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## ORIGINAL ARTICLE

# *Mycobacterium tuberculosis* and *M. bovis* infection in Feedlot Deer (*Cervus unicolor swinhoei* and *C. nippon taiouanus*) in Taiwan

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**KEYWORDS**

*Cervus nippon taiouanus*;  
*Cervus unicolor swinhoei*;  
*Mycobacteria* spp.;  
Polymerase chain reaction;  
Sequence analysis

**Background/purpose:** *Mycobacterium bovis* frequently infects wild and farm deer species with tuberculosis. This study investigated mycobacterial infection in two native deer species *Cervus unicolor swinhoei* (Formosan Sambar, Sambar) and *C. nippon taiouanus* (Formosan Sika, Sika).

**Methods:** Based on different sampling sources of 19 intradermal tuberculin test (ITT) Sambar, mycobacterial infection and/or species were detected by acid-fast stain, duplex polymerase chain reaction (PCR) and multiplex nested PCR (mnPCR) methods, traditional mycobacterial culture and gross lesion. Blood samples of 167 Sambar deer and 147 Sika deer were then tested by duplex PCR and mnPCR methods to investigate the prevalence of mycobacterial infection. Sequence variations of these mycobacterial species were analyzed as well.

**Results:** Duplex PCR and mnPCR assays could differentiate between MTBC (*M. bovis* and *M. tuberculosis*) and *M. avium*, as well as between *M. bovis* and *M. tuberculosis*, respectively. These PCR methods showed a higher detection rate than traditional culture and matched the gross lesions examined in 19 ITT-examined Sambar. Therefore, the mycobacterial infection in blood samples of 314 deer samples was detected using these PCR methods. Duplex PCR and mnPCR showed an identical prevalence of 16.1% in Sambar and 8.2% in Sika and a significant difference in prevalence between these two deer species. *M. bovis* and *M. tuberculosis* were the species detected in feedlot Sambar and Sika. *M. tuberculosis* was found only and first in Sambar fed in central Taiwan. Sequence analysis revealed diverse genetic variations in *M. bovis* and *M. tuberculosis* associated with deer subspecies.

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**Conclusion:** Multiplex PCR methods were established, and *M. bovis* and *M. tuberculosis* were identified in feedlot deer in Taiwan. Sequence variations indicated diverse sources of both mycobacterial species.

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## Introduction

Unlike *Mycobacterium tuberculosis*, which only causes human tuberculosis (TB), *M. bovis* is a zoonotic pathogen that infects humans and domestic animals. This infection causes bovine TB, thus posing a major economic and public health problem for the animal husbandry industry. In North America, bovine tuberculosis is a major infectious disease in cervids.<sup>1</sup> In Taiwan, *Cervus unicornis swinhoei* (Sambar) and *C. nippon taiouanus* (Sika) are farmed for health and medicine (antlers and blood), food (venison), and leather products (hide). Although infection rates of *M. bovis* in deer declined from 4.1% in 1996 to 0.2% in 2006,<sup>2</sup> *M. tuberculosis* has not yet been reported in deer.

As a relatively slow-growing bacteria, *M. bovis* can be identified by acid-fast bacilli (AFB) staining, traditional mycobacterial culture, and intradermal tuberculin test (ITT). Of these methods, AFB staining is the fastest, yet requires more than  $10^4$  /mL bacteria in a clinical sample.<sup>3</sup> Although traditional culture method is golden standard, 8 to 12 weeks are required<sup>4</sup> and inappropriate sampling produces a false negative outcome.<sup>5</sup> As the conventional test for diagnosing TB,<sup>6</sup> ITT method has a high sensitivity (84%) and specificity (80%) in detecting Mycobacteria from infected animals, yet lacks the ability to differentiate *M. bovis* from *M. avium* and other Runyon groups III and IV mycobacteria.<sup>7</sup> Additionally, thin skins (1–3 mm)<sup>8</sup> or commonly mixed infection with *M. avium*<sup>9,10</sup> or *M. avium* ssp. *paratuberculosis*<sup>11</sup> in deer significantly lowers the sensitivity of the ITT test. In addition to the *M. tuberculosis* complex (MTBC), infection with nontuberculous mycobacteria (NTM) has also been described.<sup>12</sup> Therefore, a molecular diagnostic method such as polymerase chain reaction (PCR) has been developed to differentiate MTBC from NTM.<sup>13,14</sup> Aimed at controlling TB annually to eliminate ITT-positive animals, a preventative program in Taiwan from 1996 to 2006 gradually reduced mycobacterial infection for dairy cattle, dairy goats and deer from 0.37% to 0.16%, 0.5% to 0.2% and 4.1% to 0.2%, respectively.<sup>2</sup> However, ITT-positive deer are not forced to be eliminated from the field and sanitation strongly affects mycobacterial infection. This study investigates *M. bovis* and *M. tuberculosis* infection in Sambar and Sika deer collected from several farms by using ITT, mycobacterial culture, and PCR methods. The phylogenetic relation of *M. bovis* and *M. tuberculosis* associated with deer subspecies is also analyzed.

## Materials and methods

### Samples and experimental design

In Trial 1, 16 positive and three negative ITT *Cervus unicornis swinhoei* (Sambar) were sampled from five feedlot

farms. The deer were examined by necropsy for gross lesion, and their blood and tissues were sampled for bacterial culture, acid-fast stain and PCR identification as well as histopathological diagnosis. Specimens were then placed in a saturated sodium-borate solution to inhibit overgrowth of other microorganisms before shipment for culture. Next, representative gross lesions of retropharyngeal, mediastinal, tracheobronchial, and mesenteric lymph nodes, as well as the lung were determined. Finally, DNA templates of blood, lung tissues, and lymph nodes were used for PCR amplification.

In Trial 2, 167 Sambar deer and 147 Sika deer were randomly sampled from 15 Sambar and six Sika deer farms in central and southern Taiwan from May 2005 to May 2008. Twenty mL of blood were taken from the jugular vein, placed in a tube containing EDTA, and stored at 4°C for DNA purification. Finally, mycobacterial infection of both species was determined by PCR amplification of purified blood DNA.

## Mycobacterial identification

### Conventional method

#### a. Culture methods

Ten mL of sampled blood were added into BACTEC™ Myco/F Lytic Culture Vials, which were then normally placed in the BACTEC 9000 Blood Culture System and incubated at 35°C with continuous agitation for 42 days. The identification period was shortened by measuring the fluorescence of each vial at 10-minute intervals by the BACTEC fluorescent series instrument (Becton, Dickinson and Company, Sparks, MD, USA). Mycobacterial infection of each blood sample was then verified using the rate of oxygen reduction. Additionally, isolation and identification of *Mycobacterium* were followed according to the method of Thoen.<sup>15</sup>

#### b. Intradermal tuberculin test (ITT)

ITT skin test was performed by intradermal injection of 0.1 mL of 2000 IU (0.3 mg of protein) *M. bovis* PPD into lateral cervical region for each individual. Skin thickness was measured at the injection site before and 72 hours (i.e., the optimal timing to detect maximal dermal reactivity in cattle<sup>16</sup>) after injection by calipers. Moreover, test sensitivity was maximized by considering visible, measurable, or palpable responses as positive indications of TB infection.

### PCR amplification

#### a. Preparation of DNA template

DNA templates of reference *M. bovis* and *M. tuberculosis* were kindly donated by the Animal Health Research Institute, Council of Agriculture, Executive Yuan, Republic of China, Taiwan. Chromosomal DNA of other tested bacteria was prepared by centrifugation at 20,000g for 2 minutes of

overnight broth first. Pellets were then mixed with 180  $\mu$ L of lysozyme buffer (AMRESCO) (20mM Tris-HCl, 2mM EDTA, 1.2% Triton X-100; 20 mg/mL lysozyme) and reacted at 37°C for 30 minutes. Next, mycobacterial chromosomal DNA was purified by the Tissue and Cell Genomic DNA purification kit (Gene Mark, Taipei, Taiwan). For the blood sample, 10 mL of blood were mixed with 0.2% EDTA (pH 7.2), centrifuged at 3300g for 10 minutes and incubated with RBC lysis buffer for 5 minutes at room temperature. Finally, genomic DNA was purified by a blood genomic DNA purification kit (Gene Mark, Taiwan).

#### b. PCR amplification

A 50  $\mu$ L PCR reaction mixture contained 5–10 ng DNA template, 1 $\times$  PCR reaction buffer, 0.2 mM dNTPs, 1.6  $\mu$ M of each primer and 1.25 U Taq DNA polymerase. Table 1 lists all primers.

- i) **Duplex PCR.** MTBC and NTM were differentiated by a duplex PCR amplification containing two primer sets Tbc1/TbcR5 and M5/RM3<sup>17</sup> designed to amplify the 235- and 136-bp DNA fragments of gene *rpoB* encoding RNA polymerase  $\beta$ -subunit for MTBC (*M. bovis* and *M. tuberculosis*) and *M. avium*, respectively. The PCR condition was then processed with an initial denaturation at 95°C for 5 minutes, 35 cycles of 30 seconds at 95°C, 30 seconds at 68°C, 60 seconds at 72°C, and a final elongation at 72°C for 10 minutes. Finally, the PCR products were separated by 1.5% agarose.
- ii) **Multiplex nested PCR (mnPCR).** The multiplex nested PCR method was designed not only to differentiate *M. bovis* from *M. tuberculosis*, but also to increase PCR sensitivity. First PCR amplification was performed by using primer sets TBF/R and Rv3618F/R, followed by second PCR amplification with second primer sets of NTB/R and NRv3618F/R. The PCR condition was described previously.<sup>18,19</sup>
- iii) **Sensitivity and specificity of each PCR reaction.** Spiked blood samples were prepared by spiking about 10<sup>3</sup> CFU of bacteria into a normal blood sample and using a positive control. Additionally, an internal DNA control was used by amplification of a partial *spnI* DNA fragment with SI-1 and SI-2 primers.<sup>20</sup> Based on 10-fold serial dilution of known mycobacterial DNA, the

sensitivity of each PCR set was evaluated to detect four *Mycobacterium* species, which were *M. tuberculosis*, *M. bovis*, *M. avium* subsp. *paratuberculosis*, and *M. avium* subsp. *avium* (BCRC15441). Moreover, PCR specificity was tested using *Staphylococcus aureus* (BCRC10781), *Streptococcus agalactiae* (BCRC 10787), *Streptococcus uberis* (BCRC12579), *Streptococcus dysgalactiae*, *Escherichia coli*, and *Pasteurella multocida*. Furthermore, sensitivity and specificity of PCR method towards the conventional culture method to identify mycobacterial infection were evaluated.<sup>21</sup>

#### Sequence analysis of PCR products amplified from *M. bovis* and *M. tuberculosis*

Following purification of PCR products for second PCR amplification of multiplex nested PCR by the Promega PCR purification kit, the purified products were sequenced and aligned by the MegAlign program of Lasergene software (DNASRRAR, Madison, WI, USA). Accession numbers are from FJ593676 to FJ593692 for *M. tuberculosis* and from FJ409086 to FJ409111 for *M. bovis*.

#### Statistical analysis

Differences in mycobacterial infection rates among the three detection methods and samples from the two deer species were analyzed based on Chi-square testing.

## Results

#### Detection of mycobacterial infection by using Duplex PCR and mnPCR methods

The two PCR methods differed in their objectives. Duplex PCR could differentiate *M. bovis* and *M. tuberculosis* from *M. avium*, while mnPCR could differentiate *M. bovis* from *M. tuberculosis* (Tables 1 and 2). The sensitivity of mnPCR was 10-fold higher than that of duplex PCR (100 fg for mnPCR vs. 1 pg for duplex PCR) (Table 2). Owing to its high sensitivity and specificity, *M. bovis* and *M. tuberculosis* in deer were detected using mnPCR.

**Table 1** Primers used for Duplex and Multiplex nested PCR (mnPCR) amplification

| PCR method             | PCR Step | <i>Mycobacterium</i> spp.                  | Primer set         | Primer sequence (5'–3')   | Product size (bp) | Reference |
|------------------------|----------|--|--------------------|---------------------------|-------------------|-----------|
| Duplex PCR             | 1        | <i>M. bovis</i> and <i>M. tuberculosis</i> | Tbc1               | CGTACGGTCGGCGAGCTGATCCAA  | 235               | 17        |
|                        |          |  | TbcR5              | CCACCAGTCGGCGCTTGTGGGTCAA |                   |           |
|                        |          | <i>M. avium</i>                            | M5                 | GGAGCGGATGACCACCCAGGACGTC | 136               |           |
|                        |          |  | RM3                | CAGCGGGTTGTTCTGGTCCATGAAC |                   |           |
| mnPCR                  | 1        | <i>M. bovis</i>                            | TBF                | GACCACGGTGGTCCGCG         | 636               | 19        |
|                        |          |  | TBR                | CATGACCCCGCTACCG          |                   |           |
|                        |          | <i>M. tuberculosis</i>                     | Rv3618F            | ATTGCACATCCGCCCC          | 326               | 18        |
|                        |          | Rv3618R                                    | GGACAAACCCTGCCGC   |                           |                   |           |
|                        | 2        | <i>M. bovis</i>                            | NTBF               | CCCCTGATGCAAGTGCC         | 471               | 19        |
|                        |          |  | NTBR               | CCCACATCCCAACACC          |                   |           |
| <i>M. tuberculosis</i> |          | NRv3618F                                   | GCTCAACACCCGCCAATC | 224                       | 18                |           |
|                        | NRv3618R | ACATCCGCCCTACACC                           |                    |                           |                   |           |

**Table 2** The specific PCR detection of *Mycobacterium tuberculosis*, *M. bovis* and *M. avium*

| Reference strains  | Duplex PCR |        | Multiplex nested PCR |                 |
|--|------------|--------|----------------------|-----------------|
|  | Tbc1-TbcR5 | M5-RM3 | TBF-TBR              | Rv3618F-Rv3618R |
| Sensitivity  | 1 pg       | 1 pg   | 100 fg               | 100 fg          |
| <i>Mycobacterium tuberculosis</i> complex                    |            |        |                      |                 |
| <i>M. tuberculosis</i> <sup>a</sup>                          | +          | -      | -                    | +               |
| <i>M. bovis</i> <sup>a</sup>                                 | +          | -      | +                    | -               |
| Nontuberculous mycobacteria                                  |            |        |                      |                 |
| <i>M. avium</i> subsp. <i>paratuberculosis</i> <sup>a</sup>  | -          | +      | -                    | -               |
| <i>M. avium</i> subsp. <i>avium</i> (BCRC15441) <sup>b</sup> | -          | +      | -                    | -               |
| Non-mycobacteria   |            |        |                      |                 |
| <i>Staphylococcus aureus</i> (BCRC10781) <sup>b</sup>        | -          | -      | -                    | -               |
| <i>Streptococcus agalactiae</i> (BCRC10787) <sup>b</sup>     | -          | -      | -                    | -               |
| <i>Streptococcus uberis</i> (BCRC 12579) <sup>b</sup>        | -          | -      | -                    | -               |
| <i>Streptococcus dysgalactiae</i> <sup>c</sup>               | -          | -      | -                    | -               |
| <i>Escherichia coli</i> <sup>c</sup>                         | -          | -      | -                    | -               |
| <i>Pasteurella multocida</i> <sup>c</sup>                    | -          | -      | -                    | -               |

<sup>a</sup> *M. tuberculosis* was kindly provided by Animal Health Research Institute (AHRI), Council of Agriculture Executive Yuan in Taiwan.

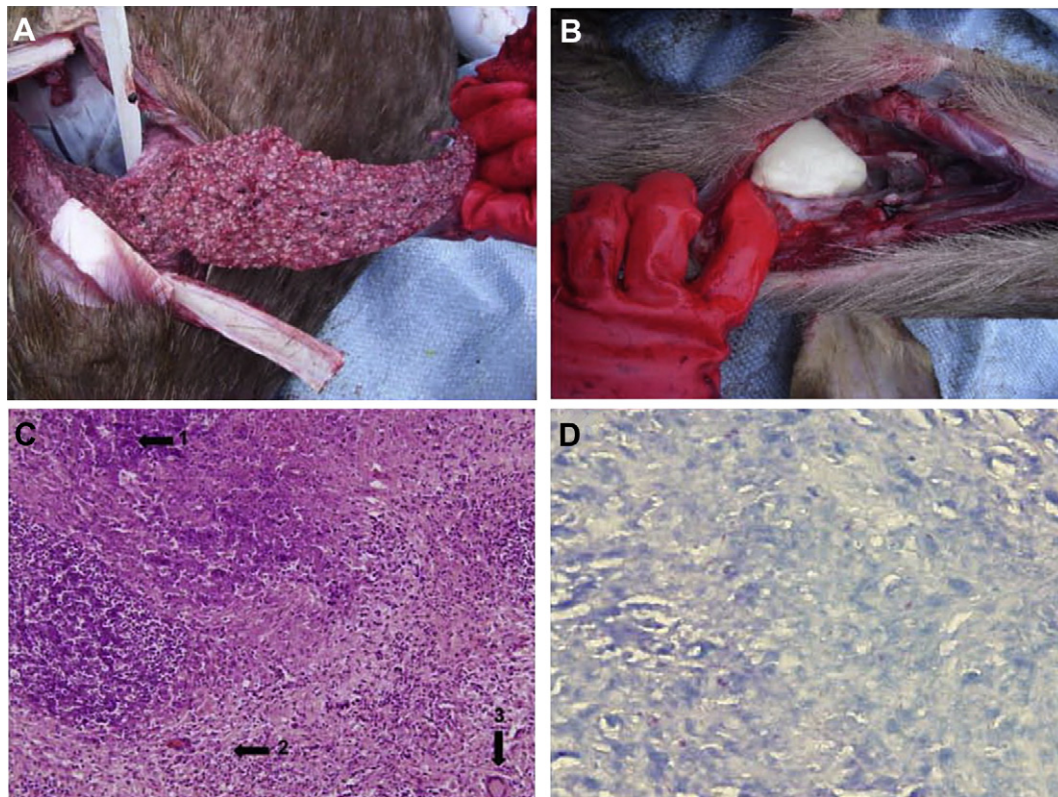
<sup>b</sup> BCRC stands for Bioresource Collection and Research Center in Taiwan.

<sup>c</sup> Clinical strains were identified by biochemical assays.

### Comparison of different detection methods for *Mycobacterium*

Necropsy examination revealed tubercle in the lung, caseous necrosis exudates in the retropharyngeal lymph

node and confluent tubercles in the lymph node (Fig. 1). Additionally, the granulomatous and acid-fast bacteria were also observed in the histopathologic section (Fig. 1). Moreover, mycobacterial infection in 19 ITT-positive and negative samples was determined using ITT, traditional



**Figure 1.** Necropsy examination of mycobacterial infected Sambar Deer. (A) Tubercle in the lung. (B) Caseous necrosis exudates in the retropharyngeal lymph node. (C) Confluent tubercles in the lymph node (200 $\times$ ). Arrow 1: caseous necrosis, Arrow 2: epithelioid cells, and Arrow 3: Langhans' giant cell. (D) Acid-fast stained bacteria in the lung (600 $\times$ ).

**Table 3** Comparison of different methods in identifying mycobacterial infection in *C. unicolor swinhoei*

| Farm    | Number | ITT   | Acid-fast stain | Culture <sup>a</sup>      | Multiplex-nest-PCR (mn-PCR) <sup>b</sup> | Gross lesion  |
|---------|--------|-------|-----------------|---------------------------|--|---|
| A       | 960311 | -     | +               | <i>M. bovis</i>           | <i>M. bovis</i>                          | Necrotic and caseous granulomas and enlarged lymph node |
|         | 960312 | +     | -               | -                         | <i>M. bovis</i>                          | -   |
|         | 960313 | +     | +               | <i>M. bovis</i>           | <i>M. bovis</i>                          | Necrotic and caseous granulomas and enlarged lymph node |
|         | 960314 | +     | -               | <i>Mycobacterium</i> spp. | -  | -   |
|         | 960316 | +     | -               | -                         | <i>M. bovis</i>                          | Necrotic and caseous granulomas                         |
|         | 960320 | +     | -               | <i>Mycobacterium</i> spp. | -  | -   |
|         | 960343 | +     | +               | <i>M. bovis</i>           | <i>M. bovis</i>                          | Necrotic and caseous granulomas and enlarged lymph node |
|         | 960344 | +     | +               | <i>M. bovis</i>           | <i>M. bovis</i>                          | -   |
|         | 960348 | -     | -               | -                         | <i>M. bovis</i>                          | Necrotic and caseous granulomas                         |
|         | B      | 96048 | +               | -                         | -  | -   |
| 96066   |        | +     | +               | <i>M. bovis</i>           | <i>M. bovis</i>                          | Lung necrotic and caseous granulomas                    |
| 96074   |        | -     | -               | -                         | -  | -   |
| C       | 98B585 | +     | -               | <i>M. bovis</i>           | <i>M. bovis</i>                          | -   |
| D       | 93H224 | +     | +               | -                         | <i>M. bovis</i>                          | -   |
|         | 95J502 | +     | -               | -                         | <i>M. bovis</i>                          | -   |
| E       | 95J474 | +     | +               | <i>M. bovis</i>           | <i>M. bovis</i>                          | Lung necrotic and caseous granulomas                    |
|         | 95J115 | +     | +               | -                         | <i>M. bovis</i>                          | Necrotic and caseous granulomas                         |
|         | 95J118 | +     | -               | <i>M. bovis</i>           | <i>M. bovis</i>                          | -   |
|         | 95J121 | +     | -               | -                         | <i>M. bovis</i>                          | -   |
| Total,% | 19     | 16    | 42.1 (8/19)     | 52.6 (10/19)              | 78.9 (15/19)                             | 42.1 (8/19)   |

<sup>a</sup> Culture samples were lymph node and lung.

<sup>b</sup> mn-PCR samples were blood, lymph node and lung.

culture, acid-fast stain, PCR amplification and gross lesion in the lungs. The prevalence of mycobacterial infection was 84.2% (16/19) for ITT, 52.6% (10/19) for culture method (8 *M. bovis* isolates and 2 unknown *Mycobacterium* spp. isolates from lymph node and lung), 42.1% (8/19) for acid-fast stain, 78.9% (15/19) for PCR amplification of blood, lung, and lymph node samples (3 ITT-negative deer were dead before blood collection), and 42.1% (8/19) for gross lesion in the lung and lymph node (Table 3). Above results were not consistent with each other for any two methods. In comparison with gold standard (infection) results, sensitivity and specificity were 83.3% and 100% for culture method, 83.3% and 28.6% for PCR amplification, and 66.7% and 100% for acid-fast stain, respectively (Table 4).

In comparison with the culture method, mnPCR revealed a higher sensitivity and lower specificity in mnPCR. Due to

time-consuming nature of the culture method, PCR method is rapid, simple and inexpensive. Therefore, mycobacterial infection in the blood samples of two feedlot deer was identified using PCR methods. Among 314 samples, Duplex PCR and mnPCR revealed an identical prevalence of 16.1% in Sambar and 8.2% in Sika, as well as a significant difference in prevalence between these two deer species (Table 5).

#### Infection of *M. bovis* and *M. tuberculosis* in both deer species

An attempt was made to eliminate possible contamination by positive samples during PCR amplification by performing each test three times and sequencing directly all PCR

**Table 4** The comparison of positive infection with Culture, mnPCR and acid-fast stain detecting method

| Positive infection <sup>a</sup> | Culture        |          | mnPCR          |          | Acid-fast stain |          |
|---------------------------------|----------------|----------|----------------|----------|-----------------|----------|
|                                 | Positive       | Negative | Positive       | Negative | Positive        | Negative |
| Positive                        | 10             | 2        | 10             | 2        | 8               | 4        |
| Negative                        | 0              | 7        | 5              | 2        | 0               | 7        |
| Total                           | 10             | 9        | 15             | 4        | 8               | 11       |
| Sensitivity                     | 83.33% (10/12) |          | 83.33% (10/12) |          | 66.67% (8/12)   |          |
| Specificity                     | 100% (7/7)     |          | 28.57% (2/7)   |          | 100% (7/7)      |          |
| Kappa                           | 0.78652        |          | 0.13072        |          | 0.59574         |          |

<sup>a</sup> Infection definition: deer was positive in acid-fast stain, culture, and gross lesion/histopathology.

**Table 5** Prevalence of *Mycobacterium tuberculosis* complex (MTBC) determined by two PCR methods in two deer species

| Cervus species                       | Duplex PCR (%) |               |                            | Multiplex nested PCR (%)   |
|--------------------------------------|----------------|---------------|----------------------------|----------------------------|
|                                      | Male           | Female        | Total                      |                            |
| <i>C. unicolor swinhoei</i> (Sambar) | 11.4 (13/114)  | 26.4 (14/53)  | 16.1 <sup>x</sup> (27/167) | 16.1 <sup>x</sup> (27/167) |
| <i>C. nippon taiouanus</i> (Sika)    | 9.9 (8/81)     | 6.1 (4/66)    | 8.2 <sup>y</sup> (12/147)  | 8.2 <sup>y</sup> (12/147)  |
| Total                                | 10.8 (21/195)  | 15.1 (18/119) | 12.4 (39/314)              | 12.4 (39/314)              |

<sup>x,y</sup> in the same column indicate significant difference between the two groups determined by the Chi square test ( $p < 0.001$ ).

products of *M. bovis* and *M. tuberculosis*. Mycobacterial infection was equally prevalent in Sambar farms (5/15; 33%) and Sika farms (2/5; 40%). *M. tuberculosis* was also identified in both deer from five farms (Table 6). Compared

to *M. tuberculosis*, *M. bovis* was more prevalent in Sambar (29.3% for *M. bovis* vs. 15.5% for *M. tuberculosis*) and equally prevalent in Sika. In Sambar, mycobacterial infection was significantly higher in central Taiwan (57.5% in

**Table 6** Prevalence of *M. bovis* and *M. tuberculosis* in Sambar and Sika from 7 ITT positive deer farms in central and south Taiwan

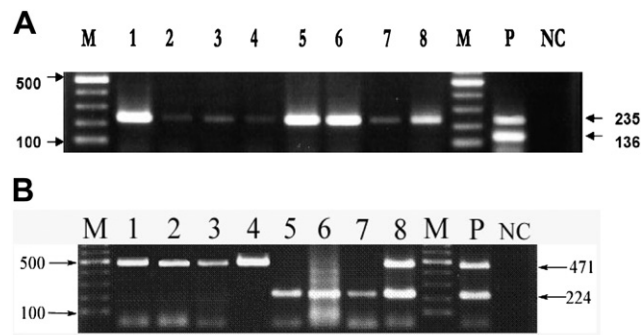
| Deer Species | Region         | Farm         | Total deer | Sample number | Mycobacterium species | Infected number | Sex       |   |   |
|--------------|----------------|--------------|------------|---------------|-----------------------|-----------------|-----------|---|---|
|              |                |              |            |               |                       |                 | F         | M |   |
| Sambar       | Central Taiwan | NT-A         | 35         | 25 (71.4%)    | MB                    | 6 (24%)         | 6         | 0 |   |
|              |                |              |            |               | MT                    | 3 (12%)         | 2         | 1 |   |
|              |                |              |            |               | Both                  | 1 (4%)          | 1         | 0 |   |
|              |                |              |            |               | Total                 | 10 (40%)        | 9         | 1 |   |
|              |                | NT-B         | 25         | 9 (36%)       | MB                    | 5 (55.6%)       | 0         | 5 |   |
|              |                |              |            |               | MT                    | 2 (22.2%)       | 1         | 1 |   |
|              |                |              |            |               | Total                 | 7 (77.8%)       | 1         | 6 |   |
|              | NB-C           | 6            | 6 (100%)   | MB            | 2 (33.3%)             | 1               | 1         |   |   |
|              |                |              |            | MT            | 4 (66.7%)             | 0               | 4         |   |   |
|              | Total          | 66           | 40 (60.6%) | Total         | 6 (100%)              | 1               | 5         |   |   |
|              |                |              |            | MB            | 13 (32.5%)            | 7               | 6         |   |   |
|              |                |              |            | MT            | 9 (22.5%)             | 3               | 6         |   |   |
|              | South Taiwan   | South Taiwan | TL-A       | 30            | 13 (43.3%)            | MB              | 2 (15.4%) | 2 | 0 |
|              |                |              |            |               |                       | MT              | 0 (0%)    | 0 | 0 |
| Both         |                |              |            |               |                       | 1 (7.7%)        | 1         | 0 |   |
| Total        |                |              |            |               |                       | 2 (15.4%)       | 2         | 0 |   |
| TL-B         |                |              | 40         | 5 (12.5%)     | MB                    | 2 (40%)         | 1         | 1 |   |
|              |                |              |            |               | MT                    | 0 (0%)          | 0         | 0 |   |
|              |                |              |            |               | Total                 | 2 (40%)         | 1         | 1 |   |
| Total        |                | 70           | 18 (25.7%) | MB            | 4 (22.2%)             | 3               | 1         |   |   |
|              |                |              |            | MT            | 0 (0%)                | 0               | 0         |   |   |
|              |                |              |            | Total         | 4 (22.2%)             | 3               | 1         |   |   |
| Sum          |                | 136          | 58 (42.6%) | MB            | 17 (29.3%)            | 10              | 7         |   |   |
|              |                |              |            | MT            | 9 (15.5%)             | 3               | 6         |   |   |
|              |                |              |            | Both          | 1 (1.7%)              | 1               | 0         |   |   |
| Sika         |                | South Taiwan | TS-A       | 79            | 79 (100%)             | MB              | 4 (5.1%)  | 2 | 2 |
|              | MT             |              |            |               |                       | 2 (2.5%)        | 0         | 2 |   |
|              | Both           |              |            |               |                       | 1 (1.3%)        | 0         | 1 |   |
|              | Total          |              |            |               |                       | 6 (7.6%)        | 2         | 4 |   |
|              | TS-B           |              | 60         | 8 (13.3%)     | MB                    | 2 (25%)         | 0         | 2 |   |
|              |                |              |            |               | MT                    | 3 (37.5%)       | 2         | 1 |   |
|              |                |              |            |               | Total                 | 5 (62.5%)       | 2         | 3 |   |
|              | Total          | 139          | 87 (62.6%) | MB            | 6 (6.9%)              | 2               | 4         |   |   |
|              |                |              |            | MT            | 5 (5.7%)              | 2               | 3         |   |   |
|              |                |              |            | Both          | 1 (1.1%)              | 0               | 1         |   |   |
|              | Total          | 139          | 87 (62.6%) | Total         | 12 (13.8%)            | 4               | 8         |   |   |

average) than in southern Taiwan (an average of 22.2%). Such a difference in prevalence among farms was also found in Sika from 7.6% in TS-A farm to 75% in TS-B farm. Mycobacterial infection was generally higher in Sambar (46.6%) than in Sika (13.8%). Additionally, *M. bovis* and *M. tuberculosis* from the same deer were isolated for both deer species.

Nucleotide variations were found mainly in 16 bp out of 401 bp (4.0%) for 25 PCR products of *M. bovis* and 9 bp out of 171 bp (5.3%) for 16 PCR products of *M. tuberculosis*. Notably, most mutations were either point mutation or deletion. Phylogenetic study of *M. bovis* and *M. tuberculosis* sequences demonstrated. Two main clusters in *M. bovis* and *M. tuberculosis*, respectively. In *M. bovis*, cluster I was separated into 11 subtypes, and the reference strain was in I-8 type with two other sequences (Fig. 3A). In *M. tuberculosis*, cluster I was categorized into two main subtypes I-1 and I-2; in addition, reference strains and the other 11 strains belonged to I-1 subtypes (Fig. 3B). The sequences from two deer species were mixed together in cluster I for both *Mycobacterium* species, except Sik2 for *M. bovis* and Sam1 of *M. tuberculosis*, which were independent of the other sequences.

## Discussion

The conventional culture method and ITT methods are time consuming and dependent of experienced users. ITT examination of TB infection is performed based on cell-mediated immune response induced by injection of PPD under skin. With the single mid-cervical skin test, ITT has



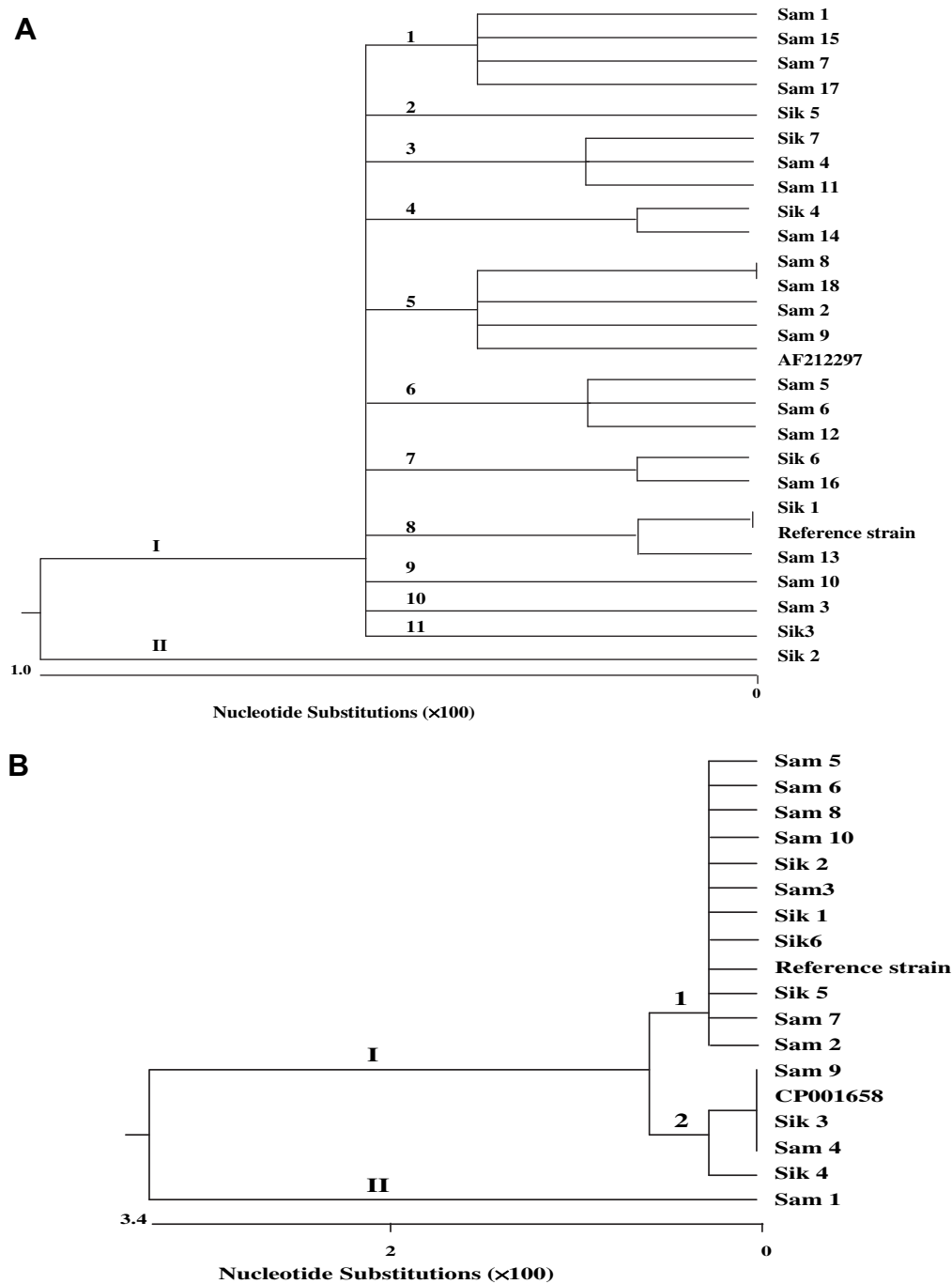
**Figure 2.** (A) Gel electrophoresis of PCR products of MTBC species amplified from animal blood or nasal swab specimens by Duplex PCR assay for identification of *M. bovis* and *M. tuberculosis* as well as *M. avium* subsp. *avium* (lanes 1–8 = positive MTBC specimens; M = 100 bp DNA ladder marker; NC = negative control; P = reference strain of *M. bovis* and *M. avium* subsp. *avium*, 235-bp product stands for *M. bovis* and *M. tuberculosis*. 136-bp PCR product represents *M. avium*) and (B) multiplex nested PCR for identification of *M. bovis* and *M. tuberculosis* (M: 100 bp DNA ladder marker, lanes 1–4: Positive specimens of *M. bovis*, lanes 5–7: *M. tuberculosis*, lane 8: positive specimens of *M. bovis* and *M. tuberculosis* detected in the same specimen, P: reference strains of *M. bovis* and *M. tuberculosis*, NC: negative control. The size of PCR product was 471 bp for *M. bovis* and 224 bp for *M. tuberculosis*).

a sensitivity of approximately 80% for TB diagnosis in deer.<sup>7,8,22</sup> However, delayed-type hypersensitivity response developed at 2 to 4 weeks after infection;<sup>23</sup> this manifests itself as a positive response to PPD, yet can be a negative result in a culture assay. According to our results, ITT-negative deer died of *M. bovis* infection (Table 3). The conventional culture method is limited in that 8 to 12 weeks<sup>4</sup> are required for culture, and a false negative is given due to sampling and minimal bacterial dose.<sup>5</sup> Deer and cattle can be infected with *M. bovis* at 500 CFU, and a low infection rate was found in sentinel node of deer.<sup>24</sup> To increase the detection rate and shorten the examination period of mycobacteria in clinical samples, PCR assays have been developed<sup>25</sup> by targeting different genes, such as 16S rRNA,<sup>26</sup> IS6110,<sup>27</sup> *mtp40* (target gene of Rv3618/Nrv3618 primer set),<sup>28–30</sup> RD9 and RD10.<sup>31</sup> In this study, both duplex PCR method and mnPCR method could identify mycobacterial infection by using blood samples with a higher sensitivity than that achieved by the ITT method (Tables 3 and 4). ITT and PCR methods may differ owing to the booster phenomenon, such as PCR assays, and errors in PPD administration and reading the results,<sup>32</sup> failure to produce an i.d. bleb, or the pseudo-negative immune response common in the early or chronic phases of TB.

In contrast to the duplex PCR method for differentiation of MTBC (including *M. tuberculosis* and *M. bovis*) and *M. avium*,<sup>17</sup> this study developed mnPCR to differentiate *M. bovis* from *M. tuberculosis* (Fig. 2, Tables 2 and 5). *M. bovis* has been reported in farm and wild deer species, with prevalence ranging from 0% in red deer (*C. elaphus*), to 2.1% in sika deer (*C. nippon*), and up to 18.5% in fallow deer (*Dama dama*).<sup>33</sup> The highest prevalence (89%) was observed in the mixture of *C. nippon* and *D. dama*.<sup>7</sup> Although previous studies have found PCR amplification of *M. tuberculosis* in humans,<sup>34,35</sup> this study described for the first time *M. tuberculosis* infection in both deer species by mnPCR amplification of blood samples (Table 5). In the present study, both mycobacterial species were also identified from blood, lung and lymph node (Tables 3–5), suggesting that *M. bovis* and *M. tuberculosis* in an activated state can enter into the lymph node, the blood stream and other tissues in the infectious state.

This study also attempted to eliminate the possible contamination of positive control in PCR amplification, with sequence analysis results demonstrating that *M. bovis* and *M. tuberculosis* could infect both deer species and are derived from diverse sources (Fig. 3, Table 5). Sequence information also revealed that *M. bovis* and *M. tuberculosis* evolved differently more faster in Sika than in Sambar; in addition, sequence variations of *M. tuberculosis* were quicker than that of *M. bovis* (Fig. 3). Although prevalence of *M. bovis* and *M. tuberculosis* were equal in Sika, *M. tuberculosis* was identified in Sambar in central Taiwan (Table 5), which has high humidity year-round. Importantly, our results demonstrate that deer may be the reservoir for *M. tuberculosis*, posing a potential public health threat by transmission of *M. tuberculosis* between deer and humans.

In conclusion, *M. bovis* and *M. tuberculosis* were identified in feedlots Sambar and Sika by mnPCR amplification from blood samples. Additionally, prevalence of both mycobacterial species differed between two deer species and between regions in Sambar. Furthermore, sequence



**Figure 3.** Dendrograms of *Mycobacterium bovis* (A) and *M. tuberculosis* (B) was constructed by using MegAlign Cluster W method of Lasergene v7.1 software (DNASRRAR, Madison, Wisconsin, USA) to analyze the sequences of PCR products amplified from blood of two feedlot deer with parameter of gap penalty of 15.00 and gap length penalty of 6.66.

variations of mycobacterial species were associated with deer species.

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