously reported

habitats of newts (Chan-ard et al., 1999; Nutphund, 2001; Chan-ard, 2003; Pomchote, 2004), e.g., small streams, ponds, and under stones or logs on the mountains with an elevation of at least 1,000 m asl, were chosen as survey sites in the above 14 locations. Upon an encounter, the newt was captured either by hand or aquatic dip net. A photograph of each captured individual was taken on site for color pattern analysis thereafter. Data regarding habitat type, altitude, coordination, population, morphology (color pattern and size) and behavior were recorded. The ecological and physical data of habitat, e.g., habitat type of stream, pond or mountain, water depth, and water and air temperatures were also recorded. Data were analyzed using SigmaStat version 2.0. Most newts were then released to their habitats but a few specimens were preserved in 95 % (v/v) ethanol for subsequent molecular and bone analyses.
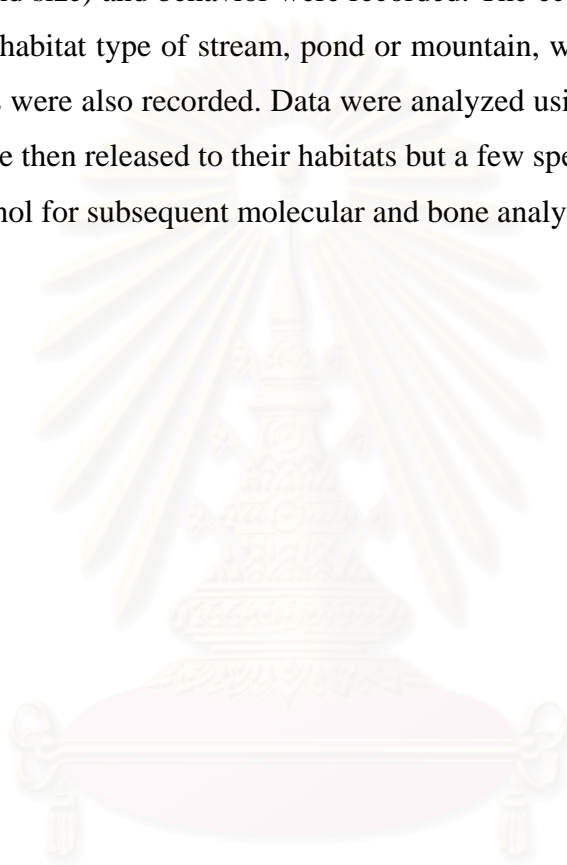
RESULTS

Distribution

      The distribution of the Himalayan newt, *T. verrucosus*, obtained by reviewing previous published literature (scientific and non-scientific), verified museum specimens, and field surveys are displayed in **Figs. 2.2** and **2.3**. Because of no previous data suggesting the distribution of these species in other areas were found, the surveys were conducted only in the north and northeast. It was concluded that this species was restricted to the north and the northeast of Thailand (16-19°N). The majority of populations were found at high altitude where the elevation is at least 1,000 m asl and thus typically at or near the top of mountains.

      From the verified specimens in museum and previously published literatures, *T. verrucosus* was found only in four provinces, namely Mae Hong Son, Chiang Mai, Nan and Loei (**Table 2.1**). Of the 14 survey locations, newts were encountered in 11 locations (**Table 2.2**). Among those 11 locations, Chiang Rai and Phitsanulok are provinces with no prior record and thus constitute new locality records.
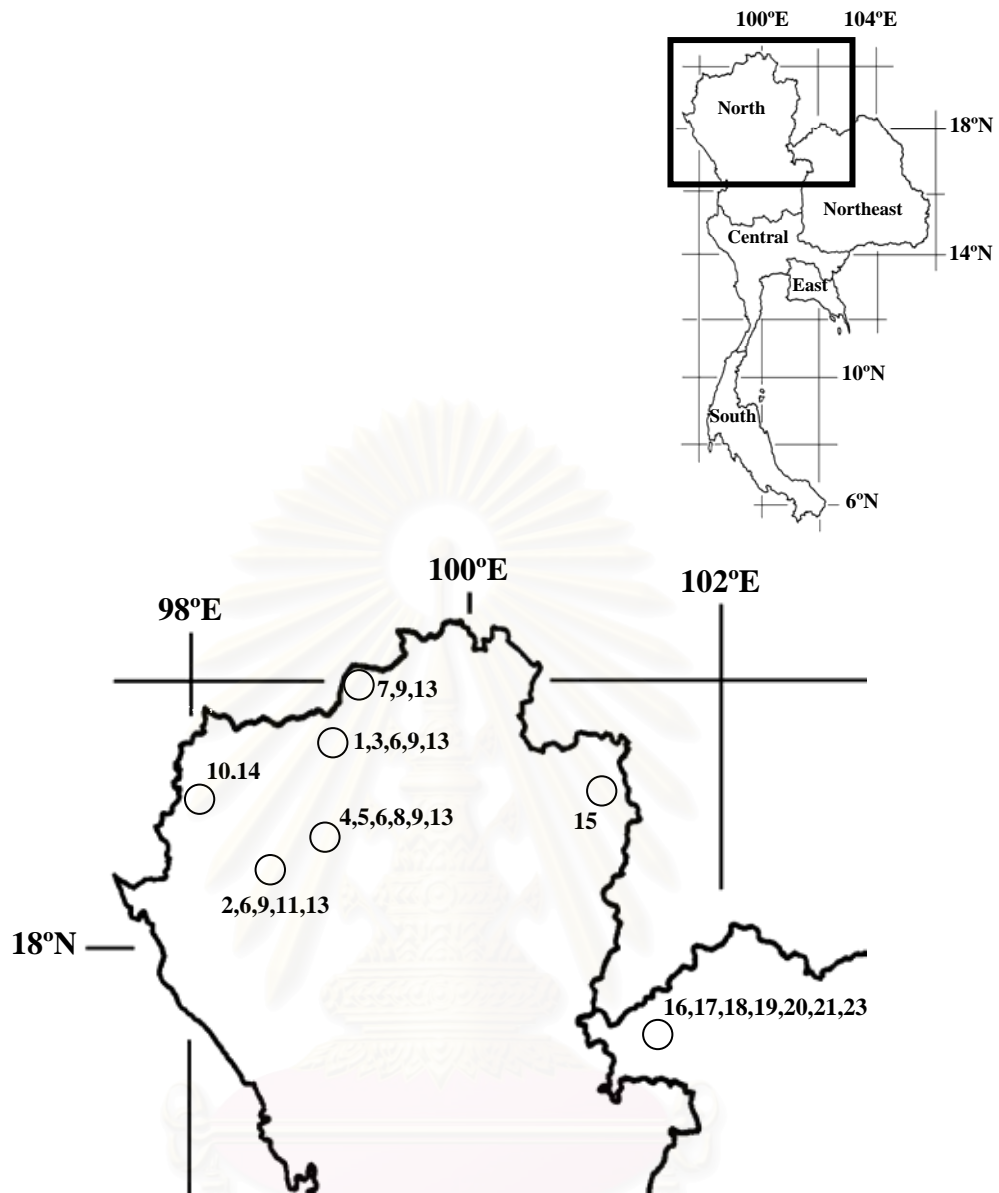
Fig. 2.2 Distribution of the Himalayan newt (*T. verrucosus*) gathered from the published literature. The numbers refer to the locations in **Table 2.1**.
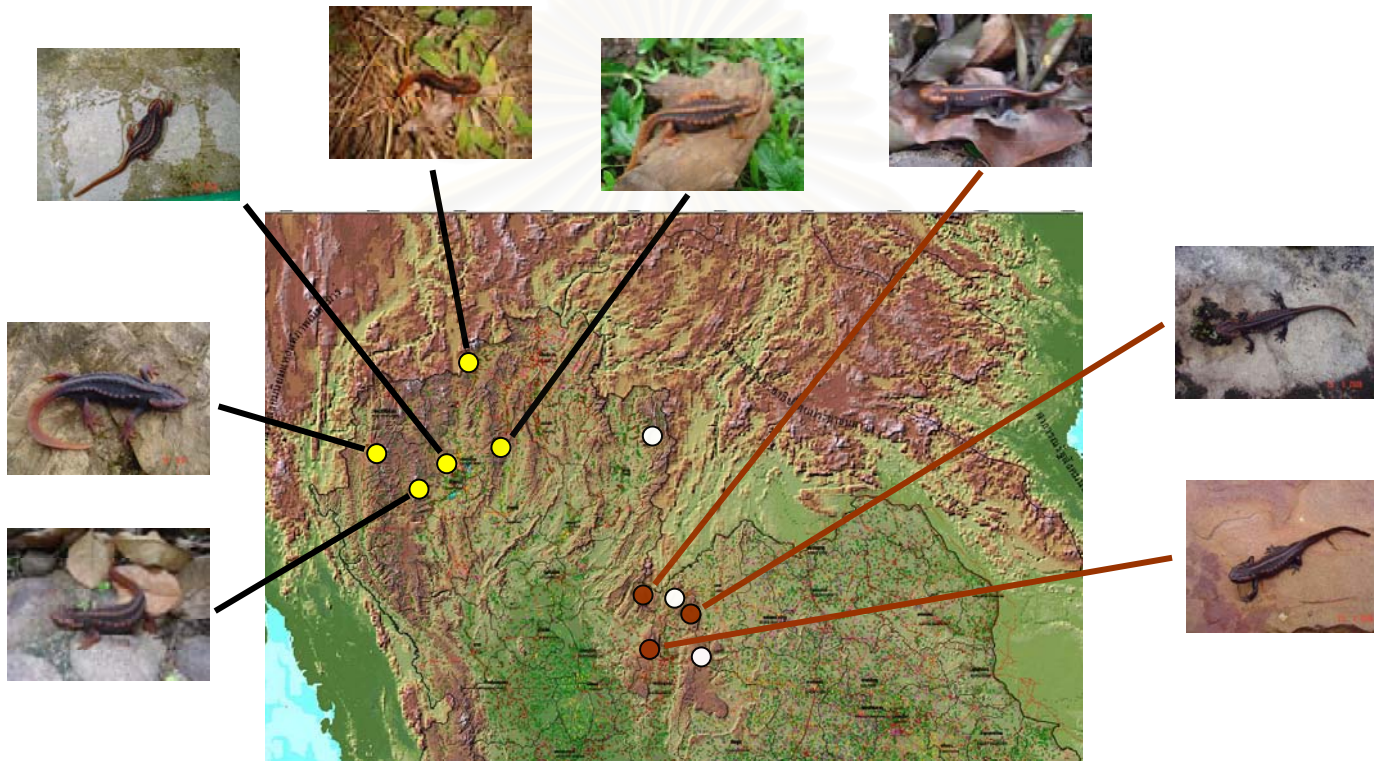
Fig. 2.3 Distribution of the Himalayan newt from our survey.

Table 2.1   Locations and number of newts reported from the published literature.

Region	No.	Province	Location	Number	Reference
North	1	Chiang Mai	Chiang Dao	40	Smith, 1924
	2	Chiang Mai	Doi Inthanon NP	1	Taylor, 1962
	3	Chiang Mai	Chiang Dao	1	Taylor, 1962
	4	Chiang Mai	Huey Kok-ma, Doi Suthep-Pui	3	Suvatti, 1965
	5	Chiang Mai	Doi Suthep	1	Beaver, 1982
	6	Chiang Mai	Doi Chiang Dao, Doi Pui, Doi Inthanon and Doi Suthep	25	Wongratana, 1984
	7	Chiang Mai	Doi Ang Khang	1	Wongratana, 1984
	8	Chiang Mai	Doi Pui	N/A	Matsui et al., 1996
	9	Chiang Mai	Doi Chiang Dao, Doi Suthep-Pui, Doi Inthanon and Doi Ang Khang	N/A	Nabhitabhata et al., 2000
	10	Mae Hong Son	Namtok Mae Surin NP	N/A	Nabhitabhata et al., 2000
	11	Chiang Mai	Doi Inthanon	N/A	Nutphund, 2001
	12	N/A	N/A	N/A	Chan-ard, 2003
	13	Chiang Mai	Doi Chiang Dao, Doi Suthep-Pui, Doi Inthanon and Doi Ang Khang	N/A	Nabhitabhata and Chan-ard, 2005
	14	Mae Hong Son	Namtok Mae Surin NP	N/A	Nabhitabhata and Chan-ard, 2005
	15	Nan	Doi Phu Ka	N/A	Nabhitabhata and Chan-ard, 2005

Table 2.1   (continued)

Region	No.	Province	Location	Number	Reference
Northeast	16	Loei	Phu Luang WS	1	Wongratana, 1984
	17	Loei	Phu Luang WS	1	Wongratana, 1984
	18	Loei	Phu Luang WS	1	Wongratana, 1984
	19	Loei	Phu Luang WS	N/A	Chan-ard et al., 1999
	20	Loei	Phu Luang WS	N/A	Nabhitabhata et al.,
	21	Loei	Phu Luang WS	N/A	2000
	22	N/A	N/A	N/A	Nutphund, 2001
	23	Loei	Phu Luang WS	N/A	Chan-ard, 2003
					Nabhitabhata and
					Chan-ard, 2005
Total number				75	

Table 2.2   Locations and the number of newts found in this survey.

Region	No.	Province	Location	Microhabitat	Coordinate (North, East)	Altitude (m)	Number found	Survey date	Temperature (°C) Water	Temperature (°C) Air	Water depth (cm)
North	1	Chiang Mai	Watershed management station, Doi Ang Khang	Small pond	19° 53' 42.4", 099° 03' 02.2"	1,492	11	December 2001	14.5	11.7	74.6
	2	Mae Hong son	Namtok Mae Surin NP	Large pool	18° 56' 27.3", 098° 04' 23.0"	1,267	8	October 2004	19.3	19.6	N/A
	3	Chiang Rai	Doi Lahnga, Khun Chae NP	Medium pool and under rocks	18° 56' 04.4", 099° 22' 47.8"	1,443	10	May 2006	21.4	21.2	N/A
	4	Chiang Mai	Phuping Rajanives Palace, Doi Suthep	Artificial pool	18° 48' 16.0", 098° 54' 09.3"	1,436	68	June 2005	20.9	23.6	16.5
	5	Chiang Mai	Doi Chang Kien, Doi Pui	N/A	N/A	N/A	1	October 2004	N/A	N/A	N/A
	6	Chiang Mai	Royal Garden Siribhume, Doi Inthanon	Agricultural pond	18° 32' 46.4", 098° 31' 13.7"	1,313	13	May 2005, June 2005	19.2	20.6	29

Table 2.2   (continued)

Region	No.	Province	Location	Microhabitat	Coordinate (North, East)	Altitude (m)	Number found	Survey date	Temperature (°C)		Water depth (cm)
									Water	Air	
North	7	Chiang Mai	Watershed management station, Doi Inthanon	Small pond	18° 30' 51.5", 098° 28' 29.3"	1,584	3	January 2003	16.2	14.4	50
	8	Chiang Mai	Doi Inthanon NP	N/A	N/A	N/A	1	January 2004	N/A	N/A	N/A
Northeast	9	Loei	Phu Suan Sai NP	Small muddy pond	17° 31' 48.9", 100° 58' 44.7"	1,119	5	May 2006	21.7	22.2	18
	10	Loei	Phu Luang WS	Small streams	17° 16' 23.9", 101° 31' 18.8"	1,515	16	July 2003, May 2006	18.9	21.5	53
	11	Phitsanulok	Phu Hin Rong Kla NP	Flooded pools	16° 59' 17.8", 101° 00' 05.4"	1,285	29	May 2003, May 2006	19.5	20.1	26.2
Total number							**165**				

Table 2.3   Locations examined but at which no newts were found in this survey.

| Region | No. | Province | Location | Microhabitat | Coordinate (North, East) | Altitude (m) | Number found | Survey date | Temperature (°C) | | Water depth (cm) |
									Water	Air	
North	12	Nan	Doi Phuka NP	N/A	19° 12' 33.0", 101° 04' 15.8"	1,130	0	May 2005	N/A	N/A	N/A
Northeast	13	Loei	Phu Reua NP	N/A	17° 29' 58.5", 101° 20' 29.8"	1,183	0	May 2006	N/A	N/A	N/A
	14	Phetchabun	Nam Nao NP	N/A	16° 45' 02.0", 101° 33' 54.6"	945	0	May 2005	N/A	N/A	N/A

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Color Pattern

From the surveys, two types of color pattern of *T. verrucosus*, broadly an orange to yellow body coloration (Type I), and a duller body coloration (Type II) were observed (**Figs. 2.4-2.14**). Interestingly, these two types were geographically separated. Type I (orange to yellow body) newts were found only in the northern part of Thailand: Doi Ang Khang, Namtok Mae Surin NP, Khun Chae NP, Phuping Rajanives Palace, Doi Suthep, Doi Pui and Doi Inthanon NP, while the Type II (dull body) newts were found only in the northeastern part of Thailand: Phu Suan Sai NP, Phu Luang WS and Phu Hin Rong Kla NP (**Table 2.2**, **Fig. 2.3**).

Female newts with the Type I (orange to yellow body) were noticeably bigger compared to those with the Type II (dull body) (**Fig. 2.15**), and in general, female newts had a significantly larger and more robust size than males (**Fig. 2.16**). For instance, at Phuping Rajanives Palace, Chiang Mai Province, the average weight of 19 females was 20.25 ± 5.72 g (ranging over 9.7 - 30.8 g) whilst that for 20 males was 9.96 ± 1.25 g (ranging over 8.1 - 12.6 g) ($p < 0.001$; Mann-Whitney Rank Sum Test). The average snout-vent length of these 19 females and 20 males was 77.77 ± 10.02 mm (ranging over 57.2 - 88.3 mm) and 63.93 ± 6.13 mm (ranging over 60.2 - 88.6 mm) ($p < 0.001$; Mann-Whitney Rank Sum Test), respectively.

Fig. 2.4   *T. verrucosus* Type I from Watershed management station, Doi
Inthanon, Chiang Mai Province.

Description of Coloration – The ground color are brown or black. The cranial crests,
parotoid glands, dorsal ridge, dorsolateral warts, limbs, tail and ventral surface are
orange to yellow coloration.

Fig. 2.5 *T. verrucosus* Type II from Phu Suan Sai NP, Loei Province.

Description of Coloration – The dorsal body, ventral surface and limbs are dull or brown coloration. The cranial crests, parotoid glands, dorsal ridge of body and tail, dorsolateral warts, lower jaw, vent, ventral ridge of tail and tip of phalanges are lighter brown than body.

Fig. 2.6 A juvenile of *T. verrucosus* (Type I) from Watershed management station, Doi Ang Khang, Chiang Mai Province.

Fig. 2.7 *T. verrucosus* (Type I) from Namtok Mae Surin NP, Mae Hong Son Province.

Fig. 2.8   *T. verrucosus* (Type I) from Doi Lahnga, Khun Chae NP, Chiang Rai
                 Province.

Fig. 2.9   *T. verrucosus* (Type I) from Phuping Rajanives Palace, Doi Suthep,
                 Chiang Mai Province.

Fig. 2.10   *T. verrucosus* (Type I) from Royal Garden Siribhume, Doi Inthanon, Chiang Mai Province.

Fig. 2.11   *T. verrucosus* (Type I) from Watershed management station, Doi Inthanon, Chiang Mai Province.

Fig. 2.12   *T. verrucosus* (Type II) from Phu Suan Sai NP, Loei Province.

Fig. 2.13   *T. verrucosus* (Type II) from Phu Luang WS, Loei Province.

Fig. 2.14 *T. verrucosus* (Type II) from Phu Hin Rong Kla NP, Phitsanulok Province.
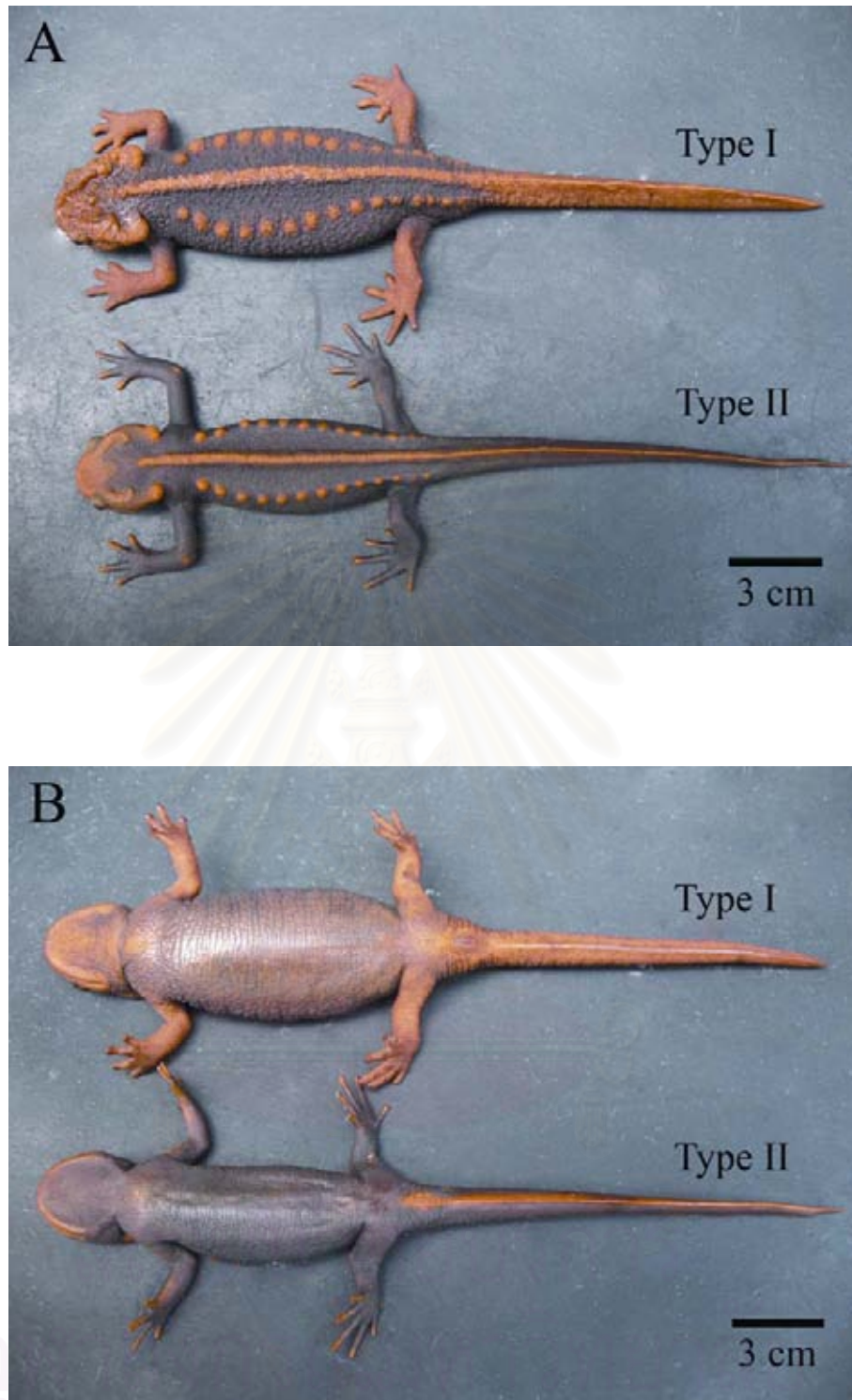
Fig. 2.15   (A) Dorsal view and (B) ventral view of Type I (orange to yellow body coloration) and Type II (dull body coloration) females of *T. verrucosus* in Thailand.
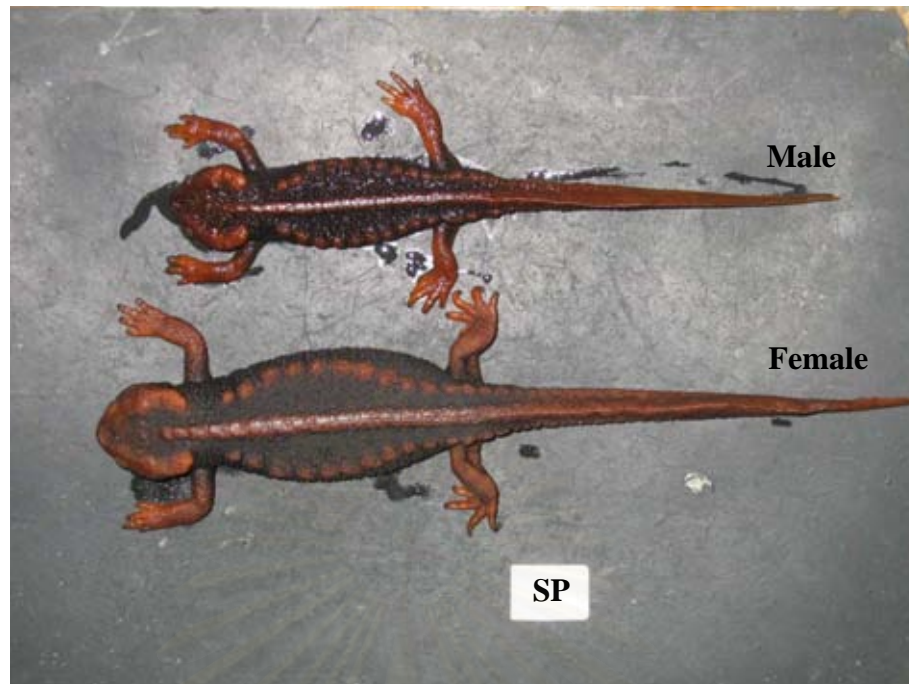
Fig. 2.16   The significant larger and more robust size of female (below) than male (above) newts. These newts were found at Royal Garden Siribhume, Doi Inthanon, Chiang Mai Province.

Habitat Uses and Foraging Behaviors

Comparing the habitats of the Himalayan newt in from the published data and these surveys in Thailand, no significant differences were observed. Moreover, the two morphotypes Type I and Type II found in the north and the northeast of Thailand also occupy the same habitats (**Figs. 2.17-2.25**). They were usually found in small slow-moving streams or ponds with either clear or muddy water conditions (**Figs. 2.26** and **2.27**), and an average depth of 38.18 ± 21.57 cm (range; 9 - 120 cm). In most instances, the bottoms were covered with small pieces of debris. The average water and air temperatures were 19.07 ± 2.37 °C (range; 13.4 - 21.7 °C), and 19.43 ± 3.86 °C (range; 9.5 - 26.0 °C), respectively. The average altitude of inhabited locations was 1,383.8 ± 147.38 m (range; 1,119 - 1,584 m).

In all cases where habitats where newts were found in contrast no fish were found, yet small water insects co-inhabited the same habitats with the newt. This may well suggests that fish are perhaps voracious predators of eggs and larvae. It remains to be determined if this is true, and if so, which fish species are responsible and thus potentially limiting to newt distribution.

As stated, in all areas where newts were found, small water insects co-inhabited the same habitats with the newt. Larvae and adults caught from the field sites and reared in laboratory at Chulalongkorn University likewise were found to forage mainly on small crustaceans such as water fleas (*Moina macrocopa*) and also showed cannibalistic behaviors among siblings of larvae with different sizes (**Fig. 2.28**), whilst adults consumed small invertebrates and vertebrates such as bloodworms (*Chironomus* sp.), mealworms (*Tenebrio molitor*) and crickets (*Acheta* sp.).

Fig. 2.17   The habitat of *T. verrucosus* at Watershed management station, Doi Ang Khang, Chiang Mai Province.

Fig. 2.18   The habitat of *T. verrucosus* at Namtok Mae Surin NP, Mae Hong Son Province.

Fig. 2.19   The habitat of *T. verrucosus* at Doi Lahnga, Khun Chae NP, Chiang
             Rai Province.

Fig. 2.20   The habitat of *T. verrucosus* at Phuping Rajanives Palace, Doi Suthep,
             Chiang Mai Province.

Fig. 2.21   The habitat of *T. verrucosus* at Royal Garden Siribhume, Doi Inthanon,
Chiang Mai Province.

Fig. 2.22   The habitat of *T. verrucosus* at Watershed management station, Doi
Inthanon, Chiang Mai Province.

Fig. 2.23   The habitat of *T. verrucosus* at Phu Suan Sai NP, Loei Province.

Fig. 2.24   The habitat of *T. verrucosus* at Phu Luang WS, Loei Province.

Fig. 2.25   The habitat of *T. verrucosus* at Phu Hin Rong Kla NP, Phitsanulok
Province.

Fig. 2.26   A clear water habitat of *T. verrucosus*; Phu Luang WS, Loei Province.

Fig. 2.27   A muddy water habitat of *T. verrucosus*; Watershed management

                   station, Doi Ang Khang, Chiang Mai Province.

Fig. 2.28   The different sizes of larval siblings cause cannibalistic behaviors.

Breeding Behaviors

In Thailand, the Himalayan newt was observed to display seasonal breeding, this being in May to July, and is the beginning of rainy season. During the breeding season, the cloacal openings of females are dominant circular while those of males are oval (**Fig. 2.29**).

Two females captured from Phu Luang WS and kept in caged boxes in May, 2006, laid 15 and 20 fertilized eggs, respectively, which were covered by a transparent jelly envelope. In nature, fertilized eggs are deposited on wet grass or plants which overhang the water surface, near small streams or ponds, and were observed in May 2004, at Phu Hin Rong Kla NP, Phitsanulok Province and in May 2006, at Phu Suan Sai NP, Loei Province (**Figs. 2.30-2.33**). Juveniles were found from August to December, 2005 at Chiang Mai Province (**Fig. 2.34**).

Fig. 2.29   The prominently oval shape of male (left) cloacal opening compared to the noticeably circular shape of female (right). Newt specimens were from Phuping Rajanives Palace, Doi Suthep, Chiang Mai Province during the breeding season.

Fig. 2.30   A pair of eggs of *T. verrucosus* (Type II) from Phu Suan Sai NP, Loei Province.

Fig. 2.31   The oviposition site of *T. verrucosus* (Type II) from Phu Suan Sai NP, Loei Province.

Fig. 2.32   An egg of *T. verrucosus* (Type II) from Phu Hin Rong Kla NP, Phitsanulok Province.

Fig. 2.33   The oviposition site of *T. verrucosus* (Type II) from Phu Hin Rong Kla NP, Phitsanulok Province.

Fig. 2.34   The nearly equal size of three juveniles found at Phuping Rajanives Palace, Doi Suthep, Chiang Mai Province.

DISCUSSION

During our field surveys, *T. verrucosus* was found at 11 locations in Thailand, of which eight represent new locality distribution records. In previous publications, this species has been reported from only four provinces of Thailand: Mae Hong Son, Chiang Mai, Nan and Loei (**Table 2.1**). The field surveys reported here adds a further two provinces: Chiang Rai and Phitsanulok. However, against this, the newts were not found at Phu Reua NP in Loei Province, Nam Nao NP in Phetchabun Province, and Doi Phuka in Nan Province. Perhaps this search did not cover appropriate habitats at those localities.

As reported by Wongratana (1984), newts were found at Phu Luang WS which was the lowest reported latitude. However, in this survey newts were encountered at Phu Hin Rong Kra NP, Phitsanulok Province, which is located at a more southerly latitude than Phu Luang WS (**Fig. 2.2**), and thus extends its known distribution in Thailand slightly southwards. Whether this represents the discovery of a previously population or a true recent range expansion southwards cannot be determined. Regardless, here this study confirms that this species currently is distributed in northern and northeastern mountain ranges in accordance with Nabhitabhata et al. (2000), Sanguansombat (2001), Chan-ard (2003), and Pomchote (2004), while Kuzmin et al. (1994), Anders et al. (1998), Nutphund (2001), Palden (2003), and Hegde and Deuti (2007) reported that this species found only northern mountain ranges.

Two types of the Himalayan newt were found with respect to different color patterns and female sizes: Type I (orange to yellow body coloration) distributed in the northern mountain ranges and Type II (dull body coloration) distributed in the northeastern mountain ranges. Type I females were relatively larger in size than Type II females, but no differences were observed in males of both types. In general, female newts are significantly larger and more robust than males, as reported by Roy and Mushahidunnabi (2001) and Hegde and Deuti (2007).

Whilst these two types of color patterns and female size differences have not been reported before, the polymorphism has only been noted, so far at least, within Thai newts. For example, Wongratana in 1984 mentioned the color of newts from one sampling population which may match the Type II of this survey (dull body

coloration), whilst Taylor (1962) reported the color of another specimen with lighter brown glands on sides of neck and dorsolateral regions were lighter brown and a tail that was generally lighter brown than body but never compared the color pattern thoroughly across the Thai populations. Moreover, the figure of two newts illustrated (Thai and Yunnan types are orange and yellow colorations, respectively) by Nutphund in 2001 were similar to the Type I in this study, whilst Chan-ard (2003) displayed a single picture that resembles the Type II in this study, and Matsui et al. (1996) also showed a single photo from the northern part of Thailand that resembles the Type I in this study. Based on these different color patterns and habitat, which support existence of these two morphotypes and their geographical separation, the differences between them as subspecies or conspecifics requires DNA analysis and, if possible, hybridization studies over several generations to confirm hybrid fertility or not for further investigation.

In agreement with the previous published documents, where most of the habitats that the species was found at were above 1,000 m asl (Smith, 1924; Taylor, 1962; Wongratana, 1984; Kuzmin et al., 1994; Matsui et al., 1996; Anders et al., 1998, Chan-ard et al., 1999; Nutphund, 2001; Palden, 2003; Pomchote, 2004; Nabhitabhata and Chan-ard, 2005; Khonsue and Pomchote, 2006; Hegde and Deuti, 2007), in this study newts habitats were always above 1,100 m asl.

Based on the data of habitat use, *T. verrucosus* utilized aquatic habitats for breeding and inhabited dry lands in the non-breeding season (Kuzmin et al., 1994; Anders et al., 1998; Khonsue and Pomchote, 2006; Hegde and Deuti, 2007). For the Thai population, this was the beginning of rainy season (Wongratana, 1984) and typically May until July, as observed from the two populations at Phu Hin Rong Khra NP and Phuping Rajanives Palace. On the other hand, Kuzmin et al. (1994) reported that *T. verrucosus* in Darjeeling in India bred in March to May and sometimes up to September. During the reproductive period, the cloacal openings of females were distinctly circular in shape whilst those of males show oval shapes, and these agree with the figure of Anders et al. (1998).

The fertilized eggs, encased in a transparent jelly envelope such that the developing embryos were visible, were in accordance with Roy and Mushahidunnabi (2001) and Hegde and Deuti (2007). The preferable locations for egg laying were under moist pieces of wood debris, as observed at Phu Hin Rong Kla NP, Phitsanulok Province on May, 2004 as a single egg, or attached to wet grass, submerged

vegetation in small streams or ponds, as observed at Phu Suan Sai NP, Loei Province on May, 2006, as a pair of eggs. Although this study only located a single and paired egg once each, the locations types of each of these two deposited eggs are in agreement with the previously reported data of Kuzmin et al. (1994) and Roy and Mushahidunnabi (2001), but in contrast not with the report by Zhao et al. (1998) that newts in this genus deposited their eggs in the water, and the report by Anders et al. (1998) that found only single and not paired eggs.

Water current is possibly one of the important governing factors regarding habitat use, since newts were not found in fast-moving streams; rather most of them inhabited areas with slow currents in agreement with Anders et al. (1998). Kuzmin et al. (1994) and Hegde and Deuti (2007) mentioned that the habitats of *T. verrucosus* were rice fields, tea gardens, meadows underlying the shores of pools and at the edge of woods, again all slow moving current habitats. In comparison, from this study, newts were found in both aquatic habitats (small streams, ponds and water falls) and up lands (under stones and wood debrises in tea garden at Chiang Mai Province). Besides, newts were discovered in both clear and muddy waters. Notice that, the aquatic habitats where newts were found were free of fish. These results may reflect that fish are voracious and effective predators of either eggs (if laid in the water) and or larvae, consistent with Anders et al. (1998). Regarding their diets, larval newts prey upon small crustaceans and insects, while adults prey on larger insects, earthworms, amphibian eggs and larvae as a reported by, for example, Kuzmin et al. (1994).

To better understand newt biology in Thailand, especially for conservation, the following information should be studied in detail: taxonomic clarification, distribution in other locations, life history, ecology, and population dynamics. These data are required to understand better the requirements to manage and conserve the key habitats which have not yet been destroyed for the purposes of cultivation or tourism, and perhaps restore other habitats to suitable niches for natural or introduced colonization's.

CONCLUSION

The Himalayan newt (*Tylototriton verrucosus*) in Thailand can be divided into two types based on color patterns, distribution and female sizes. Type I (orange to yellow body coloration) is distributed in the northern mountain ranges at Doi Ang Khang, Chiang Mai Province (the Daen Lao Range); Namtok Mae Surin NP, Mae Hong Son Province, Doi Inthanon, Doi Suthep and Doi Pui, Chiang Mai Province (the East Thanon Thong Chai Range); Khun Chae NP, Chiang Rai Province (the Western Phi Pan Nam Range). Type II (dull body coloration) is distributed in the northeastern mountain ranges at Phu Suan Sai NP, Loei Province and Phu Hin Rong Kla NP, Phitsanulok Province (the Western Phetchabun Range); Phu Luang WS, Loei Province (the Eastern Phetchabun Range). Furthermore, Type I females are larger in size than Type II females.

This species inhabits at an elevation of over 1,000 m asl and uses aquatic habitats such as mountain pools, hill streams and ponds for breeding, and inhabits dry lands such as under rocks, stones and wood debrises in the non-breeding season.

The breeding season is the beginning of rainy season. During this period, the cloacal openings of females are obviously circular but males are oval. The fertilized eggs are laid singly or in pairs on land or in water attached on submerged plants.

The larvae and adults can consume a variety of invertebrates and vertebrates including their smaller siblings.

CHAPTER III
GENETIC VARIATION OF NEWTS GENUS
Tylototriton IN THAILAND
INTRODUCTION

Salamanders and newts are amphibians that belong to the Kingdom Animalia; Phylum Chordata; Class Amphibia; Order Caudata (or Urodela). These groups can be clearly distinguished from other amphibians (frogs and caecilians) by the following characteristics: elongated bodies, present of tails in all post ova developmental stages including adults, the two pairs of limbs have an approximately equal size (except in the family Sirenidae which is lacking hindlimbs) (Halliday and Adler, 2002; Larson, Wake, and Devitt, 2006), and the differences in their skeleton and musculature (Larson et al., 2006).

10 families, 62 genera, and 548 species of salamanders are currently recognized (Frost et al., 2006), and are widespread being distributed throughout North America, Central America, Northern South America, Europe, Mediteranean, Africa, and Asia including Japan and Taiwan (Halliday and Adler, 2002). One of the most diverse groups, the family Salamandridae, is divided into two groups: the true salamanders (smooth skin) and the newts (rough skin) (Ballinger and Lynch, 1983; Griffiths, 1996; Khonsue and Pomchote, 2006; Weisrock et al., 2006).
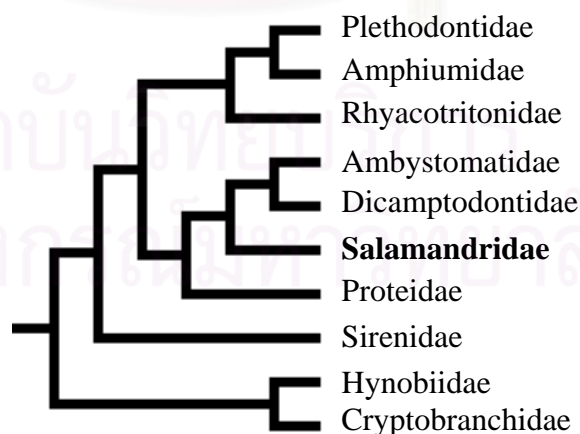
Figure 3.1   Phylogenetic relationships of salamanders based on combined molecular and morphological data (Wiens, Bonett, and Chippindale, 2005).

Newts consist of 18 genera and 73 described species in North America and Eurasia (Stuart and Papenfuss, 2002; Frost et al., 2006). The five genera in Asia are *Cynops*, *Echinotriton*, *Pachytriton*, *Paramesotriton*, and *Tylototriton* (Stuart and Papenfuss, 2002). *Tylototriton* is reported to be distributed in eastern and western China, Nepal, Sikkim and Darjeeling in India, Burma (Myanmar), northern Thailand, northern Vietnam, and Okinawa in Japan (Zhao et al., 1988). This genus includes eight currently recognized species; namely *Tylototriton verrucosus*, *T. asperrimus*, *T. kweichowensis*, *T. taliangensis*, *T. hainanensis*, *T. wenxianensis*, *T. shanjing*, and *T. vietnamensis*. In Thailand, only *T. verrucosus* Anderson 1871 (the Himalayan newt) has been recorded (e.g., Taylor, 1962; Wongratana, 1984; Matsui et al., 1996; Chan-ard et al., 1999; Nabhitabhata, Chan-ard, and Chuaynkern, 2000; Nutphund, 2001).

There are several studies on the phylogenetic relationships of Urodelan species that are associated with *Tylototriton* species. Titus and Larson (1995) studied the phylogenetic relationships within the salamander family Salamandridae using 18 species representing 14 genera and included a specimen of *T. verrucosus* (from the University of Texas-Arlington, U.S.A.) using mtDNA sequences encoding the 12S rRNA, 16S rRNA, the intervening valine tRNA and morphological characters. Veith et al. (2004) sequenced fragments of three mtDNA genes, 16S rRNA, cyt *b* and ATPase, using species from five salamandrid genera: *Pleurodeles*, *Triturus*, *Neurergus*, *Salamandra* and *Tylototriton* (*T. verrucosus* from a pet trade) to examine the plausibility of the Iberian-African vicariance hypotheses to explain the basal split within *Pleurodeles*. Weisrock et al. (2006) studied the phylogenetic relationships among salamanders of family Salamandridae (96 specimens including 61 of the 66 recognized salamandrid species and outgroups) using approximately 2,700 bases of mtDNA sequence data, including all recognized species of the genus *Tylototriton* were used, with the *T. verrucosus* specimen from Nepal (**Fig. 3.2**). Steinfartz et al. (2007) investigated the phylogenetic position of the genus *Triturus* with molecular data, based on a full sampling of *Triturus* species and a comprehensive representation of other salamandrid species using one of two Bayesian phylogenetic analyses approaches to analysis sequences derived from three mitochondrial gene fragments (12S rRNA, 16S rRNA and cyt *b*). The cyt *b* mtDNA gene fragment sequence of *T. verrucosus*, from GenBank accession number AF295685, was used.

All the above mentioned phylogenetic analyses place the genera *Tylototriton* and *Pleurodeles* as sister taxa or formed a sister clade, except for the report of Frost et

al. (2006), which suggested that the genera *Tylototriton* and *Salamandrina* formed the sister group clade within the *Pleurodeles* + (*Tylototriton* + *Salamandrina*) group.
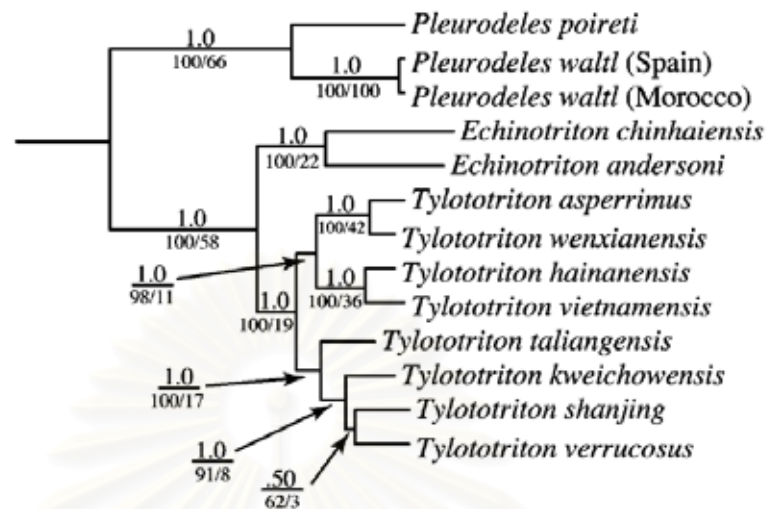
Fig. 3.2   Phylogenetic relationships for the salamandrid genera *Tylototriton*, *Echinotriton* and *Pleurodeles*. Numbers above branches are Bayesian posterior probabilities. Numbers below branches are parsimony bootstrap values (before slash) and decay indices (after slash) (Weisrock et al., 2006).

Chan, Zamudio, and Wake (2001) examined the evolutionary relationships among seven species of the three salamandrid genera: *Paramesotriton*, *Pachytriton* and *Cynops* by comparing sequences of a cyt *b* mtDNA gene fragment, using *T. verrucosus* from Jingdong, Yunnan Province, China as an outgroup (GenBank accession number AF295685).

Besides, this taxon was also used to solve the morphological problem causing substitutes or adulterants by Liu et al. (2001). They designed a pair of allele-specific primers for differentiating the high demand from Chinese crude drug, *Gecko gecko*, from its substitutes or adulterants, such as *T. verrucosus*. PCR was used based on the sequences of the mitochondrial 12S rRNA gene fragment of 17 samples from the families Gekkonidae, Agamidae, Salamandridae (including *T. verrucosus* from Tengchong, Yunnan Province, China) and Hynobiidae, respectively.

In Thailand, there have been few reports on the distribution of the Himalayan newt, *Tylototriton verrucosus*, most of which was published 20-40 years ago (e.g., Smith, 1924; Taylor, 1962; Beaver, 1982), some were in the non-scientific literatures,

and morphological descriptions (i.e., Wongratana, 1984; Nutphund, 2001; Chan-ard, 2003). However, Pomchote (2004) noted basic data that *T. verrucosus* in Thailand may be divided into two different types based on the differences in morphometry, body coloration and distribution: Type I *T. verrucosus*, with an orange to yellow body coloration similar to *T. shanjing* found in Yunnan Province, China, are distributed in the northern Thailand mountain ranges, whilst Type II *T. verrucosus*, with a dull body coloration, are distributed in the northeastern mountain ranges of Thailand and are currently not known or reported outside this region of Thailand. However, there is a surprising lack of studies concerning the population genetics of *T. verrucosus* across its reported range even though this species is listed as a near threatened species in Thailand (reported by Nabhitabhata and Chan-ard, 2005). Therefore, this study focuses on *T. verrucosus* populations, including the two morphotypes already discussed (Type I and Type II), using mtDNA sequence based analysis using two regions of differing evolutionary rates, namely a fragment of the 16S rRNA and the control or D-loop region.

MtDNA genes have been used widely for the study of population genetics and phylogenetic relationships (i.e., Brown et al., 1982; Moritz, Schneider, and Wake, 1992; Sumida, 1997; Lu et al., 2004). Although not in all, in most animals, including amphibians, the mitochondrial genome is maternally inherited, genetic recombination is rare and allelic segregation does not arise (Li and Graur, 1991; Tan and Wake, 1995). The relatively rapid evolutionary rate at the nucleotide sequence level for animal mtDNA, about 1-10 times faster than a single copy nuclear DNA depended on the gene analyzed (Brown, George, and Wilson, 1979; Vawter and Brown, 1986), and that its variation is frequently distributed among populations rather than within populations (Moritz et al., 1992; Shaffer and McKnight, 1996). Hence, mtDNA can often provide the means of distinguishing between closely related organisms at a relatively high resolution (Li and Graur, 1991). However, they are prone to problems associated with unequal gene flow, such as male biased dispersal or immigration. Given the evolutionary age of salamanders is at least 150 million years ago (Gao and Shubin 2001), especially between the sister genera *Tylototriton* and *Pleurodeles* (e.g., Titus and Larson, 1995; Carranza and Arnold, 2004; Veith et al., 2004) that separated in the late Paleogene period / first part of the Cenozoic era (Roelants et al., 2006).

The two rRNA genes found in the animal mitochondrial genome are considered unique sequences (Li and Graur, 1991). Since rRNA constitutes the non-

translated structural components of the ribosome that functions in translation of protein from mRNA (Hillis, Moritz, and Mable, 1996; Smith and Szathmary, 1999; Campbell and Reece, 2002), rRNA genes have very specific functional and structural requirements restricting their sequence evolution (Li and Graur, 1991). The evolutionary rate of 16S rRNA gene has been used to assess the phylogenetic relationships within many salamandrid groups (e.g., Steinfartz, Veith, and Tautz, 2000; Parra-Olea, Garcia-Paris, and Wake, 2002; Mattoccia, Romano, and Sbordoni, 2005).

The D-loop is the most rapidly evolving part of the mitochondrial genome (Shaffer and McKnight, 1996; Steinfartz et al., 2000; Sumida et al., 2000), and potentially provides the highest degree of resolution among recent evolutionary events and especially for closely related populations (Shaffer and McKnight, 1996; Steinfartz et al., 2000; Martinez-Solano et al., 2006).

Therefore, the combination of both mtDNA fragment sequences, within the limitations of using mtDNA only, potentially allows resolution of fairly recent to older speciation or substructuring events and consequentially they were employed to analyze the genetic variation between *T. verrucosus* populations in Thailand.

MATERIALS AND METHODS

Tissue Sampling

Tissue samples of 21 adults from seven populations, subdivided into four populations and 12 individuals of Type I *T. verrucosus*, and three populations and nine individuals of Type II *T. verrucosus* (**Table 3.1**) were obtained by removing approximately 1-2 mm^3 of the tail tip from either live or preserved specimens as detailed (Arntzen and Olgun, 2000; Mattoccia et al., 2005). The samples were preserved in 95 % (v/v) or absolute ethanol and were stored at -20 °C for DNA extraction and further analysis. For live individuals, they were released back to their habitats after tail clipping.

Table 3.1   Details of the sampling sites and numbers of specimens of *T. verrucosus* used in this study.

Region	Code of samples	Locality	Province	Coordinate (North, East)	Number
North	LK003, 004, 005	Doi Lahnga, Khun Chae NP	Chiang Rai	18° 56' 04.4", 099° 22' 47.8"	3
	PPf, g, h	Phuping Rajanives Palace, Doi Suthep	Chiang Mai	18° 48' 16.0", 098° 54' 09.3"	3
	ITNT1, T2, T3	Watershed management station, Doi Inthanon	Chiang Mai	18° 30' 51.5", 098° 28' 29.3"	3
	SPc, 11, 12	Royal Garden Siribhume, Doi Inthanon	Chiang Mai	18° 32' 46.4", 098° 31' 13.7"	3
Northeast	PK4, 5, 6	Phu Suan Sai NP	Loei	17° 31' 48.9", 100° 58' 44.7"	3
	PL007, 008, 009	Phu Luang WS	Loei	17° 16' 23.9", 101° 31' 18.8"	3
	PH019, 024, 025	Phu Hin Rong Kla NP	Phitsanulok	16° 59' 17.8", 101° 00' 05.4"	3

DNA Extraction

DNA was extracted from the tail tissue using a modified phenol-chloroform method (Sambrook and Russell, 2001). A small piece of tissue was dissected from

each sample, placed in a 1.5 ml microcentrifuge tube containing 300 µl of TEN+1 % SDS buffer (50 mM Tris-base; pH 8.0, 100 mM NaCl, 5 mM EDTA; pH 8.0, 1 % SDS (w/v)) for 15 min. The aqueous phase was removed and 335 µl of TEN+1 % SDS buffer mixed with 15 µl of proteinase K solution (7 mg/ml) was added and incubated at 55 °C for three hours or until the tissue was completely dissolved. An equal volume of phenol-chloroform (1:1 (v:v)) solution was then added to each tube, shaken vigorously, resulting milky solution. The solution was then centrifuged at 14,000 rpm for 8 min. After that, the upper aqueous phase was removed carefully and transferred to a new 1.5 ml microcentrifuge tube, and the phenol-chloroform extraction repeated. Next, 700 µl of absolute ethanol was added, inverted gently then stored at -20 °C overnight. The precipitated DNA was recovered by centrifugation at 14,000 rpm for 15 min, removal of the supernatant and washing of the pellet in 700 µl of 70 % (v/v) ethanol. The DNA pellet at the bottom of the tube was left to dry at room temperature for more than 1 hour or overnight prior to resuspension in 30 µl of TE buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0), centrifuged at 14,000 rpm for 10 sec and stored at -20 °C until further use.

Agarose Gel Electrophoresis

In order to determine the quality and to quantity the concentration of extracted DNA, agarose gel electrophoresis technique was used. For checking, the loading sample was comprised of 1 µl of the test DNA solution, 3 µl of 4x loading dye (standard stain orange G, 40 % (v/v) glycerol) and 7 µl of distilled water. A TBE-0.8 % (w/v) of agarose gel was prepared by weighting 0.4 g of GenePure LE Agarose (RESEARCH ORGANICS, INC.) and mixing with 50 ml of 1X TBE buffer (0.89 M Tris-base, 0.89 M boric acid, 0.02 M EDTA) in a 200 ml flask. The well dispersed suspension was then heated in a microwave about 2 min or until completely solubilized without any remaining agarose suspension. Next, the melted agarose was left at room temperature to cool for about 15 min, when 0.4 % (w/v) EtBr solution was added, mixed by gentle swirling until evenly dispersed and the mixed solution was poured into the sealed gel tray, of the appropriate combs inserted, air bubbles removed, and the gel was left at room temperature for about 40 min to set. Once set, the gel was placed in the horizontal electrophoretic chamber containing 1X TBE buffer in both wells and extra TBE added so as to just cover the gel. The two combs were gently removed ensuring the wells were clean and full of TBE with no trapped

air. Samples were loaded into each well using an automatic micropipette. λ *Hin*d III (Fermentas) was loaded as DNA marker set to allow determination of the molecular weight (for quality) and estimation of size of the extracted DNA, as per the manufactures instructions. After loading, electrophoresis was run at 70 V for approximately 30 min. Finally, DNA band(s) were visualized under UV light and photographed using a gel document system (Bio-Rad).

For examination of PCR reactions to resolve and visualize PCR products, a 100 bp ladder DNA marker (Fermentas) was used as a standard marker instead of the λ *Hin*d III ladder, and the TBE-agarose gel was 1.0 % (w/v) instead of 0.8 % (w/v), and resolved at 70 V for 30 min. The products were then visualized under UV light and photographed using the gel document system (Bio-Rad).

PCR Amplification

The following primers were used for PCR amplification. For the 16S rRNA, the forward (16Saf) 5´-CGCCTGTTTATCAAAAACAT 3´ and reverse (16Sbr) 5´-CCGGTCTGAACTCAGATCACGT 3´ primers were as Palumbi (1996); whilst for the control region or D-loop, the forward primer (tRNA-Pro-f) 5´ CCACTGGCACCC AArGCCAAAATTCT 3´ and reverse (12SrRNA-r) 5´ TTCTCGTATAACCGCGGT GGCTGGCA 3´ primers were manually designed from sequences of nine salamandrid species from five genera obtained from GenBank: *Andrias japonicus* (accession number AB208679), *Andrias davidianus* (AJ492192), *Ambystoma californiense* (AY659995), *Ambystoma dumerilii* (AY659994), *Ambystoma andersoni* (AY659993), *Ambystoma tigrinum tigrinum* (AY659992), *Lyciasalamandra atifi* (AF154053), *Ranodon sibiricus* (AJ419960), and *Paramesotriton hongkongensis* (AY458597), respectively. PCR amplifications were performed in a final volume of 25 μl containing 1X of PCR buffer (Real Biotech Corporation, RBC), 2 mM MgCl$_2$, 250 μM of dNTPs, 2 μM of each primer, 1 unit of *Taq* DNA polymerase and approximately 25-50 ng of genomic DNA. A negative control was used in all PCR amplifications. PCR condition for 16S rRNA amplification was as followed: an initial denaturation of 95 °C for 5 min, denaturation of 95 °C for 45 sec, annealing of 50 °C for 1 min, extention 72 °C for 1 min, and a final extension at 72 °C for 10 min. For D-loop, the amplification condition was as the following: an initial denaturation of 95 °C for 3 min, denaturation of 95 °C for 1 min, annealing of 60 °C for 1 min, extension at 72 °C for 3 min, and a final extension at 72 °C for 10 min.

PCR Product Purification

The PCR products were purified, for removing any contamination in PCR mixture, by using a MACHEREY-NAGEL PCR clean-up, Gel extraction kit. First, the DNA fragment was excised from an agarose gel carefully, and the weight of the sliced gel was determined, and it was then transferred to a clean 1.5 ml microcentrifuge tube. Second, the gel was dissolved by adding 200 μl buffer NT for each 100 mg of the gel and incubating at 50 °C until the gel was completely dissolved (about 5-10 min). A Nucleospin® Extract II column was placed into a 2 ml collecting tube and the sample was loaded then it was centrifuged for 1 min at 11,000 × g to bind the DNA to the silica residue. After discarding the flow through, the Nucleospin® Extract II column with bound DNA was washed with 600 μl buffer NT3 by centrifugation for 1 min at 11,000 × g and a further 2 min at 11,000 × g to remove residual buffer NT3. DNA was eluted from the Nucleospin® Extract II column by the addition of 30 μl of elution buffer NE (TE) and incubated at room temperature for 1 min prior to centrifugation for 1 min at 11,000 × g.

DNA Sequencing and Phylogenetic Analysis

Purified PCR products were direct sequenced by sending to the Sequencing Laboratory of Ramathibodi Hospital. All sequences were trimmed and aligned using the multiple sequence alignment program CLUSTAL X (Thompson et al., 1997) and the alignments then rechecked by eyes.

Phylogenetic relationships were analyzed by two different methods: distance using NJ (Saitou and Nei, 1987) and ML via heuristic searches, 100 random stepwise additions, TBR branch-swapping algorithm (Felsenstein, 1981). These methods were carried out using PAUP*4β10 (Swofford, 2000). ModelTest version 3.7 (Posada and Crandall, 1998) was used to determine the best sequence evolution model for the distance and ML analyses, under the AIC (Akaike, 1974). According to the results of these tests, 16S rRNA gene analysis was carried out using the TrN+I model (Tamura and Nei, 1993) for ML analysis (nucleotide frequencies 0.3621 (A), 0.2112 (C), 0.1822 (G), 0.2445 (T), proportion of invariable sites I = 0.6718, equal rates for all sites), the D-loop gene analysis also used the TrN+I model for ML analysis (nucleotide frequencies 0.2913 (A), 0.2078 (C), 0.1624 (G), 0.3385 (T), proportion of invariable sites I = 0.4790, equal rates for all sites), and the combined data of 16S

rRNA and D-loop genes were carried out using the TrN+G model for ML analysis (nucleotide frequencies 0.3188 (A), 0.2091 (C), 0.1709 (G), 0.3012 (T), proportion of invariable sites I = 0, gamma distribution shape parameter = 0.3460). Distance analysis was performed with the Tamura-Nei model with the same ModelTest parameters as that used for each ML analysis. Bootstrap supports (Felsenstein, 1985) were calculated using 2,000 replicates for NJ and 100 replicates for ML. The Hong Kong Warty newt, *Paramesotriton hongkongensis*, (AY458597) was used as an outgroup.

RESULTS

Specimen descriptions two types of color pattern of *T. verrucosus* found in Thailand.

    1.1 Type I (orange to yellow form).

Fig. 3.3   *T. verrucosus* Type I from Doi Lahnga, Khun Chae NP, Chiang Rai Province.

Description of Coloration – The ground color is brown or black. The cranial crests, parotoid glands, dorsal ridge, dorsolateral warts, limbs, tail and ventral surface are orange to yellow in coloration.

Representative of Type I Specimens

    LK003, LK004, and LK005 refer to specimens from Doi Lahnga, Khun Chae NP, Chiang Rai Province.

    PPf, PPg, and PPh refer to specimens from Phuping Rajanives Palace, Doi Suthep**,** Chiang Mai Province.

    ITNT1, ITNT2, and ITNT3 refer to specimens from Watershed management station, Doi Ang Khang, Chiang Mai Province.

    SPc, SP11, and SP12 refer to specimens from Royal Garden Siribhume, Doi Inthanon, Chiang Mai Province.

1.2 Type II (dull form).

Fig. 3.4   *T. verrucosus* Type II from Phu Luang WS, Loei Province.

Description of Coloration – The dorsal body, ventral surface and limbs are dull or brown in coloration. The cranial crests, parotoid glands, dorsal ridge of body and tail, dorsolateral warts, lower jaw, vent, ventral ridge of tail and tip of phalanges are of a lighter brown than that of the body.

Representative of Type II Specimens

PK4, PK5, and PK6 refer to specimens from Phu Suan Sai NP, Loei Province.

PL007, PL008, and PL2627 refer to specimens from Phu Luang WS, Loei Province.

PH019, PH024, and PHG refer to specimens from Phu Hin Rong Kla NP, Phitsanulok Province.

DNA Extraction

Genomic DNA of *T. verrucosus* tail tip (1-2 mm^3) was extracted by the phenol-chloroform method as described. Good quality extracted genomic DNA was obtained and the quantity of the obtained DNA was estimated at approximately 25-50 ng/µl (see **Fig. 3.5**).
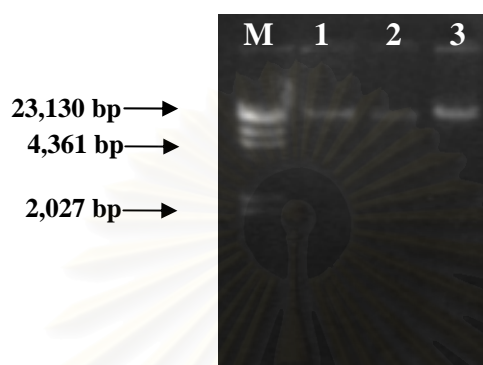
Fig. 3.5   Extracted genomic DNA resolved through a 0.8 % (w/v) agarose gel stained with EtBr. Lane M represents λ *Hin*d III (Fermentas) as DNA marker. Lanes 1-3 show individual genomic DNA from LK003, LK004 and LK005, respectively.

PCR Amplification

PCR technique is an approach for DNA amplification of specific sequence by synthesed primers that extended simultaneously using the complementary strand of DNA as template. 16S rRNA and D-loop (including partial tRNA-Pro, 12SrRNA and total tRNA-Phe sequences) regions were successfully amplified with products size of about 600 and 1,060 bp, respectively, as expected (see **Figs. 3.6** and **3.7**).
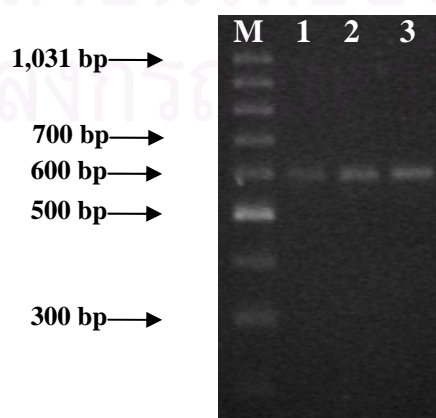
Fig. 3.6   PCR products of 16S rRNA gene resolved through a 1.0 % (w/v) agarose gel stained with EtBr. Lane M represents 100 base pair DNA ladder (Fermentas) as DNA marker. Lanes 1-3 contain the PCR products of *T. verrucosus* from PL007, PL008 and PL009, respectively.
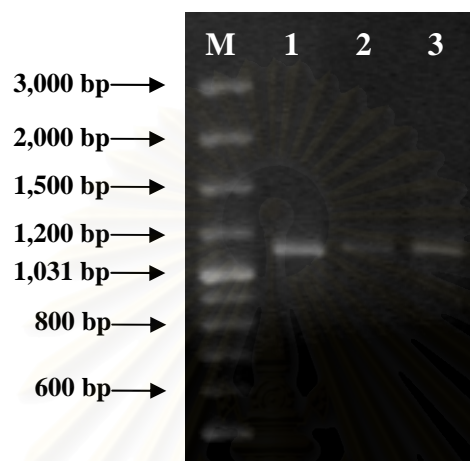
Fig. 3.7   PCR products of D-loop gene (including partial tRNA-Pro, 12SrRNA and total tRNA-Phe sequences) resolved through a 1.0 % (w/v) agarose gel stained with EtBr. Lane M represents 100 base pair DNA ladder plus (Fermentas) as DNA marker. Lanes 1-3 contain the products of *T. verrucosus* from ITNT1, ITNT2 and ITNT3, respectively.

Sequence Analysis

After the PCR products of 16S rRNA and D-loop genes from seven localities in Thailand were purified, they were sequenced in both the forward and reverse directions (leading and lagging strands). Chromas version 1.45 (Zajec, 1986) was used to check the quality of the resulted sequences and call the likely base identity for each interval (shown in **Figs. 3.8** and **3.9**). These electropherograms were however additionally checked by eyes to correct miscalls and look for secondary smaller peaks, a sign of a mixed template sequence. Mostly, in both genes the forward and reverse sequences were consistent allowing derivation of a consensus sequence. Moreover, the sequences of tRNA-Phe gene, 69 bp, were also reported (**Fig. B.1**).

For partial 16S rRNA sequences, they were completely trimmed and the consensus was kept for phylogenetic analysis. The 498-500 bases obtained is shown in **Fig. B.2**. The percentages of nitrogenous base composition (A, C, G, and T) are

summarized in **Table A.1**, and show a moderately rich A+T content for both *T. verrucosus* and *P. hongkongensis*, at 60.5 % and 61 % respectively, whilst *T. verrucosus* from northern Thailand populations revealed and average of 60.9 % A+T (60.1-62 %) comparing very well with that of the northeastern Thailand populations with and average of 60 % A+T (59.8-60.2 %).

The pairwise distances evaluated across all pairs of sequences were transformed into a distance and ranged from 0-20.238 %. Variation between individuals in the northern population (Type I) was low to medium with the highest being seen between ITNT1 and LK003, LK004 and LK005 at 3.216 %. However, within subclade variances were much lower being 0 to 1.488 %. Within individuals in the northeastern population (Type II), variation between individuals was low with the highest seen being that between PH024 and PH025 versus PL009, PK4, PK5 and PK6 at 0.411 %, although within subclade comparisons were lower at 0 to 0.203 % sequence difference. However, in contrast, the pairwise difference between individuals from the northern and northeastern populations (that is between Type I and Type II populations) revealed much higher differences ranging from 4.857 % to the highest of 7.815 % between ITNT2 and PH024 or PH025 (**Table A.3**).

With respect to the control region sequences, the complete length of *T. verrucosus* is about 729-730 bp, which is the same length as that seen in *P. hongkongensis* (730 bp), a species within another family in the Salamandridae. These *T. verrucosus* D-loop sequences were trimmed and the remaining consensus sequences (**Fig. B.3**) kept for phylogenetic analysis. The percentage nucleotide base composition (A, C, G, and T) of each is summarized in **Table A.2**. The A+T content of *T. verrucosus* and *P. hongkongensis* were 62.9 % and 64 % respectively, while comparison between the two *T. verrucosus* populations (Type I and Type II) showed that Type I *T. verrucosus* were slightly less AT rich (mean 61.9 %; range, 61.7-62.3 %) than the Type II populations (mean 64.2 % A+T; range, 64.1-64.4 %).

The pairwise distances evaluated across all pairs of sequences were transformed into a distance and found to range from 0-45.098 %. Within the northern (Type I) population, pairwise individual distances varied from low to medium, that is from 0 to 3.513 %, the highest being across the subclade between SP11 and LK003, LK004 and LK005. Within subclade pairwise distances were low, ranging from 0 to 0.841 %. Within the northeastern (Type II), pairwise distances were all low, ranging from 0 to 0.839 %, the highest being across the subclade with PL007 or PL008 versus

PK4, PK5 and PK6. However, within each subclade, pairwise distances were low (0 to 0.418 %). In contrast, pairwise distances across Type I and Type II populations were high ranging from 6.985 to 8.756 %, the highest distance being between LK003, LK004 and LK005 versus PL007 and PL008 (**Table A.4**).
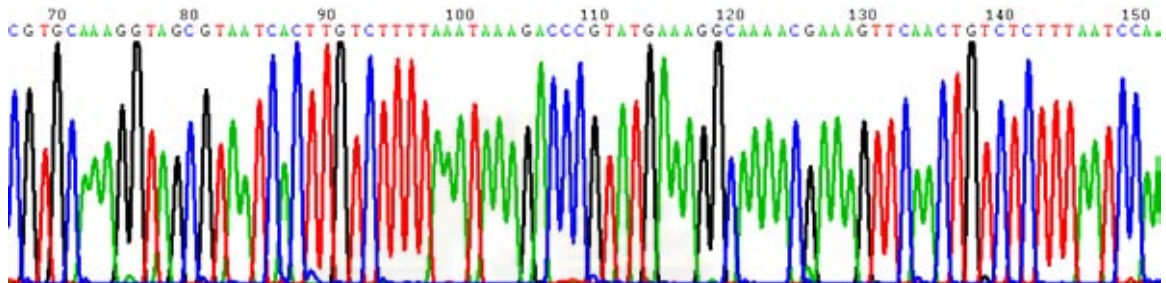
Fig. 3.8   Electropherogram of 16S rRNA sequence of *T. verrucosus* from Phu Luang WS. Green, blue, black and red colors show Adenine (A), Cytocine (C), Guanine (G), and Thymine (T), respectively.
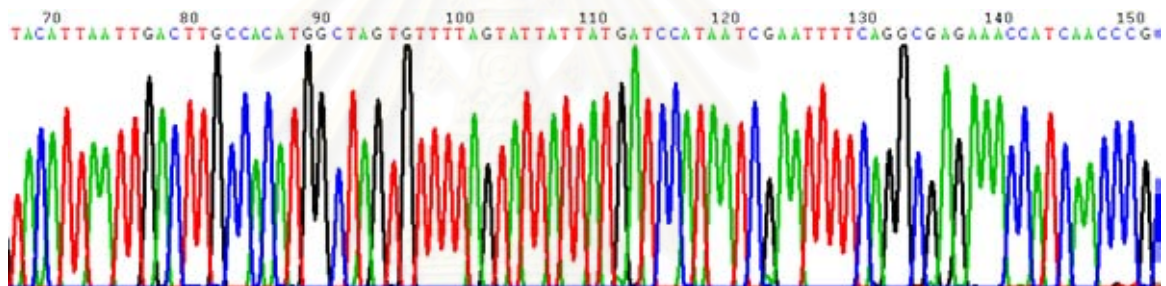
Fig. 3.9   Electropherogram of D-loop sequence of *T. verrucosus* from Phu Luang WS. Green, blue, black and red colors show Adenine (A), Cytocine (C), Guanine (G), and Thymine (T), respectively.

Phylogenetic Analysis

The sequences of the 16S rRNA fragment, the D-loop fragment and the combined data of these two gene fragments of *T. verrucosus* in Thailand (from the northern (Type I) and northeastern (Type II) mountain ranges), along with a single sequence of *P. hongkongensis* from GenBank were used for phylogenetic analysis. Phylogenetic trees were constructed via distance analysis using NJ and via character analysis using ML. In both cases, trees were assessed for branch support by bootstrap statistics which are shown on nodes of each phylogenetic tree (If they were more than 50 %).

The data derived from the 16S rRNA gene fragment sequences are presented via two phylogenetic trees, a NJ distance analysis (**Figs. 3.10**) and a ML analysis

(**Figs. 3.11**). For the NJ based distance analysis, two major groups (northern and northeastern populations) of *T. verrucosus* were separated, and these concur absolutely with the Type I and Type II morphological distinction of *T. verrucosus*. The first major group (Group A) is composed of solely of newts from the northeastern mountain ranges (Type II), with high bootstrap support (100 %), whilst the second major group (Group B) is composed entirely of newts from the northern mountain ranges (Type I). The A (northeastern or Type II) group could be divided into two subgroups, the first subgroup (subgroup 1A) being composed of PL007, PL008 and PH019 plus PH024 and PH025 as a recently slightly diverged pair. The second subgroup, subgroup 2A, is composed of PL009 and all of three of the assayed PK population. The B (northern or Type I) group could also be separated into two subgroups. The first subgroup, 1B, being composed of all the SP, ITN and PPh specimens examined plus PPg and PPf individuals as a slightly older diverged sister clade. The second subgroup, 2B, is composed of the three individuals from the LK population.

For ML analysis, essentially the same tree topology was observed with the clear split of the northern (Type I) and northeastern (Type II) samples into two separate major groups (A and B), and the same subdivision of group A (Northeastern; Type II) into two subgroups (1A and 2A), with PH024 and PH025 slightly and recently diverged within subgroup 1A, and group B into the two main subgroups 1B and 2B, as seen with the NJ based analysis. However, the only slight difference being that within group 1B the slight and recent divergence of ITNT1 and ITNT2 seen in NJ analysis was absent in the ML analysis.

The phylogenetic trees derived from the D-loop fragment sequences were also produced by both NJ (**Figs. 3.12**) and ML (**Figs. 3.13**) based analysis. Essentially the same tree topology were produced by NJ and ML analysis for the D-loop DNA fragment sequences as that seen with 16S rRNA, respectively, and by NJ compared with ML analysis of the D-loop sequences. Slight differences between D-loop region and 16S rRNA fragment sequence based phylogenetic trees based upon both NJ and ML analysis is that group A are split into three subgroups with realignments of members such that PL007 and PL008 become a unique clade (subgroup 1A) away from PH019, PH024 and PH025 (now subgroup 3).

Given the apparent high level of congruency between phylogenetic trees produced from both 16S rRNA and D-loop (control region) fragment sequences, these

two data sets were reanalyzed, after merging the two respective DNA fragments with equal weighting, using both NJ (**Figs. 3.14**) and ML (**Figs. 3.15**) based analysis. Essentially the same tree topologies were still produced with all the northern (Type I) and northeastern (Type II) samples being split perfectly into two major groups.

The A group was further split into three subgroups (1-3A), each with the same membership between NJ and ML analysis, but differ only in the relationship of each subgroup to the other between the two analytical methods. The subdivision of group B into two subgroups was congruent with both analytical methods.
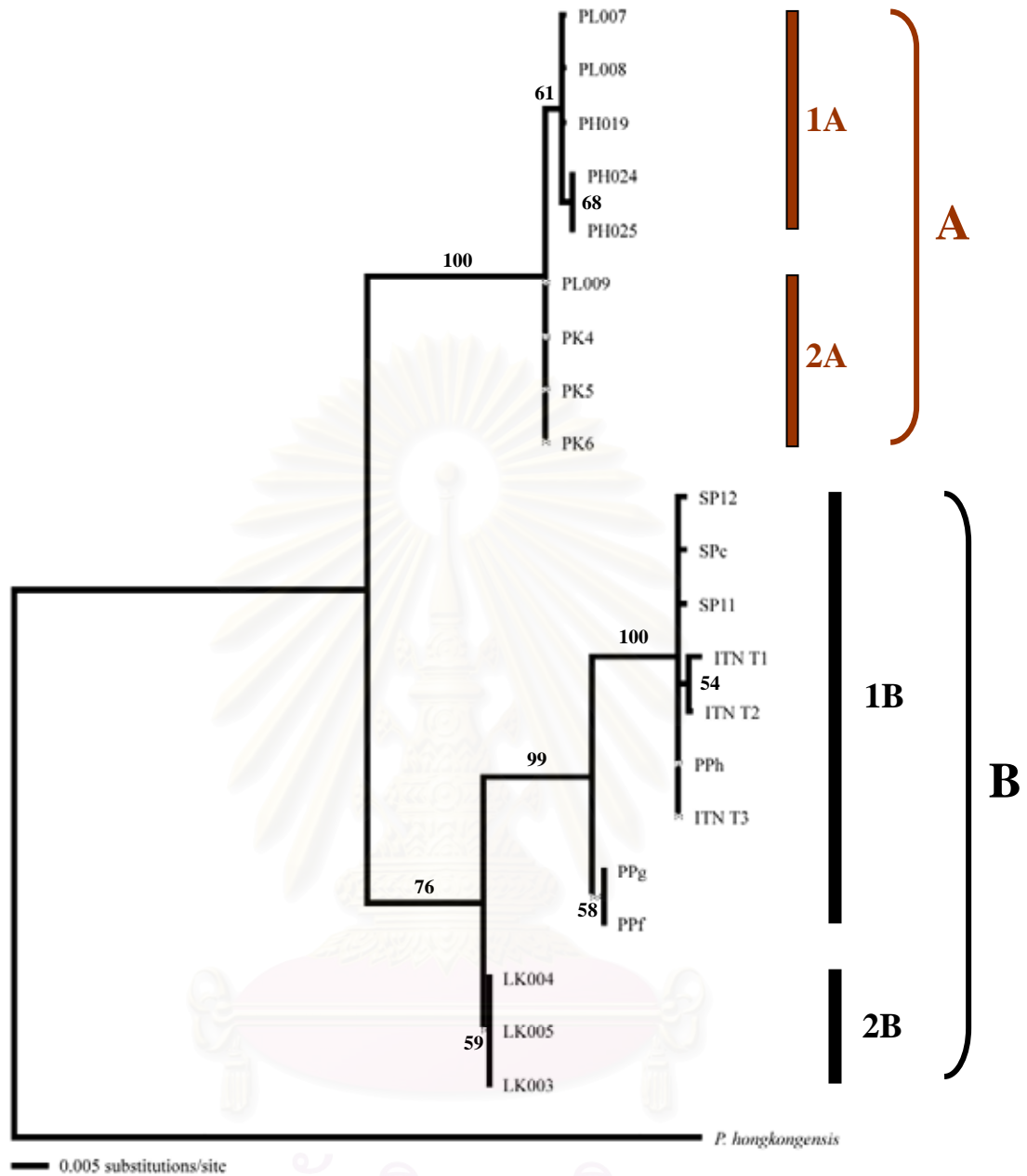
Fig. 3.10   NJ based phylogenetic tree derived from the 16S rRNA DNA fragment sequences from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup. Bootstrap supports are shown on the branches of the phylogenetic tree when > 50 %.
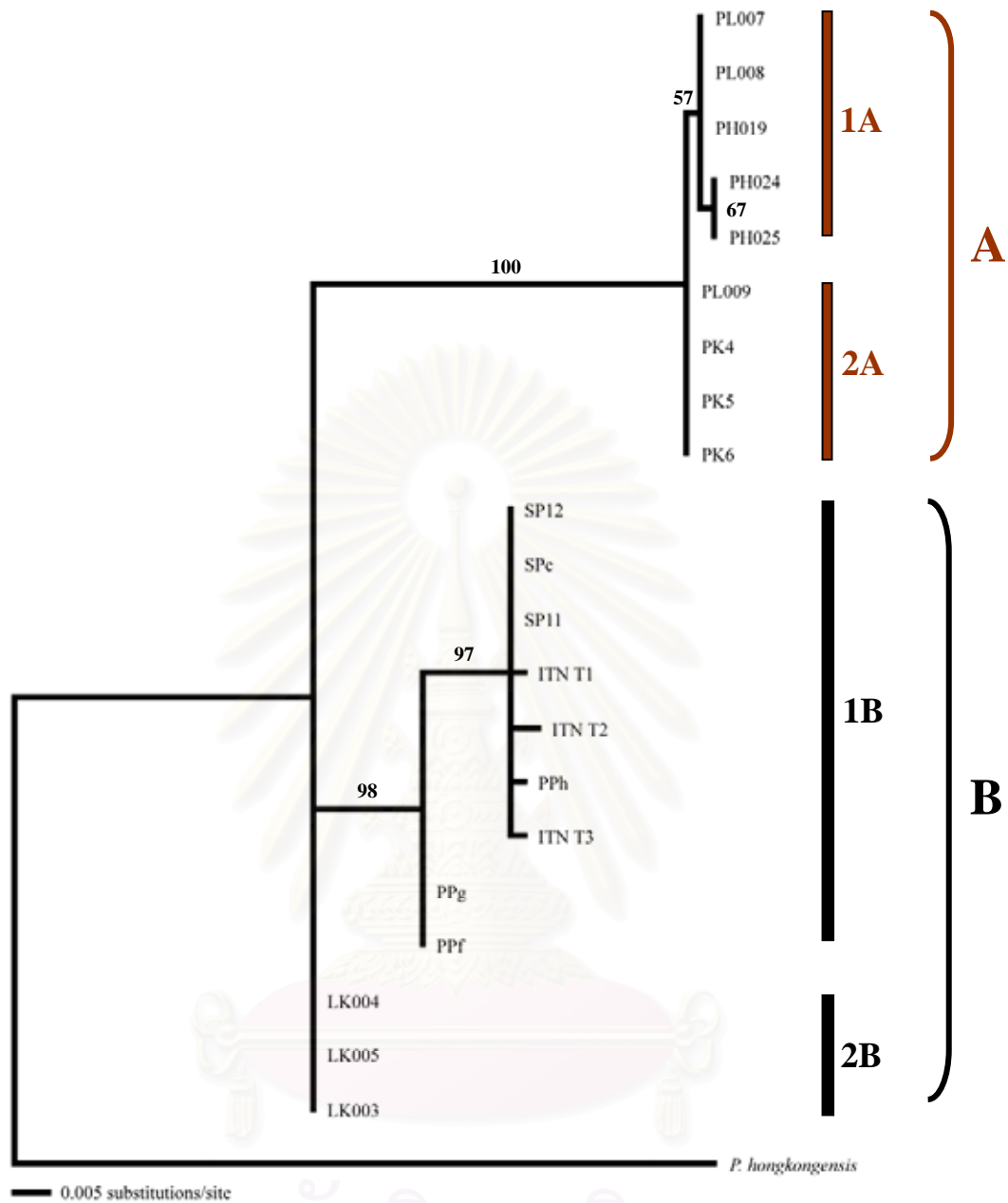
Fig. 3.11 ML based phylogenetic tree derived from the 16S rRNA DNA fragment sequences from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup. Bootstrap supports are shown on the branches of the phylogenetic tree when > 50 %.
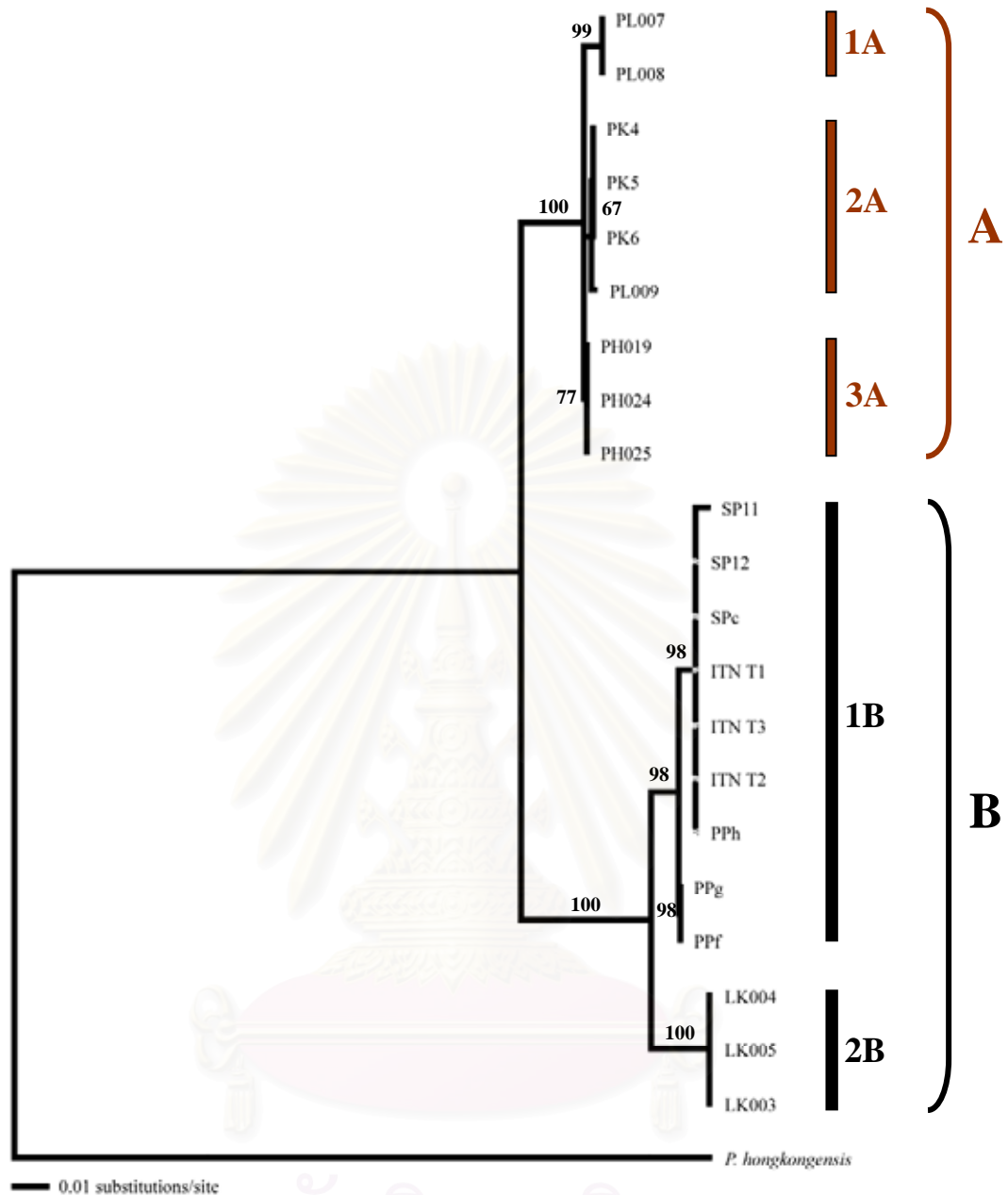
Fig. 3.12   NJ based phylogenetic tree derived from the D-loop DNA fragment sequences from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup. Bootstrap supports are shown on the branches of the phylogenetic tree when > 50 %.
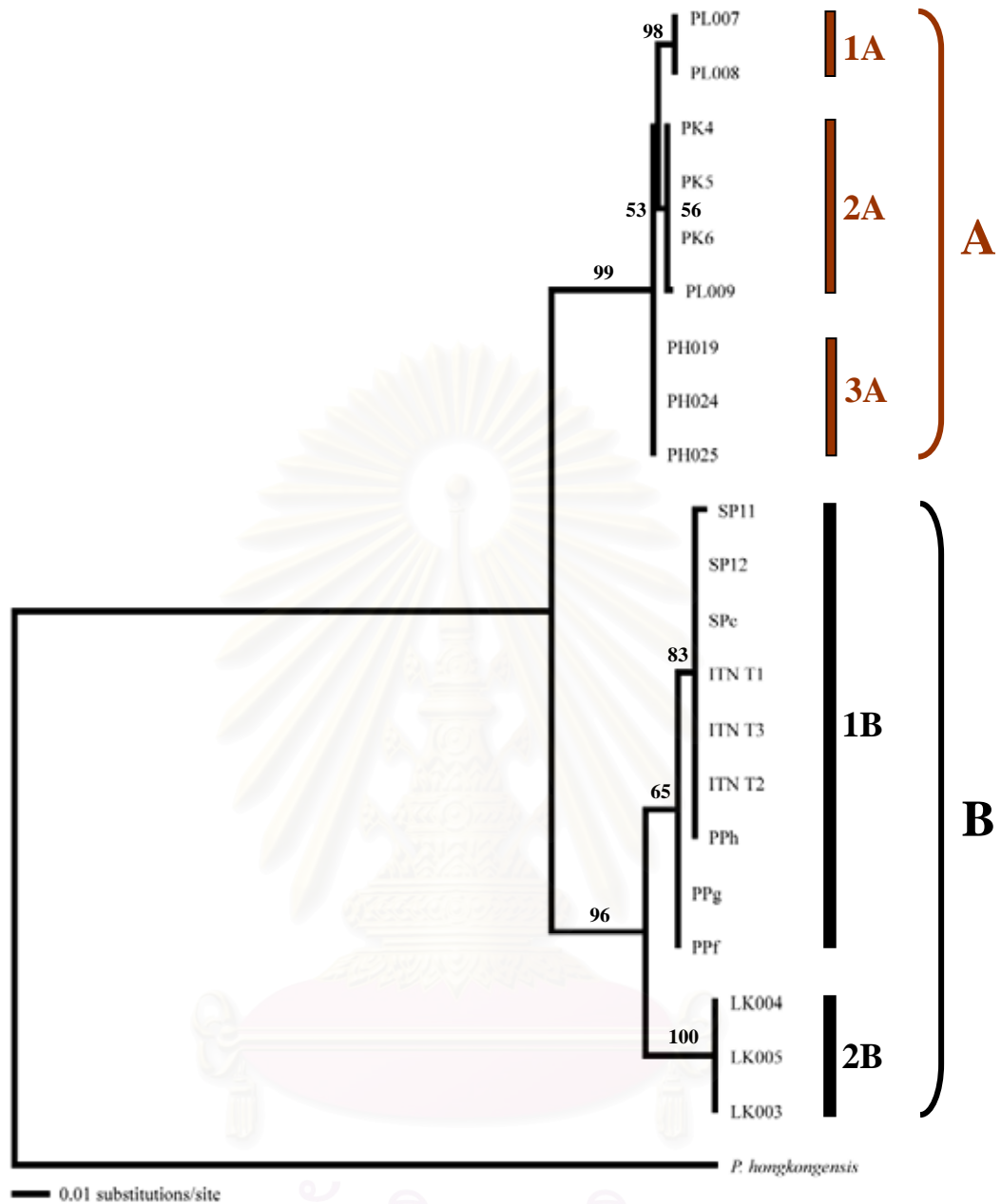
Fig. 3.13   ML based phylogenetic tree derived from the D-loop DNA fragment sequences from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup. Bootstrap supports are shown on the branches of the phylogenetic tree when > 50 %.
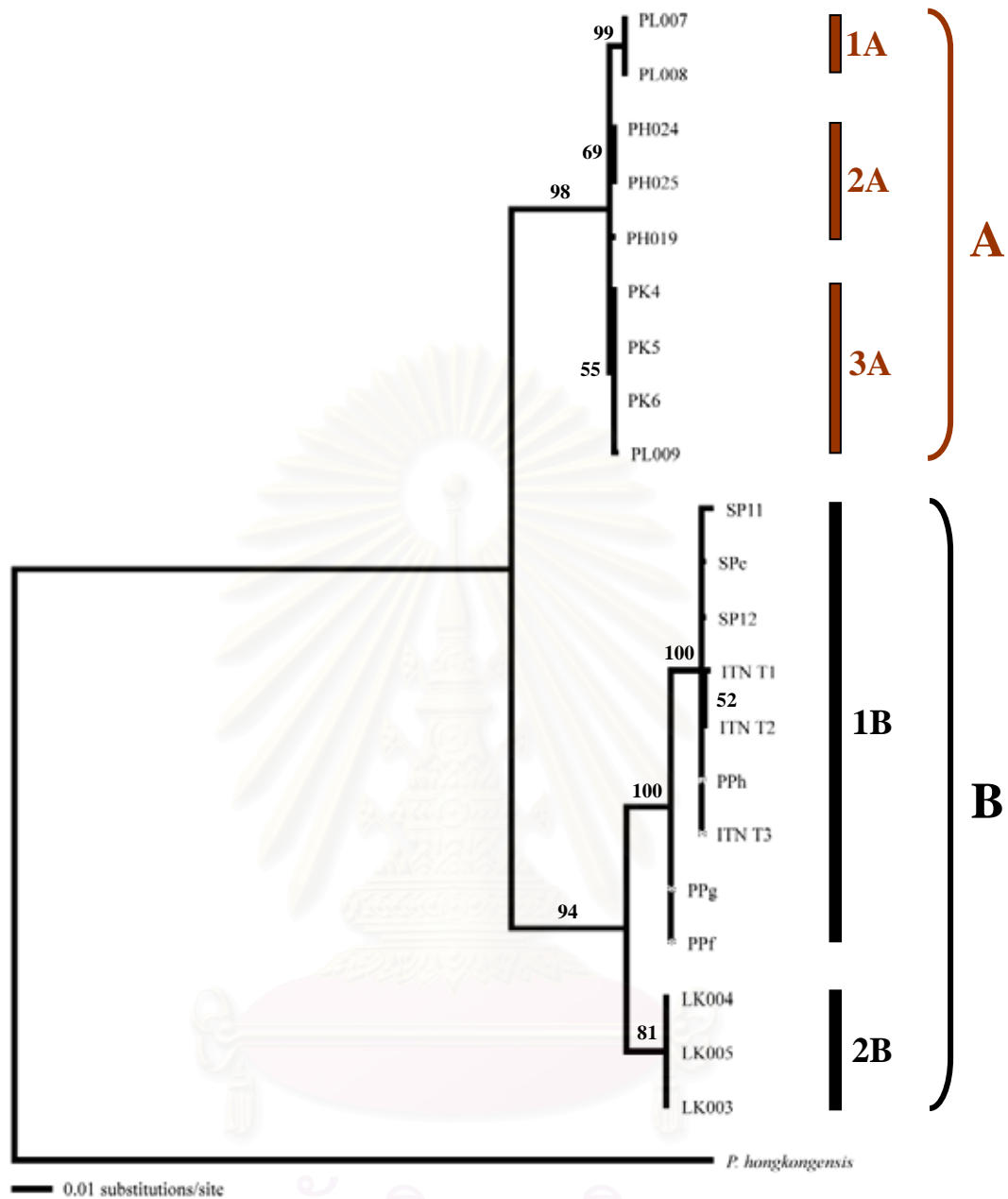
Fig. 3.14 NJ base phylogenetic tree derived from the combined 16S rRNA and D-loop DNA fragment sequences, with equal weighting, from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup. Bootstrap supports are shown on the branches of the phylogenetic tree when greater than 50 %.
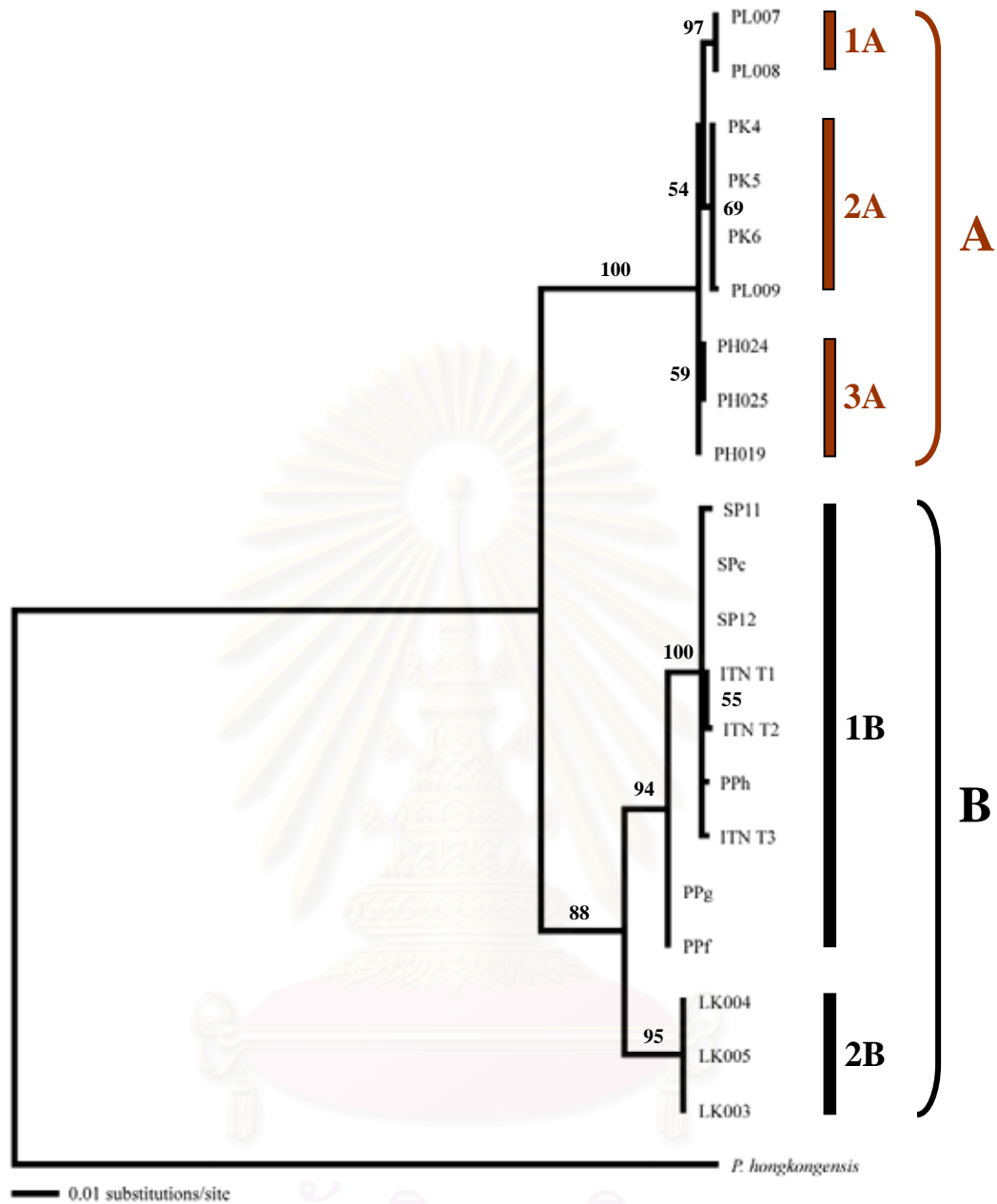
Fig. 3.15 ML base phylogenetic tree derived from the combined 16S rRNA and D-loop DNA fragment sequences, with equal weighting, from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup. Bootstrap supports are shown on the branches of the phylogenetic tree when greater than 50 %.

DISCUSSION

The phylogenetic relationships of the Himalayan newt (*Tylototriton verrucosus*) in Thailand were assessed via mtDNA sequences derived from PCR amplified fragments of the 16S rRNA and D-loop regions. MtDNA is a directly maternal inheritance (Li and Graur, 1991; Tan and Wake, 1995; Sumida, 1997; Macdonald, 2003; Kawamoto et al., 2007). As such the processes of breeding, genetic recombination or allelic segregation have no affect in contrast to nuclear DNA (Li and Graur, 1991; Tan and Wake, 1995; Macdonald, 2003). Furthermore, the evolutionary rate of change at the nucleotide sequence level for animal mtDNA is faster than that for nuclear DNA (Brown et al., 1979; Vawter and Brown, 1986; Carr, Brothers, and Wilson, 1987; Hoelzel, Hancock, and Dover, 1991). Therefore, mtDNA is generally useful for assessing relationships among populations or species that closely related (Brown et al., 1979; Li and Graur, 1991).

The 16S rRNA gene is considered a unique sequence (Li and Graur, 1991). It is a structural component of the ribosome that functions in the translation of protein from mRNA (Hillis et al., 1996; Smith and Szathmary, 1999; Campbell and Reece, 2002). Thus, it is considered a highly conserved gene (Lopez et al, 1997). In contrast, the D-loop or control region is the most rapidly evolving region in the mitochondrial genome (Shaffer and McKnight, 1996; Lopez et al., 1997; Steinfartz et al., 2000; Sumida et al., 2000), and as such is able to resolve relationships among closely related populations (Shaffer and McKnight, 1996; Steinfartzet et al., 2000; Sumida et al., 2000; Martinez-Solano et al., 2006; Kawamoto et al., 2007).

In order to study the genetic variation among *T. verrucosus*, first of all, tail tips of each newt were used for DNA extraction. A tissue sampling via tail-clipping is proper for further DNA analysis because the clipped tail tip has ability to regrow and so causes minimal damage and fitness costs to the individuals, an important consideration for a threatened or sensitive species. Moreover, clipped tail tips show faster regrowth than clipped toe tips (Arntzen, Smithson, and Oldham, 1999). There were some problems that appeared during this process, such as attaining a smeared band or degraded genomic DNA. It might be possible that some tissue samples were stored in 70 % (v/v) ethanol (should be stored in 95 % (v/v) or absolute ethanol) for a long time and or under unsuitable conditions (i. e., ambient tropical or above).

According to phylogenetic analysis by distance and ML methods, the results from partial sequences of the 16S rRNA and D-loop (control region) fragments and the combined data set of these two fragments combined with equal weighting clearly showed that there were two genetically distinct *T. verrucosus* populations, all samples segregating perfectly between the north (Type I) and northeast (Type II) of Thailand.

The A+T content of *T. verrucosus* from these partial 16S rRNA and D-loop regions were 60.5 % and 62.9 %, respectively, whilst that for *P. hongkongensis* was 61 % and 64 %, for the 16S rRNA and D-loop regions, respectively.

The maximum values of pairwise distances between the two major lineages, that is between the northern and northeastern populations (7.815 % for 16S rRNA and 8.756 % for D-loop regions) are more than the maximum values within each specimen in the northern and northeastern lineages (3.216 % for 16S rRNA and 3.513 % for D-loop regions). When comparing between the two major lineages and *P. hongkongensis* (the outgroup), the maximum values of pairwise distances of two major lineages are distinctly less than the outgroup (20.238 % for 16S rRNA and 45.098 % for D-loop regions). *P. hongkongensis* was chosen as the outgroup since it is distinctly different from *T. verrucosus*. However, if more closely related species had additionally been used as extra outgroups, such as others *Tylototriton* spp., *Echinotriton* spp., and *Pleurodeles* spp., the sequence divergences and pairwise distances would be much more likely to be comparatively able to infer if the northern and northeastern Thai samples are likely to define subspecies or species boundaries.

The phylogenetic trees obtained from the use of 16S rRNA and D-loop sequences, and the combined data set (both distance and ML methods) were almost congruent. Therefore, the phylogenetic trees derived from the combined data with equal weighting of the 16S rRNA and control region were focused upon (**Figs. 3.14** and **3.15**).

The results by both distance analysis (NJ) and character analysis (ML) congruently revealed two genetically distinct groups of *T. verrucosus* in Thailand, comprised of the northern lineage, which were all Type I samples (ITNT1, ITNT2, ITNT3, SPc, SP11, SP12, PPf, PPg, PPh, LK003, LK004 and LK005); and a distinct northeastern lineage, which were all Type II samples (PK4, PK5, PK6, PL007, PL008, PL009, PH019, PH024 and PH025).
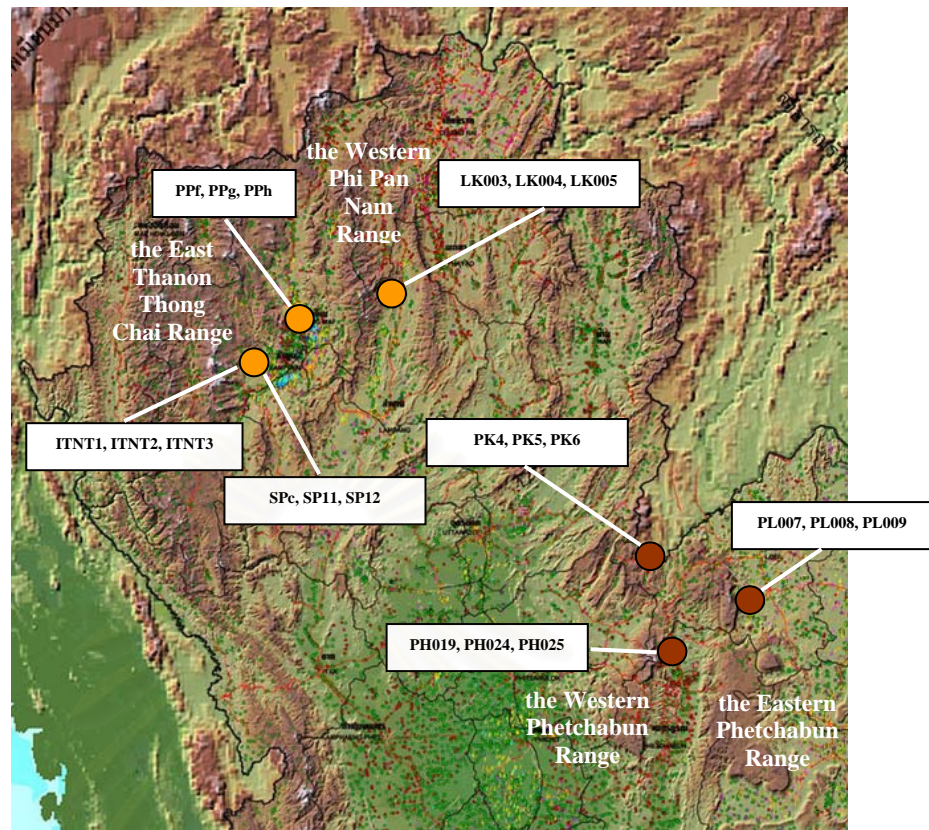
Fig. 3.16   Distribution of the Himalayan newt within the northern and northeastern mountain ranges in Thailand, as recorded in this study. The detail of samples present is given in **Table 3.1** (Figure was modified from www.gisthai.org).

From previous recorded data (i.e., Kuzmin et al., 1994; Anders et al., 1998; Pomchote et al., 2007) and this study, it was found that *T. verrucosus* are found within Thailand in the mountainous areas at an elevation of more than 1,000 m. Thus the term clade is defined as a group of populations that are geographically continuous and have the capacity for gene flow within these populations.

Within the northern (Type I) lineage, there are two major clades that are divided by mountain ranges: the East Thanon Thong Chai Range composed of all ITN, SP, and PP populations (from two sites at Doi Inthanon and one site at Doi Suthep, respectively), and the Western Phi Pan Nam Range, composed of all LK population (from Khun Chae NP).

Within the northeastern (Type II) lineage, there are three major clades: the first clade comprised of the whole PK population and PL009 (from Phu Suan Sai NP and Phu Luang WS, respectively), the second clade included the entire PH population

(from Phu Hin Rong Kla NP), and the third clade was composed of PL007 and PL008 specimens (from Phu Luang WS). However, the bootstrap support for the existence of the three clades varied between NJ and ML analysis and was not strong and thus this notion remains equivocal and in need of further verification. Indeed, these analyses are based upon maternally inherited mitochondrial DNA sequences and so to establish the presence or absence of gene flow between and within populations, as well as any isolation by distance, future research should assess codominant nuclear markers and in particular highly polymorphic loci such as nuclear microsatellite sequences.

From the landforms of Loei Province (**Fig. 3.16**), there is a connection between the Western and Eastern Phetchabun Ranges (include Phu Suan Sai NP, Phu Hin Rong Kla NP, and Phu Luang WS). Hence, each *T. verrucosus* specimen from this northeastern region might have recent common ancestor by migration of some individuals and thus a more closely related genetic structure and this may also. Then account for of the lower bootstrap support (< 69 %) for the northeastern lineage clades, but as mentioned awaits polymorphic nuclear loci analysis to resolve this hypothesis. In the meantime, the moderate bootstrap values suggest the phylogenetic relationships of these northeastern clades remain equivocal, except for the clustering of PL007 and PL008, somewhat alike the report of Parra-Olea (2002) and Lu et al. (2004) in trying to resolve the phylogenetic relationships of the salamandrid genera *Pseudoeurycea* and *Paramesotriton*, respectively. Thus, at present the northeastern (Type II) lineage is best separated into two major clades that are divided by mountain ranges: the Western Phetchabun Range composed of all PK and PH populations (from Phu Suan Sai NP and Phu Hin Rong Kla NP), and the Eastern Phetchabun Range composed of all PL populations (from Phu Luang WS).

Regardless, the data consistently indicated that *T. verrucosus* in Thailand could be divided into two types (**Figs. 3.3** and **3.4**) from genetic variation and geographic distribution that is congruent with body color morphology: Type I (orange to yellow body coloration) distributed in northern mountain ranges and Type II (dull body coloration) distributed in northeastern mountain ranges. This result accords with the basic data by Pomchote (2004), Pomchote et al. (2006a), Pomchote, Khonsue, and Pariyanont (2006b), and Pomchote et al. (2007).

Since salamanders have low migration and dispersion abilities (Staub, Brown, and Wake, 1995), populations tend to be isolated from each other by geographical barriers (Matsui et al., 2006). In addition, *T. verrucosus* were also found only at an

altitude of more than 1,000 m (Smith, 1924; Taylor, 1962; Wongratana, 1984; Matsui et al., 1996; Chan-ard et al., 1999; Nutphund, 2001; Pomchote, 2004; Nabhitabhata and Chan-ard, 2005). Consequently, geographic barrier are likely to be important limiting factors in their distribution and act as further reproductive isolating factors of the two groups by causing reduced or prevented gene flow between populations with concomitant increased genetic differentiation in isolated newt populations (Sotiropoulos et al., 2007; Marsh et al., 2008).

A more comprehensive study is now required and should include all the distribution ranges of *T. verrucosus*, plus more samples per population, and to use outgroups that more closely related to *T. verrucosus* and include other genes from both mtDNA and nuclear DNA including microsatellites. The taxonomic status of these two types of newts might then be better resolved.
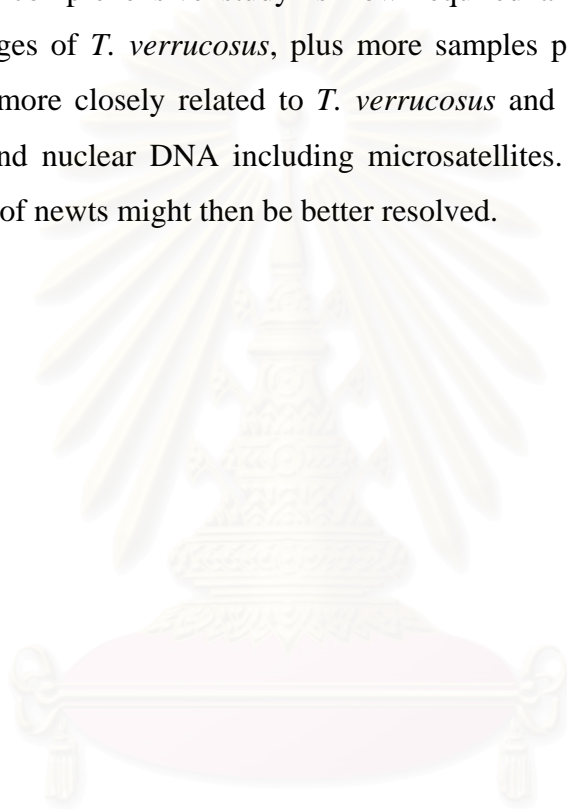
CONCLUSION

The phylogenetic relationships of the Himalayan newt (*Tylototriton verrucosus*) in Thailand are related to their geographic distributions. The species can be separated into two distinct types. Type I (orange to yellow body coloration) is distributed in the northern mountain ranges and is similar to that found across the reported Indo-china-Asia range, and Type II (dull body coloration) which is distributed in the northeastern Thailand mountain range and is currently unknown elsewhere. Within these two Thailand population types:

1) *T. verrucosus* Type I from the northern mountain range can be subdivided into two distinct populations; (i) the East Thanon Thong Chai Range (composed of populations from Doi Inthanon and Doi Suthep, Chiang Mai Province) and (ii) the Western Phi Pan Nam Range (composed of populations from Doi Lahnga, Khun Chae NP, Chiang Rai Province).

2) *T. verrucosus* Type II from northeastern mountain range can be divided into two distinct populations; (i) the Western Phetchabun Range (composed of populations from Phu Suan Sai NP, Loei Province and Phu Hin Rong Kla NP, Phitsanulok Province) and (ii) the Eastern Phetchabun Range (composed of populations from Phu Luang WS, Loei Province). However, the possibility of further division of the Western Phetchabun Range is plausible but remains equivocal awaiting further study.

CHAPTER IV
BONE MORPHOLOGY OF NEWTS GENUS
Tylototriton IN THAILAND
INTRODUCTION

The term skeleton, in a widest sense, is used to define a system of hard parts which gives the vertebrate body shape, supports its weight, offers a system of anchorage and leverage for muscles to produce movement, and protect the soft organs and tissues (Flower, 1885; Kardong, 2006).

Bones are the most imperishable of animal tissues, often retaining the exact form after other portions of an organism (such as nerve, muscle, and tissue) have completely disappeared. In the case of extinct animals, bones (typically fossilized) afford the ability to attain knowledge of the organisms from their characters (Flower, 1885). In addition, the alteration of various parts of the skeleton and especially the skull over a long time period is the key to understanding the route of vertebrate evolution. Therefore, many taxonomists have used bone-derived morphological characters for taxonomic studies.

There are several works relating bone morphologies in many groups of amphibians, for example within the order Anura, Martin (1973) examined the relationship of 11 species groups of North American *Bufo* using characteristic measurements; within the order Gymnophiona, Wilkinson and Nussbaum (1996) analyzed the phylogenetic relationships of the caecilian families; and within the order Caudata, Larson and Dimmick (1993) assessed the phylogenetic relationship of the family Salamandridae using morphological characters, included bone morphology.

There are few bone morphology studies that are directly focused on *Tylototriton verrucosus*, in contrast most work has been based upon molecular (i.e., Titus and Larson, 1995; Chan, Zamudio, and Wake 2001; Weisrock et al., 2006) or ecological (e.g., Nussbaum, 1987; Kuzmin, Dasgupta, and Smirina, 1994; Hegde and Deuti, 2007) studies.

Riese (1892) described and illustrated many parts of the axial and appendicular skeletons of *T. verrucosus* from western Yunnan Province, China, in detail. In 1928, Noble studied the fossils of salamandrid skeletons from the Miocene Ohningen beds of Switzerland and described a new species, *Tylototriton primigenius*

(sp. nov.), by comparison with *T. verrucosus*. He also presented and figured various parts of the skeleton of the latter species.

The various portions of the *T. verrucosus* skeleton that have been used in comparison with other species are summarized as the following: Ozeti and Wake (1969) studied the muscles and skeletons of the hyobranchia of salamanders and newts (family Salamandridae) among 14 genera, including *T. verrucosus*. Nussbaum and Brodie (1982) studied and illustrated a comparative anatomy of *Tylototriton andersoni* and *T. verrucosus* by comparing characters of the skull, ribs, body, limbs, fingers and toes and suggested that *T. andersoni* should be placed in a new genus, *Echinotriton* (gen. nov.). Lastly, Zhao et al. (1988) studied the salamanders in China including *T. verrucosus* and compared their characters with the newt genus *Ehinotriton* and other *Tylototriton* species (from Nussbaum and Brodie, 1982) and the relationship between this species and others, and the position of *Tylototriton* were shown via cladogram based analysis using bone characters.

Osteological comparisons between *T. verrucosus* and *T. shanjing*, which focused on measurements and descriptions of the skull and vertebrae, revealed the differentiation and separation of *T. verrucosus* from *T. shanjing* (Haller-Probst, 1988).

T. shanjing was also separated from *T. verrucosus* by Nussbaum, Brodie, and Datong (1995). They observed that some individuals of the then classified *T. verrucosus* had two distinctive color patterns. One form was dark brown dorsally, with bright orange to yellow coloration confined to the ventral edge of the tail. The other has a dark brown to black dorsal ground color with orange dorsolateral warts, limbs, ventrolateral surfaces, vertebral crest, lateral and medial crests on the head are orange color. These two color forms appeared to be allopatric with no evidence of color intergradation. They also analyzed morphometric and meristic characters and proposed the orange form as a new species, *T. shanjing*, which occurs only in western Yunnan Province, China.

There is no previous comparative bone morphology studies upon *Tylototriton verrucosus* found in Thailand, even though the external morphology such as female size, male and female body color and their distribution ranges are noticeably different (reported by Pomchote, 2004). In order to compare the difference between *T. verrucosus* populations, a skeleton comparison should compared with previous reports in the literature.

MATERIALS AND METHODS

There are several techniques for preparing and studying the removal of flesh by dermestid beetles (Hall and Russell, 1933), enzyme activity (Taylor, 1967), pre-soaker method (Ossian, 1970), sandfleas or shrimps (Friedman, 1973), bacterial maceration (Hill, 1975), X-ray (Chan et al., 2001), and cleared-and-stained (Johnson et al., 2006).

1. X-ray Study

Specimens that had been preserved in 95 % (v/v) ethanol were individually placed in a transparent box and sufficient water was added to the box to cover the whole specimen and it was left to hydrate until soft enough for proper fixation (Heyer et al., 1994), when it was surface dried by using a towel or tissue and brought to an X-ray machine. Finally, the X-ray film was examined.

2. Dry Skeleton Study

The Median-striped bullfrog (*Kaloula mediolineata*) and the Green-backed frog (*Rana erythraea*) were used for preliminary studies and comparative optimization since neither are rare nor endangered and were readily available being in laboratory. The results of these preliminary optimization trials showed that the dry skeleton method, modified from Meckvichai (2007) (Mimeographed), was the better procedure and thus was used for analysis of the valuable *T. verrucosus* samples.

The bone morphological analysis was carried out upon a total of 12 *Tylototriton verrucosus* individuals sampled from six places within four provinces in northern and northeastern Thailand: Doi Inthanon and Phuping Rajanives Palace, Doi Suthep, Chiang Mai Province; Doi Lahnga Khun Chae NP, Chiang Rai Province; Phu Suan Sai NP and Phu Luang WS, Loei Province and Phu Hin Rong Kla NP, Phitsanulok Province (**Figures 4.1** and **4.2**). Samples were anesthetized in MS222 solution and preserved in 95 % (v/v) ethanol. All of the viscera were removed using operating scissors and forceps taking care not to damage any of the hyoid apparatus, pectoral girdles, ribs, vertebrae, and pelvic girdles. Next, the specimen was placed in concentrated 2.5 – 5 % (w/v) KOH solution for 1-2 hours to digest the skin, muscles, tendons, and ligaments. The period of time required for this stage depended on the

time that the specimen had been preserved in 95 % (v/v) ethanol (more time preserved, less time digested). However, samples were monitored and checked carefully during this process as it is essential not to leave the skeleton in the KOH for too long otherwise various parts of the bones and particularly the phalange will be disarticulated or destroyed. After that, the head and four limbs were separated by operating scissors and forceps. These parts were placed in diluted KOH (approximately 0.5 % (w/v) KOH) for 2 - 3 days (the exact time and KOH concentration depending upon the condition of these parts). The skin, muscles, tendons, ligaments, and other tissues were eliminated as much as possible. Note that, the bones were not damaged by the gentle handling with forceps. The sample was gently washed with water many times to remove KOH and then each part of the prepared skeleton was separated carefully, using a toothbrush or paintbrush where required to remove remaining materials. If the bones could not be completely cleaned in this manner, they were digested with KOH and washed with water again as described. The clean bones were dried using a towel or tissue and left at normal room temperature. Finally, all pieces of bone were stored in a box and Thymol crystals were added to prevent any fungal germination. A digital vernier caliper was used to measure the various parts of bones in each specimen (**Figs. 4.3-4.7**).

Specimen descriptions of two types of color pattern of *T. verrucosus* in Thailand.

  1.1 Type I (orange to yellow form).

Figure 4.1 *T. verrucosus* Type I from Royal Garden Siribhume, Doi Inthanon, Chiang Mai Province.

Description of Coloration – The ground color is brown or black and the cranial crests, parotoid glands, dorsal ridge, dorsolateral warts, limbs, tail and ventral surface are all orange to yellow in color.

Representative Specimens of Type I

  ITNT1 and **ITNT2** refer to specimens from Watershed management station, Doi Ang Khang, Chiang Mai Province.

  LK3/5/50 and **LK** refer to specimens from Doi Lahnga, Khun Chae NP, Chiang Rai Province.

  PP2638 and **PP2643** refer to specimens from Phuping Rajanives Palace, Doi Suthep, Chiang Mai Province.

1.2 Type II (dull form).

Fig. 4.2 *T. verrucosus* Type II from Phu Luang WS, Loei Province.

Description of Coloration – The dorsal body, ventral surface and limbs are dull or brown in color. The cranial crests, parotoid glands, dorsal ridge of body and tail, dorsolateral warts, lower jaw, vent, ventral ridge of tail and tip of phalanges are all lighter brown than the body.

Representative Specimens of Type II

PK5 and **PK6** refer to specimens from Phu Suan Sai NP, Loei Province.

PLG and **PL2627** refer to specimens from Phu Luang WS, Loei Province.

PHF and **PHG** refer to specimens from Phu Hin Rong Kla NP, Phitsanulok Province.
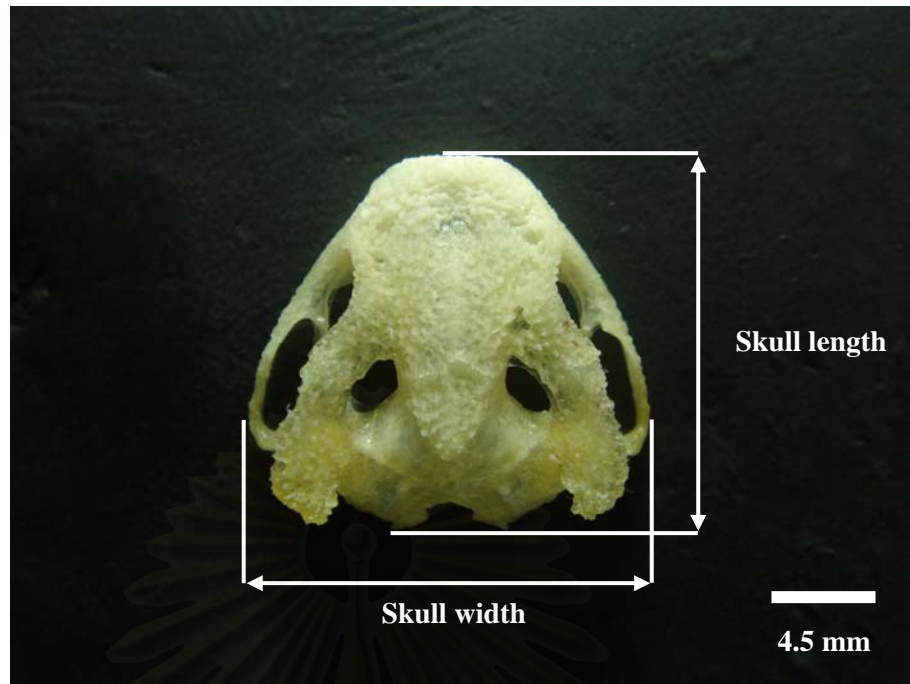
Fig. 4.3   Measurements at the dorsal side of the skull.
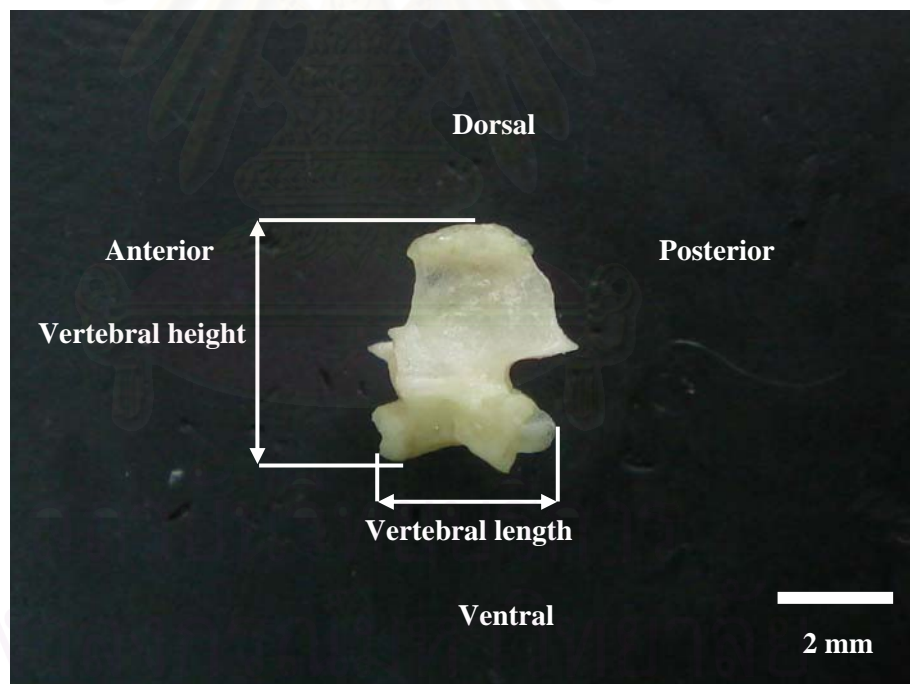
Fig. 4.4   Measurements at the lateral side of the trunk vertebra.
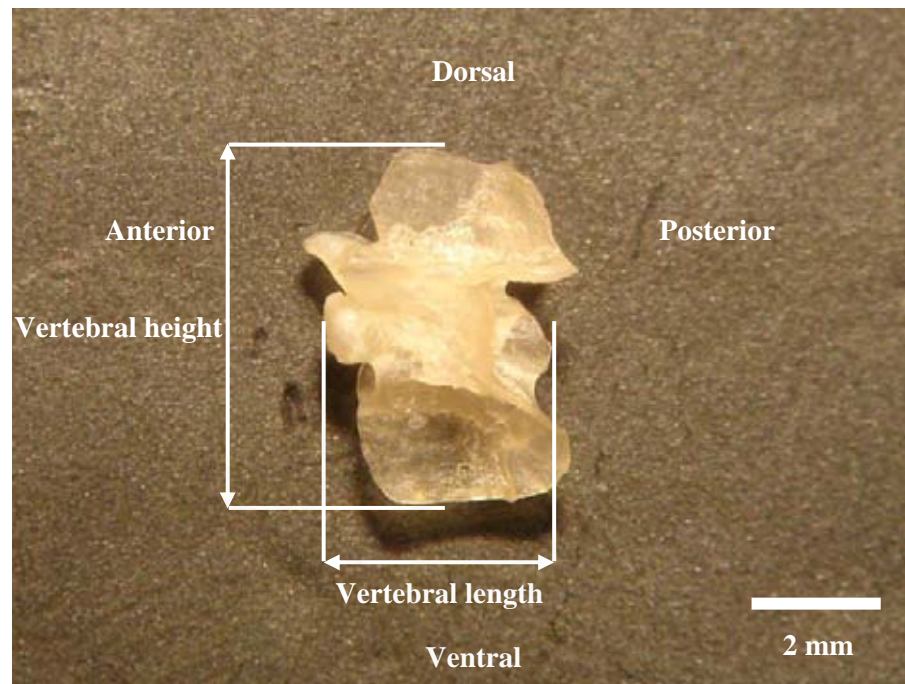
Fig. 4.5   Measurements at the lateral side of the caudal vertebra.
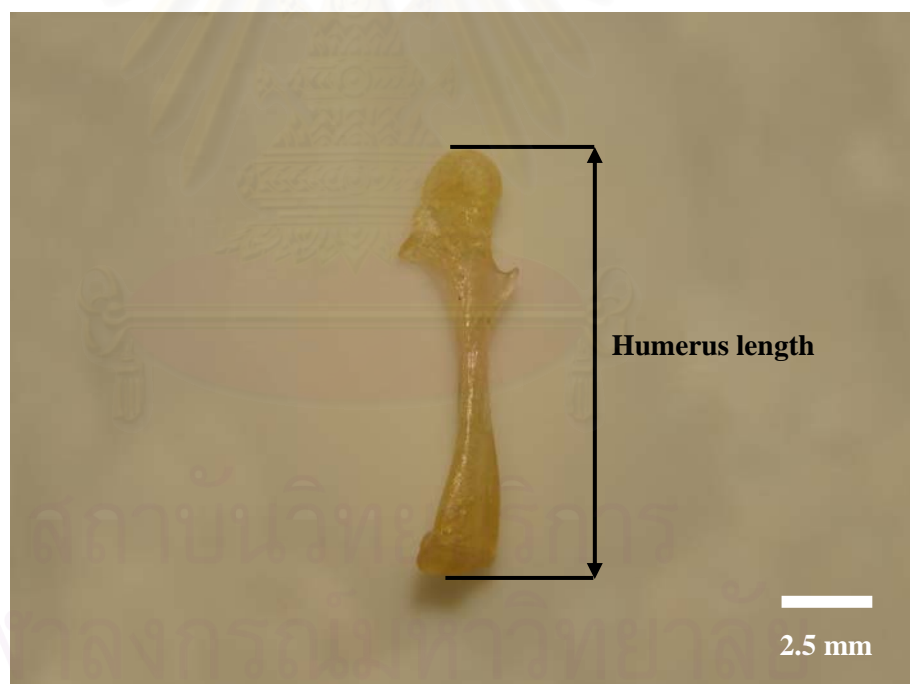
Fig. 4.6   Measurement at the dorsal side of the humerus.

Fig. 4.7   Measurement at the dorsal side of the femur.

RESULTS

1. X-ray Study

The figures obtained from the X-ray film showed a poor quality of resolution. Therefore, with these specimens at least, the dry skeleton technique was a more suitable method for bone study since good resolution is essential for accurate measurements and morphological discrimination. Thus the accuracy attained and required, justifies the extra time and materials required by the dry skeleton technique over X-ray study, and so was used throughout this study.

2. Dry Skeleton Study
2.1 The Skull (Figs. C.1-C.36)
General Form:

The skull is relatively large, broader than long (**Table 4.1**) and triangular in shape. There are two separated premaxillae. The nasal bones are broad and in contact with each other. The nasal process is short and does not reach the large frontal. The complete fronto-squamosal arch is thick, bony and strong. The skull has well-developed bony crests covering many parts of the dorsal skull. The frontal processes of maxillae are large and extended dorsally, whilst the posterior processes are long and contact with the short anterior processes of quadrates. The pterygoids extend to or nearly meet the maxillae. The vomerine teeth are triangular in shape. The two parallel rows of vomeropalatine teeth contact each other at the anteromedial of the skull and are continuous and diverge posteriorly. The dentaries have rather semicircular outlines

Table 4.1   Skull width and length measurements.

Specimen number	Skull width (mm)	Skull length (mm)
ITNT1	17.55	15.32
ITNT2	17.24	14.99
LK3/5/50	19.67	16.94
LK	17.49	15.10
PP2638	15.64	13.35
PP2643	17.10	15.15
PK5	16.07	14.39
PK6	17.03	15.44
PLG	15.37	14.41
PL2627	15.94	14.03
PHF	16.82	14.87
PHG	15.08	14.04

The differentiation in skull morphology between Type I (orange to yellow coloration) and Type II (dull coloration) is shown in Figs. 4.8 - 4.13:
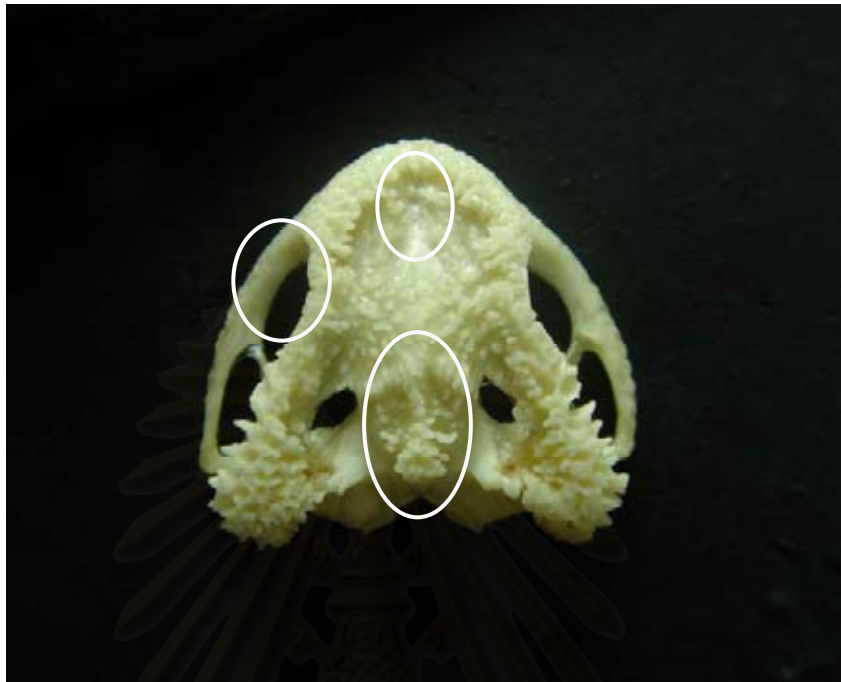
Fig. 4.8   Dorsal view of skull of ITNT1 (representative of Type I).
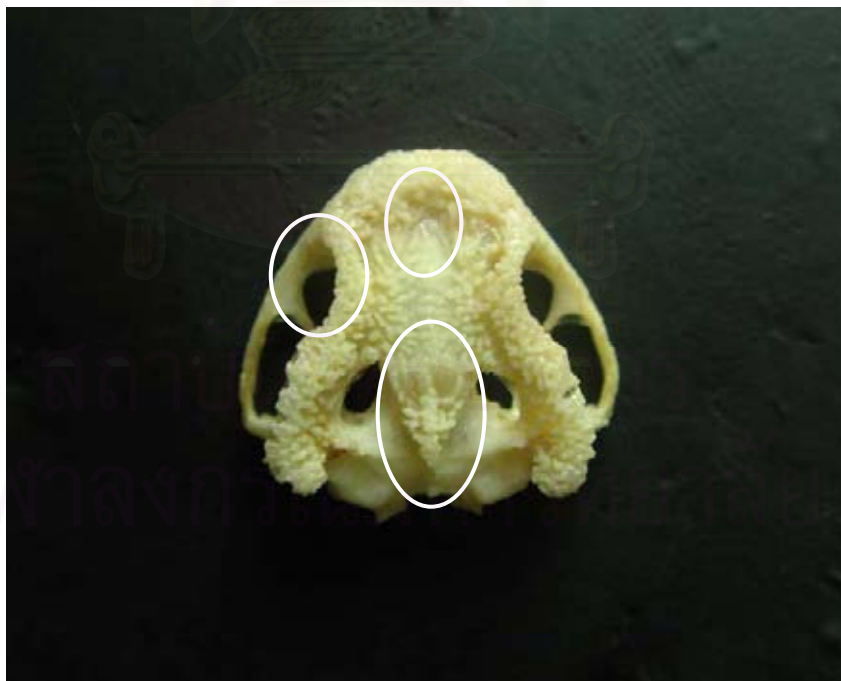
Fig. 4.9   Dorsal view of skull of PLG (representative of Type II).
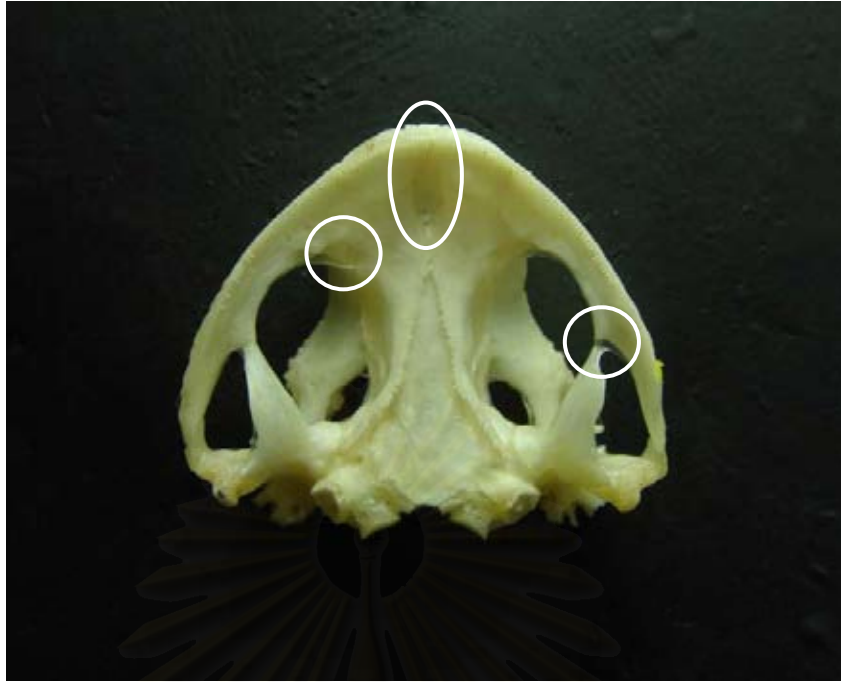
Fig. 4.10   Ventral view of skull of ITNT1 (representative of Type I).

Fig. 4.11   Ventral view of skull of PLG (representative of Type II).

Fig. 4.12   Lateral view of skull of ITNT2 (representative of Type I).

Fig. 4.13   Lateral view of skull of PLG (representative of Type II).

The secondary bony crests of Type I populations almost cover all parts of the dorsal skulls except: the posterior parts of nasals (two specimens), the posterior parts of nasals and the anterior parts of frontals (three specimens), and most parts of the nasals and frontals. However, the secondary bony crests of Type II populations cover all parts of the skulls (**Figs. 4.8** and **4.9**). The processes of the maxillae, at the upper positions that meet the pterygoids, are distinctly different between northern (Type I) and northeastern (Type II) populations: these processes express little concave shape in all Type I populations whereas those of Type II populations display a clearly concave form (**Figs. 4.8** and **4.9**). The shape of the proximal tip of the middorsal crests are also mostly different between Type I and II populations being curved in Type I populations, but angular in Type II populations (**Figs. 4.8** and **4.9**).

The ranges of the cavum can be used to separate Type I and II specimens. The ranges of Type I samples are invaded at the premaxillae, whilst Type II are not invaded (**Figs. 4.10** and **4.11**). The shape of the anterolateral vomeropalatine and their position next to the contacted areas between the vomeropalatines and maxillae, are obviously separated. Type I specimens exhibit less concave structures and little hook-shaped forms, whilst Type II specimens show distinctly concave structures like semicircles and appear clearly hook-shaped in form (except PHG showing little hook-shaped) (**Figs. 4.10** and **4.11**). The shapes of the maxillary processes where they contact with the pterygoids are another character that appears to separate Type I and II populations. In Type I, these processes are slightly projected to meet the pterygoids whereas in Type II specimens these parts are prominent projections (**Figs. 4.10** and **4.11**).

The shapes of bony crests where they attach onto the lateral sides of the frontal bones nearly differ between the two type populations. Thus the shapes are mostly convex from the continuous bony crest bases in Type I populations but, on the other hand, the shapes of Type II specimens are flattened in appearance except that sample PHF displays a slightly convex form (**Figs. 4.12** and **4.13**). The shape of the posteriorly pointed end processes of the bony crests that attach on the tympanic bones are noticeably different between these two types with the tip processes of Type I specimens almost tending to point downwardness (except specimen LK that was damaged so cannot be examined for this character) whilst, on the contrary, the tip processes of Type II specimens show both straight and upward directions to the tips (**Figs. 4.12** and **4.13**).

2.2 The Hyobranchial Skeleton (Figs. C.37-C.47)

General Form:

The Ceratohyal

The two large ceratohyals compose of both cartilaginous and bony parts. The anterior parts are expanded and cartilaginous, in contrast with the posterior parts that are generally slender and ossified. The anterior tips are slightly pointed but the posterior tips are rod-like in shape and have small cartilaginous caps. Both anterior and posterior tips are joined to the skull by a ligament.

The First Basibranchial

It is a stout and cartilaginous element. This structure bears the first and second pairs of radii anteriorly. The first pair is usually shorter and slightly smaller than the second pair. Both pairs of radii are cylindrical in shape, with decreasing diameters from anterolaterally to the tips.

The First Ceratobranchial

The first ceratobranchials are flattened and well-developed bony parts. They arise from the midpoint of the first basibranchial. The distal parts attach with cartilaginous area of the first epibranchials.

The Second Ceratobranchial

The second ceratobranchials are smaller, weaker and slenderer than the first ceratobranchial. The anterior parts are in contact with the posterior parts of the first basibranchial, while the posterolateral parts join with the cartilaginous regions that are the first epibranchial and first ceratobranchial.

The First Epibranchial

The first epibranchials are bony parts but have cartilaginous caps on the posterior tips. They articulate to the posterior tips of both the first and second ceratobranchial at the cartilaginous areas.

The differentiation of the hyobranchial skeleton between Type I (orange to yellow coloration) and Type II (dull coloration) forms of *T. verrucosus*:

The individual hyobranchial skeletons of these two types of newts are similar in general form. There are no obvious characters that could differentiate between them.

The Vertebral Column (**Figs. C.48-C.52**)

General Form:

The vertebral column is divided into five regions, namely, (i) the cervical with one vertebra, (ii) the trunk with normally thirteen vertebrae, (iii) the sacral with one vertebra, (iv) the caudosacral with generally four vertebrae, and (v) the caudal that is the most variable region with typically 19 or more vertebrae (**Table 4.2**).

The vertebrae are opisthocoelous, meaning that the centrum is convex anteriorly and concave posteriorly. The cervical vertebra differs in form distinctly from the other vertebral regions with the occipital condylus articulating with the occipital condyle, whilst the odontoid process articulates with the foramen magnum of the skull. The trunk vertebrae have dominant high neural spines and solidly developed bony plates, showing a sculptured dorsal surface, on the top of the neural spines. The trunk vertebrae have gradually extended lengths from the anterior to the posterior parts (**Table 4.3**), but their heights decrease slightly in the same direction (**Table 4.3**). The angles of projection from the axial of the transverse processes decrease from the anteriority to posteriority (**Fig. C.51**), whilst their ventral surfaces become more obviously concave. The sacral vertebra is normally the fifteenth and has the general pattern of the trunk vertebrae but it is slightly larger. Furthermore, the transverse processes and ribs are also attached with the iliums are noticeably strong and stout. The caudosacral vertebrae have a haemal arch and canal, which is more obvious in the caudal vertebrae. Their lengths and heights are short and low, respectively, from the anterior to the posterior ends (**Table 4.4**).

Table 4.2   Number of vertebral column in five regions of twelve specimens.

Specimen number	Number of vertebral column				
	Cervical	**Trunk**	**Sacral**	**Caudosacral**	**Caudal**
ITNT1	1	13	1	4	31
ITNT2	1	13	1	4	24
LK3/5/50	1	13	1	4	26
LK	1	12	1	4	23
PP2638	1	13	1	4	21
PP2643	1	13	1	4	30
PK5	1	13	1	4	28
PK6	1	13	1	4	32
PLG	1	13	1	5	21
PL2627	1	13	1	4	19
PHF	1	13	1	4	24
PHG	1	13	1	4	23

Table 4.3 1st, 4th and 12th trunk vertebral length and height measurements.

Specimen number	1st trunk length (mm)	4th trunk length (mm)	12th trunk length (mm)	1st trunk height (mm)	4th trunk height (mm)	12th trunk height (mm)
ITNT1	3.45	4.07	4.20	5.03	4.77	3.93
ITNT2	3.33	3.91	3.89	5.00	4.81	4.20
LK3/5/50	3.66	4.31	4.17	5.68	4.93	4.26
LK	3.24	3.72	4.06	5.47	5.20	4.18
PP2638	3.58	3.31	3.26	4.50	3.69	3.06
PP2643	3.45	4.02	4.24	4.50	4.22	3.45
PK5	3.83	3.34	3.55	4.45	4.29	3.77
PK6	3.88	3.76	3.96	4.55	4.94	4.09
PLG	3.88	3.77	3.80	3.66	3.62	3.06

Table 4.3   (continued)

Specimen number	1st trunk length (mm)	4th trunk length (mm)	12th trunk length (mm)	1st trunk height (mm)	4th trunk height (mm)	12th trunk height (mm)
PL2627	3.72	3.07	3.16	3.63	3.93	3.26
PHF	3.03	3.89	4.03	4.12	4.21	3.67
PHG	3.68	3.60	3.78	4.36	4.39	3.80

Table 4.4   8th, 12th and 16th caudal vertebral length and height measurements.

Specimen number	8th caudal length (mm)	12th caudal length (mm)	16th caudal length (mm)	8th caudal height (mm)	12th caudal height (mm)	16th caudal height (mm)
ITNT1	3.42	3.14	3.80	4.97	4.46	3.85
ITNT2	3.31	3.11	3.91	5.67	4.53	3.84
LK3/5/50	3.44	3.13	3.94	5.55	4.67	3.94
LK	3.65	3.34	3.99	5.06	4.21	3.77
PP2638	3.53	3.50	3.31	4.29	4.12	3.66
PP2643	3.16	3.19	3.83	5.08	4.35	3.66
PK5	3.91	3.82	3.54	4.96	3.92	3.23
PK6	3.18	3.91	3.74	5.31	4.60	4.08
PLG	3.95	3.01	3.86	4.17	3.55	3.08
PL2627	3.80	3.74	3.20	4.28	3.59	3.91
PHF	3.17	3.88	3.68	4.48	3.72	3.28
PHG	3.81	3.86	3.39	4.85	4.51	3.58

The differentiation of the vertebral column between Type I (orange to yellow coloration) and Type II (dull coloration) forms of *T. verrucosus* is shown in Figs. 4.14 - 4.17:

Fig. 4.14   Lateral view of 1st trunk vertebra of LK (representative of Type I).
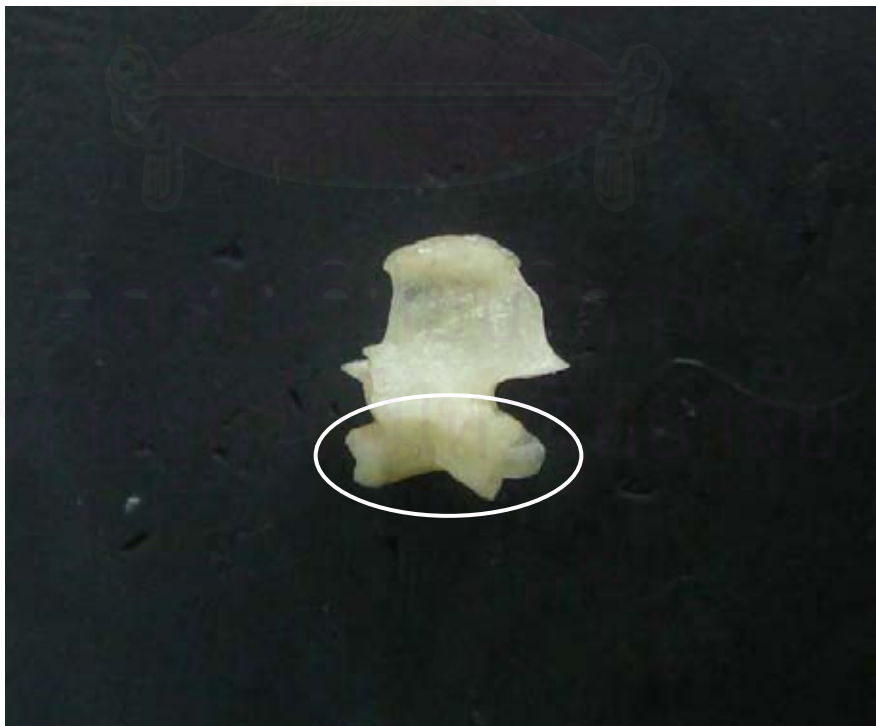
Fig. 4.15   Lateral view of 1st trunk vertebra of PHF (representative of Type II).

Fig. 4.16   Dorsal view of presacral vertebra of LK (representative of Type I).
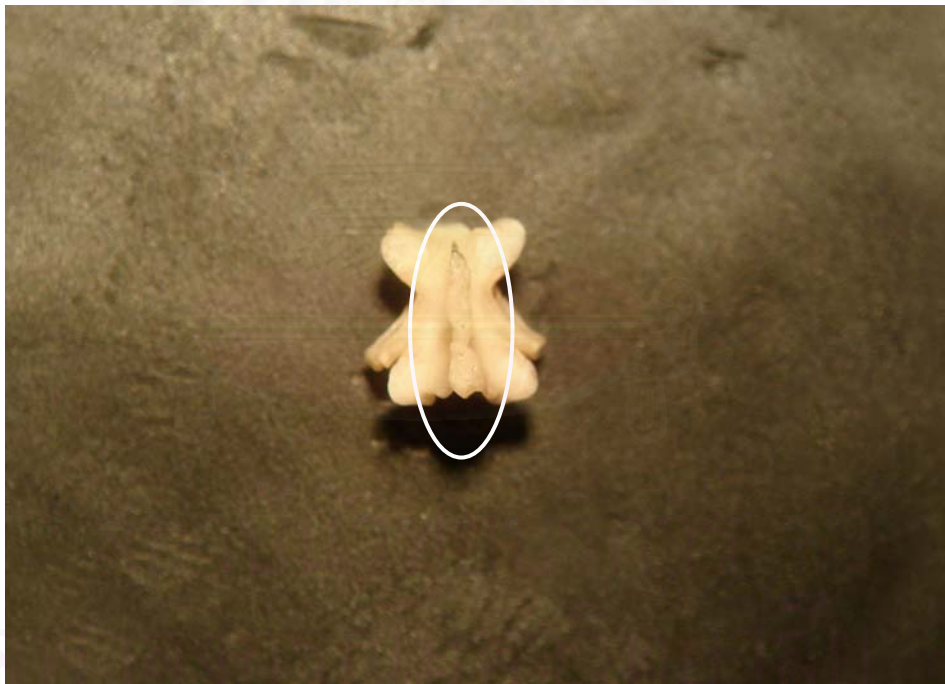
Fig. 4.17   Dorsal view of presacral vertebra of PHF (representative of Type II).

The ventral surfaces of the primary trunk vertebrae of Type I newts are less concave than Type II newts (**Figs. 4.14** and **4.15**). The shapes of bony plates on the top of neural spines of the presacral (last trunk) vertebrae are also different between the two types of newts. All Type I specimens examined revealed an arrow-like shape ("▲") whilst almost all Type II samples showed a triangle shaped point ("▲"), the exceptions being PK5 and PK6 that appear like a small arrow ("▲") in shape (**Figs. 4.16** and **4.17**). The other parts of the vertebrae were not distinct or different enough Type I and II specimens to be diagnostic or informative (with respect to just Thai *T. verrucosus* samples) characters.

The Rib (**Figs. C.53-C.64**)
General Form:

There are two heads of the rib at the proximal end, which are not clearly discerned, and they are constricted in the middle. The ribs are relatively long, sharply pointed and certain ribs are slightly curved upwards or, particularly the middle trunk ribs, downward distally. The third and fourth ribs usually have epipleural processes (**Table 4.5**) and if the epipleural processes are large, the ribs appear forked. Almost every rib is associated with a wart except for the first and second ribs. There are postsacral ribs on caudosacral vertebrae and muscles are attached by fibers along the entire length of the ribs. The ribs of the anterior and middle trunk regions are long and decrease dramatically in size posteriorly, especially the postsacral ribs.

Table 4.5   The order of rib of trunk vertebrae that appear in the epipleural processes.

Specimen number	Order of rib which forms the epipleural process
ITNT1	3 (left, right), 4 (left, right)
ITNT2	3 (left, right), 4 (left, right)
LK3/5/50	3 (left, right), 4 (left, right), 5 (left)
LK	3 (left, right), 4 (left, right)
PP2638	3 (left, right), 4 (left, right)
PP2643	3 (left, right), 4 (left, right), 5 (left, right), 7 (left)
PK5	3 (left, right), 4 (left, right)
PK6	3 (left, right), 4 (left, right)

Table 4.5   (continued)

Specimen number	Order of rib which forms the epipleural process
PLG	3 (left, right), 4 (left, right), 5 (left)
PL2627	3 (left, right), 4 (left, right), 5 (left, right)
PHF	3 (left, right), 4 (left, right), 5 (left)
PHG	3 (left, right), 4 (left, right), 5 (left)

The differentiation of ribs between Type I (orange to yellow coloration) and Type II (dull coloration) forms of *T. verrucosus*:

There are no obviously different characters between the ribs that can distinguish between these two morphotypes of newt.

The Pectoral Girdle (**Figs. C.65-C.75**)

General Form:

The Suprascapula

It is an ossified plate that attach with the scapula region and has an obvious boundary between the suprascapula and scapula. The proximal portion is thicker than the distal portion.

The Scapula

This portion is long, curved in a dorsoventral direction and is the most noticeably ossified bone in the pectoral girdle. The proximal part is greatly expanded and merges with the procoracoid and coracoid regions, whilst the distal part contacts with the suprascapula. The boundary between scapula and suprascapula and between scapula and procoracoid and coracoid regions are noticeable. There is a deep glenoid cavity at the upper posterior of the proximal part.

The Procoracoid

This portion is an anterior part of the ventral plate of the pectoral girdle and remains cartilaginous around the margins, especially at the tip (very thin cartilage), but the other parts are a little ossified in form. There is an incisura coracoidea that separates the procoracoid and coracoid regions.

The Coracoid

It is a slightly large osseous portion and extends ventrally in a convex form helping to protect the heart by overlapping of the right and left sides (**Table 4.6**). The edges this portion are a very thin cartilage, like the procoracoid portion.

Table 4.6 The pattern of overlapping coracoid portions.

Specimen number	The pattern of overlapping (see from ventral)
ITNT1	The right side is above the left side
ITNT2	The right side is above the left side
LK3/5/50	The left side is above the right side
LK	The left side is above the right side
PP2638	The right side is above the left side
PP2643	The left side is above the right side
PK5	The left side is above the right side
PK6	The left side is above the right side
PLG	The right side is above the left side
PL2627	The left side is above the right side
PHF	The right side is above the left side
PHG	The left side is above the right side

The differentiation of the pectoral girdle between Type I (orange to yellow coloration) and Type II (dull coloration) *T. verrucosus* newts:

The pectoral girdles of the newts were very variable within as well as between the two newt morphotypes with no diagnostic or informative characters (at least within these two morphotypes only).

The Forelimb

General Form:

The Humerus (**Figs. C.76-C.87**)

The humerus is usually longer than the femur (**Table 4.7**). The head merges into the expanded crista ventralis humeri which rise steeply from the shaft. At the other side of this structure, there is a crista dorsalis humeri that is a small hooked process and does not extend on to the head of humerus. The radial condyle is larger

than the ulnar condyle and they contact with the proximal tips of the radius and ulna, respectively.

The Radius (**Figs. C.88-C.99**)

The radius is completely separate from the ulna. Its length is approximately as long as the ulna. The proximal end, which meets the humerus, is distinctly smaller than the expanded distal end, which articulates with the wrist, and tapers gradually to the middle of the shaft where the diameter is the smallest.

The Ulna (**Figs. C.100-C.111**)

The proximal end, contacts with the humerus, is larger than the smaller distal end, meets the wrist, and tapers slightly to the middle of the shaft where the diameter is smallest. There is a slight bony crest running from the middle shaft to the distal part.

The Carpus (**Figs. C.112-C.121**)

The carpus is generally well-calcified and normal having seven elements (**Table 4.8**): a radiale, a large fused ulnare+intermedium (intermedioulnare), a centrale and four carpalia. The radiale articulates with the distal part of the radius, the first centrale, the centrale and the intermedioulnare. The intermedioulnare is the largest bone of this structure and supports the fourth carpalia and the centrale. The centrale articulates with all elements of the carpus. The first centrale partially supports the first metacarpal by sharing with the large fused carpale 1+2, but the latter element is the main support. Besides, the carpale 3 and 4 support the third and fourth metacarpals, respectively. The metacarpals and phalanges are like hour-glass-shaped bones with cartilaginous epiphyses. The phalangeal formulars are universally 2,2,3,2 and the distal phalanges are terminally expanded.

Table 4.7   The length of humerus and femur measurements (left side).

Specimen number	Humerus length (mm)	Femur length (mm)
ITNT1	11.60	11.73
ITNT2	11.29	10.10
LK3/5/50	13.86	10.21
LK	11.97	10.94
PP2638	9.76	8.90
PP2643	11.89	10.64

Table 4.7 (continued)

Specimen number	Humerus length (mm)	Femur length (mm)
PK5	10.89	9.29
PK6	13.04	10.46
PLG	11.30	9.33
PL2627	10.22	8.56
PHF	11.81	9.52
PHG	11.92	10.05

Table 4.8 The number of carpal elements.

Specimen number	Number of carpal elements	
	Left hand	**Right hand**
ITNT1	7	6
ITNT2	7	8
LK3/5/50	7	7
LK	7	7
PP2638	8	6
PP2643	7	8
PK5	7	7
PK6	8 (3 cartilages)	7 (2 cartilages)
PLG	7	8
PL2627	7	7
PHF	7	5
PHG	7	6

The differentiation of the forelimbs between Type I (orange to yellow coloration) and Type II (dull coloration) *T. verrucosus* **newts:**

There are no differences in the forelimbs between Type I and II newts.

The Pelvic Girdle

General Form:

The Ilium (**Figs. C.122-C.133**)

It is a club-shaped and ossified structure. The dorsal portion has cartilage at the tip and projects a little backward inclination where it is attached to the rib of sacral vertebra firmly with fibrous tissue. There is an acetabulum at the area of contact with the puboischium.

The Puboischium (**Figs. C.122-C.133**)

It is an approximately quadrangular shaped ossified shaped plate. The anterior tip of this plate is cartilaginous. The two components of the puboischium are joined by medial cartilaginous symphysis. There is a foramen obturatorium on the lateroanterior tip of the pubic portion. The lateral portion of this structure projects posteriorly forming a spine that is concave to the medial symphysis.

The Cartilago Ypsiloidea (**Figs. C.134-C.144**)

This is a Y-shaped cartilage that articulates with the anterior puboischium and lies in the middle line of the body.

The differentiation of the pelvic girdle between Type I (orange to yellow coloration) and Type II (dull coloration) *T. verrucosus* newts:

The characters of the pelvic girdles could not be used in grouping between Type I and II newts because they are too variable within samples from the same group let alone between groups and thus are neither diagnostic nor informative characters in this context.

The Hindlimb
General Form:

The Femur (**Figs. C.145-C.156**)

The head is rounded and articulates the pelvic girdle at the acetabulum. There is a solidly expanded trochanter which is directed anteromedially on the ventral surface. There are two articular condyles at the distal ends - the larger tibial condyle and the smaller fibular condyle that contact with the proximal tips of the tibia and fibula, respectively.

The Tibia (**Figs. C.157-C.168**)

The tibia is absolutely separate from the fibula. The length is approximately as long as the fibula. There is a prominent tibial crest which arises from the dorsolateral side of the shaft, of which the tip is independent from the shafted bone and looks like

a thorn. The proximal end articulates the femur and the distal end contacts with the ankle.

The Fibula (**Figs. C.169-C.180**)

The fibula is more slender than the tibia. The proximal end is smaller than the distal end. The distal end is expanded and concave at the part that is proximal to the tibia. The proximal end contacts with the femur and the distal end articulates the ankle.

The Tarsus (**Figs. C.181-C.190**)

The tarsus is typically commonly well-ossified and usually consists of eight or nine elements (**Table 4.9**): a tibiale, a large fused intermedium+fibulare (intermediofibulare) (for eight elements) or an intermedium and a fibulare (for nine elements), a centrale and five tarsalia. The tibiale is the smallest element of the proximal row of tarsal elements, and articulates with the distal part of tibia, the first centrale, the centrale and the intermedium. The intermedium supports the centrale and articulates both the tibia and the fibula. The fibulare is the largest element of the proximal row and it is fused (for eight elements) or is not fused (for nine elements) with the intermedium. The centrale articulates with all elements of the tarsus except the fifth tarsalia. The first centrale partially supports the first metatarsal by sharing with the enlarged tarsale 1+2, but the latter element is the main support. In addition, the tarsale 1+2 also supports the second metatarsal. The third, fourth and fifth tarsalia support their respective metatarsals. The metatarsals and phalanges are all hour-glass-shaped bones with cartilaginous epiphyses. The phalangeal formulars are normally 2,2,3,3,2 and the distal phalanges are terminally expanded.

Table 4.9   The number of tarsal elements.

Specimen number	Number of tarsal elements	
	Left foot	**Right foot**
ITNT1	9	7 (4 cartilages)
ITNT2	9	7
LK3/5/50	9	8
LK	8	8
PP2638	9	9
PP2643	8	8

Table 4.9   (continued)

Specimen number	Number of tarsal elements	
	Left foot	**Right foot**
PK5	8	8
PK6	8 (2 cartilages)	8 (2 cartilages)
PLG	8	8
PL2627	8	8
PHF	8	6
PHG	8	8

The differentiation of the hindlimbs between Type I (orange to yellow coloration) and Type II (dull coloration) *T. verrucosus* **newts:**

There are certain differences in the number of tarsal elements in the left foot. In Type I the number is eight or nine but in Type II the number is eight. The other structures of hindlimb are variable between the two morphotypes.

DISCUSSION

From this osteological study, the obtained data has both similarities and differences from the previously reported data about this genus and species.

The skull is large, broader than long, and triangular shape agrees with Noble (1928) and Nussbaum and Brodie (1982), but is inconsistent with Haller-Probst (1988) who reported that the skull lengths of both *Tylototriton verrucosus* and *Tylototriton shanjing* are longer than wide. The aperture nasalis of specimens in this study either could not be seen from the dorsal view, in agreement with a figure of Noble (1928) and for *T. shanjing* (Haller-Probst, 1988) or were hardly visible but could only be seen as small margins from above, which is consistent with the pictures of Riese (1892) and Nussbaum and Brodie (1982). Notice that the *T. verrucosus* specimen of Haller-Probst (1988) also showed the aperture nasalis in dorsal view about two thirds.

The premaxillae are separated agree with Nussbaum and Brodie, 1982 and the pictures of Riese, 1892 and Haller-Probst, 1988. The nasal bones are broad and in contact with each other, similar to previous reports (Zhao et al., 1988 and the pictures of Riese, 1892; Noble, 1928; Nussbuam and Brodie, 1982; Haller-Probst, 1988). The nasal processes are short and not reach the large frontals concord with other reports (Zhao et al., 1988 and the pictures of Riese, 1892; Noble, 1928; Nussbuam and Brodie, 1982; Haller-Probst, 1988).

The fronto-squamosal arches are thick, bony, and strong (Taylor, 1962; Noble, 1928; Nussbaum and Brodie, 1982; Zhao et al., 1988). The dorsolateral crests and middorsal crest are well-developed on the skull (Nussbaum and Brodie, 1982; Zhao et al., 1988 and the images of Riese, 1892; Noble, 1928; Haller-Probst, 1988). However, interestingly, specimens in this study also present unique patterns of secondary bony crests that differ from that previously reported. The secondary bony sculptures of all Type I population samples except PP2638 nearly pierce all parts and actually pierce a few parts of frontals, whilst that for Type II populations pierce all parts of the frontals and nasals. The shapes of proximal tips of middorsal crests of Type I populations are curved similar to an illustration of Riese (1892), whereas, that of Type II specimens display angular shapes.

The anterior processes of maxillae are large and the posterior processes are long in agreement with prior works (Nussbaum and Brodie, 1982; Zhao et al., 1988 and the figures of Riese, 1892; Noble, 1928). The maxillae contact with the quadrates as reported (Taylor, 1962; Noble, 1928; Nussbaum and Brodie, 1982; Zhao et al., 1988 and the pictures of Riese, 1892 and *T. verrucosus* of Haller-Probst, 1988), but are incongruence with the *T. shanjing* specimen of Haller-Probst (1988). All of the examined specimens appear both strong and weak contact between maxillae and quadrates that agree with Noble (1928), Nussbaum and Brodie (1982) and the drawing of Riese (1892). However, this study did not find individuals with small gaps between the maxillae and quadrates in contrast to Nussbaum and Brodie (1982), and the quadrates of all examined specimens did not display curved spines as reported for *T. shanjing* by Haller-Probst (1988). Rather, whilst all of the northern populations (Type I) present a small degree of concavities at the anterior processes of the maxillae at the upper positions that meet the pterygoids, in agreement with the figures of Riese (1892), Noble (1928) and Nussbaum and Brodie (1982), in contrast all of the northeastern populations (Type II) display noticeable concavities. In this study, the pterygoids both extend to (according with Taylor, 1962; genus *Tylototriton* of Noble, 1928; Zhao et al., 1988 and the figures of Riese, 1892; *T. verrucosus* and *T. shanjing* of Haller-Probst, 1988) or nearly meet the maxillae in agreement with *T. verrucosus* specimens of Noble (1928), Zhao et al. (1988) and the figure of Nussbuam and Brodie (1982). The length of maxillae projections which contact the pterygoids of Type I populations is short, like the illustrations of Riese (1892) and Noble (1928), whilst those in Type II populations are notable longer.

The vomerine teeth are triangular shape (Zhao et al., 1988), but the ranges of the Type I cavum are invaded the premaxillae and are oval and not circular as reported for *T. shanjing* (Haller-Probst, 1988), whilst the ranges of Type II are not invaded, as reported for *T. verrucosus* (Haller-Probst, 1988) and the illustrations of Riese (1892) and Noble (1928). There are three specimens (ITNT2, PP2638 and PK6) which reveal anterior margins of the vomeropalatine teeth that nearly reach the cavum, as reported for *T. verrucosus* and *T. shanjing* (Haller-Probst, 1988), but the others are far from the cavum. The two rows of vomeropalatine teeth series are long (Nussbaum and Brodie, 1982), meet together anteriorly (except ITNT2) and lay diverge posteriorly (Taylor, 1962 and the figures of Riese, 1892; Noble, 1928; Haller-Probst, 1988) in a variable form. The shapes of the anterolateral vomeropalatines near

the posterior tip where they contact the maxillae are less concave with little hook-shaped appearances in Type I samples, similar to the illustrations of Riese (1892) and Noble (1928), but are distinctly semicircular concavities and obviously hook-shaped forms in Type II samples.

The shapes of bony crests attached on the lateral sides of the frontal bones are mostly different between Type I and II populations. Type I populations show convex structures (in accord with the pictures of Riese (1892) and Noble (1928), whilst Type II populations appear as flattened structures (except PHF). Moreover, the posteriorly pointed end processes of the bony crests in Type I samples tend to point downwardness, but in Type II samples they are both straight and upward projecting. The dentaries of Type I and Type II samples are all rather semicircular in outlines like that reported for *T. shanjing* (Haller-Probst, 1988).

The hyobranchial skeletons are highly variable parts. The ceratohyals vary in the size and shape of the anteriorly cartilaginous part in each specimen with the anterior tips appearing both slightly pointed and blunt, similar to previous reports (the figures of Riese, 1892; Noble, 1928; Ozeti, 1967). However, whilst the completely cartilaginous first basibranchials relate to the drawings of Riese (1892) and Noble (1928), this is incongruent with the figure of Ozeti (1967) where they appear more slender and rod-like. Type I and Type II specimens all revealed the first pair of radii as being either shorter or the same length as the second pair with the area of their attachment to the first basibranchials being similar to the figure of Ozeti (1967), but unlike the pictures of Riese (1892) and Noble (1928). The first ceratobranchials themselves are flattened and strongly ossified parts in all specimens resembling the figures reported by Riese (1892) and Ozeti (1967), but absolutely different from an illustration of Noble (1928). The second ceratobranchials were either totally cartilaginous (in ten specimens) or partly ossified (LK and PP2643), similar to a study of Ozeti (1967), but in contrast to the report of Zhao et al. (1988) and the illustrations of Riese (1892) and Noble (1928) which report only cartilaginous elements. The first epibranchials of all Type I and Type II specimens were similar to the data of Riese (1892), Noble (1928), and Ozeti (1967).

The vertebral column was analyzed as five separate regions. The vertebrae are opisthocoelous (Noble, 1928; Haller-Probst, 1988). The cervical vertebra, or atlas, is clearly separated from the other vertebrae as reported (Francis, 1934; Haller-Probst, 1988), but differ from the figure of Riese (1892) in that they display high neural

spines, distinct bony plates, circular occipital condylus, and visualized odontoid processes (when seen from the dorsal view). The trunk vertebrae have prominent high neural spines with strongly developed sculptural bony plates (Nussbaum and Brodie, 1982). The ventral surfaces of primary trunk vertebrae of Type I newts are less concave than Type II, but both are gradually elongate from anterior to posterior parts consistent with Haller-Probst (1988). However, in contrast, their heights and angles of projections from the axial of transverse processes both decrease, whilst their ventral surfaces are noticeable more concave from anteriority to posteriority. The numbers of trunk vertebrae were 12-13 which are inconsistent with the report of Zhao et al. (1988).

The shapes of bony plates on the top of neural spines of the presacral vertebrae are different between Type I and Type II specimens. All Type I samples show a arrowhead ("▲") shape, as reported for *T. shanjing* (Haller-Probst, 1988), whilst all Type II samples except PL2627 and PHG reveal a triangular tip ("▲") like that reported for *T. verrucosus* (Haller-Probst, 1988). The sacral vertebra is slightly stronger and larger than the other trunk vertebrae (Francis, 1934). The caudosacral vertebrae are present (Riese, 1892; Zhao et al., 1988). The lengths of caudal vertebrae are short from anteriority to posteriority because the newts use the anterior parts for more locomotive than the posterior parts. The heights of the dorsal spines are low from anteriority to posteriority indicating that the newts inhabit terrestrial life styles, in agreement with Nussbaum and Brodie (1982). In addition, there are variations on many parts of several regions of vertebrae such as angle of zygaphophyses, shape of transverse processes, and shape of arches.

The ribs are long, sharply pointed, slightly curved especially on the middle trunk regions and are not distinctly separated into two heads, but constrict in the middle as reported (Noble, 1928; Zhao et al., 1988). The third ribs are bifid (Noble, 1928; Nussbaum and Brodie, 1982) and all of the Type I and Type II specimens examined had a fourth ribs with epipleural processes. In addition, six specimens have epipleural processes on the fifth and one specimen on the seventh, which may or may not agree with that of Nussbaum and Brodie (1982) since they only mentioned that the ribs have epipleural processes but not which specific ribs.

Except for a pair of first and second ribs, ribs were associated with warts as reported before (Nussbaum and Brodie, 1982). Therefore, the number of ribs could be determined from the number of warts. The anterior and middle ribs were noticeably

longer than the posterior ribs which are in contrast with the report of Zhao et al. (1988), who stated that all ribs are approximately the same lengths in the genus *Tylototriton*, but in agreement with the picture of Nussbaum and Brodie (1982). Furthermore, the ratios of lengths between ribs and vertebrae do not approach unity as reported by Zhao et al. (1988). The caudosacral vertebrae also presented ribs (Zhao et al., 1988) and muscle fibers attach along the ribs (Nussbaum and Brodie, 1982; Zhao et al., 1988).

The pectoral girdles of specimens in this study are more similar to the figure of Riese (1892) than to that of Noble (1928). In particular, the sizes of glenoid cavities in this study are smaller than those reported in Noble (1928) and the shapes of the suprascapulae are different. Besides, the detailed forms of pectoral girdles in each individual are variable, especially in the proportion of cartilage to bone at the suprascapula, procoracoid and coracoid regions. In addition, the patterns of overlapping of coracoid portions also vary (the left is ventral to the right or *vice versa*).

The forelimb comprises the normal elements found in a tetrapod forelimb (Francis, 1934). The humerus is longer than femur, except in one specimen (ITNT1). There are two lateral crests near their head portions (Francis, 1934), which are variable in size and shape. The radial condyle is larger than the ulnar condyle and the forms of the radii and ulnae are consistent with the report of Francis (1934), but differ in small details as the forms and sizes of the variable bony crests of each ulnar specimen.

The carpals are normally strongly calcified elements (Nussbaum and Brodie, 1982), but one individual (PK6) had cartilage at a radiale and the first centrale elements in both hands. The numbers of carpals are normally seven ossified elements (mostly on left hands), but the right hands were variable in the number of carpal elements ranging from five to eight elements and mostly either six or eight elements. The six elements, seen in specimens ITNT1, PP2638, and PHG, occurs from fusion of a radiale and a carpale 1, whilst eight elements, seen in specimens ITNT2, PP2643, and PLG, occurs by separation of the intermedioulnare into two pieces. The phalangeal formulas were usually 2,2,3,2 in the left hands in accord with Francis (1934), but at the right hands of four specimens did not display this formula presenting instead a decreasing number of phalanges. The metacarpals and phalanges

are all hour-glass-shaped bones with cartilaginous epiphyses and the distal phalanges are terminally expanded in agreement with Ozeti (1967).

The detailed forms of pelvic girdles in each individual are far more variable than the pectoral girdles and include variations in the shape and proportion of cartilage in the puboischium; the shapes and components of the cartilago ypsiloidea (only four specimens, ITNT2, LK/3/5/50, PP2643 and PK6) were composed of only cartilage, the others being cartilage and bone composites. Thus the pelvic girdles of some specimens resemble the figures of Riese (1892) and Noble (1928), except the appearance of the cartilago ypsiloidea of Noble (1928) absolutely differs from all of the Type I and Type II specimens examined here.

The form of the hindlimb corresponds to the forelimb except for a difference in the number of elements. The femurs have a little variation in shape and size of the trochanters, tibial and fibular condyles and agree with the report of Francis (1934), but differ in small details such as the shapes and sizes of the tibial crests of each tibial specimen and the degrees of concavities of each fibular specimen.

Generally, the tarsals are solidly ossified elements (Nussbaum and Brodie, 1982), but one individual (PK6) had cartilage at a tibiale and the first centrale elements in both feet. The numbers of tarsal elements of this study are interested. For the left feet, in Type I populations all specimens had nine elements, which accords with Nussbaum and Brodie (1982), except two specimens (LK and PP2643) which had eight. The presence of eight elements is due to a fusion of a fibulare and an intermedium. In contrast, all the Type II specimens examined had eight elements. On the right feet, there are several variations in the number of tarsal elements ranging from six to nine elements, with the most frequent being eight, especially in Type II specimens. The phalangeal formulas were normally 2,2,3,3,2 for both feet in accord with the pictures of Francis (1934) and Nussbaum and Brodie (1982). However, some individuals lack some phalanges and others lack a toe or more than one distal phalange per toe, which may be due to handicap. The metatarsals and phalanges are all hour-glass-shaped bones with cartilaginous epiphyses and the distal phalanges are terminally expanded as same as a report of Ozeti (1967).

CONCLUSION

From the osteological study among samples of the Himalayan newt, *Tylototriton verrucosus*, from several populations found in Thailand, the result shows an obvious difference in several shapes of skulls and vertebrae between the two color types of newts: Type I (orange to yellow body coloration) and Type II (dull body coloration).

For the skulls, the differences are the boundaries of secondary bony crests, the shapes of the processes of the maxillae, the shapes of proximal tips of middorsal crests, the ranges of the cavum, the shapes of anterolateral vomeropalatine, the shapes of bony crests where attach on the lateral sides of frontal bones and the shapes of posteriorly pointed end processes of bony crests that attach on the tympanic bones.

The vertebrae of these two types also can be distinguished and have different shapes of the ventral surfaces of the primary trunk vertebrae and the shapes of bony plates on the top of neural spines of the last trunk vertebrae.

Bone morphology clearly suggests that *T. verrucosus* in Thailand can be separated into two different types. Type I (orange to yellow body coloration) distributed in the Northern mountain ranges, and Type II (dull body coloration) distributed in the Northeastern mountain ranges.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

The results from this study revealed that the Himalayan newt (*Tylototriton verrucosus*) found in Thailand can be divided into two types (defined as Type I and II) based on the totally congruent differences in their geographic distribution, body coloration, phylogenetic relationships and bone morphology. Previously, *T. verrucosus* in Thailand was considered as a single species.

Field observations found that, there are two color morphotypes (Type I and Type II) in this taxon (**Figs. 2.4-2.14**) in Thailand that also show differences in average female (but not male) body size and shape. Interestingly, these two morphotypes were geographically separated and totally allopatric in Thailand. All Type II specimens occurred only in the northeastern mountains of Thailand, whilst Type I is restricted in Thailand to the northern mountains, but maybe congruent with the species in the rest of its recorded Indo-Asia range.

Type I newts show brown or black coloration on the grounds, but the cranial crests, parotoid glands, dorsal ridges, dorsolateral warts, limbs, tails and ventral surfaces are orange to yellow in color (e.g., **Fig. 2.4**). This type are distributed in the northern mountain ranges and were comprised of individuals from Doi Ang Khang, Chiang Mai Province (the Daen Lao Range); Namtok Mae Surin NP, Mae Hong Son Province, Doi Inthanon, Doi Suthep and Doi Pui, Chiang Mai Province (the East Thanon Thong Chai Range); Khun Chae NP, Chiang Rai Province (the Western Phi Pan Nam Range) (**Fig. 2.3**).

Type II newts appear dull or brown in color on the dorsal body, ventral surfaces and limbs. Their cranial crests, parotoid glands, dorsal ridges of body and tail, dorsolateral warts, lower jaws, vents, ventral ridges of tail and tip of phalanges are all lighter brown than the body (i.e., **Fig. 2.5**). Type II newts are distributed in the northeastern mountain ranges and were composed of individuals from Phu Suan Sai NP, Loei Province and Phu Hin Rong Kla NP, Phitsanulok Province (the Western Phetchabun Range); Phu Luang WS, Loei Province (the Eastern Phetchabun Range) (**Fig. 2.3**).

The ecological data of Type I and Type II newts are not different as far as these surveys revealed. They both could be found in mountainous areas at an altitude of more than 1,000 m as reported previously (Kuzmin et al., 1994; Anders et al.,

1998; Pomchote et al., 2007), they were both normally found in still-water ponds, small streams or under logs or wood debrises (**Figs. 2.17-2.25**). In general, both Type I and Type II female newts reaching sexual maturity are bigger and more robust than males (**Fig. 2.16**), in agreement with earlier reports (Roy and Mushahidunnabi, 2001; Hegde and Deuti, 2007), and during the breeding season the cloacal openings of females are clearly circular while those of males are oval. Moreover, the larvae of both types can consume both small invertebrates and vertebrates including their small siblings (Pomchote, 2004).

16S rRNA and D-loop fragment sequences of mtDNA have been popularly used for assessing relationships among populations or species that are closely related (e.g., Hedges et al., 1995; Kawamoto et al., 2006), including to resolve the relationships among salamander populations (i.e., Garcia-Paris and Wake, 2000; Matsui et al., 2007).

The percentage sequence divergences in and between Type I and Type II newts were variable both among each population and between populations. Some individuals in each type did not show any difference in both gene fragments with others in the same type, whilst others did reveal differences but all individuals from Type I showed significant sequence differences with all individuals from Type II (**Tables A.3** and **A.4**). Thus the percentage sequence divergences between all comparisons between the northern and northeastern populations were more than that between individuals within each individual population and reached a maximum of 7.815 % and 8.756 % for 16S rRNA and D-loop regions, respectively, which is not dissimilar to the 9.5 % sequence divergence between *T. verrucosus* and *T. taliangensis* at the cyt *b* mtDNA sequence (Chan et al., 2001). The level of the highest mtDNA sequence divergence between the Type I and Type II of *T. verrucosus* in Thailand was clearly of the same extent as that found between *Tylototriton* species, in support of the notion that Type I and Type II maybe different species or undergoing speciation, although of course this does not establish this.

The phylogenetic trees obtained from the combined data set of 16S rRNA and D-loop fragment sequences, with equal weighting, via both NJ based distance analysis (**Figs. 3.14)** and ML based character analysis (**Figs. 3.15**) revealed that the Type I and Type II newts form two distinct and separate groups suggesting they are genetically distinct populations and supporting, but not establishing, that they are distinct species. Further support could be derived from analysis of other populations of *T. verrucosus*

from across its range and from other species within the genus, which time and availability of samples precluded from being evaluated in this study. However, within these two major clades, the Type I or northern clade and the Type II or northeastern clade, there were at least four well supported subclades, two from the northern and two from the northeastern lineages that were clearly congruent with their distribution based upon their geographic habitats on mountain ranges (**Fig 3.16**), supporting geographical isolation by distance due to mountain ranges.

Skeleton bone morphology based characters have been used for taxonomic studies by several taxonomists (e.g., Killebrew, 1979; Trueb and Massemin, 2000). The bone is the most imperishable component compared with other organs (i.e., nerve, muscle, and tissue), thus a change of a various parts of them for a long time period is the key to understanding the route of animal evolution (Flower, 1885).

In this study, there were some characteristic differences that were conserved within each clade (Type I and Type II) but could clearly distinguish between them, consistent with the notion that these two clades have been separated for some time. However, due to sample availability and time constraints, this study only compared Type I and Type II specimens and no other either geographical isolates of *T. verrucosus* or, importantly, no other closely related and distant species. Thus, informative characters that are conserved within both Type I and Type II would be missed, biasing the character interpretation towards only discriminating characters and masking similarities. However, it does highlight useful discriminatory characters for verification in future studies and so these are listed as follows.

The differences of skulls between Type I and Type II include the boundaries of secondary bony crests, the shapes of maxillary processes, the shapes of proximal tips of middorsal crests, the cavum ranges, the shapes of the anterolateral vomeropalatines, the shapes of the maxillary processes that contact with the pterygoids, the shapes of the bony crests attached to the lateral sides of the frontal bones, and the shapes of the posteriorly pointed end processes of the bony crests attached to the tympanic bones (**Figs. 4.8-4.13**), were all informative characters that clearly distinguished between Type I and Type II individuals. The vertebrae also could used to distinguish these two types of newt such as the concave degree of the ventral surfaces of the primary trunk vertebrae and the shapes of bony plates on the top of neural spines of the last trunk vertebrae (**Figs. 4.14-4.17**).

Note that, there are some differences in the number of tarsal elements in the

left foot found between both of the two newt types (**Table 4.9**), whereas the other bony parts display more variation within the same populations as well as between other populations. From the osteological comparisons between *T. verrucosus* and *T. shanjing* (Haller-Probst, 1988), the skulls and vertebrae of these two species can be obviously distinguished. Some characters of his specimens in both these two species were similar and different from Thai specimens in this work. The differences of skull characters among the two types of Thai specimens had a higher degree of difference than those between *T. verrucosus* and *T. shanjing*. On the other hand, the differences in the vertebrae among the two newt types of Thai specimens had a lower degree of difference than that between *T. verrucosus* and *T. shanjing*. Several characters of the skulls and vertebrae could be used to be an evidence to show the patterns of geographic distribution.

The data from the patterns of distribution, molecular and morphological characters within *T. verrucosus* recorded in Thailand show remarkable congruence in splitting the Type I and Type II morphotypes into separate populations coincident with their geographical distribution and mountain range (habitat) barriers, raising the probability that more than a single species of this taxon may be represented.

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APPENDICES

Appendix A

Table A. 1 Percentage nucleotide base composition of 16S rRNA sequences from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup.

Region	Samples	A	C	G	T
North	LK003	37.1	20.3	17.7	24.9
	LK004	37.1	20.3	17.7	24.9
	LK005	37.1	20.3	17.7	24.9
	PPf	36.7	20.8	17.8	24.6
	PPg	36.7	20.8	17.8	24.6
	PPh	36.5	21.6	18.0	23.8
	ITNT1	36.5	22.0	17.8	23.6
	ITNT2	36.5	21.8	17.8	23.8
	ITNT3	36.5	21.6	18.0	23.8
	SPc	36.5	21.8	18.0	23.6
	SP11	36.5	21.8	18.0	23.6
	SP12	36.5	21.8	18.0	23.6
	Mean	**36.7**	**21.3**	**17.9**	**24.2**
Northeast	PK4	36.0	21.0	18.8	24.2
	PK5	36.0	21.0	18.8	24.2
	PK6	36.0	21.0	18.8	24.2
	PL007	36.0	21.2	18.8	24.0
	PL008	36.0	21.2	18.8	24.0
	PL009	35.9	21.0	18.8	24.2
	PH019	36.0	21.2	18.8	24.0
	PH024	36.0	21.4	18.8	23.8
	PH025	36.0	21.4	18.8	23.8
	Mean	**36.0**	**21.2**	**18.8**	**24.0**
Outgroup	*P. hongkongensis*	35.5	20.4	18.6	25.5

Table A.2   Percentage nucleotide base composition of D-loop sequences from 21
T. verrucosus samples and *P. hongkongensis* as the outgroup.

Region	Samples	A	C	G	T
North	LK003	28.1	21.5	16.1	34.2
	LK004	28.1	21.5	16.1	34.2
	LK005	28.1	21.5	16.1	34.2
	PPf	27.8	21.5	16.3	34.4
	PPg	27.8	21.5	16.3	34.4
	PPh	27.7	21.9	16.4	34.0
	ITNT1	27.7	21.9	16.4	34.0
	ITNT2	27.7	21.9	16.4	34.0
	ITNT3	27.7	21.9	16.4	34.0
	SPc	27.7	21.9	16.4	34.0
	SP11	27.7	21.9	16.4	34.0
	SP12	27.7	21.9	16.4	34.0
	Mean	**27.8**	**21.7**	**16.3**	**34.1**
Northeast	PK4	29.1	20.1	15.6	35.2
	PK5	29.1	20.1	15.6	35.2
	PK6	29.1	20.1	15.6	35.2
	PL007	29.1	20.3	15.6	35.0
	PL008	29.1	20.3	15.6	35.0
	PL009	29.1	20.0	15.6	35.3
	PH019	29.1	20.1	15.7	35.0
	PH024	29.1	20.1	15.7	35.0
	PH025	29.1	20.1	15.7	35.0
	Mean	**29.1**	**20.2**	**15.6**	**35.1**
Outgroup	*P. hongkongensis*	29.6	19.7	16.2	34.4

Table A.3   The Tamura-Nei distance (%) of 16S rRNA sequences from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup.

Samples	PL007	PL008	PH019	PH024	PH025	PL009	PK4	PK5
PL007	-	-	-	-	-	-	-	-
PL008	0.000	-	-	-	-	-	-	-
PH019	0.000	0.000	-	-	-	-	-	-
PH024	0.203	0.203	0.203	-	-	-	-	-
PH025	0.203	0.203	0.203	0.000	-	-	-	-
PL009	0.203	0.203	0.203	0.411	0.411	-	-	-
PK4	0.203	0.203	0.203	0.411	0.411	0.000	-	-
PK5	0.203	0.203	0.203	0.411	0.411	0.000	0.000	-
PK6	0.203	0.203	0.203	0.411	0.411	0.000	0.000	0.000
SP12	7.552	7.552	7.552	7.182	7.182	7.190	7.180	7.180
SPc	7.552	7.552	7.552	7.182	7.182	7.190	7.180	7.180
SP11	7.552	7.552	7.552	7.182	7.182	7.190	7.180	7.180
ITNT1	7.812	7.812	7.812	7.440	7.440	7.447	7.437	7.437
ITNT2	7.441	7.441	7.441	7.815	7.815	7.094	7.084	7.084
PPh	7.183	7.183	7.183	7.555	7.555	6.839	6.829	6.829
ITNT3	7.183	7.183	7.183	7.555	7.555	6.839	6.829	6.829
PPg	6.249	6.249	6.249	6.587	6.587	5.935	5.925	5.925
PPf	6.249	6.249	6.249	6.587	6.587	5.935	5.925	5.925
LK004	5.145	5.145	5.145	5.454	5.454	4.857	4.848	4.848
LK005	5.145	5.145	5.145	5.454	5.454	4.857	4.848	4.848
LK003	5.145	5.145	5.145	5.454	5.454	4.857	4.848	4.848
P. hongkongensis	17.240	17.240	17.240	17.213	17.213	17.296	17.270	17.270

Table A.3   (continued)

Samples	PK6	SP12	SPc	SP11	ITNT1	ITNT2	PPh	ITNT3
PL007	-	-	-	-	-	-	-	-
PL008	-	-	-	-	-	-	-	-
PH019	-	-	-	-	-	-	-	-
PH024	-	-	-	-	-	-	-	-
PH025	-	-	-	-	-	-	-	-
PL009	-	-	-	-	-	-	-	-
PK4	-	-	-	-	-	-	-	-
PK5	-	-	-	-	-	-	-	-
PK6	-	-	-	-	-	-	-	-
SP12	7.180	-	-	-	-	-	-	-
SPc	7.180	0.000	-	-	-	-	-	-
SP11	7.180	0.000	0.000	-	-	-	-	-
ITNT1	7.437	0.201	0.201	0.201	-	-	-	-
ITNT2	7.084	0.406	0.406	0.406	0.203	-	-	-
PPh	6.829	0.203	0.203	0.203	0.406	0.201	-	-
ITNT3	6.829	0.203	0.203	0.203	0.406	0.201	0.000	-
PPg	5.925	1.279	1.279	1.279	1.488	1.260	1.051	1.051
PPf	5.925	1.279	1.279	1.279	1.488	1.260	1.051	1.051
LK004	4.848	2.993	2.993	2.993	3.216	2.940	2.718	2.718
LK005	4.848	2.993	2.993	2.993	3.216	2.940	2.718	2.718
LK003	4.848	2.993	2.993	2.993	3.216	2.940	2.718	2.718
P. hongkongensis	17.270	19.842	19.842	19.842	20.190	20.238	19.894	19.894

Table A.3 (continued)

Samples	PPg	PPf	LK004	LK005	LK003	*P. hongkongensis*
PL007	-	-	-	-	-	-
PL008	-	-	-	-	-	-
PH019	-	-	-	-	-	-
PH024	-	-	-	-	-	-
PH025	-	-	-	-	-	-
PL009	-	-	-	-	-	-
PK4	-	-	-	-	-	-
PK5	-	-	-	-	-	-
PK6	-	-	-	-	-	-
SP12	-	-	-	-	-	-
SPc	-	-	-	-	-	-
SP11	-	-	-	-	-	-
ITNT1	-	-	-	-	-	-
ITNT2	-	-	-	-	-	-
PPh	-	-	-	-	-	-
ITNT3	-	-	-	-	-	-
PPg	-	-	-	-	-	-
PPf	0.000	-	-	-	-	-
LK004	1.500	1.500	-	-	-	-
LK005	1.500	1.500	0.000	-	-	-
LK003	1.500	1.500	0.000	0.000	-	
P. hongkongensis	16.015	16.015	11.957	11.957	11.957	-

Table A.4 The Tamura-Nei distance (%) of D-loop sequences from 21 *T. verrucosus* samples and *P. hongkongensis* as the outgroup.

Samples	PL007	PL008	PK4	PK5	PK6	PL009	PH019	PH024
PL007	-	-	-	-	-	-	-	-
PL008	0.000	-	-	-	-	-	-	-
PK4	0.839	0.839	-	-	-	-	-	-
PK5	0.839	0.839	0.000	-	-	-	-	-
PK6	0.839	0.839	0.000	0.000	-	-	-	-
PL009	0.697	0.697	0.139	0.139	0.139	-	-	-
PH019	0.699	0.699	0.418	0.418	0.418	0.277	-	-
PH024	0.699	0.699	0.418	0.418	0.418	0.277	0.000	-
PH025	0.699	0.699	0.418	0.418	0.418	0.277	0.000	0.000
SP11	8.383	8.383	8.205	8.205	8.205	8.384	8.036	8.036
SP12	8.023	8.023	7.845	7.845	7.845	8.024	7.678	7.678
SPc	8.023	8.023	7.845	7.845	7.845	8.024	7.678	7.678
ITNT1	8.023	8.023	7.845	7.845	7.845	8.024	7.678	7.678
ITNT3	8.023	8.023	7.845	7.845	7.845	8.024	7.678	7.678
ITNT2	8.023	8.023	7.845	7.845	7.845	8.024	7.678	7.678
PPh	8.023	8.023	7.845	7.845	7.845	8.024	7.678	7.678
PPg	7.321	7.321	7.149	7.149	7.149	7.322	6.985	6.985
PPf	7.321	7.321	7.149	7.149	7.149	7.322	6.985	6.985
LK004	8.756	8.756	8.229	8.229	8.229	8.404	8.057	8.057
LK005	8.756	8.756	8.229	8.229	8.229	8.404	8.057	8.057
LK003	8.756	8.756	8.229	8.229	8.229	8.404	8.057	8.057
P. hongkongensis	41.364	41.364	40.301	40.301	40.301	40.319	40.001	40.001

Table A.4 (continued)

Samples	PH025	SP11	SP12	SPc	ITNT1	ITNT3	ITNT2	PPh
PL007	-	-	-	-	-	-	-	-
PL008	-	-	-	-	-	-	-	-
PK4	-	-	-	-	-	-	-	-
PK5	-	-	-	-	-	-	-	-
PK6	-	-	-	-	-	-	-	-
PL009	-	-	-	-	-	-	-	-
PH019	-	-	-	-	-	-	-	-
PH024	-	-	-	-	-	-	-	-
PH025	-	-	-	-	-	-	-	-
SP11	8.036	-	-	-	-	-	-	-
SP12	7.678	0.279	-	-	-	-	-	-
SPc	7.678	0.279	0.000	-	-	-	-	-
ITNT1	7.678	0.279	0.000	0.000	-	-	-	-
ITNT3	7.678	0.279	0.000	0.000	0.000	-	-	-
ITNT2	7.678	0.279	0.000	0.000	0.000	0.000	-	-
PPh	7.678	0.279	0.000	0.000	0.000	0.000	0.000	-
PPg	6.985	0.841	0.558	0.558	0.558	0.558	0.558	0.558
PPf	6.985	0.841	0.558	0.558	0.558	0.558	0.558	0.558
LK004	8.057	3.513	3.207	3.207	3.207	3.207	3.207	3.207
LK005	8.057	3.513	3.207	3.207	3.207	3.207	3.207	3.207
LK003	8.057	3.513	3.207	3.207	3.207	3.207	3.207	3.207
P. hongkongensis	40.001	45.098	43.564	43.564	43.564	43.564	43.564	43.564

Table A.4   (continued)

Samples	PPg	PPf	LK004	LK005	LK003	*P. hongkongensis*
PL007	-	-	-	-	-	-
PL008	-	-	-	-	-	-
PK4	-	-	-	-	-	-
PK5	-	-	-	-	-	-
PK6	-	-	-	-	-	-
PL009	-	-	-	-	-	-
PH019	-	-	-	-	-	-
PH024	-	-	-	-	-	-
PH025	-	-	-	-	-	-
SP11	-	-	-	-	-	-
SP12	-	-	-	-	-	-
SPc	-	-	-	-	-	-
ITNT1	-	-	-	-	-	-
ITNT3	-	-	-	-	-	-
ITNT2	-	-	-	-	-	-
PPh	-	-	-	-	-	-
PPg	-	-	-	-	-	-
PPf	0.000	-	-	-	-	-
LK004	3.203	3.203	-	--	-	-
LK005	3.203	3.203	0.000	-	-	-
LK003	3.203	3.203	0.000	0.000	-	-
P. hongkongensis	43.533	43.533	43.533	43.533	43.533	-

Appendix B

Fig. B.1   A 69 character matrix of 21 *T. verrucosus* samples based on partial
tRNA-Phe sequences. Asterisks (*) represent conserved nucleotide
residues across all samples.

```
                ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10        20        30        40        50        60
PH024           GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
PH019           GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
PK4             GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
PL009           GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
PH025           GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
PL007           GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
PK5             GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
PK6             GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
PL008           GTTAATGTAG CTTATAT-AA AGCGTGGCAC TGAAGATGCT AAGATGGATT ATAAAAAAAT
LK003           GTTAATGTAG CTTATATTAA AGTATGGCAC TGAAAATGCT AAGATAGATT TTAAAACA-T
LK004           GTTAATGTAG CTTATATTAA AGTATGGCAC TGAAAATGCT AAGATAGATT TTAAAACA-T
LK005           GTTAATGTAG CTTATATTAA AGTATGGCAC TGAAAATGCT AAGATAGATT TTAAAACA-T
PPf             GTTAGTGTAG CTTATATTAA AGCACGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
PPg             GTTAGTGTAG CTTATATTAA AGCACGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
SP11            GTTAGTGTAG CTTATATTAA AGCATGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
SP12            GTTAGTGTAG CTTATATTAA AGCATGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
PPh             GTTAGTGTAG CTTATATTAA AGCATGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
SPc             GTTAGTGTAG CTTATATTAA AGCATGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
ITNT3           GTTAGTGTAG CTTATATTAA AGCATGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
ITNT1           GTTAGTGTAG CTTATATTAA AGCATGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
ITNT2           GTTAGTGTAG CTTATATTAA AGCGTGGCAC TGAAAATGCC AAGATAGATT TTAAGACA-T
Clustal Consens **** ***** ******* ** **    ***** **** ****    ***** ****    *** * * *


                ....|....|
                         70
PH024           CTCATGAACA
PH019           CTCATGAACA
PK4             CTCATGAACA
PL009           CTCATGAACA
PH025           CTCATGAACA
PL007           CTCATGAACA
PK5             CTCATGAACA
PK6             CTCATGAACA
PL008           CTCATGAACA
LK003           CTCATGAACA
LK004           CTCATGAACA
LK005           CTCATGAACA
PPf             CTCATGAACA
PPg             CTCATGAACA
SP11            CTCATGAACA
SP12            CTCATGAACA
PPh             CTCATGAACA
SPc             CTCATGAACA
ITNT3           CTCATGAACA
ITNT1           CTCATGAACA
ITNT2           CTCATGAACA
Clustal Consens **********
```

Fig. B.2 A 498-500 character matrix of 21 *T. verrucosus* samples and 1 *P. hongkongensis* based on partial 16S rRNA sequences. Asterisks (*) represent conserved nucleotide residues across all samples.

```
                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         10         20         30         40         50         60
PL007            GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
PL008            GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
PH019            GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
PH024            GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
PH025            GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
PL009            GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
PK4              GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
PK5              GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
PK6              GGTCCCGCCT GCCCGGTGAC TA-TTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
SP12             GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
SPc              GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
SP11             GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
ITNT1            GGTCCCGCCT CCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
ITNT2            GGTCCCGCCT CCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
PPh              GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
ITNT3            GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
PPg              GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
PPf              GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TTATGACCGT GCAAAGGTAG
LK004            GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
LK005            GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
LK003            GGTCCCGCCT GCCCAGTGAC TAATTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
P. hongkongensis GGTCCCGCCT GCCCAGTGAT TAATTTCAAC GGCCGCGGTA TCATGACCGT GCAAAGGTAG
Clustal Consens  ********** *** ****   ** *******  ********** * ******** **********


                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         70         80         90        100        110        120
PL007            CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
PL008            CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
PH019            CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
PH024            CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
PH025            CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
PL009            CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
PK4              CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
PK5              CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
PK6              CGTAATCACT TGTCTTTTAA ATAAAGACCC GTATGAAAGG CAAAACGAAA GTTCAACTGT
SP12             CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
SPc              CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
SP11             CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
ITNT1            CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
ITNT2            CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
PPh              CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
ITNT3            CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
PPg              CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
PPf              CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
LK004            CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
LK005            CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
LK003            CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAAACGAAA GTTCAACTGT
P. hongkongensis CGTAATCACT TGTCTTTTAA ATAAAGACCT GTATGAAAGG CAAGACGAAA GCTCAACTGT
Clustal Consens  ********** ********** *********  ********** *** ****** * ********
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Fig. B.2   (continued)

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     130        140        150        160        170        180
PL007            CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PL008            CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PH019            CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PH024            CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PH025            CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PL009            CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PK4              CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PK5              CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PK6              CTCTTTAATC CAATCAATGA AACTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
SP12             CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AACATATAAG
SPc              CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AACATATAAG
SP11             CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AACATATAAG
ITNT1            CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AACATATAAG
ITNT2            CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AACATATAAG
PPh              CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AACATATAAG
ITNT3            CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AACATATAAG
PPg              CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
PPf              CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
LK004            CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
LK005            CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
LK003            CTCTTTAATC CAATCAATGA AATTTATCTT TTCGTGCAGA AGCGGAAATA AATATATAAG
P. hongkongensis CTCTTTAATC TAATCAGTGA AATTAATTTC TCCGTGCAGA AGCGAAGATA ATTATATAAG
Clustal Consens  ********** ***** *** ** * ** *  * ********* **** * *** *   *******


                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                     190        200        210        220        230        240
PL007            ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CCATAATAGT C-TTAACCAA
PL008            ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CCATAATAGT C-TTAACCAA
PH019            ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CCATAATAGT C-TTAACCAA
PH024            ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CCACAATAGT C-TTAACCAA
PH025            ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CCACAATAGT C-TTAACCAA
PL009            ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CTATAATAGT C-TTAACCAA
PK4              ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CTATAATAGT C-TTAACCAA
PK5              ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CTATAATAGT C-TTAACCAA
PK6              ACGAGAAGAC CCTATGGAGC TTTAAACACA CTATTAACTA CTATAATAGT C-TTAACCAA
SP12             ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATCAACTA CTACAATA-T T-TCAACCAA
SPc              ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATCAACTA CTACAATA-T T-TCAACCAA
SP11             ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATCAACTA CTACAATA-T T-TCAACCAA
ITNT1            ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATCAACTA CTACAATA-T T-TCAACCAA
ITNT2            ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATCAACTA CTATAATA-T T-TCAACCAA
PPh              ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATCAACTA CTATAATA-T T-TCAACCAA
ITNT3            ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATCAACTA CTATAATA-T T-TCAACCAA
PPg              ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATTAACTA CTATAATA-T T-TCAACCAA
PPf              ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATTAACTA CTATAATA-T T-TCAACCAA
LK004            ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATTAACTA TTATAATA-T T-CCAACCAA
LK005            ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATTAACTA TTATAATA-T T-CCAACCAA
LK003            ACGAGAAGAC CCTATGGAGC TTTAAATACA CTATTAACTA TTATAATA-T T-CCAACCAA
P. hongkongensis ACGAGAAGAC CCTGTGGAGC TTCAAATATA A-ATTAATTA TACATTTATT CACCATCCAA
Clustal Consens  ********** *** ****** ** *** * *  ** ** **      ** *    * ****
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Fig. B.2   (continued)

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                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       250        260        270        280        290        300
PL007              CAGGCAAAAA ATTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
PL008              CAGGCAAAAA ATTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
PH019              CAGGCAAAAA ATTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
PH024              CAGGCAAAAA ATTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
PH025              CAGGCAAAAA ATTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
PL009              CAGGCAAAAA -TTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
PK4                CAGGCAAAAA ATTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
PK5                CAGGCAAAAA ATTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
PK6                CAGGCAAAAA ATTACAAACT ACATAGCCAT AATATGAGTT TTGGGTTGGG GCGACCACGG
SP12               CAGGAAAAAA ATAA-AACCC ACATAGCCAT GATATAAGTT TTTGGTTGGG GCGACCGCGG
SPc                CAGGAAAAAA ATAA-AACCC ACATAGCCAT GATATAAGTT TTTGGTTGGG GCGACCGCGG
SP11               CAGGAAAAAA ATAA-AACCC ACATAGCCAT GATATAAGTT TTTGGTTGGG GCGACCGCGG
ITNT1              CAGGAAAAAA ATAA-AACCC ACATAGCCAT GATATAAGTT TTTGGTTGGG GCGACCGCGG
ITNT2              CAGGAAAAAA ATAA-AACCC ACATAGCCAT GATATAAGTT TTTGGTTGGG GCGACCGCGG
PPh                CAGGAAAAAA ATAA-AACCC ACATAGCCAT GATATAAGTT TTTGGTTGGG GCGACCGCGG
ITNT3              CAGGAAAAAA ATAA-AACCC ACATAGCCAT GATATAAGTT TTTGGTTGGG GCGACCGCGG
PPg                CAGGAAAAAA ATAA-AACCC ACATAGCTAT AATATAAGTT TTTGGTTGGG GCGACCGCGG
PPf                CAGGAAAAAA ATAA-AACCC ACATAGCTAT AATATAAGTT TTTGGTTGGG GCGACCGCGG
LK004              CAGGAAAAAA ATAA-AACC- ACATAGCTAT AATATAAGTT TTTGGTTGGG GCGACCACGG
LK005              CAGGAAAAAA ATAA-AACC- ACATAGCTAT AATATAAGTT TTTGGTTGGG GCGACCACGG
LK003              CAGGAAAAAA ATAA-AACC- ACATAGCTAT AATATAAGTT TTTGGTTGGG GCGACCACGG
P. hongkongensis   CAGGAGAAAA ATAAAGACGT ATAT---TAT AATTCAAATT TTCGGTTGGG GCGACCACGG
Clustal Consens    ****  ****  * *  *    * **    **  **   * ** ** ******* ****** ***

                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                       310        320        330        340        350        360
PL007              AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
PL008              AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
PH019              AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
PH024              AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
PH025              AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
PL009              AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
PK4                AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
PK5                AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
PK6                AGAAAAAAAA ATCCTCCGAG ATAAGTAATT TAGAGCAACA ATTCGAAAAT TAAAACATTT
SP12               AGAAAAGAA ACCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
SPc                AGAAAAGAA ACCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
SP11               AGAAAAGAA ACCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
ITNT1              AGAAAAGAA ACCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
ITNT2              AGAAAAGAA ACCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
PPh                AGAAAAGAA ACCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
ITNT3              AGAAAAGAA ACCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
PPg                AGAAAAGAA ATCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
PPf                AGAAAAGAA ATCCTCCGAG ATAAACAATC TAGAATAACA CTTCAAAAAT TAAAACATTT
LK004              AGAAAAGAA ATCCTCCGAG ATAAACAATT TAGAATAACA CTTCAAAAAT TAAAACATTT
LK005              AGAAAAGAA ATCCTCCGAG ATAAACAATT TAGAATAACA CTTCAAAAAT TAAAACATTT
LK003              AGAAAAGAA ATCCTCCGAG ATAAACAATT TAGAATAACA CTTCAAAAAT TAAAACATTT
P. hongkongensis   AAGAAAAAAT ATCCTCCGAG ATAAGCAATT TAGAGCTACA CTTCAAAAAT TAAAACATTT
Clustal Consens    *  **** *  * ******** ****  ***  ****   ***  *** ***** **********
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Fig. B.2   (continued)

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                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         370        380        390        400        410        420
PL007              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PL008              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PH019              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PH024              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PH025              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PL009              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PK4                ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PK5                ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PK6                ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
SP12               ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
SPc                ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
SP11               ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
ITNT1              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
ITNT2              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PPh                ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
ITNT3              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PPg                ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
PPf                ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
LK004              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
LK005              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
LK003              ATTATAAATG ATCCACTAAG TGACCAACGA ACCAAGTTAC CCTAGGGATA ACAGCGCAAT
P. hongkongensis   ATTATAAATG ATCCACCAAG TGACCAACGA ACCAAGTTAC CCCAGGGATA ACAGCGCAAT
Clustal Consens    ********** ****** *** ********** ********** ** ******* **********


                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         430        440        450        460        470        480
PL007              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PL008              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PH019              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PH024              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PH025              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PL009              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PK4                CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PK5                CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PK6                CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
SP12               CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
SPc                CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
SP11               CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
ITNT1              CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
ITNT2              CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PPh                CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
ITNT3              CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PPg                CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
PPf                CCTTTCTAAG AGTCCCTATC GACGAACGGG TTTACGACCT CGATGTTGGA TCAGGACACC
LK004              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
LK005              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
LK003              CCTTTCTAAG AGTCCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
P. hongkongensis   CCTTTCTAAG AGTTCATATC GACGAATGGG TTTACGACCT CGATGTTGGA TCAGGACACC
Clustal Consens    ********** *** * **** ****** *** ********** ********** **********
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Fig. B.2 (continued)

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                      ....|....| ....|....| ...
                              490        500
PL007           CAAATGGTGC AGCCGCTATA AA-
PL008           CAAATGGTGC AGCCGCTATA AA-
PH019           CAAATGGTGC AGCCGCTATA AA-
PH024           CAAATGGTGC AGCCGCTATA AA-
PH025           CAAATGGTGC AGCCGCTATA AA-
PL009           CAAATGGTGC AGCCGCTATA AA-
PK4             CAAATGGTGC AGCCGCTATA AA-
PK5             CAAATGGTGC AGCCGCTATA AA-
PK6             CAAATGGTGC AGCCGCTATA AA-
SP12            CAAATGGTGC AGCCGCTATA AA-
SPc             CAAATGGTGC AGCCGCTATA AA-
SP11            CAAATGGTGC AGCCGCTATA AA-
ITNT1           CAAATGGTGC AGCCGCTATA AA-
ITNT2           CAAATGGTGC AGCCGCTATA AA-
PPh             CAAATGGTGC AGCCGCTATA AA-
ITNT3           CAAATGGTGC AGCCGCTATA AA-
PPg             CAAATGGTGC AGCCGCTATA AA-
PPf             CAAATGGTGC AGCCGCTATA AA-
LK004           CAAATGGTGC AGCCGCTATA AA-
LK005           CAAATGGTGC AGCCGCTATA AA-
LK003           CAAATGGTGC AGCCGCTATA AA-
P. hongkongensis CAAATGGTGC AGCCGCTATT AAA
Clustal Consens ********** *********  **
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Fig. B.3 A 722-726 character matrix of 21 *T. verrucosus* samples and 1 *P. hongkongensis* based on control region sequences. Asterisks (*) represent conserved nucleotide residues across all samples.

```
                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          10         20         30         40         50         60
PL007             CCTAAGATCA GAGTAGCGCG AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
PL008             CCTAAGATCA GAGTAGCGCG AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
PK4               CCTAAGATCA GAGTAGCGCT AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
PK5               CCTAAGATCA GAGTAGCGCT AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
PK6               CCTAAGATCA GAGTAGCGCT AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
PL009             CCTAAGATCA GAGTAGCGCG AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
PH019             CCTAAGATCA GAGTAGCGCG AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
PH024             CCTAAGATCA GAGTAGCGCG AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
PH025             CCTAAGATCA GAGTAGCGCG AAGGACATAT TATGTTTA-T AGTACATTAA TTGACTTGCC
SP11              CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGCATA-T AGTACATTAA TTGACTTGCC
SP12              CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGCATA-T AGTACATTAA TTGACTTGCC
SPc               CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGCATA-T AGTACATTAA TTGACTTGCC
ITNT1             CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGCATA-T AGTACATTAA TTGACTTGCC
ITNT3             CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGCATA-T AGTACATTAA TTGACTTGCC
ITNT2             CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGCATA-T AGTACATTAA TTGACTTGCC
PPh               CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGCATA-T AGTACATTAA TTGACTTGCC
PPg               CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGTATA-T AGTACATTAA TTGACTTGCC
PPf               CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGTATA-T AGTACATTAA TTGACTTGCC
LK004             CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGTATA-T AGTACATTAA TTGACTTGCC
LK005             CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGTATA-T AGTACATTAA TTGACTTGCC
LK003             CCTAAGATCA GAGTAGCGCG GAGGACATAT TATGTATA-T AGTACATTAA TTGACTTGCC
P. hongkongensis  CCCGTAGTTT TTGTAATG-A GAGTGCATAC TATGCTTAAT AGTGCATTCA TCTACTTGCC
Clustal Consens   **       *     *** *      ** **** **** ** * *** **** * *  *******


                  ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                          70         80         90        100        110        120
PL007             ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
PL008             ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
PK4               ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
PK5               ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
PK6               ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
PL009             ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
PH019             ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
PH024             ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
PH025             ACATGGCTAG TGTTTTAGTA TTATTATGAT CCATAATCGA ATTTTCAGGC GAGAAACCAT
SP11              ATACGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
SP12              ATACGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
SPc               ATACGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
ITNT1             ATACGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
ITNT3             ATACGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
ITNT2             ATACGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
PPh               ATACGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
PPg               ATATGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
PPf               ATATGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
LK004             ATATGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
LK005             ATATGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
LK003             ATATGGCTAG TGTTTTAGTA TTATTAGGAT CCATAATCTA ATTTTCAGGC GAGAAACCAC
P. hongkongensis  AAACGTCTT- TGTTTTGGTA TTATTAGGAT TGTTGACCTT ACCCTCAGGC GAGAAATCAC
Clustal Consens   * * * **    ****** *** ****** ***    * * *    *   ****** ****** **
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Fig. B.3   (continued)

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                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           130        140        150        160        170        180
PL007              CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CTTCAATTGT AGAGTGTTTT
PL008              CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CTTCAATTGT AGAGTGTTTT
PK4                CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
PK5                CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
PK6                CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
PL009              CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CTTCAATTGT AGAGTGTTTT
PH019              CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CTTCAATTGT AGAGTGTTTT
PH024              CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CTTCAATTGT AGAGTGTTTT
PH025              CAACCCGCCC ACCATGACCC TCGTTCCAAA ATTTCCAGGA CTTCAATTGT AGAGTGTTTT
SP11               CAACCCGCCC ACAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
SP12               CAACCCGCCC ACAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
SPc                CAACCCGCCC ACAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
ITNT1              CAACCCGCCC ACAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
ITNT3              CAACCCGCCC ACAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
ITNT2              CAACCCGCCC ACAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
PPh                CAACCCGCCC ACAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
PPg                CAACCCGCCC ACAACGACCC TCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
PPf                CAACCCGCCC ACAACGACCC TCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
LK004              CAACCCGCCC CTAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
LK005              CAACCCGCCC CTAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
LK003              CAACCCGCCC CTAACGACCC CCGTTCCAAA ATTTCCAGGA CCTCAATTGT AGAGTGTTTT
P. hongkongensis   CAACCCGCCC CCCACGCTAC TCGTTTAGAA ACTTCAAGGA CYTCAATTGT AGAGTGTCTT
Clustal Consens    ********** * * *   ****  ** * *** **** * ******** ******* **


                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                           190        200        210        220        230        240
PL007              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
PL008              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
PK4                ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
PK5                ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
PK6                ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
PL009              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
PH019              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
PH024              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
PH025              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGAACA TACCTAGTAG AGTTTCTATC
SP11               ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
SP12               ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
SPc                ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
ITNT1              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
ITNT3              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
ITNT2              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
PPh                ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
PPg                ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
PPf                ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
LK004              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
LK005              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
LK003              ACGTTTATTT TCA---ACAG CTGGTTTGAA TCTATGGACA AACCTAGTAG AGTTTCTATC
P. hongkongensis   TTTACTATTT ATACAGGCAG TTGGTTTGAA TCTATGAACA TTGATAGTAG AGTTTCTATC
Clustal Consens       *****   *   ***  ******** ****** ***    ****** **********
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Fig. B.3 (continued)

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                     ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         250        260        270        280        290        300
PL007            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTATCC TTGACCCCGC
PL008            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTATCC TTGACCCCGC
PK4              ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTATCC TTGACCCCGC
PK5              ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTATCC TTGACCCCGC
PK6              ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTATCC TTGACCCCGC
PL009            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTATCC TTGACCCCGC
PH019            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
PH024            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
PH025            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
SP11             ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
SP12             ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
SPc              ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
ITNT1            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
ITNT3            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
ITNT2            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
PPh              ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
PPg              ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
PPf              ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
LK004            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
LK005            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
LK003            ATTTATCTTT AAGAGGCCTC TGGTAAAATG CTTTCAATAT CGTCGTACCC TTGACCCCGC
P. hongkongensis ATTTCTTTTT AAGAGGCCTC TGGTAAAATG CTTACTGTAC TAGATGGCCC ATGATCA-GC
Clustal Consens  **** * *** ********** ********** *** * **        **  *** *  **


                     ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         310        320        330        340        350        360
PL007            ATAACTGTCT TAGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PL008            ATAACTGTCT TAGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PK4              ATAACTGTTT TGGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PK5              ATAACTGTTT TGGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PK6              ATAACTGTTT TGGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PL009            ATAACTGTTT TGGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PH019            ATAACTGTTT TGGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PH024            ATAACTGTTT TGGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PH025            ATAACTGTTT TGGACTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
SP11             ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
SP12             ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
SPc              ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
ITNT1            ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
ITNT3            ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
ITNT2            ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PPh              ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PPg              ATAACTGTTT TGGATTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
PPf              ATAACTGTTT TGGATTGCAT TTAGTATTTT TTTCTCTCTG TGAAGTCAAT GCCCCATACA
LK004            ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAGGTCAAT GCCCCATACA
LK005            ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAGGTCAAT GCCCCATACA
LK003            ATAACTGTTT TGGATTGCAT TTGGTATTTT TTTCTCTCTG TGAGGTCAAT GCCCCATACA
P. hongkongensis AGAACTGATC TGGCCTGCAT TCA-TTTTTT TTTTTCTCTG TGAAGTCAAT CCCCCATAAA
Clustal Consens  * *****    * * ***** *   * **** *** ****** *** ******  ******* *
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Fig. B.3   (continued)

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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         370        380        390        400        410        420
PL007            GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAACTTAACA
PL008            GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAACTTAACA
PK4              GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAATTTAACA
PK5              GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAATTTAACA
PK6              GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAATTTAACA
PL009            GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAATTTAACA
PH019            GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAATTTAACA
PH024            GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAATTTAACA
PH025            GTCTTGAGCC GGGGTACGTA A-TCTAAGCC TGAGCATATA GTGAAAATGT AAATTTAACA
SP11             GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
SP12             GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
SPc              GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
ITNT1            GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
ITNT3            GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
ITNT2            GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
PPh              GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
PPg              GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
PPf              GTCTTGAGCC GGGGTATCCG G-CCTAAGCC TGCGCATGGT CTGCAAATGT ATACTTAACA
LK004            GTCTTGAGCC GGCACACCTG GGCCTAAATC TGAGCATG-C CTGCAAATGT ATATTTAACA
LK005            GTCTTGAGCC GGCACACCTG GGCCTAAATC TGAGCATG-C CTGCAAATGT ATATTTAACA
LK003            GTCTTGAGCC GGCACACCTG GGCCTAAATC TGAGCATG-C CTGCAAATGT ATATTTAACA
P. hongkongensis GTCTTGAATC GG--CACTAT ACTAGAGACC TGAACATGGA TTGCAGATGT AGGGTTACAA
Clustal Consens  *******  * **    *          *   *  ** ***      ** * **** *   *** *
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                 ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         430        440        450        460        470        480
PL007            GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
PL008            GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
PK4              GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
PK5              GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
PK6              GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
PL009            GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
PH019            GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
PH024            GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
PH025            GATAAGGAAT GGGTACAATA ATATTCATGT TGTTCGGACA TACTAATTTT TTCCCTCTAA
SP11             GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
SP12             GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
SPc              GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
ITNT1            GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
ITNT3            GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
ITNT2            GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
PPh              GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
PPg              GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
PPf              GATATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
LK004            GGTATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
LK005            GGTATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
LK003            GGTATTGAGT GGGGCGCATA ATATTCATGT TGTTCGGACA TAATAATTTT TTCCCTCTAA
P. hongkongensis AGTATAAAAT CGCGGATAAA ATATTCATGT TATAGGGACA TAGCATTTAT TTCCCCCTAA
Clustal Consens    **   * *  *      * * ********** * *  ***** **  * ** * ***** ****
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Fig. B.3   (continued)

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                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         490        500        510        520        530        540
PL007            AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PL008            AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PK4              AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PK5              AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PK6              AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PL009            AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PH019            AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PH024            AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PH025            AGCAAGGGAT ATATT-CTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
SP11             AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
SP12             AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
SPc              AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
ITNT1            AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
ITNT3            AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
ITNT2            AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PPh              AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PPg              AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
PPf              AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGTTTTTCTA AGAATACTTA
LK004            AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGCTTTTCTA AGAATACTTA
LK005            AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGCTTTTCTA AGAATACTTA
LK003            AGCAAGAGAC ATATT-CTTT TCCTATTTCC CCCCGGAGCT AGCTTTTCTA AGAATACTTA
P. hongkongensis AACAAGGGAT ATATTATTTT TCCTGTTTCC CCCCGGAGCT AGTTTTTCTA ATAATATAGA
Clustal Consens  * **** **  *****  *** **** ***** ********** ** ******* * ****     *


                    ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         550        560        570        580        590        600
PL007            TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
PL008            TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
PK4              TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
PK5              TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
PK6              TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
PL009            TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
PH019            TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
PH024            TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
PH025            TTTTGAATAT CATCTAATTC TATTTTTTGA TAAACCCCCC CACCCCCAAA TGTTAGTCTT
SP11             TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
SP12             TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
SPc              TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
ITNT1            TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
ITNT3            TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
ITNT2            TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
PPh              TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
PPg              TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
PPf              TTTTGAATAT CATCTAATTC TATTTTTTGG TTAACCCCCC CACCCCCAAA TGTTAGTCTC
LK004            TTTTGAATAT CATCTAATTC TATTTTTTGA TTAACCCCCC CACCCCCAAA TGTTAGTCTC
LK005            TTTTGAATAT CATCTAATTC TATTTTTTGA TTAACCCCCC CACCCCCAAA TGTTAGTCTC
LK003            TTTTGAATAT CATCTAATTC TATTTTTTGA TTAACCCCCC CACCCCCAAA TGTTAGTCTC
P. hongkongensis TTTTGAATAT TATCTAATTT TATTTTTGGA T-AACCCCCC TACCCCCAAA TATCCGTCTA
Clustal Consens  ********** ******* * ******* *  * ******** ********  * *   ****
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Fig. B.3   (continued)

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                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         610        620        630        640        650        660
PL007              ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
PL008              ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
PK4                ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
PK5                ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
PK6                ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
PL009              ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
PH019              ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
PH024              ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
PH025              ATCAGTATGT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCTTCA TACTTACGAC
SP11               ATCGGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
SP12               ATCAGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
SPc                ATCAGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
ITNT1              ATCAGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
ITNT3              ATCAGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
ITNT2              ATCAGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
PPh                ATCAGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
PPg                ATCAGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
PPf                ATCAGTATCT ACAACTCTTT AGTCAACCCC CGAAACTAAA AAGACCTTTA TACTTACGAC
LK004              ATCAGTATCT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCATTA TACTTACGAC
LK005              ATCAGTATCT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCATTA TACTTACGAC
LK003              ATCAGTATCT ACAACTTTTT AGTCAACCCC CGAAACTAAA AAGACCATTA TACTTACGAC
P. hongkongensis   ATCAGTATGA GAAACTTTTT TAGCCAACCC CAAGACTAAA AAGACCTACA TACCTACGAC
Clustal Consens    *** ****    **** ***     * * *** * * ****** ******   * *** ******


                   ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
                         670        680        690        700        710        720
PL007              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAT TTTTTTGATT GTTAT-TACA
PL008              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAT TTTTTTGATT GTTAT-TACA
PK4                ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAA TTTTTTGATT GTTAT-TACA
PK5                ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAA TTTTTTGATT GTTAT-TACA
PK6                ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAA TTTTTTGATT GTTAT-TACA
PL009              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAA TTTTTTGATT GTTAT-TACA
PH019              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAA TTTTTTGATT GTTAT-TACA
PH024              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAA TTTTTTGATT GTTAT-TACA
PH025              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTT-GAAAA TTTTTTGATT GTTAT-TACA
SP11               ATTAGACTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
SP12               ATTAGGCTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
SPc                ATTAGGCTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
ITNT1              ATTAGGCTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
ITNT3              ATTAGGCTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
ITNT2              ATTAGGCTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
PPh                ATTAGGCTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
PPg                ATTAGGCTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
PPf                ATTAGGCTTA TGCT-AGAAA AATACCACTT TTTTTAAAAG TTTTTTGATT GTTAT-TACA
LK004              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTA-AAAAG TTTTTTGATT GTTAT-TACA
LK005              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTA-AAAAG TTTTTTGATT GTTAT-TACA
LK003              ATTAGGCTTA TGCT-AGAAA AATACTACTT TTTA-AAAAG TTTTTTGATT GTTAT-TACA
P. hongkongensis   AATAGGTTAG TACTGAGCAA AATAAGATTT TTTT-----G TTAATAAATT TTTATATACA
Clustal Consens    * ***   *   * ** ** ** ****  * ** ***      ** * *** **** ****
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Fig. B.3   (continued)

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                     . . . . | . . . . | . . . .
                                730
PL007            GTGTTACACT GTAA
PL008            GTGTTACACT GTAA
PK4              GTGTTACACT GTAA
PK5              GTGTTACACT GTAA
PK6              GTGTTACACT GTAA
PL009            GTGTTACACT GTAA
PH019            GTGTTACACT GTAA
PH024            GTGTTACACT GTAA
PH025            GTGTTACACT GTAA
SP11             GTGTTACACT GTAA
SP12             GTGTTACACT GTAA
SPc              GTGTTACACT GTAA
ITNT1            GTGTTACACT GTAA
ITNT3            GTGTTACACT GTAA
ITNT2            GTGTTACACT GTAA
PPh              GTGTTACACT GTAA
PPg              GTGTTACACT GTAA
PPf              GTGTTACACT GTAA
LK004            GTGTTACACT GTAA
LK005            GTGTTACACT GTAA
LK003            GTGTTACACT GTAA
P. hongkongensis GTATTACACT GTAA
Clustal Consens  ** ******* ****
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Appendix C

Figs. C.1-C.6 The dorsal view of skull of *T. verrucosus* Type I.

4.5 mm

Fig. C.1 ITNT1	**Fig. C.2 ITNT2**

Fig. C.3 LK3/5/50	**Fig. C.4 LK**
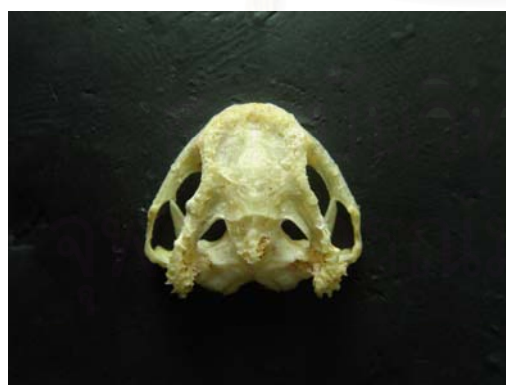
Fig. C.5 PP2638	**Fig. C.6 PP2643**

Figs. C.7-C.12   The dorsal view of skull of *T. verrucosus* Type II.

Fig. C.7 PK5

Fig. C.8 PK6

Fig. C.9 PLG

Fig. C.10 PL2627

Fig. C.11 PHF

Fig. C.12 PHG

Figs. C.13-C.18   The ventral view of skull of *T. verrucosus* Type I.

4.5 mm

Fig. C.13 ITNT1

Fig. C.14 ITNT2

Fig. C.15 LK3/5/50

Fig. C.16 LK

Fig. C.17 PP2638

Fig. C.18 PP2643

Figs. C.19-C.24   The ventral view of skull of *T. verrucosus* Type II.

Fig. C.19 PK5

Fig. C.20 PK6

Fig. C.21 PLG

Fig. C.22 PL2627

Fig. C.23 PHF

Fig. C.24 PHG

Figs. C.25-C.30   The lateral view of skull of *T. verrucosus* Type I.

4.5 mm

Fig. C.25 ITNT1

Fig. C.26 ITNT2

Fig. C.27 LK3/5/50

Fig. C.28 LK

Fig. C.29 PP2638

Fig. C.30 PP2643

Figs. C.31-C.36   The lateral view of skull of *T. verrucosus* Type II.

4.5 mm

Fig. C.31 PK5

Fig. C.32 PK6

Fig. C.33 PLG

Fig. C.34 PL2627

Fig. C.35 PHF

Fig. C.36 PHG

Figs. C.37-C.41   The certain part of hyobranchial skeleton of *T. verrucosus* Type I.

Fig. C.37 ITNT1

Fig. C.38 ITNT2

Fig. C.39 LK3/5/50

Fig. C.40 LK

Fig. C.41 PP2643

Figs. C.42-C.47   The certain part of hyobranchial skeleton of *T. verrucosus* Type II.

Fig. C.42 PK5

Fig. C.43 PK6

Fig. C.44 PLG

Fig. C.45 PL2627

Fig. C.46 PHF

Fig. C.47 PHG

Fig. C.48 Cervical vertebrae of *T. verrucosus*: a) dorsal, b) ventral, c) anterior, d) posterior, e) lateral view of ITNT1, ITNT2, LK3/5/50, LK, PP2638, PP2643, PK5, PK6, PLG, PL2627, PHF and PHG (from left to right side).
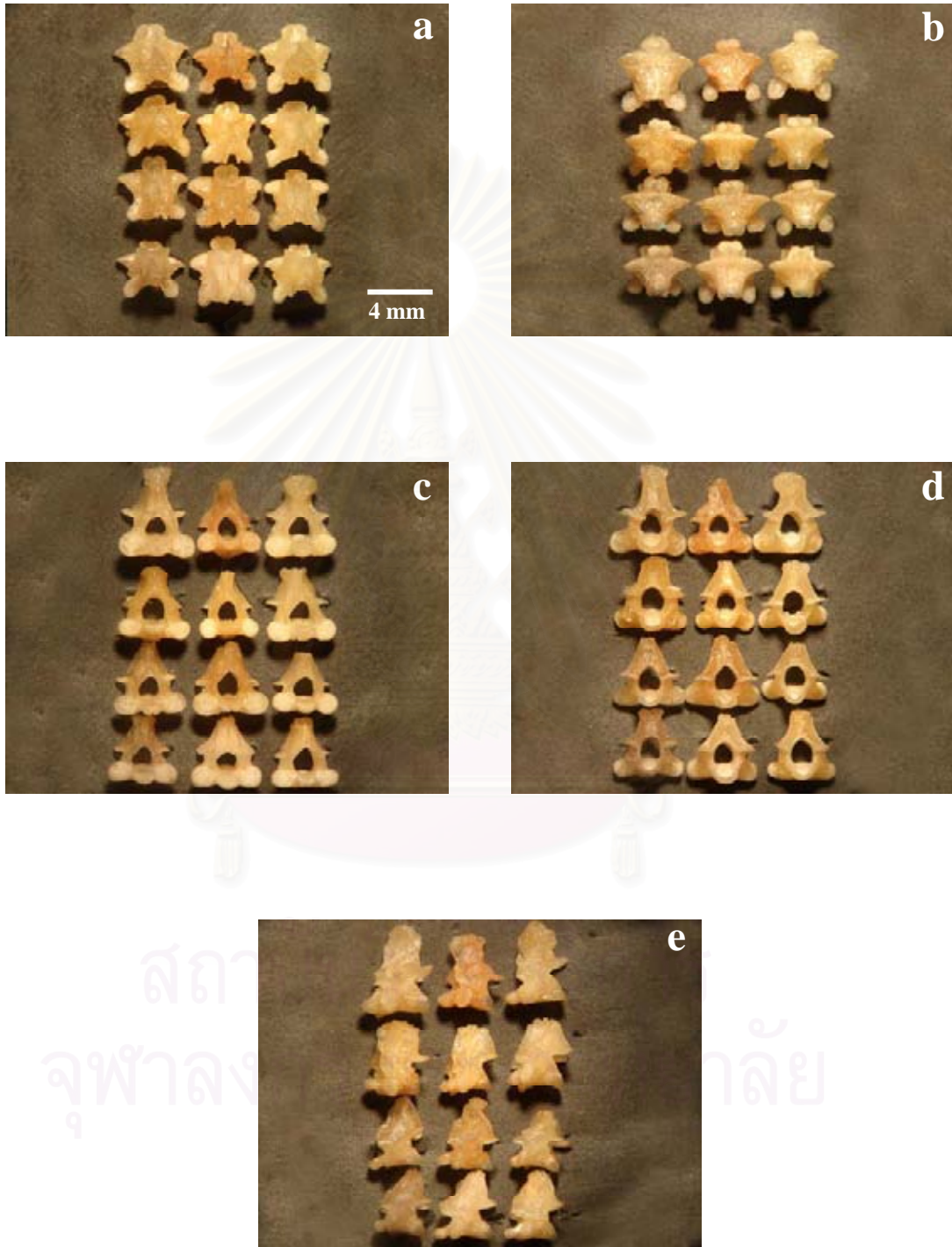
Fig. C.49   Primary trunk vertebrae of *T. verrucosus*: a) dorsal, b) ventral, c) anterior, d) posterior, e) lateral view of ITNT1, ITNT2, LK3/5/50, LK, PP2638, PP2643, PK5, PK6, PLG, PL2627, PHF and PHG (from left to right side).
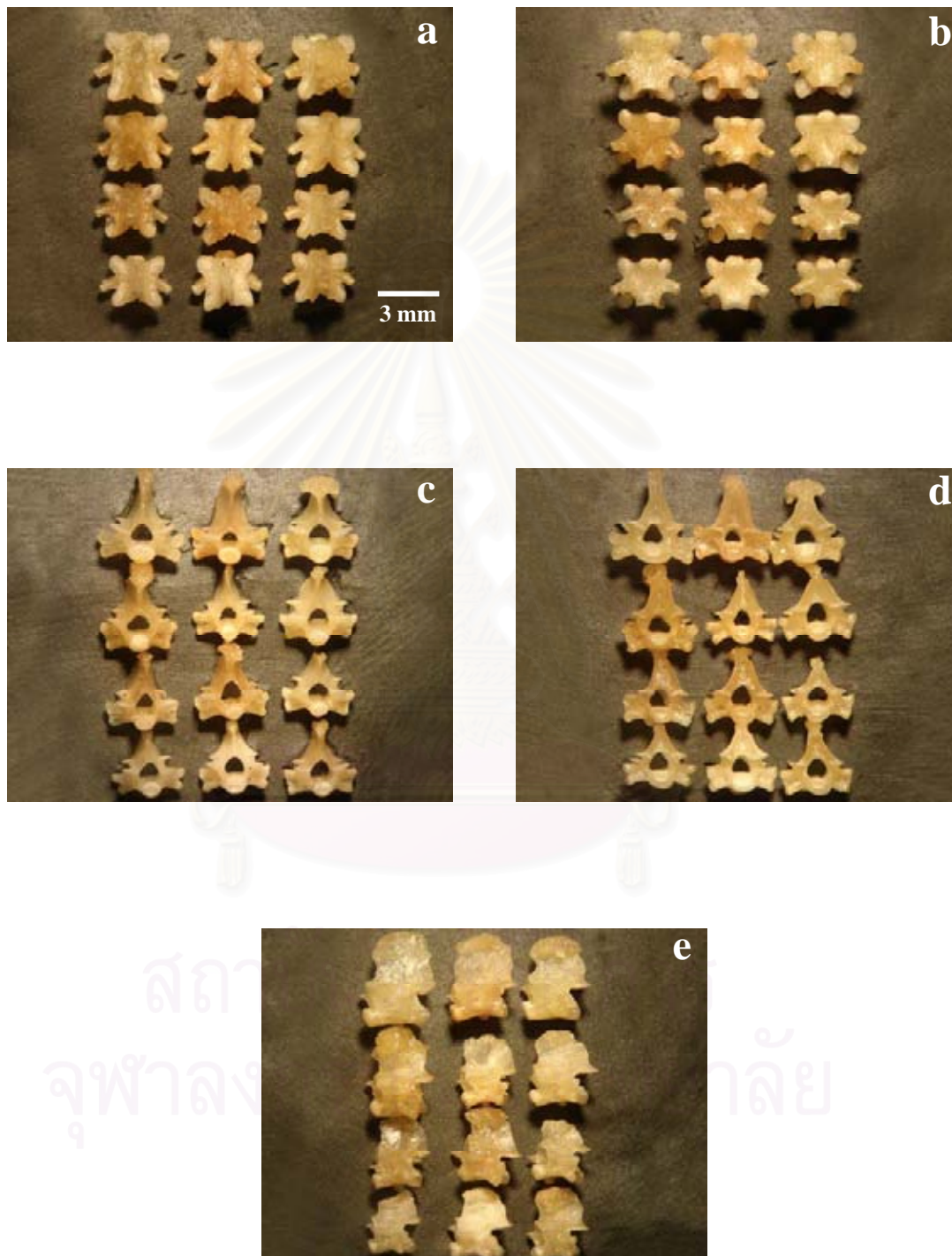
Fig. C.50 Sacral vertebrae of *T. verrucosus*: a) dorsal, b) ventral, c) anterior, d) posterior, e) lateral view of ITNT1, ITNT2, LK3/5/50, LK, PP2638, PP2643, PK5, PK6, PLG, PL2627, PHF and PHG (from left to right side).
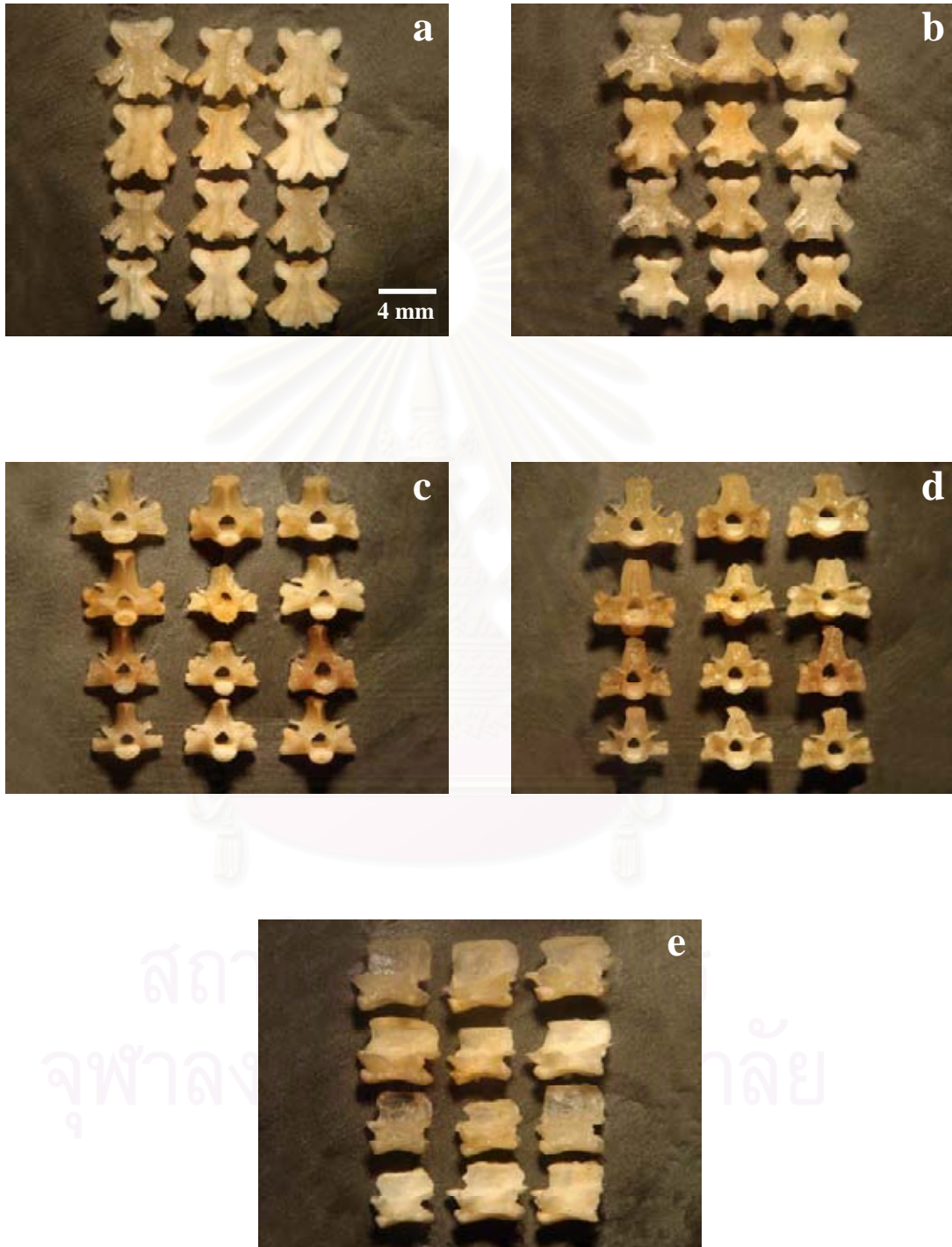
Fig. C.51   1st, 4th and 12th trunk vertebrae of *T. verrucosus*: a) dorsal, b) ventral, c) lateral view of ITNT2; d) dorsal, e) ventral and f) lateral view of PK6.
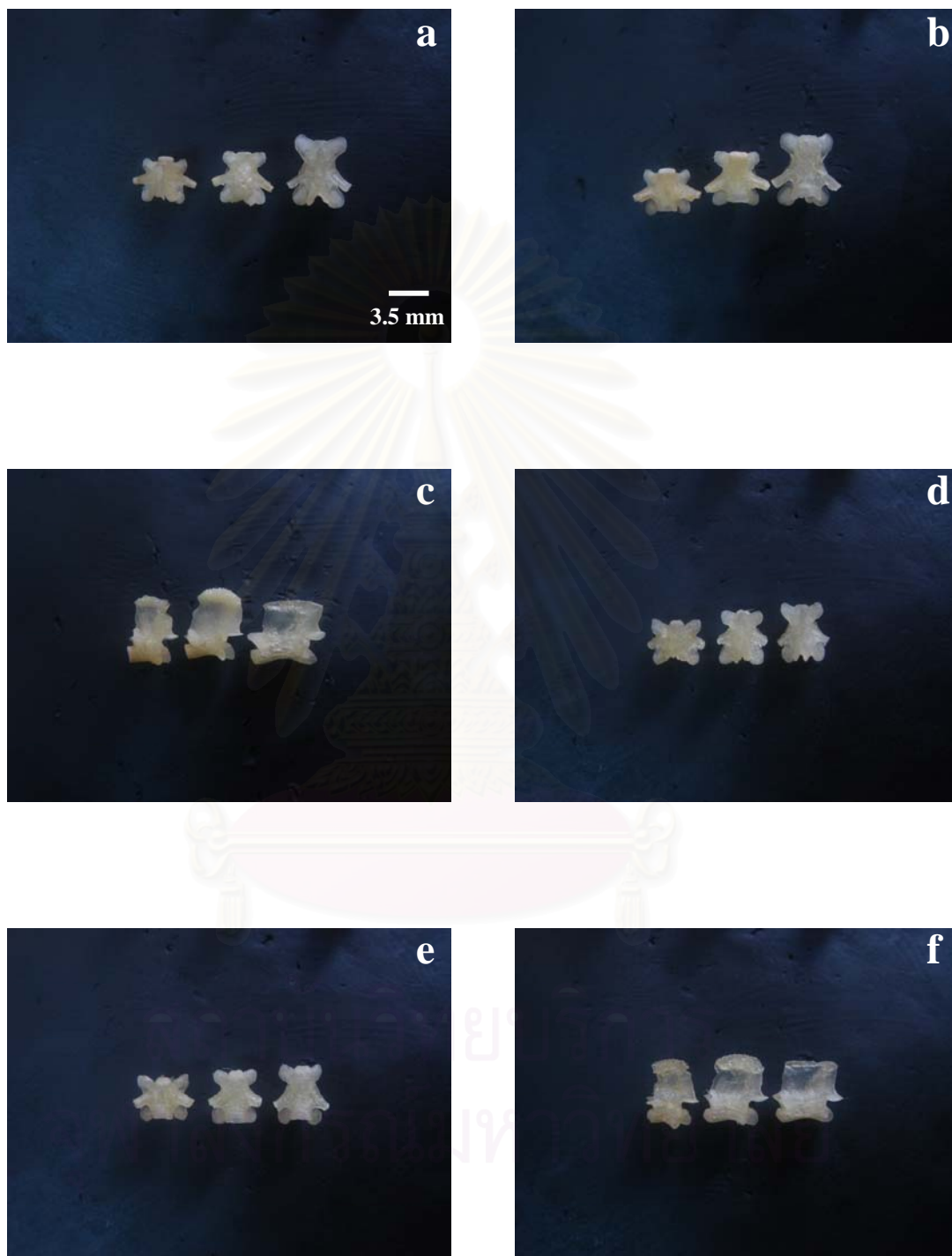
Fig. C.52   Dorsal view of the last trunk vertebrae of *T. verrucosus* of ITNT1,
ITNT2, LK3/5/50, LK, PP2638, PP2643, PK5, PK6, PLG, PL2627,
PHF and PHG (from left to right side).

Figs. C.53-C.58   The certain ribs of *T. verrucosus* Type I. The number in each
figure refers to the sequence of vertebral ribs.
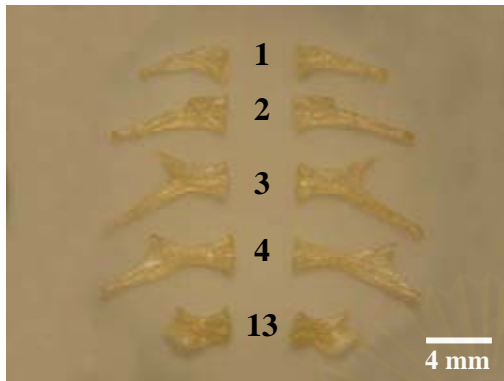
Fig. C.53 ITNT1
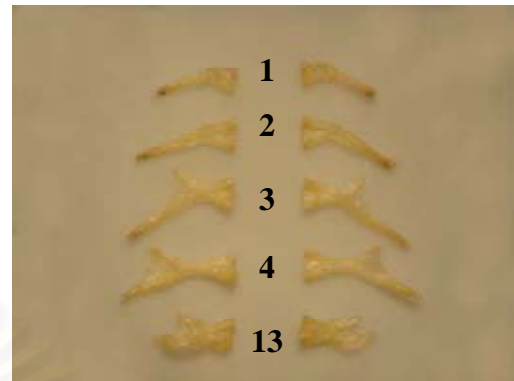
Fig. C.54 ITNT2

Fig. C.55 LK3/5/50

Fig. C.56 LK
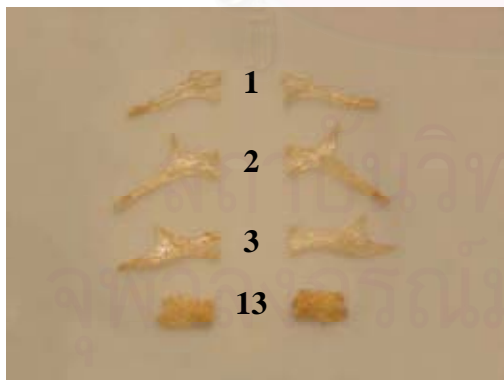
Fig. C.57 PP2638
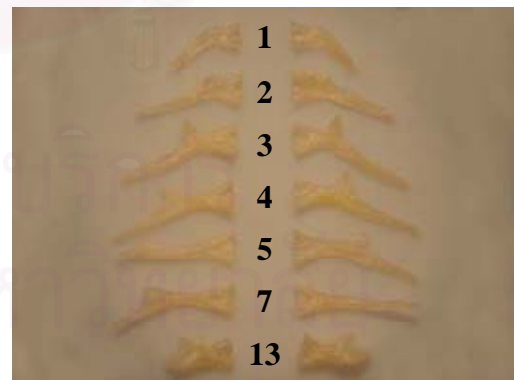
Fig. C.58 PP2643

Figs. C.59-C.64   The certain ribs of *T. verrucosus* Type II. The number in each figure refers to sequence of vertebral ribs.
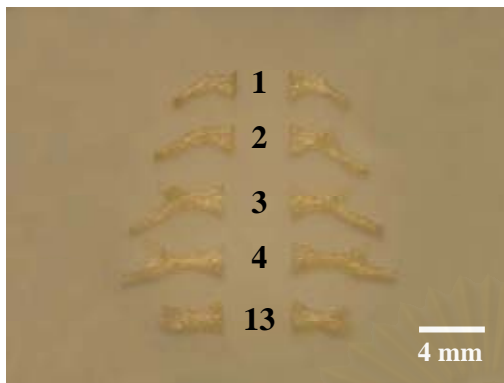
Fig. C.59 PK5

Fig. C.60 PK6
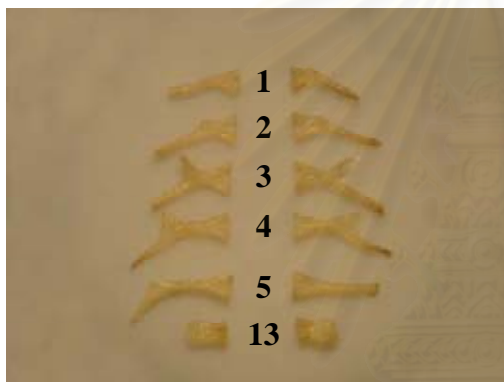
Fig. C.61 PLG

Fig. C.62 PL2627

Fig. C.63 PHF
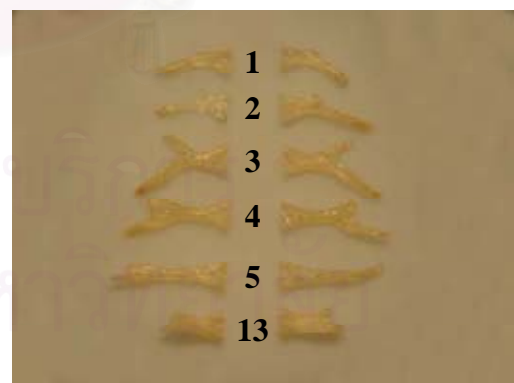
Fig. C.64 PHG

Figs. C.65-C.69 The ventral view of the pectoral girdle of *T. verrucosus* Type I.
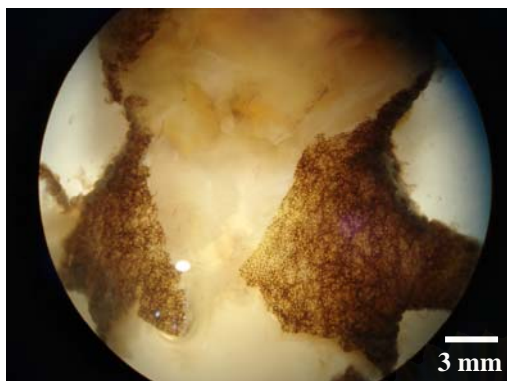
Fig. C.65 ITNT1
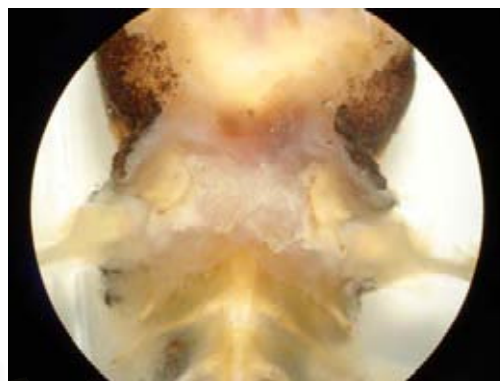
Fig. C.66 ITNT2
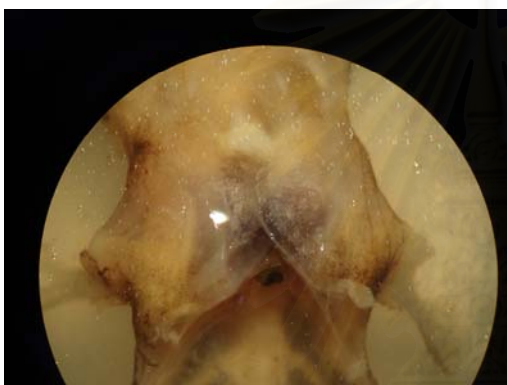
Fig. C.67 LK3/5/50
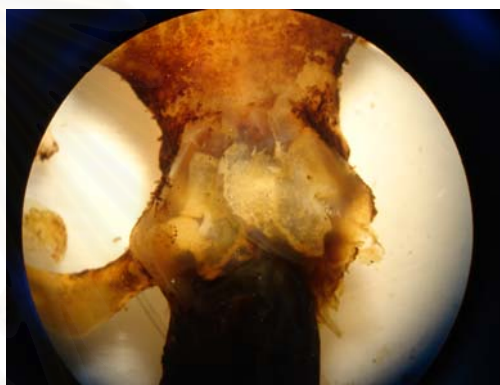
Fig. C.68 LK
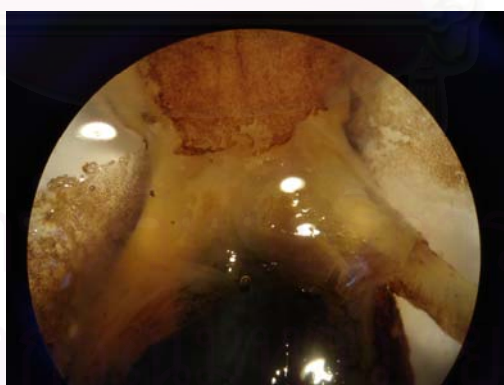
Fig. C.69 PP2643

Figs. C.70-C.75 The ventral view of the pectoral girdle of *T. verrucosus* Type II.

Fig. C.70 PK5
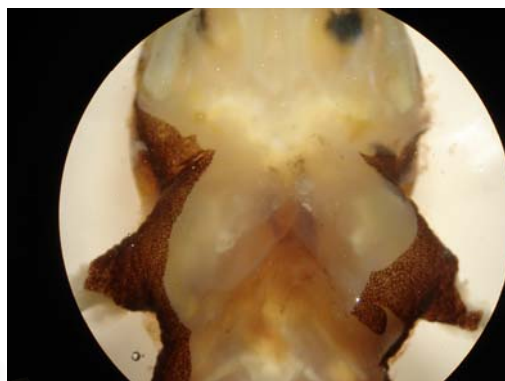
Fig. C.71 PK6

Fig. C.72 PLG
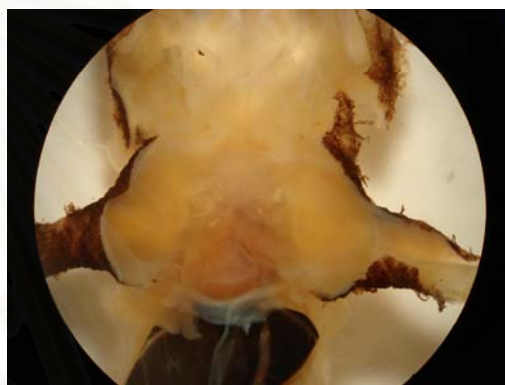
Fig. C.73 PL2627

Fig. C.74 PHF

Fig. C.75 PHG

Figs. C.76-C.81   The left humerus of *T. verrucosus* Type I.

2.5 mm

Fig. C.76 ITNT1

Fig. C.77 ITNT2
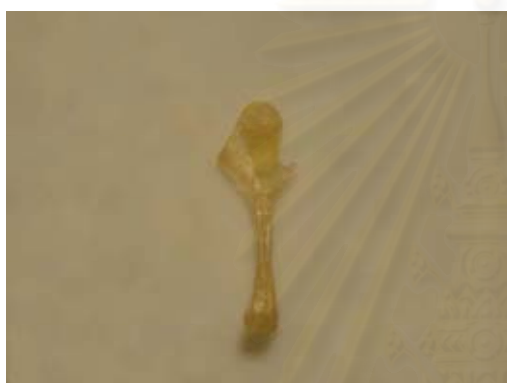
Fig. C.78 LK3/5/50
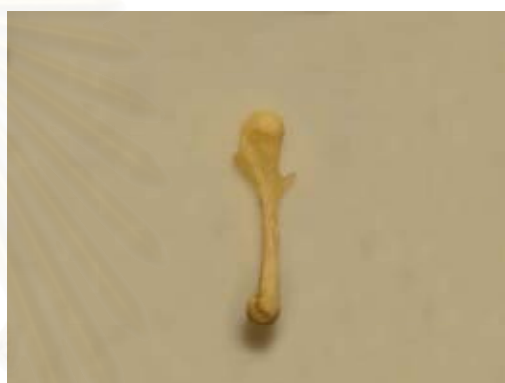
Fig. C.79 LK

Fig. C.80 PP2638
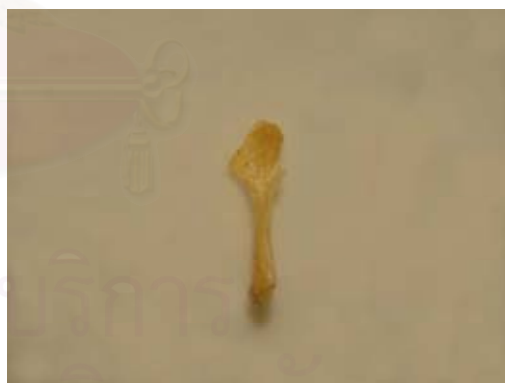
Fig. C.81 PP2643

Figs. C.82-C.87 The left humerus of *T. verrucosus* Type II.

Fig. C.82 PK5

Fig. C.83 PK6

Fig. C.84 PLG

Fig. C.85 PL2627

Fig. C.86 PHF
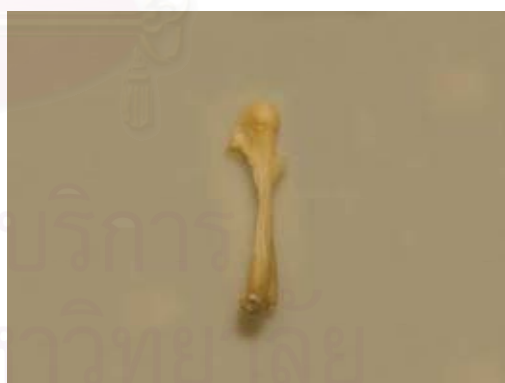
Fig. C.87 PHG

Figs. C.88-C.93   The left radius of *T. verrucosus* Type I.

Fig. C.88 ITNT1

Fig. C.89 ITNT2

Fig. C.90 LK3/5/50

Fig. C.91 LK

Fig. C.92 PP2638

Fig. C.93 PP2643

2.5 mm
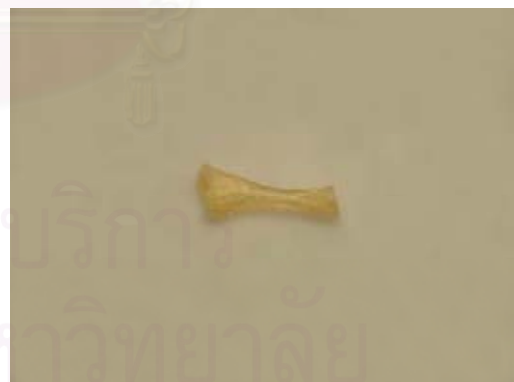
Figs. C.94-C.99   The left radius of *T. verrucosus* Type II.

2.5 mm
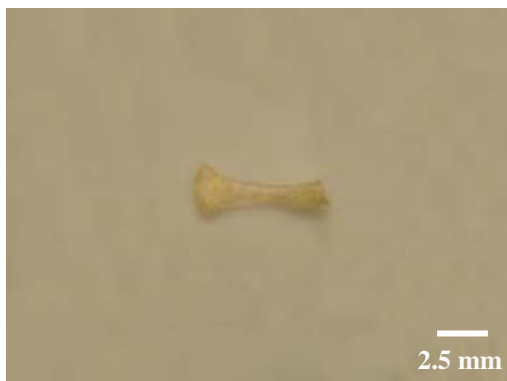
Fig. C.94 PK5   **Fig. C.95 PK6**

Fig. C.96 PLG   **Fig. C.97 PL2627**
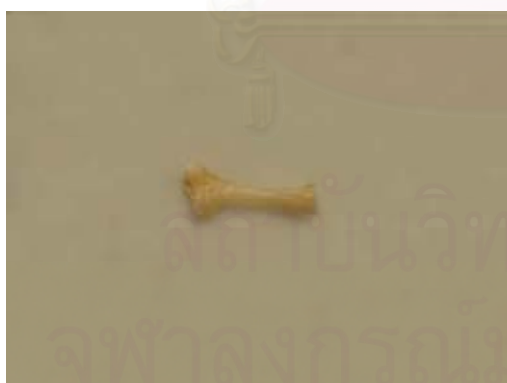
Fig. C.98 PHF   **Fig. C.99 PHG**

Figs. C.100-C.105   The left ulna of *T. verrucosus* Type I.

2.5 mm

Fig. C.100 ITNT1

Fig. C.101 ITNT2

Fig. C.102 LK3/5/50

Fig. C.103 LK
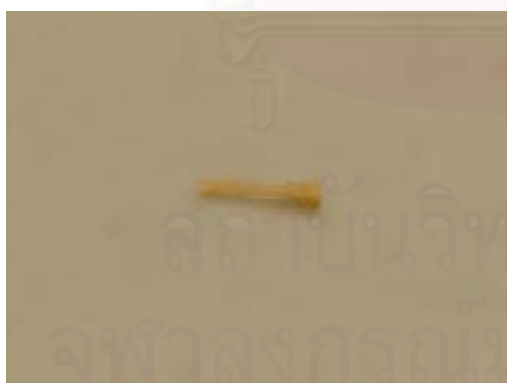
Fig. C.104 PP2638
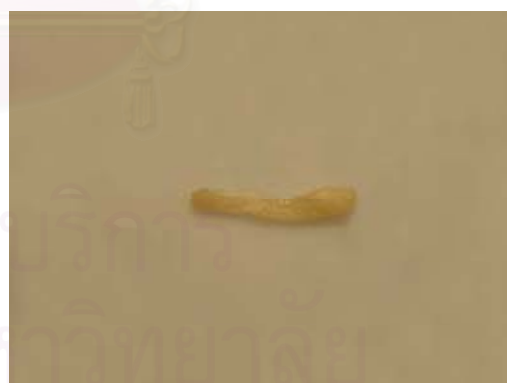
Fig. C.105 PP2643

Figs. C.106-C.111   The left ulna of *T. verrucosus* Type II.

2.5 mm

Fig. C.106 PK5

Fig. C.107 PK6

Fig. C.108 PLG

Fig. C.109 PL2627
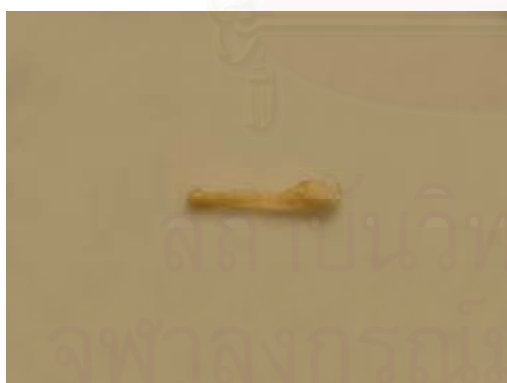
Fig. C.110 PHF
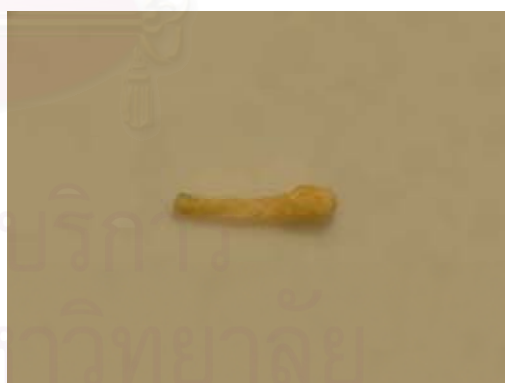
Fig. C.111 PHG

Figs. C.112-C.116   The left carpal skeleton of *T. verrucosus* Type I.

Fig. C.112 ITNT1

Fig. C.113 ITNT2

Fig. C.114 LK3/5/50
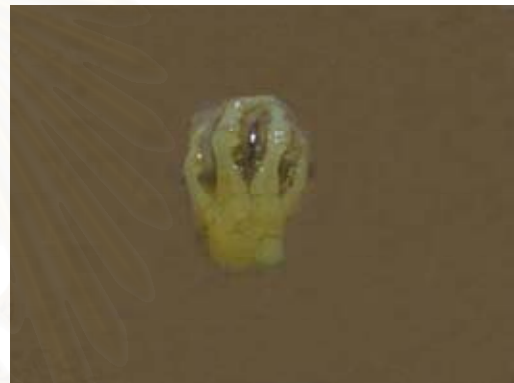
Fig. C.115 LK

Fig. C.116 PP2643

Figs. C.117-C.121   The left carpal skeleton of *T. verrucosus* Type II.

Fig. C.117 PK5

Fig. C.118 PK6

Fig. C.119 PLG

Fig. C.120 PL2627

Fig. C.121 PHF

Figs. C.122-C.127   The pelvic girdle of *T. verrucosus* Type I.

Fig. C.122 ITNT1

Fig. C.123 ITNT2

Fig. C.124 LK3/5/50

Fig. C.125 LK

Fig. C.126 PP2638

Fig. C.127 PP2643

Figs. C.128-C.133 The pelvic girdle of *T. verrucosus* Type II.

Fig. C.128 PK5

Fig. C.129 PK6
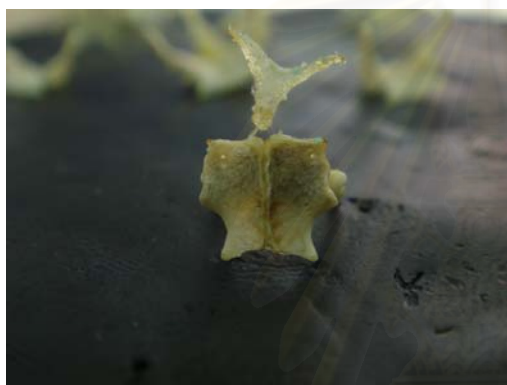
Fig. C.130 PLG

Fig. C.131 PL2627

Fig. C.132 PHF

Fig. C.133 PHG

Figs. C.134-C.138 The cartilago ypsiloidea of *T. verrucosus* Type I.

Fig. C.134 ITNT1

Fig. C.135 ITNT2

Fig. C.136 LK3/5/50
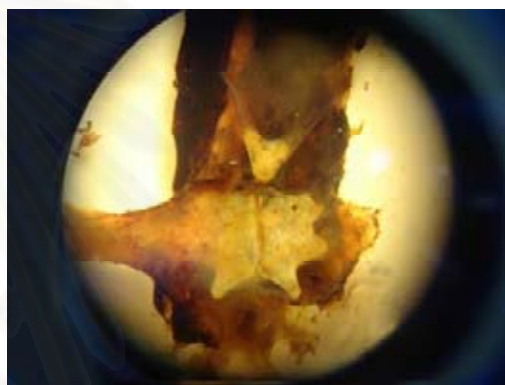
Fig. C.137 LK

Fig. C.138 PP2643

Figs. C.139-C.144   The cartilago ypsiloidea of *T. verrucosus* Type II.

Fig. C.139 PK5

Fig. C.140 PK6
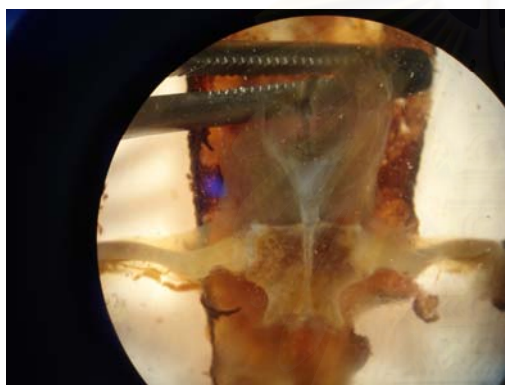
Fig. C.141 PLG
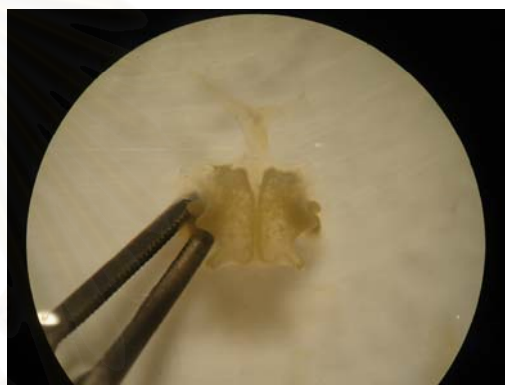
Fig. C.142 PL2627

Fig. C.143 PHF

Fig. C.144 PHG

Figs. C.145-C.150   The left femur of *T. verrucosus* Type I.

2.5 mm

Fig. C.145 ITNT1

Fig. C.146 ITNT2

Fig. C.147 LK3/5/50
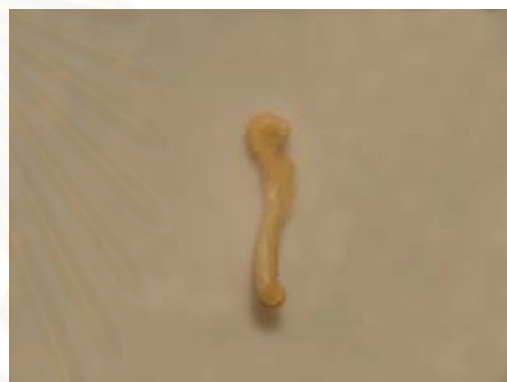
Fig. C.148 LK

Fig. C.149 PP2638
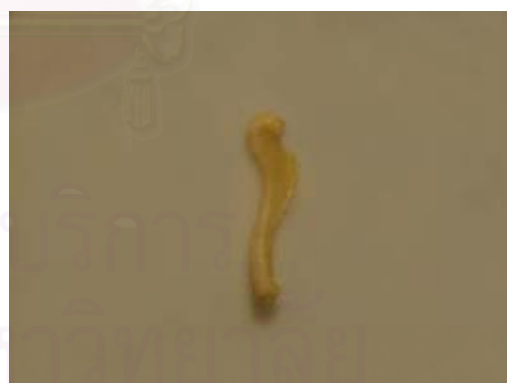
Fig. C.150 PP2643

Figs. C.151-C.156   The left femur of *T. verrucosus* Type II.

Fig. C.151 PK5

Fig. C.152 PK6

Fig. C.153 PLG

Fig. C.154 PL2627

Fig. C.155 PHF
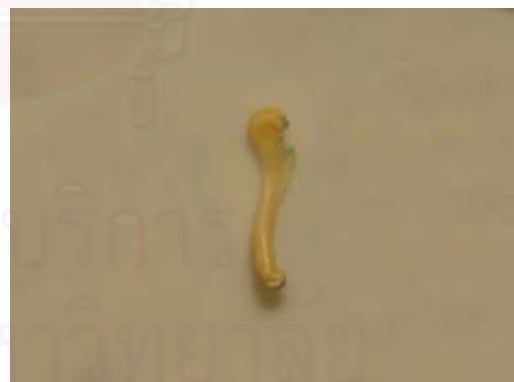
Fig. C.156 PHG

Figs. C.157-C.162   The left tibia of *T. verrucosus* Type I.

Fig. C.157 ITNT1

Fig. C.158 ITNT2

Fig. C.159 LK3/5/50

Fig. C.160 LK

Fig. C.161 PP2638
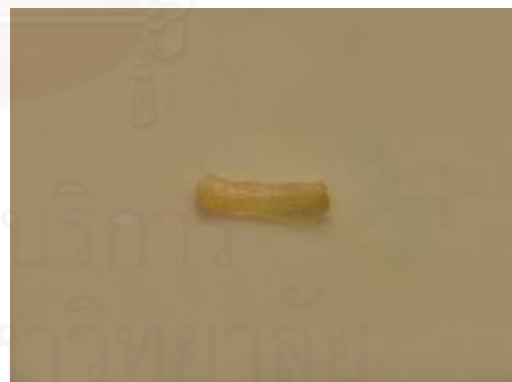
Fig. C.162 PP2643

Figs. C.163-C.168   The left tibia of *T. verrucosus* Type II.

2.5 mm

Fig. C.163 PK5

Fig. C.164 PK6

Fig. C.165 PLG
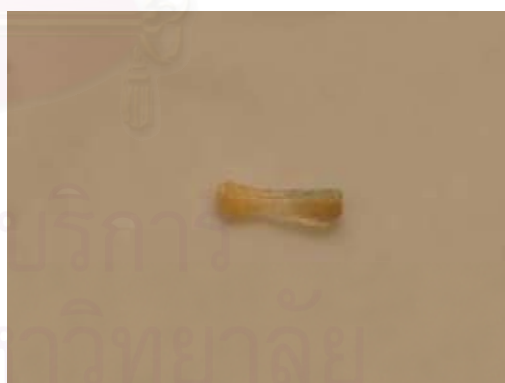

Fig. C.166 PL2627
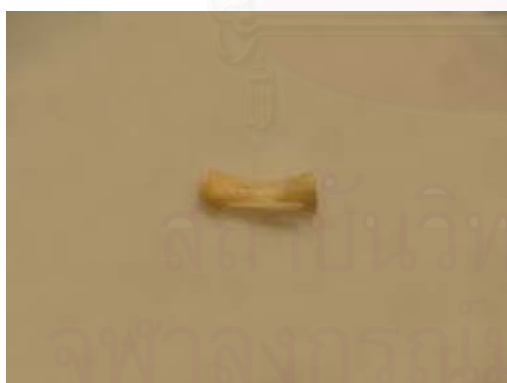
Fig. C.167 PHF

Fig. C.168 PHG

Figs. C.169-C.174   The left fibula of *T. verrucosus* Type I.

Fig. C.169 ITNT1

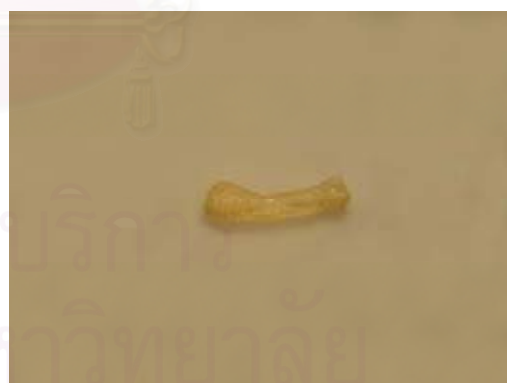**Fig. C.170 ITNT2**

Fig. C.171 LK3/5/50

Fig. C.172 LK

Fig. C.173 PP2638

Fig. C.174 PP2643

Figs. C.175-C.180   The left fibula of *T. verrucosus* Type II.

Fig. C.175 PK5

Fig. C.176 PK6

Fig. C.177 PLG

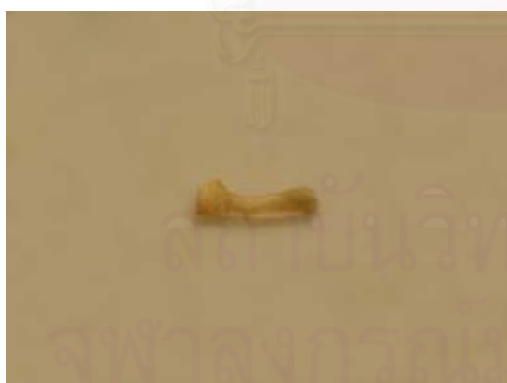**Fig. C.178 PL2627**

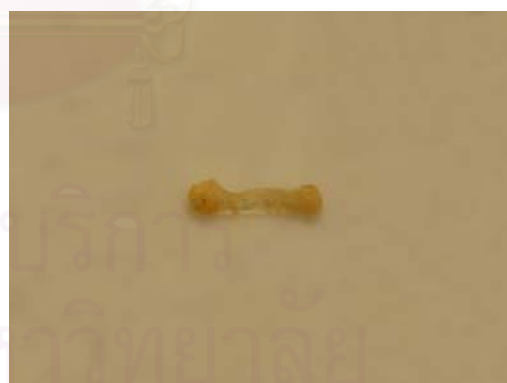**Fig. C.179 PHF**

Fig. C.180 PHG

Figs. C.181-C.185   The left tarsal skeleton of *T. verrucosus* Type I.

Fig. C.181 ITNT1

Fig. C.182 ITNT2

Fig. C.183 LK3/5/50

Fig. C.184 LK

Fig. C.185 PP2643

Figs. C.186-C.190   The left tarsal skeleton of *T. verrucosus* Type II.

Fig. C.186 PK6

Fig. C.187 PLG

Fig. C.188 PL2627

Fig. C.189 PHF

Fig. C.190 PHG

BIOGRAPHY

Mr. Porrawee Pomchote was born on February 11th, 1982 in Chiang Mai Province. He graduated with a Bachelor's Degree of Science in Biology in 2005 from the Department of Biology, Faculty of Science, Chulalongkorn University. At present, he is a graduate candidate in the Master's Degree Program in Zoology at the Department of Biology, Faculty of Science, Chulalongkorn University.

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