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Analysis of the Genetic Relationships among Thai Gibbon Species Using AFLP Markers

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Abstract: Cytogenetic studies of three gibbon species using conventional banding patterns were investigated showing 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 1669 scorable bands were generated. The resulting bands were used for dendrogram construction. From the dendrogram, the individuals of *Hylobates lar* are closely related to *H. agilis* more than *H. pileatus* with a bootstrap value of 78%. Averages of inter-specific genetic similarity values among all gibbon species studied are 67.15% (between *H. lar* and *H. pileatus*) to 71.03% (between *H. lar* and *H. agilis*). In summary three gibbon species show genetic stability within a species. The development of specific molecular markers of a species is beneficial for genetic differentiation of this group of primates.

Key words: AFLP, genetic relationships, gibbon, *Hylobates*

INTRODUCTION

The hylobatidae (gibbons) are a relatively small and morphologically homogeneous group of primate species living in closed canopy rain forest throughout Southeast Asia (Takacs *et al*., 2005). They consist of 12 species and are subdivided into 4 morphologically and karyologically distinct genera, namely *Hylobates* (diploid number (2n) = 44) containing *H. lar*, *H. pileatus*, *H. agilis*, *H. moloch*, *H. muelleri* and *H. klossii*, *Hoolock* (2n = 38) containing *H. hoolock*, *Nomascus* (2n = 52) containing *N. concolor*, *Nomascus* sp. cf. *nasutus*, *N. gabriellae* and *N. leucogenys* and *Symphalangus* (2n = 50) containing *S. syndactylus* (Geissmann, 2002). Three of these have been seen in Thailand including *H. lar*, *H. pileatus* and *H. agilis* that are only in the lar group.

Scientists have little understanding of the biogeographic history of gibbons, largely because of their sparse fossil record (Geissman, 2002; Takacs *et al*., 2005). A limited understanding of the environmental history of Thailand further compounds the problem. Researchers have proposed few scenarios to describe the radiation of gibbons. Moreover, habitat loss and fragmentation, habitat degradation, hunting (food, medicine and sport) and illegal trade (medicine, pets) are the top four which have seriously threatened genetic resources of gibbons throughout their ranges.

The genetic relationships among different gibbon species are still controversially discussed with limited material. The currently accepted classification of gibbons is mainly based on morphological studies (Geissmann, 2002), while their cytogenetics and molecular genetics have become important tools (Tanomtong *et al*., 2005; 2006; Chaveerach *et al*., 2006; Tanee *et al*., 2006). As part of a study on the complete genetic relationships of Hylobatidae, Thai gibbons play a key role, because they are endangered species and the small amount of 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

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

In this study we investigated the genetic relationships among *H. agilis*, *H. lar* and *H. pileatus* that are in the lar group using the AFLP technique in order to clarify the relationships among these three gibbon species.

MATERIALS AND METHODS

Sample collection: Blood samples from 2 black handed gibbons (*H. agilis* from Southern Thailand), 5 white handed gibbons (*H. lar* Northeastern Thailand) and 3 pileatus gibbons (*H. pileatus* from Northeastern Thailand) were collected (Fig. 1). The samples were divided into two groups, the first for cytogenetic analysis and the second for molecular analysis.

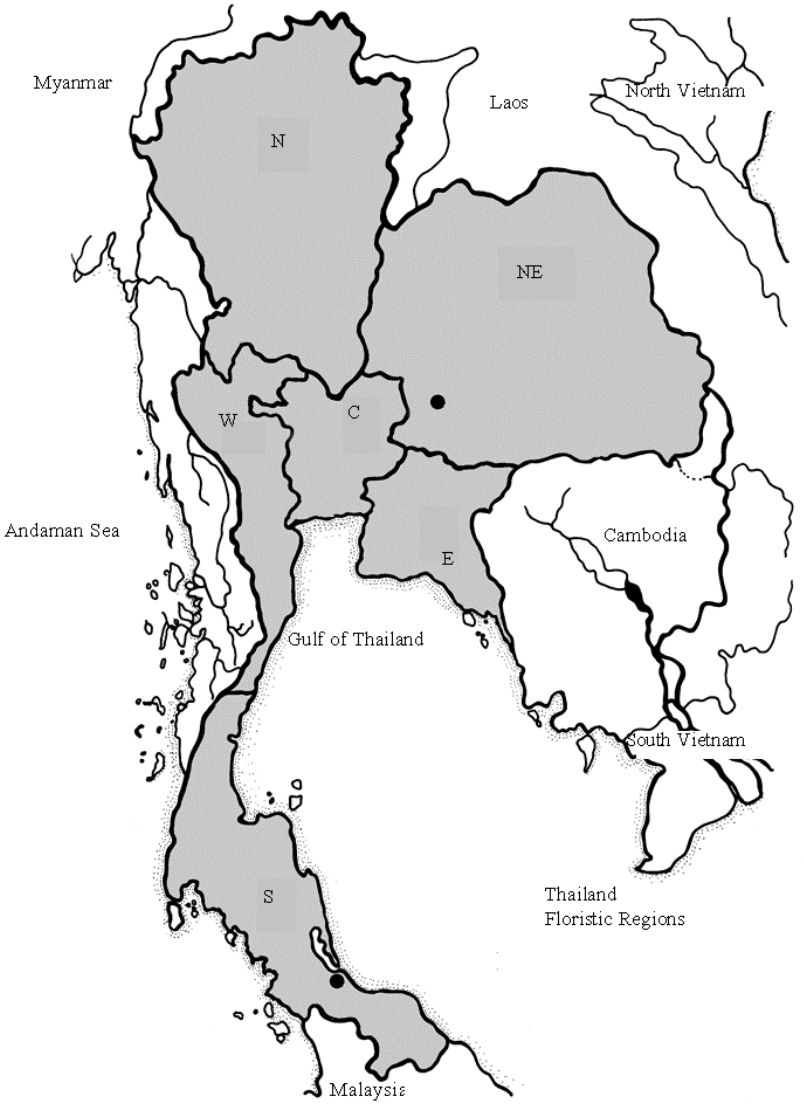

Fig. 1: Map of Thailand indicating sample collection of gibbons (●), where N is Northern Thailand, NE is Northeastern Thailand, C is Central Thailand, W is Western Thailand, E is Eastern Thailand and S is Southern Thailand

Cytogenetic analysis: Blood samples were subjected to cytogenetic studies by lymphocyte culture of whole blood. 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.

AFLP reaction: The procedures of 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 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. 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™ Silver Staining Kit (Invitrogen, USA).

Data analysis: Each AFLP band was considered as an independent character and the bands were scored visually as having each band either absent (0) and present (1) 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 among *Hylobates lar*, *H. agilis* and *H. pileatus* individuals using Ochiai similarity coefficients which were then converted to a genetic distance matrix. Based on the genetic distance matrix, cluster analyses were performed and the corresponding dendrograms were constructed for the three gibbon species using the Single Linkage Cluster method. Cophenetic correlations were computed from the clustering matrix in order to get the best fit dendrogram. All of these analyses were performed using the Fingerprinting II program (BioRad, USA).

RESULTS

Results from lymphocyte culture of whole blood and conventional staining of the three gibbons species, namely *Hylobates lar*, *H. agilis* and *H. pileatus* 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 three gibbon species is from a male of *H. pileatus* as shown in Fig. 2.

AFLP fragments were established for five individuals of *H. lar*, two individuals of *H. agilis* and three individuals of *H. pileatus*. Seven primer combinations generated a total of 1669 scorable bands, averaging at 238 bands per primer. Of these bands, 32.59% (544 bands) were polymorphic. Figure 3 presents AFLP fingerprinting of three gibbons with seven primer combinations.

Fig. 2: Diploid chromosome number of male *H. pileatus*, 2n = 44 consisting of 42 autosomes and 2 sex chromosomes

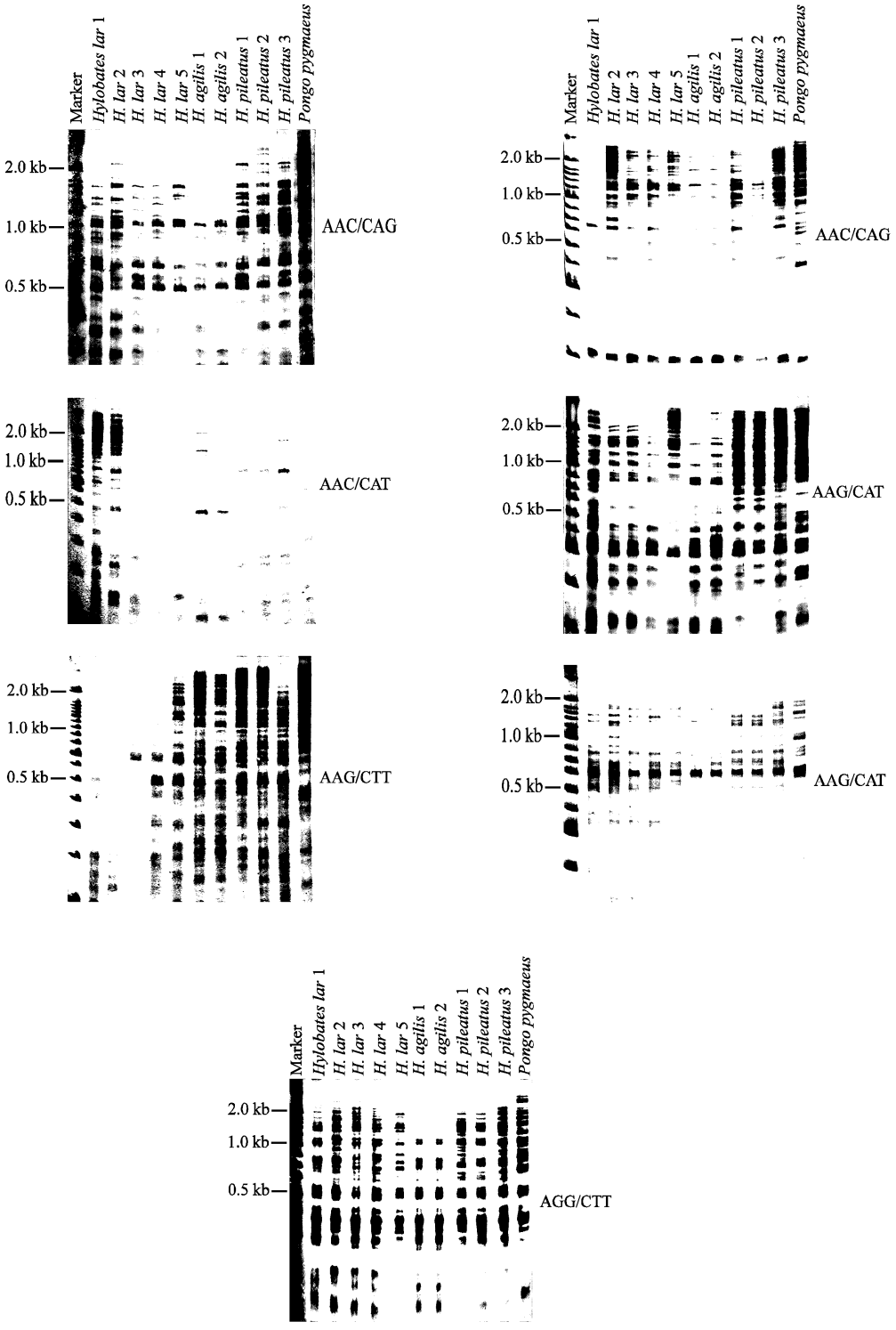

Fig. 3: Specific banding pattern of three gibbon species with the seven successful primer combinations

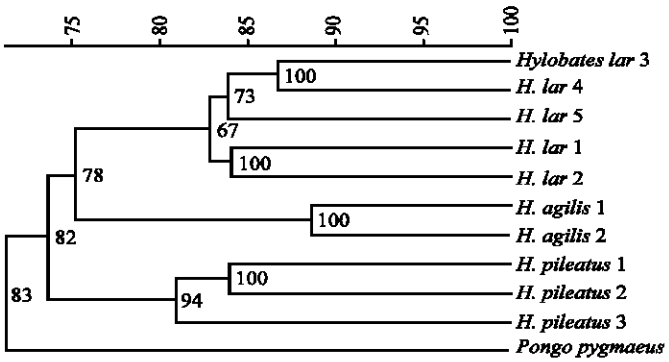

Fig. 4: Dendrogram depicting the seven AFLP primer combinations produced by single linkage cluster analysis that are used to clarify the genetic relationships among the three gibbon species

Table 1: Average similarity index values based on AFLP analysis of Hylobatid species and *P. pygmaeus*

Specics	*H. lar*	*H. agilis*	*H. pileatus*	*Pongopygmæus*
Hilobates lar	78.61			
H. agilis	71.03	88.70		
H. pileatus	67.15	69.97	81.43	
P. pygmæus	62.36	64.00	69.30	100.00

A dendrogram was constructed based on the AFLP scoring data and using single linkage cluster analysis. The three hylobatid species are clustered together, whereas *Pongo pygmaeus* is separated as an outgroup with a bootstrap value of 83%. Within the hylobatid cluster, *H. lar* is more closely related to *H. agilis* than *H. pileatus* (Fig. 4). Average values of inter-specific similarity index (S) among all hylobatid studied species are 67.15% (between *H. lar* and *H. pileatus*) to 71.03% (between *H. lar* and *H. agilis*) (Table 1).

DISCUSSION

The identity of diploid chromosome number (2n = 44) of three gibbon species agrees with Ankel-Simons (2000) and Geissmann (2002), reflecting the close genetic relationships among species of gibbons. Moreover, Ankel-Simons (2000) suggested that chromosome polymorphism and inter-specific chromosomal variability are common among prosimian primates. Primate cytogenetics can still not reconstruct reliably the relatedness of gibbon species. The AFLP molecular marker was thus used to elucidate their relatedness. The advantage of AFLP fingerprints is that they are highly reproducible in the samples studied.

The sample studied contained a small number of some species such as *H. agilis* because blood samples are difficult to collect from primates in the forest. However, at least two samples could be used for preparing DNA fingerprints. Within the *Hylobates* species (Fig. 4), *H. lar* is very closely related to *H. agilis* more than *H. pileatus* with high bootstrap value (78%) and high average S-value (71.03%). This result is supported by other work using DNA sequences (Garza and Woodruff, 1992; Hayashi *et al*., 1995; Takacs *et al*., 2005), morphology (Kimble and Martin, 1993) and by vocal data (Geissmann, 2002).

Based on the geography of the Khao Yai National Park, Thailand, two members of the lar group, *H. lar* and *H. pileatus*, meet and occasionally hybridize. In the Khao Yai overlap zone, pure *H. lar* can be found 5 km into the *pileatus* side of the zone and pure *H. pileatus* go 9-12 km into the *lar* side. The change of the morphological index from 90% *lar* to 90% *pileatus* occurs over about 9 km (Kimbel and Matin, 1993). However, the genetic relationships between *H. lar* and *H. pileatus* is low with a 67.15% S value, while the S value between *H. agilis* and *H. pileatus* is 69.97%. Therefore, we suggest that although *H. lar* and *H. pileatus* have overlapping habitation zones and habitat has been lost, they still show genetic stability within a species. In order to resolve the genetic relationships within the *lar* group properly, it is necessary to study a larger sample and cover distributed areas, 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 gibbons. As AFLP analysis does not require prior genetic information of the taxa studied and AFLP fingerprints are reproducible, the technique should be of value in genetic analysis of a wide variety of primates (Chaveerach *et al*., 2006; Tanee *et al*., 2006). Moreover, the seven successful primer combinations can be used to sufficiently determine genetic differences among gibbon species.

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