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Taxonomic Status of the White-head Langur (*Trachypithecus francoisi leucoscephalus*) Inferred from Allozyme Electrophoresis and Random Amplified Polymorphism DNA (RAPD)

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Bo Ding, Hai-peng Li, Ya-ping Zhang, Zimin Liu and Yi Wei (2000) Taxonomic status of the white-head langur (*Trachypithecus francoisi leucocephalus*) inferred from allozyme electrophoresis and random amplified polymorphism DNA (RAPD). *Zoological Studies* **39**(4): 313-318. Six sample specimens of *Trachypithecus francoisi* and 3 of *T. leucocephalus* were analyzed by use of allozyme electrophoresis and random amplified polymorphism DNA (RAPD) in order to clarify the challenged taxonomic status of the white-head langur. Among the 44 loci surveyed, only 1 locus (PGM-2) was found to be polymorphic. Nei's genetic distance was 0.0025. In total, thirty 10-mer arbitrary primers were used for RAPD analysis, of which 22 generated clear bands. Phylogenetic trees were constructed based on genetic distances using neighbor-joining and UPGMA methods. The results show that *T. francoisi* and *T. leucocephalus* are not monophyletic. *T. francoisi* from Guangxi, China and Vietnam could not be clearly distinguished, and they are not divided into 2 clusters. A *t*-test was performed to evaluate between genetic distances within and between *T. leucocephalus* and *T. francoisi* taxa groups. The statistical test shows that the taxa group within *T. leucocephalus* and *T. francoisi* does not significantly differ from that between *T. leucocephalus* and *T. francoisi* at the 5% level. Our results suggest that the level of genetic differentiation between *T. leucocephalus* and *T. francoisi* is relatively low. Recent gene flow might exist between *T. francoisi* and *T. leucocephalus*. Combining morphological features, geographical distribution, allozyme data, RAPD data, and mtDNA sequences, we suggest that the white-head langur might be a subspecies of *T. francoisi*.

Key words: *Trachypithecus*, White-head langur, Genetic differentiation, Classification.

The black leaf monkey or langur (*Trachypithecus francoisi*) is distributed from S. China to N. Vietnam and Laos, and east of the Mekong R. (Wolfheim 1983, Ma et al. 1989, Corbet and Hill 1992). It was first described by Pousargues in 1898 from specimens collected in Longzhou, southern Guangxi Province, China.

The white-head leaf monkey (*Trachypithecus leucocephalus*) was discovered in 1952 in southwestern Guangxi, China. Its habitat is restricted to only 4 counties, i.e., Changzuo, Longzhou, Ningming, and Fusui, in southern Guangxi. The distribution area is about 1600 km^2 and the habitat area is about 200 km^2. Its population size is about 400~1400 (Wang and Jiang 1995, Liu and Wei 1995). The white-head langur is distributed in a narrow triangular area along the southern Zuo River, the eastern Ming River, and along northwestern Shiwanda Mt. The white-head langur and the black langur seem to have their own habitats, and they are separated by the Zuo and Ming Rivers and the Shiwanda Mt. range (Jiang et al. 1991). Tan (1957) named it as a new species, *T. leucocephalus*. Li and Ma (1980) revised Tan's new species to a subspecies of *T. francoisi*, and referred to it as *T. f. leucocephalus*. Brandon-Jones (1984) stated that *T. leucocephalus* of southwestern Guangxi was a species distinct from *T. francoisi*. But Eudey (1987) did not accept Brandon-Jones' view.

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Burton et al. (1995) agreed with the viewpoint of Li and Ma (1980).

All of the viewpoints mentioned above are mainly based on morphological characters and geographical distribution. New genetic evidence from protein and molecular information might be useful in further elucidating the phylogeny and taxonomic status of the white-head langur.

Allozyme electrophoresis is a simple but useful approach for population genetic analysis. Random amplified polymorphic DNA (RAPD) has the potential for analyzing phylogenetic relationships among and within closely related species (Williams et al. 1990, Welsh et al. 1991).

Herein we investigate the genetic differences between *T. francoisi* and *T. f. leucocephalus* by using allozyme and RAPD techniques in order to clarify the taxonomic status of the white-head langur.

MATERIALS AND METHODS

Sample sources and DNA extraction

Blood samples from 3 white-head langurs (*T. leucocephalus* 1, 2, and 3, from Guangxi, China), and liver tissue samples from 6 black langurs (*T. francoisi* 1, 2, and 3, from Guangxi, China; *T. francoisi* 4, 5, and 6 from Vietnam) and 1 Phayre's langur (*T. phayrei*, from Yunnan, China) were collected.

High-molecular weight DNA was prepared by using the methods modified from Zhang and Ryder (1993). Briefly, DNA was extracted from lymphocytes or by adding DNA extraction buffer (10 mM Tris-HCl, 10 mM EDTA, 10 mM NaCl, 1.0% SDS, 0.2 mg/ml Proteinase K, pH 8.0), and was extracted with an equal volume of phenol for 12 h, phenol-chloroform (1:1) for 1 h, and chloroform for 15 min. The DNA was precipitated from the aqueous phase by adding 1 volume of isopropanol. The fibrous DNA precipitate was rinsed in 1 ml of 70% ethanol and dried. The DNA was dissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and diluted to about 10 ng/ul.

Allozyme electrophoresis

Samples were analyzed by horizontal starch electrophoresis. The starch concentration was 12%, and 7 buffer systems for different proteins and isozymes were used: (I) Tris-citrate, pH 7.0; (II) Tris-borate-EDTA, pH 8.6; (III) Tris-borate-Na_2EDTA, pH 8.0; (IV) Tris-borate-citrate-LiOH, pH 8.0; (V) borate-NaOH, pH 8.6; (VI) Tris-borate-citrate-NaOH, pH 7.8; and (VII) Tris-citrate, pH 8.7. Our histochemical staining methods followed Pasture (1988). Forty-two blood proteins and isozymes encoded by 44 genetic loci were examined. They are acid phosphatase (ACP), adenosine deaminase (ADA), alcohol dehydrogenase (ADH), adenylate kinase (AK), albumin (ALB), alkaline phosphatase (ALP), carbonate dehydrogenase (CAR), catalase (CAT), ceruloplasmin (CP), diaphorase (DIA), esterase-1,2,3,D (ES-1,2,3, D), fructokinase (FK), fumarase (FUM), glyceraldehydephosphate dehydrogenase (GAPD), glucose-6-phosphate dehydrogenase (G6PD), glutamate oxaloacetate transaminase (GOT), 3-hydroxybutyrate dehydrogenase (HBDH), hemoglobin-α,β (HB-α,β), haptoglobin (HAP), hexokinase (HK), isocitrate dehydrogenase (IDH), leucine aminopeptidase (LAP), lactate dehydrogenase-A,B (LDH-A, B), malate dehydrogenase-1,2 (MDH-1,2), mannosephosphate isomerase (MPI), purinenucleoside phosphorylase (NP), peptidase-B (PEP-B), peroxidase (PER), 6-phosphogluconate dehydrogenase (6PGD), phosphoglucomutase-1,2 (PGM-1,2), phosphohexose isomerase (PHI), pyruvate kinase (PK), protein-α_2 (P-α_2), sorbitol dehydrogenase (SDH), transferrin (TF), tetrazolium oxidase (TO), and xanthine dehydeogenase (XDH).

Nei's genetic distances (*D* values) were estimated by the methods of Pasteur et al. (1988).

RAPD analysis

1) PCR reaction: *Taq*-DNA polymerase was from the Sino-American Biotechnology, China. Oligonucleotide 10-mer primers were from Operon Technologies, USA (Table 2). PCR was performed with a volume of 10 ul consisting of 10 mM Tris-HCl, pH 8.9, 50 mM MgCl, 100 uM of dNTPs, 0.2 uM of primer, 10 ng of genomic DNA, and 0.5 units of *Taq*-DNA polymerase, using a Satratagene amplifier. PCR consisted of 40 cycles performed as follows: denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min, and extension at 72 °C for 2 min. The first cycle included a 3-min pre-denaturation, and the final cycle included an additional 5-min extension. We followed the procedures of Chen and Zhang (1997) to ensure the reproducibility of PCR.

2) Genetic distance (*D*) of pairwise comparisons between individuals was calculated based on the proportion of shared bands: $D = 1 - F$, $F = 2N_{xy}/(N_x + N_y)$, where N_x is the number of bands in individual *x*; N_y is the number of bands in individual *y*; and N_{xy} is the number of bands shared by individuals *x* and *y*. The index *F*, expressed as a percentage, ranges

Table 1. Genetic distances (*D*) matrix of *T. phayrei*, *T. leucocephalus*, and *T. francoisi* based on RAPD data of 22 primers

	T.p.	*T.l.*1	*T.l.*2	*T.l.*3	*T.f.*1	*T.f.*2	*T.f.*3	*T.f.*4	*T.f.*5	*T.f.*6
T.p	–									
*T.l.*1	0.359	–								
*T.l.*2	0.430	0.194	–							
*T.l.*3	0.465	0.237	0.285	–						
*T.f.*1	0.480	0.323	0.259	0.274	–					
*T.f.*2	0.392	0.228	0.271	0.269	0.277	–				
*T.f.*3	0.403	0.220	0.312	0.262	0.270	0.162	–			
*T.f.*4	0.449	0.215	0.294	0.242	0.193	0.262	0.175	–		
*T.f.*5	0.521	0.407	0.333	0.364	0.283	0.327	0.303	0.262	–	
*T.f.*6	0.473	0.228	0.333	0.224	0.294	0.286	0.250	0.308	0.416	–

from 0 (no shared bands) to 100% (identical bands in *x* and *y*).

3) Phylogenetic estimation: Neighbor-joining (N-J) and UPGMA (unweighted pair-group method with arithmetic average) analyses were performed using Neighbor program in Phylip software package vers. 3.5c (Saitou 1987, Felsenstein 1993) based on a distance matrix.

4) The degree of polymorphism was quantified using Shannon's index of phenotypic diversity: $H_o = -\Sigma\pi_i ln\pi_i$, where π_i is the frequency of phenotype *i* (King and Schaal 1989). H_o can be calculated and compared for different populations. Let $H_{pop} = (\Sigma H_o)/n$ be the average diversity over *n* different populations, and let $H_{sp} = -\Sigma\, l\, \pi\, n\, \pi$ be the diversity calculated from the phenotypic frequencies, π, in all populations considered together. Then, the proportion of diversity present within populations, H_{pop}/H_{sp}, can be compared with that between populations $(H_{sp} - H_{pop})/H_{sp}$.

RESULTS

Among 44 loci examined, only 1 locus (PGM-2) was found to be polymorphic in the white-head and black langurs. The gene frequencies in the white-head langur and black langur are 0.17/0.83 and 0.50/0.50, respectively. So Nei's genetic distance is 0.0025.

Thirty PCR primers of arbitrary sequences, 10 nucleotides in length, were screened, and 22 were used for phylogenetic analysis. Each primer amplified 3 to 9 prominent DNA fragments. Only clear bands were scored for further calculation. In total, 125 bands were detected by the 22 primers, 114 of which were variable. No monomorphism was shown in the 22 primers. The percentage of polymorphism is very high, with 13 of the 22 primers showing 100% polymorphism.

Phylogenetic relationship

The *F* indices were transformed into a distance matrix (Table 1), then phylogenetic relationships were computed based on the distance matrix.

Although we used *T. phayrei* as the outgroup to root the tree, the N-J tree is an unrooted tree. The UPGMA tree shares the same topology with the N-J tree (Fig. 1). Two clusters were observed in our N-J tree: *T. leucocephalus* 1 and 2; and *T. francoisi* 1, 2, 3, 4, 5, 6 and *T. leucocephalus* 3.

The phenotypic frequencies detected with the 22 primers were calculated and used in estimating

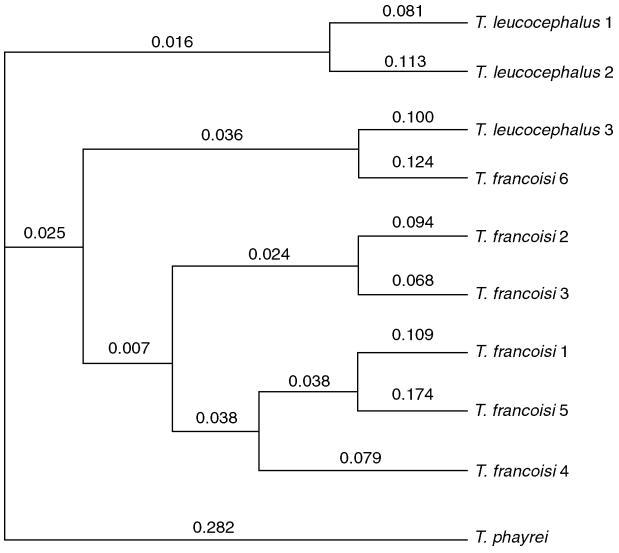

Fig. 1. The Neighbor-joining tree derived from distance analysis using Neighbor in the program, Phylip, based on genetic distances. The length of each branch is indicated above the branch. *T. phayrei* was used as the outgroup.

diversity (H_o) within population types (Table 2). A *t*-test was carried out to evaluate differences between genetic distances within and between *T. leucocephalus* and *T. francoisi* taxa groups. The statistical result shows that the variation within *T. leucocephalus* and *T. francoisi* is not significantly different from the taxa group between *T. leucocephalus* and *T. francoisi* (p = 0.433).

Primers OPC15 and OPC16 detected the most variability within the population, while primer OPC1 detected the least. An assessment of the proportion of diversity present within populations, H_{pop}/H_{sp}, and between populations, $(H_{sp} – H_{pop})/H_{sp}$, indicates that, on average, most of the diversity (73%) is detected within populations. However, the distribution of variability between and within populations varies with different primers (Table 2).

DISCUSSION

Our results show that under the described conditions, individual RAPD phenotypes were highly reproducible. A high level of polymorphism was observed among *T. leucocephalus* and *T. francoisi* individuals. It appears that the RAPD technique is useful for phylogenetic studies between *T. leucocephalus* and *T. francoisi* populations.

Up to now, the classification of the white-head langur has remained controversial. Tan (1957) proposed that the white-head langur should be recognized as *T. leucocephalus*. Li and Ma (1980) recognized the white-head langur as a subspecies of *T. francoisi*, based on a field survey of the geographical distribution of both Francois' langurs and the white-head langurs in southern Guangxi Province. Li (1993) found that elevation coincides with habitat use, and thus separated white-cheek *(T. francoisi*) from white-head langurs (*T. leucocephalus*). So he classified them as separate species. All viewpoints mentioned above are mainly based on gross morphological features and geographic distributions.

Trachypithecus leucocephalus and *T. francoisi* share a similar habitat in the karst hill region. Their diets and social behaviors are very similar. Morphologically intermediate types can be found along the banks of the Zuo River. Some groups contain individuals with white hair on the head and a black tail. Other groups contain individuals with black hair on the head and a white tail. The proportion of white to black color varies among individuals. Based on information from historical geology, some parts of the Zuo and Ming Rivers are very narrow, which do not form a strictly natural barrier. Furthermore,

Table 2. Partitioning of the genetic diversity within and between the *T. leucocephalus* and *T. francoisi* populations for 22 primers

Primer	H_o		H_{sp}	H_{pop}	H_{pop}/H_{sp}	$(H_{sp} – H_{pop})/H_{sp}$
	T. leucocephalus	*T. francoisi*				
OPC2	0.64	0.30	0.56	0.47	0.84	0.16
OPC5	1.00	0.30	0.71	0.65	0.92	0.08
OPC6	0.00	1.21	1.08	0.61	0.57	0.43
OPC9	0.64	0.57	0.62	0.61	0.99	0.01
OPC10	0.46	1.10	1.13	0.78	0.69	0.31
OPC11	0.37	0.57	0.63	0.47	0.75	0.25
OPC14	0.27	1.32	1.11	0.80	0.72	0.28
OPC19	0.27	0.50	0.43	0.39	0.90	0.10
OPC7	0.27	0.27	0.27	0.27	1.00	0.00
OPC8	1.62	2.34	2.29	1.98	0.87	0.13
OPD2	0.37	1.08	1.06	0.73	0.69	0.31
OPD3	0.00	1.46	1.63	0.73	0.45	0.55
OPD1	0.37	0.27	0.44	0.32	0.73	0.27
OPC1	0.37	0.64	1.03	0.34	0.33	0.67
OPD4	0.91	0.30	1.31	0.61	0.47	0.53
OPD5	0.27	1.19	1.07	0.73	0.68	0.32
OPD6	0.91	1.00	1.11	0.96	0.86	0.14
OPD7	0.91	1.51	1.52	1.21	0.79	0.21
OPC15	0.54	0.42	0.47	0.48	1.02	–0.02
OPC16	0.54	0.42	0.47	0.48	1.02	–0.02
OPC17	0.73	0.96	1.08	0.85	0.79	0.21
OPC18	0.54	0.91	0.83	0.73	0.88	0.12
Average	0.55	0.85	0.95	0.69	0.73	0.27

Shiwanda Mt. is not a strict barrier either. So the above natural barriers can not prevent gene flow between the white-head and the black langurs.

Wang et al. (1997) sequenced the ND3-ND4 region of mtDNA from *T. leucocephalus* and *T. francoisi*. Their results showed a genetic similarity between *T. leucocephalus* and *T. francoisi*. The genetic distance was 1.6% between *T. leucocephalus* and *T. francoisi*, and 8.5% between *T. francoisi* and *T. phayrei*.

Our results show that *T. leucocephalus* 3 and the 6 *T. francoisi* specimens are included in 1 cluster. *T. leucocephalus* and *T. francoisi* are not monophyletic. The *T. francoisi* from Guangxi and Vietnam could not be distinguished clearly and are not divided into a single cluster. Also, the allozyme data show that *T. leucocephalus* and *T. francoisi* share almost all the loci surveyed (except for locus PGM-2), and the genetic distance is only 0.0025.

Our statistical test results demonstrate that the difference between taxa groups of *T. leucocephalus* and *T. francoisi* is not significant at the 5% level. Our results suggest that the difference between *T. leucocephalus* and *T. francoisi* is within the species level. Recent gene flow might exist between *T. leucocephalus* and *T. francoisi*.

Furthermore, we collected some data of gene frequencies from macaques (*Macaca* sp.). The mean of intraspecific Nei's genetic distances among different populations of *Macaca mulatta* was 0.0049 ± 0.0008 (S.E.), and 99% confidence intervals were 0.0023-0.0074 (unpubl. data, the number of genetic loci surveyed was 36). And that of interspecific distances among *Macaca* was 0.3053 ± 0.0439 (S.E.), and 99% confidence intervals were 0.1903-0.4204 (calculated from data of Fooden 1989, the number of genetic loci was 24). The genetic distance between white-head and black langurs falls into the interval of the former.

Combining the morphological features, geographical distribution, allozyme and RAPD data, DNA sequencing, and historical geology, we agree with Li and Ma's (1980) viewpoint, i.e., the white-head langur is not a valid separate species. It is better to regard *T. leucocephalus* as a subspecies of *T. francoisi*. It should be pointed out that the sample size used in present study is small; more samples should be used to improve the statistical analysis accuracy of the genetic differentiation level and gene flow between *T. leucocephalus* and *T. francoisi*.

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基於同功酶電泳和隨機擴增多態 DNA (RAPD)探討白頭葉猴之分類地位

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為了澄清白頭葉猴的分類地位，我們用同功酶電泳和隨機擴增多態 DNA (RAPD)技術對 6 隻黑葉猴和 3 隻白頭葉猴進行了分析。蛋白電泳檢測的 44 個遺傳座位中僅有一個磷酸葡萄糖異構酶 2 (phosphoglucomutase 2, PGM-2)座位發現多態。 Nei 氏遺傳距離為 0.0025 。共有 30 個 10 基隨機引物用於RAPD 分析，其中 22 個引物能夠產生清晰的條帶。基於遺傳距離用 Neighbor-joining 和 UPGMA 方法構建了系統發育樹。結果表明，白頭葉猴和黑葉猴沒有分別單獨形成單系群。來自廣西和越南的黑葉猴沒有形成兩分枝，不能被清晰地區分開。白頭葉猴和黑葉猴種內和種間遺傳距離檢驗表明，種內和種間差異不顯著 (5% 水平)。我們的結果顯示，白頭葉猴和黑葉猴之間遺傳分化水平相當低，它們之間可能存在近期的基因互流。結合形態學特徵、地理分布、同功酶、 RAPD 和粒線體 DNA 序列數據，我們認為白頭葉猴可能是黑葉猴的一個亞種。

關鍵詞：葉猴屬，白頭葉猴，遺傳分化，分類。

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