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Genetic Relationships of Langur Species Using AFLP Markers

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Abstract: Cytogenetic studies of five langur species using conventional banding pattern were investigated. All species studied have an identical number of 44 diploid chromosomes, they are assumed to have common evolutionary relationships. For in depth study, molecular markers were assessed using the Amplified Fragment Length Polymorphism (AFLP) method. With seven successful primer combinations, a total of 1043 scorable bands were generated. The percentage of polymorphic bands for each primer ranged from 48.60 to 94.12%. The resulting bands were used for dendrogram construction. From the dendrogram, the individuals of *Trachypithecus* species are grouped into two major clusters, *T. phayrei* is clustered with *T. obscurus*, while *T. cristatus* is clustered with *T. francoisi*. The bootstrap value between two groups is 94%. The other cluster, *Presbytis femoralis* is separated from the *Trachypithecus* species with a bootstrap value of 94%. Averages of inter-specific genetic similarity values among all langur species studied are 70.16% (between *T. obscurus* and *P. femoralis*) to 88.12% (between *T. phayrei* and *T. obscurus*). In summary *T. phayrei* might be a subspecies of *T. obscurus*. The development of specific molecular markers of a species is beneficial for genetic differentiation of this group of primates.

Key words: AFLP, genetic relationships, *Presbytis, Trachypithecus*

INTRODUCTION

The diversity of living colobines is portrayed by the recognition in most classifications of some 30 species. They have been grouped into 4 or 9 genera and these genera are often arranged in two clusters, one African and one Asian. The phylogenetic separation of the Asian colobines is less clearly united than the African species. The Asian colobines, commonly referred to as langurs, are comprised of 6 genera (*Semnopithecus*, *Trachypithecus*, *Presbytis*, *Pygathrix*, *Nasalis* and *Simias*) and about 31 species (Oates *et al*., 1994). In Thailand, 2 genera and 4 species are recognized, *Presbytis femoralis*, *Trachypithecus cristatus*, *T. phayrei* and *T. obscurus*.

There is still very little scientific data available on the distribution and status of primates in Thailand. In recent years, a number of surveys has been conducted and our knowledge has increased considerably. However, at this time no comprehensive work has been published which compiles all of the data on this subject from the whole country. This lack of knowledge about the status and distribution of leaf monkeys in Thailand poses a serious problem in terms of how to conduct a long-term conservation program.

The genetic relationships among different colobine species are still controversially discussed with unavailable material. The currently accepted classification of langurs is mainly based on morphological studies, while their cytogenetics (Tanomtong *et al*., 2006) and molecular genetics have become important tools (Nadler *et al*., 2003). As part of a study on the complete genetic relationships of Colobinae, the Thai langurs play a key role, because of the high number of endemic species and the low amount of currently available data.

The Amplified Fragment Length Polymorphism (AFLP) technique is one DNA fingerprinting procedure which uses PCR to amplify a limited set of DNA fragments from a specific DNA sample. Since AFLP analysis does not require prior genetic information of the taxa studied, it should be of value in phylogenetic analysis of a wide variety of organisms (Vos *et al*., 1995). Furthermore, AFLP phenotypes are highly reproducible (Ovilo *et al*., 2000; Liu *et al*., 2005) and thus, reliable (Aggarwal *et al*., 1999; Sudupak *et al*., 2004). The AFLP markers have been widely applied in plant and animal studies and have the potential for analyzing genetic variation and phylogenetic relationships among and within closely related species (Aggarwal *et al*., 1999; Mace *et al*., 1999; Loh *et al*., 2000;

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Despres *et al*., 2002; Pelser *et al*., 2003; Banfer *et al*., 2004; Chen *et al*., 2004; Sudupak *et al*., 2004; Ude *et al*., 2003; Wang *et al*., 2004; Liu *et al*., 2005).

From the advantage of AFLP above, we investigate the genetic relationships of langur species in Thailand using the AFLP technique in order to clarify their taxonomic status.

MATERIALS AND METHODS

Sample collection: Blood samples from 5 dusky leaf monkeys (*T. obscurus* from Southern Thailand), 3 phayre's leaf monkeys (*T. phayrei* 1 from Northern Thailand, *T. phayrei* 2 from central Thailand and *T. phayrei* 3 from Northeastern Thailand), 3 silvered leaf monkeys (*T. cristatus* from Northeastern Thailand), a francois's langur (*T. froncoisi* from Nakhonrachasima zoo, Thailand, it is not common species in Thailand), a banded leaf monkey (*P. femoralis* from Northeastern Thailand) and a slow loris (*Nycticebus coucang*) as outgroup were collected. The collected locations are shown on a map of Thailand (Fig. 1). The samples were divided into two groups, the first for cytogenetic analysis and the second for molecular analysis.

Cytogenetic analysis: The cultured cells were examined by the colchicines-hypotonic fixation-air drying technique followed by a conventional technique (Tanomtong *et al*., 2005). Chromosomal checks were performed with 20 cells of each individual by light microscopy.

Molecular analysis

DNA isolation: Genomic DNA was extracted from blood samples following Sambrook and Russel (2001). The quality and quantity of extracted DNA was assessed by 0.8% agarose gel electrophoresis and spectrophotometry.

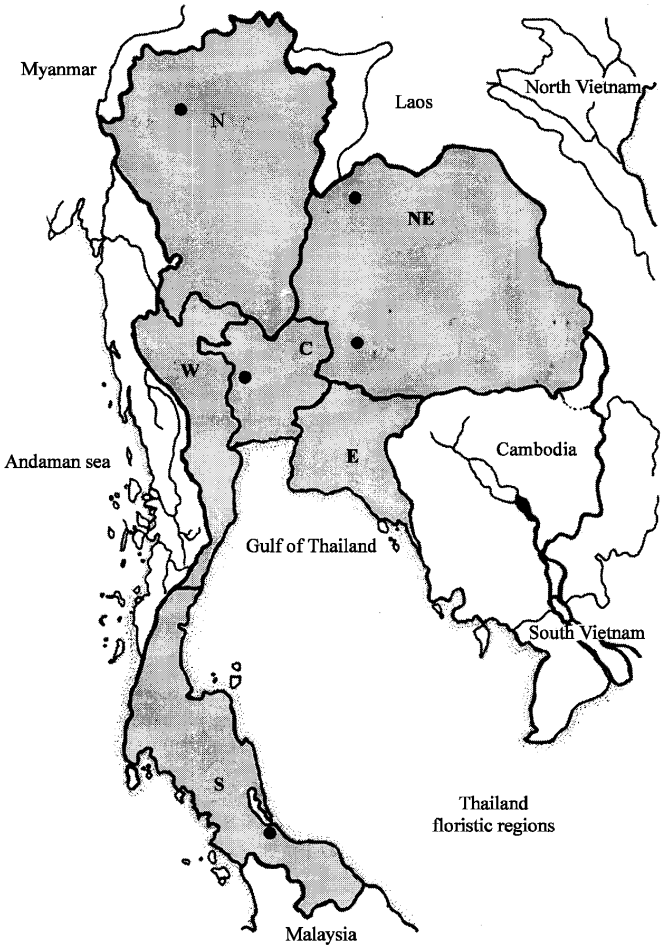

Fig. 1: Map of Thailand indicating sample collection of langurs (●), where N is Northern Thailand, NE is Northeastern Thailand, C is Central Thailand, W is Western Thailand, E is Eastern Thailand and S is Southern Thailand

AFLP reaction: The procedures of the AFLP method (Vos *et al.*, 1995) were performed according to the protocol of the Kit (AFLP^R Analysis System I, Invitrogen, USA). After adaptor ligation and preselective amplification, selective amplification was conducted with 28 primer combinations and seven primer combinations: E-ACC/M-CAG, E-AAC/M-CAT, E-AAC/M-CAG, E-AAG/M-CAT, E-AAG/M-CTT, E-AGG/M-CTT and E-AGC/M-CAT were successful. The PCR products amplified with different primer combinations were loaded onto 6.0% denaturing polyacrylamide gels and electrophoresed for 3 h and detected by Silver Quest^{TM} Silver Staining Kit (Invitrogen, USA).

Data analysis: Each AFLP band was considered as an independent character and the bands were scored visually as either absent (0) or present (1) for each band across all samples with the same primer pairs. Qualitative differences in band intensity were not considered. With the band data, a pair-wise genetic similarity matrix was generated between five langur species using Ochiai similarity coefficients which were then converted to a genetic distance matrix. Based on the genetic distance matrix, cluster analyses were performed and corresponding dendrograms were constructed for five langur species using the Single linkage Cluster method. Cophenetic correlations were computed from the clustering matrix in order to get the best fit dendrogram. All these analyses were done using the Fingerprinting II program (BioRad, USA).

RESULTS

Results from lymphocyte culture of whole blood and conventional staining of the five langur species, namely *Presbytis femoralis*, *Trachypithecus cristatus*, *T. froncoisi*, *T. phayrei* and *T. obscurus* indicate that all species have an identical number of 44 diploid chromosomes (2n) consisting of 42 autosomes and 2 sex chromosomes. A representative chromosome of the five langur species is from a female of *T. cristatus* chromosome as shown in Fig. 2.

AFLP fragments were established for three individuals of *T. phayrei*, five individuals of *T. obscurus*, three individuals of *T. cristatus*, an individual of *T. froncoisi* and an individual of *P. femolaris*. Seven primer combinations yielded a total of 1043 scorable bands and averaged 149 per primer. Of these bands, 61.74% were polymorphic. Percentages of Polymorphic Bands (PPB) for each primer ranged from 48.60% to 94.12%. Primer *Eco*RI-AAG/*Mse*I-CTT combinations produced the highest number of bands (179), while primer *Eco*RI-AGC/*Mse*I-CAT combinations produced a minimum number of bands (119) (Table 1). Figure 3 presents AFLP fingerprinting of five langur species with seven primer combinations.

A dendrogram was constructed based on the AFLP scoring data and using single linkage cluster ananlysis (Fig. 4). Within langur species, they are separated into two groups, *Presbytis* and *Trachypithecus*,

Table 1: A summary of AFLP primers, number of bands scored, number of polymorphic bands and percentage of polymorphisms for amplification profiles of five species of Thai langurs

AFLP primers	No. of band scored	No. of polymorphism bands	Percentage of polymorphism
*Eco*RI-ACC/*Mse*I-CAG	167	85	50.90
*Eco*RI-AAC/*Mse*I-CAT	178	97	54.49
*Eco*RI-AAC/*Mse*I-CAG	130	72	55.38
*Eco*RI-AAG/*Mse*I-CAT	129	84	65.12
*Eco*RI-AAG/*Mse*I-CTT	179	87	48.60
*Eco*RI-AGC/*Mse*I-CAT	119	112	94.12
*Eco*RI-AGG/*Mse*I-CTT	141	107	75.89
Total	1043	644	61.74

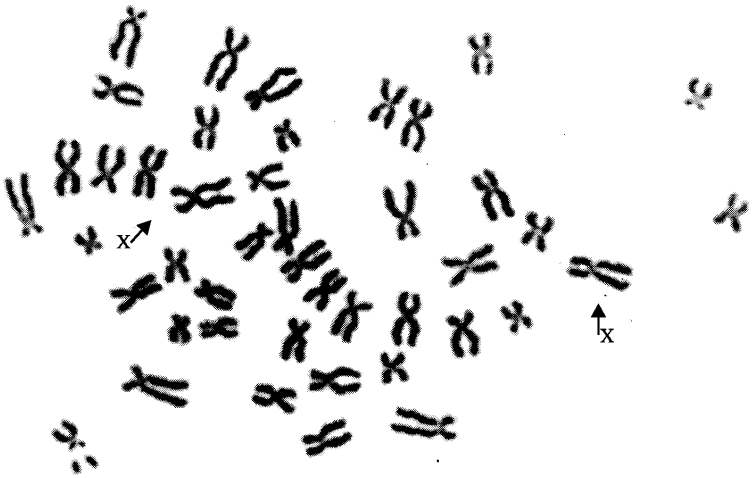

Fig. 2: Diploid chromosome number of female *T. cristatus*, 2n = 44 consisting of 42 autosomes and 2 sex

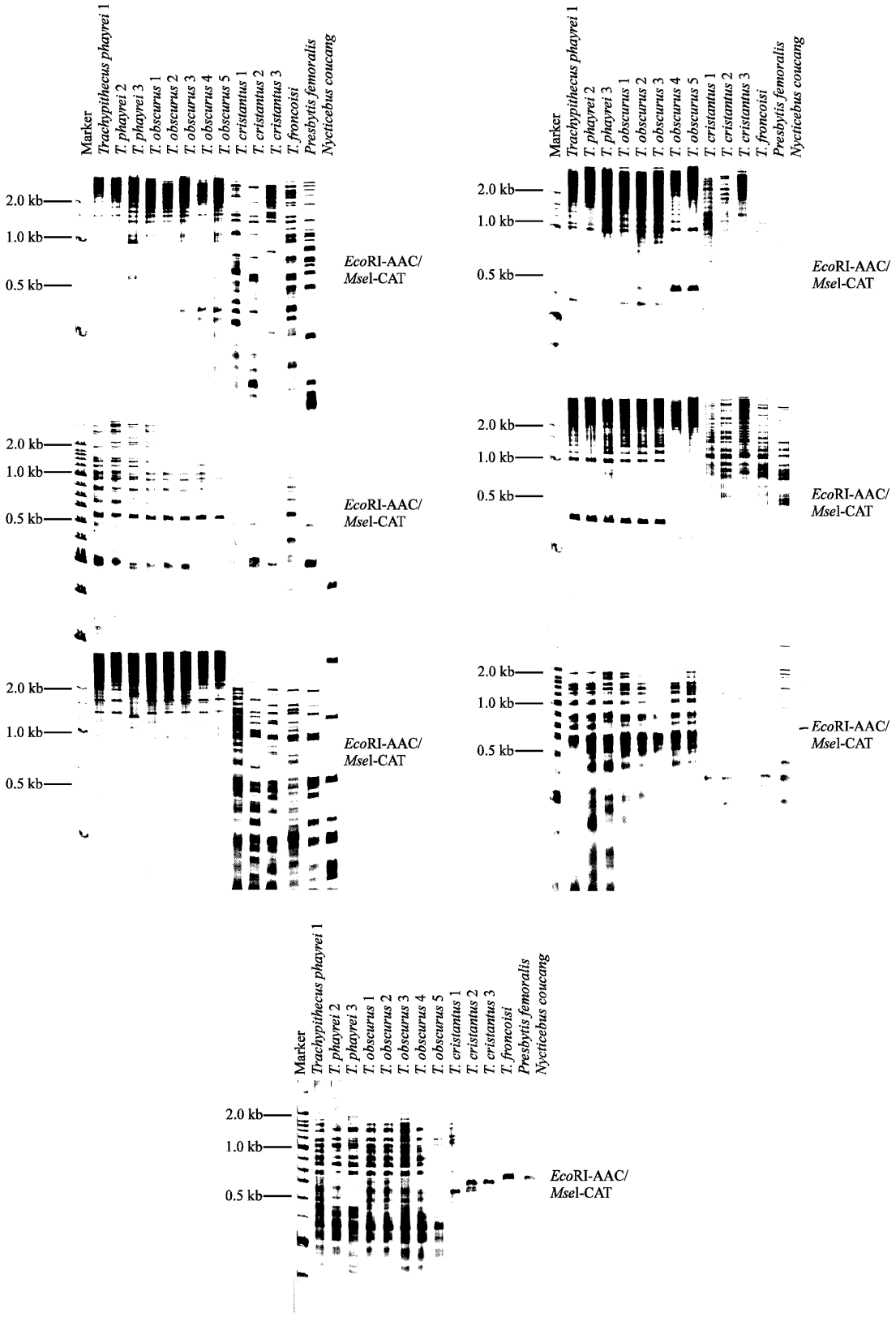

Fig. 3: Specific banding patterns of five langur species with the seven successful primer combinations

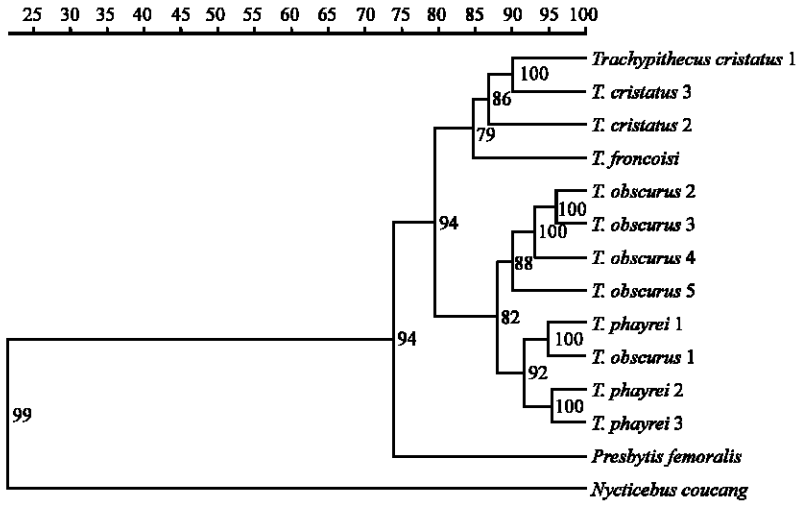

Fig. 4: Dendrogram depicting the seven AFLP primer combinations produced by single linkage cluster analysis that is used to clarify the genetic relationships of five langur species

Table 2: The average similarity index values based on the AFLP analysis of five langur species and *N. coucang*

Species	*T. cristatus*	*T. francoisi*	*T. obscurus*	*T. phayrei*	*P. femolaris*	*N. coucang*
Trachypithecus cristatus	87.50					
T. francoisi	84.3	100				
T. obscurus	79.55	76.78	91.29			
T. phayrei	80.22	76.2	88.12	92.20		
Presbytis femolaris	78.57	79.8	70.16	71.57	100	
Nycticebus coucang	23.03	21.60	22.04	22.20	27.40	100

whereas the outgroup *N. coucang* accession is segregated from the langurs with a high bootstrap value of 99%. The *Presbytis* group contains only species *P. femoralis* derived from *Trachypithecus* group with 94% bootstrap support. Within the *Trachypithecus* group, *T. phayrei* is clustered with *T. obscurus*, while *T. cristatus* is clustered with *T. francoisi* with 82% bootstrap support. Averages values of inter-specific genetic similarity (S) among all langur studied species are 70.16% (between *T. obscurus* and *P. femoralis*) to 88.12% (between *T. phayrei* and *T. obscurus*) (Table 2).

DISCUSSION

The identity of diploid chromosome number (2n = 44) of five langur species from cytogenetic studies agrees with Chiarelli (1963), Ponsa *et al.* (1983), Bigoni *et al.* (1997), Nie *et al.* (1998) and Wienberg (2005), reflecting the close genetic relationships among species of langurs. The chromosome number does not contain information sufficient for completely resolving the genetic relationships of langurs, so the AFLP molecular marker was used to elucidate. AFLP fingerprints were highly reproducible in samples studied. A high level of polymorphism was found among langur species. The *Presbytis* species formed a distinct cluster (Fig. 4) that only links with the *Trachypithecus* species at a level of 75.02% similarity. Within the genus *Trachypithecus*, *T. phayrei* is very closely related to *T. obscurus* based on the highest S value, 88.12%, while the lowest S value within species is 87.50% (in *T. cristatus*). Moreover, these two species are not a monophyletic group. The *T. phayrei* from Central, Northern and Northeastern Thailand could not be distinguished clearly and are not divided into a single cluster with high (82%) bootstrap support.

Based on the morphology, a more judicious solution is to divide the ring species into components reflecting their predominant pelage color: Silver (*T. villosus*), grey (*T. barbei*) and brown (*T. obscurus*). Brandon-Jones *et al.* (2004) therefore treats typical *T. phayrei* as a subspecies of *T. obscurus*, while Pocock (1935) treats it as a species. However, Pocock rightly acknowledged that it is very similar to typical *T. obscurus* from the southern part of the Malay Peninsula.

We agree with Brandon-Jones *et al.* (2004) viewpoint, i.e., the phayre's leaf monkey is not a valid separate species. It is better to regard *T. phayrei* as a subspecies of *T. obscurus*. In order to resolve the genetic relationships between *T. obscurus* and *T. phayrei*

properly, it is necessary to study a larger sample, as well as to consider comparative behavioral studies. Thus, these results suggest that genetic analysis based on AFLP fingerprinting has a good capacity for study of species diversity, especially in langurs. As AFLP analysis does not require prior genetic information of the taxa studied and AFLP fingerprints are reproducible, this technique should be of value in genetic analysis of a wide variety of primates. Moreover, the seven successful primer combinations can be sufficiently used to determine genetic differences of five langur species.

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