VNU Journal of Science: Natural Sciences and Technology, Vol. 32, No. 1S (2016) 329-335

Assessment of Genetic Relationship of some Horseshoe Bats (Chiroptera: Rhinolophidae) in Vietnam Using Cytochromoxydase Subunit I (*COI*) Gene Sequence

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Received 11 August 2016
Revised 25 August 2016; Accepted 09 September 2016

Abstract: DNA barcoding was used to examining the genetic relationship between some *Rhinolophus* bat taxa (*R. malayanus, R. cf. malayanus, R. marshalli, R. cf. marshalli*) in Vietnam using cytochrome oxidase-I (*COI*) gene sequence. Through this study, we constructed the phylogenic trees and analysed genetic relationships between some *Rhinolophus* taxa collected in Vietnam. The obtained phylogenetic tree showed two well-defined clusters. The genetic distances between species varied from 2.7% to 16.3%. The smallest distances were recorded between species from the same group whereas the largest distances were between species from the different groups. Genetic data supported the previous conclusion based on morphological classification of *R. malayanus, R. cf. malayanus, R. marshalli, R. cf. marshalli*.

Keywords: Genetic relationship, *COI* gene, *Rhinolophus*, Vietnam.

1. Introduction

Mitochondrial DNA is widely used as a tool in identifying species, evaluating genetic and phylogenetic relationships in different taxa and applying to conserve biodiversity [1, 8]. Recently, mitochondrial DNA are also used as an useful tool in bat researches, including describing new taxa [9], revealing cryptic species [10, 11] and classifying different bat species [12-14].

In Vietnam, only a few researches have used mitochondrial DNA for genetic analysis and classification of bat species [9], 15 17][17]. Among mitochondrial DNA sequences, the Cytochrome oxidase - I (*COI*) sequence is considered a reliable, cost-effective and accessible solution for species identification [18]. In this study, we aimed to evaluate the genetic variation and phylogenetic relationships of some species of the genus *Rhinolophus* (horseshoe bat) in Vietnam by analyzing the sequence of *COI*.

2. Materials and Methods

Materials: 9 samples of *Rhinolophus* bat species collected from different locations in

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Vietnam (Table 1) were used in this study. The samples were collected from the muscle of the vouchers or from the patagium of the released bats and preserved in 95% ethanol.

Table 1. Samples collected and used in this study

Symbol	Samples	Location
B2	*R.* cf. *malayanus*	KienGiang province
B3	*R. malayanus*	Quang Tri province
B4	*R. malayanus*	Quang Tri province
B5	*R.* cf. *malayanus*	KienGiang province
B6	*R.* cf. *malayanus*	KienGiang province
B9	*R. marshalli*	ThanhHoa province
B10	*R. marshalli*	ThanhHoa province
B12	*R.* cf. *marshalli*	Lam Dong province
B13	*R.* cf. *marshalli*	KonTum province

DNA extraction: Total DNA was extracted according to the Sambrook [19] with the following steps. Firstly, each sample was added with 600 µl of tissue lysis buffer (contains 0.1M NaCl, 0.05M EDTA pH8, 0.05M Tris-HCl pH8, 1% (w/v) SDS). The sample was then grinded and added with 15 µl proteinase K (20mg/ml) before being incubated overnight at 56◦C. The sample was then added with 600 µl Phenol-Chloroform-Isoamyl alcohol (PCI) (25:24:1 v/v) and gently mixed 3 minutes before centrifuging at 12000 rpm for 15 minutes at 4◦C. The supernatant was tranferred to a new 1.5 ml microcentrifuge tube and added with NaOAC 3M pH 4 (1:10 v/v the sample) and ethanol 100% (2:1 v/v the sample), then incubated at -20◦C overnight. After that, the sample was centrifuged at 12000 rpm for 15 minutes at 4◦C. The supernatant was discarded and the DNA pellet was dissolved with 500 µl ethanol 70% before centrifuging at 12000 rpm for 15 minutes at 4◦C. The supernatant was discarded and the DNA pellet was air-dried to drain off any excess ethanol. DNA pellet was dissolved in 50 µl TE buffer (Tris-HCl 0.01M pH8, EDTA 0.5M pH8) and stored at -20◦C. To check the quality of the extracted DNA, samples were analyzed by DNA electrophoresis on agarose gel and stained with FloroSafe before being visualized under UV Light.

PCR amplification of COI gene: *COI* gene was amplified by universal primers: VF1d (5'–TTCTCAACCAACAARGAYATYGG-3') and VR1d (5'–TAGACTTCTGGGTGGCCRAARAAYCA-3') [20]. The amplicons were approximately 700 bp in length. PCRs (polymerase chain reactions) were carried out in 20 µl volumes. Each reaction contained 6 to 7 µl of Deionized distilled water (DDW), 1 µl of each primer (10 µM), 10 µl of 2xPCR Master mix Solution (i-Taq) (iNtRON), and 1 to 2 µl of DNA template. The reactions were run under the thermal cycle of an initial denaturation at 94◦C for 4 min followed by 35 cycles of 94^0C for 30 s, 60^0C for 30 s, 72^0C for 1 min, and a final elongation cycle at 72^0C for 5 min. PCR products were checked using electrophoresis on a 2% agarose gel.

DNA sequencing: PCR products were purified using MEGA quick-spin TMTotal Fragment DNA Purification Kit (iNtRON). Purified DNA samples were sent to the 1st Base Company (Singapore) for sequencing. The sequencing was performed in 1 direction using the forward primer. The results were analyzed by Sequencer v.4.1. The DNA sequences were

checked authenticity by comparing with the data in Genbank using Blast tools in website http://blast.ncbi.nlm.nih.gov/Blast.cgi [8, 21].

Phylogenetic relationships were reconstructed based on 9 COI sequences generated in this research and from 15 COI sequences of reference bat species obtaind from GenBank (Table 2). The phylogenetic tree was constructed using Maximum Likelihood (ML) with a Kimura-2-parameter (K2P) substitution model, and Maximum Parsimony. Bootstrap support based on 1000 replicates was estimated. All analyses were performed in MEGA 6.0 [22].

Table 2. GenBank accession numbers

No.	Species names	Genbank No. (*COI*)	Voucher numbers for this study
1.	*R. affinis*	GU684798	
2.	*R. macrotis*	HM541601	
3.	*R. malayanus*	HM541619	ROM MAM 118045
4.	*R. malayanus*	HM541620	ROM MAM 118046
5.	*R. malayanus*	HM541621	ROM MAM 118077
6.	*R. malayanus*	HM541622	ROM MAM 118082
7.	*R. malayanus*	HM541623	ROM MAM 118104
8.	*R. malayanus*	HM541624	CMF980210-04
9.	*R. marshalli*	HM541625	HZM 4.35974
10.	*R. marshalli*	HM541626	EBD 23915
11.	*R. marshalli*	HM541627	EBD 24975
12.	*R. marshalli*	HM541629	ROM MAM 117825
13.	*R. paradoxolophus*	HM541668	
14.	*R. philippinensis*	HM541772	
15.	*R. stheno*	HM541823	

3. Results and discussion

3.1. Total DNA extraction

Total DNA was extracted and analyzed in 1% agarose gel (**Fig. 1**). Although all bands are smear, the total DNA bands of all samples with the theoretical size, more than 10kb. The clearly bands indicate that DNA concentration is quite high. Therefore, these DNA can be used for PCR amplification of COI gene.

3.2. PCR amplification of COI gene

All PCR products appeared with only one clear, bright band, in the expected size (**Fig. 2**). It suggests that we successfully amplified COI genes from 9 *Rhinolophus* samples, PCR reaction used primers with high specificity. After PCR products were purified, they were sent to the 1st Base Company (Singapore) for DNA sequencing. The sequencing was performed in 1 direction using the forward primer.

3.3. Phylogenetic analysis

The genetic distances between species analysed in this research varied from 2.7% to

16.3% (Table 3). These distances are higher than the sequence divergence among *Rhinolophus* species reported by Guillén *et al* [23] (1.5%-15%). The smallest distances were recorded between species from the same group whereas the largest distances were between species from the different group. The mean genetic distance between species was larger between groups than within groups.

The maximum likelihood (ML) tree recovered two well-defined clusters composed of *R. malayanus*, *R. affinis*, *R. stheno* in the first cluster in *R. megaphyllus* species group, and *R. philippinensis*, *R. marshalli*, *R. paradoxolophus*, *R. macrotis* in the second cluster in *R. philippinensis* species group (Fig. 2).

Within the first cluster, *R. malayanus* forms a well-supported monophyletic cluster and itself separates into two clusters. B2, B5, and B6 samples (*R.* cf. *malayanus*) are genetically close with bootstrap support 98%. Moreover, in pairwise distance analysis, they are exactly alike with the number of base differences per site is 0% (Table 3). COI sequences of *R.* cf. *malayanus* samples (B2, B5, and B6) differed from COI sequences of *R. malayanus* by 2.3-2.9%. The difference might appear among different species belong to the *Rhinolophus* species [22]. The difference of COI sequences between *R.* cf. *malayanus* and *R. affinis*, *R. stheno* (which belong to *R. megaphyllus* group) is over 12%. This result agreed with a previous study revealing a significant different between *R.* cf. *malayanus* specimens and *R. malayanus* specimens based on morphological study [1]. Morphological and genetic analysis suggest that *R.* cf. *malayanus (*B2, B5, and B6) might belong to another taxa, close to *R. malayanus*. This findings should be confirmed with more intensive studies in near future.

Within the second *cluster*, *R.marshalli*, *R. paradoxolophus* and *R. macrotis* form a sub-*cluster* whereas *R. philippinensis* itself forms a sub-*cluster*. Of all the *R. marshalli* samples collected in this study (B9, B10, B12, B13), the samples B12, and B13 form a well-supported sister relationship with *R. marshalli* HM541626 (bootstrap support 89%); B9 is closer to *R. marshalli* HM541625 and *R. marshalli* HM541625 whereas B10 itself is separated from all other samples and as well as from published sequences of *R. marshalli*, *R. paradoxolophus* and *R. macrotis*. Samples in this cluster slightly differed from each other by 0.3-3.2%. In contrast, they significantly differed from *R. philippinensis* by over 11% (Table 3).

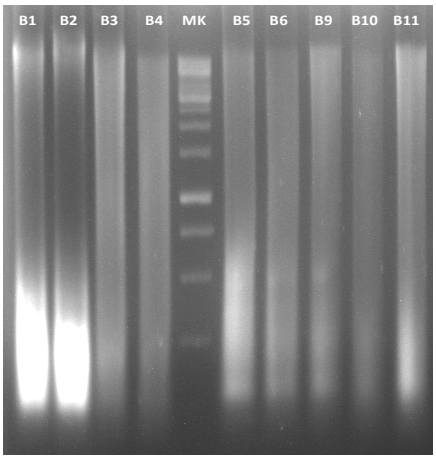

Fig.1. The total DNA extraction of Rhinolophus samples in 1% agarose gel marker 1 kb.

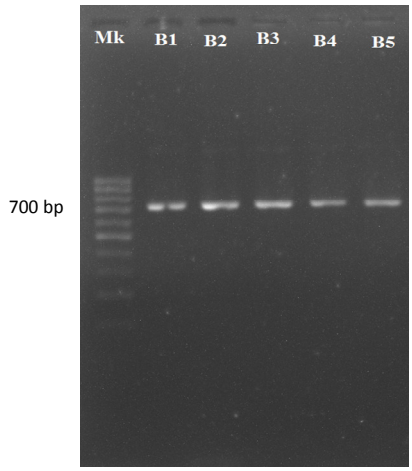

Fig.2. The PRC productions in 2% agarose gel electrophoresis (Lane MK represented marker 100bp).

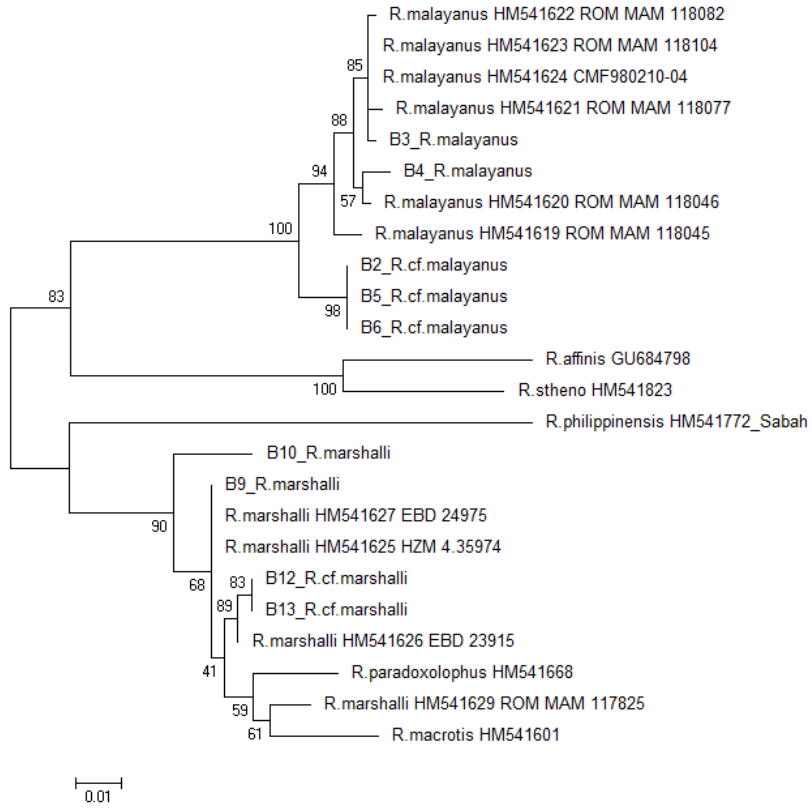

Fig. 3. Maximum likelihood tree of *COI* gene in *R. megaphyllus* and *R. philippinensis* species group.

Table 3. Percentage of differences per site among COI sequences using Pairwise Distances

No.	Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	B2_R.cf.malayanus																							
2	B3_R.malayanus	0.027																						
3	B4_R.malayanus	0.028	0.013																					
4	B5_R.cf.malayanus	0.000	0.027	0.025																				
5	B6_R.cf.malayanus	0.000	0.027	0.025	0.000																			
6	R.malayanus_HM541621_ROM_MAM_118077	0.029	0.005	0.013	0.029	0.029																		
7	R.malayanus_HM541622_ROM_MAM_118082	0.027	0.003	0.011	0.028	0.028	0.005																	
8	R.malayanus_HM541623_ROM_MAM_118104	0.026	0.002	0.010	0.026	0.026	0.003	0.002																
9	R.malayanus_HM541624_CMF980210-04	0.026	0.002	0.010	0.026	0.026	0.003	0.002	0.000															
10	R.malayanus_HM541620_ROM_MAM_118046	0.026	0.008	0.006	0.026	0.026	0.009	0.008	0.006	0.006														
11	R.malayanus_HM541619_ROM_MAM_118045	0.023	0.015	0.013	0.023	0.023	0.017	0.015	0.014	0.014	0.014													
12	R.affinis_GU684798	0.132	0.138	0.143	0.133	0.133	0.134	0.132	0.134	0.134	0.137	0.139												
13	R.stheno_HM541823	0.130	0.135	0.139	0.131	0.131	0.129	0.131	0.130	0.129	0.133	0.137	0.072											
14	B9_R.marshalli	0.102	0.113	0.108	0.102	0.102	0.119	0.117	0.115	0.115	0.109	0.112	0.136	0.125										
15	B10_R.marshalli	0.108	0.112	0.107	0.107	0.107	0.117	0.116	0.114	0.114	0.108	0.113	0.136	0.124	0.022									
16	B12_R.cf.marshalli	0.104	0.116	0.111	0.105	0.105	0.119	0.118	0.116	0.116	0.110	0.113	0.139	0.127	0.009	0.032								
17	B13_R.cf.marshalli	0.105	0.115	0.111	0.104	0.104	0.122	0.120	0.118	0.118	0.112	0.115	0.139	0.128	0.009	0.032	0.000							
18	R.marshalli_HM541629_ROM_MAM_117825	0.111	0.112	0.112	0.110	0.110	0.111	0.110	0.108	0.108	0.104	0.111	0.135	0.125	0.022	0.038	0.024	0.024						
19	R.marshalli_HM541627_EBD_24975	0.105	0.115	0.110	0.104	0.104	0.114	0.113	0.111	0.111	0.105	0.110	0.132	0.123	0.000	0.025	0.010	0.010	0.021					
20	R.marshalli_HM541626_EBD_23915	0.111	0.122	0.117	0.110	0.110	0.120	0.119	0.117	0.117	0.111	0.116	0.135	0.126	0.006	0.032	0.003	0.003	0.022	0.006				
21	R.marshalli_HM541625_HZM_4.35974	0.112	0.121	0.116	0.112	0.112	0.124	0.119	0.119	0.119	0.112	0.119	0.140	0.124	0.000	0.022	0.014	0.014	0.026	0.000	0.010			
22	R.macrotis_HM541601	0.117	0.123	0.122	0.117	0.117	0.126	0.125	0.123	0.123	0.117	0.123	0.149	0.134	0.037	0.055	0.037	0.037	0.031	0.037	0.037	0.043		
23	R.paradoxolophus_HM541668	0.113	0.117	0.115	0.112	0.112	0.117	0.116	0.114	0.114	0.108	0.116	0.134	0.128	0.027	0.043	0.031	0.030	0.031	0.027	0.027	0.033	0.042	
24	R.philippinensis_HM541772_Sabah	0.148	0.156	0.154	0.147	0.147	0.155	0.157	0.155	0.155	0.155	0.151	0.163	0.152	0.117	0.119	0.118	0.117	0.116	0.117	0.117	0.128	0.120	0.119

4. Conclusion

Genetic analysis of some Rhinolophus bat taxa (R. malayanus, R. cf. malayanus, R. marshalli, R. cf. marshalli) in Vietnam using COI gene sequences agreed with the morphological classification of these Rhinolophus bat taxa. This preliminary result suggests that R. cf. malayanus (B2, B5, and B6) might belong to another taxa, close to R.

malayanus. This findings should be confirmed with more intensive studies in near future. This result also indicated that COI gene can be used as molecular marker to analyze genetic relationship among bat species.

Acknowledgements

This research is funded by the Vietnam National University, Hanoi (VNU) under project number QG.15.19. We thank all staff in Departments of Genetics and Vertebrate Zoology for their assistance.

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Đánh giá mối quan - hệ di truyền của một số loài dơi lá mũi (Chiroptera: Rhinolophidae) ở Việt Nam sử dụng trình tự gen Cytochrom Oxydase Subunit I (*COI*)

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Tóm tắt: Phương pháp DNA barcoding đã được sử dụng để đánh giá mối quan hệ di truyền giữa một số loài dơi lá mũi ở Việt Nam thuộc giống *Rhinolophus*, với việc sử dụng gene cytochrome oxidase-I (*COI*). Nghiên cứu này đã xây dựng được cây quan hệ di truyền và phân tích mối quan hệ di truyền giữa các mẫu thu được trong nghiên cứu với nhau và so với một số trình tự được công bố trên Genbank. Cây quan hệ di truyền tách thành hai nhánh rõ ràng. Khoảng cách di truyền giữa các loài thay đổi từ 2.7% đến 16.3%. Những khoảng cách di truyền nhỏ nhất được ghi nhận giữa các loài trong cùng một nhóm loài trong khi những khoảng cách lớn nhất xuất hiện giữa các loài không cùng nhóm loài với nhau. Dẫn liệu về di truyền cũng phù hợp với kết quả nghiên cứu về hình thái đã được công bố trước đó.

Từ khóa: Mối quan hệ di truyền, gen *COI, Rhinolophus*, Vietnam.