Multiplex PCR Assay for Identification of Mycobacterial Species Isolated from Liquid Cultures

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We developed a 2-step multiplex polymerase chain reaction (PCR) assay to rapidly identify the most common mycobacterial species isolated from positive liquid cultures as follows. In the first step, a multiplex PCR-A assay was used to differentiate *Mycobacterium tuberculosis* complex from nontuberculous mycobacterial (NTM) species. In the second step, 2 parallel multiplex PCR-B/C assays were used to identify *M. avium*, *M. intracellulare*, *M. kansasii*, *M. fortuitum*, *M. abscessus/chelonae*, *M. goodii*, and *M. terrae*. The multiplex PCR assays were tested on a total of 147 liquid cultures, including 15 reference strains and 4 mixed cultures. The results were compared with those obtained by PCR-restriction enzyme analysis of the *hsp65* gene (PRA-*hsp65*) and species-specific PCR. For 129 of 143 cultures yielding a single isolate, the results of the multiplex PCR matched those of species-specific PCR and PRA-*hsp65* except for 8 *M. intracellulare* isolates and 6 other NTM species not represented by the multiplex PCR in this study. Moreover, the multiplex PCR detected the coexistence of 2 mycobacterial species in 4 mixed cultures, whereas PRA-*hsp65* misidentified the mixed cultures as a single species. The data demonstrated that multiplex PCR assays may be easy and useful for the rapid identification of most common mycobacterial species in liquid cultures, particularly in mixed cultures.

Key Words: Multiplex PCR; Nontuberculous mycobacteria (NTM); Culture media

Introduction

Tuberculosis still remains an important public health problem in Korea, where the prevalence of active pulmonary tuberculosis per 100,000 has decreased from 5,065 to 1,032 in the last 30 years.¹ In addition, nontuberculous mycobacteria (NTM) are increasingly encountered in the clinical laboratory, due to the dissemination of HIV, the increased use of immunosuppressive

drugs, and the improvement in detection techniques such as liquid cultures.² The correct and rapid diagnosis of mycobacteria to the species level is extremely important because the treatment of tuberculosis differs from the treatment of diseases caused by NTM, and adequate therapy taken in time can reduce the spread of the disease and the costs of hospitalization.

Since it was first introduced, the BACTEC 460 TB radiometric system (Becton Dickinson, USA) has been the gold standard for rapid detection of mycobacteria by use of the BACTEC 12B liquid medium containing ¹⁴C-labeled palmitic acid substrate. On the basis of CO₂ evolution, this system has increased recovery rates and

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has decreased the time to detection of mycobacteria from 8 weeks with conventional cultures to 2 weeks. The BACTEC MGIT 960 system (BD, USA), the newer broth-based method that has recently been developed, offers rapid time to detection similar to that of the BACTEC 460 TB system while using fully automated, high-capacity, noninvasive, nonradiometric, and self-contained incubator readers.² However, these broth-based systems lack appropriate diagnostic tests for rapid differentiation of different species of mycobacteria. Additional techniques such as high-performance liquid chromatography,³ Gen-Probes,^{4,6} and sequencing based on the 16S rRNA gene^{7,8} can provide rapid differentiation of a wide variety of mycobacteria growing in liquid cultures. However, these procedures require additional expensive equipment and remain limited to reference laboratories or they require several probes and cover a limited number of mycobacterial species.

Recently, polymerase chain reaction (PCR) techniques have provided new possibilities for the rapid identification of mycobacteria growing in liquid cultures. A wide variety of species-specific sequences, which can be candidate targets for single PCR to differentiate mycobacteria, have been reported in mycobacteria as follows: MPB70 in *M. tuberculosis* complex,^{9,10} DT6 in *M. avium*,^{6,11} DT1 in *M. intracellulare*,^{6,11} p6123 in *M. kansasii*,^{12,13} superoxide dismutase gene in *M. fortuitum*,¹⁴ 16S-23S rRNA internal transcribed spacer region in *M. gordonae*,¹⁵ IS1395 in *M. xenopi*,¹⁶ and 16S rRNA hypervariable regions in *M. terrae* and *M. chelonae*.⁷ Considering the relatively large number of mycobacterial species-specific target genes presently characterized, at least 8 or more separate PCR reactions are required for identification of mycobacterial species isolated from liquid cultures by using a conventional PCR procedure. This is potentially expensive and labor-intensive and increases the risk of cross-contamination.

To overcome these limitations and to develop a rapid and reliable method to identify most common clinical mycobacterial isolates growing in liquid cultures, 2-step multiplex PCR assays were designed. In the first step, a

multiplex PCR-A was initially used for detection of all mycobacterial species and differentiation of *M. tuberculosis* complex from NTM species. In the second step, 2 parallel multiplex PCR-B and PCR-C assays were used to allow simultaneous detection and identification of 7 different mycobacterial species such as *M. avium*, *M. intracellulare*, *M. kansasii*, *M. fortuitum*, *M. gordonae*, *M. chelonae*/*M. abscessus*, and *M. terrae*.

Materials and Methods

1. Reference strains and clinical isolates

Clinical mycobacterial isolates (n=132, Table 1) were retrieved from laboratory collections obtained between 1994 and 2005 at the Department of Laboratory Medicine, Chonnam National University Hospital (Gwangju,

Table 1. Reference mycobacterial strains used in the multiplex PCR assays

Mycobacterial species	Strains	No. of isolates
<i>M. abscessus</i>	Clinical Strain	1
<i>M. avium</i>	ATCC 25291	1
	Clinical strain	36
<i>M. bovis</i>	ATCC 19210	1
<i>M. bovis</i> BCG	French strain 1173P2	1
<i>M. chelonae</i>	ATCC 35749	1
	Clinical strain	2
<i>M. fortuitum</i>	ATCC 49403	1
	ATCC 6841	1
	Clinical strain	3
<i>M. gordonae</i>	ATCC 14470	1
	Clinical strain	18
<i>M. intracellulare</i>	ATCC 13950	1
	Clinical strain	28
<i>M. kansasii</i>	ATCC 12478	1
	Clinical strain	2
<i>M. marinum</i>	ATCC 927	1
	Clinical strain	2
<i>M. scrofulaceum</i>	ATCC 19981	1
<i>M. smegmatis</i>	ATCC 19420	1
<i>M. szulgai</i>	ATCC 35799	1
<i>M. terrae</i>	ATCC 15755	1
	Clinical strain	1
<i>M. tuberculosis</i> H37Rv	ATCC 27294	1
<i>M. tuberculosis</i> complex	Clinical strain	35
<i>M. tuberculosis</i> + <i>M. avium</i>	Clinical strain	3
<i>M. avium</i> + <i>M. kansasii</i>	Clinical strain	1
Total		147

Korea). Fifteen mycobacterial reference strains, listed in Table 1, were included in this study. The reference strains were obtained from the Korean Institute of Tuberculosis (KIT), the Korean National Tuberculosis Association (KNTA), Seoul, Korea. Reference strains and clinical isolates were subcultured in MGIT tubes (BD), and the MGIT tubes were read by using the BACTEC MGIT 960 system (BD). When the tubes give positive signals, an aliquot was withdrawn from the tubes for acid-fast bacilli (AFB) staining. The AFB-positive isolates were subjected to DNA extraction.

2. Multiplex PCR assay

Mycobacterial DNA was extracted from MGIT culture media by using the heat lysis method as described previously.¹⁷ The multiplex primers were designed according to the recommendations described previously.¹⁸ Table 2 lists the combinations of primer sets for the multiplex PCR assays. For all multiplex PCR and species-specific PCR, amplification was performed in a DNA thermal cycler (GeneAmp PCR 9600; Perkin-Elmer, USA). A 5 μ L aliquot of each DNA sample was used in a total

volume of 50 μ L PCR mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM dNTPs, optimal amounts of primers (12.5 ~25.0 pmol), and 1 U of DynaZyme DNA polymerase (Finzymes, Oy, Finland). The multiplex PCR conditions were optimized as follows: in the first cycle, denaturation at 95°C for 5 min; 35 cycles consisting of denaturation at 94°C for 15 sec, annealing at 65°C for 15 sec, and extension at 72°C for 30 sec; and in the final cycle, extension at 72°C for 10 min. Electrophoretic separation of each PCR product (10 μ L) was performed in 2% agarose gel slabs in Tris-borate buffer [0.5×TBE; 0.04 M Tris-borate (pH 8.4), 1 mM EDTA] at 100 V for 30 min. The gels were stained in ethidium bromide solution (0.5 μ g/mL) and the products were visualized in a transilluminator under UV light. Reference strains were run as positive controls, and deionized water was used as a negative control. For comparison of product lengths, 1 μ g of 100 bp ladder (Bio-Rad, USA) was used as a molecular size marker.

Table 2. Oligonucleotide sequences used in this study

Multiplex PCR	Species	Target gene	Primer	Sequences (5' to 3')	Product size (bp)
A	<i>Mycobacterium</i> genus	16S rRNA	Forward	CACATGCAAGTCGAACGGAAGGC	565 ~ 590
		generic	Reverse	GCCCGTATCGCCCGCACGKCACA	
	<i>M. tuberculosis</i> complex	MPB70	Forward	GCCTCGAACAATCCGGAGTTGACAA	385
			Reverse	GAGGCATTAGCACGCTGTCAATC	
B	<i>M. avium</i>	DT6	Forward	GGGACCTAACGTAGGCATCCGTG	187
			Reverse	GGGTAGCGTTGAAGTACCAGGT	
	<i>M. intracellulare</i>	DT1	Forward	TGGCTGGCCATTACGAAGGAGT	648
			Reverse	CGGTCCGACAGGCCTTCCTCG	
	<i>M. kansasii</i>	p6123	Forward	GAACAGCACCTCGGCGAACTCG	347
			Reverse	GTGGGCTCGGATATGGCGGTCC	
	<i>M. fortuitum</i>	SOD	Forward	CCAAGCTCGATGAGGCGCGG	275
			Reverse	CCGATCGCCCAGGCTGTCTGT	
C	<i>Mycobacterium</i> genus	16S rRNA	Forward	CACATGCAAGTCGAACGGAAGGC	565 ~ 590
		generic	Reverse	GCCCGTATCGCCCGCACGKCACA	
	<i>M. chelonae</i> *	16S rRNA	Forward	ACCACACACTTCATGGTGAGTGG	441
		hypervariable	Reverse	GCCCGTATCGCCCGCACGCKCAC	
	<i>M. goodii</i>	16S-23S ITS	Forward	AAGACCGGGTGACGACAACAA	221
			Reverse	TGCGCCCTTAGACACTTACAAAC	
	<i>M. terrae</i>	16S rRNA	Forward	CACATGCAAGTCGAACGGAAGG	149
		hypervariable	Reverse	CCACCACAGAACATGCATCCCAT	

M., mycobacterium; SOD, superoxide dismutase; rRNA, ribosomal RNA; ITS, internal transcribed spacer.

**M. abscessus* can be also detected by PCR using the primers specific for *M. chelonae* but cannot be discerned from *M. chelonae*.

3. PCR restriction fragment length polymorphism analysis (PRA) of *hsp65*

PCR-restriction enzyme analysis of the *hsp65* gene (PRA-*hsp65*) was performed by the following modification of the original procedure as described previously.¹⁹ Briefly, an aliquot (5 μ L) of each template DNA was used in 50 μ L of the PCR mixture containing 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM MgCl₂, 0.1% Triton X-100, 0.2 mM dNTPs, 12.5 pmol of each primer TB11 (5'-ACCAACGATGGTGTGTCCAT-3') and TB12 (5'-CTTGTCGAACCGCATACCCT-3'), and 2 U of DynaZyme DNA polymerase (Finzymes). The amplification was performed as follows: in the first cycle, denaturation at 95°C for 5 min; 35 cycles consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 60 sec; and in the final cycle, extension at 72°C for 10 min. For *Bst*EII (Promega, USA) and *Hae*III (Promega) digestions, 10 μ L of each PCR product was added to a mixture containing 1 μ L (20 U) of each enzyme, 3 μ L of restriction buffer, and 16 μ L of deionized water. The mixture was incubated for 60 min at 60°C for *Bst*EII digestion and 37°C for *Hae*III digestion. Electrophoretic separation of digested products was performed for 60 min at 100 V on 3% agarose gels, which were photographed under UV light after staining with ethidium bromide. Isolates were identified by using the PRA algorithm as described previously.²⁰

Results

In order to validate the performance of the multiplex PCR assays for the detection and identification of members of the genus *Mycobacterium* and the differentiation of mycobacteria to the species level, a 2-step approach was performed for the reference strains listed in Table 1. In the first step, to distinguish *M. tuberculosis* complex from NTM, sample DNA was amplified by multiplex PCR-A with the primer pairs (A) listed in

Fig. 1. Multiplex PCR assay for the differentiation of *M. tuberculosis* complex from nontuberculous mycobacteria (NTM) species and the identification of 8 NTM species. The multiplex PCR-A gives both common (565~590 bp) and specific (385 bp) bands from *M. tuberculosis* complex, whereas it produces only a common band from each NTM species. The multiplex PCR-B gives each specific product from *M. avium* (187 bp), *M. intracellulare* (648 bp), *M. kansasii* (347 bp), and *M. fortuitum* (275 bp), respectively. The multiplex PCR-C gives common amplicon (565~590 bp) from all mycobacteria and each specific amplicon from *M. abscessus*/*M. chelonae* complex (441 bp), *M. gordonae* (221 bp), and *M. terrae* (149 bp), respectively. Lanes A, B, and C mean multiplex PCR-A, multiplex PCR-B, and multiplex PCR-C, respectively. Lane M, 100-bp ladder DNA size marker; lanes 1~3, *M. tuberculosis* H37Rv ATCC 27294; lanes 4~6, *M. avium* ATCC 25291; lanes 7~9, *M. intracellulare* ATCC 13950; lanes 10~12, *M. fortuitum* ATCC 6841; lanes 13~15, *M. kansasii* ATCC 12478; lanes 16~18, *M. gordonae* ATCC 14470; lanes 19~21, *M. chelonae* ATCC 35749; lanes 22~24, *M. abscessus* clinical strain; lanes 25~27, *M. terrae* ATCC 15755.

Table 2. In the second step, to differentiate NTM to the species level, DNA extracts were amplified by multiplex PCR-B and PCR-C in 2 parallel reactions by using each set of primer pairs (B and C) listed in Table 2. As shown in Fig. 1, multiplex PCR-A amplified 2 products of ~590 bp and 385 bp from *M. tuberculosis* ATCC 27294, whereas it amplified only a single ~590 bp product from NTM species. The multiplex PCR-B produced 648 bp, 347 bp, 275 bp, and 187 bp bands

Table 3. Summarized results of the multiplex PCR, species-specific PCR and PRA *hsp65* for identification of mycobacteria growing from a total of 147 liquid cultures

Mycobacterial species	No. of cultures	No. of results identified by										Species-specific PCR	PRA <i>hsp65</i>
		Type of multiplex PCR and each amplicon size (bp)											
		A		B				C					
		590	385	648	347	275	187	590	441	221	149		
MTBC	38	38	38	-	-	-	-	38	-	-	-	38	38
<i>M. avium</i>	37	37	-	-	-	-	37	37	-	-	-	37	37
<i>M. intracellulare</i>	29	29	-	21	-	-	-	29	-	-	-	21	29
<i>M. kansasii</i>	3	3	-	-	3	-	-	3	-	-	-	3	3
<i>M. fortuitum</i>	5	5	-	-	-	5	-	5	-	-	-	5	5
<i>M. chelonae</i>	4	4	-	-	-	-	-	4	4	-	-	4	4
<i>M. gordonae</i>	19	19	-	-	-	-	-	19	-	19	-	19	19
<i>M. terrae</i>	2	2	-	-	-	-	-	2	-	-	2	2	2
<i>M. marinum</i>	3	3	-	-	-	-	-	3	-	-	-	-	3
<i>M. scrofulaceum</i>	1	1	-	-	-	-	-	1	-	-	-	-	1
<i>M. smegmatis</i>	1	1	-	-	-	-	-	1	-	-	-	-	1
<i>M. szulgai</i>	1	1	-	-	-	-	-	1	-	-	-	-	1
MTBC+ <i>M. avium</i>	3	3	3	-	-	-	3	3	-	-	-	3	-
<i>M. avium</i> + <i>M. fortuitum</i>	1	1	-	-	-	1	1	3	-	-	-	1	-
Total	147	147	41	21	3	6	41	147	4	19	2	133	143

MTBC, *M. tuberculosis* complex; PRA, PCR-restriction enzyme analysis.

from *M. intracellulare*, *M. kansasii*, *M. fortuitum*, and *M. avium* reference strains, respectively. The multiplex PCR-C was able to identify reference strains as *M. chelonae*/*M. abscessus*, *M. gordonae*, or *M. terrae* according to their product sizes of ~590 bp and 441 bp, ~590 bp and 221 bp, or ~590 bp and 149 bp, respectively (Fig. 1).

We tested the multiplex PCR assay on 147 liquid cultures of mycobacteria, which included 15 reference mycobacterial species. To determine and verify a specific gene amplicon by the multiplex PCR assays, each species-specific PCR and PRA-*hsp65* were performed by using the same reaction conditions as for the multiplex PCR. The results obtained from the clinical isolates and reference strains by using the multiplex PCR, species-specific PCR, and PRA-*hsp65* are shown in Table 3. All mycobacteria subjected to the multiplex PCR exhibited a positive Mycobacterium genus amplicon of ~590 bp. Of the 147 cultures, 143 yielded 1 isolate, whereas 4 grew 2 mycobacterial species. In 129 of the 143 culture tubes yielding a single isolate, the multiplex PCR results were equivalent as determined by single PCR analysis and PRA-*hsp65* except for 8 *M. intracellulare* isolates.

Fig. 2. Multiplex PCR assay for the detection of the coexistence of 2 mycobacterial species growing in mixed cultures. In the mixed culture of *M. tuberculosis* complex and *M. avium*, the multiplex PCR-A gives both common (565~590 bp) and specific (385 bp) bands from *M. tuberculosis* complex, the multiplex PCR-B gives a specific product from *M. avium* (187 bp), and the multiplex PCR-C gives only the common band. In the mixed culture of *M. avium* and *M. fortuitum*, the multiplex PCR-A and C gives only the common band (565~590 bp) from the mixed culture, the multiplex PCR-B gives 2 separate products from *M. avium* (187 bp) and *M. fortuitum*. Lanes A, B, and C mean multiplex PCR-A, multiplex PCR-B, and multiplex PCR-C, respectively. Lane M, 100-bp ladder DNA size marker; lanes 1~3, mixed culture of *M. tuberculosis* complex and *M. avium*; lanes 4~6, mixed culture of *M. avium* and *M. fortuitum*.

These isolates were not amplified by multiplex PCR nor by species-specific PCR with DT1 primers for *M. intracellulare*. For the other 6 cultures yielding no

amplicon by the multiplex PCR, PRA-*hsp65* identified the following species not represented by the multiplex PCR primer sets: *M. marinum* ATCC 927, 2 *M. marinum* clinical strains, *M. scrofulaceum* ATCC 19981, *M. smegmatis* ATCC 19420, and *M. szulgai* ATCC 35799. The multiplex PCR revealed 2 mycobacterial species in the remaining 4 mixed cultures (Table 3). The mixed cultures from patients who reported a history of repetitively treated pulmonary tuberculosis grew *M. tuberculosis* complex plus *M. avium* (n=3) or *M. avium* plus *M. fortuitum* (n=1) (Fig. 2). However, these cultures were unidentified or misidentified as a single species by PRA-*hsp65*.

Discussion

This study shows that the advantages of multiplex PCR are considerable, as compared with other recent and innovative molecular procedures, such as techniques for analyzing PCR products by sequencing analysis,^{8,21} restriction enzyme analysis,^{19,20,22,23} or hybridization assays.^{8,24,25} Among these methods, PRA-*hsp65* as proposed by Telenti et al.¹⁹ is the best approach for differentiating a wide variety of mycobacterial species, but it is time-consuming and it is difficult to read the results visually. In addition, PRA-*hsp65* is not very sensitive and it cannot detect the coexistence of different mycobacterial species in liquid cultures. Among 132 clinical isolates in this study, 6 were not identified by PRA-*hsp65* because of mixed cultures of 2 different mycobacterial species and novel PRA patterns not present in the reference algorithm.

In contrast, the multiplex PCR assays of this study and the study of Klemen et al.¹⁵ could differentiate these different isolates even when 2 or 3 mycobacterial species were present in a sample. Moreover, the method can be extended easily. As soon as specific DNA sequences are identified in other species of mycobacteria, a protocol can be formulated and another primer set for a multiplex PCR can be added. The insertion sequences

IS1395 and IS2404/2406 were found in *M. xenopi* and *M. ulcerans*, respectively.^{16,26,27} These may be candidates for targets in a multiplex PCR because the entire procedure, including sample preparation, can be completed within one working day. It is also more rapid and easy to interpret the results than PRA-*hsp65*.

However, there are 2 limitations of the multiplex PCR-B. First, there is no primer for the 16S rRNA gene as a common mycobacterial positive control primer in the primer mixture of multiplex PCR-B, whereas such a primer pair for the 16S rRNA gene is included in the primer mixture of multiplex PCR-A and PCR-C. To overcome this limitation, the multiplex PCR-B should be performed in parallel with multiplex PCR-C. Second, the multiplex PCR-B assay has a low sensitivity for detection of *M. intracellulare*. The data for *M. intracellulare* showed that the multiplex PCR-B detected only 21 of 29 cultures (72.4%), indicating that the failure was due to the absence of the target gene DT1 in some clinical isolates of *M. intracellulare* or PCR inefficiency caused by a relatively large amplicon size. We performed species-specific PCR using DT1 primers for those 8 *M. intracellulare* isolates. The species-specific PCR also failed, revealing that there was no DT1 gene in those isolates. A study in Guinea-Bissau, West Africa, reported that none of 5 isolates identified as *M. intracellulare* by 16S rRNA sequencing was DT1-positive.²⁸ Our study as well as that of Koivular et al.²⁸ contradict the earlier reports^{6,11} on the usefulness of DT1 PCR as an identification method for *M. intracellulare*. Although the multiplex PCR-B using DT1 primers was unable to detect some *M. intracellulare* isolates in cultures, this problem was solved by another approach such as PRA-*hsp65* because these isolates were checked by the multiplex PCR-C and underwent further evaluation according to the algorithm shown in Fig. 3.

To identify mycobacterial species growing from liquid media by use of these multiplex PCR assays, we suggest the detection algorithm shown in Fig. 3. The first step is to culture the mycobacteria with the BACTEC MGIT 960 system. If the cultures are positive in the

Fig. 3. Algorithm for the rapid identification of common clinical mycobacterial isolates growing in positive liquid cultures to the species level by use of multiplex PCR assays. Abbreviations: AFB, acid-fast bacilli; MTBC, *M. tuberculosis* complex; MAV, *M. avium*; MIN, *M. intracellulare*; MKA, *M. kansasii*; MFO, *M. fortuitum*; MGO, *M. gordonae*; MCH, *M. chelonae*; MTE, *M. terrae*; NTM, nontuberculous mycobacteria.

system, proceed to the next step. The next step is to perform AFB staining. If AFB is present, proceed to multiplex PCR-A. If AFB are absent and the samples are contaminated with other bacteria, discard the samples. The third step is to perform the multiplex PCR-A for differentiation of *M. tuberculosis* complex from NTM. If both 385 bp and ~590 bp (common) bands or only 385 bp bands are present, the results are *M. tuberculosis* complex. If only the common band is present, proceed to the multiplex PCR-B and PCR-C. The fourth step is to perform the multiplex PCR-B and PCR-C. If each specific band is present or if both specific and common bands are present, the results are *M. avium*, *M. intracellulare*, *M. kansasii*, *M. fortuitum*, *M. gordonae*, or *M. terrae* except *M. chelonae*. If only the common band is present, the result is other NTM and proceed to further evaluation. If the *M. chelonae* band is present, proceed to further evaluation. The multiplex PCR-C is unable to discern between these 2 species, because *M. chelonae* shares the primer sequence of 16S rRNA hypervariable region with *M. abscessus* for the multiplex PCR-C assay (Fig. 1).

In conclusion, the multiplex PCR assays in the

present study may be an easy and reliable method for the rapid detection and differentiation of commonly encountered mycobacterial species in liquid cultures, even in those in which different species coexist.

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