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MOLECULAR PHYLOGENY OF THE OLD WORLD PORCUPINES (FAMILY HYSTRICIDAE) USING MITOCHONDRIAL CYTOCHROME *B* GENE

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Abstract: The Old World porcupines (Family Hystricidae) are generally large terrestrial rodents. The major threats to their survival includes over-hunting primarily due to high demand for their meat that is thought to be of high medicinal value. This threat is further impacted by mass habitat destruction where forest is converted into agricultural land or human settlements. Despite their large and unique appearance, little is known with regards to their intrafamilial phylogenetic relationships. This is hampered by the limited DNA sequences that are available on public databases for species identification and comparison. In this study, the phylogenetic relationships among eight out of eleven extant species of Hystricidae were examined using a partial cytochrome *b* gene of the mitochondrial DNA. The first reference record of DNA sequence for all four species of porcupines in Malaysia (*Atherurus macrourus*, *Hystrix brachyura*, *Thecurus crassispinis*, and *Trichys fasciculata*) were made available in GenBank database. These reference sequences are crucial for species identification in a forensic case framework. The phylogeny supported the monophyly of the family Hystricidae. Except for *Thecurus*, the genera within Hystricidae: *Atherurus*, *Hystrix*, and *Trichys* formed distinct groups supporting their genus status with *Trichys* forming the basal group. Based on the positioning of *Thecurus crassispinis* within the *Hystrix* species group in the phylogeny tree, we suggested that *Thecurus crassispinis* to be systematically classified as *Hystrix crassispinis*. Within the genus *Hystrix*, further studies are needed to elucidate the relationships by including the remaining three species within the genus (*Hystrix javanica*, *Hystrix pumila*, and *Hystrix sumatrae*). Furthermore within *Hystrix brachyura*, additional studies are needed to investigate the regional populations structuring within their range countries in Southeast Asia to assist in the sustainable management and conservation of the species.

Keywords: Hystricidae, reference DNA sequence data, *Thecurus crassispinis*, phylogenetic relationship, mitochondrial cytochrome *b*, sustainability.

Introduction

The Old World porcupines (Hystricidae) belongs to the infraorder Phiomorpha, which is distinct from the infraorder Caviomorpha found native to the American continent. Hystricidae consists of eleven extant species from possibly four genera (*Hystrix*, *Atherurus*, *Thecurus* and *Trichys*). Eight species are Asiatic in origin while the remaining three can be found in Africa and the Mediterranean (Figure 1). Members within this family are generally large terrestrial rodents characterized by their unique spines covering their whole body.

In Malaysia four species exist, namely *Atherurus macrourus* (brush-tailed porcupine), *Hystrix brachyura* (Malayan porcupine), *Thecurus crassispinis* (thick-spined porcupine), and *Trichys fasciculata* (long-tailed porcupine) (Wilson & Reeder, 2005; IUCN, 2016; Phillipps & Phillipps, 2016) (Figure 2). *A. macrourus* are nocturnal rat-like species and have long slender body and almost the entire body are covered by

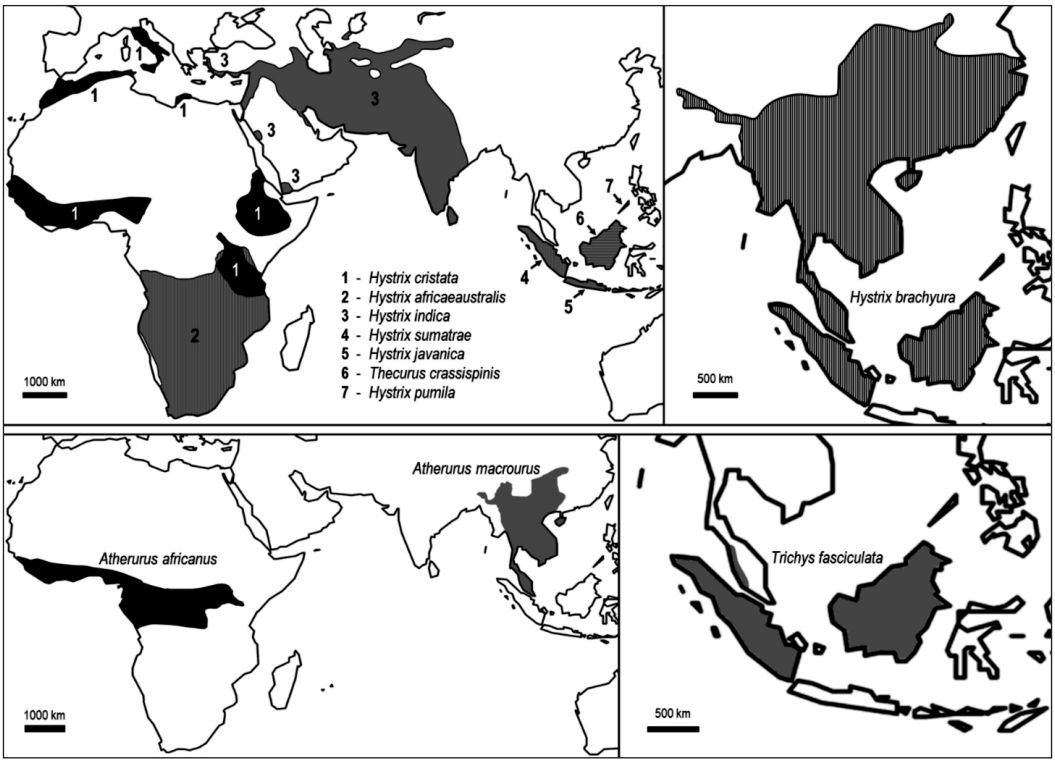

Figure 1: Distribution map of all 11 species of the Old World porcupines (Hystricidae) (adapted from IUCN, 2016)

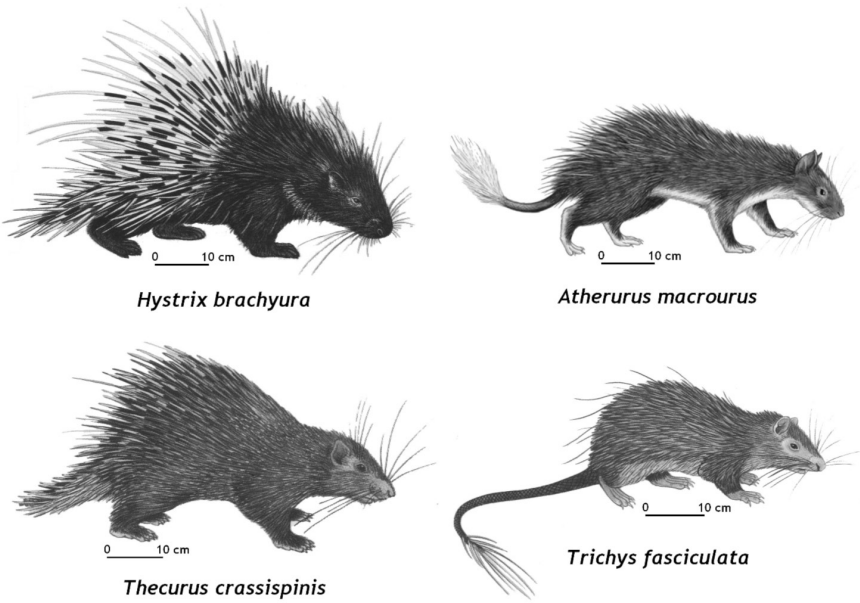

Figure 2: The illustration of four Hystricidae species of Malaysia (illustration of *H. brachyura*, *T. crassispinis* and *T. fasciculata* by Karen Phillips as adapted from Payne & Francis (2007) while *A. macrourus* was illustrated by Ginn Choe as taken from Choe (2006))

quills; black-brown to grey-brown on the upper back and white to light brown on the underside (Choe, 2006). They can be found burrowing in cavities and holes among tree roots along river banks (Khan, 1992; Choe, 2006). *H. brachyura* are characterized by their unique white and black band quills and can be easily distinguished from *T. crassispinis* which have dark brownish quills. Both species can be found in various forest habitats as well as agriculture plantations (Payne & Francis, 2007). *T. fasciculata* are the smallest species within Hystricidae resembling a large spiny rat. The upperparts are brownish while the undersides are whitish in color and they are found in various forest types and also cultivated lands (Payne & Francis, 2007).

In Southeast Asia (SEA), due to their large size, Hystricidae are considered high in economic value and mostly exploited as food source and used for medicinal purposes (Molur *et al*., 2005; Zainuddin, 2006). In Peninsular Malaysia particularly, *H. brachyura* is bred commercially and are increasingly recognized as an alternative food source (Zainuddin, 2006). Since 2005, the Department of Wildlife and National Parks (DWNP) of Peninsular Malaysia have initiated the Malayan Porcupine Commercial Breeding Project in collaboration with the local communities to ensure sustainable commercial breeding system of *H. brachyura* population in captivity through the use of advance biotechnology applications (DWNP, 2013).

In Peninsular Malaysia, under the new Wildlife Conservation Act (2010), *A. macrourus* and *H. brachyura* are protected species under the First Schedule while *Trichys fasciculata* is a totally protected species under the Second Schedule. Although all four Hystricidae species in Malaysia are classified as Least Concern in the recent IUCN (2016) assessment, overhunting of wild populations as well as habitat loss and degradation were cited as the major threats to this rodent family (Molur *et al*., 2005). In some Asian countries, Hystricidae are considered as agriculture pest and are facing threats due to illegal population controls by locals (Macdonald & Barrett, 1993).

Despite the widespread exploitation on members within this rodent family, little is known on their evolutionary, taxonomic and phylogenetic relationships (Huchon & Douzery, 2001; Sallam *et al.,* 2009). Van Weers (1979) provided the taxonomic identification keys based on morphological characters to identify the genera within Hystricidae. However, several arguments on their systematics were identified. In particular, the argument on whether *Thecurus* warrants a genus rank were addressed by Van Weers (1978, 1979, 2005). Phillipps and Phillipps (2016) classified the species as *Thecurus crassispinis* while several authors classified the species as *Hystrix* (*Thecurus*) *crassispinis* in which they noted that *Thecurus* is a subgenus (Van Weers, 1978; 1979; 2005; Wilson & Reeder, 2005).

Illegal hunting and trade of porcupines have recently been on the rise in Peninsular Malaysia. Since 2010, several enforcement cases involving porcupines have been reported (DWNP, 2010; 2011; 2013). Most of these cases involved animal parts which have limited or no morphological identification. Thus, the use of molecular data are needed in such instances to accurately identify the species and possibly their origin. Unfortunately, no reference sequence data is currently available in the public domain (GenBank, EMBL, DNA Data Bank of Japan or BOLD) for all four species of Hystricidae from Malaysia. Without a sequence database of local wildlife species, species identification process would be complicated and impossible (Kim, 2005). Hence, this study was initiated to provide reference DNA sequence data for the purpose of species identification in forensic cases as well as to provide insight into the phylogenetic relationships among the Hystricidae.

Methodology

Sample Collection and Laboratory Analyses

A total of 18 individuals from four species of porcupines: *A. macrourus* (N=9), *H. brachyura* (N=5), *Thecurus crassispinis* (N=1), and *Trichys fasciculata* (N=3) from Malaysia were sampled

(Table 1). All individuals were identified with the identification keys provided by Khan (1992) and Payne and Francis (2007). Approximately 1 ml of blood samples were collected by qualified veterinarian and preserved in FTA® cards (Whatman, UK). Total genomic DNA was extracted from all samples using the DNeasy Blood and Tissue Kit (Qiagen, Germany). DNA extracted products were then subjected to PCR using a universal primer pair to amplify the partial cytochrome *b* (Cyt *b*) segment of the mitochondrial DNA gene (mtDNA) known as CYTB1 (5' – CCA TCC AAC ATC TCA GCA TGA TGA AA – 3') and CYTB2 (5' – GCC CCT CAG AAT GAT ATT TGT CCT CA – 3') (Kocher *et al*., 1989). The PCR amplification profile and subsequent DNA purification follows Ryan & Esa (2006) while cycle sequencing reaction were done according to Rovie-Ryan *et al*. (2008). DNA sequencing was conducted

Table 1: List of samples and GenBank sequences used in this study. Asterisk (*) indicates the 18 sequences produced in this study

No	Genus/ Species	Origin	GenBank Acc No.
1	*Atherurus africanus*	Nigeria	HQ450774
2	*A. africanus*	Nigeria	HQ450775
3	*A. africanus*	Cameroon	KJ193296
4	*A. africanus*	Cameroon	KJ193297
5	*A. africanus*	Cameroon	KJ193298
6	*A. africanus*	Cameroon	KJ193299
7	*A. africanus*	Cameroon	KJ193300
8	*A. africanus*	Ghana	KJ193301
9	*A. africanus*	Ghana	KJ193302
10	*A. africanus*	Cameroon	KJ193304
11	*A. macrourus*	Peninsular Malaysia (PM)	KX580782*
12	*A. macrourus*	PM	KX580783*
13	*A. macrourus*	PM	KX580784*
14	*A. macrourus*	Negeri Sembilan, PM	KX580785*
15	*A. macrourus*	PM	KX580786*
16	*A. macrourus*	PM	KX580787*
17	*A. macrourus*	PM	KX580788*
18	*A. macrourus*	PM	KX580789*
19	*A. macrourus*	PM	KX580790*
20	*A. macrourus*	NA	FJ931121
21	*Hystrix africaeaustralis*	NA	X70674
22	*Hystrix brachyura*	Johor, PM	KX580791*
23	*H. brachyura*	Johor, PM	KX580792*
24	*H. brachyura*	Negeri Sembilan, PM	KX580793*
25	*H. brachyura*	Negeri Sembilan, PM	KX580794*
26	*H. brachyura*	Sarawak, Malaysia	KX580795*
27	*H. brachyura*	Thailand	JQ991599
28	*H. cristata*	Italy	FJ472565

29	*H. cristata*	Italy	FJ472566
30	*H. cristata*	Tunisia	FJ472567
31	*H. cristata*	Libya	FJ472568
32	*H. cristata*	Morocco	FJ472569
33	*H. cristata*	Morocco	FJ472570
34	*H. cristata*	Eritrea	FJ472571
35	*H. cristata*	Eritrea	FJ472572
36	*H. cristata*	Ethiopia	FJ472573
37	*H. cristata*	Tanzania	FJ472574
38	*H. cristata*	Burkina Faso	FJ472575
39	*H. cristata*	Namibia	FJ472576
40	*H. cristata*	Namibia	FJ472577
41	*H. cristata*	Namibia	FJ472578
42	*Hystrix indica*	India	JN794531
43	*Thecurus crassispinis*	Sabah, Malaysia	KX580796*
44	*Trichys fasciculata*	PM	KX580797*
45	*T. fasciculata*	PM	KX580798*
46	*T. fasciculata*	PM	KX580799*
47	*Ctenodactylus vali*	NA	AJ389532
48	*Fukomys damarensis*	NA	KT321364
49	*Massoutiera mzabi*	NA	AJ389533
50	*Petromus typicus*	NA	DQ139935
51	*Thryonomys swinderianus*	South Africa	AJ301644

using both the forward and reverse primers to authenticate the sequence.

Sequence Alignment and GenBank Sequences

Multiple alignments of the sequences were done and ambiguous flanking region were identified and removed with the program Geneious 5.6.7 (Drummond *et al*., 2012). BLAST analysis (Altschul *et al*., 1990) was conducted to check for sequence similarity. Following the authenticity test by Li and Zhang (2005) the sequences were also checked for Numts by translating the sequences into amino acid sequences. The absence of stop codon or indels conclude that the sequences were derived from mtDNA fragments rather than nuclear transpositions.

For the construction of phylogenetic relationships, eight out of eleven species of Hystricidae available in the GenBank were used in phylogenetic analyses (Table 1). DNA sequences representing the other main branches (other than Hystricidae) within the Infraorder of Hystricognathi were also obtained from GenBank which consists of Bathyergidae (*Fukomys damarensis*), Petromuridae (*Petromus typicus*), and Thryonomyidae (*Thryonomys swinderianus*).

Sequence Analyses and Phylogenetic Tree Construction

Sequence characterizations such as conserved sites (CS), variable site (VS), and parsimony-informative sites (PIS) were checked using MEGA7 (Kumar *et al*., 2016). Molecular diversity indices including the number of haplotypes (NHap), haplotype diversity (*Hd*), and genetic diversity (π) (Nei, 1987) were calculated using DnaSP 5.10 (Librado & Rozas,

2009). Genetic distances among the sequences were calculated using the Kimura 2-parameter model in MEGA7.

Phylogenetic trees were constructed using neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods as implemented in MEGA7, and the Bayesian inference (BI) as implemented in BEAST 2.0 (Bouckaert *et al*., 2014). For the ML analysis, to determine the best substitution model to run the ML tree, the dataset were tested for goodness of fit on 24 models of evolution as implemented in MEGA7. To assess the robustness of the NJ, MP, and ML trees, bootstrapping (Felsenstein, 1985) with 2,000 replicates were conducted. For the BI analysis, two independent runs each with 10 million Markov chain Monte Carlo (MCMC) generations (sub-sampled every 1000 generations) were conducted using the following settings: HKY85 substitution model with four gamma category counts (Hasegawa *et al*., 1985), strict clock, and Yule model (Heled & Drummond, 2012). TRACER 1.6 (Rambaut *et al*., 2014) were used to assess convergence of all parameters. The log and tree files from both runs were then combined using LogCombiner 2.1.2 available within the BEAST 2.0 package. TreeAnnotator 2.1.2 (available within the BEAST 2.0 package) was then used to create a consensus tree from the combined tree files with a burn-in of 10% and a posterior probability limit of 0.5.

Results

Sequence Characteristics, Diversity and Distance

Upon trimming of ambiguous flanking region, all 18 samples yielded 307-bp of sequence length. For the BLAST search result, all the *A. macrourus* showed 98% - 99% of sequence similarity to a Cyt *b* sequence of *A. macrourus* (FJ931121) in GenBank of unknown locality. However for *H. brachyura*, the closest match given was with one *H. brachyura* sample from Thailand (JQ991599) and several *H. cristata* sequences all with 93% of sequence similarity. Similarly, for *Thecurus crassispinis*, the closest match given was to the sequences of *H. cristata* with 93% of sequence similarity. For *Trichys fasciculata* on the other hand, the closest match given was to *Jaculus jaculus* (Lesser Egyptian jerboa) (JX885140) with 83% of sequence similarity. All sequences were deposited in GenBank with the accession number KX580782 - KX580799.

Table 2 summarizes the sequence characteristics and the molecular diversity indices calculated for the dataset used in this study. In total, the Hystricidae (N= 46; excluding outgroup species) showed 198 CS, 109 VS, and 102 PIS. Both the *Atherurus* and *Hystrix* showed similar sequence characteristics while *Trichys* showed least variation in the sequences. Excluding *Thecurus*, π were 0.093, 0.059, and 0.002, respectively for *Atherurus*, *Hystrix*, and *Trichys*.

Genetic distances calculated between and within the eight Hystricidae species are shown in Table 3. Among genera, *Atherurus* is genetically distant from *Hystrix* at 17.1% and from *Trichys* at 22.5% while *Hystrix* is distant to *Trichys* at 22.0% (data not shown). In general, species distances within Hystricidae ranged between 4.0% - 23.2%. Within the genus *Hystrix*, genetic distances recorded a range between 4.0% - 9.4%. We also calculated the genetic distances between the regional populations of *H. brachyura* from Peninsular Malaysia, Borneo, and Indochinese. Results showed that the peninsula population differed from the Bornean population by 4.4% and to the Indochinese population by 7.1% while the Bornean population differed from the Indochinese population by 7.0% of genetic distance.

Phylogenetic Relationships

The phylogenetic relationships of Hystricidae are summarized in Figure 3. All four methods of phylogenetic trees construction produced similar topologies and thus was represented by the ML tree (-log likelihood= -2075.37). In general, the tree separated three genera of Hystricidae (*Atherurus*, *Hystrix*, and *Trichys*) into their respective genus groups except for

Table 2: Sequence characteristics (as calculated using MEGA7) and standard molecular diversity indices (calculated using DnaSP) calculated for the dataset used in this study. CV= conserved sites; VS= variable sites; PIS= parsimony-informative sites; NHap= number of haplotypes; *Hd*= haplotype diversity; π= nucleotide diversity

Family/Genus/ Species	N	Sequence Characteristics			Diversity Indices		
		CV	VS	PIS	NHap	*Hd*	π
Atherurus	20	242	65	58	12	0.905	0.093
A. africanus	10	276	31	25	7	0.004	0.036
A. macrourus	10	298	9	0	5	0.667	0.006
Hystrix	23	240	67	42	18	0.972	0.059
H. africaeaustralis	1	-	-	-	-	-	-
H. brachyura	6	274	5	25	6	1.000	0.039
H. cristata	14	283	24	18	9	0.923	0.028
H. indica	1	-	-	-	-	-	-
Thecurus crassispinis	1	-	-	-	-	-	-
***Trichys* (*T. fasciculata*)**	3	306	1	0	2	0.067	0.002
Hystricidae	46	198	109	102	32	0.975	0.123

Table 3: Genetic distances (in percentages, %) as calculated using the Kimura 2-parameter model (Kimura, 1980) for between (below the diagonal) and within (in grey shaded column) the Hystricidae species used in this study

No.	Species	1	2	3	4	5	6	7	8
1	*Atherurus africanus*	3.9							
2	*Atherurus macrourus*	17.9	0.6						
3	*Hystrix africaeaustralis*	19.8	15.9	-					
4	*Hystrix brachyura*	17.3	16.1	8.4	3.9				
5	*Hystrix cristata*	18.9	15.8	4.0	8.9	2.6			
6	*Hystrix indica*	16.0	14.1	6.9	7.6	9.1	-		
7	*Thecurus crassispinis*	19.2	15.1	7.3	8.0	9.4	6.9	-	
8	*Trichys fasciculata*	22.9	22.1	23.2	22.3	21.9	22.7	20.0	0.2
9	Outgroup	27.9	24.6	27.1	26.0	25.6	24.9	25.0	27.4

Thecurus with moderate bootstrap support. *Trichys* which was represented by a monotypic species of *Trichys fasciculata* formed the basal group.

Within the genus *Hystrix*, with the exception of the single sequence of *H. africaeaustralis* (X70674), all the species clustered within their respective species groups. However, due to the small number of sequences representing *Thecurus crassispinis* and *H. indica* (one sequence for each species), their phylogenetic position within the genus *Hystrix* remains unresolved.

Discussion

In this study, we present the first sequence records for all four species of porcupines from Malaysia using the partial Cyt *b* gene segment. Moreover, the single sequence of the Bornean thick-spined porcupine (KX580796) generated in this study is the first sequence record for the species in the GenBank database. The fact that the

BLAST search could not give a strong sequence similarity for the Malaysian porcupine species (except *A. macrourus*) indicated that efforts towards generating the Malaysian Wildlife DNA Sequence Database are imperative. This database is especially important for the purpose of species identification in wildlife forensic cases.

Phylogenetically, the species within each genera of Hystricidae were well grouped within their respective genus except for *Thecurus*. At the basal, *Trichys* is the least specialized species

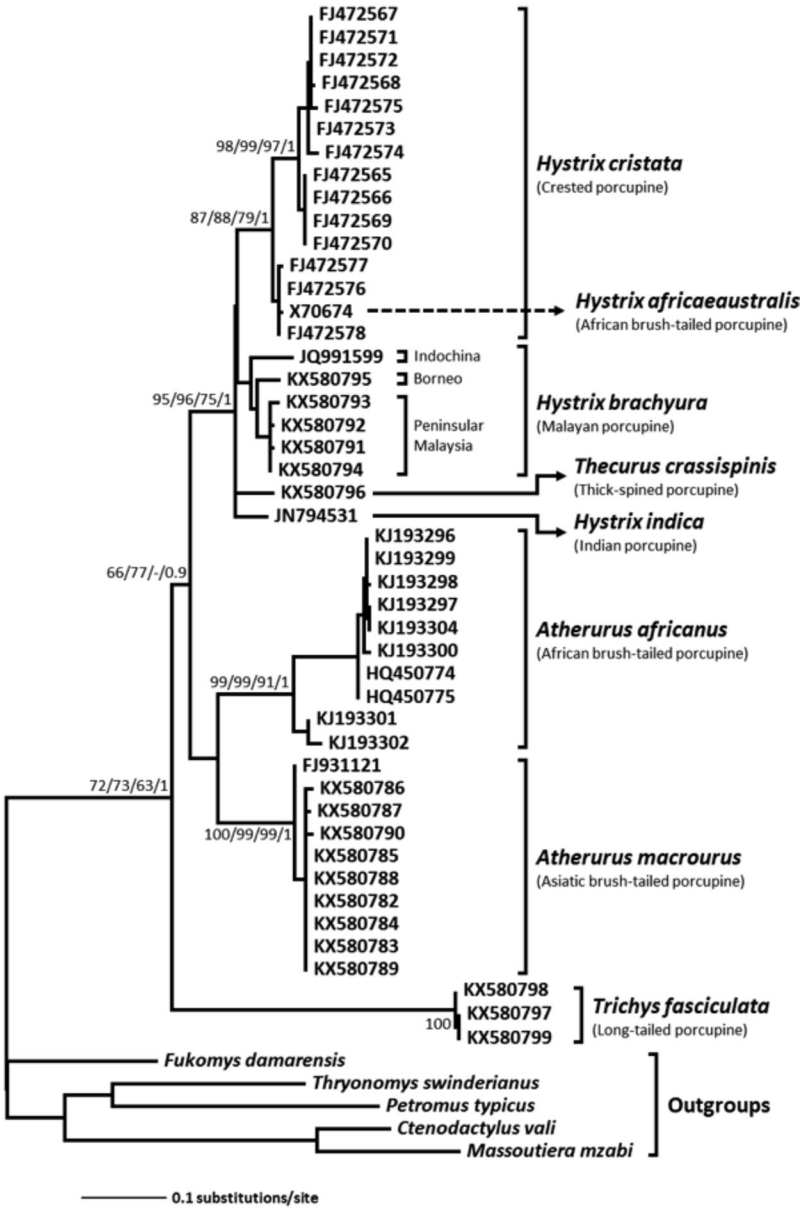

Figure 3: Phylogenetic relationships among the species within the Family Hystricidae as constructed from the 307-bp fragment of the mtDNA cytochrome *b* gene segment represented by the maximum likelihood (ML) tree. Only branches with bootstrap support above than 50% were shown.

within the Hystricidae (Van Weers, 2005) and was hypothesized to have first evolved in the Paleocene (Huchon & Douzery, 2001). Within *Atherurus*, although the two species (*A. africanus* and *A. macrourus*) occurs allopatrically and are genetically distant (17.9%), the node that separates both species was weakly supported (<50% of bootstrap support). Within *Hystrix*, the unresolved phylogenetic positioning of *H. indica* showed that further studies are needed to unlock the relationships using more samples as well as using more powerful markers such as the control region segment of the mtDNA gene. In addition, future studies should include the other three valid species *of Hystrix* (*H. javanica*, *H. pumila*, and *H. sumatrae*) to provide a better view into their phylogenetic relationships. Understanding their evolution would provide valuable insight into the historical biogeography of SEA.

The obscure position of the single sequence of *H. africaeaustralis* within *H. cristata* could be a case of misidentification as the sample was given as a gift to Din-Pow *et al*. (1993). Both species occurred sympatrically in East Africa (Congo, Tanzania, and Uganda) but Skinner and Smithers (1990) noted that they do not hybridize in the wild. This finding shows that the Cyt *b* segment used is a powerful molecular marker to distinguish among porcupine species. Nevertheless, the use of multi-genetic marker could provide more insight into the phylogenetic relationships as well as potential hybridization events especially using molecular markers that provide different genetic information (such as Y-DNA for paternal and microsatellite for biparental information).

As for the monotypic genus of *Thecurus*, Van Weers (1978; 1979; 2005) and Wilson and Reeder (2005) classified the species as *Hystrix* (*Thecurus*) *crassispinis* in which they noted that *Thecurus* is a subgenus. Recently, Phillipps and Phillipps (2016) classified the species as *Thecurus crassispinis* while IUCN (2016) placed it within the genus *Hystrix*. Van Weers (1978; 1979) argues the generic status of *Thecurus* and noted that insufficient diagnostic characters to distinguish *Thecurus* as a distinct genus from *Hystrix* thus suggesting that this taxon belongs to the rank of subgenus. Based on the phylogenetic analyses (see Figure 3), the species falls within the *Hystrix* species group thus we argue the validity of placing the thick-spined porcupine in the genus *Thecurus* as proposed by Phillipps and Phillipps (2016). We tentatively suggest that it should be systematically classified as *Hystrix crassispinis*.

Interestingly within *H. brachyura*, the sequences from Peninsular Malaysia, Borneo, and Indochinese (Thailand) showed evidence of population structuring although were weakly supported. Several other studies working on other animal taxa such as primates (Tosi *et al*., 2002), bats (Tingga & Abdullah, 2012), felids (Luo *et al*., 2014), fishes (Ryan & Esa, 2006; Tan *et al*., 2012), and herpetofauna (Inger & Voris, 2001) have also pointed out the structuring among the regional populations in SEA particularly the insular regions of the Sundaland. Thus, further studies to investigate the population structuring within *H. brachyura* are needed. Such studies are important to assist in the conservation and genetic management of both wild and captive populations particularly for the sustainability of the commercial breeding programs.

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