ORIGINAL ARTICLE

Efficiency of the Mitochondrial DNA Markers (CO*I*, cyt *b*) and a Nuclear DNA Marker (RAG*I*) in Molecular Identification of Zoonotic Diseases' Hosts

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Abstract

Background: Morphological and allozyme studies are not remarkably efficient in identification of cryptic and unknown species; therefore, the differences between intra-and interspecific genetic variation (DNA barcoding) have been applied in recent decades. Applying molecular markers has been common for identification of taxa, so that suitable marker choice representing high divergence is a crucial issue to reveal taxonomic status of the taxa in this approach.

Methods: In this analytical study, the performance of two mitochondrial markers including cytochrome *c* oxidase subunit *I* (CO*I*) and cytochrome *b* (cyt *b*) was compared with nuclear recombination activating protein *I* locus (RAG*I*), and their efficiency in identification of mammal taxa as the host of zoonotic diseases was evaluated. The CO*I*, cyt *b*, and RAG*I* sequences were retrieved from GenBank. Intra-and interspecific genetic distances were estimated and compared at the species level. The variances in genetic divergence were also calculated and compared between the markers.

Results: Our results showed a wide gap between intra-and interspecific genetic distances for both CO*I* and cyt *b* markers and less apparent gap for RAG*I*, indicating that this nuclear marker is less proper for species delimitation in DNA barcoding.

Conclusion: We concluded that in the case of multiple sequences available CO*I*, contributes to accurate differentiation at the species level, showing a significant gap between intra-and interspecific genetic distances and may play an important role as DNA barcoding marker.

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Introduction

In applied biology, correct identification of taxa is a crucial issue. It can be a serious challenge when species are medically important and in close relationship with human public health, e.g. identification of the hosts and vectors of zoonotic diseases. Lack of dichotomous identification keys for many host and vector species necessitates interdisciplinary studies for the diagnosis of taxa in zoonotic diseases. Molecular systematics has been a developing field of study since 1990s.[1, 2] Investigation of evolutionary patterns of molecular

markers for identification of new lineages has been become predominant in mammal taxonomic works.[3, 4] Phylogenetic relationships can be understood from analyses of molecular data.[2, 5, 6] This approach is also non-invasive, especially considering its role in identification of mammals with conservation priorities.[7-12] Nuclear and mitochondrial markers are also accurate and efficient tools for biodiversity monitoring[13] and assessing ecological conditions of species based on the new branch of ecology called molecular ecology.[14, 15] On the other hand, various studies have focused on molecular markers for recognizing mammalian cryptic or complex species[16-20] and population structure within the species.[21-26] Molecular markers have also been suggested as valuable tools for comparison of unidentified specimens and describing new taxa.[27-29] Recently, DNA barcoding using short DNA sequences has become popular for species delimitation among biologists.[30-34] This approach has been accepted among molecular ecologists to assess biodiversity, especially within small invertebrates.[35-37] Some DNA markers comprising mitochondrial protein coding cytochrome oxidase subunit *I* (CO*I*), cytochrome *b* (cyt *b*),[38-43] and recombination activating protein *I (*RAG*I*) were used as a rapid, automatable, and non-invasive criteria for identification of vertebrate species.[44-49]

In the present study, the efficiency of two mtDNA markers (CO*I* and cyt *b*) and a nuclear marker RAG*I* in mammalian host identification was compared. Intra-and interspecific genetic variations have been compared for mammalian species, especially in the presence or absence of barcoding gap.[50-52] We aimed to represent the possible dependence of the intraspecific genetic divergences to the number of sequences per species, and compare the mean variability within and between species among these three markers.

Materials and Methods

Data Obtain and How to Use Them

The CO*I*, cyt *b*, and RAG*I* sequences were selected and retrieved from GenBank (less than 150 sequences per species randomly and at least two sequences from each species) as available on the 11th December 2016. These sequences included 15526 CO*I* sequences, 34371 cyt *b* sequences, and 3088 RAG*I* sequences. All the sequences were aligned using MAFFT ver.7.402, multiple alignment program for amino acid or nucleotide sequences;[53] stop codons were removed from each marker. Presumably, inappropriate sequences (low quality sequences) were visually recognized and eliminated. Additionally, the sequences shorter or longer than most homologous sequences were removed from these markers before alignment. A large number of mitochondrial sequences of species were omitted for two markers and only their haplotypes were used in this study. The haplotypes were determined by DnaSP ver.5.0 software,[54] for the analysis of nucleotide polymorphism from the aligned DNA sequence data.

The final matrix included 660 CO*I* sequences with a length of more than 630 base pairs (bp) for each sequence, 4943 cyt *b* sequences with a length of more than 910 bp for each sequence and 417 RAG*I* sequences with a length of more than 1060 bp for each sequence. The number of sequences and species used in this study were summarized in Table 1.

Data Analysis

The sequences were double-checked visually and the genetic distances were estimated in MEGA ver.7.402[55] using Kimura two-parameter (K2P) models.[56] K2P is the most effective model when genetic distances are low.[57] This model has been widely used in barcoding studies. For each marker, MEGA ver.7.402 software was applied to execute phylogenetic tree using Neighbour-joining (NJ) method with K2P model to exclude erroneous sequences. Intra-and interspecific K2P genetic distances were calculated at the species level based on output matrix of MEGA ver.7.402; these distances were compared for each species using SPD1.1, Format-converter program.[57] The variability within and between species were compared among the three markers. To assess the effect of sample size on intraspecific divergences for each marker, a regression analysis was employed. The variances in intraspecific divergences were also calculated using Analysis of Variance (ANOVA), and compared between markers. When necessary, data were log-transformed, and the differences were considered statistically significant when $P<0.05$ in a two-tailed test. All data were analyzed using SPSS 16 software and their graphs were plotted. In addition, for calculation and comparison of the mean value of the mass data corresponding to each marker, the R program was applied. The significant differentiation between the markers was tested by means of one-way ANOVA.

Results

The results showed that the mean of inter specific genetic distances in mammals were generally more than intraspecific genetic distances for each marker (Table 2). For the three markers CO*I*, cyt *b*, and RAG*I,* regression analysis results were $R^2=0.00$, $P=0.47$; $R^2=0.004$, $P=0.06$; and $R^2=0.008$, $P=0.46$, respectively. The regression analyses showed that the mean of divergence within the species was not dependent on the sample sizes per species for each marker. Totally, intraspecific K2P distances for these markers were ranged from zero to 35.10% (CO*I*: 0–12.06%, RAG*I*: 0–11.89% and cyt *b*: 0–35.10%) and interspecific K2P distances ranged from zero to 46.6% (RAG*I*: 0–28.58%, CO*I*: 0–36.62% and cyt *b*: 0–46.60%).

Table 1: Number of DNA sequences and mammal taxa used in the molecular analyses

	Individuals	Species	Genera	Families	Orders
All mammals		6495	1314	167	27
CO*I*	660	228	168	79	26
cyt *b*	4943	1915	634	125	27
RAG*I*	417	295	196	40	12

Table 2: The mean intra-and interspecific genetic distances for the three molecular markers of mammals used in the present study. The values are in percentage (%)

Marker	Mean		
	COI	cyt b	RAGI
Intraspecific variation	0.44±0.01	2.6±0.30	0.48±0.02
Interspecific variation	25.00±4.42	28.00±5.46	13.00±2.05

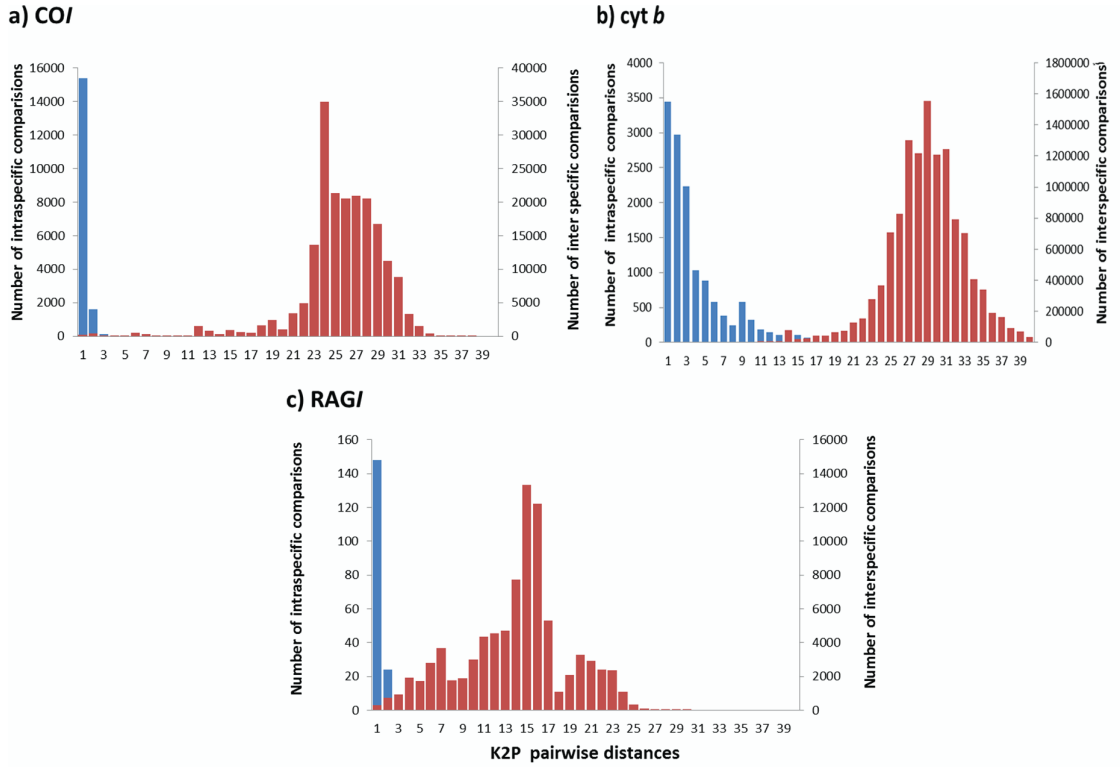

Figure 1: Intraspecific (red) and interspecific (blue) variation of mitochondrial and nuclear markers in K2P distances of mammals (a) CO*I*, (b) cyt *b*, (c) RAG*I*.

The mean and standard deviation (SD) for intraspecific K2P distances of CO*I* are 0.44%±0.01 for species with more than two sequences (mean=6.17; range of sequences=2-133; number of species=82), respectively. Intrageneric K2P distances were nearly 6-fold higher than the mean intraspecific K2P distances (Figure 1a). For cyt *b*, the mean and SD for intraspecific K2P distances in species with more than two sequences (mean=4.1; range of sequences=2-67; number of species=943) were 2.6%±0.30, respectively. The mean intrageneric K2P distances compared with intraspecific K2P distances were nearly 38 times higher (Figure 1b). The mean and SD for intraspecific K2P distances of RAG*I* were 0.48%±0.02 for species with more than two sequences (mean=2.54; range of sequences=2-9; number of species=68), respectively. For this gene, intrageneric K2P distances were approximately 28 times higher than intraspecific K2P distances (Figure 1c).

According to Figure 1, there were large gaps between intra-and interspecific divergences for both CO*I* and cyt *b* markers. These gaps are less in RAG*I* in comparison to the other two markers; furthermore, the gap is very distinctive in CO*I* compared to cyt *b*. The large gap of K2P distances between the intra- and interspecies levels suggests that these mtDNA markers are able to discriminate most of the mammal species by only a few specimens.

Discussion

Selecting the Appropriate Markers

In this study, DNA sequences were used to compare the efficiency of two mitochondrial markers, CO*I* and cyt *b*, besides a nuclear marker RAG*I* in identification of mammal species. For DNA barcoding, the difference between intra-and interspecific genetic variation should be clear[33, 50, 58] and distinct distance between intra-and interspecific divergence regarding to selected marker is essential. In the ideal DNA barcoding, the overlap between two of these values should not exist[57] although lack of standard level of divergence and inconsistency in the rate of evolution between different segments of the genome within and between taxa may challenge the correct taxonomy.[50, 59] Indeed, when intraspecific genetic distances are greater than interspecific genetic distances because of shared haplotypes in different species, failure in barcoding will occur.[60, 61] The results showed that CO*I* gene was the most proper marker for species delimitation based on the intra-and interspecific variation in the DNA sequences and is the most appropriate gene for identification of mammal species. There is a clear gap between intra-and interspecific divergence for CO*I* sequences, and cyt *b* also shows relative gaps. The average intraspecific CO*I* divergence for Didelphid marsupials was 2.0% relatively five times less than interspecific variation within each genus (11.2%).[62] This value was 1.0% within species and 10.1% between species for small mammals.[63] Clare et al.[64] also reported that the mean K2P CO*I* sequence distance between the congeners (7.80%) was 13 times higher than the mean divergence within species (0.60%). This gap is not very distinct for the RAG*I*, indicating that nuclear markers may be less proper for delimitation of the species boundaries in DNA barcoding. Liu et al.[65] also confirmed that applying nuclear markers of ray finned fishes failed to correctly identify the species. Nuclear DNA compared to mtDNA encounters relatively slower mutation rate. However, Zardoya and Meyer, 1996[66] mentioned that different length and mutation rates between various markers cannot account for their different performance in determining phylogenetic relationships. On the other hand, the "density of lineage creation events in time", " phylogenetic depth", and " completeness of taxa representation" are more effective in making correct phylogeny and consequent determination of species boundaries.[24, 66] In spite of the point that nuclear markers are not suitable for taxonomic studies and could not rapidly and properly separate the closely related cryptic species,[67] they are suitable for addressing the relationships among classes and orders rather than species levels in the phylogenetic studies.[68] Several studies on the DNA barcoding of animals suggest that CO*I* can truly identify the species in more than 95% of cases.[32, 58] The efficacy of CO*I* gene for identification of mammal species was also previously assessed for identification of Guyanan bats (more than 93%;[69]) and the species within Praomyini rodents (100%;[70]).

Origin of High Intraspecific Genetic Distances

The unexpected high intraspecific genetic distances calculated in the study, e.g. between African baboons *Papio cynocephalus* (18% to 35%), were related to mislabeling of the sequences in GenBank (compare the GenBank accession numbers EU885420 to EU885463 with the labels in the reference[71]). Additionally, considerable intraspecific genetic divergence calculated between some bats *Myotis mystacinus* (20%; AB106605 versus AY665141, AY665167, AY665166, and AY665140;[72]), and *M. formosus* (19.3%; EU434932 versus EF555234, EF555235, and EU434933;[73]), and insectivores *Crocidura* sp. (17%; FJ814026 versus EU122221, EU122220, and EU122219;[74]) are related to cryptic diversity and complex species. However, substantial intraspecific genetic distances estimated in pocket gophers of the genus *Cratogeomys* were attributed to a function of time and genetic differences between subpopulations.[75]

Pitfalls and Debates Over DNA Barcoding

There has been a long debate on the concept of DNA taxonomy,[76, 77] in which the role of traditional morphological approach in delimitation of species boundaries was overlooked. In spite of the efficiency of mitochondrial CO*I* for identification of unidentified specimens to the species level, the marker is useless at deeper phylogenetic levels due to its constraints.[78] Set aside the advantages of using genetic markers, pitfalls of applying DNA barcoding in identification of specimens should be taken into consideration.[79] This approach has been sensitively dependent on the methodology and the result interpretation.[34, 59, 80] As a source, potential incompatibility between mitochondrial and nuclear DNA in the case of introgression[77] and also probability of recombination of DNA[81-83] which may cause inconsistency between nuclear and mitochondrial markers should be considered. Moreover, distance-based DNA barcoding may encounter a failure due to hybridization and in the case of incomplete lineage sorting.[77] From the major criticisms of DNA barcoding is the species concept which is still focusing on species-level identifications.[50, 52, 83]

DNA Barcoding as a Tool for Host-Vector Identification

Medical studies on zoonotic and vector-borne diseases can take advantage of DNA barcoding to understand the life cycle of pathogens. The utility of DNA barcoding for the identification of species

with great medical importance was reviewed in Ondrejicka et al.'s study.[84] Alcaide et al.[85] also tested the applicability of molecular barcoding to identify the hosts from the midgut content of blood-feeding arthropods. This approach can also be used for unknown host detection of vector born diseases.[86] Having its special benefits, e.g. costs, this screening tool has been currently developed and promoted to the next-generation sequencing (NGS) metabarcoding methodology which is capable of detecting a wide range of vectors and hosts.[87]

Conclusion

In this study, we showed that there were clear gaps between intra-and interspecific genetic distances at CO*I* and cyt *b* in large groups of mammals. Furthermore, our results showed that there was a special difference in the intraspecific genetic distances among the mitochondrial and nuclear markers. The efficiency of DNA barcoding and the applicability of the DNA taxonomy depend on the barcoding gap and the accuracy of taxonomic identification is related to the thresholds implemented to determine each level of taxonomic ranking for species and genera.

Conflict of Interest: None declared.

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