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PHYLOGENETIC RELATIONSHIPS OF THE MALAY PENINSULA OTTERS (*LUTRA SUMATRANA*, *LUTROGALE PERSPICILLATA*, AND *AONYX CINEREUS*) BASED ON DNA SEQUENCES OF MITOCHONDRIAL D-LOOP REGION

M. K. A Rosli[1], P. Abdul-Patah[1,§], S. M. F. Syed-Shabthar[1], M. N. Burhanuddin[§], T. Sekiguchi[α], H. Sasaki[β], M. N. Shukor[*], S. Yaakop[*] and B. M. Md-Zain[1¥].

[1]School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia
[§]Department of Wildlife and National Parks (Peninsular Malaysia), Km 10, Jalan Cheras, Kuala Lumpur, 50664, Malaysia
[α]Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan
[β]Chikushi Jogakuen University Junior College, 2-12-1 Ishizaka, Dazaifu 818-0192, Japan
[¥]Corresponding author: (abgbadd@ukm.edu.my; abgbadd1966@yahoo.com)

ABSTRACT

Aonyx cinereus, *Lutrogale perspicillata* and *Lutra sumatrana* are three species of the Old World Otter that can be found in Peninsular Malaysia. The main objective of this research was to determine the phylogenetic relationships among these three otter genera using 401 bp DNA sequences of the D-loop region. Tree topologies were reconstructed using the Neighbor-Joining (NJ) and the Maximum Parsimony (MP) methods. Both NJ and MP trees showed that the three Otter species (*A. cinereus*, *L. perspicillata*, and *L. sumatrana*) formed their own monophyletic clade. The results also showed that *L. perspicillata* and *A. cinereus* were sister clades and closely related, which is supported by the bootstrap values of 85% (NJ) and 84% (MP). The two species also had a close genetic distance value of 0.031. The clades, consisting of both *A.cinereus* and *L. perspicillata*, were formed in a distinct group from *L. sumatrana*, which was found to be grouped with *L. perspicillata* in previous morphological studies. The independent monophyletic clade of *L. sumatrana* was supported by high bootstrap values. These results imply that *L. perspicillata* does not belong to the same group as *L. sumatrana* which has been concluded in several previous molecular studies.

Key words: Otter, *Aonyx cinereus*, *Lutrogale perspicillata*, *Lutra sumatrana*, Malaysian otter, mtD-loop region.

INTRODUCTION

Otters are semi-aquatic mammals that belong to the family Mustalidae and to the subfamily Lutrinae (Hussain 1999). Most of these charismatic species are top predators in their respective communities and are threatened due to a combination of habitat loss, hunting, and pollution (Mason and MacDonald 1990). A total of 13 otter species are found in the world comprising seven distinct genera consisting of three major clades based on nuclear and mitochondrial markers (Koepfli and Wayne 1998). The three clades are: the sea and river otters of Eurasia and Africa (*Aonyx*, *Enhydra*, *Hydrictis*, *Lutra*, and *Lutrogale*), the marine and river otters from North, central, and South America (*Lontra*), and a basal lineage of *Pteronura brasiliensis* (Koepfli and Wayne 1998; Koepfli *et al.* 2008a; Koepfli *et al.* 2008b). In addition, Wilson and Reeder (2005) also recognize that there are 13 species in the Lutrinae subfamily.

Three species of otters can be found throughout the Peninsular Malaysia: *Aonyx cinereus*, *Lutra sumatrana*, and *Lutrogale perspicillata* (Shariff 1984; Burhanuddin 1990; Hussain *et al.* 2011). All three species are represented by three different genera in which *A. cinereus* originates from the genus *Aonyx*, *L. perspicillata* originates from the genus *Lutrogale*, and *L. sumatrana* originates from the genus *Lutra* (Duplaix 1975; Hwang and Lariviere 2005; IUCN 2010; Hussain *et al.* 2011). Based on previous systematic studies of morphological data, *Lutra* and *Lutrogale* are often grouped together in the Lutrini clade, and *Amblonyx* is grouped with *Aonyx* in the Aonychini clade (van Zyll de Jong 1987; van Zyll de Jong 1991; Willemsen 1992). However, molecular data has indicated that *L. perspicillata* is a sister clade of *A. cinerius* in a higher clade that is different from *L. sumatrana*, which is a species that was previously closely related to *L. perspicillata* morphologically (Koepfli *et al.* 2008).

Only few studies available involving the genetic aspects of otters in Peninsular Malaysia with a focus on population genetics of *A. cinereus* (Rosli *et al*. 2014). Molecular systematic research in Malaysia mainly focused on other mammals, such as cattle, human, primates, etc. (Md-Zain *et al.* 2010a; Md-Zain *et al.* 2010b; Vun *et al.* 2010; Rosli *et al.* 2011a; Rosli *et al.* 2011b). Mitochondrial D-loop is a non-coding region that

has been extensively used in phylogenetic studies (Ang *et al.* 2011; Vianna *et al.* 2011). In this study, the mtD-loop region was selected due to its effectiveness in portraying relationships between species and population levels (Lim *et al.* 2010; Rosli *et al.* 2014). The main objective of this study was to portray the phylogenetic relationships of all three otter species in Peninsular Malaysia using the mitochondrial DNA sequences of the D-loop region.

MATERIALS AND METHODS

Samples and DNA isolation. Sampling was conducted in several Peninsular Malaysia states such as Terengganu, Perak, Kedah, Penang, Johor, Malacca, and Pahang (Fig. 1). The 27 collectedsamples represented all three Malaysian otter species: *L. sumatrana*, *A. cinereus*, and *L. perspicillata*. All samples (Table 1) were obtained with the help of various parties, such as the Department of Wildlife and National Parks (Wildlife and National Parks), the National Zoo of Malaysia, the museum of Zoology, the Universiti Kebangsaan Malaysia (UKM). Tissues of road kill and fecal samples were used in this study. Fecal samples were preserved in 70% ethanol solution to prevent damage. Tissue samples were stored in freezer at a temperature of -80°C. The total genomic DNA was extracted using a standard extraction kit and the protocol provided by QIAGEND Neasy Tissue or Stool Kit (Qiagen).

DNA amplification. A polymerase chain reaction (PCR) was performed using a 25 µl reaction mixture containing 1µl of genomic DNA, 2.5µlPCR Buffer 10X, 1 µl 50 mM$MgCl_2$, 0.5µl 10mMdNTP mix, 1.5 µl each of 10 pmol/µl primer, and 4 units of *Taq* DNA Polymerase (Vivantis) in PTC-100 Thermal Cycler (MJ Research Inc.). The partial mtD-loop fragment of approximately 450 base pairs was amplified using forward primer tanaD-F (ACCATCAGCACCCAAAGCTG) and reverse primer tanaD-R (GGGCTGATTAGTCATTAGTCCA TC) (Masuki *et al.* 2008). The PCR conditions were as follows: 4 minutes denaturation at 94°C followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 56°C, 1 minute at 72°C, and a final 7 minutes extension at 72°C before cooling to 4°C for 10 minutes in thethermal cycler.DNA from the PCR products was purified using a Vivantis G-F1 PCR Clean-up Kit (Vivantis) and was sent directly to a private laboratory (First Base Sdn. Bhd) for sequencing.

Phylogenetic analysis. The sequencing results were exported as FASTA sequence files. The mtD-loop sequences of studied samples were aligned using the Clustal W multiple alignment algorithm of Bio Edit and the outgroup sequences of palm civet (*Paradoxurus hermaphroditus*). All sequences were analyzed using PAUP4.0b10 (Swofford 2002) and MrBayes 3.1 (Huelsenbeck *et al.* 2001) for phylogeny reconstruction. Two methods of analysis in the PAUP included: (1) Neighbor-joining (NJ) using the Kimura 2-parameter (Kimura 1980) model, and (2) Maximum Parsimony (MP) with stepwise addition 1000 replicates in a heuristic search (Swofford 2002). All trees were subjected to a bootstrap value of 1000 replicates. A genetic distance analysis was performed using the Kimura 2-parameter in PAUP4.0b10.

RESULTS

DNA from 30 samples was extracted, amplified, and sequenced. Sequences of 401 base pairs of hypervariable mtD-loop region were aligned. Prior to the sequence analysis, the Basic Local Alignment Search Tool (BLAST) was used to compare the nucleotide of sequence databases (NCBI) and to calculate the statistical significance of the matches. All DNA sequences matched the genus *Lutrinae* DNA sequences in the online database GenBank with a maximum identical index of between 90-99%.

Sequence polymorphism. From the 30 aligned DNA sequences, a portion of the 347 bp representing 31.9% of the complete mtD-loop region were included for further analysis. From the analysis, 91 variable sites were detected in which 11 variable characters were parsimony-uninformative and 80 characters were parsimony-informative. This portion of the 23.1% of informative sites suggested that D-loop is not a conserved region in the mtDNA. The sequence analysis for the polymorphism of the mtD-loop sequences are summarized in Table 2.

Phylogenetic construction

Neighbor-joining. NJ trees were constructed using the Kimura-2-parameter algorithm and bootstrap test. NJ topology showed a clear separation between the outgroup (civet) and the ingroup (Fig. 2). All three otter species were grouped in a major clade that was different from the outgroup. The ingroup was divided into two main clades (Clade A and B). Clade A was divided into two subclades (Subclade I and II). Subclade I is a monophyletic clade of *L. perspicillata* with a bootstrap value of 99%, while Subclade II is a monophyletic clade of *A. cinereus* with a bootstrap value of 68%. Clade B is a monophyletic clade of *L. sumatrana* with a bootstrap value of 98%.

Maximum parsimony. An MP tree was constructed by performing a heuristic search with 1000 replications. The tree shared the same topology with NJ with the exception of a higher bootstrap value (Fig. 3). The bootstrap value for Subclade I is 100%, the value for Subclade II is 94%, and the value for Clade B is 100%. Based on the MP tree, several values of indexes were obtained. The values are: a Consistency index of CI=0.8947, a Homoplasy index of HI=0.1053, a Retention index of RI=0.9694, a Rescaled

consistency index of RC=0.8673, and a tree length of 114.

Genetic distance analysis. A genetic distance analysis was performed using the Kimura-2-Parameter algorithm. Pairwise distances between samples from all three different genuses were calculated as shown in Table 3. The results showed that the biggest genetic distance value was obtained between *A. cinereus* and *L. sumatrana* with a value of 0.070. The closest genetic distance value was between *L. perspicillata* and *A. cinereus* with a value of 0.031. For the genetic distance between otter species and the outgroup, the values obtained were 0.254 (*L. perspicillata*), 0.232 (*L. sumatrana*), and 0.229 (*A. cinereus*).

Table 1. List of genetic samples used in this study.

No.	Sample Code	Species	Location	Type of Sample
1	*L. perspicillata* TP 7	*L. perspicillata*	Tanjung Piandang	Feaces
2	*L. perspicillata* TP 6	*L. perspicillata*	Tanjung Piandang	Feaces
3	*L. perspicillata* TP 5	*L. perspicillata*	Tanjung Piandang	Feaces
4	*L. perspicillata* TP 12	*L. perspicillata*	Tanjung Piandang	Feaces
5	*L. perspicillata* TP 13	*L. perspicillata*	Tanjung Piandang	Feaces
6	*L. perspicillata* KG 15	*L. perspicillata*	Kuala Gula	Feaces
7	*L. perspicillata* KG 16	*L. perspicillata*	Kuala Gula	Feaces
8	*L. perspicillata* KG 19	*L. perspicillata*	Kuala Gula	Feaces
9	*L. perspicillata*SP 3	*L. perspicillata*	Sungai paka	Feaces
10	*L. perspicillata*SP 4	*L. perspicillata*	Sungai paka	Feaces
11	*L. perspicillata*KJMN	*L. perspicillata*	Kg Jelutong Malim Nawar	Feaces
12	*L. perspicillata*KPS 5	*L. perspicillata*	Kampung Padang Salim	Feaces
13	*L. perspicillata*KPS 6	*L. perspicillata*	Kampung Padang Salim	Feaces
14	*A. cinereus*Sampel7	*A. cinereus*	Kuala Gula	Feaces
15	*A. cinereus*Sampel8	*A. cinereus*	Kuala Gula	Feaces
16	*A. cinereus*TM 12	*A. cinereus*	Tumpat	Feaces
17	*A. cinereus*TM 14	*A. cinereus*	Tumpat	Feaces
18	*A. cinereus*TM 15	*A. cinereus*	Tumpat	Feaces
19	*A. cinereus*PN 5	*A. cinereus*	Penarik	Feaces
20	*A. cinereus*PN 1	*A. cinereus*	Penarik	Feaces
21	*A. cinereus*PN 93	*A. cinereus*	Penarik	Feaces
22	*L. sumatrana* PI	*L. sumatrana*	Paya Indah	Feaces
23	*L. sumatrana*JHR	*L. sumatrana*	Johor	Tissues
24	*L. sumatrana*ZM	*L. sumatrana*	Zoo Melaka	Tissues
25	*L. sumatrana* BR	*L. sumatrana*	Bera	Tissues
26	*L. sumatrana*KR	*L. sumatrana*	Kampar	Tissues
27	*L. sumatrana*PKN	*L. sumatrana*	Pekan	Tissues
28	CIVET PNG 29705 4	*P. hermaphroditus*	Penang	Feaces
29	CIVET PNG 30185 4	*P. hermaphroditus*	Penang	Feaces
30	CIVET PNG 30185 3	*P. hermaphroditus*	Penang	Feaces

Table 2.Summary of DNA variations along the sequences across taxa.

Characters	Value
Total	347
Constant	256
Variation	91
Parsimony uninformative	11
Parsimony informative	80
A nucleotide frequency	0.296
T nucleotide frequency	0.292
C nucleotide frequency	0.247
G nucleotide frequency	0.165
Tree length	114

Table 3. Genetic distance of the three otter species of Malay Peninsular using Kimura 2 Parameter.

	L. perspicillata	*A. cinerea*	*L. sumatrana*
L. perspicillata	-		
A. cinereus	0.031	-	
L. sumatrana	0.062	0.070	-
P. hermaphroditus	0.254	0.229	0.232

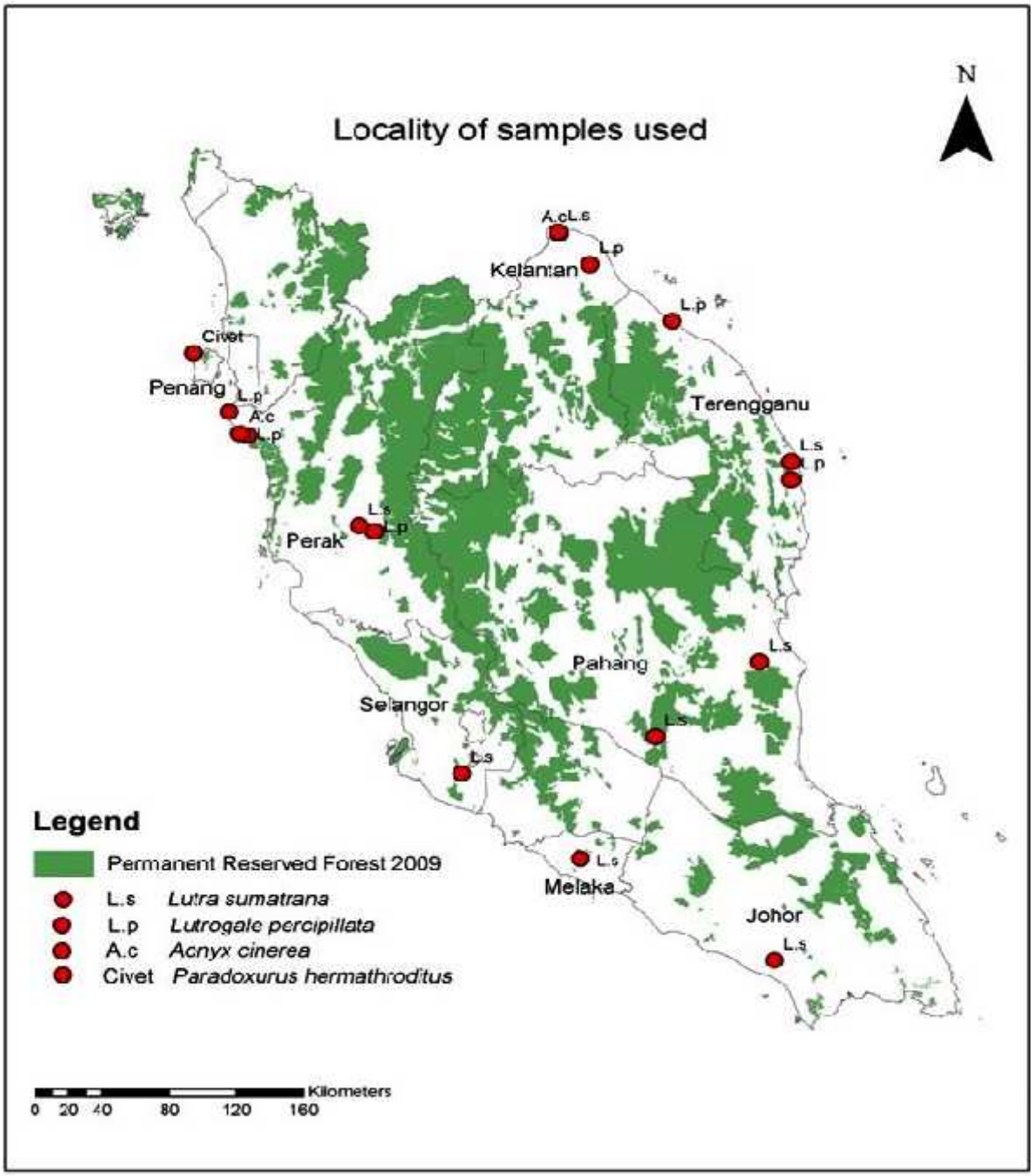

Fig. 1. Sampling sites for the three otter species used in this study.

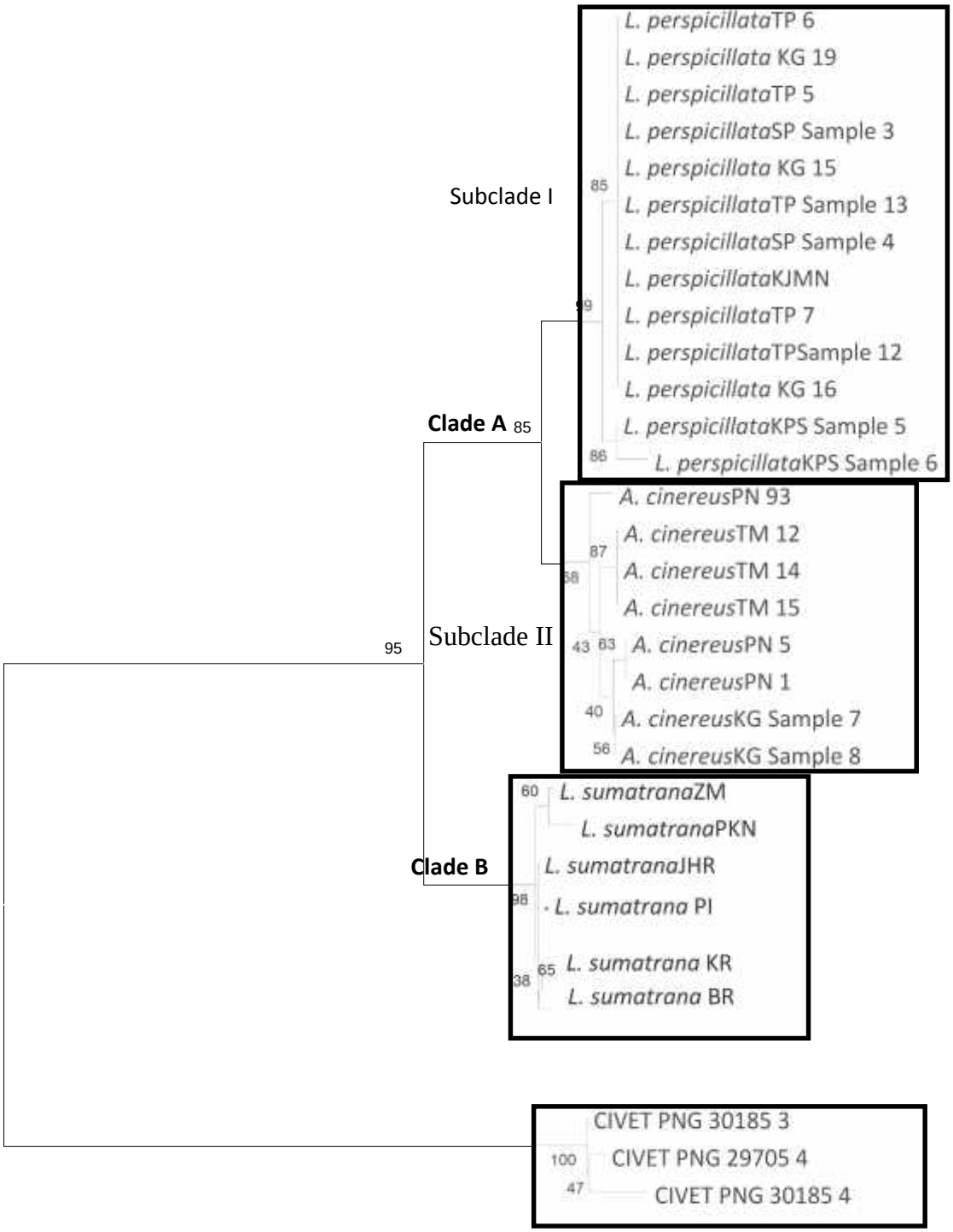

Fig. 2.Neighbor-Joining tree. Number at branches indicates the bootstrap values.

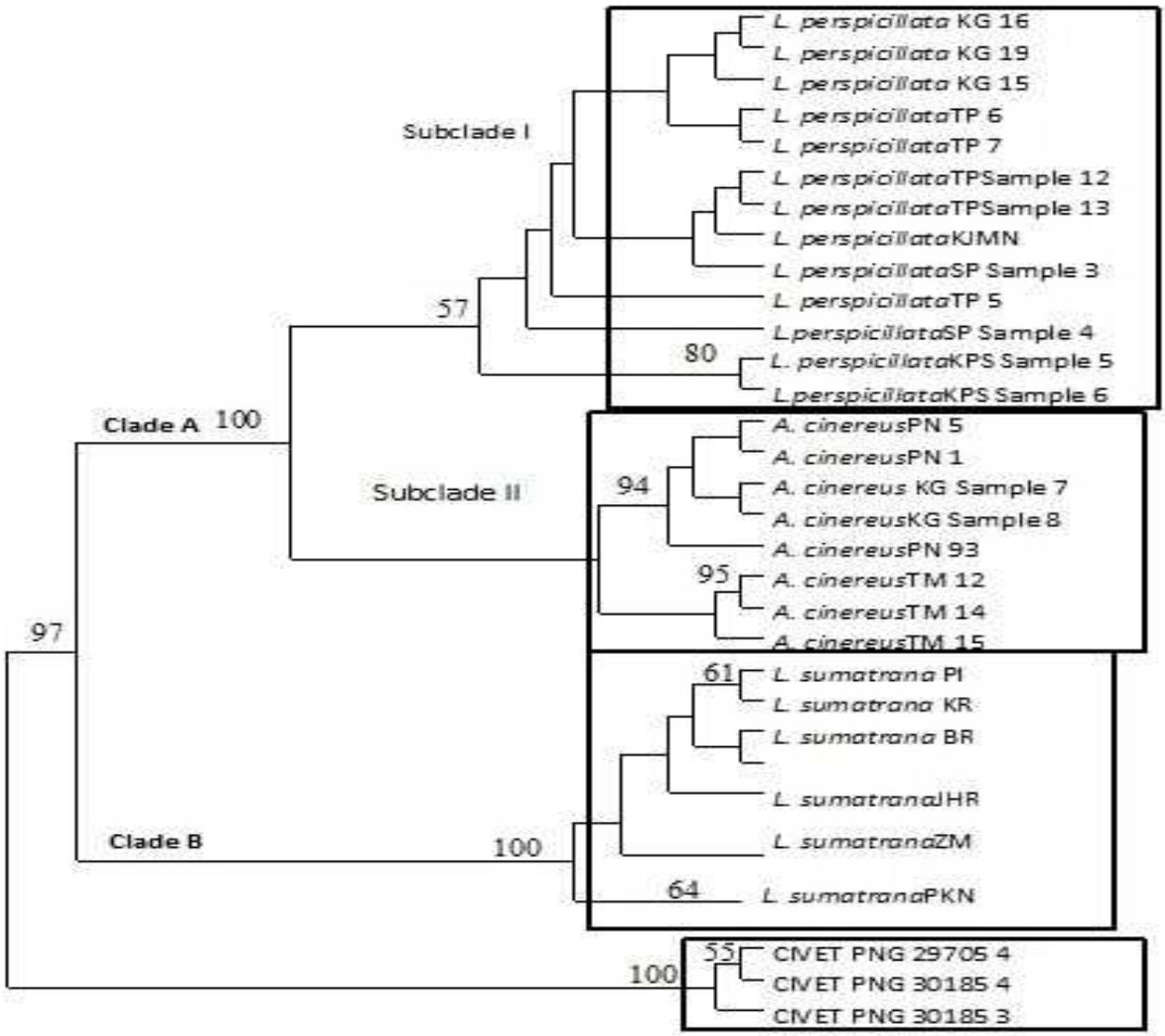

Fig. 3.Maximum Parsimony tree. Number at branches indicates the bootstrap values.

DISCUSSION

A total length of 401 bp partial mtD-loop sequences were sequenced and edited using BioEdit software. In the sequence variation analysis, the selected region showed a significant DNA polymorphism with 111 characters from a total of 212 variable characters that were parsimony informative. This shows that the mtD-loop region is an effective locus to study DNA variation characteristics of otter species in a systematic study (Vianna *et al.* 2010; Vianna *et al*. 2011).

In this study, NJ and MP phylogenetic tree topologies clustered each otter species of *A. cinereus*, *L. perspicillata*, and *L. sumatrana* in a monophyletic group, which was separate from an external group of *P. hermaphroditus*. Between the three species, two major clades were formed. Clade A consisted of *L. perspicillata*, which is also the sister group of *A. cinereus*, and Clade B consisted of *L. sumatrana*. The grouping of Clade A was supported by a high bootstrap value of 85% for the NJ tree and 84% for the MP tree, which concurs with the results obtained by Koepfli *et al.* (2008a) that show similar results of *L. perspicillata* and *A. cinereus* being sister groups; however, both species have significant differences in morphology characters and ecological aspects, such as body mass and diet selection (Hwang and Larivierie 2005). According to Koepfli *et al.* (2008a), there are some possible explanations for this grouping based on continuous observation: a) hybridization between the two species may occur and is not impossible since the two species have the same chromosome number of 2n = 38, b) both species share a similar brain structure with the presence of a larger rear sigmoid gyrus that provides a higher tactile sensitivity

that influences similarities in foraging activity for both species, and c) *Lutrogale* have teeth structures that are suitable for eating shelled foods, which is a diet similar to *A. cinereus*, based on extinct *Lutrogale paleoleptonyx* and *Lutrogale robusta* fossils found in Java, Indonesia.

Furthermore, the results of this study indicated that the separation of the genus *Lutra* was represented by *L. sumatrana* with *Lutrogale* and supported by a high genetic distance value of 6.2%. This research study supports the findings of Koepfli *et al.*2008a and Koepfli *et al.* 2008b. The DNA variation results from this study can further strengthen the position of *L. perspicillata* in the genus *Lutrogale* and indicate that the classification of *L. perspicillata* in the *Lutrogale* genus is not based solely on morphological differences in the species of the genus *Lutra* as was reported by Pocock (1941), Harris (1968), Willemsem (1980, 1992) and Hwang and Lariviere (2005). To further support this statement, further research involving samples of the species *L. lutra*is needed. Koepfli*et al.* (2008a) stated that they did not support the hypotheses that *L. perspicillata* is a subgenus of *Lutra* or that the species evolved from a common ancestor of *Lutra*. Instead, the results show that *Lutrogale* shared the same ancestor as *Aonyx* and further proposed that *Lutrogale*be classified in the *Aonyxini*group.

The position of *L. sumatrana* in Clade B was supported by a high bootstrap value of 98%. The classification and taxonomic status of *L. sumatrana* are still unresolved. The status of this species was disputed and classified as a subspecies of *L. lutra*, known as *L. lutrabarang*, based on a high morphological similarity (Koepfli *et al.*2008a); however, in this study, *L. sumatrana* formed its own monophyletic group based on DNA studies conducted by Koepfli *et al.* (2008a,b), and *L. sumatrana* was recognized as a valid species. According to Koepfli *et al.* (2008a), the skulls of *L. lutrabarang* from several different locations are shorter than the skulls of *L. sumatrana* based on morphological data. Additionally, Sivasothi and Burhanuddin (1994) also found out that *L. sumatrana* possessed a more flattened and longer skull in comparison with *L. lutra*. To validate these conclusions, further studies should be conducted to include samples from *L. lutra barang* that can be found scattered throughout Thailand, Vietnam, and Sumatra (Pocock 1941; Sivasothi and Burhanuddin 1994).

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