國立臺灣大學生命科學院生態學與演化生物學研究所

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Doctoral Dissertation

臺灣黑熊(*Ursus thibetanus formosanus*)之保育遺傳研究

Conservation genetics of Formosan black bears (*Ursus thibetanus formosanus*)

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國立臺灣大學博士學位論文
口試委員會審定書

臺灣黑熊(*Ursus thibetanus formosanus*)之保育遺傳研究

Conservation genetics of Formosan black bears (*Ursus thibetanus formosanus*)

本論文係石芝菁君（學號D94b44002）在國立臺灣大學生態學與演化生物學研究所完成之博士學位論文，於民國107年1月2日承下列考試委員審查通過及口試及格，特此證明

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所長 高 文 媛 （簽名）

誌謝

多年前考上高考，我從一個研究海洋生物分子演化的研究生進入公職，很幸運的有機會先後在行政院農業委員會特有生物研究保育中心與臺北市立動物園任職，於是我從海洋走向陸地、開始接觸陸域哺乳動物。尤其初在臺北市立動物園工作時，負責野生動物收容中心和溫帶動物區，接觸到動物園內十數隻的亞洲黑熊或稱臺灣黑熊，這些動物因為收容救傷緣故背景不明，想要藉由遺傳方法解決黑熊血緣疑慮、讓這些動物更有保育意涵的動機，開啟了我的博士班研究生涯。幾經耽擱、停頓、再繼續，在許多人的協助鼓勵下終於能完成這本博士論文，心情筆墨難以形容，更有許多人需要感謝。

最終能夠完成論文，首先一定要感謝指導教授李玲玲老師，雖然我一語成讖、果然因為工作沒法專心學業，拖了許久才完成論文研究，老師一路包容、從沒放棄。無數次的討論、反覆修改我一定難以閱讀的英文稿件、耐心指導完成論文發表，在我身上花了如此多的時間。黃美秀老師讓我進入黑熊研究的領域，一直與我討論黑熊生態與保育問題；更慷慨的讓一個在動物園工作、接觸圈養動物遠多於野外族群的學生，分析自己千辛萬苦從野外採集來的黑熊樣本。李壽先老師指導了所有分子遺傳相關的分析；並提供實驗室的空間，讓我完成了大部分微衛星體的實驗。沒有幾位老師在各個面向的指導與協助，這份論文沒有辦法完成。另外，也要感謝王穎老師與洪志銘老師對論文內容的斧正，擔任我的口試委員並提供了許多精闢的建議；以及于宏燦老師與吳海音老師於博士資格考階段，對論文架構的指導。

這份論文與我在動物園的工作密切結合，臺北市立動物園是最強大的後盾，提供了黑熊樣本與研究環境。感謝歷任的陳寶忠園長、葉傑生園長與金仕謙園長，一直給予支持並在博士班初期給予公假修課。感謝在博士班後期不得不頻繁請假

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撰寫論文時，慷慨同意代理、讓我在工作上沒有後顧之憂的彭仁隆助理研究員與朱孝芬研究員；張廖年鴻助理研究員除了同樣代理了我許多動物組組長時的工作外，更協助黑熊粒線體資料的親緣分析並與我分享博士班求學的經驗。張明雄副研究員在許多人都已經不敢詢問論文進度的時候，仍苦口婆心提醒我一定要安排工作與論文的優先序位。另外，特別要感謝初念博士班時動物園的動物組組長、現任的林務局林華慶局長，不但在動物園時鼓勵我攻讀博士、給予極大的研究空間與資源；更在幾經人事變遷後，只要有機會聯繫，一定先關心我的論文進度。長官們的期許、同事們的支持，讓我即便耽擱停頓，卻從不願放棄完成論文。

來自各方的黑熊樣本是這份論文得以完成的關鍵，感謝特生中心楊吉宗前副主任及其他中心同仁，提供特生中心的黑熊樣本並協助私人黑熊樣本的採集；屏東科技大學保育類野生動物收容中心，協助進行收容黑熊的採集與提供來自越南的黑熊樣本；高雄壽山動物園亦是提供黑熊樣本的重要夥伴，這幾個單位也都在遺傳分析完成後，一起參與了因應而生的臺灣黑熊域外保育計畫。另外，中國四川成都 CITES 辦公室的龔繼恩先生，協助安排亞洲動物基金龍橋亞洲黑熊救護中心的樣本採集;中國林業科學研究院森林生態環境與保護研究所的劉芳副教授，提供了自己博士班研究所蒐集的四川野外黑熊樣本；北京大學生命科學學院羅述金研究員，在其於美國國家癌症中心研究期間，提供俄羅斯的黑熊樣本，一併予以致謝。

能夠完成龐大數量黑熊樣本的分析，特別要感謝臺北市立動物園遺傳親緣實驗室歷年的工作夥伴，包括謝育錚小姐、侯宣伊小姐、陳慧倫研究助理等人在分生實驗上的協助。儲瑞華博士、黃傳景博士與郭浩志博士，於他們在臺大工作的時期，分別指點我哺乳動物毛髮 DNA 粹取、排遺 DNA 粹取、微衛星體分析與協助部分黑熊粒線體實驗；也要感謝李壽先老師實驗室前後多位學生與助理，在微衛星體判讀與數據分析的協助。感謝李玲玲老師實驗室的學弟白任暉協助修飾

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部分英文文稿；馮建瑄、陳威辰兩位學弟妹協助口試場地準備與口試過程紀錄。學校行政程序上，也感謝所辦楊月鈴助教一直以來的耐心提醒與細心協助。

漫長的博士班生涯，學業、工作與生活能夠平衡並堅持下去，朋友們的支持不可或缺。謝謝林秋娟、李明玲、吳倩菊與林秀穎，她們是我工作上的長期戰友、更是我很幸運擁有的好友，我們背景專業不同但同樣先後面臨家庭與學業瓶頸，除了分享經驗、互相鼓勵，更感謝她們軟硬兼施的督促我完成學業。摯友吳松霖與林柏芬夫妻，長期當我的心情垃圾桶並一直為我禱告；松霖更是我研究上的好夥伴，協助完成論文中許多的統計分析。王惟芬學姐除了願意聽我設法兼顧學業與工作的甘苦，並且協助了早期稿件的英文潤飾。

爸爸、媽媽和家人們是我最大的支柱，他們即便不了解我在忙什麼、不明白一個博士學位為何要讀這麼久，仍然只有無條件的支持。在多次因為撰寫論文而決定休學時，頻頻我希望不要休學，一直表示學費不是問題，有困難爸爸媽媽可以幫忙的，這麼可愛、這麼讓人感動。如果得到博士學位有任何榮耀，這都是屬於我的家人。

最後，要感謝所有參與這本論文的黑熊們，無論你們是生活在人為照養環境或是活躍於野外山林間，希望這份論文對得起你們所供獻的樣本，並且對現在與未來的黑熊保育有所幫助。

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摘要

臺灣黑熊(*Ursus thibetanus formosanus*)是亞洲黑熊(*U. thibetanus*)分布於臺灣的特有亞種。臺灣黑熊的族群數量與分布範圍因為棲地的縮減、破碎化及獵捕而持續減少，分布於其他地理區域的亞洲黑熊也遭遇同樣的生存威脅。為了建立適當的保育策略，我們必須優先了解這個物種在族群內與族群間的遺傳多樣性與親緣關係。由於不同地理區的亞洲黑熊亞種間外表型態的差異並不明顯，難以利用表型的差異來區分，這樣的研究資料更顯得重要。

本研究主要的目的是釐清臺灣黑熊與其他地區亞洲黑熊的遺傳變異程度與親緣關係，以了解臺灣黑熊族群的遺傳定位與分化狀況。論文有四項主要的工作，首先是篩選適當的亞洲黑熊微衛星體基因座分子標記；其次是量化評估黑熊毛髮與排遺樣本在不同採樣時間與保存方法下 DNA 萃取的成功率，以建立黑熊樣本採集與保存的標準作業流程；接著利用粒線體 DNA 控制區域及篩選出的微衛星體分子標記進行臺灣黑熊與其他地區亞洲黑熊的遺傳分析；最後也利用同樣遺傳方法釐清臺灣地區圈養黑熊的遺傳狀況。

論文首先自 33 個具有微衛星體基因座的臺灣黑熊 DNA 序列中，篩選出 10 個具有專一性及多型性的 4 重複序列微衛星體分子標記。量化評估黑熊毛髮與排遺採樣與保存方法的結果，顯示了在亞熱帶環境下，DNA 增幅的成功率隨樣本放置於野外的時間及增幅標的 DNA 的長度增加而下降；但採集排遺內外不同位置並不影響排遺樣本中 DNA 增幅的成功率。另外，浸泡酒精的排遺樣本於採集後有否進行冷凍保存處理，對一週內樣本小片段 DNA 的增幅沒有影響，但將影響長片段 DNA 的增幅效果。粒線體 DNA 控制區域部分序列的遺傳分析結果顯示，日本黑熊與臺灣黑熊形成兩個單系群；東北黑熊在與西南黑熊混雜的支序中自成一群；而東南亞的西藏黑熊樹型複雜、並未形成單系群。微衛星體分子標記的遺傳結構分析顯示，臺灣黑熊、西南黑熊、東北黑熊與東南亞的西藏黑熊這 4 個黑熊亞種

doi:10.6342/NTU201800373

各自分群。最後，針對圈養黑熊的遺傳分析顯示，7 隻圈養黑熊具有臺灣黑熊獨特的粒線體單型，其中 3 個體的微衛星體分析確認其臺灣黑熊的亞種分類。

本研究的結果提供了亞洲黑熊亞種鑑別與擬定保育管理單位的明確基礎，並可做為臺灣黑熊保育與經營管理重要的參考資料。

關鍵詞：臺灣黑熊、遺傳親緣、非侵入式採樣、域外保育、粒線體 DNA、微衛星體

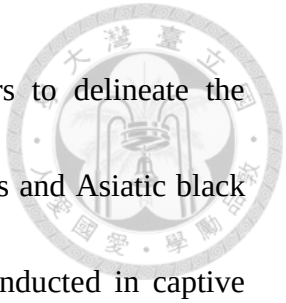
doi:10.6342/NTU201800373

Abstract

The Formosan black bear (*Ursus thibetanus formosanus*) is an endemic subspecies of the Asiatic black bear (*U. thibetanus*) inhabiting Taiwan. Habitat degradation and fragmentation, as well as poaching have caused a decrease in its population and distribution. Similar threats to populations of Asian black bears have taken place elsewhere in their range. To establish proper conservation strategies for the species, a priority research is to reconstruct its evolutionary history and examine genetic diversity within and among its populations, especially when identification of Asiatic black bear subspecies by morphological characters is vague and controversial.

The objectives of my study were to apply molecular techniques to delineate the phylogenetic relationships of Formosan black bears and other subspecies, and to assess genetic status of the Formosan black bears. My dissertation included 4 major aspects. The first part was to select appropriate microsatellite genetic markers for genetic analyses of Asiatic black bears. The second part was to quantitatively evaluate the effects of sample age and storage techniques on success rates of DNA extraction from various types of samples, i.e. bear hair and feces. Such results would facilitate the development of standard operation procedures for collection and storage of these samples before analysis. Thirdly, I applied the mitochondrial DNA control region and

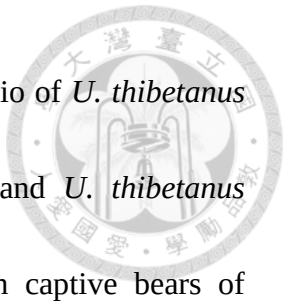
doi:10.6342/NTU201800373



microsatellite markers developed in this study as genetic markers to delineate the phylogenetic relationship and genetic status of Formosan black bears and Asiatic black bears from other areas. Lastly, the same genetic analyses were conducted in captive bears to reveal the genetic ancestry of captive Asiatic black bears in Taiwan.

In my study, ten polymorphic microsatellite markers were developed for the Formosan black bear from a partial genomic library enriched for GAAA repeat and were used to examine the polymorphism in bear populations. The evaluation results showed that the amplification success rates decreased with sample age and amplicon size in both hair and faecal DNA, but did not show differences among different sampling locations of faeces in subtropical Taiwan. The immediate freezing of ethanol-soaked faecal samples in the field were not so critical in affecting DNA quality of short fragments from samples collected within a week but the effect of immediate freezing was significant for longer mtDNA fragments. The mitochondrial DNA analyses indicated that the Japanese black bears (*U. thibetanus japonicus*) and the Formosan black bears (*U. thibetanus formosanus*) formed two distinct clades. The northeastern Asia population (*U. thibetanus ussuricus*) formed a group within the clade containing a mixture of bears from southwestern China (*U. thibetanus mupinensis*). And the bears from southeastern Asia were not monophyletic. In addition, the population structure

doi:10.6342/NTU201800373



analysis of tetramicrosatellite loci showed a clear subdivision scenario of *U. thibetanus formosanus*, *U. thibetanus mupinensis*, *U. thibetanus ussuricus*, and *U. thibetanus thibetanus*. Finally, in the results of captive bear analyses, seven captive bears of unknown origin showed the unique mtDNA haplotypes of the Formosan black bear. And three of them were verified as the Formosan black bear subspecies according to microsatellite data.

The results of this study have provided an explicit basis for subspecies identification for Asiatic black bears and important information for conservation and management of Formosan black bears.

KEYWORDS: Formosan black bear, phylogenetic relationship, noninvasive genetic sampling, *ex situ* conservation, mitochondrial DNA, microsatellite

doi:10.6342/NTU201800373

Table of Contents

doi:10.6342/NTU201800373

doi:10.6342/NTU201800373

doi:10.6342/NTU201800373

List of Figures

doi:10.6342/NTU201800373

doi:10.6342/NTU201800373

List of Tables

doi:10.6342/NTU201800373

Chapter 1 Overview of the dissertation

1.1 Overview of the dissertation

The Asiatic black bear (*Ursus thibetanus*) has a wide distribution in southern and eastern Asia spanning from Pakistan to Russian Far East of Asian continent, and the surrounding islands, including Japan and Taiwan (Servheen et al. 1999; Wozencraft 2005; Garshelis and Steinmetz 2016). This medium-sized bears occupy a variety of forested habitats from near sea level to an elevation of 4,300 m (Garshelis and Steinmetz 2016).

Like many other bear species, the Asiatic black bear has been threatened by habitat loss and poaching, and is listed as “Vulnerable” by the IUCN Red List of Threatened Species since 1990 (Garshelis and Steinmetz 2016). It is also an Appendix I species of the Convention of International Trade on Endangered Species of Wild Fauna and Flora (CITES) (CITES 2017).

Seven subspecies of *U. thibetanus* have been recognized, including *U. thibetanus ussuricus* inhabiting southern Siberia, northeastern China, and Korean peninsula, *U. thibetanus japonicus* inhabiting Japan, *U. thibetanus formosanus* inhabiting Taiwan, *U. thibetanus mupinensis* inhabiting southwestern China, *U. thibetanus laniger* inhabiting Himalaya area, *U. thibetanus gedrosianus* inhabiting Pakistan, and the nominate

doi:10.6342/NTU201800373

subspecies *U. thibetanus thibetanus* (Hou and Hu 1997; Ma et al. 1998; Wozencraft 2005).

Traditionally taxonomic differences are based on diagnostic morphological characters, and combinations of measurements, particularly from skulls (Kitchener 2010). However, the subspecies of Asiatic black bears can be recognized only in accordance with their geographic distribution (Wozencraft 2005). The morphological differences among these subspecies reported were from few specimens and description of these differences are vague (Hwang et al. 2008; Kitchener 2010). For examples, Heptner et al. (1998) distinguished *U. thibetanus ussuricus* from other subspecies by its largest skull measurements, pure black hair, and long fur. Hu (1995) compared the differences among *U. thibetanus thibetanus*, *U. thibetanus mupinensis*, and *U. thibetanus laniger* by vague descriptions of body sizes, length of hair, and the pattern of chest mark. Therefore, it is difficult to determine the origin of individual bear specimen based on its morphological characteristics.

Taxonomy is essential for conservation and the implementation of protective legislation (O'Brien and Mayr 1991; Kitchener 2010). Lack of taxonomic delimitation in the wild may result in loss of unique populations, or the recognition of too many subspecies may prevent mixing of depleted gene pools owing to local population

doi:10.6342/NTU201800373

bottlenecks. According to the subspecies concept defined by O'Brien and Mayr (1991), subspecies is defined to include populations below the species level that share a distinct geographic distribution, a group of phylogenetically concordant characters, and a unique natural history relative to other subdivisions of the species. And if a population of a species is genetically distinctive by strong phylogenetic structuring of mitochondrial DNA (mtDNA) variation and nuclear alleles from the others due to long-term evolutionary isolation, it should be treated as an ‘evolutionarily significant unit’ (Ryder 1986; Moritz 1994).

In recent years, the development of molecular techniques allows us to examine genetic variation of animal species distributed over wide geographical areas regardless of sex, age, and local phenotypic responses to the environment which have greatly benefited taxonomy and systematics (Frankham et al. 2002; Van Dike 2008a; Kitchener 2010). Due to the wide distribution and the controversially morphological traits of subspecies identification of Asiatic black bears, the information about the genetic status and genetic partitions is important for the conservation of these subspecies or populations. After all, the conservation strategy for this species will be bound to knowledge of its taxonomy.

The Formosan black bear (*U. thibetanus formosanus*) is considered an endemic

doi:10.6342/NTU201800373

subspecies of Asiatic black bears inhabiting Taiwan (Wozencraft 2005). Again, its subspecies status was based on geographic distribution and limited information on morphological differences. Similar to other Asiatic black bear subspecies, habitat degradation and fragmentation, as well as poaching, have caused a decline in its population (Wang 1990, 1999; Hwang and Wang 2006). In the conservation of endangered Formosan black bears, molecular genetic techniques could help conservation biologists to define and identify its subspecies status and the management units for conservation more clearly by their genetic constituency.

A few studies have been conducted to examine the genetic status of Formosan black bears. Chu et al. (2000) analyzed the mtDNA control region and cytochrome b of the Asiatic black bears in Taipei Zoo. Chen and Yang (2002) compared partial gene sequences of mitochondrial 12S rRNA and 16S rRNA among 11 captive Asian black bears in Taiwan. Wu et al. (2015) tentatively indicated the black bear from Taiwan was highly nested within the southern East Asian continental population with only one individual in their analysis. However, few bear specimens from Taiwan had been analyzed in all these three studies and the geographical information of captive bear specimens may also be controversial. Therefore, the phylogenetic relationship and genetic status of Formosan black bears remain unclear.

doi:10.6342/NTU201800373

Moreover, the *ex situ* conservation for possible reintroduction of the Formosan black bears in the future may also suffer from lack of knowledge about the taxonomy of subspecies. In captivity, hybridization may occur due to wrong taxonomy of subspecies. Descendants of such captive populations would be unavailable for reintroduction to avoid genetically introgression in the wild populations, ultimately wasting resources for breeding program in *ex situ* conservation (Frankham et al. 2002; Van Dike 2008b; Kitchener 2010). Thus, the subspecies taxonomy of Formosan black bears should be clarified in genetics for both *in situ* and *ex situ* conservation.

Before studying the genetic status of Formosan black bears and other Asiatic black bears, it is critical to develop suitable genetic markers for better application of genetic methods in assessing genetic partitions, defining the evolutionary significant units for conservation management, and improving the taxonomic designations (Moritz 1994; Beebee and Rowe 2008). The mitochondrial DNA fragments are useful in addressing questions about species identification, population structure and phylogenetic research (Waits et al. 1999; Murphy et al. 2002; Roon et al. 2003), whereas the microsatellites of nuclear DNA have utility in individual identification (Murphy et al. 2002), kinship analysis, gene flow, and demographic studies (Roon et al. 2003; DeMay et al. 2013). Thus, these two kinds of molecular markers would be used in the genetic analyses of

doi:10.6342/NTU201800373

Asiatic black bears in this dissertation.

Some microsatellite genetic markers have been developed and used in the genetic studies of Ursid. Most of these markers are dinucleotide loci (Paetkau et al. 1995; Taberlet et al. 1997; Paetkau et al. 1998; Kitahara et al. 2000; Wu et al. 2010). Two studies reported tetranucleotide loci, which are considered better due to fewer stutter bands and less scoring ambiguity (Hung et al. 2004), from American black bears (*Ursus americanus*) (Meredith et al. 2009; Sanderlin et al. 2009). There is no report on the tetranucleotide microsatellite loci for Asiatic black bears. Therefore, the development of tetranucleotide microsatellites should provide an ideal genetic tool kit to study the population genetics of the endangered Formosan black bears and other Asiatic black bear subspecies.

In addition, noninvasive methods have been recommended for collecting samples of wide-ranging and illusive rare carnivores such as the Formosan black bears. For effective application of noninvasive genetic analysis in subtropical Taiwan, it is important to identify the variables which may affect the DNA quality of noninvasive samples, such as faeces or hair. Most studies evaluating the quality and DNA amplification success of noninvasive faeces or hair samples were conducted on brown bears (*Ursus arctos*) in temperate regions (Murphy et al. 2002; Murphy et al. 2007;

doi:10.6342/NTU201800373

Stenglein et al. 2010). However, few were on bears in regions with different climatic conditions, for instance, tropics and subtropics. Genetic studies using faecal and hair samples of wild populations have been carried out initially in the Formosan black bear. Therefore, a pilot study is recommended to determine DNA degradation rates in this system and to develop the appropriate noninvasive protocol (Taberlet et al. 1999; Renan et al. 2012; DeMay et al. 2013).

Therefore, the aims of this dissertation were to develop appropriate tools for Asiatic black bear genetic studies and to clarify genetic status of the Formosan black bear. The dissertation ws organized into the next four chapters.

In Chapter 2, ten novel easy-scored polymorphic tetranucleotide repeat (GAAA) microsatellite markers were developed and evaluated for their polymorphism in the Formosan black bears. These microsatellite loci could be applied as molecular tools for genetic analyses of the Formosan black bears and other Asiatic black bears.

To reinforce the optimization of noninvasive sampling approaches in the Asiatic black bear research in subtropical Taiwan, in Chapter 3, the effects of multiple variables on amplification success rate of mitochondrial DNA (mtDNA) extracted from the Asiatic black bear faeces and hair were quantitatively evaluated. The results showed that the amplification success rates decreased with sample age and amplicon size in both

doi:10.6342/NTU201800373

hair and faecal DNA, but did not show differences between two faecal preservation methods, i.e. storage in ethanol then frozen or kept at room temperature, in shorter fragments, and among different sampling locations of faeces. It suggests that careful selection of primers for suitable PCR product sizes depending on sample conditions could optimize success rates of genetic analysis in noninvasive genetic research.

In Chapter 4, mitochondrial phylogeny of bear specimens collected from Taiwan, mainland China, Russia, Vietnam, and Thailand were conducted based on partial mitochondrial DNA control region and its 5'-flanking region to assess the genetic status of the Asiatic black bear populations, and elucidate the unclear genetic taxonomy of the Formosan black bear. The mitochondrial DNA analyses supported the Formosan black bears formed a unique monophyletic group. In addition, the population structure analysis of tetramicrosatellite loci was employed to indicate a clear subdivision scenario of these four subspecies, *U. thibetanus formosanus*, *U. thibetanus mupinensis*, *U. thibetanus ussuricus*, and *U. thibetanus thibetanus*.

Finally, in Chapter 5, a pilot study of genetic analysis on both mitochondrial DNA and microsatellite loci from captive bear specimens was conducted to reveal the genetic ancestry of captive Asiatic black bears in Taiwan. In this study, seven captive bears of unknown origin showed the unique mtDNA haplotypes of the Formosan black bear. And

doi:10.6342/NTU201800373

three of them had a single verified subspecies ancestry of the Formosan black bear based on microsatellite data. Given the fact that the size of the wild population is critically small and that the bears of native origin are kept in different zoos, institutes, and rescue centers in Taiwan, these institutions are highly encouraged to cooperate with each other in implementing an *ex situ* breeding plan for the conservation of this subspecies.

In summary, these studies enhanced genetic tool for conservation genetic studies of the Formosan black bear. They also revealed the level of genetic variation among different populations of Asiatic black bears and provided an explicit basis for subspecies identification of the Formosan black bear. Such information will be important and beneficial for both *in situ* and *ex situ* conservation of this Asiatic bear species in the future.

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Chapter 2 Ten novel tetranucleotide microsatellite DNA markers from Asiatic black bear, *Ursus thibetanus*

2.1 Abstract

Ten polymorphic microsatellite markers were developed for the endangered Formosan black bear (*Ursus thibetanus formosanus*) from a partial genomic library enriched for GAAA repeat. Polymorphism of these loci was evaluated in 27 Formosan black bear specimens of unknown relationship. The number of alleles per locus ranged from five to fifteen and the observed heterozygosity of each locus ranged from 0.556 to 0.889. These loci should provide useful molecular tools to study conservation genetics of the Formosan black bear and other Asiatic black bears.

2.2 Introduction and methods

The Formosan black bear (*Ursus thibetanus formosanus*) is an endemic subspecies of Asiatic black bear inhabiting Taiwan (Wozencraft 2005). Similar to all other Asiatic black bears, degradation and fragmentation of habitat as well as poaching have caused a decrease in population and distribution of the Formosan black bear (Wang 1999; Hwang and Wang 2006). To formulate proper conservation strategies, it is important to understand the genetic diversity and genetic structure within and among populations of this subspecies.

doi:10.6342/NTU201800373

In this study, we reported ten novel easy-scored polymorphic tetranucleotide repeat (GAAA) microsatellite loci from the Formosan black bear. We followed the protocol developed by Hsu et al. (2003) to enrich microsatellite-contained fragment in a partial genomic library. The library was constructed from genomic DNA which extracted from tissue sample of a Formosan black bear individual using the proteinase K-chloroform method (Sambrook et al. 1989). Microsatellite-enriched PCR (polymerase chain reaction) library was ligated into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α. A total of 880 clones were lifted to Hybond-N+ membranes (Amersham Pharmacia Biotech) and hybridized with [γ32P] ATP end-labelled (GAAA)10 oligonucleotides, then 56 hybridized clones were sequenced using DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Bioscience) on a MegaBACE 1000 autosequencer (Amersham Bioscience). Sequences were proofread using software SEQUENCER 4.2 (Gene Codes). We found 47 clones with microsatellite motif, of which 33 loci containing more than 10 units of GAAA motif were chosen to design the PCR primers.

All forward primers were 5’-tailed with an M13-tail (5'-GGAAACAGCTATGACCAT-3') or a CAG-tag (5'-CAGTCGGGCGTCATCA-3') (Schuelke 2000; Boutin-Ganache et al. 2001). DNA extracted from tissue samples of 17

doi:10.6342/NTU201800373

Formosan black bears and from faecal samples of ten Formosan black bears with unknown relationship were used to characterize these 33 loci. PCRs were set up in 10-μL reaction volumes containing 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.05 μM of tailed forward primer, 0.12 μM of reverse primer, 0.18 μM of fluorescent-labelled M13 or CAG-tag primer that were labeled with HEX, FAM or TAMRA fluorescent dyes, 0.2 U Taq DNA polymerase (Biotech), and around 30 ng genomic DNA. The PCR condition was 95 °C for 4 min, then 40 cycles at 95 °C for 30 s, 30 s at the optimal annealing temperature of each primer pair (Table 2-1) and 72 °C for 20 s, followed by a final extension at 72 °C for 7 min. The PCR products were electrophoresed in a MegaBACE 1000 autosequencer (Amersham Biosciences). Sizes of alleles were scored with software GENETIC PROFILER 2.0 (Amersham Biosciences).

2.3 Results and discussions

Twenty three loci that appeared difficult to score or monomorphic were excluded from subsequent analyses. Genotype frequencies of ten loci were analysed using CERVUS 2.0 (Marshall et al. 1998) to calculate the observed and expected heterozygosities. Tests for departure from Hardy–Weinberg equilibrium and linkage equilibrium between pairs of loci were performed using GENEPOP 3.4 (Raymond and

doi:10.6342/NTU201800373

Rousset 1995). Polymorphism assessment at these ten microsatellite loci is summarized in Table 2-1. The number of alleles per locus ranged from 5 to 15 and the observed heterozygosities ranged from 0.556 to 0.889. There was no evidence for large allele dropout and null alleles detecting by MICRO-CHECKER (van Oosterhout et al. 2004) in all ten loci. Four loci (UT23, UT25, UT35 and UT38) represented significant differences between the observed and expected heterozygosities ($P < 0.05$), which are probably due to genetic drift driven by Formosan black bear's small population size. No significant deviation from linkage equilibrium was detected after Bonferroni correction.

With microsatellites that isolated from Japanese black bear (*U. thibetanus japonicus*) (Kitahara et al. 2000) and other Ursids (Paetkau et al. 1995; Taberlet et al. 1997), the tetranucleotide microsatellites we isolated should provide an ideal genetic tool kit to study the population genetics of the endangered Formosan black bear and other Asiatic black bears that are also under threat.

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Tables

Table 2-1 Characterization of the ten microsatellite loci of Formosan black bear (*Ursus thibetanus formosanus*).

Locus	Core motif	Primer sequence (5'−3')	*N*	T_a (°C)	MgCl2 (mM)	No. of alleles	Allele size(bp)[a]	H_O	H_E	*P*-values (HWE)	GenBank Accession no.
UT1	$(GAAA)_9GGGA(GAAA)_{10}$	F: CAG- AGCAACTCTTCTCAGATGTTCACAAA R: CCCAGGTCAGCACTTGGCATAC	27	64	2.5	5	176-192	0.556	0.584	0.461	FJ640076
UT3	$(GAAA)_{18}$	F: CAG- AAGACATACAGAAGCCAAGACTAG R: TACTCAATTACAAAGGATAACTATA	25	56	2.5	7	256-282	0.640	0.776	0.186	FJ640077
UT4	$(GAAA)_6GAGA(GAAA)_{11}$	F: M13- GAGTTATTGGCACTAAAATCTAATG R: CTGCAAATCCCTGCTCAACTTTC	27	56	2.5	7	157-182	0.704	0.814	0.107	FJ640078
UT23	$(GAAA)_{10}GA(GAAA)_{22}$	F: M13- GCTGGATACATCATCCTGGCTC R: GGAATCAAGTTCGGCATCGGG	27	62	2.5	12	349-382	0.778	0.881	0.040*	FJ640079
UT25	$(GAAA)_2(GA)_{12}$ $(GAAA)_{16}$	F: M13- GCTCAGGGCGTGATCCCAGAG R: GGCTCCCCTGCACTAGAGATTTAAC	27	62	2.5	6	314-333	0.704	0.720	0.011*	FJ640080
UT29	$(GAAA)_2AA(GAAA)_{17}$	F: CAG- GACATTGCCTTTTACAGAGCAG R: GGGCAGATCTCAACCACCATAAGC	27	64	2.5	8	204-236	0.889	0.788	0.058	FJ640081
UT31	$(GAAA)_{17}GG(GAAA)_3$	F: CAG- AATAAACTGATGCAGCCATACTAG R: CTGCCACTGAATCTTCTGATCTTAG	26	64	2.5	15	315-369	0.846	0.909	0.560	FJ640082
UT35	$(GAAA)_{15}$	F: CAG- ACTCCCTAGTAAGTAGAAAGCACAC R: CCCACAGGATGGGCTCAAGAA	27	64	2.5	7	218-247	0.630	0.825	0.022*	FJ640083
UT36	$(GAAA)_{16}$	F: CAG- AGACTCAGGAAGTCTGGAGTGGGA R: CTTTCGGCTCAGGGATCGAGC	27	62	2.5	7	276-309	0.630	0.727	0.154	FJ640084
UT38	$(GAAA)_{24}$	F: M13- ATTATTGATGAGCAGGGACAG R: CTAAAGCAACAACATGTGAATG	27	56	2.5	10	196-232	0.778	0.839	0.039*	FJ640085

Abbreviations: *N*, number of individuals genotyped; T_a, PCR annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy–Weinberg equilibrium and *, $P < 0.05$. [a] Allele size includes the additional size of tails added to forward primer.

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Chapter 3 Evaluation on the effects of ageing factor, sampling and preservation methods on Asiatic black bear (*Ursus thibetanus*) noninvasive DNA amplification

3.1 Abstract

Noninvasive genetic sampling allows studying wildlife without having to catch, handle or even observe individuals. In this study, factors which may affect the quality of noninvasive samples of Asiatic black bear (*Ursus thibetanus*) in the subtropical areas were identified. We collected hair and faecal samples from captive Asiatic black bears and quantitatively evaluated the effects of hair age (from fresh to 60 days), faeces age (from fresh to 14 days), faeces sampling locations (i.e. sample collected from either the surface, inside or a mixture of both the surface and inside of faeces), and faeces preservation methods (frozen or kept at room temperature in 95% ethanol) on amplification success rates of mitochondrial DNA fragments of different sizes (450bp, 900bp, and 1600bp). The results showed that the amplification success rates decreased with sample age and amplicon size in both hair and faecal DNA. In subtropical environment, there was no significant difference between amplification success of DNA extracted from fresh and 7-day-old samples of either the hair or faeces. The amplification success rates were not influenced by sampling location of faeces. For

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faeces preserved in 95% ethanol, the amplification success appeared unaffected by frozen at -20 °C or kept at room temperature in shorter mtDNA fragments, but was significantly influenced when amplicon size was 1600bp. The results of this study will reinforce the optimization of noninvasive sampling approaches in Asiatic black bear research, especially in the subtropics.

3.2 Introduction

Noninvasive genetic sampling has been proven a powerful tool for investigating populations of wildlife, particularly those elusive, rare, and free-ranging species roaming in large areas (Roon et al. 2003; Broquet et al. 2007). Through a set of genetic procedures, noninvasive genetic sampling allows the study of the biology of wildlife without having to catch, handle, or even observe individuals (Piggott and Taylor 2003; Broquet et al. 2007). Researchers could integrate various noninvasive techniques in monitoring trends of wildlife populations, especially in large carnivores, for the purposes of management and conservation (Waits and Paetkau 2005; Schwartz et al. 2007; De Barba et al. 2010). Conservation biologists, for instance, have routinely used noninvasive genetic methods to monitor the long-term population trends of the brown bears in North America (Woods et al. 1999; Broquet et al. 2007).

Noninvasive DNA could be retrieved from various types of wildlife samples

doi:10.6342/NTU201800373

include hair, faece, urine, shed feather, buccal cells from food, snake skin, eggshells et al. (Sloane et al. 2000; Valiere and Taberlet 2000; Vigilant et al. 2001; Broquet et al. 2007; Beja-Pereira et al. 2009). Faeces and shed hair are more easily collected, and thus are often used as the noninvasive genetics materials (Broquet et al. 2007; Renan et al. 2012). Despite the many advantages, a major limitation of noninvasive faeces or hair samples is the low quantities of host DNA which is often highly degraded (Waits and Paetkau 2005) and often leads to low PCR amplification rates.

The quantity and quality of faecal and hair DNA can be affected by sample age (Murphy et al. 2007; Santini et al. 2007; Vynne et al. 2012), environmental conditions (e.g. humidity, temperature, exposure to the sun or rain) (Murphy et al. 2007; Michalski et al. 2011), or technical factors, including sampling location, i.e. whether sample were collected from the surface or inside of faeces (Piggott and Taylor 2003; Stenglein et al. 2010) and storage method (Santini et al. 2007; Panasci et al. 2011). DNA extraction protocol and amplicon size, the fragment length of amplified DNA makers can also affect the quantity and quality of faecal and hair DNA extracted, thus the success rate of amplification (Piggott et al. 2004; Buchan et al. 2005; Hoffman and Amos 2005; Broquet et al. 2007). Previous studies which had evaluated factors affecting the DNA quality and amplification success rates of faecal and hair samples suggest that success

doi:10.6342/NTU201800373

rates will be the highest when samples are fresh and dry or preserved in low temperature (Farrell et al. 2000; Lucchini et al. 2002; Piggott 2004; DeMay et al. 2013).

However, DNA degradation rates can differ among taxa and even within species under different climatic or operational conditions (DeMay et al. 2013). The lack of a quantitative comparison of studies in various animal-environment systems makes it difficult to decide which protocol is the most suitable for a given system (Beja-Pereira et al. 2009; Renan et al. 2012). Those general trends are not necessarily transferable across species or study sites (DeMay et al. 2013) and may be of limited applicability to new studies. Therefore, pilot studies are still recommended for each system to determine DNA degradation rates and the appropriate noninvasive protocol (Taberlet et al. 1999; Renan et al. 2012; DeMay et al. 2013).

Noninvasive genetic sampling is often applied in Ursid research. Most studies evaluating the quality and DNA amplification success of noninvasive faeces or hair samples were conducted on brown bears (*Ursus arctos*) in temperate regions (Murphy et al. 2002; Murphy et al. 2007; Stenglein et al. 2010), but few were on bears in regions with different climatic conditions. DNA samples collected under high temperature and humidity in the tropics and the subtropics may be particularly susceptible to degradation (Wasser et al. 1997; Bayes et al. 2000; Vynne et al. 2012). Only a few studies comparing

doi:10.6342/NTU201800373

storage treatments or extraction methods have been conducted in tropical forests, and most of them were limited to primates, ungulates and canids (Nsubuga et al. 2004; Vallet et al. 2008; Soto-Calderon et al. 2009; Vynne et al. 2012). Comparative studies using DNA of faeces and hair in Ursid have not been performed and the effectiveness of methods for preserving samples has not been evaluated in the tropics or subtropics.

Our study focuses on the Formosan black bear (*Ursus thibetanus formosanus*), an endemic subspecies of Asiatic black bear inhabiting Taiwan, a subtropical island (Wozencraft 2005). Similar to all other Asiatic black bear subspecies, habitat degradation and fragmentation, as well as poaching, have caused a decrease in the population and distribution of the Formosan black bear (Hwang and Wang 2006; Hwang and Garshelis 2007; Hwang et al. 2010). To formulate proper conservation strategies, it is important to understand the genetic diversity and genetic structure of this subspecies (Shih et al. 2009). For efficient application of noninvasive genetic analysis, it is necessary to identify the variables which may affect the DNA quality and further DNA amplification success in this system.

The main objective of this study is to quantitatively evaluate the effect of multiple variables on amplification success rate of mitochondrial DNA (mtDNA) extracted from Asiatic black bear faeces and hair. When using faeces as noninvasive DNA sources,

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subsamples are often taken from species producing larger faeces instead of collecting the entire faeces in the field (Stenglein et al. 2010). Since few studies have experimentally tested samples taken from the different parts of faeces, we examined the impact of sampling locations, e.g. from the surface or inside of faeces. Soaking faeces in ethanol and silica desiccation are widely employed for faecal DNA preservation (Wasser et al. 1997; Frantzen et al. 1998; Santini et al. 2007). In a subtropical region like Taiwan, ethanol preservation should be more preferable than silica desiccation because high temperature and humidity may hinder the effect of desiccation of silica (Murphy et al. 2002). Although transportation of frozen samples from the field to the laboratory would be difficult in field research (Nsubuga et al. 2004), the effect of immediate freezing of ethanol-soaked samples in DNA preservation was also evaluated in this study.

In this study, we collected hair and faecal samples from captive Asiatic black bears in subtropical Taiwan to assess the effects of sample age (over a 60-day period for hair and a 14-day period for faeces) and faecal preservation methods (frozen or kept at room temperature in 95% ethanol) on amplification success rates of different mtDNA amplicon size. The results of this pilot study will allow us to make recommendations for optimal noninvasive sampling protocols and to provide sampling and storage guidelines for field researchers conducting noninvasive genetic sampling of Asiatic black bears in

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the subtropics.

3.3 Materials and methods

3.3.1 Experiment design, sample collection and preservation

The specimens for different treatments and evaluation were collected from 5 captive Asiatic black bears (2 males, 3 females) at Taipei Zoo and all treatments were done in the zoo as well. These bears were on a mainly vegetarian diet. Faecal samples were collected and treated with an average temperature of 26.4 °C and average relative humidity of 71% (climate data from the Central Weather Bureau, Taiwan). Hair samples were collected and tested in Taipei Zoo with an average temperature and relative humidity of 28.37 °C and 74%, respectively. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Taipei Zoo.

Fresh faeces were collected in less than 12 hours after being deposited by the 5 bears, then immediately transported to a semi-outdoor flat ground where they would not be directly exposed to rain and sunlight (to simulate the condition under canopy in the wild). For age and sampling location treatments, 1-ml of faecal samples were taken with wooden sticks from inside, surface and inside-surface mixture of faeces at 0 (which means fresh), 1, 3, 7, 14 days post collection from the bear facilities. After each sampling, the remaining faeces were left undisturbed and subsequent samples were

doi:10.6342/NTU201800373

collected from undisturbed portions of the faeces. All faecal samples were soaked in 4-ml of 95% ethanol (Wasser et al. 1997; Murphy et al. 2002; Panasci et al. 2011), then frozen at -20 °C or kept at ambient room temperature in the laboratory for 2 weeks to serve as samples to test the effects of 2 different storage conditions. The sample size of each age, sampling location and storage method treatment was 10, with 2 from each of the 5 bears.

In the treatment of hair age, hair specimens with follicles were collected from captive bears while the animals were in narcosis for health check-ups. We designed 5 hair age treatments: fresh hair and hair of 7, 14, 30, and 60-day old, which were hair laying outdoors under partial tree shade for different amount of time after being collected from the bears to imitate hair collected from the hair-trap. Each treatment included 15 samples (10 hair follicles for each sample) which were also collected from different bears equally.

3.3.2 DNA extraction and PCR amplification

All faecal and hair samples of respective treatments were then preserved at -80 °C (Murphy et al. 2000) and DNA was extracted from these samples within 2 weeks to reduce the effect of long storage time. Faecal samples were extracted with methods detailed in Hung et al. (2004), which was modified from a

doi:10.6342/NTU201800373

hexadecyltrimethylammonium bromide (CTAB)-based extraction (Parsons et al. 1999). Hair DNA extractions were carried out by the traditional phenol-chloroform procedure (Kocher et al. 1989).

The amplification success may depend on the length of target amplified fragment. Thus all extracts were amplified of mitochondrial control region and its flanking regions using 3 primer pairs for different length of amplified segments: (1)1600bp, CB-Z, 5'-ATGAATTGGAGGACAACCAGT-3' (Matsuhashi et al. 1999) and D4, 5'-AGGCATTTTCAGTGCCTTGCTTTG-3' (Matsuhashi et al. 1999); (2) 900bp, CB-Z and Ut-Dr, 5'- TGCGTACATATGCGTACATAT-3' (designed in this study); (3) 450bp, UT-1, 5'-TGATCACCAGGCCTCGAGAAA-3' (Ishibashi and Saitoh 2004) and Ut-Dr. PCR amplifications were carried out using an ASTEC Thermal Cycler PC-808 in a total volume of 20 μL reaction mixture containing: 2 μL of faecal DNA extract and 0.5 μL of hair DNA extract respectively, 1× PCR buffer(including 1.5mM $MgCl_2$), 0.5μM of each primer, 200μM dNTP and 0.5 U of Taq DNA polymerase (Supertherm Taq, JMR). The PCR thermal profile included an initial denaturation of 10 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C, and a postcycling final extension at 72 °C for 10 min. A reagent with negative control to test contamination and a positive control to confirm proper PCR conditions were included in each group of PCR

doi:10.6342/NTU201800373

reactions.

The PCR products were electrophoresed on 1% agarose gels and visualized using ethidium bromide staining under UV light to score each PCR sample amplification/non-amplification for target DNA fragment. All samples that failed to produce a positive amplification were attempted to amplify for a second time to avoid random non-amplification (Murphy et al. 2007).

3.3.3 Data analyses

The amplification success rates for each treatment and each mtDNA fragments were calculated as percentage of the positive amplification number divided by the total number of PCR attempts.

Faecal DNA amplification results were firstly evaluated using the Friedman test to assess the effect of sampling location (sampling from inside, surface and inside-surface mixture of faeces) on amplification success in 6 preservation method and amplicon size combinations (2 preservation methods and 3 mtDNA fragments of different length). Next we used the Wilcoxon test to examine the differences between two faecal preservation methods (frozen at -20 °C and kept at room temperature). The Mann-Whitney U test was used to test the differences between amplification success rates of faecal and hair samples of 0-day and 7-day-old. The Friedman test, Wilcoxon

doi:10.6342/NTU201800373

test and Mann-Whitney U test were all computed using StatView 5.0 software (SAS Institute Inc.) and the results were considered statistically significant if the P-value was smaller than 0.05. Later the Page's trend test was performed on both faecal and hair DNA amplification results to test whether there were trends across sample ages and amplicon sizes.

3.4 Results

3.4.1 Influence of faecal sampling locations

Fresh bear faeces were soft, moist, smelly, and contained indigestible fibers and seeds. One-day-old faeces kept their original shape and remained moist with a slightly dry surface. Three-day-old faeces kept their shape but were dry in the surface and soft inside. Seven-day-old faeces were hard, dry, and moldy. At the 14th day, the faeces became flaky and the remains contained mostly fibers. Therefore, we could collect samples from 3 sampling locations (surface, inside, and surface-inside mixture) successfully for all faecal samples except those that were 14 days old, from which we only collected a sample of surface-inside mixture.

The results showed that sampling locations had no significant effect on amplification success rate regardless of preservation method and amplicon size (Friedman test, P = 0.145 - 0.926) (Table 3-1). Therefore, the data of 3 sampling

doi:10.6342/NTU201800373

locations were pooled to calculate the average values before examining the results of faecal preservation method and age of treatments.

3.4.2 Influence of preservation methods, age of faecal samples and amplicon size

The amplification success appeared unaffected by preservation methods (frozen at -20 °C or kept at room temperature in 95% ethanol) in shorter 450bp and 900bp mtDNA fragments from samples within a week ($P = 0.330$ for 450bp and $P = 0.090$ for 900bp, Wilcoxon test), but was significantly influenced when amplicon size was 1600bp ($P = 0.011$). In samples aged from fresh to 7 days, the amplification success rates of 1600bp fragment were higher in frozen samples (53.33% to 6.67%) than the room temperature samples (26.67% to 0%) (Fig. 3-1). Amplification success rates of 1600bp fragment dropped to zero for DNA extracted from 14-day-old faecal samples regardless of the storage method used (Fig. 3-1). Although PCR amplification success rates of 450bp and 900bp amplicons of the 7-day old frozen samples were higher than those of 14-day old samples as expected, an unexpected result was found in the PCR amplification success rates of 450bp and 900bp amplicons in 14-day old samples at room temperature, which were higher than that of the 7-day old samples (Table 3-1 and Fig. 3-1(b)).

When examining the trends of amplification success rates using average values of

doi:10.6342/NTU201800373

subsamples from 3 sampling locations by Page's trend test, both data of frozen and room temperature samples showed a significantly declining trend with increasing age of faeces (frozen samples: $L=163.5 > 160$ ($k=5$, $b=3$, $\alpha=0.001$), $P < 0.001$; room temperature samples, $L=162 > 160$ ($k=5$, $b=3$, $\alpha=0.001$), $P < 0.001$) and size of amplicon (frozen samples: $L=177 > 172$ ($k=3$, $b=13$, $\alpha=0.001$), $P < 0.001$; room temperature samples, $L=179.5 > 172$ ($k=3$, $b=13$, $\alpha=0.001$), $P < 0.001$). PCR performances on DNA extracted from fresh versus 14-day-old faecal samples declined from 100% to 40% for 450bp fragments, from 93.33% to 40% for 900bp fragments, and from 53.33% to 0% for 1600bp fragments in frozen samples (Fig. 3-1(a)); and from 100% to 60% for 450bp, from 93.33% to 40% for 900bp, and from 16.67% to 0% for 1600bp fragments in room temperature samples (Fig. 3-1(b)).

3.4.3 Influence of hair age and amplicon size

There was no significant difference between mtDNA amplification success of DNA extracted from fresh and 7-day-old samples of either the hair or faeces. In the results of hair treatments, Page trend test also showed a significantly decreasing trend of amplification success rates with both hair age and amplicon size (hair age: $L=157.5 > 155$ ($k=5$, $b=3$, $\alpha=0.01$), $P < 0.01$; amplicon size: $L=68.5 > 68$ ($k=3$, $b=5$, $\alpha=0.01$), $P < 0.01$). The amplification success rate of 450bp fragment was 53.33% even when the

doi:10.6342/NTU201800373

hair samples had been in an outdoor environment for 60 days (Table 3-2, Fig. 3-2). But for 900bp fragment, the success rates decreased to 80% for 7-day-old samples, 53.33% for 14-day-old samples and 0% after 30 days (Table 3-2, Fig. 3-2). Furthermore, the 1600bp fragment could only be amplified from fresh hair samples with 86.67% success rate (Table 3-2, Fig. 3-2).

3.5 Discussions

When collecting faecal samples in the field, most molecular scatology studies suggested sampling the outer portions of the faeces because a greater number of intestinal epithelial cells could be present (Albaugh et al. 1992; Flagstad Ø et al. 1999; Stenglein et al. 2010; Wasser et al. 2011). Stenglein et al. (2010) indicated that the sampling location had a significant effect on nuclear DNA quality of brown bear and wolf scats, and the outer part of the faecal samples had higher DNA quality. Our results show that sampling locations of faeces have no significant effect on mtDNA amplification success rate. Such discrepancy in the results may be due to 2 potential reasons. First, the mtDNA and nuclear DNA may have differential decay rates and patterns (Berger et al. 2001; Foran 2006; Soto-Calderon et al. 2009; DeMay et al. 2013). Second, DNA decay rates and patterns may be different under different climatic condition (Panasci et al. 2011). Most of the studies regarding the effects of sampling

doi:10.6342/NTU201800373

locations were conducted in the temperate region (Stenglein et al. 2010; Wasser et al. 2011). Faeces exposed to the subtropical environment of high temperature and humidity in our study might have an effect particularly on the outer portions of faeces, and likely counterbalance the advantage of having more and better quality intestinal epithelial cells on the surface.

Our results showed that preservation methods did not affect the amplification success rates of 450bp and 900bp mtDNA fragments from samples collected within a week. However, the success rates of 1600 bp fragment from the frozen samples was significantly higher than those samples stored at room temperature. Similar to our findings, Santini et al. (2007) suggested that wolf scats stored in 95% ethanol at -20 °C had the best nuclear DNA quality comparing to those stored in 95% ethanol at room temperature, dried at -20 °C, and in GUS at room temperature. Santini et al. (2007) further indicated the disparities between samples kept frozen and at room temperature increased over time, e.g. 98% positive PCRs at -20 °C and 55% successful PCRs at room temperature after 6 months. However, the non-linear decrease in the amplification success rates of DNA extracted from samples stored at room temperature (Fig. 3-1(b)) was inconsistent with the results of the frozen samples, even though the declining trend of the amplification success rates of DNA with increasing age was statistically

doi:10.6342/NTU201800373

significant, which may suggest that the performances of preservation at room temperature may be less predictable than those of frozen samples. Such results suggest that although immediate freezing of faecal samples is often difficult in the field, researchers should consider it especially when amplification of longer mtDNA fragment is critical for their research. In any case, freezing ethanol-soaked samples is highly recommended after the samples are brought back to the laboratory.

Most of the studies regarding the impact of sample age on faecal mtDNA amplification indicated that the amplification success generally decreased over time (Farrell et al. 2000; Murphy et al. 2007; Soto-Calderon et al. 2009; DeMay et al. 2013). Our results are consistent with these studies and those studies that showed a decreasing trend in the amplification success rates with increasing amplicon size (Broquet et al. 2007; DeMay et al. 2013). Furthermore, we found that for the samples as old as 14 days the amplification success rates of mtDNA remained to be at least 40% for the 450bp and 900bp fragments; in contrast, 1600bp fragment could not be amplified from faecal samples older than 7 days (Fig. 3-1). Information on rates of faecal DNA degradation regarding sample age and amplicon size in this study allowed researchers to choose better strategies for collecting noninvasive samples and choose suitable markers depending on the conditions of faeces in the field to balance the costs and output of

doi:10.6342/NTU201800373

laboratory work. When faecal samples are of older age, smaller mtDNA fragments are expected to have higher amplification success rates and may therefore be favored in genetic studies. If larger mtDNA sequences with increased resolution are needed for phylogenetic research (Waits et al. 1999), the noninvasive genetic materials need to be extracted within a certain time frame.

The results of amplification success rates showing no significant difference between hair and faeces at 0 and 7 days in our study are consistent with the comparative review of Broquet et al. (2007). Broquet et al. (2007) mentioned that greater inhibitor concentrations may counterbalance the advantage of larger target DNA amount in faecal samples. Regarding the effects of hair age and amplicon size on amplification success rates of DNA from hair samples, Roon et al. (2003) demonstrated that DNA of hair degraded with time when the samples were preserved using silica desiccant and -20 °C freezing. Broquet et al. (2007) reviewed the relationship between mtDNA amplification success and fragment length of hair samples in 2 published papers (Vigilant 1999; Roon et al. 2003) and indicated the shorter fragments lead to higher amplification success. However, few studies had measured the rates of hair DNA degradation regarding sample age in outdoor environment without preservation and amplicon size like our study, which indicated that amplification success rates significantly decreased with both

doi:10.6342/NTU201800373

hair age and amplicon size. In addition, the amplification success rates we found were lower in comparison to the rates in Roon et al. (2003), which might suggest the impact of high temperature and humidity on the quality and degradation rates of DNA of hair samples collected in the subtropics. Researchers conducting noninvasive analyses in the subtropics therefore can consider the DNA amplification success rates from hair samples of different ages revealed in this study and design suitable intervals for hair collection to get appropriate DNA materials.

The mtDNA fragments are useful in addressing questions about species identification, population structure, and phylogenetic research (Waits et al. 1999; Murphy et al. 2002; Roon et al. 2003), whereas the microsatellites of nDNA have utility in individual identification, kinship analysis, gene flow, and demographic studies (Murphy et al. 2002; Roon et al. 2003; DeMay et al. 2013). Mitochondrial DNA and nuclear DNA may have differential decay rates (Foran 2006; Soto-Calderon et al. 2009) and some studies have suggested using mtDNA as a screening for further nDNA analyses (Hung et al. 2004; Vynne et al. 2012). Our study examined the amplification success rates of mtDNA from faecal and hair samples but the decay rates of nDNA in the subtropics remain unanswered. Consequently, it would be necessary to examine the amplification success rates of nDNA from various non-invasive materials in the future.

doi:10.6342/NTU201800373

In addition, some studies have indicated that diet may influence target DNA quantity and genetic analysis of faeces (Murphy et al. 2003; Nsubuga et al. 2004; Panasci et al. 2011; Vynne et al. 2012; DeMay et al. 2013). Vynne et al. (2012) further suggested that the effect of diet should be considered especially in studies of species with highly varied diets. Asiatic black bears are omnivorous animals and the diet of the Formosan black bear in the subtropical Taiwan does change seasonally (Hwang et al. 2002). Although the faecal samples of this study were deposited from zoo bears with a mainly vegetarian diet, the components of the diet were not the same as the natural diet of bears in the wild. Therefore, evaluation of faecal DNA degradation under different natural diet of the bears is recommended in future studies.

Our study is the first one to quantitatively evaluate mtDNA degradation of noninvasive hair and faecal samples of Ursid animal in the subtropics. The discrepancy of results between our study and the comparative research in temperate region suggests the importance of pilot study for a new study system. In conclusion, our results demonstrated that faeces and hair could be applied as noninvasive samples for the Asiatic black bears under subtropical climate. We suggest that the amplification success rates are not influenced by sampling location of faeces in subtropical environment. The immediate freezing of ethanol-soaked faecal samples in the field are not so critical in

doi:10.6342/NTU201800373

affecting DNA quality of short fragments from samples collected within a week but the effect of immediate freezing is significant for longer mtDNA fragments. We also found that although it may be challenging to amplify longer mtDNA fragments from older faecal and hair samples, shorter fragments could be successfully amplified. Researchers collecting noninvasive samples in similar taxa and field conditions should consider the DNA degradation rates revealed in this study. Careful selection of primers for suitable PCR product sizes depending on sample conditions could optimize success rates of genetic analysis and save both time and financial cost in noninvasive genetic research.

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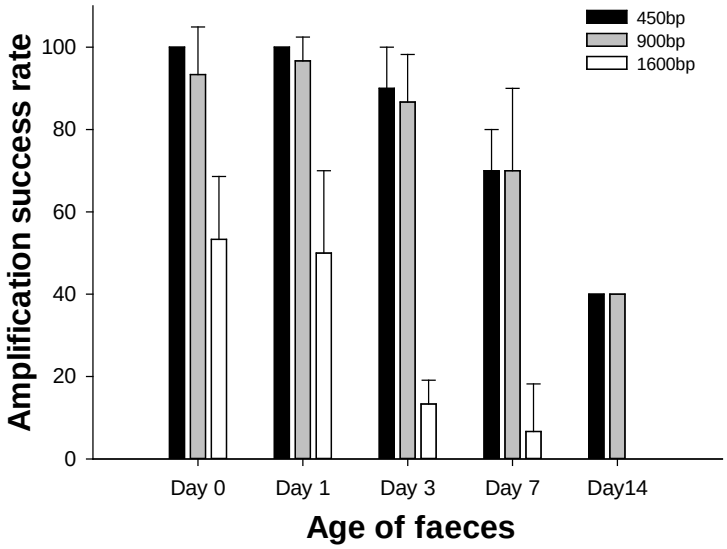
Figures

(a)



(b)

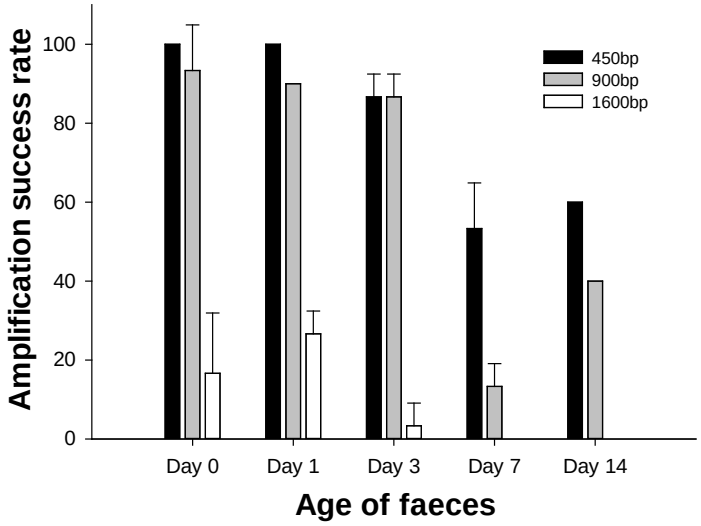


Fig. 3-1 The amplification success rate of faecal samples of different age kept (a) frozen and (b) under room temperature with different amplicon sizes.
Data are the average values of samples collected from 3 different sampling locations, i.e. from the surface, inside, and surface-inside mixture of faeces, except for the 14-day-old faeces, from which only a surface-inside mixture sample was taken.

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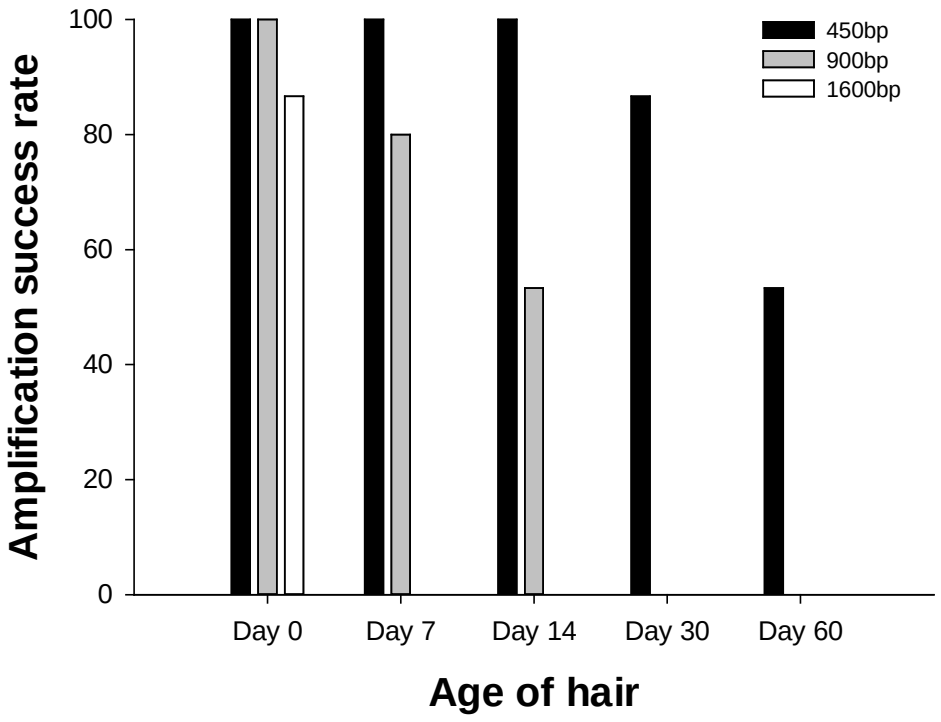

Fig. 3-2 The amplification success rate of DNA from different hair age with different amplicon sizes.

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Tables

Table 3-1 Comparisons of the effects of sampling locations on the PCR amplification success rates of faecal DNA collected from samples of different ages, stored by different methods and extracts for mtDNA control region fragments of different sizes.

S: samples from surface of faeces. I: samples from inside of of faeces, and M: samples from inside-surface mixture of faeces; F: frozen at -20 °C and R: kept at room temperature. *P*-values were the results of the Friedman test.

Age (day)	Storage Method	Amplification success rate (%)											
		450bp				900bp				1600bp			
		S	I	M	*P*-value	S	I	M	*P*-value	S	I	M	*P*-value
0	F	100(10/10)	100(10/10)	100(10/10)	0.607	100(10/10)	100(10/10)	80(8/10)	0.926	70(7/10)	40(4/10)	50(5/10)	0.145
1		100(10/10)	100(10/10)	100(10/10)		100(10/10)	90(9/10)	100(10/10)		50(5/10)	30(3/10)	70(7/10)	
3		90(9/10)	100(10/10)	80(8/10)		80(8/10)	100(10/10)	80(8/10)		10(1/10)	10(1/10)	20(2/10)	
7		60(6/10)	70(7/10)	80(8/10)		50(5/10)	70(7/10)	90(9/10)		20(2/10)	0(0/10)	0(0/10)	
14		-	-	40(4/10)		-	-	40(4/10)		-	-	0(0/10)	
0	R	100(10/10)	100(10/10)	100(10/10)	0.607	80(8/10)	100(10/10)	100(10/10)	0.717	30(3/10)	0(0/10)	20(2/10)	0.150
1		100(10/10)	100(10/10)	100(10/10)		90(9/10)	90(9/10)	90(9/10)		30(3/10)	30(3/10)	20(2/10)	
3		90(9/10)	80(8/10)	90(9/10)		90(9/10)	80(8/10)	90(9/10)		10(1/10)	0(0/10)	0(0/10)	
7		40(4/10)	60(6/10)	60(6/10)		20(2/10)	10(1/10)	10(1/10)		0(0/10)	0(0/10)	0(0/10)	
14		-	-	60(6/10)		-	-	40(4/10)		-	-	0(0/10)	

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Table 3-2 The PCR amplification success rates of different hair ages and amplicon sizes.

Age (day)	Amplification success rate (%)		
	450bp	900bp	1600bp
0	100(15/15)	100(15/15)	86.67(13/15)
7	100(15/15)	80(12/15)	0(0/15)
14	100(15/15)	53.33(8/15)	0(0/15)
30	86.67(13/15)	0(0/15)	0(0/15)
60	53.33(8/15)	0(0/15)	0(0/15)

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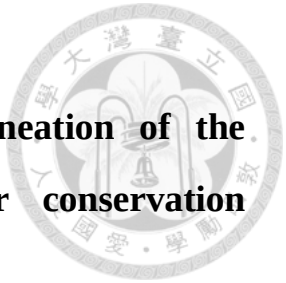

Chapter 4 Genetic comparison and subspecies delineation of the Asiatic black bears (*Ursus thibetanus*) and their conservation implications

4.1 Abstract

The Asiatic black bear (*Ursus thibetanus*) has a wide distribution in Asia. However, habitat degradation and poaching have caused a decline in its population and distribution throughout its geographical range. To develop proper conservation strategies for the species, one of the research priorities should be to examine the level of genetic variation within and among its subspecies and populations. To delineate the phylogenetic relationship and genetic status among Asiatic black bears, a mitochondrial phylogeny study was conducted based on partial mitochondrial DNA control region and its 5'-flanking region of bear specimens collected from Taiwan, mainland China, Russia, Vietnam, and Thailand. The mitochondrial DNA analyses indicated that the Japanese black bears (*U. thibetanus japonicus*) and the Formosan black bears (*U. thibetanus formosanus*) formed two distinct clades. The northeastern Asia population (*U. thibetanus ussuricus*) formed a group within the clade containing a mixture of bears from southwestern China (*U. thibetanus mupinensis*). And the bears from southeastern Asia were not monophyletic. In addition, ten tetramicrosatellite loci were employed to

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compare genetic variation among four subspecies of the Asiatic black bear, i.e. *U. thibetanus formosanus*, *U. thibetanus mupinensis*, *U. thibetanus ussuricus*, and *U. thibetanus thibetanus*. The population structure analysis indicated a clear subdivision scenario of these four subspecies. This study revealed the level of genetic variation among different populations of the Asiatic black bears and provided an explicit basis for subspecies identification, which is important for future *in situ* and *ex situ* conservation of the species.

4.2 Introduction

The Asiatic black bear (*Ursus thibetanus*) has a wide distribution in Asia spanning from Pakistan to Russian Far East of the Asian continent, and the surrounding islands, including Japan and Taiwan (Servheen et al. 1999; Wozencraft 2005; Garshelis and Steinmetz 2016). Habitat degradation and poaching have caused a decline in its population and distribution throughout its geographical range (Servheen et al. 1999; Ishibashi and Saitoh 2004; Hwang and Wang 2006; Kim et al. 2011; Garshelis and Steinmetz 2016). This medium-sized bear is threatened in much of its native habitat and has been listed as an Appendix I species by the Convention of International Trade Pact on Endangered Species of Wild Fauna and Flora (CITES) (CITES 2017).

There are seven subspecies of *U. thibetanus* have been recognized (Wozencraft

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2005), including *U. thibetanus ussuricus* inhabiting southern Siberia, northeastern China, and Korean peninsula, *U. thibetanus japonicus* inhabiting Japan, *U. thibetanus formosanus* inhabiting Taiwan, *U. thibetanus mupinensis* inhabiting southwestern China, *U. thibetanus laniger* inhabiting Himalaya area, *U. thibetanus gedrosianus* inhabiting Pakistan, and the nominate subspecies *U. thibetanus thibetanus*. However, the subspecies of Asiatic black bears can be recognized only in accordance with their geographic distribution (Wozencraft 2005). The morphological differences among these subspecies reported were from few specimens and description of these differences are vague (Hwang et al. 2008; Kitchener 2010). For examples, Heptner et al. (1998) distinguished *U. thibetanus ussuricus* from other subspecies by its largest skull measurements, pure black hair, and long fur. Hu (1995) compared the differences among *U. thibetanus thibetanus*, *U. thibetanus mupinensis*, and *U. thibetanus laniger* by vague descriptions of body sizes, length of hair, and the pattern of chest mark. Therefore, it is difficult to determine the origin of individual bear specimen based on its morphological characteristics.

According to O'Brien and Mayr (1991), subspecies is defined to include populations below the species level that share a distinct geographic distribution, a group of phylogenetically concordant characters, and a unique natural history relative to other

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subdivisions of the species. The information about the genetic status and genetic partitions of different subspecies and populations is important not only for the taxonomy but also for conservation of the Asiatic black bear, since the conservation strategy for this species needs to consider its subspecies and population status. For instance, if a population of a species is genetically distinctive by strong phylogenetic structuring of mtDNA variation and nuclear alleles from the others due to long-term evolutionary isolation, it should be treated as an 'evolutionarily significant unit' (Ryder 1986; Moritz 1994) and be managed separately due to a high priority for conservation (Moritz 1994; Crandall et al. 2000).

Some molecular phylogeographic studies on the genetic condition of *U. thibetanus* have been conducted. Yasukochi et al. (2009) analyzed the left domain of the mitochondrial control region (about 240 bp) from specimens of the Asiatic black bear in Japan and the Asian continent. The results indicated that the Japanese population formed a distinct clade from the Asian continental populations. In the study of Kim et al. (2011), the mitochondrial phylogeny based on DNA sequences (615 bp) of mitochondrial D-loop region among the Asiatic black bears in Japan, Southeast Asia (Vietnam), Russian Far East, and North Korea suggested that the Asiatic black bear populations from Russian Far East and North Korea form a single evolutionary unit

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distinct from populations from Japan and Southeast Asia. Wu et al. (2015) reconstructed a phylogenetic tree of mitochondrial D-loop sequences that included the Japanese population, the southern East Asian continental population (Chinese and Vietnamese population), and the northern East Asian continental population (Russian and North Korean population). Their resulting tree also indicated that the Japanese subspecies had diverged from other Asian black bears, and bears from other parts of East Asia, such as mainland China, Taiwan, and Korea, were intermingled with bears from Southeast Asia.

However, these previous phylogeographic studies of the Asiatic black bears mainly focused on the northern East Asian continental population in Russian Far East and North Korea (*U. thibetanus ussuricus*), the Japanese population (*U. thibetanus japonicus*), and bear specimens from Southeast Asia (mostly Vietnamese population, *U. thibetanus thibetanus*) and were based on mtDNA of maternal inheritance (Avise et al. 1987). Few bear specimens from southwestern China (*U. thibetanus mupinensis*) and Taiwan (*U. thibetanus formosanus*) have been analyzed. Therefore, the phylogenetic relationship of the Asiatic black bears in southern East Asian continental and Taiwan populations with populations in other Asia region remained unclear.

Clarification of the genetic status of the Asian black bear subspecies is important not only for advancing knowledge of the phylogeny but also for conservation of the

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species. The geographical information of some bear specimens in previous studies from this area may be controversial. For instance, Chu et al. (2000) analyzed the mtDNA control region and cytochrome b of the Asiatic black bears kept in the Taipei Zoo and found that the genetic composition of a few individuals that were known to originate from the Yushan area of Taiwan was different from others that were assumed to have come from different parts of Taiwan or even from other countries.

Thus, it is also a conservation priority to clarify the uncertain genetic status of Asiatic black bears, especially populations in the southern East Asian continent, such as *U. thibetanus mupinensis*, and Taiwan for better management of both *in situ* and *ex situ* populations.

The aims of this study was, therefore, to delineate the phylogenetic relationship and genetic status among Asiatic black bears, particularly populations from the southern East Asia continent and Taiwan, by examining partial mitochondrial DNA (mtDNA) control region and its 5'-flanking region (partial cytochrome b, tRNA-Thr, and tRNA-Pro) and ten tetramicrosatellite loci of specimens collected from various parts of Taiwan, southwestern China, Southeast Asia (mainly Vietnam and Thailand), as well as northeastern China and Russia.

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4.3 Materials and methods

4.3.1 Sample collection and genomic DNA extraction

In this study, we collected different sample types of Asiatic black bear specimens, including DNA, tissue, hair, fur, and feces from Taiwan, southwestern China, and other Asia regions. For validly delineating the phylogenetic relationship among Asiatic black bears, only voucher specimens from wild individuals or from captive bears verified as wild-born from a specific geographic locale (defined as Luo et al. (2004)) of *U. thibetanus formosanus* and *U. thibetanus mupinensis* were used in this study. Although the bear specimens of Vietnam, Thailand, northeastern China and Russia are with limited information of the exact original locality, it was certain that the specimens are from native animals based on those countries by collectors' records.

After excluding fecal specimens from identical bear by the microsatellite analysis, a total of 77 samples of individual bears could be successfully sequenced their mtDNA control region fragment and were used in the following analyses (Fig. 4-1 and Table 4-1), including 46 samples from Taiwan (42 samples from central and southern Taiwan, and four samples were from northern Taiwan) (Fig. 4-2), 16 samples from the southwestern China (Sichuan), one sample from the northeastern China (Liaoning), five samples from Russia, and nine samples from Southeast Asia (Vietnam, eight and

doi:10.6342/NTU201800373

Thailand, one) (Fig. 4-1). Of these samples, seven were collected in the form of genomic DNA, 20 were blood samples, six were hair, three were fur, and 41 were faeces. These samples were presumably labeled as the following subspecies based on their geographical location: *U. thibetanus formosanus* (from Taiwan), *U. thibetanus mupinensis* (from Sichuan of China), *U. thibetanus ussuricus* (from the northeastern China and Russia), and *U. thibetanus thibetanus* (from Vietnam and Thailand).

In addition, other mitochondrial D-loop sequences of 20 haplotypes (119 bears) of *U. thibetanus japonicas*, 26 bears of *U. thibetanus ussuricus*, 14 bears of *U. thibetanus thibetanus*, and a sequence of *U. thibetanus mupinensis* (from Yunnan of China) from previous studies (Ishibashi and Saitoh 2004; Yu et al. 2007; Hwang et al. 2008; Choi et al. 2010; Kim et al. 2011) (Table 4-2) were employed in the overall phylogenetic analyses. One sequence of American black bear (*U. americanus*) (GenBank Accession, AF303109) (Delisle and Strobeck 2002) was also included as outgroup. Lengths of these sequences are from 615 bp to 706 bp (Table 4-2).

Total genomic DNA from blood and fur was extracted using a standard proteinase K digestion and phenol-chloroform extraction procedure (Kocher et al. 1989; Sambrook et al. 1989). Faecal samples were extracted with methods detailed in Hung et al. (2004), which was modified from a hexadecyltrimethylammonium bromide (CTAB)-based

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extraction (Parsons et al. 1999). Hair DNA extractions were carried out by the traditional phenol-chloroform procedure (Kocher et al. 1989).

4.3.2 mtDNA DNA amplification and sequencing

According to Matsuhashi et al. (1999) and Ishibashi and Saitoh (2004), there is a variable region on the 5' side of mitochondrial control region and its 5'-flanking region. This mitochondrial DNA fragment have been used in a lot of previous bear phylogenetic studies (Matsuhashi et al. 1999; Ishibashi and Saitoh 2004; Ohnishi et al. 2009; Yasukochi et al. 2009; Kim et al. 2011; Wu et al. 2015). In this study, all extracts of bear samples were first amplified for about 900 bp of this highly variable mtDNA control region and its 5'-flanking region (partial cytochrome b, tRNA-Thr, and tRNA-Pro) using polymerase chain reaction (PCR) with a PCR primer pair: CB-Z, 5'-ATGAATTGGAGGACAACCAGT-3' (Matsuhashi et al. 1999) and Ut-Dr, 5'-TGCGTACATATGCGTACATAT-3' (designed based on sequences of the Asiatic black bears in Shih et al. (2017)) (Fig. 4-3). PCR amplifications were carried out using the ASTEC Thermal Cycler PC-808 or Thermal Cycler PC-818 in a total volume of 50 μL reaction mixture containing: 1 μL of DNA extract, 1× PCR buffer (including 1.5mM $MgCl_2$), 0.5μM of each primer, 200μM dNTP and 0.5 U of Taq DNA polymerase (Supertherm Taq, JMR). The PCR thermal profile included an initial denaturation of 10

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min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 ˚C, and a postcycling final extension at 72 °C for 10 min. All amplifications included a negative control without template DNA to test contamination and a positive control to confirm proper PCR conditions. Each PCR product was electrophoresed on 1% agarose gels and visualized using ethidium bromide staining under UV light to score the amplified fragment length.

After purified with the HiYield Gel/PCR DNA Fragments Extraction Kit (RBC Bioscience, Taipei, Taiwan), PCR products were used as template for direct sequencing. Sequences of all PCR products were obtained in both directions with the same primers, CB-Z and Ut-Dr in PCR amplification by the multiple fluorescent dyes method using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). In addition, another primer UT-1, 5'-TGATCACCAGGCCTCGAGAAA-3' (Ishibashi and Saitoh 2004) was also used for sequencing (Fig. 4-3). All sequences were manually inspected and a consensus sequence of each sample was generated using the program SeqMan of DNAStar software (DNA STAR, Inc., Madison, WI, USA). Consequently, for the purpose of phylogenetic analysis, sequences in our study were cut to about 700bp to encompass the majority of the published sequence data (Ishibashi and Saitoh 2004; Ohnishi et al. 2009)(Table 4-2).

doi:10.6342/NTU201800373

DNA samples which could be successfully sequenced mtDNA target fragment were then genotyped for microsatellite loci. The samples that could not be amplified for mtDNA fragment were considered invalid and excluded from further analyses.

4.3.3 Microsatellite Genotyping

For microsatellite analysis, ten tetranucleotide polymorphic microsatellite loci (UT1, UT3, UT4, UT23, UT25, UT29, UT31, UT35, UT36, and UT38) originally developed for the Formosan black bear (*U. thibetanus formosanus*) were amplified by PCR using those PCR primer pairs described in Shih et al. (2009). In preliminary test, we also amplified the other six dinucleotide microsatellite loci (G10L, G10M, G10X, MSUT2, MSUT6, and MSUT7) designed for the American black bear and Asiatic black bear (Paetkau et al. 1995; Kitahara et al. 2000), but the genotyping data of those loci were not used in further microsatellite analyses because of the stutter bands and scoring ambiguity that usually happen with dinucleotide loci (Hung et al. 2004).

PCRs were carried out using the ASTEC Thermal Cycler PC-808 or Thermal Cycler PC-818 in 10-μL reaction volumes containing 1× PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.05 μM of tailed forward primer, 0.12 μM of reverse primer, 0.18 μM of fluorescent-labelled M13 or CAG-tag primer that were labeled with HEX, FAM, or TAMRA fluorescent dyes, 0.2 U Taq DNA polymerase (Biotech), and around

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30 ng genomic DNA. The PCR thermal profile was 95 °C for 4 min, then 40 cycles at 95 °C for 30 s, 30 s at the optimal annealing temperature of each primer pair according to Shih et al. (2009) and 72 °C for 20 s, followed by a final extension at 72 °C for 7 min. Each PCR amplification run included 2 negative controls of only sterile water and PCR reagents without DNA template to check for contamination.

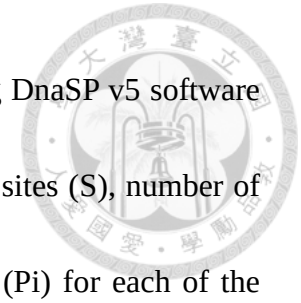
PCR products were electrophoresed in a MegaBACE 1000 autosequencer (Amersham Biosciences). Sizes of alleles were scored with the analysis software GENETIC PROFILER 2.0 (Amersham Biosciences). Consensus genotypes were constructed using scoring data obtained from 3 or more genotypes (Hedmark and Ellegren 2006).

4.3.4 Data analysis

mtDNA sequence analysis

Sequences (about 700bp) were aligned using the software MEGA7 (Kumar et al. 2016) with published sequences from 20 haplotypes (119 bears) of *U. thibetanus japonicas*, 26 bears of *U. thibetanus ussuricus*, 14 bears of *U. thibetanus thibetanus*, a sequence of *U. thibetanus mupinensis*, and a sequence of American black bear (*U. americanus*) (Delisle and Strobeck 2002; Ishibashi and Saitoh 2004; Yu et al. 2007; Hwang et al. 2008; Choi et al. 2010; Kim et al. 2011) (Table 4-2).

doi:10.6342/NTU201800373



Preliminary analysis of genetic diversity was conducted using DnaSP v5 software (Librado and Rozas 2009) to calculate the number of polymorphic sites (S), number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (Pi) for each of the subspecies. According to the definition of Ishibashi and Saitoh (2004), the numbers of Ts at a T-repeat site in target mtDNA control region sequences were used in defining different haplotypes but the T-repeat site variation was not taken into account in calculation of genetic diversity (Ishibashi and Saitoh 2004). The genetic distances within and among subspecies and the outgroup species *U. americanus* were calculated using MEGA7 with the Kimura 2-parameter model (Kimura 1980).

Phylogenetic reconstructions among unique mtDNA haplotypes were assessed using three approaches, neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI). NJ and ML analyses were conducted using PAUP* version 4 beta (Swofford 2001) with the best fitting model of sequence evolution, TIM3+I+G model determined by the Akaike information criterion (AIC) in jModeltest 2.1.5 (Posada 2008). The nodal support was assessed by 1000 bootstrap replicates with NJ option for NJ and heuristic search for ML. In addition, BI analyses were inferred using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with the HKY+I+G model, as selected under the Bayesian information criterion (BIC) in jModeltest 2.1.5. Two

doi:10.6342/NTU201800373

independent runs with 4 Markov chains were performed for 2,000,000 generations and assigned sampling frequency an every 100 generations. About 25% of sampling trees were discarded (the burn-in step) after estimating with a conservative approach. Then a consensus tree was calculated using the remaining 30,002 trees (which log-likelihoods converged to stable values). Bayesian posterior probabilities (BPP) were used to measure the nodal support of BI tree. Trees were routed with outgroup species *U. americanus*.

Microsatellite analysis

The allelic richness (number of alleles), observed (H_O) and expected (H_E) heterozygosity for each locus were calculated for the measurement of genetic polymorphism in each of the four subspecies, *U. thibetanus formosanus*, *U. thibetanus mupinensis*, *U. thibetanus ussuricus*, and *U. thibetanus thibetanus* using the software CERVUS 3.0 (Kalinowski et al. 2007). For both observed and expected heterozygosity, mean heterozygosity was obtained as an arithmetic average of heterozygosities at all loci.

Tests for departure from Hardy–Weinberg equilibrium (HWE) of each locus were performed using the web version of GENEPOP 4.2 (http://genepop.curtin.edu.au/) (Raymond and Rousset 1995; Rousset 2008). The software MICRO-CHECKER (Van

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Oosterhout et al. 2004) was used to check the presence of allele dropout and null alleles in all loci. The data of loci deviating from HWE or with large allele dropout and null alleles were not included in the later analysis.

A Bayesian clustering analysis of STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003) was applied to assess population structure and to test the most possible subdivision number of microsatellite data set. The number of genetically distinct clusters (K) was set from 1 to 10. Runs were conducted under the admixture model for ten independent simulations for each K with correlated allele frequencies using an MCMC method with 1,000,000 iterations and burn-in of 10,000 generations. The most likely number of clusters (K) was determined depending on the log of the posterior probability of data [LnP(K)] and the average rate of change (ΔK) for each value of K as described by Evanno et al. (2005) and implemented in the software Structure Harvester v.6.93 (Earl and vonHoldt 2012).

4.4 Results

4.4.1 Genetic diversity of the mtDNA control region

The mtDNA control region and its 5'-flanking region sequences of 77 Asiatic black bears in this study were 703 to 706 bp in length because of the T-repeat number variation (Table 4-3). The base substitutions and the T-repeat number variation defined a

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total of 21 mtDNA haplotypes, including five haplotypes from *U. thibetanus formosanus*, six haplotypes from *U. thibetanus mupinensis*, three haplotypes from *U. thibetanus ussuricus*, and seven haplotypes from *U. thibetanus thibetanus* (Table 4-3). The mtDNA control region and its 5'-flanking region sequences of 77 Asiatic black bears in this study had been deposited to GenBank as accession numbers MG004595-MG004671 (Table 4-3).

The results of genetic diversity analysis by DnaSP v5 in five subspecies of the Asiatic black bear (based on mtDNA control region fragments in this study and sequences reported from previous studies, Table 4-2) showed that the numbers of polymorphic sites of the species ranged from 4 to 42 and the numbers of haplotypes defined by the base substitutions and the T-repeat-number variation were from 5 to 20 (Table 4-4). Among the five Asiatic black bear subspecies, *U. thibetanus formosanus* from Taiwan had the least number of polymorphic sites, haplotype diversity, and nucleotide diversity (4, 0.205, and 0.00053) (Table 4-4). *U. thibetanus thibetanus* from southeast Asia showed the highest genetic diversity level, the number of polymorphic sites, haplotype diversity, and nucleotide diversity were 42, 0.972, and 0.01706, respectively (Table 4-4).

doi:10.6342/NTU201800373

4.4.2 Genetic distance of subspecies

The results of genetic distances calculated by MEGA7 indicated that within subspecies distance of *U. thibetanus formosanus* was the shortest (0.00034) and *U. thibetanus thibetanus* showed the highest value (0.01660) (Table 4-4). As for pairwise distance among subspecies, the distance between *U. thibetanus ussuricus* and *U. thibetanus mupinensis* was the shortest (0.00293) and the distances between black bear in Japan and other subspecies were the longest (from 0.04415 to 0.04793) (Table 4-4).

4.4.3 Phylogenetic relationship of mtDNA haplotypes

The neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) phylogenetic trees inferred from mtDNA haplotypes showed congruent topologies (Fig. 4-4, Fig. 4-5, and Fig. 4-6). All three trees highlighted the southeastern Asian subspecies *U. thibetanus thibetanus* to have complex topology. Some haplotypes of *U. thibetanus thibetanus* were placed at the basal position of tree topology (as American black bear was used as outgroup) and the others intermingled with bears of other subspecies in later clades. In addition, the East Asian continent subspecies (*U. thibetanus mupinensis* and *U. thibetanus ussuricus*), the Japan subspecies (*U. thibetanus japonicas*), and Taiwan subspecies (*U.thibetanus formosanus*) formed three monophyletic clades which coincided with their geographic distribution. In the East

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Asian continent clade (79% NJ bootstrap support and 1.00 BI BPP support), most haplotypes of *U. thibetanus ussuricus* formed a distinct group. The monophyletic clade of Japan subspecies was supported with both 100% NJ and ML bootstrap values and 1.00 BI BPP support. All haplotypes of Taiwan subspecies formed a distinctive monophyletic group (79% NJ bootstrap, 73% ML bootstrap and 1.00 BI BPP support). Among the five unique haplotypes of Formosan black bears, two of them were from northern Taiwan and the other three haplotypes are from central and southern regions. Meanwhile, a *U. thibetanus mupinensis* haplotype, CWG2 from Yu et al. (2007) fell into the basal position of tree topology intermingling with the southeastern Asian subspecies *U. thibetanus thibetanus* in all 3 trees.

4.4.4 Genetic diversity of microsatellite loci

Of 77 bear samples used for mtDNA phylogenetic analysis, 71 samples that could be genotyped for more than seven in ten tetranucleotide microsatellite loci were used in further microsatellite analyses (Fig. 4-1 and Fig. 4-2). The allelic richness, observed heterozygosity (H_O), and expected heterozygosity (H_E) in each loci of the four subspecies, *U. thibetanus formosanus*, *U. thibetanus mupinensis*, *U. thibetanus ussuricus*, and *U. thibetanus thibetanus* are given in Table 4-5. Average alleles per locus (mean of ten loci examined) were 12 for *U. thibetanus formosanus*, 10.9 for *U.*

doi:10.6342/NTU201800373

thibetanus thibetanus, 13.6 for *U. thibetanus mupinensis*, and 5.9 for *U. thibetanus ussuricus* (Table 4-5). The numbers of alleles were higher in *U. thibetanus formosanus* and *U. thibetanus mupinensis*. In the calculation of ten loci, both of the average H_O and H_E of *U. thibetanus thibetanus* (0.772 and 0.938) were the highest among these four subspecies (Table 4-5). Departure from Hardy–Weinberg equilibrium was found at three loci (UT3, 4, 38) for *U. thibetanus formosanus*, three loci (UT1, 3, 23) for *U. thibetanus thibetanus*, three loci (UT3, 23, 31) for *U. thibetanus mupinensis*, and at UT23 for *U. thibetanus ussuricus* (Table 4-5). In addition, there was no evidence for large allele dropout detecting by MICRO-CHECKER in all ten loci. The data of UT3 and UT23 were not included in the STRUCTURE analysis due to the deviation from HWE in most of subspecies in analysis.

4.4.5 Subdivision of subspecies from STRUCTURE

Results of STRUCTURE analysis and Structure Harvester in microsatellite data showed that the five subdivision scenario (K=5) had the highest posterior probability (mean Ln = -2575.43) and higher ΔK value ($\Delta K = 34.4186$) (Fig. 4-7). These five subdivisions were *U. thibetanus mupinensis*, *U. thibetanus ussuricus*, *U. thibetanus thibetanus*, and two subdivisions of *U. thibetanus formosanus* (Fig. 4-8). The results also showed that there was gene flow between *U. thibetanus mupinensis* and *U.*

doi:10.6342/NTU201800373

thibetanus thibetanus.

In addition, 46 Asiatic black bears of Taiwan subspecies were sequenced the mtDNA control region and its 5'-flanking region sequences in this study and five haplotypes were defined (Fig. 4-4, Fig. 4-5 and Fig. 4-6). Two haplotypes, TW14 and TW17 were from bears in Lala Mountain Reserve and Shei-Pa National Park of northern Taiwan (Fig. 4-2), and other three haplotypes were from bears of central and southern Taiwan. In these 46 bear specimens, 41 specimens were genotyped for more than seven in ten tetranucleotide microsatellite loci and were used in STRUCTURE analysis with bear specimens from other subspecies. Results of STRUCTURE analysis suggested two subdivisions of *U. thibetanus formosanus* (Fig. 4-8). One of these two subdivisions indicated that eight bear specimens from central and southern Taiwan, and others were from northern, central and southern Taiwan. These two subdivisions did not show a geographical pattern.

4.5 Discussions

The results of phylogeographic analysis of mtDNA haplotypes indicate a good agreement with the subspecies classifications of the Asiatic black bears reported by some previous studies (Hwang et al. 2008; Yasukochi et al. 2009; Kim et al. 2011; Wu et al. 2015). According to all of the NJ, ML, and BI phylogenetic trees, the Asiatic black

doi:10.6342/NTU201800373

bears in Japan were genetically a different clade from bears of other areas of Asia in their mtDNA characters (Fig. 4-4, 4-5 and 4-6). Even though combined with mtDNA haplotypes of the *U. thibetanus mupinensis* as the East Asian continent clade, most haplotypes of *U. thibetanus ussuricus* formed a distinct group as well (Fig. 4-4, 4-5 and 4-6). In addition, the result of STRUCTURE analysis of microsatellite data in our study also indicated a robust subdivision between *U. thibetanus ussuricus* from Russia and other three Asiatic black bear subspecies (Fig. 4-8). Furthermore, our tree topology supports the results of Kim et al. (2011) and Wu et al. (2015) indicating the independent evolutionary history of *U. thibetanus japonicas* and *U. thibetanus ussuricus*, although the geographic distance of these two subspecies is short. However, in contrast to the result of Japanese population at the basal position of tree topology in Wu et al. (2015), our results, which were based on more sample sizes from different areas, showed that it is the southeastern Asian subspecies *U. thibetanus thibetanus* at the basal position of tree topology.

The genetic data in this study also supported that *U. thibetanus formosanus* from Taiwan was distinctly different from other Asiatic black bear subspecies. MtDNA analysis showed that the Formosan black bears were monophyly (Fig. 4-4, 4-5 and 4-6) and microsatellite analysis also supported the subdivision of Taiwan population scenario

doi:10.6342/NTU201800373

(Fig. 4-8). Wu et al. (2015) indicated that the black bear from Taiwan is part of the southern East Asian continental population. However, their conclusion was tentative on only one bear specimen from Taiwan. Our study is the first to use sufficient sample size of the Formosan black bears ranging from various parts of Taiwan, and provide clear genetic evidence for the subspecies designation of *U. thibetanus formosanus* from the Asian continental populations. Based on our results, this island bear population should be regarded as an evolutionarily significant unit (ESU). Both *in situ* and *ex situ* management strategies for this bear subspecies should concern about the genetic information and subspecies delineation clarified in this study. It is highly recommended that the genetic status of the captive population should be closely examined and the non-native bears should be removed from breeding program to avoid genetic introgression of smuggling animals of other subspecies.

In addition, habitat degradation and poaching have caused a decline in the population of *U. thibetanus formosanus* (Wang 1990, 1999; Hwang and Wang 2006). The declining population and the low genetic diversity in comparison to other subspecies (Table 4-4), with only five mtDNA haplotypes, may reduce the potential for adaptation and increase the risk of local extinction of this endemic subspecies in the future due to genetic and demographic factors (Frankham et al. 2002). Microsatellite

doi:10.6342/NTU201800373

data seem to indicate that there are subdivisions within the Formosan black bear population (Fig. 4-8). However, these two subdivisions did not show a geographical pattern. Due to the limited number of bear specimens from northern Taiwan, and the significant deviation of three of ten loci from Hardy–Weinberg equilibrium, which probably due to the genetic drift driven by Formosan black bear's small population size, our inclusion of no genetic differentiation in *U. thibetanus formosanus* warrant further study.

Similar to the findings of Kim et al. (2011), we found that the Asiatic black bears in Southeast Asia, *U. thibetanus thibetanus* appeared to have the highest genetic diversity (Table 4-4) and had complicated subpopulation structure according to complex tree topology on mtDNA haplotypes (Fig. 4-4, 4-5, 4-6). Due to the basal position and the complexity of *U. thibetanus thibetanus* in tree topology, we agree with the inference of Kim et al. (2011) and Wu et al. (2015) that the ancestral distribution area of Asiatic black bear is Southeast Asia. However, because few genetic analyses have been conducted on the west continental bear populations (i.e., *U. thibetanus laniger* and *U. thibetanus gedrosianus*), this inference of bears' ancestral distribution should be tentative based on present studies.

In *U. thibetanus mupinensis*, the subspecies status is not well defined. The

doi:10.6342/NTU201800373

haplotypes of *U. thibetanus mupinensis* combined with the *U. thibetanus ussuricus* as the East Asian continent clade (Fig. 4-4, 4-5, 4-6) and the microsatellite data supported that there was gene flow between *U. thibetanus mupinensis* and *U. thibetanus thibetanus* (Fig. 4-8). This result is similar to the finding of Hwang et al. (2008) which indicating a split tree topology of two *U. thibetanus mupinensis* populations. We infer the gene flow between *U. thibetanus mupinensis* and *U. thibetanus thibetanus* is derived from their continuous geographical range. It should be noted that, a haplotype of *U. thibetanus mupinensis*, CWG2 was fell in basal position of tree topology with other *U. thibetanus thibetanus* haplotypes (Fig. 4-4, 4-5, 4-6). This haplotype was from the study of Yu et al. (2007) with unknown geographical information. This unreasonable result perhaps is a consequence of limited information of sample locality. It also reveals the importance of voucher specimens in genetic analysis.

In conclusion, our study demonstrated the clear genetic subspecies designation of the Formosan black bears and the Japanese black bears. Therefore, the conservation policies for endangered Asiatic black bears in these areas should concern about the genetic information revealed in this study and consider these endemic bear populations as different unique evolutionarily significant units. For *ex situ* management strategies, a comprehensive genetic assessment of the captive population is needed. Moreover, the

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possible substructure in *U.thibetanus thibetanus* and *U.thibetanus formosanus* and the unwell defined subspecies status of *U. thibetanus mupinensis* arising from this study remain unanswered. To clarify remaining uncertainties of the evolutionary history of Asiatic black bears and to inform management strategies in *U.thibetanus thibetanus*, *U.thibetanus formosanus*, and *U. thibetanus mupinensis*, more extensive genetic analyses on voucher specimens with geographical variation are necessary in the future.

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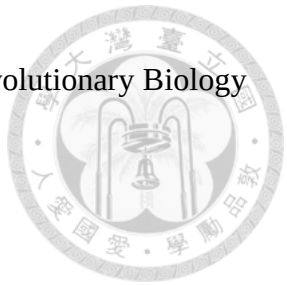

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Figures

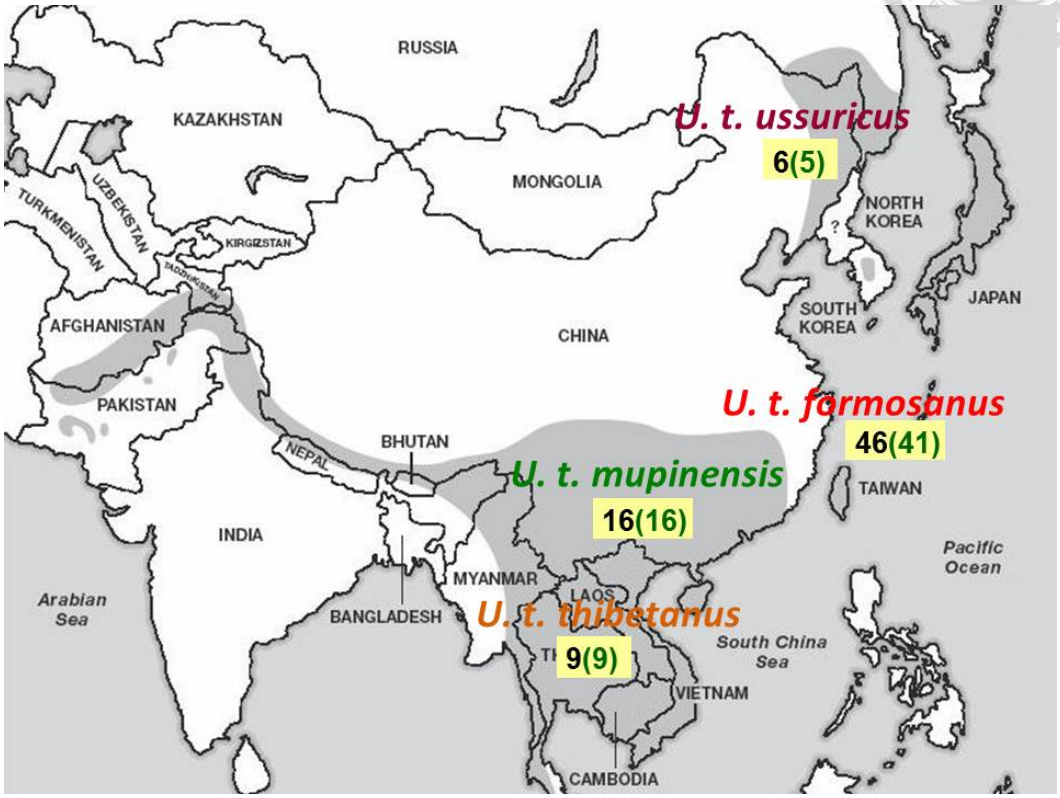

Fig. 4-1 Samples distribution of *Ursus thibetanus* corresponding to traditional subspecies designation.

The numbers under subspecies names indicated bear sample sizes used for mtDNA analyses and microsatellite analyses (in parentheses) examined in this study. The background map revealed geographic distribution of Asiatic black bears (Servheen et al., 1999).

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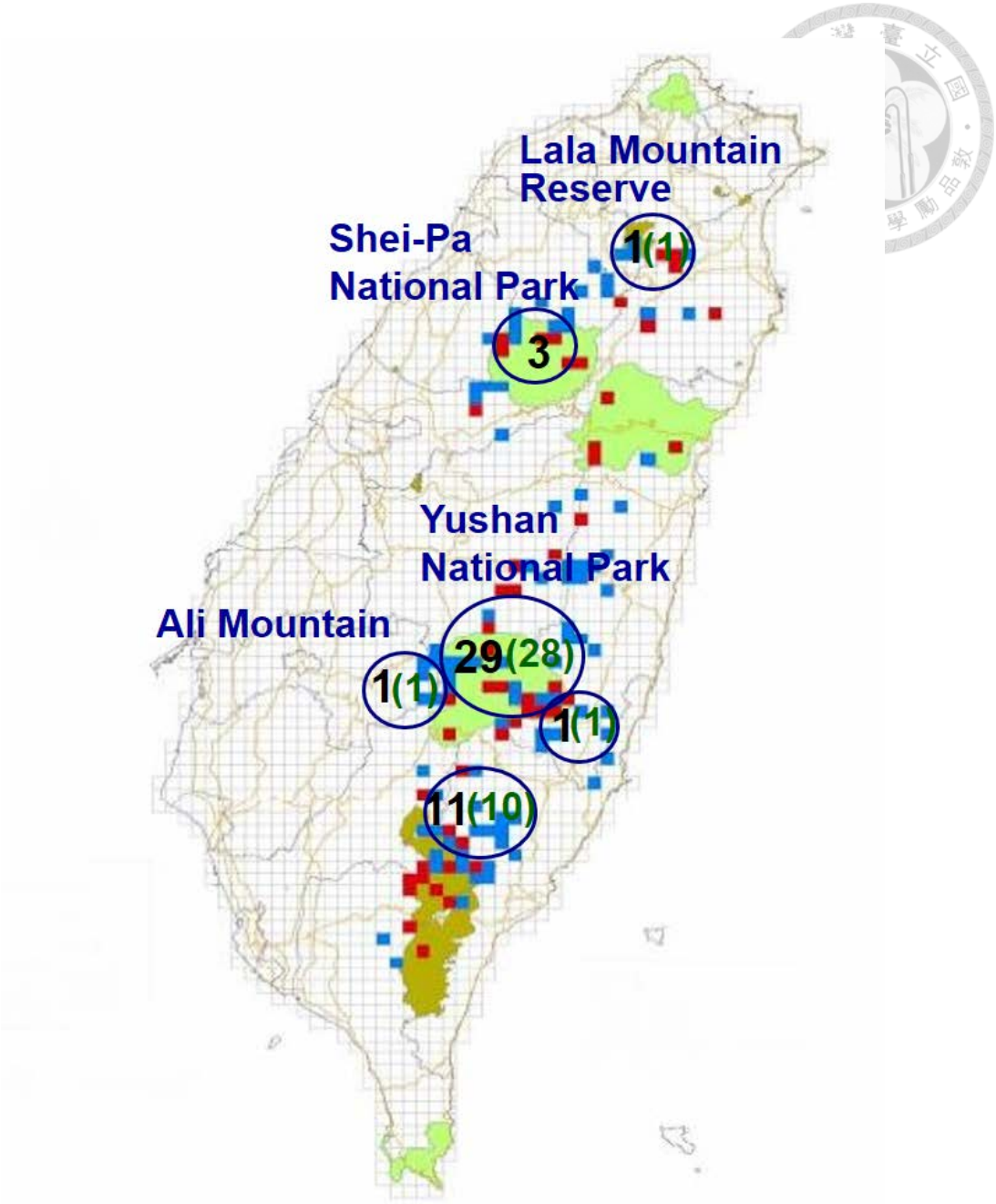

Fig. 4-2 Samples distribution of *U.thibetanus formosanus* in Taiwan.
The numbers in different map areas indicated bear sample sizes used for mtDNA analyses and microsatellite analyses (in parentheses). The background map of geographic distribution of Formosan black bears was from Hwang and Wang (2006).

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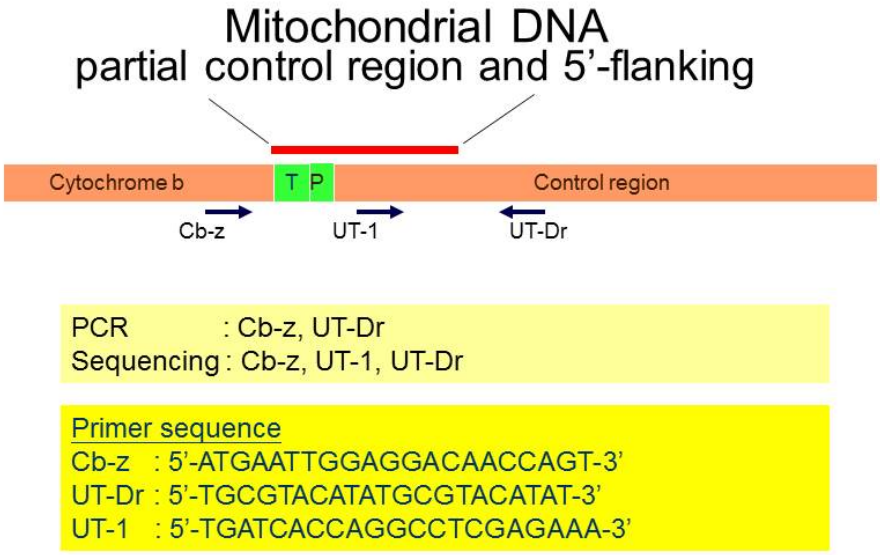

Fig. 4-3 The primer frame for mtDNA partial control region and its 5'-flanking region. Arrows indicated positions of primers using in PCR and sequencing. T, threonine-tRNA gene; P, proline-tRNA gene.

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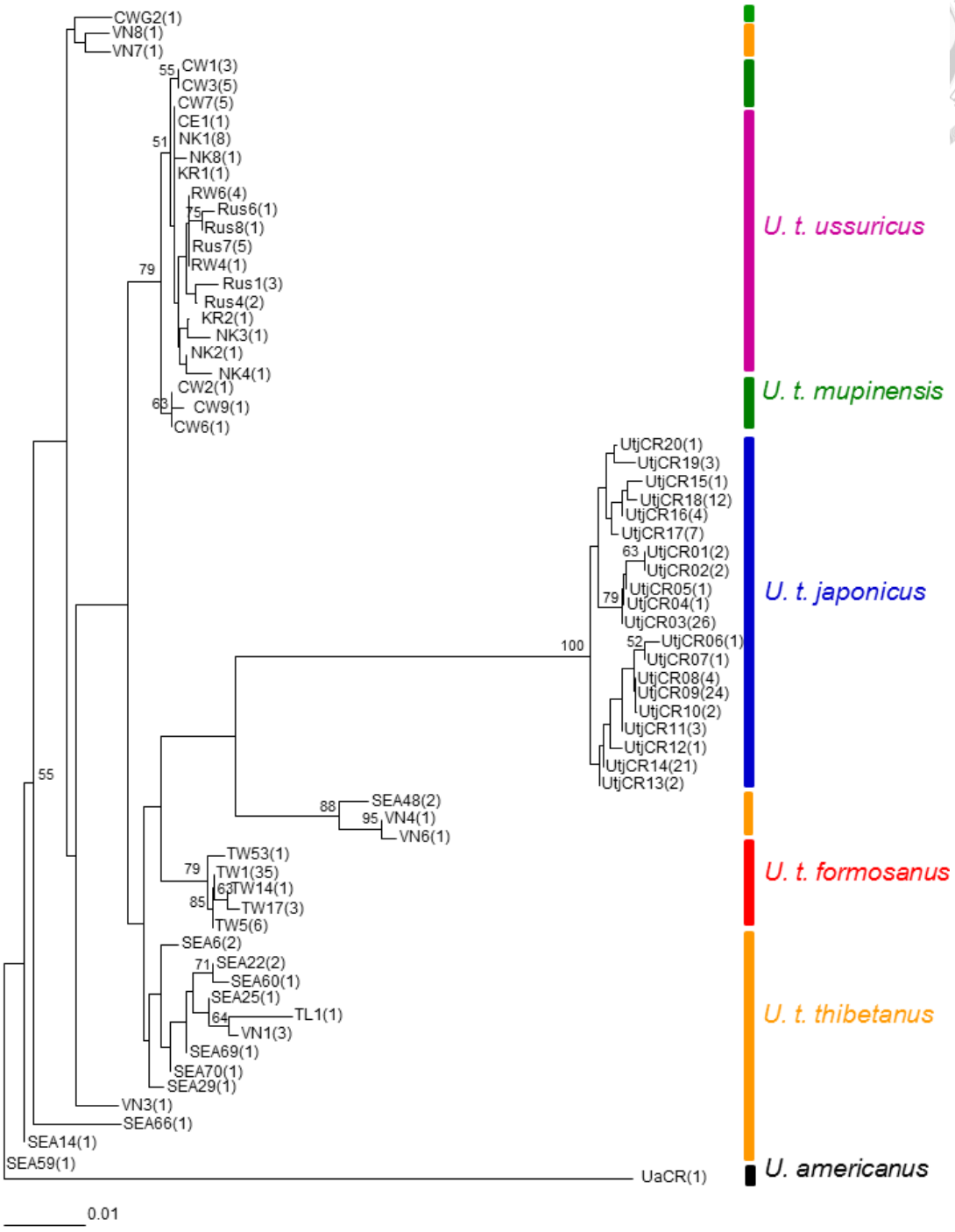

Fig. 4-4 Phylogenetic relationships based on neighbor-joining analysis among the Asiatic black bear mtDNA haplotypes.

Bootstrap supports were provided above branch at node for the divergence of lineages. The bear sample sizes with each haplotype were showed in parentheses.

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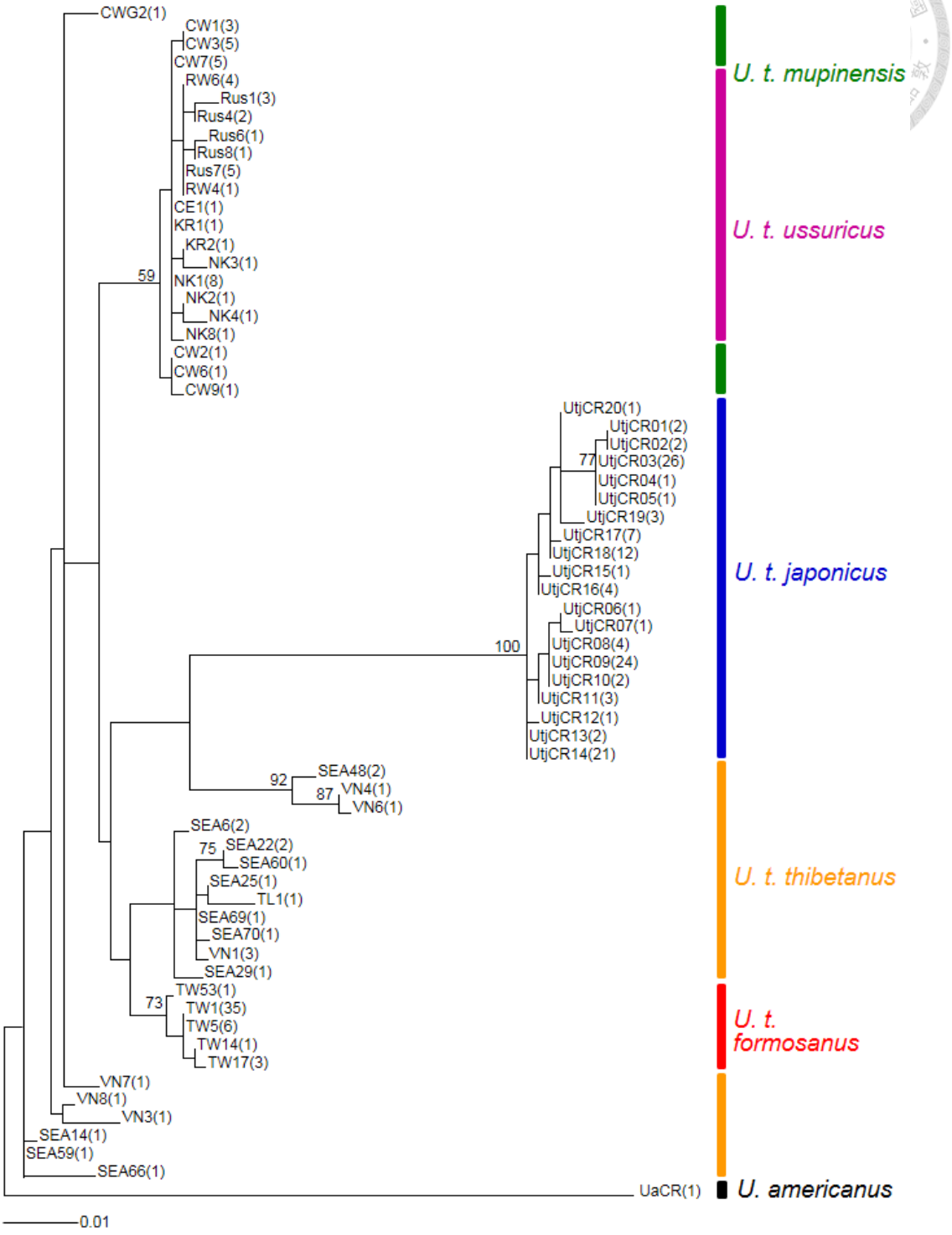

Fig. 4-5 Phylogenetic relationships based on Maximum Likelihood analysis among the Asiatic black bear mtDNA haplotypes.

Bootstrap supports were provided above branch at node for the divergence of lineages. The bear sample sizes with each haplotype were showed in parentheses.

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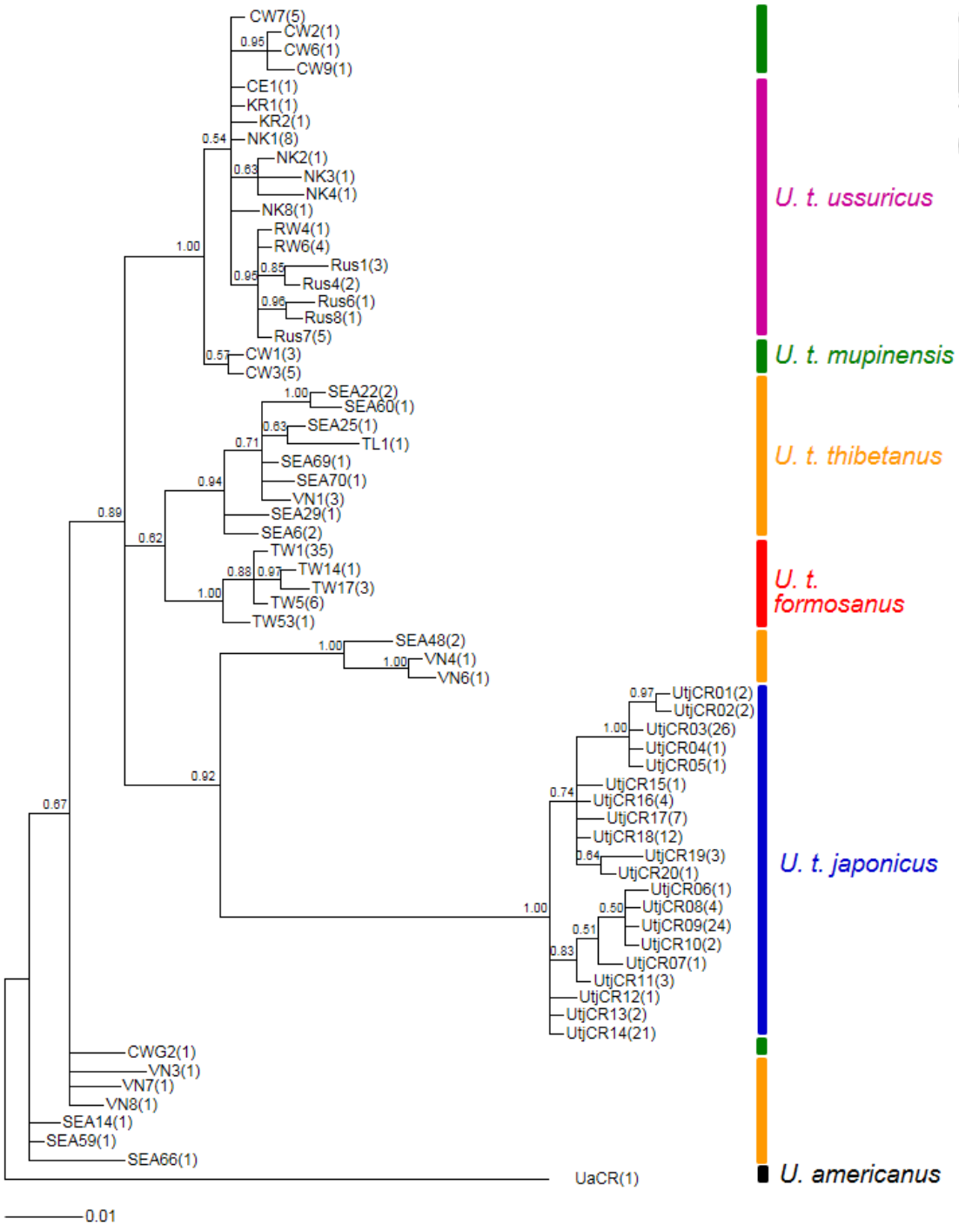

Fig. 4-6 Phylogenetic relationships based on Bayesian inference analysis among the Asiatic black bear mtDNA haplotypes.

Bayesian posterior probabilities were provided above branch at node for the divergence of lineages. The bear sample sizes with each haplotype were showed in parentheses.

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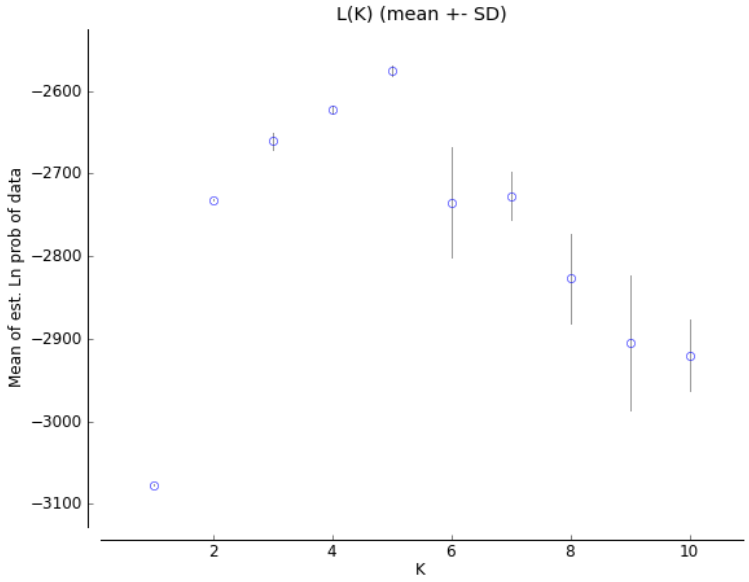
(a)



(b)

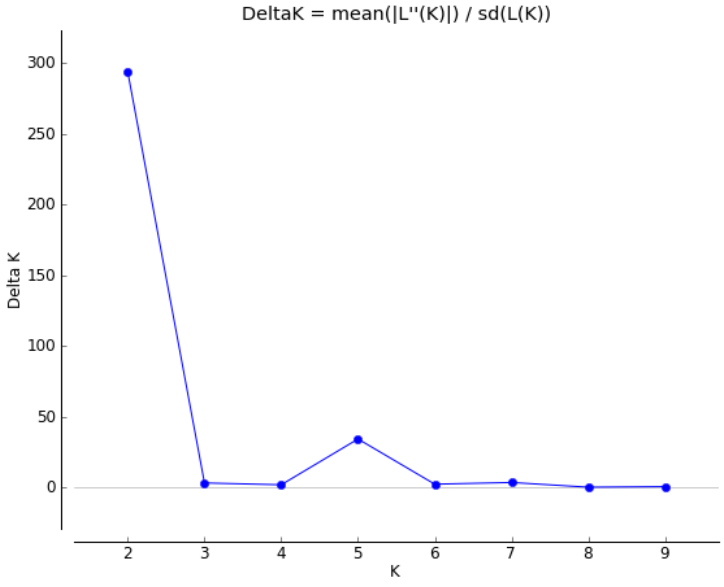


Fig. 4-7 The log of the posterior probability [LnP(K)] and the average rate of change (Delta K, ΔK) for each value of K based on microsatellite data of 4 subspecies of Asiatic black bears.

(a) Mean estimated LnP(K) of possible clusters (K) from 1 to 10; (b) ΔK based on rate of change of LnP (K) between successive K values.

doi:10.6342/NTU201800373

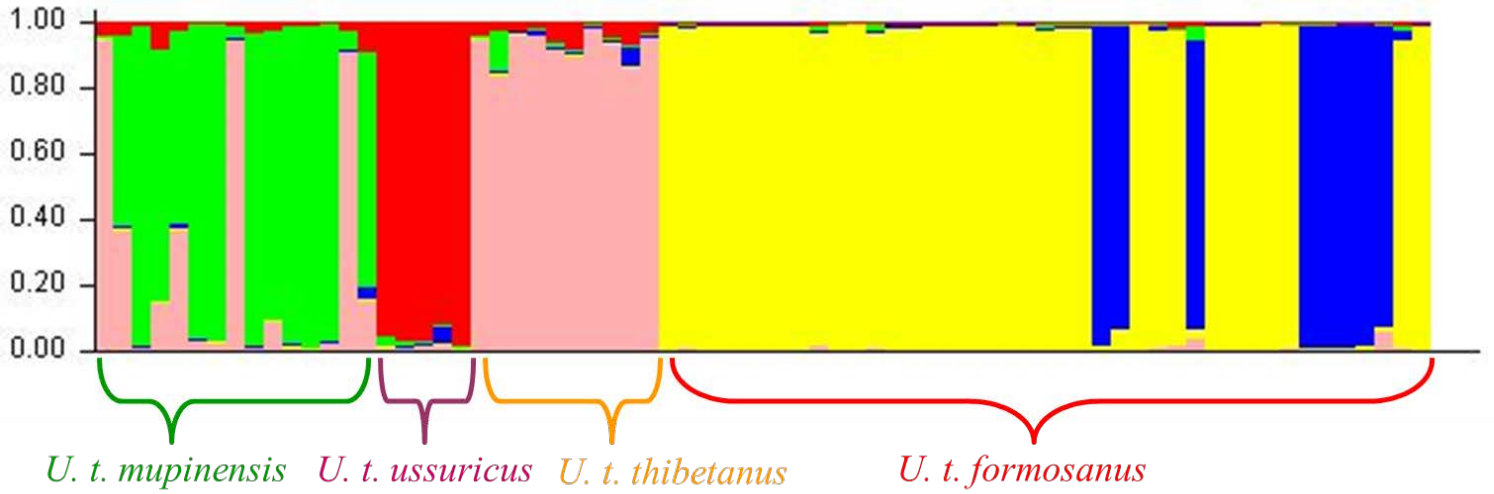


Fig. 4-8 Bayesian population genetic structure of 4 subspecies of Asiatic black bears, including *U. thibetanus formosanus*, *U. thibetanus mupinensis*, *U. thibetanus ussuricus*, and *U. thibetanus thibetanus* using STRUCTURE 2.3.4.
Bar plot showed the population structure of clustering result with the highest posterior probability, K=5.

doi:10.6342/NTU201800373

Tables

Table 4-1 Samples of *Ursus thibetanus* analyzed in this study.

Subspeies designation	Sample Location	Number of Individuals	Sample Status[a]	Sample Source	MtDNA Haplotype (no. of individuals)
U.thibetanus formosanus	Taiwan	43	W	Pingtung University of Science and Technology, Taiwan (Mei-Hsiu Hwang)	TW1(35), TW5(6), TW53(1), TW14(1), TW17(3)
	Taiwan	1	C	Taipei Zoo, Taiwan	
	Taiwan	2	C	Taiwan Endemic Species Research Institute, Taiwan	
U. thibetanus mupinensis	China (Sichuan)	12	W	Pingtung University of Science and Technology (Mei-Hsiu Hwang), Taiwan	CW1(3), CW3(5), CW7(5)
	China (Sichuan)	2	C	Animals Asia Foundation, (Heather J. Bacon); CITES Chengdu (Jien Gong), China	CW6(1)
	China (Sichuan)	2	W	Peking University (Fang Liu), China	CW2(1), CW9(1)

doi:10.6342/NTU201800373

U. thibetanus thibetanus	Tailand	1	C	Taipei Zoo, Taiwan	TL1(1)
	Vietnam	6	U	Pingtung Wildlife Rescue Center (collected from CatTien, Vietnam), Taiwan	VN1(3), VN3(1), VN4(1), VN6(1)
	Vietnam	2	U	Taipei Zoo (collected from bears of a circus from Vietnam), Taiwan	VN7(1), VN8(1)
U. thibetanus ussuricus	China (Liaoning)	1	C	Animals Asia Foundation, (Heather J. Bacon); CITES Chengdu (Jien Gong), China	CE1(1)
	Russia	5	3W2U	National Cancer Institute (Shu-Jin Luo and Stephen J. O'Brien), USA	RW4(1), RW6(4)

[a] Sample Status: W, wild bears; C, captive bears; U, unknown status.

doi:10.6342/NTU201800373

Table 4-2 MtDNA partial control region and its 5'-flanking sequences of *Ursus thibetanus* from other studies.

Species/ Subspeies	Number of sequences	Sequence ID	Length(bp)	Genbank Accession	Reference
U. thibetanus japonicas	20	UtjCR01 ~ UtjCR20	702 ~ 706	AB101520 ~ AB101539	Ishibashi et al.(2004)
U. thibetanus mupinensis	1	CWG2	705	EF196661	Yu et al.(2007)
U. thibetanus ussuricus	1	KR1	705	EF667005	Hwang et al.(2007)
	1	KR2	703	EF681884	Choi et al.(2010)
	12	Rus1 ~ Rus12	677	EU264506 ~ EU264527, HM135178	Kim et al.(2011)
	12	NK1 ~ NK12	677	HM135178, EU264503 ~ EU264519	Kim et al.(2011)
U. thibetanus thibetanus	14	SEA6 ~ SEA70	615	HM135185 ~ HM135190	Kim et al.(2011)
U. americanus	1	UaCR	704	AF303109	Delisle and Strobeck(2002)

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Table 4-3 GenBank accession number of mtDNA partial control region and its 5'-flanking region of Asiatic black bear specimens sequenced in this study.

Subspecies designation	Specimen ID	Length(bp)	Accession no.
U.thibetanus formosanus	TW1*	704	MG004640
	TW2	704	MG004639
	TW3	704	MG004638
	TW4	704	MG004637
	TW5*	705	MG004636
	TW6	704	MG004635
	TW7	704	MG004634
	TW8	704	MG004633
	TW9	705	MG004632
	TW10	704	MG004631
	TW11	705	MG004630
	TW12	704	MG004629
	TW14*	705	MG004628
	TW17*	704	MG004627
	TW18	704	MG004626
	TW19	704	MG004625
	TW21	704	MG004624
	TW22	704	MG004623
	TW23	704	MG004622
	TW24	704	MG004621
	TW25	704	MG004620
	TW31	704	MG004619
	TW33	705	MG004618
	TW34	704	MG004617
	TW35	704	MG004616
	TW38	704	MG004615
	TW39	704	MG004614
	TW40	704	MG004613
	TW41	704	MG004612
	TW42	704	MG004611

doi:10.6342/NTU201800373

	TW43	704	MG004610
	TW44	704	MG004609
	TW46	704	MG004608
	TW47	704	MG004607
	TW49	704	MG004606
	TW50	704	MG004605
	TW52	704	MG004604
	TW53*	704	MG004603
	TW54	705	MG004602
	TW55	704	MG004601
	TW56	704	MG004600
	TW57	704	MG004599
	TW58	704	MG004598
	TW59	704	MG004597
	TW60	704	MG004596
	TW61	705	MG004595
U. thibetanus mupinensis	CW1*	704	MG004671
	CW2*	705	MG004670
	CW3*	705	MG004669
	CW4	704	MG004668
	CW5	705	MG004667
	CW6*	704	MG004666
	CW7*	704	MG004665
	CW9*	705	MG004664
	CW10	704	MG004663
	CW11	704	MG004662
	CW12	705	MG004661
	CW13	704	MG004660
	CW15	705	MG004659
	CW16	705	MG004658
	CW17	704	MG004657
	CW18	704	MG004656
U. thibetanus ussuricus	CE1*	704	MG004655
	RW1	704	MG004654

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	RW2	704	MG004653
	RW3	704	MG004652
	RW4*	703	MG004651
	RW6*	704	MG004650
U. thibetanus thibetanus	TL1*	705	MG004649
	VN1*	705	MG004648
	VN2	705	MG004647
	VN3*	705	MG004646
	VN4*	706	MG004645
	VN5	705	MG004644
	VN6*	706	MG004643
	VN7*	704	MG004642
	VN8*	706	MG004641

*Haplotypes used in phylogenetic analyses.

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Table 4-4 DNA polymorphism and genetic distance between bears of different subspecies.

The pairwise genetic distances were calculated based on the Kimura 2-parameter model. The analyses included bear samples sequenced in this study and sequences from previous studies. (n: sample sizes, S: number of polymorphic sites, h: number of haplotypes, Hd: haplotype diversity, Pi: nucleotide diversity.)

Subspecies	n	S	h[a]	Hd	Pi	Genetic distance					
						U. thibetanus formosanus	*U. thibetanus thibetanus*	*U. thibetanus mupinensis*	*U. thibetanus ussuricus*	*U. thibetanus japonicus*	*U. americanus*
U. thibetanus formosanus	**46**	**4**	**5(4)**	**0.205**	**0.00053**	**0.00034**	**0.01701**	**0.01370**	**0.01471**	**0.04793**	**0.06238**
U. thibetanus thibetanus	**23**	**42**	**18(17)**	**0.972**	**0.01706**	**-**	**0.01660**	**0.01659**	**0.01777**	**0.04541**	**0.05979**
U. thibetanus mupinensis	**17**	**14**	**7(5)**	**0.713**	**0.00357**	**-**	**-**	**0.00308**	**0.00293**	**0.04415**	**0.05849**
U. thibetanus ussuricus	**32**	**11**	**15(11)**	**0.810**	**0.00267**	**-**	**-**	**-**	**0.00165**	**0.04591**	**0.05998**
U. thibetanus japonicus	**119 (20 haplotypes)**	**13**	**20(14)**	**0.958**	**0.00515**	**-**	**-**	**-**	**-**	**0.00429**	**0.07515**

[a] Numbers in parentheses showed the results of haplotype numbers from DnaSP v5 software which were not included haplotypes defined by the number of Ts at a T-repeat site in analyzed sequences.

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Table 4-5 Genetic polymorphism of microsatellite loci in 4 Asian black bear subspecies including observed/expected heterozygosities and values of allelic richness in parentheses.

Locus	***U. t. formosanus*** **(n=41)**	***U. t. thibetanus*** **(n=9)**	***U. t. mupinensis*** **(n=16)**	***U. t. ussuricus*** **(n=5)**
UT1	0.615/0.578 (6)	0.778/0.935* (10)	0.813/0.764 (8)	0.200/0.378 (3)
UT3	0.579/0.800* (12)	0.500/0.967* (12)	0.333/0.935* (10)	0.800/0.956 (8)
UT4	0.634/0.829* (12)	0.889/0.889 (9)	0.938/0.863 (10)	1.000/0.889 (6)
UT23	0.846/0.911 (22)	0.556/0.935(*) (11)	0.500/0.952* (17)	0.400/0.933(*) (7)
UT25	0.756/0.716 (6)	0.889/0.967 (13)	0.867/0.975 (21)	0.800/0.867 (7)
UT29	0.878/0.836 (13)	0.556/0.935 (10)	1.000/0.907 (12)	0.600/0.733 (4)
UT31	0.892/0.896 (17)	0.889/0.967 (13)	0.563/0.940* (18)	0.800/0.911 (7)
UT35	0.732/0.837 (8)	1.000/0.928 (11)	0.938/0.849 (14)	1.000/0.929 (6)
UT36	0.667/0.804 (11)	0.889/0.908 (9)	0.813/0.861 (13)	0.600/0.644 (4)
UT38	0.780/0.866(*) (13)	0.778/0.948 (11)	0.813/0.891 (13)	0.800/0.933 (7)
Mean(10)	0.738/0.807 (12)	0.772/0.938 (10.9)	0.758/0.894 (13.6)	0.700/0.817 (5.9)
Mean(8)[a]	0.774/0.795 (10.75)	0.834/0.935 (10.75)	0.843/0.881 (13.625)	0.725/0.786 (5.5)

* locus deviated from HWE

[a] excluding UT3, UT23 for deviation from HWE.

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Chapter 5 Genetic status of captive Asiatic black bears in Taiwan and the conservation implication of *ex situ* population management

5.1 Abstract

Due to its critically small population size, *ex situ* breeding programs of the Formosan black bear (*Ursus thibetanus formosanus*) have become an important part of its conservation strategies. To ensure individuals in the captive program are of native origin, genetic analysis on both mitochondrial DNA and microsatellite loci from captive bear specimens was conducted to reveal the genetic ancestry of captive Asiatic black bears in Taiwan. In this study, we identified seven captive bears of unknown origin which showed the unique mtDNA haplotypes of Formosan black bears. And three of them had a single verified subspecies ancestry of the Formosan black bear in microsatellite data. These bears of native origin were kept in different zoos, institutes, and rescue centers in Taiwan. Genetic analysis conducted in our study is important in helping relevant these institutions to cooperate and better plan for *ex situ* conservation of the Formosan black bears.

5.2 Introduction

Although the Formosan black bear (*Ursus thibetanus formosanus*) has been listed as an endangered species under the Wildlife Conservation Act of Taiwan since 1989,

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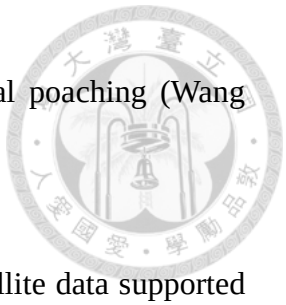

their population has declined due to habitat degradation and illegal poaching (Wang 1990, 1999; Hwang and Wang 2006).

Based on the results of Chapter 4, both mtDNA and microsatellite data supported that the Formosan black bear was distinctly different from other Asiatic black bears in their genetics, suggesting that this island bear population should be regarded as an evolutionarily significant unit (ESU). These results have important implications for the conservation of the Formosan black bears, i.e. both *in situ* and *ex situ* management strategies should treat this endemic bear subspecies as a separate management unit from other Asiatic black bear populations.

As wild populations of endangered species continue to decline, successful *ex situ* breeding and management has gradually become important. According to the recommendations from IUCN (1987), *ex situ* populations for conservation of threatened species should be founded before wild populations drop to below 1,000 individuals to avoid serious genetic impacts, such as high inbreeding levels with less wild founders and the detrimental effect of removing animals from the wild populations (Frankham et al. 2002). The small population sizes of the Formosan black bear renders *ex situ* breeding programs to be considered as a part of its conservation strategies.

When considering an *ex situ* breeding program for conserving threaten species,

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hybridization between unrecognized subspecies or species should be avoided. Wrong taxonomy of subspecies often results in inadvertent hybridization in captivity. Descendants of such captive populations would be unavailable for reintroduction to avoid genetically introgression in the wild populations. A case of wrong subspecies taxonomy in *ex situ* conservation program that had happened in the Asian lion *Panthera leo persica* Species Survival Plans (SSP) of the Association of Zoos and Aquariums (AZA) was totally compromised by unrecorded hybridization with African lions (O'Brien et al. 1987; Frankham et al. 2002; Kitchener 2010).

According to the most recent records of the Taiwan Forestry Bureau, there are about 30 captive Asiatic black bears in Taiwan. Most of them were rescued, confiscated, or abandoned animals and their descendants with questionable origin and life history information. For an *ex situ* breeding program to be successful, it is necessary to ensure that all individuals included in the program are of known taxonomy (WAZA 2005). Thus taxonomy of the captive Asiatic black bears in Taiwan should be clarified in advance and bears of native origin must be identified to increase the number of founders in a breeding program.

Two previous studies had been conducted on the genetic status of captive Asiatic black bears in Taiwan. Chu et al. (2000) analyzed the mitochondrial DNA (mtDNA)

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control region (532 bp) and cytochrome b (397 bp) of the Asiatic black bears in the Taipei Zoo. Chen and Yang (2002) compared partial gene sequences of mitochondrial 12S rRNA (391 bp) and 16S rRNA (425 bp) among 11 captive Asian black bears in Taiwan. However, both studies did not analyze all captive bears in Taiwan and few voucher specimens with verified geographic origins had been included. Therefore, the genetic status of captive Asiatic black bears in Taiwan remains unclear.

In this study, we collected specimen of bears from different facilities in Taiwan and conducted a genetic analysis on both mitochondrial DNA and microsatellite loci to reveal the genetic ancestry of these captive Asiatic black bears.

5.3 Materials and methods

5.3.1 Sample collection and DNA extraction

We collected samples of 30 captive Asiatic black bears from zoos, rescue centers, and private owners in Taiwan (Table 5-1). Eight of these 30 samples were genomic DNA, nine were blood, five were hair, and eight were feces. Two fur specimens of confiscated bear paws from the Pingtung University of Science and Technology were also collected and analyzed (Table 5-1). Total genomic DNA of each specimens was extracted using the same methods as described in Chapter 4.

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5.3.2 mtDNA sequencing and Microsatellite Genotyping

Following the procedures of mtDNA amplification and sequencing described in Chapter 4, all extracts of bear samples were first amplified for around 900 bp of the highly variable mtDNA control region and its 5'-flanking region using PCR with the primer pair, CB-Z (Matsuhashi et al. 1999) and Ut-Dr (designed in Shih et al. (2017)). Later sequences of all PCR products were obtained in both directions with the 3 primers, CB-Z, Ut-Dr, and UT-1 (Ishibashi and Saitoh 2004). The same as the voucher bear samples examined in Chapter 4, all sequences in this study were cut to about 700bp to encompass the majority of the published sequence data for the purpose of phylogenetic analysis.

Ten tetranucleotide polymorphic microsatellite loci (UT1, UT3, UT4, UT23, UT25, UT29, UT31, UT35, UT36, and UT38) (Shih et al. 2009) were amplified by PCR and genotyped with the procedures described in Chapter 4.

5.3.3 Data analysis

mtDNA sequence analysis

All sequences of captive bears and paws in this study were aligned with 77 sequences of voucher Asiatic black bears, 61 published sequences of Asiatic black bears, and a sequence of American black bear described in Chapter 4. Then phylogenetic

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reconstructions were assessed using three approaches, neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) with the same procedures described in Chapter 4. NJ and ML analyses were conducted using PAUP* version 4 beta (Swofford 2001) with the best fitting TIM3+I+G model. BI analyses were inferred using MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) with the HKY+I+G model. In BI analyses, two independent runs with four Markov chains were performed for 4,000,000 generations and assigned sampling frequency an every 100 generations. Trees were all routed with outgroup species *U. americanus*.

Microsatellite analysis

Genotypes from eight microsatellite loci were analyzed by a Bayesian clustering analysis in STRUCTURE 2.3.4 (Pritchard et al. 2000; Falush et al. 2003) using the prior population options obtained from the voucher bears. Five subdivision scenario (K=5) was set according to the results of the Bayesian clustering analysis of voucher bear specimens in Chapter 4. The genotype data of UT3 and UT23 were not included in the STRUCTURE analysis due to the deviation from Hardy–Weinberg equilibrium (HWE) in voucher bears.

5.3.4 Subspecies assignment

Based on the genetic status of subspecies obtained from voucher bears described

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in Chapter 4, mtDNA haplotypes of bears with unknown genetic origin were assigned maternal lineage ancestry based on its phylogenetic relationship to the voucher bear subspecies first. Then, the Bayesian clustering analysis in STRUCTURE based on 8 microsatellite loci was used to assign the biparental genetic ancestry.

Bear samples were considered to have the purebred Formosan black bear ancestry if they were consistently supported by both mitochondrial lineage and microsatellite genotype assignment results. Bear individuals with genetic origin of *U. thibetanus formosanus* in mtDNA, but admixed origins in microsatellite analysis were also identified. Others were categorized as bears with admixed origins or with purebred origin of other subspecies in both mtDNA and microsatellite analyses. Bear specimens with only mitochondrial data were considered to have incomplete evidence in maternal lineage only.

5.4 Results

The mtDNA control region and its 5'-flanking region sequences (703 to 706 bp in length) of all samples of 30 captive Asiatic black bears and two paws were successfully amplified and sequenced. In these 32 bear samples, three bear specimens and a paw specimen could not be genotyped for more than seven in ten tetranucleotide microsatellite loci and were not used in further microsatellite analyses.

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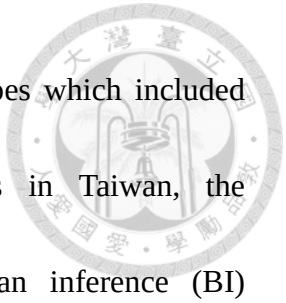

In the phylogenetic relationship analyses of mtDNA haplotypes which included the source-unknown samples collected from various facilities in Taiwan, the neighbor-joining (NJ), maximum likelihood (ML), and Bayesian inference (BI) phylogenetic trees showed identical topologies (Fig. 5-1, Fig. 5-2, and Fig. 5-3). Seven source-unknown bears including TWC25, TWC26, TWC27, TWC21, TWC22, TWC23, and TWC29 show the unique haplotypes of the Formosan black bears (Fig. 5-1, Fig. 5-2, and Fig. 5-3). Others are not in the Taiwan clade and commingled with haplotypes of southeastern Asia subspecies (*U. thibetanus thibetanus*) and southwestern China subspecies (*U. thibetanus mupinensis*). None of source-unknown samples are in the clades of the northeastern Asian continent subspecies (*U. thibetanus ussuricus*) or the Japan black bears (*U. thibetanus japonicas*) (Fig. 5-1, Fig. 5-2, and Fig. 5-3).

In the Bayesian clustering analysis of STRUCTURE with microsatellite loci including source-unknown samples, three of seven bears assigned in the mtDNA Taiwan clade showed a single verified subspecies ancestry of the Formosan black bear, including TWC25, TWC26, and TWC27 (Fig. 5-4). Except for TWC16, TWC17, TWC18, and TWC19 (which were genetically assigned to *U. thibetanus mupinensis*), most of others were of admixed origin (Fig. 5-4).

We assigned three bear samples, TWC25, TWC26, and TWC27 to Asiatic black

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bears with purebred origin of *U. thibetanus formosanus*, TWC21, TWC22, TWC23, and TWC29 to bears with admixed subspecies origins including *U. thibetanus formosanus*, TWC16, TWC17, TWC18, and TWC19 to bears with purebred origin of *U. thibetanus mupinensis*, and determined 17 had admixed subspecies origins of *U. thibetanus thibetanus* and *U. thibetanus mupinensis*. TWC9, TWC10, TWC30, and TWQP5 were bear samples with admixed origins in mtDNA analyses but without enough microsatellite data for analysis (Table 5-1).

5.5 Discussions

In the 30 origin-unknown captive Asiatic black bears tested in this study, only three bears were with purebred origin of *U. thibetanus formosanus*, four were *U. thibetanus formosanus* in maternal lineage ancestry but with admixed origins of *U. thibetanus thibetanus* or *U. thibetanus mupinensis* in biparental microsatellite analysis. Namely, more than two thirds of the captive Asiatic black bears in Taiwan were not of native *U. thibetanus formosanus* origin. Due to the geographical adjacency among Taiwan, China, and southeastern Asia and the frequently legal and illegal trade among these countries, it may be reasonable that most bears of admixed origins or non-native origins are with southeastern Asia subspecies (*U. thibetanus thibetanus*) and southwestern China subspecies (*U. thibetanus mupinensis*) ancestries. In addition,

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according to the information provided by the owners of these bear specimens, TWC4, TWC16, TWC17 (TWC18 and TWC19 are offspring of TWC16 and TWC17), and TWC28 were bought from indigenous people or mountain areas in Taiwan. However, genetic analysis revealed that these six captive Asiatic black bears were not native bear subspecies. This discordance may demonstrate the necessity of genetic analyses in subspecies identification.

In this study, we developed a method to assess subspecies ancestry of the Asiatic black bears with uncertain background, especially to identify native Formosan black bear from other subspecies. It should be a powerful tool for *ex situ* conservation of the Formosan black bear to increase the number of purebred bears suitable for conservation breeding. However, our method could not clarify the difference between southeastern Asia subspecies (*U. thibetanus thibetanus*) and southwestern China subspecies (*U. thibetanus mupinensis*). According to the results described in Chapter 4, it may be due to the gene flow between *U. thibetanus mupinensis* and *U. thibetanus thibetanus*. It will be worth of conducting further studies with more effective genetic markers to increase the accuracy of subspecies identification between these subspecies in the future.

For *ex situ* conservation breeding program of the Formosan black bear, TWC25, TWC26, and TWC27 in this study and three voucher bears in captivity described in

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Chapter 4 (TW14, TW17, and TW18) should be the core founders because of their purebred origin of *U. thibetanus formosanus*. Four bears (TWC21, TWC22, TWC23, and TWC29) with maternal lineage ancestry of *U. thibetanus formosanus* but admixed origins in biparental nuclear genealogy may result from asymmetric breeding between two subspecies in captivity. These four bears with partial *U. thibetanus formosanus* origin may be included as of second priority for conservation breeding if there are not enough young breeders in the breeding program. Other bears of admixed origins are suggested to avoid breeding unless they are used for developing animal husbandry techniques.

Genetic analysis conducted in our study is important in helping relevant organizations to cooperate and better plan for *ex situ* conservation of the Formosan black bears. Given the fact that the critically small size of wild population and that the bears of native origin are kept in different organizations, these institutions are highly encouraged to cooperate with each other in implementing an *ex situ* breeding plan for the conservation of this subspecies.

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Figures

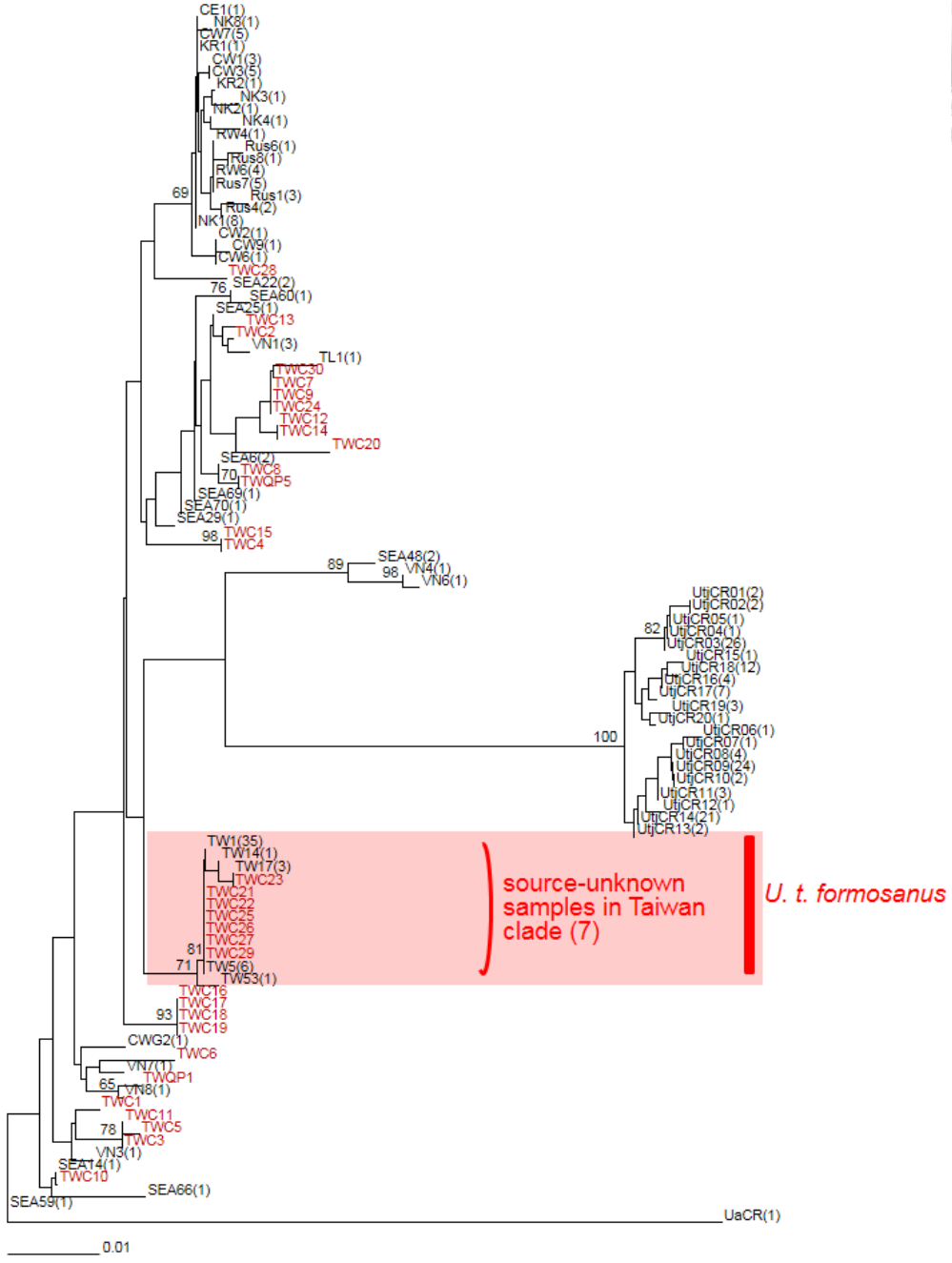

Fig. 5-1 Phylogenetic relationships of source-unknown bear samples and voucher samples based on neighbor-joining analysis.

Bootstrap supports were provided above branch at node for the divergence of lineages. The bear sample sizes with each voucher haplotype were showed in parentheses. The source-unknown bear samples were highlighted with dark red color.

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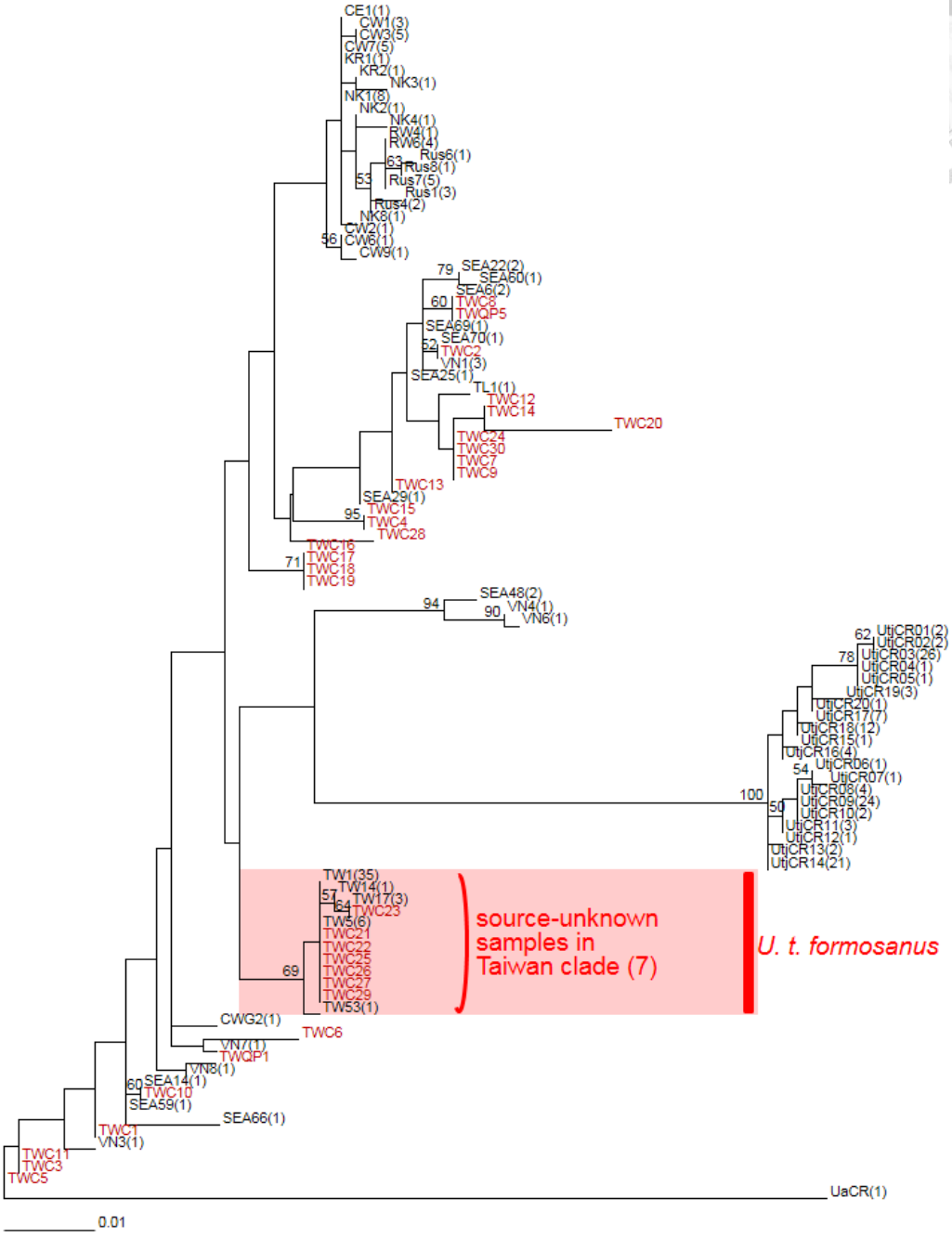

Fig. 5-2 Phylogenetic relationships of source-unknown bear samples and voucher samples based on Maximum Likelihood analysis.

Bootstrap supports were provided above branch at node for the divergence of lineages. The bear sample sizes with each voucher haplotype were showed in parentheses. The source-unknown bear samples were highlighted with dark red color.

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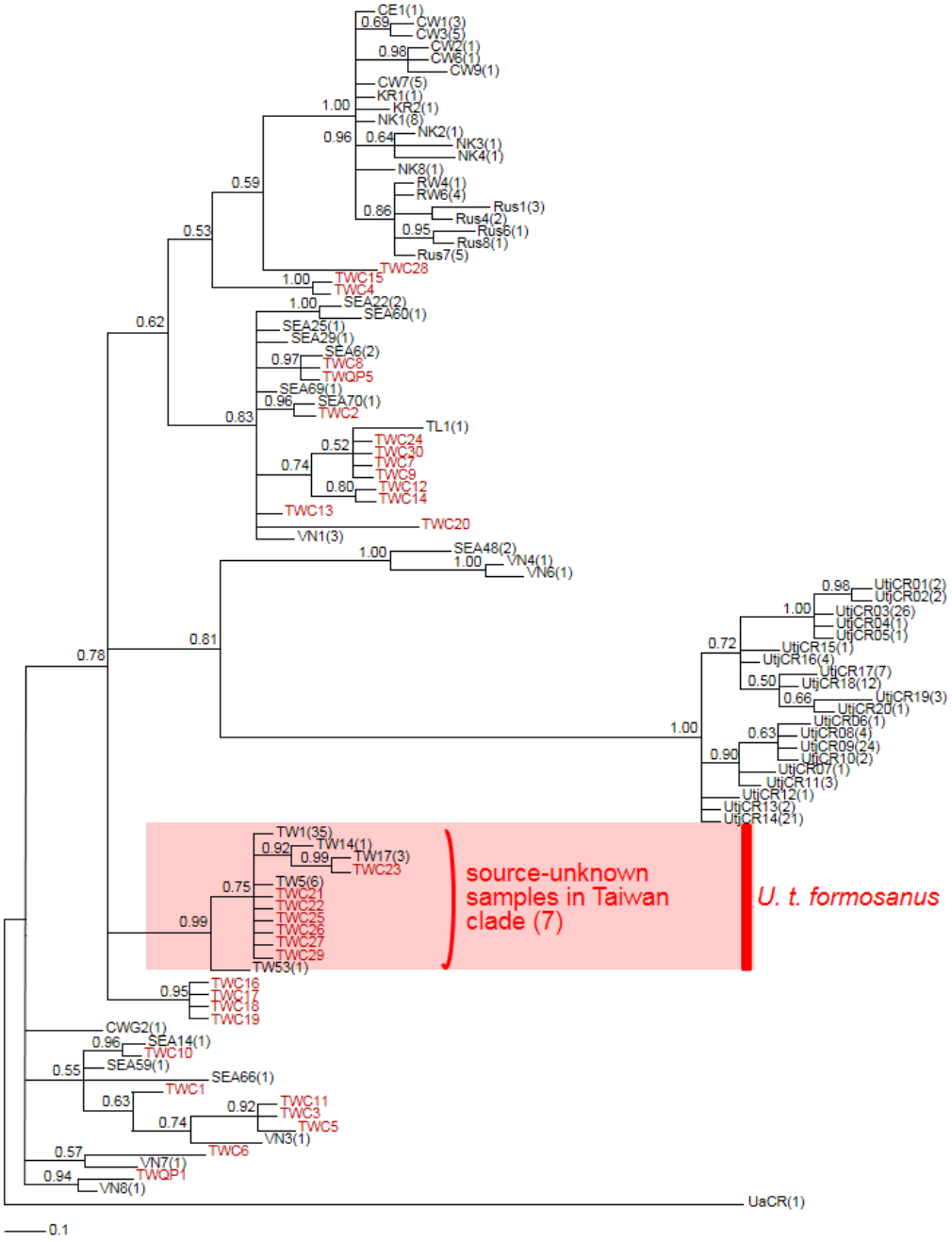

Fig. 5-3 Phylogenetic relationships of source-unknown bear samples and voucher samples based on Bayesian inference analysis.

Bayesian posterior probabilities were provided above branch at node for the divergence of lineages. The bear sample sizes with each voucher haplotype were showed in parentheses. The source-unknown bear samples were highlighted with dark red color.

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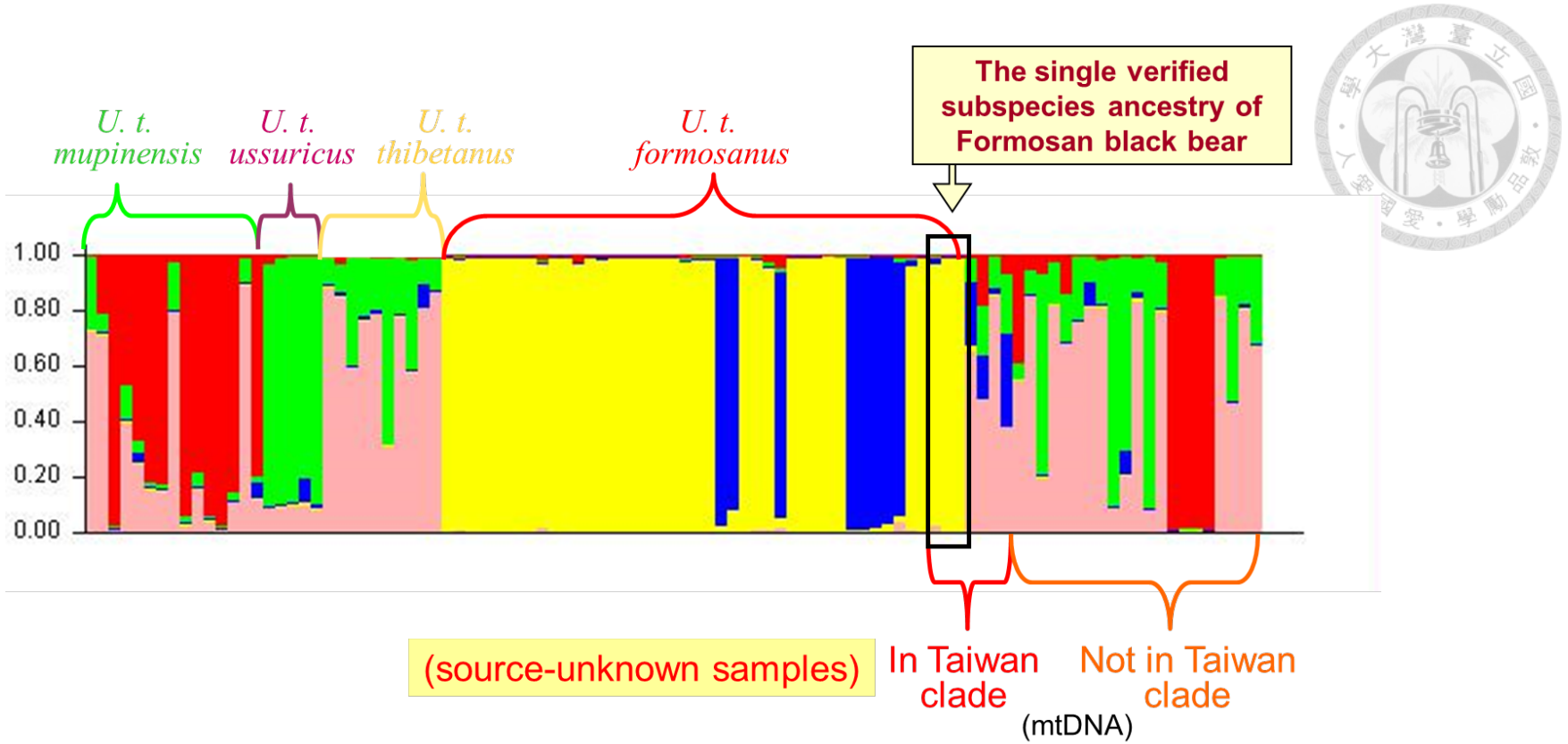

Fig. 5-4 Bayesian population genetic structure of source-unknown bear samples and voucher samples of 4 subspecies of Asiatic black bears using STRUCTURE 2.3.4.

Bar plot showed the population structure of clustering result with the highest posterior probability, K=5.

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Tables

Table 5-1 Genetic ancestries of captive Asiatic black bear samples/ paw specimens used in this study.

Code	Name/Local ID	Source/Owner	Suspected Origin[a]	Genetic Origin[a]
(Asiatic black bears with purebred origin of *U. thibetanus formosanus* in both mtDNA and microsatellite analyses, n=3)				
TWC25	愛德華	Taipei Zoo	U/ *formo*	*formo*
TWC26	六龜(♂)	Pingtung Wildlife Rescue Center	U/ *formo*	*formo*
TWC27	六龜(♀)	Pingtung Wildlife Rescue Center	U/ *formo*	*formo*
(Asiatic black bears with origin of *U. thibetanus formosanus* in mtDNA, but admixed origins in microsatellite analysis, n=4)				
TWC21[b]	黑皮	Shousan Zoo	U	*formo*/*thibe*/*mupi*
TWC22[b]	波比	Shousan Zoo	U	*formo thibe*/*mupi*
TWC23	阿妹	Shousan Zoo	U	*formo*/*thibe*/*mupi*
TWC29	寶貝	Shousan Zoo	U	*formo*/*thibe*/*mupi*
(Asiatic black bears with admixed origins or with purebred origin of other subspecies in both mtDNA and microsatellite analyses, n=21)				

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TWC1	小敏	Taipei Zoo	U	*thibe*/*mupi*
TWC2	嘉女	Taipei Zoo	U	*thibe*/*mupi*
TWC3	黑梅蓓	Taipei Zoo	U	*thibe*/*mupi*
TWC4	小熊	Taipei Zoo	U/*formo*	*thibe*/*mupi*
TWC5	嘉男	Taipei Zoo	U	*thibe*/*mupi*
TWC6	小黑	Taipei Zoo	U	*thibe*/*mupi*
TWC7	寶貝	Taipei Zoo	U	*thibe*/*mupi*
TWC8	嘟嘟	Taipei Zoo	U	*thibe*/*mupi*
TWC11	卡特	Taiwan Endemic Species Research Institute	U	*thibe*/*mupi*
TWC12	黑妞	Taiwan Endemic Species Research Institute	U	*thibe*/*mupi*
TWC13	阿財	Taiwan Endemic Species Research Institute	U	*thibe*/*mupi*
TWC14	小妞	Taiwan Endemic Species Research	U	*thibe*/*mupi*

doi:10.6342/NTU201800373

		Institute		
TWC15	元元	Taiwan Endemic Species Research Institute	U	*thibe*/*mupi*
TWC16	皮皮	Private owner (Teng-Zheng, Li)	U/*formo*	*mupi*
TWC17	乖乖	Private owner (Teng-Zheng, Li)	U/*formo*	*mupi*
TWC18[b]	平平	Private owner (Teng-Zheng, Li)	U/*formo*	*mupi*
TWC19[b]	安安	Private owner (Teng-Zheng, Li)	U/*formo*	*mupi*
TWC20	龍谷	Private farm (Long-Gu)	U	*thibe*/*mupi*
TWC24	乖乖	Shousan Zoo	U	*thibe*/*mupi*
TWC28	梅山-1	Private owner (Jin-Xiu, Lin),	U/*formo*	*thibe*/*mupi*
TWQP1	六龜山產店腳掌	Pingtung University of Science and Technology (Mei-Hsiu Hwang)	U/*formo*	*thibe*/*mupi*

(Asiatic black bears with admixed origins in mtDNA analyses but without enough microsatellite data for analysis, n=4)

TWC9	阿里	Taipei Zoo	U	*thibe*/*mupi*/U
TWC10	日月	Taipei Zoo	U	*thibe*/*mupi*/U

doi:10.6342/NTU201800373

TWC30	阿胖	Shousan Zoo	U	*thibe*/*mupi*/U
TWQP5	蔡 O 松案右後腳掌	Pingtung University of Science and Technology (Mei-Hsiu Hwang)	U	*thibe*/*mupi*/U

[a] Subspecies Code: *formo*, *U.thibetanus formosanus*; *mupi*, *U. thibetanus mupinensis*; *thibe*, *U. thibetanus thibetanus*; U, unknown.

[b] TWC21 and TWC22 are Offsprings of TWC29; TWC18 and TWC19 are Offsprings of TWC16 and TWC17.

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Appendix Publications

A. Shih C-C, Huang C-C, Li S-H, Hwang M-H, Lee L-L (2009) Ten novel tetranucleotide microsatellite DNA markers from Asiatic black bear, *Ursus thibetanus*. Conservation Genetics 10:1845-1847

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DOI 10.1007/s10592-009-9830-3

TECHNICAL NOTE

Ten novel tetranucleotide microsatellite DNA markers from Asiatic black bear, *Ursus thibetanus*

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Abstract Ten polymorphic microsatellite markers were developed for the endangered Formosan black bear (*Ursus thibetanus formosanus*) from a partial genomic library enriched for GAAA repeat. Polymorphism of these loci was evaluated in 27 Formosan black bear specimens of unknown relationship. The number of alleles per locus ranged from 5 to 15 and the observed heterozygosity of each locus ranged from 0.556 to 0.889. These loci should provide useful molecular tools to study conservation genetics of the Formosan black bear and other Asiatic black bears.

Keywords Tetranucleotide microsatellite · Tailed primers · *Ursus thibetanus* · Formosan black bear

The Formosan black bear (*Ursus thibetanus formosanus*) is an endemic subspecies of Asiatic black bear inhabiting Taiwan (Wozencraft 2005). Similar to all other Asiatic black bears, degradation and fragmentation of habitat as well as poaching have caused a decrease in population and distribution of the Formosan black bear (Wang 1999; Hwang and Wang 2006). To formulate proper conservation strategies, it is important to understand the genetic diversity and genetic structure within and among populations of this subspecies.

In this study, we reported ten novel easy-scored polymorphic tetranucleotide repeat (GAAA) microsatellite loci from the Formosan black bear. We followed the protocol developed by Hsu et al. (2003) to enrich microsatellite-contained fragment in a partial genomic library. The library was constructed from genomic DNA which extracted from tissue sample of a Formosan black bear individual using the proteinase *K*-chloroform method (Sambrook et al. 1989). Microsatellite-enriched PCR (polymerase chain reaction) library was ligated into pGEM-T Easy vector (Promega) and transformed into *Escherichia coli* DH5α. A total of 880 clones were lifted to Hybond-N + membranes (Amersham Pharmacia Biotech) and hybridized with [γ^{32}P] ATP end-labelled $(GAAA)_{10}$ oligonucleotides, then 56 hybridized clones were sequenced using DYEnamic ET Dye Terminator Cycle Sequencing Kit for MegaBACE (Amersham Bioscience) on a MegaBACE 1000 autosequencer (Amersham Bioscience). Sequences were proofread using software SEQUENCER 4.2 (Gene Codes). We found 47 clones with microsatellite motif, of which 33 loci containing more than 10 units of GAAA motif were chosen to design the PCR primers.

All forward primers were 5′-tailed with an M13-tail (5′-GGAAACAGCTATGACCAT-3′) or a CAG-tag (5′-CAG TCGGGCGTCATCA-3′) (Schuelke 2000; Boutin-Ganache et al. 2001). DNA extracted from tissue samples of 17 Formosan black bears and from faecal samples of ten Formosan black bears with unknown relationship were used to characterize these 33 loci. PCRs were set up in

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Table 1 Characterization of the ten microsatellite loci of Formosan black bear (*Ursus thibetanus formosanus*)

Locus	Core motif	Primer sequence ($5'$–$3'$)	N	T_a (°C)	$MgCl_2$ (mM)	No. of alleles	Allele size (bp)[a]	H_O	H_E	P-values (HWE)	GenBank Accession no
UT1	$(GAAA)_9GGGA(GAAA)_{10}$	F: CAG-AGCAACTCTTCTCAGATGTTCACAAA R: CCCAGGTCAGCACTTGGCATAC	27	64	2.5	5	176–192	0.556	0.584	0.461	FJ640076
UT3	$(GAAA)_{18}$	F: CAG-AAGACATACAGAAGCCAAGACTAG R: TACTCAATTACAAAGGATAACTATA	25	56	2.5	7	256–282	0.640	0.776	0.186	FJ640077
UT4	$(GAAA)_6GAGA(GAAA)_{11}$	F: M13-GAGTTATTGGCACTAAAATCTAATG R: CTGCAAATCCCTGCTCAACTTTC	27	56	2.5	7	157–182	0.704	0.814	0.107	FJ640078
UT23	$(GAAA)_{10}GA(GAAA)_{22}$	F: M13-GCTGGATACATCATCCTGGCTC R: GGAATCAAGTTCGGCATCGGG	27	62	2.5	12	349–382	0.778	0.881	0.040*	FJ640079
UT25	$(GAAA)_2(GA)_{12}$ $(GAAA)_{16}$	F: M13-GCTCAGGGCGTGATCCCAGAG R: GGCTCCCCTGCACTAGAGATTTAAC	27	62	2.5	6	314–333	0.704	0.720	0.011*	FJ640080
UT29	$(GAAA)_2AA(GAAA)_{17}$	F: CAG-GACATTGCCTTTTACAGAGCAG R: GGGCAGATCTCAACCACCATAAGC	27	64	2.5	8	204–236	0.889	0.788	0.058	FJ640081
UT31	$(GAAA)_{17}GG(GAAA)_3$	F: CAG-AATAAACTGATGCAGCCATACTAG R: CTGCCACTGAATCTTCTGATCTTAG	26	64	2.5	15	315–369	0.846	0.909	0.560	FJ640082
UT35	$(GAAA)_{15}$	F: CAG-ACTCCCTAGTAAGTAGAAAGCACAC R: CCCACAGGATGGGCTCAAGAA	27	64	2.5	7	218–247	0.630	0.825	0.022*	FJ640083
UT36	$(GAAA)_{16}$	F: CAG-AGACTCAGGAAGTCTGGAGTGGGA R: CTTTCGGCTCAGGGATCGAGC	27	62	2.5	7	276–309	0.630	0.727	0.154	FJ640084
UT38	$(GAAA)_{24}$	F: M13-ATTATTGATGAGCAGGGACAG R: CTAAAGCAACAACATGTGAATG	27	56	2.5	10	196–232	0.778	0.839	0.039*	FJ640085

N, number of individuals genotyped; T_a, PCR annealing temperature; H_O, observed heterozygosity; H_E, expected heterozygosity; HWE, Hardy–Weinberg equilibrium; *, $P < 0.05$

[a] Allele size includes the additional size of tails added to forward primer

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10 μl reaction volumes containing 1 × PCR buffer, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.05 μM of tailed forward primer, 0.12 μM of reverse primer, 0.18 μM of fluorescent-labelled M13 or CAG-tag primer that were labeled with HEX, FAM or TAMRA fluorescent dyes, 0.2 U *Taq* DNA polymerase (Biotech), and around 30 ng genomic DNA. The PCR condition was 95°C for 4 min, then 40 cycles at 95°C for 30 s, 30 s at the optimal annealing temperature of each primer pair (Table 1) and 72°C for 20 s, followed by a final extension at 72°C for 7 min. The PCR products were electrophoresed in a MegaBACE 1000 autosequencer (Amersham Biosciences). Sizes of alleles were scored with software GENETIC PROFILER 2.0 (Amersham Biosciences).

Twenty-three loci that appeared difficult to score or monomorphic were excluded from subsequent analyses. Genotype frequencies of ten loci were analysed using CERVUS 2.0 (Marshall et al. 1998) to calculate the observed and expected heterozygosities. Tests for departure from Hardy–Weinberg equilibrium and linkage equilibrium between pairs of loci were performed using GENEPOP 3.4 (Raymond and Rousset 1995). Polymorphism assessment at these ten microsatellite loci is summarized in Table 1. The number of alleles per locus ranged from 5 to 15 and the observed heterozygosities ranged from 0.556 to 0.889. There was no evidence for large allele dropout and null alleles detecting by MICRO-CHECKER (van Oosterhout et al. 2004) in all ten loci. Four loci (UT23, UT25, UT35 and UT38) represented significant differences between the observed and expected heterozygosities ($P < 0.05$), which are probably due to genetic drift driven by Formosan black bear's small population size. No significant deviation from linkage equilibrium was detected after Bonferroni correction.

With microsatellites that isolated from Japanese black bear (*U. thibetanus japonicus*) (Kitahara et al. 2000) and other Ursids (Paetkau et al. 1995; Taberlet et al. 1997), the tetranucleotide microsatellites we isolated should provide an ideal genetic tool kit to study the population genetics of the endangered Formosan black bear and other Asiatic black bears that are also under threat.

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B. Shih C-C, Wu S-L, Hwang M-H, Lee L-L (2017) Evaluation on the effects of ageing factor, sampling and preservation methods on Asiatic black bear (*Ursus thibetanus*) noninvasive DNA amplification. Taiwania 62:363-370

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Evaluation on the effects of ageing factor, sampling and preservation methods on Asiatic black bear (*Ursus thibetanus*) noninvasive DNA amplification

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ABSTRACT: Noninvasive genetic sampling allows studying wildlife without having to catch, handle or even observe individuals. In this study, factors which may affect the quality of noninvasive samples of Asiatic black bear (*Ursus thibetanus*) in the subtropical areas were identified. We collected hair and faecal samples from captive Asiatic black bears and quantitatively evaluated the effects of hair age (from fresh to 60 days), faeces age (from fresh to 14 days), faeces sampling locations (i.e. sample collected from either the surface, inside or a mixture of both the surface and inside of faeces), and faeces preservation methods (frozen or kept at room temperature in 95% ethanol) on amplification success rates of mitochondrial DNA fragments of different sizes (450bp, 900bp, and 1600bp). The results showed that the amplification success rates decreased with sample age and amplicon size in both hair and faecal DNA. In subtropical environment, there was no significant difference between amplification success of DNA extracted from fresh and 7-day-old samples of either the hair or faeces. The amplification success rates were not influenced by sampling location of faeces. For faeces preserved in 95% ethanol, the amplification success appeared unaffected by frozen at -20 °C or kept at room temperature in shorter mtDNA fragments, but was significantly influenced when amplicon size was 1600bp. The results of this study will reinforce the optimization of noninvasive sampling approaches in Asiatic black bear research, especially in the subtropics.

KEY WORDS: DNA preservation, Faecal DNA, Noninvasive genetic sampling, Hair DNA, *Ursus thibetanus*.

INTRODUCTION

Noninvasive genetic sampling has been proven a powerful tool for investigating populations of wildlife, particularly those elusive, rare, and free-ranging species roaming in large areas (Roon *et al.* 2003; Broquet *et al.* 2007). Through a set of genetic procedures, noninvasive genetic sampling allows the study of the biology of wildlife without having to catch, handle, or even observe individuals (Piggott and Taylor 2003; Broquet *et al.* 2007). Researchers could integrate various noninvasive techniques in monitoring trends of wildlife populations, especially in large carnivores, for the purposes of management and conservation (Waits and Paetkau 2005; Schwartz *et al.* 2007; De Barba *et al.* 2010). Conservation biologists, for instance, have routinely used noninvasive genetic methods to monitor the long-term population trends of the brown bears in North America (Woods *et al.* 1999; Broquet *et al.* 2007).

Noninvasive DNA could be retrieved from various types of wildlife samples include hair, faece, urine, shed feather, buccal cells from food, snake skin, eggshells *et al.* (Sloane *et al.* 2000; Valiere and Taberlet 2000; Vigilant *et al.* 2001; Broquet *et al.* 2007; Beja-Pereira *et al.* 2009). Faeces and shed hair are more easily collected, and thus are often used as the noninvasive genetics materials (Broquet *et al.* 2007; Renan *et al.* 2012). Despite the many advantages, a major limitation of noninvasive faeces or hair samples is the low quantities of host DNA which is often highly degraded (Waits and Paetkau 2005) and often leads to low PCR amplification rates.

The quantity and quality of faecal and hair DNA can be affected by sample age (Murphy *et al.* 2007; Santini *et al.* 2007; Vynne *et al.* 2012), environmental conditions (e.g. humidity, temperature, exposure to the sun or rain) (Murphy *et al.* 2007; Michalski *et al.* 2011), or technical factors, including sampling location, i.e. whether sample were collected from the surface or inside of faeces (Piggott and Taylor 2003; Stenglein *et al.* 2010) and storage method (Santini *et al.* 2007; Panasci *et al.* 2011). DNA extraction protocol and amplicon size, the fragment length of amplified DNA makers can also affect the quantity and quality of faecal and hair DNA extracted, thus the success rate of amplification (Piggott *et al.* 2004; Buchan *et al.* 2005; Hoffman and Amos 2005; Broquet *et al.* 2007). Previous studies which had evaluated factors affecting the DNA quality and amplification success rates of faecal and hair samples suggest that success rates will be the highest when samples are fresh and dry or preserved in low temperature (Farrell *et al.* 2000;

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Lucchini *et al.* 2002; Piggott 2004; DeMay *et al.* 2013).

However, DNA degradation rates can differ among taxa and even within species under different climatic or operational conditions (DeMay *et al.* 2013). The lack of a quantitative comparison of studies in various animal-environment systems makes it difficult to decide which protocol is the most suitable for a given system (Beja-Pereira *et al.* 2009; Renan *et al.* 2012). Those general trends are not necessarily transferable across species or study sites (DeMay *et al.* 2013) and may be of limited applicability to new studies. Therefore, pilot studies are still recommended for each system to determine DNA degradation rates and the appropriate noninvasive protocol (Taberlet *et al.* 1999; Renan *et al.* 2012; DeMay *et al.* 2013).

Noninvasive genetic sampling is often applied in Ursid research. Most studies evaluating the quality and DNA amplification success of noninvasive faeces or hair samples were conducted on brown bears (*Ursus arctos*) in temperate regions (Murphy *et al.* 2002; Murphy *et al.* 2007; Stenglein *et al.* 2010), but few were on bears in regions with different climatic conditions. DNA samples collected under high temperature and humidity in the tropics and the subtropics may be particularly susceptible to degradation (Wasser *et al.* 1997; Bayes *et al.* 2000; Vynne *et al.* 2012). Only a few studies comparing storage treatments or extraction methods have been conducted in tropical forests, and most of them were limited to primates, ungulates and canids (Nsubuga *et al.* 2004; Vallet *et al.* 2008; Soto-Calderon *et al.* 2009; Vynne *et al.* 2012). Comparative studies using DNA of faeces and hair in Ursid have not been performed and the effectiveness of methods for preserving samples has not been evaluated in the tropics or subtropics.

Our study focuses on the Formosan black bear (*Ursus thibetanus formosanus*), an endemic subspecies of Asiatic black bear inhabiting Taiwan, a subtropical island (Wozencraft 2005). Similar to all other Asiatic black bear subspecies, habitat degradation and fragmentation, as well as poaching, have caused a decrease in the population and distribution of the Formosan black bear (Hwang and Wang 2006; Hwang and Garshelis 2007; Hwang *et al.* 2010). To formulate proper conservation strategies, it is important to understand the genetic diversity and genetic structure of this subspecies (Shih *et al.* 2009). For efficient application of noninvasive genetic analysis, it is necessary to identify the variables which may affect the DNA quality and further DNA amplification success in this system.

The main objective of this study is to quantitatively evaluate the effect of multiple variables on amplification success rate of mitochondrial DNA (mtDNA) extracted from Asiatic black bear faeces and hair. When using faeces as noninvasive DNA sources, subsamples are often taken from species producing larger faeces instead of collecting the entire faeces in the field (Stenglein *et al.* 2010). Since few studies have experimentally tested samples taken from the different parts of faeces, we examined the impact of sampling locations, e.g. from the surface or inside of faeces. Soaking faeces in ethanol and silica desiccation are widely employed for faecal DNA preservation (Wasser *et al.* 1997; Frantzen *et al.* 1998; Santini *et al.* 2007). In a subtropical region like Taiwan, ethanol preservation should be more preferable than silica desiccation because high temperature and humidity may hinder the effect of desiccation of silica (Murphy *et al.* 2002). Although transportation of frozen samples from the field to the laboratory would be difficult in field research (Nsubuga *et al.* 2004), the effect of immediate freezing of ethanol-soaked samples in DNA preservation was also evaluated in this study.

In this study, we collected hair and faecal samples from captive Asiatic black bears in subtropical Taiwan to assess the effects of sample age (over a 60-day period for hair and a 14-day period for faeces) and faecal preservation methods (frozen or kept at room temperature in 95% ethanol) on amplification success rates of different mtDNA amplicon size. The results of this pilot study will allow us to make recommendations for optimal noninvasive sampling protocols and to provide sampling and storage guidelines for field researchers conducting noninvasive genetic sampling of Asiatic black bears in the subtropics.

MATERIALS AND METHODS

Experiment design, sample collection and preservation

The specimens for different treatments and evaluation were collected from 5 captive Asiatic black bears (2 males, 3 females) at Taipei Zoo and all treatments were done in the zoo as well. These bears were on a mainly vegetarian diet. Faecal samples were collected and treated with an average temperature of 26.4 °C and average relative humidity of 71% (climate data from the Central Weather Bureau, Taiwan). Hair samples were collected and tested in Taipei Zoo with an average temperature and relative humidity of 28.37 °C and 74%, respectively. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Taipei Zoo.

Fresh faeces were collected in less than 12 hours after being deposited by the 5 bears, then immediately transported to a semi-outdoor flat ground where they would not be directly exposed to rain and sunlight (to simulate the condition under canopy in the wild). For age and sampling location treatments, 1-ml of faecal samples were taken with wooden sticks from inside, surface and inside-surface mixture of faeces at 0 (which means fresh), 1, 3, 7, 14 days post collection from the

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bear facilities. After each sampling, the remaining faeces were left undisturbed and subsequent samples were collected from undisturbed portions of the faeces. All faecal samples were soaked in 4-ml of 95% ethanol (Wasser *et al.* 1997; Murphy *et al.* 2002; Panasci *et al.* 2011), then frozen at -20 °C or kept at ambient room temperature in the laboratory for 2 weeks to serve as samples to test the effects of 2 different storage conditions. The sample size of each age, sampling location and storage method treatment was 10, with 2 from each of the 5 bears.

In the treatment of hair age, hair specimens with follicles were collected from captive bears while the animals were in narcosis for health check-ups. We designed 5 hair age treatments: fresh hair and hair of 7, 14, 30, and 60-day old, which were hair laying outdoors under partial tree shade for different amount of time after being collected from the bears to imitate hair collected from the hair-trap. Each treatment included 15 samples (10 hair follicles for each sample) which were also collected from different bears equally.

DNA extraction and PCR amplification

All faecal and hair samples of respective treatments were then preserved at -80 °C (Murphy *et al.* 2000) and DNA was extracted from these samples within 2 weeks to reduce the effect of long storage time. Faecal samples were extracted with methods detailed in Hung *et al.* (2004), which was modified from a hexadecyltrimethylammonium bromide (CTAB)-based extraction (Parsons *et al.* 1999). Hair DNA extractions were carried out by the traditional phenol-chloroform procedure (Kocher *et al.* 1989).

The amplification success may depend on the length of target amplified fragment. Thus all extracts were amplified of mitochondrial control region and its flanking regions using 3 primer pairs for different length of amplified segments: (1)1600bp, CB-Z, 5′-ATGAATTGGAGGACAACCAGT-3′ (Matsuhashi *et al.* 1999) and D4, 5′-AGGCATTTTCAGTGCCTTGCTTTG-3′ (Matsuhashi *et al.* 1999); (2) 900bp, CB-Z and Ut-Dr, 5′- TGCGTACATATGCGTACATAT-3′ (designed in this study); (3) 450bp, UT-1, 5′-TGATCACCAGGCCTCGAGAAA-3′ (Ishibashi and Saitoh 2004) and Ut-Dr. PCR amplifications were carried out using an ASTEC Thermal Cycler PC-808 in a total volume of 20 μL reaction mixture containing: 2 μL of faecal DNA extract and 0.5 μL of hair DNA extract respectively, 1× PCR buffer(including 1.5mM MgCl2), 0.5μM of each primer, 200μM dNTP and 0.5 U of Taq DNA polymerase (Supertherm Taq, JMR). The PCR thermal profile included an initial denaturation of 10 min at 95 °C, 35 cycles of 1 min at 95 °C, 1 min at 60 °C and 2 min at 72 °C, and a postcycling final extension at 72 °C for 10 min. A reagent with negative control to test contamination and a positive control to confirm proper PCR conditions were included in each group of PCR reactions.

The PCR products were electrophoresed on 1% agarose gels and visualized using ethidium bromide staining under UV light to score each PCR sample amplification/non-amplification for target DNA fragment. All samples that failed to produce a positive amplification were attempted to amplify for a second time to avoid random non-amplification (Murphy *et al.* 2007).

Data analyses

The amplification success rates for each treatment and each mtDNA fragments were calculated as percentage of the positive amplification number divided by the total number of PCR attempts.

Faecal DNA amplification results were firstly evaluated using the Friedman test to assess the effect of sampling location (sampling from inside, surface and inside-surface mixture of faeces) on amplification success in 6 preservation method and amplicon size combinations (2 preservation methods and 3 mtDNA fragments of different length). Next we used the Wilcoxon test to examine the differences between two faecal preservation methods (frozen at -20 °C and kept at room temperature). The Mann-Whitney U test was used to test the differences between amplification success rates of faecal and hair samples of 0-day and 7-day-old. The Friedman test, Wilcoxon test and Mann-Whitney U test were all computed using StatView 5.0 software (SAS Institute Inc.) and the results were considered statistically significant if the *P*-value was smaller than 0.05. Later the Page's trend test was performed on both faecal and hair DNA amplification results to test whether there were trends across sample ages and amplicon sizes.

RESULTS

Influence of faecal sampling locations

Fresh bear faeces were soft, moist, smelly, and contained indigestible fibers and seeds. One-day-old faeces kept their original shape and remained moist with a slightly dry surface. Three-day-old faeces kept their shape but were dry in the surface and soft inside. Seven-day-old faeces were hard, dry, and moldy. At the 14th day, the faeces became flaky and the remains contained mostly fibers. Therefore, we could collect samples from 3 sampling locations (surface, inside, and surface-inside mixture) successfully for all faecal samples except those that were 14 days old, from which we only collected a sample of surface-inside mixture.

The results showed that sampling locations had no significant effect on amplification success rate regardless of preservation method and amplicon size (Friedman test, $P = 0.145 - 0.926$) (Table 1). Therefore,

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Table 1 Comparisons of the effects of sampling locations on the PCR amplification success rates of faecal DNA collected from samples of different ages, stored by different methods and extracts for mtDNA control region fragments of different sizes. S: samples from surface of faeces. I: samples from inside of faeces, and M: samples from inside-surface mixture of faeces; F: frozen at -20 °C and R: kept at room temperature. *P*-values were the results of the Friedman test.

Age (day)	Storage Method	Amplification success rate (%)											
		450bp				900bp				1600bp			
		S	I	M	*P*-value	S	I	M	*P*-value	S	I	M	*P*-value
0	F	100(10/10)	100(10/10)	100(10/10)	0.607	100(10/10)	100(10/10)	80(8/10)	0.926	70(7/10)	40(4/10)	50(5/10)	0.145
1		100(10/10)	100(10/10)	100(10/10)		100(10/10)	90(9/10)	100(10/10)		50(5/10)	30(3/10)	70(7/10)	
3		90(9/10)	100(10/10)	80(8/10)		80(8/10)	100(10/10)	80(8/10)		10(1/10)	10(1/10)	20(2/10)	
7		60(6/10)	70(7/10)	80(8/10)		50(5/10)	70(7/10)	90(9/10)		20(2/10)	0(0/10)	0(0/10)	
14		-	-	40(4/10)		-	-	40(4/10)		-	-	0(0/10)	
0	R	100(10/10)	100(10/10)	100(10/10)	0.607	80(8/10)	100(10/10)	100(10/10)	0.717	30(3/10)	0(0/10)	20(2/10)	0.150
1		100(10/10)	100(10/10)	100(10/10)		90(9/10)	90(9/10)	90(9/10)		30(3/10)	30(3/10)	20(2/10)	
3		90(9/10)	80(8/10)	90(9/10)		90(9/10)	80(8/10)	90(9/10)		10(1/10)	0(0/10)	0(0/10)	
7		40(4/10)	60(6/10)	60(6/10)		20(2/10)	10(1/10)	10(1/10)		0(0/10)	0(0/10)	0(0/10)	
14		-	-	60(6/10)		-	-	40(4/10)		-	-	0(0/10)	

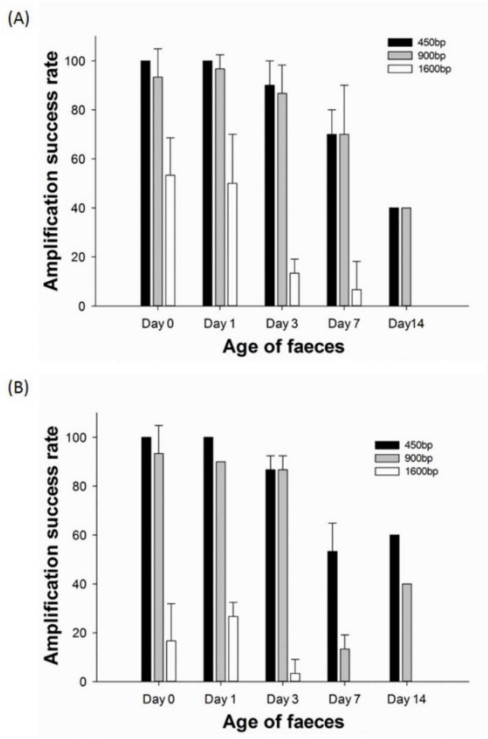

Fig. 1 The amplification success rate of faecal samples of different age kept (A) frozen and (B) under room temperature with different amplicon sizes. Data are the average values of samples collected from 3 different sampling locations, i.e. from the surface, inside, and surface-inside mixture of faeces, except for the 14-day-old faeces, from which only a surface-inside mixture sample was taken.

the data of 3 sampling locations were pooled to calculate the average values before examining the results of faecal preservation method and age of treatments.

Influence of preservation methods, age of faecal samples and amplicon size

The amplification success appeared unaffected by preservation methods (frozen at -20 °C or kept at room temperature in 95% ethanol) in shorter 450bp and 900bp mtDNA fragments from samples within a week (P = 0.330 for 450bp and P = 0.090 for 900bp, Wilcoxon test), but was significantly influenced when amplicon size was 1600bp (P = 0.011). In samples aged from fresh to 7 days, the amplification success rates of 1600bp fragment were higher in frozen samples (53.33% to 6.67%) than the room temperature samples (26.67% to 0%) (Fig. 1). Amplification success rates of 1600bp fragment dropped to zero for DNA extracted from 14-day-old faecal samples regardless of the storage method used (Fig. 1). Although PCR amplification success rates of 450bp and 900bp amplicons of the 7-day old frozen samples were higher than those of 14-day old samples as expected, an unexpected result was found in the PCR amplification success rates of 450bp and 900bp amplicons in 14-day old samples at room temperature, which were higher than that of the 7-day old samples (Table 1 and Fig. 1(B)).

When examining the trends of amplification success rates using average values of subsamples from 3 sampling locations by Page's trend test, both data of frozen and room temperature samples showed a significantly declining trend with increasing age of faeces (frozen samples: $L=163.5 > 160$ ($k=5$, $b=3$, $\alpha=0.001$), $P<0.001$; room temperature samples, $L=162 > 160$ ($k=5$, $b=3$, $\alpha=0.001$), $P<0.001$) and size of amplicon (frozen samples: $L=177 > 172$ ($k=3$, $b=13$, $\alpha=0.001$), $P < 0.001$; room temperature samples, $L=179.5 > 172$ ($k=3$, $b=13$, $\alpha=0.001$), $P<0.001$). PCR performances on DNA extracted from fresh versus 14-day-old faecal samples declined from 100% to 40% for 450bp fragments, from 93.33% to 40% for 900bp fragments, and from 53.33% to 0% for 1600bp fragments in frozen samples (Fig. 1(A)); and from

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100% to 60% for 450bp, from 93.33% to 40% for 900bp, and from 16.67% to 0% for 1600bp fragments in room temperature samples (Fig. 1(B)).

Influence of hair age and amplicon size

There was no significant difference between mtDNA amplification success of DNA extracted from fresh and 7-day-old samples of either the hair or faeces. In the results of hair treatments, Page trend test also showed a significantly decreasing trend of amplification success rates with both hair age and amplicon size (hair age: $L=157.5>155$ ($k=5$, $b=3$, $\alpha=0.01$), $P<0.01$; amplicon size: $L=68.5>68$ ($k=3$, $b=5$, $\alpha=0.01$), $P<0.01$). The amplification success rate of 450bp fragment was 53.33% even when the hair samples had been in an outdoor environment for 60 days (Fig. 2). But for 900bp fragment, the success rates decreased to 80% for 7-day-old samples, 53.33% for 14-day-old samples and 0% after 30 days (Fig. 2). Furthermore, the 1600bp fragment could only be amplified from fresh hair samples with 86.67% success rate (Fig. 2).

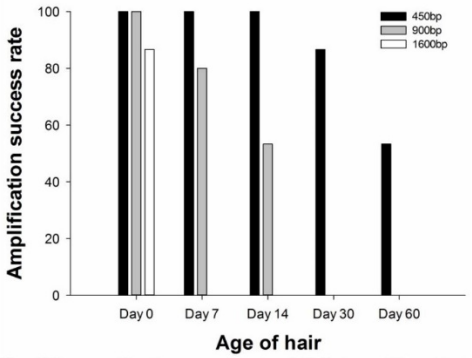

Fig. 2 The amplification success rate of DNA from different hair age with different amplicon sizes.

DISCUSSION

When collecting faecal samples in the field, most molecular scatology studies suggested sampling the outer portions of the faeces because a greater number of intestinal epithelial cells could be present (Albaugh *et al.* 1992; Flagstad Ø *et al.* 1999; Stenglein *et al.* 2010; Wasser *et al.* 2011). Stenglein *et al.* (2010) indicated that the sampling location had a significant effect on nuclear DNA quality of brown bear and wolf scats, and the outer part of the faecal samples had higher DNA quality. Our results show that sampling locations of faeces have no significant effect on mtDNA amplification success rate. Such discrepancy in the results may be due to 2 potential reasons. First, the mtDNA and nuclear DNA may have differential decay rates and patterns (Berger *et al.* 2001; Foran 2006; Soto-Calderon *et al.* 2009; DeMay *et al.* 2013). Second, DNA decay rates and patterns may be different under different climatic condition (Panasci *et al.* 2011). Most of the studies regarding the effects of sampling locations were conducted in the temperate region (Stenglein *et al.* 2010; Wasser *et al.* 2011). Faeces exposed to the subtropical environment of high temperature and humidity in our study might have an effect particularly on the outer portions of faeces, and likely counterbalance the advantage of having more and better quality intestinal epithelial cells on the surface.

Our results showed that preservation methods did not affect the amplification success rates of 450bp and 900bp mtDNA fragments from samples collected within a week. However, the success rates of 1600 bp fragment from the frozen samples was significantly higher than those samples stored at room temperature. Similar to our findings, Santini *et al.* (2007) suggested that wolf scats stored in 95% ethanol at -20 °C had the best nuclear DNA quality comparing to those stored in 95% ethanol at room temperature, dried at -20 °C, and in GUS at room temperature. Santini *et al.* (2007) further indicated the disparities between samples kept frozen and at room temperature increased over time, e.g. 98% positive PCRs at -20 °C and 55% successful PCRs at room temperature after 6 months. However, the non-linear decrease in the amplification success rates of DNA extracted from samples stored at room temperature (Fig. 1(B)) was inconsistent with the results of the frozen samples, even though the declining trend of the amplification success rates of DNA with increasing age was statistically significant, which may suggest that the performances of preservation at room temperature may be less predictable than those of frozen samples when the storage time exceeds one week. Such results suggest that although immediate freezing of faecal samples is often difficult in the field, researchers should consider it especially when amplification of longer mtDNA fragment is critical for their research. In any case, freezing ethanol-soaked samples is highly recommended after the samples are brought back to the laboratory.

Most of the studies regarding the impact of sample age on faecal mtDNA amplification indicated that the amplification success generally decreased over time (Farrell *et al.* 2000; Murphy *et al.* 2007; Soto-Calderon *et al.* 2009; DeMay *et al.* 2013). Our results are consistent with these studies and those studies that showed a decreasing trend in the amplification success rates with increasing amplicon size (Broquet *et al.* 2007; DeMay *et al.* 2013). Furthermore, we found that for the samples as old as 14 days the amplification success rates of mtDNA remained to be at least 40% for the 450bp and 900bp fragments; in contrast, 1600bp fragment could not be amplified from faecal samples older than 7 days (Fig. 1). Information on rates of

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faecal DNA degradation regarding sample age and amplicon size in this study allowed researchers to choose better strategies for collecting noninvasive samples and choose suitable markers depending on the conditions of faeces in the field to balance the costs and output of laboratory work. When faecal samples are of older age, smaller mtDNA fragments are expected to have higher amplification success rates and may therefore be favored in genetic studies. If larger mtDNA sequences with increased resolution are needed for phylogenetic research (Waits *et al.* 1999), the noninvasive genetic materials need to be extracted within a certain time frame.

The results of amplification success rates showing no significant difference between hair and faeces at 0 and 7 days in our study are consistent with the comparative review of Broquet *et al.* (2007). Broquet *et al.* (2007) mentioned that greater inhibitor concentrations may counterbalance the advantage of larger target DNA amount in faecal samples. Regarding the effects of hair age and amplicon size on amplification success rates of DNA from hair samples, Roon *et al.* (2003) demonstrated that DNA of hair degraded with time when the samples were preserved using silica desiccant and -20 °C freezing. Broquet *et al.* (2007) reviewed the relationship between mtDNA amplification success and fragment length of hair samples in 2 published papers (Vigilant 1999; Roon *et al.* 2003) and indicated the shorter fragments lead to higher amplification success. However, few studies had measured the rates of hair DNA degradation regarding sample age in outdoor environment without preservation and amplicon size like our study, which indicated that amplification success rates significantly decreased with both hair age and amplicon size. In addition, the amplification success rates we found were lower in comparison to the rates in Roon *et al.* (2003), which might suggest the impact of high temperature and humidity on the quality and degradation rates of DNA of hair samples collected in the subtropics. Researchers conducting noninvasive analyses in the subtropics therefore can consider the DNA amplification success rates from hair samples of different ages revealed in this study and design suitable intervals for hair collection to get appropriate DNA materials.

The mtDNA fragments are useful in addressing questions about species identification, population structure, and phylogenetic research (Waits *et al.* 1999; Murphy *et al.* 2002; Roon *et al.* 2003), whereas the microsatellites of nDNA have utility in individual identification, kinship analysis, gene flow, and demographic studies (Murphy *et al.* 2002; Roon *et al.* 2003; DeMay *et al.* 2013). Mitochondrial DNA and nuclear DNA may have differential decay rates (Foran 2006; Soto-Calderon *et al.* 2009) and some studies have suggested using mtDNA as a screening for further nDNA analyses (Hung *et al.* 2004; Vynne *et al.* 2012). Our study examined the amplification success rates of mtDNA from faecal and hair samples but the decay rates of nDNA in the subtropics remain unanswered. Consequently, it would be necessary to examine the amplification success rates of nDNA from various non-invasive materials in the future.

In addition, some studies have indicated that diet may influence target DNA quantity and genetic analysis of faeces (Murphy *et al.* 2003; Nsubuga *et al.* 2004; Panasci *et al.* 2011; Vynne *et al.* 2012; DeMay *et al.* 2013). Vynne *et al.* (2012) further suggested that the effect of diet should be considered especially in studies of species with highly varied diets. Asiatic black bears are omnivorous animals and the diet of the Formosan black bear in the subtropical Taiwan does change seasonally (Hwang *et al.* 2002). Although the faecal samples of this study were deposited from zoo bears with a mainly vegetarian diet, the components of the diet were not the same as the natural diet of bears in the wild. Therefore, evaluation of faecal DNA degradation under different natural diet of the bears is recommended in future studies.

Our study is the first one to quantitatively evaluate mtDNA degradation of noninvasive hair and faecal samples of Ursid animal in the subtropics. The discrepancy of results between our study and the comparative research in temperate region suggests the importance of pilot study for a new study system. In conclusion, our results demonstrated that faeces and hair could be applied as noninvasive samples for the Asiatic black bears under subtropical climate. We suggest that the amplification success rates are not influenced by sampling location of faeces in subtropical environment. The immediate freezing of ethanol-soaked faecal samples in the field are not so critical in affecting DNA quality of short fragments from samples collected within a week but the effect of immediate freezing is significant for longer mtDNA fragments. We also found that although it may be challenging to amplify longer mtDNA fragments from older faecal and hair samples, shorter fragments could be successfully amplified. Researchers collecting noninvasive samples in similar taxa and field conditions should consider the DNA degradation rates revealed in this study. Careful selection of primers for suitable PCR product sizes depending on sample conditions could optimize success rates of genetic analysis and save both time and financial cost in noninvasive genetic research.

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