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臺灣梅花鹿品系之分析

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摘　要

本計畫是梅花鹿復育研究計畫的一部份，目的為推測梅花鹿復育族群的遺傳結構，並找出分辨純種台灣梅花鹿之方法，以便日後進行種源引入時，可判別鹿隻的血統。將梅花鹿各亞種之粒線體基因群的三個片段，利用聚合　鏈反應放大並分析其序列。從粒線體細胞色素b基因的5'端獲得264個鹽基的序列八條，其來源為四隻台灣梅花鹿（Cervus nippon taiouanus）、一隻C.n.hortulorum、一隻日本梅花鹿（C.n.nippon）、一隻C.n.pseudaxis及一隻C.elaphus xanthopygus。自粒線體細胞色素b基因的3'端獲得375個鹽基的序列十一條，其來源為八隻台灣梅花鹿（C.n.taiouanus）、一隻C.n.hortulorum、一隻日本梅花鹿（C.n.nippon）、一隻C.n.pseudaxis及一隻C.elaphus xanthopygus。所有個體的粒線體基因型皆不相同，表示在復育鹿群的基因庫內有很大的變異度。台灣梅花鹿之細胞色素b基因在靠近3'端的部份都有三個鹽基消失（即缺少一個氨基酸），只有一隻例外。此特徵加上序列分析之結果顯示，除了上述例外的一隻外，所有被取樣之台灣梅花鹿均屬於單一起源的群體。本結果認為此一例外的個體亦屬於台灣梅花鹿，但不絕對的確定。本研究使用之方法可用來分辨不同的梅花鹿亞種。

前　言

梅花鹿（Cervus nippon）有許多亞種，原生於亞洲東部，其中包括已在野外絕跡的台灣特有亞種－台灣梅花鹿（C.n.taiouanus）。在墾丁國家公園內進行之台灣梅花鹿復育計畫，即利用台北市立動物園之梅花鹿作為復育之起始族群，嘗試恢復台灣梅花鹿的野外族群。對此類型的復育計畫而言，有一項必需進行之工作，即為了解復育族群的遺傳結構，以確保野放之族群具有足夠的遺傳變異度。由於墾丁之鹿群完全來自同一個飼養族群，故上述之工作變得更為重要。本計

畫的研究目標之一即為推測復育鹿群的變異程度。

本計畫的另一個目標是想確定運用遺傳研究的方法可否分辨出梅花鹿的各個亞種，其重要性是因為未來可能必需自其他飼養族群引進新個體，以增加復育族群的遺傳變異度。而台灣的其他鹿群多半分散在各養鹿場中，許多已經和自日本及其他地方引進之其他亞種雜交，因此辨別鹿隻的血統漸為重要，以避免將外來基因引入復育族群中。墾丁另一飼養鹿群為來自東海的不明血統鹿群，本群亦列入研究以測試本遺傳研究方法的適用性。

有關梅花鹿遺傳學之研究，以往多集中於核型鑑定及同工異構酶比較（Feldhamer,1982b;van Tuinen,1983;Sung,et al.,1985;Sung, et al.,1988)這些方法在某些鹿種的族群辨別上很有用，但在梅花鹿上卻顯出很小的差異性。原因可能是對偶基因數相當少及樣本數太少，而非真的缺乏遺傳差異。

本研究利用直接比較DNA 序列的方法來比較個體的遺傳差異，針對粒線體染色體上的D 環（displacement loop:D-loop）片段及粒線體細胞色素b 基因，使用聚合酶鏈反應（PCR）將之放大然後分析其序列。由於粒線體基因群是遺傳自母親，且不像核染色體有每代重組的現象，因此粒線體的血統可保存許多世代。若有一不同的粒線體血統，例如非台灣梅花鹿被引入一族群中，在許多世代後仍可以此法分辨出來。然而必需注意的一點是，引入之外來個體必需是雌性才能利用此種辨別方法。

材料與方法

一、DNA樣本之取得

於1990年秋，自台灣兩個不同的梅花鹿族群（分飼於三個鹿群）取得51個樣本。三個鹿群中有兩群飼養於墾丁國家公園，其中一群來自東海大學，血統不明，此群共收集27個樣本。墾丁另一群梅花鹿是自台北市立動物園引入並繁殖者，一般認為是純種台灣梅花鹿，此群共收集13個樣本。樣本包括血液及毛髮樣本。在墾丁畜試所將30個全血樣離心取得濃縮之白血球，再冷凍乾燥以避免DNA 分解。另有10個血樣是以血液染在布料上乾燥取得。同時自台北市立動物園取得11個樣本，包括血染樣本及全血緩衝溶液混合液。由於墾丁之復育鹿群是來自台北市立動物園，因此在遺傳學上是屬於同一個族群。

其他梅花鹿亞種：C.n.hortulorumm 與 C.n.pseudaxis，及一種與梅花鹿血緣相近的鹿：C. elaphus xanthopygus，其DNA 樣本自紐約動物學會的George Amato處取得。日本梅花鹿（C.n.nippon）的組織樣本則自Sendai大學的Seiki Takatsuki處取得。

從血液與組織樣本中取得DNA 的方法是利用K 蛋白酶消化細胞，phenol/chloroform 混合液萃取之，再用乙醇沉澱出來（詳細步驟見附錄一）。

二、聚合酶鏈反應及序列分析

利用聚合酶鏈反應將粒線體DNA 中含D 環的區域或細胞色素b 基因的序列分離出來（Saiki,et al.,1988,附錄二），第一次步驟使用primer L15926 及H16498將粒線體基因群中一含580 個鹽基的片段放大，此片段含有D 環前397 個鹽基，放大結果得到一單股的D 環DNA，再利用下列各種primer結合物進行不對稱放大：L15926／H16498，L1／H16498；L2／H16498，L15926／H2及L1／H2。PCR 之條件為94° 一分鐘，60° 一分鐘，72° 三分鐘，40個循環。

細胞色素b DNA之取得係利用primer L14841及H15915放大一段含1074個鹽基的片段，然後利用其他 primer 製造單股DNA 。所使用之primer結合物如下：L14841／H15915，L15408／H15767 及 L15609／H15915。各primer的序列詳見表一。PCR 之條件為94° 一分鐘，50° 一分鐘，72° 一分鐘，40個循環。

三、資料分析

將細胞色素b 序列排列比較，若某特定鹽基位置有至少兩個個體有變異，即視為有區別的特徵並分析。每三個鹽基序列或稱為密碼，可對應出細胞色素b 蛋白質內的一個氨基酸，而密碼中的第三個位置其突變速率通常較第一及第二位置快，所以在序列比較中第三位置差異要比第一及第二位置差異為多。由於第三位置經多次突變而不易分析，故變化較慢之第一及第二位置在演化分析上較有用。將各序列編成目錄並標出有差異的位置，然後利用電腦軟體推算導致目前之序列差異所需之最少突變次數，所得之結果為一演化樹，代表被取樣個體之可能的血統關係。

本報告之結果是使用PHYLIP之簡化繹算法（Felsenstein,1987）獲得，其信賴值使用同一個軟體之bootstrap 選項指定到演化樹中，用不同的序列及不同的個體數得到各種可能之演化樹。

結果與討論

一、D 環序列

部份放大使用各種 primer 對整個D 環進行放大，結果顯示梅花鹿的D 環長度大約為1000個鹽基。

分析17隻鹿之D 環區前397 個鹽基，包括 Dybowski's 梅花鹿，

Tonkin 梅花鹿，日本梅花鹿及 Asian wapiti，發現在梅花鹿之間均無任何差異，僅在梅花鹿與Asian wapiti間有兩個差異，因此在梅花鹿族群內無法以D 環來做遺傳變異的分析，此397 個鹽基序列詳見圖一。

二、細胞色素b 序列

此基因之5'端有264 個鹽基被放大分析，共取樣八隻鹿，結果列於圖二；3'端之375 個鹽基大部份被放大分析，共取樣11隻鹿，結果列於圖三。

在264 個鹽基片段中有37個變異的鹽基，其中兩個是密碼中的第一位置，三個是第二位置，32個是第三位置；變異中第一位置有一個，第二位置有一個，第三位置有八個均只出現在一個個體上，故在種源演化的研究上無法提供資訊；另一個第一位置變異及五個第三位置變異亦無法利用，因其在一隻鹿或多隻鹿基因上的位置無法確定。

在375 個鹽基片段中有72個變異：19個在第一位置，4 個在第二位置，49個在第三位置；變異中第一位置有六個及第三位置有22個無法提供資訊；而兩個第一位置變異及三個第三位置變異無法利用，因其在一隻鹿或多隻鹿基因上的位置無法確定。

上述序列中都沒有完全相同者，故八隻台灣梅花鹿之粒線體基因群均不同。此結果顯示在復育鹿群內其粒線體基因型具有相當大的遺傳變異程度。雖然樣本數少且無核型的分析，但結果明顯指出復育鹿群目前的基因庫內應仍有足夠的遺傳變異度。然而此結果並不表示基因庫不需再增大，只能說目前可能不會有近親交配的重大危險。

所有的台灣梅花鹿除了TPZ34外，均有一特徵即是在序列的15613號位置上有一個氨基酸消失，此種消失突變相當罕見，並指出在這種鹿的早期歷史中某個時期，必定發生了一次這種突變。只有台灣的鹿有這種特徵，但並非所有台灣的鹿皆有此特徵。

對此發現有兩種解釋，一是此種消失突變發生在梅花鹿到達台灣之後，因此在台灣有兩種粒線體基因型；另一種可能性是此種突變發生在梅花鹿到達台灣之前，因此所有的台灣梅花鹿皆應有此種特徵，而TPZ34 沒有這種特徵，故不是純種台灣梅花鹿。

為了找出是何種可能性，因此演化樹之推演是利用電腦軟體PHYLIP 對三組不同的數據進行最嚴格的篩選，結果列於圖四。圖四a 代表12個個體的演化樹，這些個體在細胞色素b 基因的5'端有九個變異，這個演化樹只有一個分枝，表示台灣梅花鹿和其他的鹿在大於90%的50 bootstrap trials 時分開。圖四b 代表有21個變異的10個個體的演化樹，有四個分枝發生在90%的50 bootstrap trials 。圖四c 代表在第一及第二位置有五個變異的九個個體之演化樹，只有一個分枝發生在大於90%的50 bootstrap trials 時。

這三個演化樹都明顯指出，台灣梅花鹿除了TPZ34 外均屬於同一

獨立的血統群（表示單一起源），因為他們皆有同一種消失突變。TPZ34 在圖四a 中被歸入台灣梅花鹿，但在圖四c 中則否，由於圖四c 是使用第一及第二位置變異來推演的，因此應具有較大的重要性，然而可茲利用之變異太少可能減低了此演化樹的重要性。

結　　論

結果明顯顯示，除了TPZ34 外，所有被取樣之梅花鹿，包括東海的鹿群均屬於同一起源，亦即這些鹿都是純種台灣梅花鹿，而TPZ34 的演化位置則不明。這個個體可能是台灣梅花鹿在粒線體基因中發生復原而有不同的基因型，也可能牠本來就是外來種。但由於TPZ34 是動物園中的個體，有相當明確的家譜，因此認為TPZ34 仍屬於台灣梅花鹿，而此亞種有兩種不同的粒線體基因型。由於TPZ34 有外來基因群的可能性仍未排除，因此進一步的研究採取更多的樣本可能可以解決此疑問。

表一．用來放大及分析梅花鹿D-loop及細胞色素b 基因的primers

Name of primer	Sequence
(D-loop)	
L15926[a]	5'-TCAAAGGTTACACCAGTCTTGTAAAC-3'
L1	5'-CAAGGAAGAAGCTCCAGCTCCACC-3'
H1	5'-GGTGGAGCTGGAGCTTCTTCCTTG-3' (complement to L1)
L2	5'-TTGTACATTATGCACCCAATGC-3'
H2	5'-GCATTGGGTGCATAATGTACAA-3' (complement to L1)
H16498[a]	5'-CCTGAAGTAGGAACCAGATG-3'
(cytochrome *b*)	
L14841[a]	5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'
H15149D[b]	5'-CCTCAGAATGATATTTGTCCTCA-3'
L15408[c]	5'-ATAGACAAAATCCCATTCCA-3'
L15609	5'-ATTCTACGATCCATCCCAAACAAACT-3'
H15767	5'-ATGAAGGGATGTTCTACTGGTTG-3'
H15915[c]	5'-GGAATTCATCTCTCCGGTTTACAAGAC-3'

H and L refer to light and heavy strand. Numbers refer to the position of the 3' end of the oligonucleotide according to the numbering system for the human sequence (Anderson, *et al.* 1989). L1, H1, L2, and H2 are specific for sika deer. Their positions are noted in Figure 1.

[a]Primers described in Kocher, *et al.* (1989).

[b]Described in Irwin, *et al.* (1991) and modified according to fallow deer sequence.

[c]Described in Irwin, *et al.* (1991).

[d]Described in Edwards, *et al.* (1991).

圖一．梅花鹿粒線體D-loop的前397 個鹽基序列

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AAATTTTTGT TATGGGGCAT TTACAAGATT TTTAATTCCT ATGTTAGCAT TAAAATAACC
TGATCTATCA TAAATCTCTA TGTATTTCCA TGGATACCTG CACCTCCACA TTAATGTGTA
CATATATATC ATATCATGCA TAATATGTAT ATTTGTACAT TATGCACCCA ATGCATATAA
GCATGTATGT TAAATAATAT TGCAGAATAC ATAAATATTA TTGATTGTAC ATAGTTCATT
AAGTCAAATC AATTCTAGTC AACATGCATA TGATATCCAA AAGATCACAA ACGTCATCAG
CAGGCTGCAT GAAACCGTCA CCCCACTTGG CAGGTGTACC TCTTCTCATT CCAGGCCCAT
AGGTTGTGGA GGTTTCTATT GGATGAACTT TA
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            L   T   G   L   F   L   A   M   H   Y   T   S   D   T   M   T   A   F   S   S   V   T   H
Dama        CTC ACA GGC CTA TTC CTA GCA ATA CAC TAC ACA TCA GAT ACA ATA ACA GCA TTC TCC TCT GTC ACC CAT
Awap        CTC ACA GGC CTA TTC CTA GCA ATA CAC TAT ACA TCC GAT ACA ATA ACA GCA TTT TCC TCT GTC ACC CAT
Dyb         CTT ACA GGC CTA TTC CTA GCA ATA CAC TAT ACA TCT GAC ACA ATA ACA GCA TTT TCC TCT GTC ACC CAT
Mt. Goyo    CTT NCA GGC CTA TTC CTA GCA ATA CAC TAT AYA TCY GAC ACA ATA ACA GCA TTC TCC TCT RTC ACY CAT
Tonkin      NTC ACA GGC CTA TTC CTA GCA ATA CAC TAT ACA TCT GAC ACA ATA ACA GCA TTC TCC TCT GTC ACC CAT
TH916       CAT ACA GGC CTA TTC CTA GCA ATA CAC TAT ACA TCC GAY ACA ATA ACA GCA TTC TCC TCC GTY ACT CAT
TPZ19       CTC ACA GGC CTA TTC CTA GCA ATA CAC TAT ATA TCC GAC ACA ATA ACA GCR TTC TCC TCC ATC ACT CAT
TPZ34       CTA ACA GGC CTA TTC CTA GCA ATA CAC TAT ATA TCC GAC ACA ATA ACA GCA TTC TCC TCC RTC ACT CAT
TPZ49       CTA ACA GGC CTA TTC CTA GCA ATA CAC TAT ATA TCC TAC ACA ATA ACA GCR TTC TCC TCC ATC ACT CAT
      14879

            I   C   R   D   V   N   Y   G   W   I   I   R   Y   M   H   A   N   G   A   S   I   F   F
Dama        ATC TGC CGA GAC GTC AAC TAC GGC TGA ATC ATC CGA TAC ATG CAC GCA AAC GGA GCA TCA ATA TTC TTT
Awap        ATC TGT CGA GAT GTC AAT TAT GGT TGA ATT ATT CGA TAC ATA CAC GCA AAC GGG GCA TCA ATA TTT TTT
Dyb         ATC TGT CGA GAT GTC AAC TAT GGT TGA ATT ATC CGA TAC ATA CAC GCA AAC GGG GCA TCA ATA TTT TTC
Mt. Goyo    ATC TGT CGA GAY GTC AAC TAT GGY TGA ATT ATC CGA TAC ATA CAY GCA AAC GGA GCA TCA ATA TTC TTC
Tonkin      ATC TGT CGA GAT GTC AAT TAT GGT TGA ATT ATT CGA TAC ATA CAC GCA AAC GGG GCA TCA ATA TTC TTC
TH916       ATC TGC YGA GAT GTC AAT TAT GGC TGA ATC ATT CGA TAT ATA CAC GCA AAC GGG GCA TCA ATA TTT TTC
TPZ19       ATC TGC TGA GAC GTC AAT TAT GGC TGA ATT ATC CGA TAC ACA CAT GCA AAC GGA GCA TCA ATA TTC TTC
TPZ34       ATC TGC YGA GAY GTC AAY TAT GGC TGA ATT ATC YGA TAC ATA CAT GCA AAC GGA GCA TCA ATA TTC TTC
TPZ49       ATT TGC TGA GAC GTC AAT TAT GGC TGA ATT ATC TGA TAC ACA CAT GCA AAC GGA GCA TCA ATA TTC TTC
      14948

            I   C   L   F   M   H   V   G   R   G   L   Y   Y   G   S   Y   M   F   L   E   T   W
Dama        ATC TGC CTA TTT ATG CAT GTA GGA CGA GGC CTA TAC TAT GGA TCG TAT ATA TTC CTA GAA ACT TGA
Awap        ATC TGC CTA TTT ATA CAT GTA GGA CGA GGC CTA TAC TAC GGG TCA TAT ACT TTT CTA GAG ACT TGA
Dyb         ATC TGC CTA TTC ATA CAT GTA GGA CGA GGC CTG TAC TAC GGA TCA TAC ACT TTT CTA GAG ACT TGA
Mt. Goyo    ATC TGC CTA TTC ATA CAT GTA GGA CGA GGC CTG TAC TAC GGA TCA TAT ACY TTT CTA GAG ACT TGA
Tonkin      ATC TGC CTA TTC ATA CAT GTA GGA CGA GGC CTG TAC TAC GGA TCA TAT ACT TTT CTA GAG ACT TGA
TH916       ATC TGT CTA TTT ATA CAC GTA GGA CGA GGC CTG TAC TAC GGA TCA TAC ACC TTT CTA GAG ACT TGA
TPZ19       ATC TGY CTA TTC ATA CAT GTA GGG CAA GGC CTG TAC TAT GGG TCA TAY ACC TTC CTA GAG ACT TGA
TPZ34       ATC TGC CTA TTC ATA CAT GTA GGR CGA GGC CTG TAC TAC GGA TCA TAT ACT TTT CTA GAG ACT TGA
TPZ49       ATC TGT CTA TTT ATA CAT GTA GGG CAA GGT CTA TAC TCT GGG TCA TAC ACC TTC CTA GAA ACT TGA
      15017
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圖二. 九頭鹿之細胞色素b 基因5'端的264 個鹽基序列。代號說明：TH,東海大學；TPZ,北市立動物園；Tonkin,Tonkin 梅花鹿；Mt.Goyo, 日本梅花鹿 (C.n.nippon) ；Dyb,Dybowski's 梅花鹿；Awap,Asian wapiti；Dama, Fallow deer(Dama dama),取自Irwin,et al.,(1991)。N,Y及R 表示不確定的鹽基：N表完全不知道，Y表T 或 C，R表G 或 A。推測DamaDama的氨基酸序列亦列於圖中。數字代表每個鹽基與人類氨基酸序列編號系統的對應值(Anderson,et al.1989)。

```
            S  P  D  V  L  G  D  P  D  N  Y  T  P  A  N  P  L  N  T  P  P  L  I
Dama        TCA CCA GAC GTG TTA GGA GAC CCC GAC AAC TAC ACA CCA GCT AAC CCA CTC AAC ACT CCT CCA CTT ATT
Awap        GCA CCA GAC YTG CTY GGA GAC CCA GAY AAC TAY ACY CCA GCA AAY CCA CTC AAC ACA CCC CCT CAC ATY
Dyb         GCA CCA GAC YTG CTY GGA GAC CCA GAY AAC TAY ACY CCA GCA AAY CCA CTC AAC ACA CCC CCT CAC ATY
Mt Goyo     GCA CCA GAC TTG CTC GGA GAC CCA GAY AAC TAC ACT CCA GCA AAY CCA CTC AAY ACA CCC CCT CAY ATT
Tonkin      GCA CCA GAC CTR CTY GGA GAY CCA GAY AAC TAY ACY CCA GCA AAY CCA CTC AAC ACA CCC CCT CAC ATC
TH916       GCA CCA GAC YTG CTY GGA GAC CCA GAT AAC TAY ACT CCA GCA AAY CCA CTC AAT ACA CCC CCT CAT ATT
TH912       GCA CCA GAC TTG CTC GGA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TH914       GCA CCA GAC TTG CTC GGA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TH915       ACA CCA GAC TTG CTC GCA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TPZ16       GTA CCA GAC TTG CTC GGA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TPZ19       GCA CCA GAC TTG CTC GGA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TPZ34                                       TAC ACN CCA GCA AAN CCA CTC AAC ACA CCC CCT CAC ATN
TPZ35       ACA CCA GAC TTG CTC RCA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
      15481

            K  P  E  W  Y  F  L  F  A  Y  A  I  L  R  S  I  P  N  K  L  G  G  V
Dama        AAG CCA GAA TGA TAT TTC CTA TTC GCA TAC GCA ATC CTA CGA TCA ATC CCT AAC AAA CTA GGA GGA AGC
Awap        AAR CCY GAA TGA TAC TTC CTA TTT GCA TAC GCA ATC CTA CGA TCA ATT CCC AAY AAA CTA GGA GGA AGC
Dyb         AAR CCY GAA TGA TAC TTC CTA TTT GCA TAC GCA ATC CTA CGA TCA ATT CCC AAC AAA CTA GGA GGA GTN
Mt Goyo     AAR YCY GAA TGA TAC TTC CTA TTT GCA TAY GCA ATT CTA CGA TCA ATT CCC AAY AAA YTA GGA GG
Tonkin      AAA CCC GAA TGA TAC TTC CTA TTT GCA TAC GCA ATC CTA CGA TCA ATT CCC AAY AAA CTA GGA GGA GTN
TH916       AAG TCY GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TH912       AAG TCC GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TH914       AAG TCC GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TH915       AAG CCC AAA TGG TAC TTT CTA TTT GCA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TPZ16       AAG TCC GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TPZ19       AAG TCC GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TPZ34       AAA CCC GAA TGG TAC TTC CTA TTT GCA TAC GCA ATT CTA CGA TCA ATT CCC AAY AAA CTA GGA GGA G
TPZ35       AAG CCC AAA TGG TAC TTT CTA TTT GCA TAT GCA ATT CTA CGA TCA ATT CCC
      15550                                                                    15613

            L  A  L  V  L  S  I  L  I  L  I  F  M  P  L  L  H  T  S  K  Q  R  S
Dama        CTA GCA CTA GTC CTC TCT ATT CTC ATC CTT ATC TTT ATA CCC CTG CTC CAC ACA TCC AAA CAA CGA AGC
Awap        TTA GYC CTA GTC TCA TCY ATC CTA ATC TTG ATT CTC ATG CCT CTT CTT CAC ACG TCC AAA CAA CGC AGC
Dyb         NYA GYC CTA GTC TCA TCC ATC CTA ATC TTG ATT CTC ATG CCT CTT CTT CAC ACG TCC AAA CAA CGC AGC
Mt Goyo                                                                 TYC AAA CAA CGC AGC
Tonkin      TTA GYC CTA GTC TCA TCT ATC CTA ATC TTG ATT CTC ATG CCT CTT CTT CAC ACG TCC AAA CAA CGC AGC
TH916       CT                                                      AC ACG TCC AAA CAA CGC AGC
TH912       CTA GCC CTA ATG TTC TCT ATC TTA GTT CTA ATT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA TGA AGC
TH914       CTA GCC CTA ATC TTC TCT GTC TTA GTT CTA ATT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA TGA AGC
TH915       CTA GCC CTA ATC TTC TCT ATC TTA GTT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA TGA AGC ATG ATA
TPZ16       CTA GCC CTA ATC TTC TCT ATC TTA GTT CTA ATT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA TGA AGC
TPZ19       CTA GCC CTA ATC TTC TCT ATC TTA GTT CTA ATT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA T
TPZ34                   TC TCA TCC ATC CTA ANN NNG ATT CTC ATG CCT CTT CTT CAC ACG TCC AAA CAA CGC AGC
      15619
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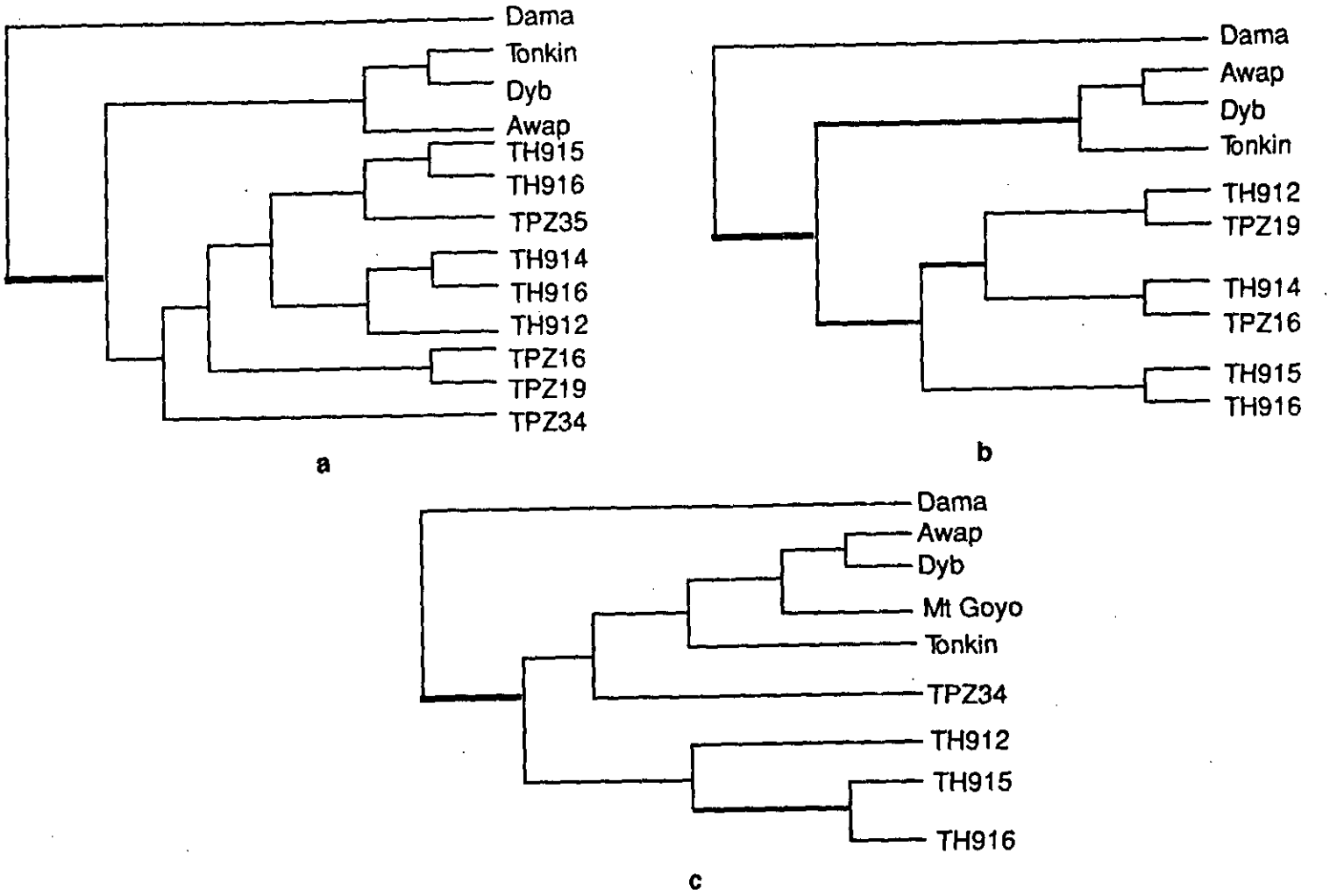

圖四．使用鹿隻鹽基序列差異中三種不同的狀況，作出之最簡易的演化樹：a)12個個體，9個差異；b)10個個體，21個差異；c)9個個體，在第一及第二位置有五個差異。代號說明如圖二及圖三。粗黑線表示分枝出現在45或以上的50 bootstrap trials(PHYLIP)。

```
            M   M   F   R   P   F   S   Q   C   L   F   W   I   L   V   A   D   L   L   T   L   T   W
Dama        ATG ATA TTT CGA CCA TTC AGC CAA TGC TTA TTT TGA ATC CTT GTA GCA GAC CTA CTA ACA CTA ACA TGA
Awap        ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATT TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
Dyb         ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATC TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
Mt Goyo     ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATG TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
Tonkin      ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATC TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
TH916       ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATC TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
TH912       ATG ATA TTC CGA CCA TTC AGT CAA TGC CTG TTC TGA AT
TH914       ATG ATA TTC CGA CCA
TH915       ATG ATA TTC CGA CCA TTC AAT CAA TGC CTG TTC TGA ATT TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
TPZ16       ATG ATA TTC CGA CCA TTC AGT CAA TGC CTG TG
TPZ34       ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATC TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
        15688

            I   G   G   Q   P   V   E   H   P   F   I   I   I   G   Q   L   A   S   I   L   Y   F   L
Dama        ATC GGA GGG CAA CCA GTT GAA CAC CCA TTT ATT ATC ATT GGA CAA CTA GCA TCT ATC TTA TAT TTT CTC
Awap        ATT GGA GGA CAG CCA GTT GAA TAC CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT GTC TTA TAC TTT TTC
Dyb         ATT GGA GGA CAA CCA GTT GAA TAT CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT GTT CTA TAC TTT TTC
Mt Goyo     ATT GGA GGA CAA CCA GTT GAA TAA
Tonkin      ATT GGA GGA CAA CCA GTT GAA TAC CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT ATC CTA TAC TTT TTC
TH916       ATT GGA GGA CAA CCA GTT GAA TAC CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT ATC TTA TAC TT
TH915       ATC GGA GGA CAA CCA GTA G
TPZ34       ATT GGA GGA CAA CCA GTT GAA TAT CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT GTT CTA TAC TTT TTC
        15757

            I   I   L   V   L   M   P   A   T   S   T   I   Q   N   N   L   L   K   W   R
Dama        ATT ATT CTA GTA CTA ATA CCA GCC ACC AGC ACA ATC CAG AAT AAC CTT CTA AAA TGA AGA
Awap        ATT ATC CTA GTC CTT ATA CCA ATT ACC AGC ACA ATC GAA AAC AAC CTC CTA AAA TGA AAA
Dyb         ATT ATC CTA GTC CTT ATA CCA ATT ACC AGC ACA ATC GAA AAT AAC CTC CTA AAA TGA AGA
Tonkin      ATT ATT CTG GTC CTT ATA CCA ATC ACC AGC ACA ATC GAA AAT AAC CTC CTA AAA TGA AGA
TPZ34       ATT ATC CTA GTC CTT ATA CCA ATT ACC AGC ACA ATC GAA AAT AAC CTC CTA AAA TGA AGA
        15826
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圖三．十一頭鹿之細胞色素b基因3'端的405個鹽基序列。代號說明：TH，東海大學；TPZ，台北市立動物園；Tonkin,Tonkin 梅花鹿；Mt. Goyo，日本梅花鹿（C.n.nippon）；Dyb,Dybowski's 梅花鹿；Awap, Asian wapiti，Dama，Fallow deer(Dama dama),取自Irwin,et al., (1991)。N,Y及R 表示不確定的鹽基：N表完全不知道，Y表T 或 C，R 表G 或 A。推測Dama Dama 的氨基酸序列亦列於圖中。數字代表每個鹽基與人類氨基酸序列編號系統的對應值(Anderson,et al.1989)。

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附 錄 一 . DNA的 萃 取

Reagents

1 M Tris-HCl, pH 7.5
10% SDS
0.5 M Na_2EDTA, pH 8.0
5.0 M NaCl
3 M NaAcetate
Proteinase K, 10 mg/ml in H_2O
Tris buffered phenol/chloroform (1:1) extraction solvent
Absolute ethanol
TE buffer, pH 7.5 (10 mM Tris-HCl, 1 mM EDTA)
TA buffer (.04M Tris-HCl, 1.142 mL/L acetic acid)

Digest buffer (0.5 ml, made from above solutions):

10 mM Tris-HCl	5 µl
10 mM EDTA	10 µl
50 mM NaCl	5 µl
2% SDS	100 µl
sterile deionized H_20	380 µl

Procedure

1. Suspend 0.2 ml buffered whole blood in 0.5 ml digest buffer. Add 15 µl proteinase K solution. Incubate one hour at 56°C or overnight at 37°C

2. Add 0.5 ml buffered phenol/chloroform. Mix thoroughly by inversion or vortexing. Centrifuge 1-2 minutes in microfuge (12,000 g).

3. Remove aqueous upper layer and transfer to new tube. Repeat extraction until interface is clear; usually 2-3 extractions.

4. Remove final aqueous layer and concentrate by ethanol precipitation as follows: add 1/10 volume of 3M Na acetate, then 2.5 volumes of cold ethanol. Store at -20°C at least one hour. Collect DNA by centrifugation, 10-30 minutes, in microfuge.

5. Remove ethanol solution, wash DNA with 1 ml 70% ethanol, reduce temperature to -20°, then spin in microfuge 15 minutes. Remove supernatant and evaporate to dryness. Resuspend DNA in 20 µl TE buffer.

DNA was visualized on a 1% agarose (SeaChem) gel in TA buffer. 1 µl of purified DNA was loaded onto the gel and electrophoresed at 60V for one hour. The gel was stained with ethidium bromide and photographed under UV light.

附錄二．聚合酶鏈反應

The polymerase chain reaction (PCR) allows the rapid amplification of selected DNA segments. Purified DNA is mixed with oligonucleotide primers, a thermostable DNA polymerase, and deoxynucleotides (the building blocks of DNA). Primers are short oligonucleotide sequences which bind to single stranded DNA in the reaction mixture, forming short double strands at which the polymerase can initiate replication. The mixture is heated to denature the purified DNA, cooled to an annealing temperature at which allows the primer binds to the single stranded template, then heated to the temperature (72° for *Taq* polymerase) at which the DNA polymerase functions most efficiently. The polymerase, starting at the primer, duplicates the original DNA strand. The mixture is heated again to denature the newly formed strands, then cooled again to allow another cycle of annealing and polymerization. Typically, 25 to 40 cycles are performed in a PCR thermal cycler, resulting in a many-fold increase in the number of copies of the target DNA. Both double stranded and single stranded amplifications are possible. In a double stranded amplification two primers which initiate transcription on opposite strands of the target DNA are used. A double stranded segment between the two primers is then amplified and can be used in further PCR reactions. In a single stranded amplification one of the two primers is reduced in concentration, usually 50 to 100 times less than the other primer, so that the PCR product contains many copies of the single stranded DNA which has been amplified by the primer at normal concentration. This single stranded product can then be sequenced directly (Gyllensten and Erlich, 1988).

PCR protocol

Reagents

10x reaction buffer	500 mM KCl 100 mM Tris-HCl, pH 8.3 25 mM $MgCl_2$ 0.1% (w/v) gelatin
100 mM deoxynucleotide mix	25 mM each dATP, dCTp, dGTP, dTTP
PCR primers	10 µM solutions of each primer
Taq polymerase	5 units/µl

Procedure

2.5 µl 10x reaction buffer
2.5 µl each 10 µM primer (final concentration 0.25 µM)
1 µl 100 mM dNTPs (final concentration 0.25 mM)
1 µl *Taq* polymerase
16.9 µl sterile, double distilled H_2O
1 µl DNA solution

Final volume = 25 µl

A premix was prepared containing all of the above except the DNA. This is placed into a 0.5 ml microfuge tube, then the DNA is added. Two drops of mineral oil were placed on top of the sample to prevent evaporation. For single stranded amplifications one of the primers was reduced in concentration by a factor of 75. PCR conditions were as follows: denaturing at 94°C for 1 minute, annealing at 45-60°C for 1 minute, extension at 72°C for 3 minutes. Thirty to 40 cycles were performed. Five µl aliquots of each PCR product were visualized on 2% NuSieve agarose gels in a Tris-acetate buffer. Gels are run at 70V for one to two hours, stained with ethidium bromide and photographed under a UV light.

Purified double stranded PCR products were obtained by cutting the ethidium bromide stained band from the agarose gels and placing them in 100-250 µl of sterile double distilled H_2O. DNA was eluted from the agarose fragment by heating the sample to 70° to melt the agarose. One µl aliquots were then used for asymmetric PCR amplifications.

An analysis of the population structure of Formosan sika deer (*C. n. taiouanus*) in Kenting National Park by amplification and direct sequencing of the mitochondrial cytochrome *b* gene

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Abstract: This project was undertaken as part of the Sika Deer Reintroduction Project in order to assess the genetic structure of the reintroduction herd in Taiwan, and to develop a methodology for differentiating sika which belong to the subspecies *C. n. taiouanus* from those which do not so that future introductions can be made using only this subspecies. The polymerase chain reaction was used to amplify and sequence three segments of the mitochondrial genome from a number of sika deer (*Cervus nippon*). Eight 264 base sequences from the 5' end of the mitochondrial cytochrome *b* gene were obtained from four *C. n. taiouanus*, one *C. n. hortulorum*, one *C. n. nippon*, one *C. n. pseudaxis*, and one *C. elaphus xanthopygus*. Eleven 375 base sequences from the 3' end of the mitochondrial cytochrome *b* gene from eight *C. n. taiouanus*, one *C. n. hortulorum*, one *C. n. nippon*, one *C. n. pseudaxis*, and one *C. elaphus xanthopygus*. No individuals share identical mitochondrial genotypes: hence the reintroduction herd gene pool shows considerable variation. There is a three base deletion (one amino acid) near the 3' end of the cytochrome *b* gene in all of the *C. n. taiouanus* individuals except one. This deletion, and analysis of sequence differences (by parsimony), confirm that all of the deer from Taiwan, except that one individual, belong to a single related (monophyletic) group. The analysis suggests, but does not confirm, that the one anomalous individual is also a member of this group. The techniques described in this report are capable of differentiating the various sika subspecies.

Introduction

Numerous subspecies of sika deer (*Cervus nippon*) are native to East Asia, including one endemic to Taiwan (Feldhamer, 1982a; Whitehead, 1972). The subspecies on Taiwan, *C. n. taiouanus*, is extinct in the wild (McCullough, 1978). The Sika Deer Reintroduction Project will reestablish wild populations of deer on Taiwan, starting in Kenting National Park, with individuals descended from the herd at the Taipei Zoo. For reintroduction projects of this type it is desirable to have some knowledge of the genetic structure of the reintroduction population to ensure adequate genetic variability in the released animals. This is particularly important in Kenting since the entire reintroduction herd is descended from a single captive population. One goal of the research described in this report was to obtain an estimate of the degree of genetic variation in the reintroduction herd.

A second goal was to determine if genetic studies can differentiate the various subspecies of sika deer. This is important because it may be desirable to increase the genetic diversity of reintroduced populations using individuals from other captive herds. Since most such herds on Taiwan are on deer farms, and many have been interbred with non-*taiouanus* deer from Japan and elsewhere, the identification of deer with non-*taiouanus* ancestry is important to ensure that no non-native genetic material enters the reintroduced deer population. Individuals from a second captive herd at Kenting, originally at Tunghai University and of unknown ancestry, were studied to test the applicability of such genetic studies for this goal.

Past studies of genetic variation in sika have relied on karyotyping and allozyme comparisons (Feldhamer, 1982b; van Tuinen, 1983; Sung, *et al.*, 1985; Sung, *et al.*, 1988). Both types of studies have proven useful in distinguishing populations of some deer species (Dratch, 1991), but the sika studies have shown little variation, probably due to the relatively small number of alleles and the small numbers of individuals examined rather than to actual lack of genetic diversity.

This project uses a more direct approach for genetic comparison of individuals: direct comparison of DNA sequences. In particular, small segments of the mitochondrial displacement loop (D-loop) and the mitochondrial cytochrome *b* gene were amplified using the polymerase chain reaction, then sequenced. Since the mitochondrial genome is maternally inherited and does not recombine with each generation as does the nuclear genome, mitochondrial lineages are conserved for many generations. Thus distinct mitochondrial lineages, such as those from non-Taiwan deer, can be identified even many generations after they have been introduced into a population. However, it is important to note that, because mitochondrial DNA is maternally inherited, identification of deer with non-*taiouanus* ancestry by using mitochondrial sequences is only valid if some of the introduced deer were female.

Materials and Methods

DNA samples. During the Fall of 1990 40 samples were collected from two distinct sika populations in three different herds in Taiwan. Two of these herds are at Kenting National Park. One herd was originally at Tunghai university and is of unknown ancestry. Samples from 27 individuals in this herd were collected. The second Kenting herd is descended from the herd at the Taipei Zoo, and is believed to be pure *C. n. taiouanus*. Thirteen samples were collected from individuals of this herd. Both blood and fur samples were collected from the Kenting individuals. Blood samples from 30 individuals were centrifuged to separate leukocytes (white blood cells) from whole blood at the Kenting Livestock Research Laboratory, then the concentrated leukocytes were lyophilized to prevent degradation of the DNA. Stains from whole blood were also made and preserved on cloth. For the remaining ten individuals only blood stains on cloth were collected. In addition, eleven blood samples from the herd at the Taipei zoo (representing every individual there) were provided by the zoo. These were preserved as stains and by mixture

with a buffered detergent solution. Since the reintroduction herd at Kenting is descended from the Taipei Zoo herd the two are, genetically, a single population.

Genomic DNA samples from two other sika subspecies, *C. n. hortulorum* (Dybowski's sika, native to northeastern China) and *C. n. pseudaxis* (Tonkin sika, native to Vietnam), as well as one closely related congener, *C. elaphus xanthopygus* (Asian wapiti, native to northern East Asia) were obtained from George Amato of the New York Zoological Society. A tissue sample from one *C. n. nippon* (Japanese sika, native to Honshu island) was obtained from Seiki Takatsuki of Sendai University.

Genomic DNA was prepared from blood and tissue samples by digestion with proteinase K, phenol/chloroform extraction, and ethanol precipitation (see Appendix I).

PCR and sequencing. Mitochondrial sequences containing the D-loop region or the cytochrome *b* gene were isolated using the polymerase chain reaction (Saiki, *et al.*, 1988, detailed in Appendix II). Single stranded D-loop DNA was obtained by first amplifying a 580 base piece of the mitochondrial genome containing the first 397 D-loop bases with primers L15926 and H16498, then using these primers and other internal primers for asymmetric amplification. The following primer combinations were used to produce single stranded DNA: L15926/H16498, L1/H16498; L2/H16498, L15926/H2, and L1/H2. PCR conditions were 94° for 1 minute, 60° for 1 minute, 72° for 3 minutes, 40 cycles.

Cytochrome *b* DNA was similarly obtained: first, a 1074 base fragment containing most of the cytochrome *b* gene was amplified using the primers L14841 and H15915, then single stranded products were made using those and other internal primers. Primer combinations were as follows: L14841/H15915, L15408/H15767, and L15609/H15915. Primer sequences are listed in Table 1. PCR conditions were 94° for 1 minute, 50° for 1 minute, 72° for 3 minutes, 40 cycles.

Table 1. Primers used for amplification and sequencing of sika D-loop and cytochrome *b* genes

Name of primer	Sequence
(D-loop)	
L15926[a]	5'-TCAAAGGTTACACCAGTCTTGTAAAC-3'
L1	5'-CAAGGAAGAAGCTCCAGCTCCACC-3'
H1	5'-GGTGGAGCTGGAGCTTCTTCCTTG-3' (complement to L1)
L2	5'-TTGTACATTATGCACCCAATGC-3'
H2	5'-GCATTGGGTGCATAATGTACAA-3' (complement to L1)
H16498[a]	5'-CCTGAAGTAGGAACCAGATG-3'
(cytochrome *b*)	
L14841[a]	5'-AAAAAGCTTCCATCCAACATCTCAGCATGATGAAA-3'
H15149D[b]	5'-CCTCAGAATGATATTTGTCCTCA-3'
L15408[c]	5'-ATAGACAAAATCCCATTCCA-3'
L15609	5'-ATTCTACGATCCATCCCAAACAAACT-3'
H15767	5'-ATGAAGGGATGTTCTACTGGTTG-3'
H15915[c]	5'-GGAATTCATCTCTCCGGTTTACAAGAC-3'

H and L refer to light and heavy strand. Numbers refer to the position of the 3' end of the oligonucleotide according to the numbering system for the human sequence (Anderson, *et al.* 1989). L1, H1, L2, and H2 are specific for sika deer. Their positions are noted in Figure 1.
[a]Primers described in Kocher, *et al.* (1989).
[b]Described in Irwin, *et al.* (1991) and modified according to fallow deer sequence.
[c]Described in Irwin, *et al.* (1991).
[d]Described in Edwards, *et al.* (1991).

Data analysis. Cytochrome *b* sequences were aligned and compared. Bases for which at least two individuals varied were treated as discrete character states for analysis. Each three base sequence, or codon, codes for a single amino acid in the cytochrome *b* protein. The third positions of codons tend to mutate more rapidly than the first and second positions, hence there are many more third position differences than first and second position differences in the sequences described in this report. Because third positons may mutate many times and thus obscure analysis, the more slowly changing first and second positions are often more useful for evolutionary analysis. Sequences were analyzed by compiling a list of the individuals and positions which vary, then using a computer program to deduce the least number of mutation events which might lead to the observed sequences. The program output is a tree which represents a *possible* lineage history for the individuals being examined.

For this report trees were constructed using the parsimony algorithm of PHYLIP (Felsenstein, 1987) and confidence values were assigned to the lineages using the bootstrap option of the same program. Trees were constructed using various components of the sequences and varying numbers of individuals, as described in Results and Discussion.

Results and Discussion

D-loop sequences. Some amplifications using primers flanking the entire D-loop were performed. These indicate that the entire sika deer D-loop is about 1000 bases long.

The first 397 bases of the D-loop region were sequenced in 17 deer, including the Dybowski's, Tonkin, and Japanese sika; and the Asian wapiti. There were no nucleotide differences between any of the sika deer, and only two differences between the sika and Asian wapiti, hence the D-loop was not useful for any analysis of genetic variation between sika populations. This 397 base sequence is listed in Figure 1.

Figure 1. The first 397 bases of the sika deer mitochondrial D-loop

```
AAATTTTTGT TATGGGGCAT TTACAAGATT TTTAATTCCT ATGTTAGCAT TAAAATAACC
TGATCTATCA TAAATCTCTA TGTATTTCCA TGGATACCTG CACCTCCACA TTAATGTGTA
CATATATATC ATATCATGCA TAATATGTAT ATTTGTACAT TATGCACCCA ATGCATATAA
GCATGTATGT TAAATAATAT TGCAGAATAC ATAAATATTA TTGATTGTAC ATAGTTCATT
AAGTCAAATC AATTCTAGTC AACATGCATA TGATATCCAA AAGATCACAA ACGTCATCAG
CAGGCTGCAT GAAACCGTCA CCCCACTTGG CAGGTGTACC TCTTCTCATT CCAGGCCCAT
AGGTTGTGGA GGTTTCTATT GGATGAACTT TA
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Cytochrome b sequences. A 264 base segment at the 5' end of the gene was amplified and sequenced for eight deer. These are listed in Figure 2. In addition, most of a 375 base sequence at the 3' end of the gene was amplified and sequenced from 11 deer. These are shown in Figure 3.

In the 264 base segment there are 37 variable bases. Of these two are at codon first positions, three at second positions, and 32 at third positions. One of the first, one second, and eight third position differences were only in one individual and were not

phylogenetically informative. One of the first and five of the third position differences were not used due to ambiguity at that position in one or more deer. In the 375 base segment there were 72 variable bases: 19 first position, 4 second position, and 49 third position. Of these six first position and 22 third position bases were not informative, and two first and three third position bases were ambiguous in one or more deer.

None of the sequences are completely identical, thus none of the eight *C. n. taiouanus* share identical mitochondrial genomes. This indicates that there is considerable genetic diversity, at least in the mitochondrial genome, of the reintroduction project herd. Although this sample is small, and does not include nuclear genes, it strongly suggests that there is considerable diversity in the gene pool of the reintroduction herd, and that this herd should have adequate genetic diversity for the reintroduction project for the immediate future. However, this does not mean that this gene pool should never be augmented, only that there is probably no immediate danger of inbreeding depression in these animals.

The most interesting characteristic of these sequences is that in all of the Taiwan deer, except TPZ34, there is an amino acid (i.e. three base) deletion at position number 15613. Deletion events such as this are extremely rare, and indicate a single event some time during the history of these deer. Only deer from Taiwan have the deletion, but not all of the Taiwan deer have it.

There are two interpretations for this finding. One is that this deletion occurred after the sika arrived on Taiwan so that there are at present two mitochondrial lineages on the island, one with and one without the deletion. The other possibility is that the deletion occurred before the deer arrived on Taiwan, and that all pure *C. n. taiouanus* have the deletion. If the latter is so then TPZ34 must have a non-native mitochondrial type and therefore is not pure *C. n. taiouanus*.

In order to assess these two possibilities evolutionary trees were constructed using the parsimony option of the computer program PHYLIP (Felsenstein, 1987) on three different subsets of the data. These are shown in Figure 4. Figure 4a shows a parsimony tree for 12

individuals which used nine variable sites in the 5' region of the cytochrome *b* gene. Only one branch of the tree, the split between the Taiwan deer and the other sika, occurred on greater than 90% of 50 bootstrap trials. Figure 4b shows a parsimony tree for 10 individuals at 21 sites. Four branches occurred on 90% of 50 bootstrap trials. Figure 4c shows a parsimony tree for nine individuals at five variable first and second position sites. Only one branch occurred on greater than 90% of 50 bootstrap trials.

All three trees clearly show that all of the Taiwan deer, except TPZ34, belong to a single distinct lineage group (i.e. they are monophyletic), as is expected since they all share a deletion. TPZ34 is grouped with the other Taiwan deer in Figure 4a, but not in Figure 4c. Figure 4c is based only on first and second position differences, and normally these would be given more weight than other trees. However, the small number of sites used reduces the value of this tree. It is suggestive, but not conclusive.

Conclusion. The data clearly show that, with the exception of TPZ34, all of the surveyed deer from Taiwan, including the Tunghai herd, are monophyletic: they are all native Formosan sika. The phylogenetic position of a single deer, TPZ34, is unclear. This individual may be pure *C. n. taiouanus* and possess a different mitochondrial genome of a type which predates the amino acid deletion, or it may have an introduced genotype. The data, in consideration with the fact that this animal is a member of the zoo herd, which has a fairly good lineage history, suggest that it is a member of *C. n. taiouanus*, and that there are two distinct mitochondrial lineages within this subspecies. However, the possibility that this individual has a non-native mitochondrial genome cannot at present be ruled out. Further studies which include more individuals are currently under way to clarify this situation.

Figure 2

```
            L   T   G   L   F   L   A   M   H   Y   T   S   D   T   M   T   A   F   S   S   V   T   H
Dama        CTC ACA GGC CTA TTC CTA GCA ATA CAC TAC ACA TCA GAT ACA ATA ACA GCA TTC TCC TCT GTC ACC CAT
Awap        CTC ACA GGC CTA TTC CTA GCA ATA CAC TAT ACA TCC GAT ACA ATA ACA GCA TTT TCC TCT GTC ACC CAT
Dyb         CTT ACA GGC CTA TTC CTA GCA ATA CAC TAT ACA TCT GAC ACA ATA ACA GCA TTT TCC TCT GTC ACC CAT
Mt. Goyo    CTT NCA GGC CTA TTC CTA GCA ATA CAC TAT AYA TCY GAC ACA ATA ACA GCA TTC TCC TCT RTC ACY CAT
Tonkin      NTC ACA GGC CTA TTC CTA GCA ATA CAC TAT ACA TCT GAC ACA ATA ACA GCA TTC TCC TCT GTC ACC CAT
TH916       CAT ACA GGC CTA TTC CTA GCA ATA CAC TAT ACA TCC GAY ACA ATA ACA GCA TTC TCC TCC GTY ACT CAT
TPZ19       CTC ACA GGC CTA TTC CTA GCA ATA CAC TAT ATA TCC GAC ACA ATA ACA GCR TTC TCC TCC ATC ACT CAT
TPZ34       CTA ACA GGC CTA TTC CTA GCA ATA CAC TAT ATA TCC GAC ACA ATA ACA GCA TTC TCC TCC RTC ACT CAT
TPZ49       CTA ACA GGC CTA TTC CTA GCA ATA CAC TAT ATA TCC TAC ACA ATA ACA GCR TTC TCC TCC ATC ACT CAT
      14879

            I   C   R   D   V   N   Y   G   W   I   I   R   Y   M   H   A   N   G   A   S   I   F   F
Dama        ATC TGC CGA GAC GTC AAC TAC GGC TGA ATC ATC CGA TAC ATG CAC GCA AAC GGA GCA TCA ATA TTC TTT
Awap        ATC TGT CGA GAT GTC AAT TAT GGT TGA ATT ATT CGA TAC ATA CAC GCA AAC GGG GCA TCA ATA TTT TTT
Dyb         ATC TGT CGA GAT GTC AAC TAT GGT TGA ATT ATC CGA TAC ATA CAC GCA AAC GGG GCA TCA ATA TTT TTC
Mt. Goyo    ATC TGT CGA GAY GTC AAC TAT GGY TGA ATT ATC CGA TAC ATA CAY GCA AAC GGA GCA TCA ATA TTC TTC
Tonkin      ATC TGT CGA GAT GTC AAT TAT GGT TGA ATT ATT CGA TAC ATA CAC GCA AAC GGG GCA TCA ATA TTC TTC
TH916       ATC TGC YGA GAT GTC AAT TAT GGC TGA ATC ATT CGA TAT ATA CAC GCA AAC GGG GCA TCA ATA TTT TTC
TPZ19       ATC TGC TGA GAC GTC AAT TAT GGC TGA ATT ATC CGA TAC ACA CAT GCA AAC GGA GCA TCA ATA TTC TTC
TPZ34       ATC TGC YGA GAY GTC AAY TAT GGC TGA ATT ATC YGA TAC ATA CAT GCA AAC GGA GCA TCA ATA TTC TTC
TPZ49       ATT TGC TGA GAC GTC AAT TAT GGC TGA ATT ATC TGA TAC ACA CAT GCA AAC GGA GCA TCA ATA TTC TTC
      14948

            I   C   L   F   M   H   V   G   R   G   L   Y   Y   G   S   Y   M   F   L   E   T   W
Dama        ATC TGC CTA TTT ATG CAT GTA GGA CGA GGC CTA TAC TAT GGA TCG TAT ATA TTC CTA GAA ACT TGA
Awap        ATC TGC CTA TTT ATA CAT GTA GGA CGA GGC CTA TAC TAC GGG TCA TAT ACT TTT CTA GAG ACT TGA
Dyb         ATC TGC CTA TTC ATA CAT GTA GGA CGA GGC CTG TAC TAC GGA TCA TAC ACT TTT CTA GAG ACT TGA
Mt. Goyo    ATC TGC CTA TTC ATA CAT GTA GGA CGA GGC CTG TAC TAC GGA TCA TAT ACY TTT CTA GAG ACT TGA
Tonkin      ATC TGC CTA TTC ATA CAT GTA GGA CGA GGC CTG TAC TAC GGA TCA TAT ACT TTT CTA GAG ACT TGA
TH916       ATC TGT CTA TTT ATA CAC GTA GGA CGA GGC CTG TAC TAC GGA TCA TAC ACC TTT CTA GAG ACT TGA
TPZ19       ATC TGY CTA TTC ATA CAT GTA GGG CAA GGC CTG TAC TAT GGG TCA TAY ACC TTC CTA GAG ACT TGA
TPZ34       ATC TGC CTA TTC ATA CAT GTA GGR CGA GGC CTG TAC TAC GGA TCA TAT ACT TTT CTA GAG ACT TGA
TPZ49       ATC TGT CTA TTT ATA CAT GTA GGG CAA GGT CTA TAC TCT GGG TCA TAC ACC TTC CTA GAA ACT TGA
      15017
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Figure 2. Aligned sequences for 264 bases near the 5' end of the cytochrome b gene for nine deer. Labels refer to: TH, Tunghai University; TPZ, Taipei Zoo; Tonkin, Tonkin sika; Mt. Goyo, Japanese sika (*C. n. nippon*); Dyb, Dybowski's sika; Awap, Asian wapiti; Dama, Fallow deer (*Dama dama*), sequence from Irwin, *et al.*, (1991). N, Y, and G refer to unresolved bases. N, completely unresolved; Y, T or C; R, G or A. The putative amino acid sequence for *Dama dama* is shown. Numbers refer to the position of each base according to the numbering system for the human sequence (Anderson, *et al.* 1989).

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            S   P   D   V   L   G   D   P   D   N   Y   T   P   A   N   P   L   N   T   P   P   L   I
Dama        TCA CCA GAC GTG TTA GGA GAC CCC GAC AAC TAC ACA CCA GCT AAC CCA CTC AAC ACT CCT CCA CTT ATT
Awap        GCA CCA GAC YTG CTY GGA GAC CCA GAY AAC TAY ACY CCA GCA AAY CCA CTC AAC ACA CCC CCT CAC ATY
Dyb         GCA CCA GAC YTG CTY GGA GAC CCA GAY AAC TAY ACY CCA GCA AAY CCA CTC AAC ACA CCC CCT CAC ATY
Mt Goyo     GCA CCA GAC TTG CTC GGA GAC CCA GAY AAC TAC ACT CCA GCA AAY CCA CTC AAY ACA CCC CCT CAY ATT
Tonkin      GCA CCA GAC CTR CTY GGA GAY CCA GAY AAC TAY ACY CCA GCA AAY CCA CTC AAC ACA CCC CCT CAC ATC
TH916       GCA CCA GAC YTG CTY GGA GAC CCA GAT AAC TAY ACT CCA GCA AAY CCA CTC AAT ACA CCC CCT CAT ATT
TH912       GCA CCA GAC TTG CTC GGA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TH914       GCA CCA GAC TTG CTC GGA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TH915       ACA CCA GAC TTG CTC GCA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TPZ16       GTA CCA GAC TTG CTC GGA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TPZ19       GCA CCA GAC TTG CTC GGA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
TPZ34                                           TAC ACN CCA GCA AAN CCA CTC AAC ACA CCC CCT CAC ATN
TPZ35       ACA CCA GAC TTG CTC RCA GAC CCA GAT AAC TAC ACT CCA GCA AAC CCA CTC AAT ACA CCC CCT CAT ATT
      15481

            K   P   E   W   Y   F   L   F   A   Y   A   I   L   R   S   I   P   N   K   L   G   G   V
Dama        AAG CCA GAA TGA TAT TTC CTA TTC GCA TAC GCA ATC CTA CGA TCA ATC CCT AAC AAA CTA GGA GGA AGC
Awap        AAR CCY GAA TGA TAC TTC CTA TTT GCA TAC GCA ATC CTA CGA TCA ATT CCC AAY AAA CTA GGA GGA AGC
Dyb         AAR CCY GAA TGA TAC TTC CTA TTT GCA TAC GCA ATC CTA CGA TCA ATT CCC AAC AAA CTA GGA GGA GTN
Mt Goyo     AAR YCY GAA TGA TAC TTC CTA TTT GCA TAY GCA ATT CTA CGA TCA ATT CCC AAY AAA YTA GGA GG
Tonkin      AAA CCC GAA TGA TAC TTC CTA TTT GCA TAC GCA ATC CTA CGA TCA ATT CCC AAY AAA CTA GGA GGA GTN
TH916       AAG TCY GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TH912       AAG TCC GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TH914       AAG TCC GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TH915       AAG CCC AAA TGG TAC TTT CTA TTT GCA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TPZ16       AAG TCC GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TPZ19       AAG TCC GAA TGG TAC TTC CTA TTT GAA TAT GCA ATT CTA CGA TCA ATT CCC AAT AAA TTA GGA --- GTC
TPZ34       AAA CCC GAA TGG TAC TTC CTA TTT GCA TAC GCA ATT CTA CGA TCA ATT CCC AAY AAA CTA GGA GGA G
TPZ35       AAG CCC AAA TGG TAC TTT CTA TTT GCA TAT GCA ATT CTA CGA TCA ATT CCC
      15550                                                                                 15613

            L   A   L   V   L   S   I   L   I   L   I   F   M   P   L   L   H   T   S   K   Q   R   S
Dama        CTA GCA CTA GTC CTC TCT ATT CTC ATC CTT ATC TTT ATA CCC CTG CTC CAC ACA TCC AAA CAA CGA AGC
Awap        TTA GYC CTA GTC TCA TCY ATC CTA ATC TTG ATT CTC ATG CCT CTT CTT CAC ACG TCC AAA CAA CGC AGC
Dyb         NYA GYC CTA GTC TCA TCC ATC CTA ATC TTG ATT CTC ATG CCT CTT CTT CAC ACG TCC AAA CAA CGC AGC
Mt Goyo                                                                         TYC AAA CAA CGC AGC
Tonkin      TTA GYC CTA GTC TCA TCT ATC CTA ATC TTG ATT CTC ATG CCT CTT CTT CAC ACG TCC AAA CAA CGC AGC
TH916       CT                                                                  AC ACG TCC AAA CAA CGC AGC
TH912       CTA GCC CTA ATG TTC TCT ATC TTA GTT CTA ATT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA TGA AGC
TH914       CTA GCC CTA ATC TTC TCT GTC TTA GTT CTA ATT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA TGA AGC
TH915       CTA GCC CTA ATC TTC TCT ATC TTA GTT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA TGA AGC ATG ATA
TPZ16       CTA GCC CTA ATC TTC TCT ATC TTA GTT CTA ATT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA TGA AGC
TPZ19       CTA GCC CTA ATC TTC TCT ATC TTA GTT CTA ATT CTT ATA CCC CTA CTC CAT ACA TCT AAA CAA T
TPZ34                   TC TCA TCC ATC CTA ANN NNG ATT CTC ATG CCT CTT CTT CAC ACG TCC AAA CAA CGC AGC
      15619
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          M   M   F   R   P   F   S   Q   C   L   F   W   I   L   V   A   D   L   L   T   L   T   W
Dama      ATG ATA TTT CGA CCA TTC AGC CAA TGC TTA TTT TGA ATC CTT GTA GCA GAC CTA CTA ACA CTA ACA TGA
Awap      ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATT TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
Dyb       ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATC TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
Mt Goyo   ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATG TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
Tonkin    ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATC TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
TH916     ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATC TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
TH912     ATG ATA TTC CGA CCA TTC AGT CAA TGC CTG TTC TGA AT
TH914     ATG ATA TTC CGA CCA
TH915     ATG ATA TTC CGA CCA TTC AAT CAA TGC CTG TTC TGA ATT TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
TPZ16     ATG ATA TTC CGA CCA TTC AGT CAA TGC CTG TG
TPZ34     ATG ATA TTC CGA CCA TTC AGC CAA TGC CTA TTC TGA ATC TTA GTA GCA GAC CTA CTA ACA CTC ACA TGA
      15688

          I   G   G   Q   P   V   E   H   P   F   I   I   I   G   Q   L   A   S   I   L   Y   F   L
Dama      ATC GGA GGG CAA CCA GTT GAA CAC CCA TTT ATT ATC ATT GGA CAA CTA GCA TCT ATC TTA TAT TTT CTC
Awap      ATT GGA GGA CAG CCA GTT GAA TAC CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT GTC TTA TAC TTT TTC
Dyb       ATT GGA GGA CAA CCA GTT GAA TAT CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT GTT CTA TAC TTT TTC
Mt Goyo   ATT GGA GGA CAA CCA GTT GAA TAA
Tonkin    ATT GGA GGA CAA CCA GTT GAA TAC CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT ATC CTA TAC TTT TTC
TH916     ATT GGA GGA CAA CCA GTT GAA TAC CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT ATC TTA TAC TT
TH915     ATC GGA GGA CAA CCA GTA G
TPZ34     ATT GGA GGA CAA CCA GTT GAA TAT CCC TTT ATT ATT ATT GGA CAA CTA GCA TCT GTT CTA TAC TTT TTC
      15757

          I   I   L   V   L   M   P   A   T   S   T   I   Q   N   N   L   L   K   W   R
Dama      ATT ATT CTA GTA CTA ATA CCA GCC ACC AGC ACA ATC CAG AAT AAC CTT CTA AAA TGA AGA
Awap      ATT ATC CTA GTC CTT ATA CCA ATT ACC AGC ACA ATC GAA AAC AAC CTC CTA AAA TGA AAA
Dyb       ATT ATC CTA GTC CTT ATA CCA ATT ACC AGC ACA ATC GAA AAT AAC CTC CTA AAA TGA AGA
Tonkin    ATT ATT CTG GTC CTT ATA CCA ATC ACC AGC ACA ATC GAA AAT AAC CTC CTA AAA TGA AGA
TPZ34     ATT ATC CTA GTC CTT ATA CCA ATT ACC AGC ACA ATC GAA AAT AAC CTC CTA AAA TGA AGA
      15826
```

Figure 3. Aligned sequences for 405 bases at the 3' end of the cytochrome b gene for 11 deer. Labels refer to: TH, Tunghai University; TPZ, Taipei Zoo; Tonkin, Tonkin sika; Mt. Goyo, Japanese sika (*C. n. nippon*); Dyb, Dybowski's sika; Awap, Asian wapiti; Dama, Fallow deer (*Dama dama*), sequence from Irwin, *et al.*, 1991. N, Y, and G refer to unresolved bases: N, completely unresolved; Y, T or C; R, G or A. The putative amino acid sequence for *Dama dama* is shown. Numbers refer to the position of each base according to the numbering system for the human sequence (Anderson, *et al.* 1989).

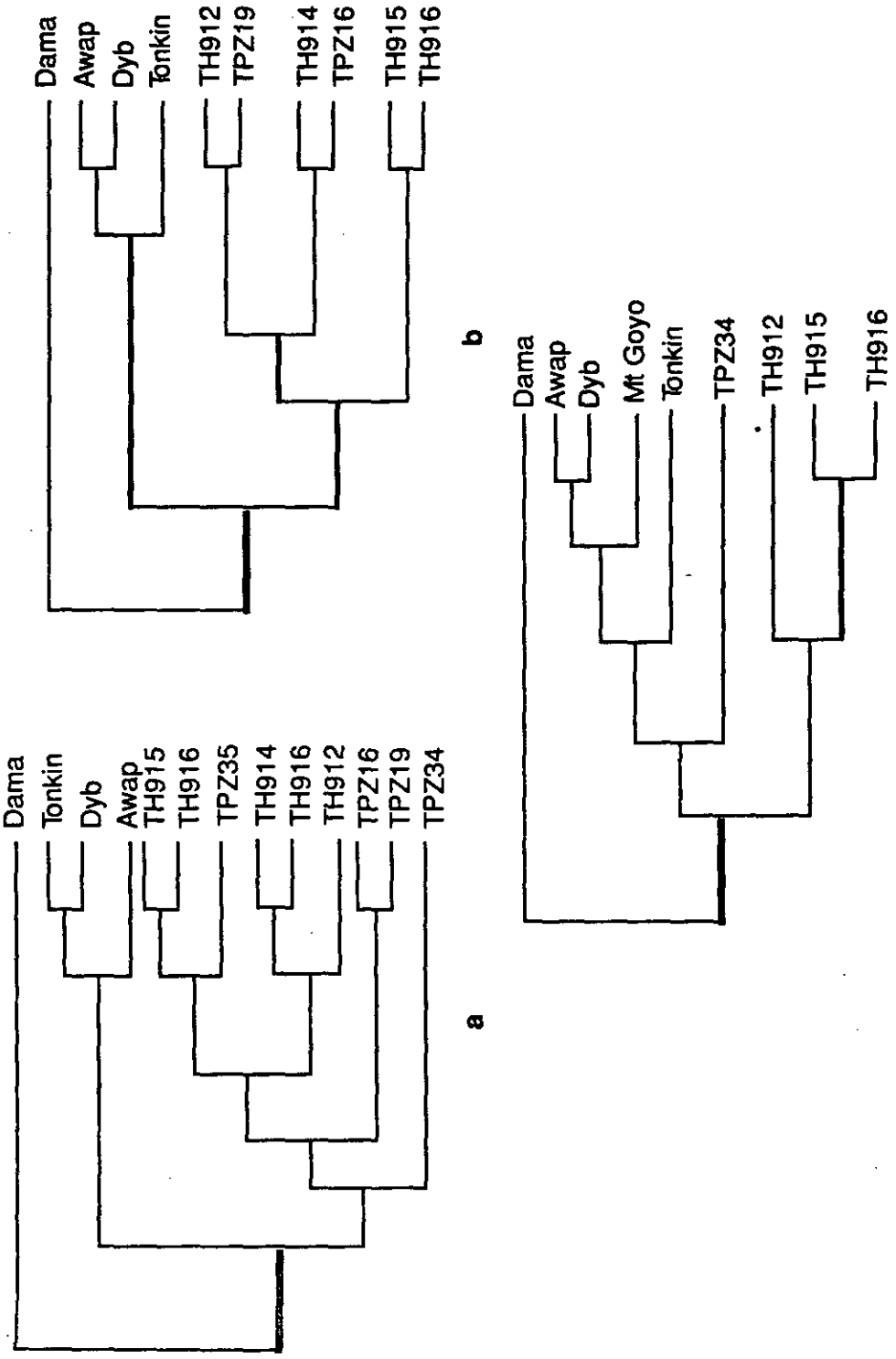

Figure 4. The most parsimonius (PHYLIP) trees for three different character state sets based on sequence differences in deer: a) 12 individuals, 9 sites, b) 10 individuals, 21 sites, c) 9 individuals at 5 first and second position sites. Abbreviations as for Figures 2 and 3. Heavy lines indicate branches supported by 45 or more of 50 bootstrap trials (PHYLIP).

Appendix I: DNA extraction

Reagents

1 M Tris-HCl, pH 7.5
10% SDS
0.5 M Na_2EDTA, pH 8.0
5.0 M NaCl
3 M NaAcetate
Proteinase K, 10 mg/ml in H_2O
Tris buffered phenol/chloroform (1:1) extraction solvent
Absolute ethanol
TE buffer, pH 7.5 (10 mM Tris-HCl, 1 mM EDTA)
TA buffer (.04M Tris-HCl, 1.142 mL/L acetic acid)

Digest buffer (0.5 ml, made from above solutions):

10 mM Tris-HCl	5 µl
10 mM EDTA	10 µl
50 mM NaCl	5 µl
2% SDS	100 µl
sterile deionized H_20	380 µl

Procedure

1. Suspend 0.2 ml buffered whole blood in 0.5 ml digest buffer. Add 15 µl proteinase K solution. Incubate one hour at 56°C or overnight at 37°C

2. Add 0.5 ml buffered phenol/chloroform. Mix thoroughly by inversion or vortexing. Centrifuge 1-2 minutes in microfuge (12,000 g).

3. Remove aqueous upper layer and transfer to new tube. Repeat extraction until interface is clear; usually 2-3 extractions.

4. Remove final aqueous layer and concentrate by ethanol precipitation as follows: add 1/10 volume of 3M Na acetate, then 2.5 volumes of cold ethanol. Store at -20°C at least one hour. Collect DNA by centrifugation, 10-30 minutes, in microfuge.

5. Remove ethanol solution, wash DNA with 1 ml 70% ethanol, reduce temperature to -20°, then spin in microfuge 15 minutes. Remove supernatant and evaporate to dryness. Resuspend DNA in 20 µl TE buffer.

DNA was visualized on a 1% agarose (SeaChem) gel in TA buffer. 1 µl of purified DNA was loaded onto the gel and electrophoresed at 60V for one hour. The gel was stained with ethidium bromide and photographed under UV light.

Appendix II: the Polymerase Chain Reaction

The polymerase chain reaction (PCR) allows the rapid amplification of selected DNA segments. Purified DNA is mixed with oligonucleotide primers, a thermostable DNA polymerase, and deoxynucleotides (the building blocks of DNA). Primers are short oligonucleotide sequences which bind to single stranded DNA in the reaction mixture, forming short double strands at which the polymerase can initiate replication. The mixture is heated to denature the purified DNA, cooled to an annealing temperature at which allows the primer binds to the single stranded template, then heated to the temperature (72° for *Taq* polymerase) at which the DNA polymerase functions most efficiently. The polymerase, starting at the primer, duplicates the original DNA strand. The mixture is heated again to denature the newly formed strands, then cooled again to allow another cycle of annealing and polymerization. Typically, 25 to 40 cycles are performed in a PCR thermal cycler, resulting in a many-fold increase in the number of copies of the target DNA. Both double stranded and single stranded amplifications are possible. In a double stranded amplification two primers which initiate transcription on opposite strands of the target DNA are used. A double stranded segment between the two primers is then amplified and can be used in further PCR reactions. In a single stranded amplification one of the two primers is reduced in concentration, usually 50 to 100 times less than the other primer, so that the PCR product contains many copies of the single stranded DNA which has been amplified by the primer at normal concentration. This single stranded product can then be sequenced directly (Gyllensten and Erlich, 1988).

PCR protocol

Reagents

10x reaction buffer	500 mM KCl 100 mM Tris-HCl, pH 8.3 25 mM $MgCl_2$ 0.1% (w/v) gelatin
100 mM deoxynucleotide mix	25 mM each dATP, dCTp, dGTP, dTTP
PCR primers	10 µM solutions of each primer
Taq polymerase	5 units/µl

Procedure

2.5 µl 10x reaction buffer
2.5 µl each 10 µM primer (final concentration 0.25 µM)
1 µl 100 mM dNTPs (final concentration 0.25 mM)
1 µl *Taq* polymerase
16.9 µl sterile, double distilled H_2O
1 µl DNA solution

Final volume = 25 µl

A premix was prepared containing all of the above except the DNA. This is placed into a 0.5 ml microfuge tube, then the DNA is added. Two drops of mineral oil were placed on top of the sample to prevent evaporation. For single stranded amplifications one of the primers was reduced in concentration by a factor of 75. PCR conditions were as follows: denaturing at 94°C for 1 minute, annealing at 45-60°C for 1 minute, extension at 72°C for 3 minutes. Thirty to 40 cycles were performed. Five µl aliquots of each PCR product were visualized on 2% NuSieve agarose gels in a Tris-acetate buffer. Gels are run at 70V for one to two hours, stained with ethidium bromide and photographed under a UV light.

Purified double stranded PCR products were obtained by cutting the ethidium bromide stained band from the agarose gels and placing them in 100-250 µl of sterile double distilled H_2O. DNA was eluted from the agarose fragment by heating the sample to 70° to melt the agarose. One µl aliquots were then used for asymmetric PCR amplifications.

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