行政院國家科學委員會專題研究計畫 成果報告

建立鹿的標靶比較基因組晶片並用來篩檢和鑑定與鹿茸生長有關的基因
研究成果報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 100-2311-B-040-001-
執 行 期 間 ：100 年 08 月 01 日至 101 年 07 月 31 日
執 行 單 位 ：中山醫學大學生物醫學科學學系（所）

計 畫 主 持 人：李月君

計畫參與人員：此計畫無其他參與人員

公 開 資 訊 ：本計畫可公開查詢

中 華 民 國 101 年 10 月 30 日

中 文 摘 要 ： 摘要

鹿茸每年再生的特性不僅可作為研究組織再生很好的動物模式，同時鹿茸在傳統中藥中是屬於珍貴的藥材。在台灣，高產鹿茸是業者所追求之目標。提高鹿隻的產茸能力方法多由育種、營養及改變環境因素來達成，而目前主要以育種方面著手。可見高產鹿茸是屬於一種遺傳相關的性狀，因此可利用分子選種的方式幫助品種之改良。新近已發展一個快速且經濟的 DNA 晶片用來篩檢動物的性狀基因座，但迄今未有關鹿的 DNA 晶片應用被報導。

我們實驗室最近已完成 3 種台灣特有鹿類(台灣水鹿、台灣梅花鹿、台灣山羌)的核型比對和跨物種的 BAC-原位螢光雜交定位。還有我們也建立了涵蓋 4 倍山羌基因組的 BAC 基因組資料庫及建構了平均 0.1Mb 解析度的山羌-牛-人的比較基因組圖譜。以我們所具備的專門技能及資源，可以建造屬於鹿類的第一代比較基因組晶片，並用來篩檢及定位與高產鹿茸有關的性狀基因座。

主要目標:

1. 建造屬於鹿類的第一代標靶比較基因組晶片
2. 篩檢和分析與高產鹿茸有關的性狀基因座的拷貝數變化。

方法:

1. 收集高產鹿茸、正常產鹿茸及低產鹿茸的各十隻的臺灣水鹿血液，並抽取其基因組 DNA。
2. 從我們實驗室現有的山羌的 BAC-DNA 基因組資料庫中，篩選出 10000 山羌 BAC 菌株 含有大於 80kb 的 DNA 片段。
3. 利用 phi29 polymerase 擴大 10000 山羌 BAC DNA，再打點於玻片上，製成屬於鹿類的第一代比較基因組晶片。
4. 利用自製的標靶比較基因組晶片分析和鹿茸產能有關的性狀基因座其拷貝數變化。
5. 定序此候選基因並與牛的參考基因序列比較其可能的基因同源性。

結果:

1. 我們已製造可用的屬於鹿類的第一代比較基因組晶片。
2. 藉由自製的第一代鹿類比較基因組晶片，我們已找到 14 個和鹿茸產能性狀有關的可能基因的拷貝數變化。
3. 經由和牛的參考基因序列比較其可能的基因同源性，發現這些 14 個可能基因數變化中有 6 個是和鹿茸的生長有極度相關性的。

意義:
經由實驗證實本計劃中所建造的比較基因組晶片是可用的，且可提供快速及有效地篩選高產鹿茸的鹿隻的一種分子選種晶片。另外，此次研究發現與鹿茸產能有關的性狀基因，將來更進一步的研究能夠闡明鹿茸每年生長的機制，以更加了解完整的骨骼組織再生的機制，希望有朝一日可應用於復健醫學的骨骼重建。

關鍵詞: 鹿茸、比較基因組晶片、基因座的拷貝數變化、山羌的 BAC 基因組資料庫

中文關鍵詞： 鹿茸、比較基因組晶片、基因座的拷貝數變化、山羌的 BAC 基因組資料庫

英文摘要： Abstract:
Deer antler not only provides an animal model for studying mammalian tissue regeneration but also is a highly economic product used in traditional Chinese medicine and nutrients. Through selective breeding and natural mating study suggested that the high yielding of velvet antler is a genetic related trait. Recently, a rapid and economical DNA microarray approach has been applied in high-resolution mapping the quantitative trait loci (QTL) of in some animals but not yet including deer species.
Specific Aims:
1. Establishing the first generation DNA chip for cervidic genomic microarray
2. Analyzing the copy number changes (CNCs) related to the QTL of deer antler growth.
Methods:
1. Blood collection and isolation of blood genomic DNA from 30 heads of Sambar.
2. Selecting 10000 muntjac BACs containing more than 80 kb insert.
3. phi29 polymerase amplifying the BACs and spotting on the slide to generate the cervid-specific BAC microarray.
4. CNCs analyses of deer with different production of velvet antler using the home-made microarray.
5. BAC-end sequencing the candidate BACs and blast

the sequence similarity with bovid reference sequences.
Results:
1. We had constructed the first cervid-specific BAC-based microarray.
2. We used the homemade microarray to analyze the CNCs related to antler production efficiency. We found the CNCs of 14 BACs.
3. Among 14 BACs, 6 BACs corresponding gene have highly related to antler growth.
Significances:
The results of this study suggested that the analyzing system of CNC (BAC-based aCGH) is feasible. The home-made first cervid-specific BAC-based microarray provides useful and powerful cervid chips for the selection of ’giant antler breed’. In the future, further exploring these candidate genes, it will elucidate the genomic factors affecting the annually regeneration of deer antler and shed more lights on the mechanism of complete bone tissue re-growth which has great potential in rehabilitated medicine.

英文關鍵詞： deer antler, aCGH chip, copy number changes, Indian muntjac BAC library

報告內容：

Introduction and literature review:

Deer antler not only provides a model for studying mammalian tissue regeneration but also is a highly economic product used in traditional Chinese medicine and nutrients. To raising the antler production, there are several strategies applied in farm such as interspecies hybrids, selective breeding, hormone control, nutrition, and day-length control etc. (Li, et al. 2001; Price et al. 2005; Suttie et al. 1989; Du and Bai 2007). Through selective breeding and natural mating, the progeny always inherited high production of velvet antler from buck parent with high production of velvet antler (Wu,畜產專刊 44 期). This suggested that the high yielding of velvet antler is a genetic related trait. Although, understanding the genes underlying important economical traits and quantitative traits is the basic footstone for breeding of farmed animals. Unfortunately, little genomic information is available in deer species.

Recently, a rapid and economical DNA microarray approach has been applied in high-resolution mapping the quantitative trait loci (QTL) of Drosophila (Lai et al. 2007). This result showed 18 QTLs affecting the variation in lifespan of Drosophila. Additionally, SNP microarray and aCGH chip has been used extentively in studying the SNPs and copy number changes (CNCs) related to the complex disease of human, since human genome sequences almost complete (Redon et al. 2006). All DNA chips are based on the informative genome sequences of species. However, the genomic information of deer species is few and no complete whole genomic sequences of deer is available in any public gene bank.

There are 3 species of indigenous deers in Taiwan (Formosan sambar, Formosan sika deer, and Formosan muntjac). Formosan sambar and Formosan sika deer have high economic value for antler production in Asian area. There have been some studies regarding the morphology, ecology, behavior, habitat, reproduction and breeding of these deer species reported (楊和陳 1994; 吳 et al. 2004; 王 et al. 2008; Chan et al. 2009). However, the genomic study of Formosan sambar and Formosan sika deer is few except of Formosan muntjac (Lin et al. 2004 and Chiang et al. 2004). Understanding the genomics of Formosan sambar and Formosan sika deer is a fundamental and beneficial subject for improving this agricultural resource and for understanding the mechanism of annually re-growth of deer antler. The first step of studying the genome of an organism is to characterize its karyotype. In our preliminary study, we have identified karyotypes of 3 indigenous deer species in Taiwan (Lin et al. 2004; Chiang et al. 2004; unpublished data 2011). Furthermore, the karyotypic comparison among sambar, sika deer, and muntjac has been achieved by G-banding and cross-species FISH analysis (appendix). Additionally, we had constructed the Indian muntjac genomic BAC library with 4x coverage (Lin et al. 2008). Based on these resources, in this study, we established the 10000 cervidae-specific BAC-based array CGH chip for mapping the QTL related to the annual re-growth of deer antler. Furthermore, we got 14 BAC clones with differential copy numbers between high and low antler production of deer in this study.

Specific Aims:

1. Establishing the first generation DNA chip for cervidic genomic microarray using muntjac (cervidae family) BAC clones (BAC libray).
2. Analyzing the copy number changes (CNCs) related to the QTL of deer antler annual re-growth using the generated cervidic BAC-based array CGH chip.
3.

Materials and methods:

Blood collection and isolation of blood genomic DNA:

The morphology or phenotype of deer species were determined by veterinarian (an Associate-PI in this project) and farmer. 30 Sambar deer were divided based on the 3 different production of antler (high, normal and low). The blood collection was performed by veterinarian. The isolation of blood genomic DNA was performed using whole genomic DNA isolation kit (QUIAGEN).

Screening and selecting BACs within the candidate chromosomal regions with CNCs.

According to the results of chromosome-based cross-species CGH, the candidate chromosomal regions with CNCs was identified. Based on our BAC-FISH map, the BACs in the candidate

chromosomal regions were selected. All selected BACs were BAC-end sequenced and analyzed first to make sure the BACs with unique DNA sequences. The BAC-end with unique DNA sequences was selected for primer designation. Subsequently, we will perform BAC library screening for several rounds to get contiguous BACs in the candidate chromosomal regions by PCR-screening using the primer pairs in the BAC-end. The physical position of BACs with positive PCR signals were confirmed by cytogenetic FISH mapping.

Amplification of BACs and generating the BAC-based microarray chip:

10000 BACs containing more than 80kb insert DNA were extracted using the Qiagen R.E.A.L. Prep 96 Plasmid kit and amplified by phi29 polymerase using Ex0-resistant random primers in separate reactions as described (Fiegler et al. 2003; Thomas et al. 2003). Two ug of amplified BACs were resuspended in 10 ul of 100mM Sodium phosphate solution and spotted onto Corning UltraGAPS slides using an OmniGrid Accent Microarrayer (GeneMachine) with 16-printing pins. Array chips were then subjected to a series of validation procedures described elsewhere (Thomas et al. 2003), including self-self and sex-mismatch hybridizations with germline DNA isolated from Sambar with normal karyotype and normal antler production.

CNCs analyses of deer with different production of velvet antler

Test (high or low antler production) and reference (normal antler production) DNA probes were labeled with Cyanine3-dCTP or Cyanine5-dCTP (Perkin Elmer) as required, using a BioPrime Array CGH Labeling System (Invitrogen) following the manufacturer's recommendations. Hybridization was performed as described (Li et al. 2006). Arrays were scanned using a GenePix4000B at 10-μm resolution and analyzed with GenePix Pro 4.1 (Axon). Each spot position was automatically located, and manual adjustments were made as necessary. Spots with poor morphology and those impinged by fluorescent debris were excluded from further analysis. In each instance a minimum of 98% of clones need to pass the exclusion criteria. Fluorescence intensities were calculated for each spot after local background subtraction, and normalized to a mean 1:1 ratio on the autosomal clones; ratios of normalized values were than established. The mean fluorescence ratio of each duplicate were then converted to a log2 ratio in order to weight genomic gains and losses equally, and plotted graphically. Following standard conventions for CGH analysis, clones demonstrating a test:reference fluorescence ratio greater than 1.25:1 (gain) or less than 0.75:1 (loss) were classed as CNCs. Dye-flip aCGH also was performed for each test deer. The significant CNCs with antler production efficiency were scored by comparing CNCs from 3 different groups (high vs. normal antler production; low vs. normal antler production; and high vs. low antler production) under no prior knowledge of the expected copy number.

Sequencing the BACs with the candidate CNCs:

The confirmed BACs with the candidate CNCs were full sequenced and blast to the genome sequences of other species in public available gene bank for orthologous gene similarity assay.

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Results and discussions:

Karyotype of Sambar deer:

We had collected and finished the karyotype analysis of 32 sambar blood samples. The tested sambar has the complete karyotype of sambar species, with exception of one deer having a hybrid karyotype from red deer. We will exclude this sample for the next experiment. The complete karyotype of Sambar deer has 2n=62 chromosomes with 4 pairs of bi-armed autosomes, 26 pairs of acrocentric autosomes, the large X chromosome, and the smallest bi-armed Y chromosome (Figure 1). The genomic DNA of 31 Sambars has been isolated for the next aCGH analysis.

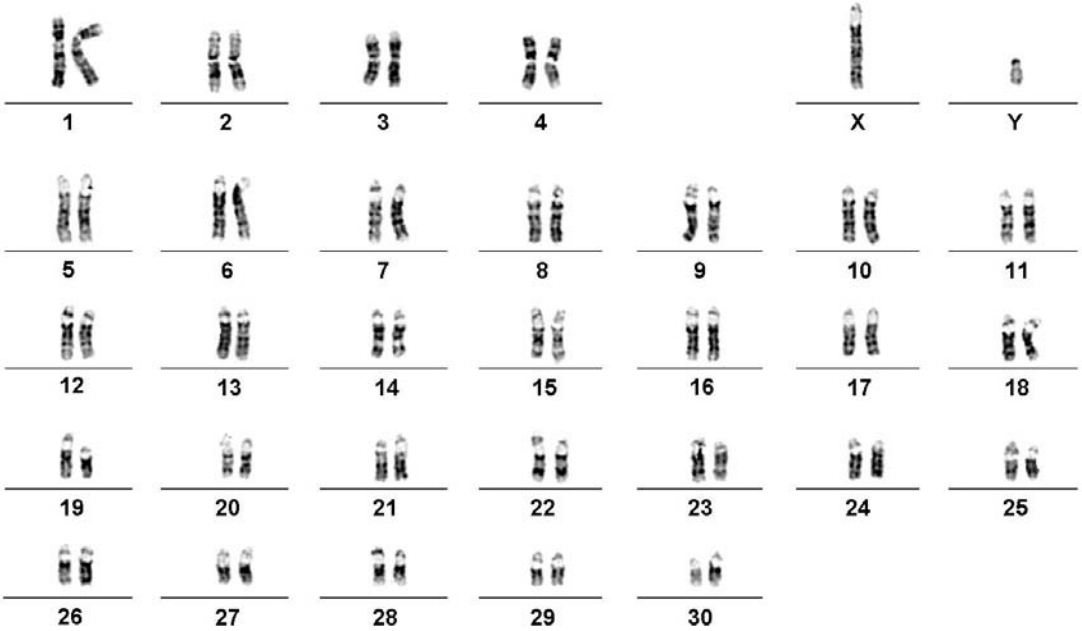


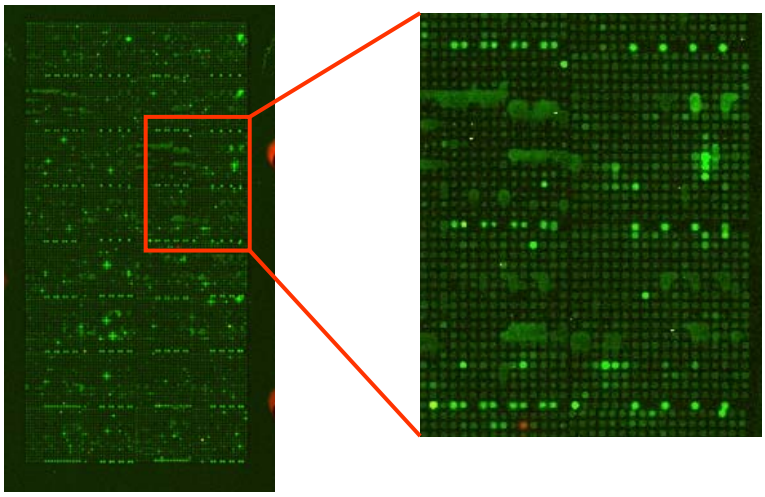
Figure 1: The karyotype of Formosan sambar deer by GTG-banding.

Fabrication of the first generation cervid specific BAC-based microarray:

We chose 10000 IM-BAC clones containing more than 80kb insert DNA. After purification and

amplification of BACs, BACs were spotted onto Corning UltraGAPS slides using an OmniGrid Accent Microarrayer (GeneMachine) attached with 32-printing pins. The part of the spotting experiment was conducted by the Microarray Core Facility, Institute of Molecular Biology, Academia Sinica. However, there was a problem of washing station causing the diffusion of spots in the first printing (Figure 2A). The diffusion problems of spots had been solved in the second printing (Figure 2B).

A:



B:

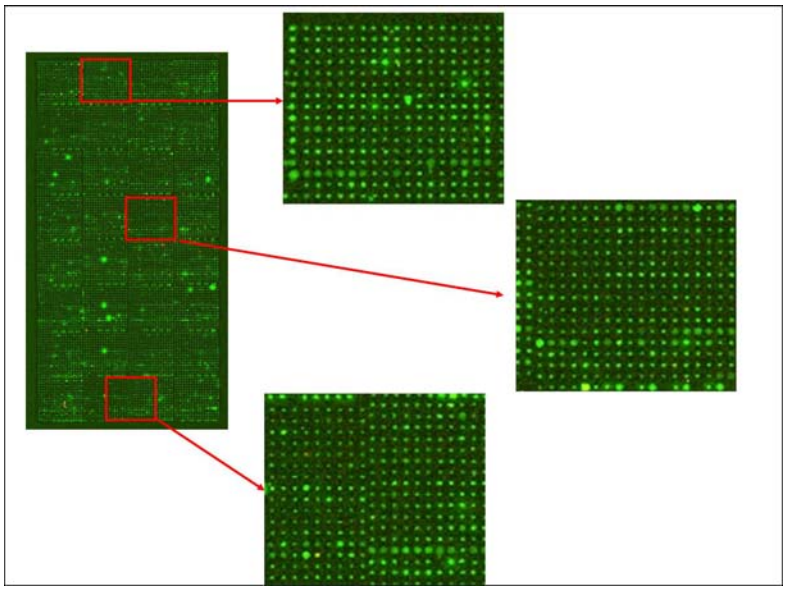


Figure 2: The first generation cervid specific BAC-based microarray covering 10000 BACs. Each BAC was spotted on the amine-silane slide (Corning UltraGaps Coated slides) by OminGrid attached with 32 quill pins. There are 32 subarrays and each subarray has 324 dots (18*18 dots). The dot spacing is 250 um in horizontal and 250 um in vertical. A: the prescanning picture of the first printing chip. B: the prescanning picture of the second printing chip.

CNCs analyses of deer with different production of velvet antler

We used the homemade cervid specific BAC-based microarray to analyze the CNCs related to antler production efficiency. 3 groups of deer were categorized into high, normal, and low production of antler. The significant CNCs with antler production efficiency were scored by comparing CNCs from 3 different groups (high vs. normal antler production; low vs. normal antler production; and high vs. low antler production). There are 14 BACs with significant copy number differences between deer with high vs. low antler production.

Sequencing the BACs with the candidate CNCs:

We further sequencing the both BAC-end of 14 candidate BACs and blast their sequences with

cow reference sequences. The results (Table 1) showed two BACs (1212H12 and 1207H1) are related to osteochondrocyte development; 1208H9 is related to the multicellular organismal development; 3 BACs (1212B7, 1206H8, and 1205E8) are related to nutrient absorption and cell growth. These correspond genes are related to antler growth. Therefore, this result suggested the analyzing system of CNC (BAC-based aCGH) is feasible.

Clone number	Correspondent to cow gene
1213D8	Resolvase-like gene
1213B7	Spermatogenesis-associated protein13 isoform1
1212B7	Solute carrier organic anion transporter family 3A1 member
1212G4	Resolvase-like gene
1212H12	Dymeclin
1210D7	Receptor expression-enhancing protein 3
1208B8	Unrelated
1208H9	PREL1 domain-containing protein 2
1208F1	Unrelated
1207H1	Protein osteopotentia homolog isoform 2
1206H8	Cytosolic β-gluosidase
1205B4	Teashirt homolog 3
1205E8	Nicotinamide phosphoribosyltransferase
1205F4	Uncharacterized proteins

In the future, we need to confirm the CNCs of these candidate genes on more deer by real-time PCR. If we could find more candidate genes related to antler growth, 1. we can make a powerful chips in selection breed of deer species with high antler production; 2. we can shed more lights on the mechanism of annually regeneration of deer antler.

國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/29

國科會補助計畫	計畫名稱: 建立鹿的標靶比較基因組晶片並用來篩檢和鑑定與鹿茸生長有關的基因
	計畫主持人: 李月君
	計畫編號: 100-2311-B-040-001- 學門領域: 動物學

無研發成果推廣資料

100 年度專題研究計畫研究成果彙整表

計畫主持人：李月君	計畫編號：100-2311-B-040-001-
計畫名稱：建立鹿的標靶比較基因組晶片並用來篩檢和鑑定與鹿茸生長有關的基因	

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	無

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：□已發表 □未發表之文稿 □撰寫中 ■無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

原本此一計畫是申請兩年期的計畫，最後只獲得壹年期的計畫補助，由於經費問題，實驗只進行至預計一年可完成的部份。若需要有完整的結果，且達到發表的結果，需要等到貴院再次補助研究經費才能進行另一年期的研究部分。

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

此一第一年期的研究成果，已有初步的結果: 經由自製的鹿的 aCGH 晶片結果分析，所得到的基因是非常可能具有調控鹿茸生長的基因。 非常可惜的事，並沒有獲得第二年的補助，所以無法繼續研究證實所找到的基因，在巨大鹿茸生長中所扮演的角色，其如何調控鹿茸生長的機制。 如果能獲得經費的補助，此次研究就可以繼續研究證實所找到的基因在巨大鹿茸生長中所扮演的角色，進而作為篩選及培育有巨大鹿茸的鹿隻，提高鹿茸產量的經濟效益。