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CONTROL REGION-mtDNA HETEROGENEITY OF KALIMANTAN FALSE GHARIAL (*Tomistoma schlegelii*) POPULATION: A PRELIMINARY STUDY

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ABSTRACT

The preliminary genetic study on Kalimantan "false gharial" from the wild was reported. Eleven tail scutes were collected from eleven individuals that originally consisting of two individuals from Kapuas River, one individual from Sentarum Lake, Jelai River, Mapam River, Perian Lake, and Lamandau River, two individuals from Barito River and three individuals from Mahakam River. PCR amplifying and sequencing 451 nucleotides in average that can be aligned at the same length of control region mitochondrial DNA. Among 11 individuals found eight polymorphic sites that consisted four haplotypes (A, B, C, and D) respectively, which is haplotype A is dominant. Based on phylogenetic tree that constructed by Tamura-Nei parameter, false gharial population in Kalimantan can be divided into two population groups; there were Central-Eastern Kalimantan population group and Western Kalimantan population group. Based on the hypothesis of landmasses separating between central-eastern Kalimantan and western Kalimantan that known as Schwaner Mountains, the genetic distance D = 1.53% was expected to be equal to 20 million years.

Key words: *False Gharial,* ***Tomistoma schlegelii****, mitochondrial DNA, Kalimantan*

INTRODUCTION

The tomistoma or "false gharial" (*Tomistoma schlegelii*) is one of the most unusual and little known of the crocodilians. The species was widely distributed in Southeast Asia, but currently the species is limited to know only from Sumatra and Kalimantan, Peninsular Malaysia and Sarawak (Ross, 1998). The species is listed in appendix I CITES with data deficient under IUCN criteria (IUCN, 1996). Bezuijan *et al.* (1998) suggests the status of false gharial is critically endangered in Sumatra. Actually, the population status of false gharial in Kalimantan and Sumatra are not different. The crocodiles inhabited mainly in the majority of riverine and lakes areas that have been in a severe degradation from human activities, such as fishing, logging and cultivation; where all of the activities were damaging the land and water ecosystem, including the crocodile habitat (Ross *et al.*, 1998).

Previous genetic studies of false gharial have been limited to captive animal. Studied on protein analyses have been done by Densmore (1983), Densmore & Owen (1989) and Densmore & White (1991); DNA fingerprinting by Aggarwal *et al.* (1994); and immunological data by Hass *et al.* (1992) and Densmore & Dessauer (1984).

The purpose of this study is to survey the pattern and extent of genetic heterogeneity among the remaining population by sequencing mitochondria DNA (mt DNA) control region, which is the highly variable part of mtDNA. The preliminary study was started by false gharial population in Kalimantan (West, Central and East).

MATERIALS AND METHODS

Tail scutes were collected from 11 individuals, consisting of two wild-caught false gharial from Kapuas River and one wild-caught from Sentarum Lake in West Kalimantan; one wild-caught from Jelai River, Mapam River, Lamandau River, and Barito River in Central Kalimantan; one wild-caught from Perian Lake and three wild-caught captive individuals from Mahakam River in East Kalimantan. One muscle-extracted DNA sample was obtained from wild-caught *Crocodylus porosus* that collected from Kapuas River for using as an out-group. The tissues sample sampling sites were shown in Figure 1.

A 20% solution of dimethyl sulfoxide (DMSO) saturated with salt (NaCl) and 95% ethanol were used to preserve tail scute samples. All of the samples were storage in freezer (-20º C) before analyzing. DNA analyses of all tissue samples were conducted in Molecular Zoology Laboratory, University of Queensland, Brisbane, Australia.

DNA Extraction

Approximately one gram small cut of each tail scute sample was digested with 500 μL lysis buffer pH 8, 25 μL

0.5% SDS and 25 μL 10 mg/μL proteinase K incubated in 55º C water bath for at least 12 hours.

The DNA solution was extracted with phenol/ chloroform/isoamyl alcohol mixture, and then precipitated with 100% ethanol. The DNA is pelleted after the precipitation step, washed with 70% ethanol, and resuspended in super distillated water and then storage in freezer (-20º C).

PCR

To guard against contamination, PCR reactions were set up at PCR bench with equipment that only used for the reactions and negative controls were conducted with every PCR reaction. A 500 bp segment of control region mtDNA was amplified using primers L15463 [5'-CGCTGGCCTTGTAAGACAGA-3'] and H15941 [5'-GAGGAAAGAATGTCCAGGC-3']. PCR amplification was performed in 30 μL reactions using 1 μL DNA extraction, 6 μL 5× Q solution, 3 μL 10× PCR buffer, 0.3 μL dNTP 10 mM, 1.2 μL primers 10 mM, 0.25 μL taq DNA polymerase 2 u/μL, and 17.05 μL distillate water. Reaction, in a Perkin Elmer GeneAMP 9700 programmable DNA. Thermocycler, were preceded by a 2-min denaturation at 94º C, followed by 35 cycles of 20-sec denaturation at 94º C, 20-sec annealing at 48º C, 45-sec extension at 72º C; after 35 cycling the reaction was ending at 72º C extension for 5-min, and after that the reaction was stored at 10º C. Amplifications were electrophoresed on 1.5% regular agarose, stained with ethidium bromide, and bands visualized under UV.

Purification

Spin through method was used to purify the target bands. The method were proceeded by cutting the target bands in 1.3% regular agarose in 0.75 × TA buffer, and the cutting gel were spin at 9000 rpm (= 906.39 g) for 12 min by using filter paper.

Sequencing

Dye-labeled termination method (Perkin-Elmer ABI Prism Dye Terminator) was used in sequencing reaction. The reactions were set up at PCR bench to avoid contamination. Sequencing reaction was 15 μL reaction using 6.0 Dye, 2.4 μL L15463, and composition of DNA and water were depending on concentration of purified DNA target. All amplified products were sequenced in the forward direction using primer L15463 to identify haplotype.

The PCR works were run by a 1-min denaturation at 94º C, followed by 27 cycles of 10-sec denaturation at 94º C, 15-sec annealing at 50º C, 4-min extension at 60º C; after 27 cycling the reaction were stored at 10º C. Sodium acetate/ethanol method was used to precipitate DNA. Sequencing of all amplified products was carried out at Australian Genome Research Facility.

Data Analysis

Sequences were aligned and edited with SEQUENCHER version 3.0. Clustal X was used in alignment nucleotides of edited sequences. To calculate the average number of nucleotide differences (k), nucleotide diversity (p), and to estimate population subdivision at the nucleotide level (West Kalimantan Group and East-Central Kalimantan Group) followed Wen-Hsiung Li & Graur (1991); and to calculate haplotype diversity followed Nei (1987). MacClade version 3.06 was used to optimize nucleotide characters in phylogenic analyses. To estimate evolutionary distance among samples Tamura-Nei distance matrix was used, and phylogenetic tree was constructed using Neighbor-Joining (NJ) and Maximum Parsimony (MP) procedures with the program PAUP. The tree was rooted using salt-water crocodile (*C. porosus*) as an out-group.

RESULTS

Genetic Variation

The control region sequence results using primer L15463 produced 451 base pairs in average that can be aligned at the same length (see Appendix 1). Among 11 individuals found eight polymorphic sites that consisted four haplotypes (A, B, C, and D) respectively. The frequencies of all haplotypes were shown in Table 1. However, three individuals from East Kalimantan and four individuals from Central Kalimantan consisted haplotype A and just one individual from Central Kalimantan (Mapam River) has haplotype B. In this study, haplotype A is dominant among the others haplotypes (B, C, and D). In general the total value of haplotype diversity of 4 haplotypes of false gharial in Kalimantan (West, Central and East Kalimantan) were $h = 0.5714$ respectively.

Population Structure

Based on phylogenetic tree that constructed by Tamura-Nei parameter (see Table 2), false gharial population in Kalimantan can be divided into two population groups; there were Central-Eastern Kalimantan population group and Western Kalimantan population group (see Figure 2). All of bases changing between two population groups were transition (T ↔ G or G ↔ A). However, there was only

one transversion between haplotype A and B (T ↔ G) at position 449, whereas between haplotype C and D just have one transition (C ↔ T) at position 32. The average transition/transversion ratio between two population groups was 5.0. Haplotype diversity within Central-Eastern Kalimantan population was h= 0.6258; whereas within Western Kalimantan population was h= 1.1504. The average number of nucleotide differences was k = 1.09, whereas nucleotide difference in Central-Eastern Kalimantan population group was k = 0.23 and in Western Kalimantan population group was k = 0.21. Nucleotide diversity of total samples is p = 0.2326 respectively; however the nucleotide diversities between the population groups were p = 0.0133 for Central-Eastern Kalimantan population group and p = 0.0035 for Western Kalimantan population group. The values of haplotype diversity and nucleotide diversity were estimated from small number of individuals, giving the result was bias. In order to reduce the bias data it is necessary to increase some more individuals.

DISCUSSION

In this study the number of individuals that had been analyzed were small; however, the 1000 bootstrap value using Neighbor-Joining (NJ) between Central-Eastern Kalimantan population and Western Kalimantan population was 100% (Figure 3). The average of genetic divergence between Central-Eastern Kalimantan population group and Western Kalimantan population group was also high (genetic distance D = 1.53%) compared to the genetic divergence within the two population groups (Central-Eastern Kalimantan population group D = 0.23%; Western Kalimantan D = 0.21%). According to Menzies & Kushlan (1991), the history of landmasses separation can determine molecular clock of the American crocodile *Crocodylus acutus* in the Caribbean, which is D = 1% was equal to 40-80 million years ago (Myr). Historically, the central Borneo mountains began to rise in the early Miocene (20 Myr) (Hall, 1991), at this time lowland masses in Western Kalimantan began to separate with the lowland masses in Central-Eastern Kalimantan by arising of Schwaner Mountains. The Schwaner Mountains was the natural barrier for the two population of false gharial. Based on the landmasses separating, the genetic distance D = 1.53% was expected to be equal to 20 million years.

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Table 1. False Gharial polymophic position between West Kalimantan population group and Central-Eastern Kalimantan population group. ( ) = number of tissues samples

Sample	Locality	Region	Haplotype		Sequence Position							
			Type	Frequency	16	32	89	150	204	327	351	449
Mahakam (3)	Mahalam River	East Kalimantan	A		T	C	C	T	G	T	C	T
Perian (1)	Mahakam River tributary	East Kalimantan	A		•	•	•	•	•	•	•	•
Lamandau (1)	Lamandau River	Central Kalimantan	A	0,6364	•	•	•	•	•	•	•	•
Barito (1)	Barito River	Central Kalimantan	A		•	•	•	•	•	•	•	•
Jelai (1)	Jelai River	Central Kalimantan	A		•	•	•	•	•	•	•	•
Mapam (1)	Mapam River	Cantral Kalimantan	B	0,0909	•	•	•	•	•	•	•	G
Kapuas-1 (1)	Kapuas River	West Kalimantan	C	0,0909	C	•	T	C	A	C	T	•
Kapuas-2 (1)	Kapuas River	West Kalimantan	D		C	T	T	C	A	C	T	•
Sentarum (1)	Kapuas River tributary	West Kalimantan	D	0,1818	C	T	T	C	A	C	T	•

Table 2. Pair wise percent nucleotide differences (k) among four haplotypes of false gharial in Kalimantan based on Tamura-Nei parameter (below diagonal) and total number differences of nucleotide among haplotypes (above diagonal).

Haplotype	A	B	C	D
A	-	1	6	7
B	0,23	-	7	8
C	1,28	1,56	-	1
D	1,50	1,78	0,21	-

Table 3. Nucleotide diversity (p) among four haplotypes of False Gharial in Kalimantan based on Tamura-Nei parameter.

π	A	B	C	D
A	-			
B	0,0133	-		
C	0,0740	0,0129	-	
D	0,1735	0,0294	0,0035	-

Figure 1. Island of Kalimantan showing major and tissue sample sampling sites

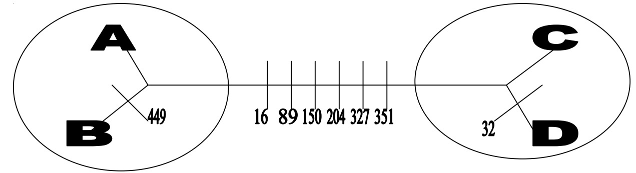

Figure 2. Parsimony network of Kalimantan false gharial haplotypes and position of polymorphic sites. Haplotype A and B denote Central-Eastern Kalimantan population group and C and D denote Western Kalimantan population group.

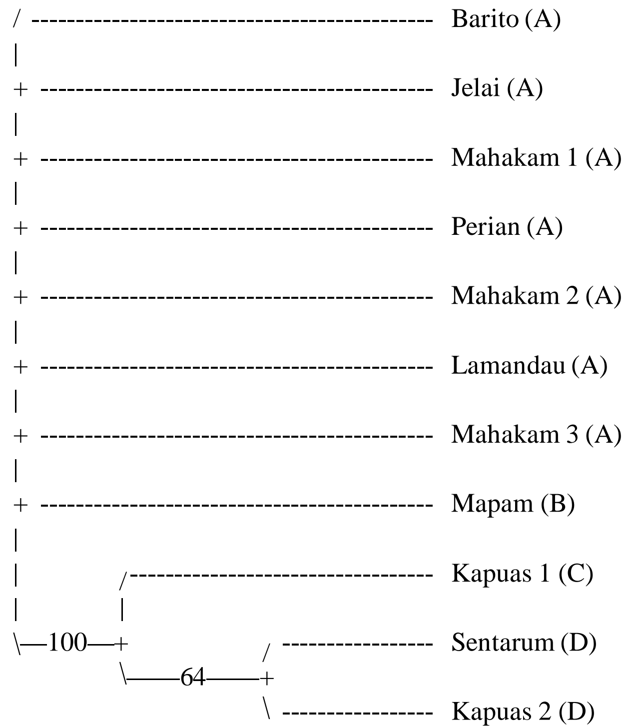

Figure 3. Bootstrap with heuristic search. Number of bootstrap 1000. Bootstrap 50% majority-rule consensus tree. A, B, C, and D are haplotypes

Appendix 1. False Gharial 451 base pairs of the control region sequence results using primer L15463

Locality	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Barito	C	C	C	A	A	G	A	G	T	G	T	C	A	C	T	T	A	C	T	C	A	A	G	A	A	G	G	C	A	G	A	C	A	C	A	A	T	C	C	T	G	C	T	C	C	A	C	T	G	G	C	C	C	C	C	A	A	A	G	C
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Locality	61	52	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120
Barito	C	A	A	C	A	T	T	C	T	C	A	C	T	A	A	A	C	T	A	C	T	T	C	T	T	G	A	A	C	A	C	C	A	T	T	T	C	A	C	A	A	T	C	C	C	G	T	C	A	T	C	G	T	A	G	C	T	T	A	A
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Locality	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180
Barito	A	T	A	C	T	A	A	A	G	C	A	C	A	A	C	A	C	T	G	A	A	A	A	T	G	T	T	A	C	T	A	T	G	G	A	C	A	G	A	C	T	T	G	T	C	C	C	G	C	A	T	G	A	C	A	A	C	A	A	A
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Locality	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240
Barito	C	T	G	C	T	A	A	A	A	C	A	A	C	C	C	A	A	C	C	T	A	C	G	G	T	G	G	C	A	A	T	A	A	A	C	C	T	T	T	G	C	C	A	T	T	G	T	T	T	T	T	A	A	A	G	A	T	A	T	T
Jelai	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Locality	241	142	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300
Barito	A	T	G	T	A	C	C	C	C	C	T	C	T	C	T	G	G	T	A	T	G	T	A	T	T	A	C	T	A	G	G	C	A	T	T	C	A	T	T	T	A	T	T	T	A	C	C	A	C	T	A	A	C	A	C	C	C	A	T	C
Jelai	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Locality	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360
Barito	C	T	C	T	A	G	T	T	C	T	T	A	T	T	A	A	T	C	A	A	C	A	C	C	T	C	T	C	G	T	G	A	A	A	T	C	A	T	C	A	T	C	C	A	T	T	G	T	A	T	C	C	A	T	A	C	T	A	A	C
Jelai	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Locality	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380
Barito	T	C	T	T	A	C	T	A	G	T	C	T	C	A	A	G	T	C	C	A
Jelai	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Perian	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lamandau	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mapam	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Kapuas 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sentarum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Kapuas 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Locality	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400
Barito	T	A	T	C	T	A	G	A	T	A	T	A	C	T	C	A	C	A	T	T
Jelai	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Perian	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lamandau	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mapam	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Locality	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420
Barito	C	A	T	T	G	C	T	C	T	T	C	T	T	A	G	A	G	A	C	C
Jelai	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Perian	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Lamandau	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mahakam 3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Mapam	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Kapuas 1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Sentarum	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Kapuas 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

Locality	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451
Barito	T	C	T	G	G	T	T	A	T	C	A	C	T	C	T	C	A	T	G	T	A	C	T	C	T	T	C	T	T	G	C
Jelai	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Perian	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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Lamandau	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
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